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5-Hydroxymethylcytosine (5hmC) and Ten-eleven translocation 1–3 (TET1–3) proteins in the dorsal root ganglia of mouse: Expression and dynamic regulation in neuropathic pain

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Abstract

Epigenetic mechanisms are increasingly implicated in chronic pain pathology. In this study, we demonstrate that the novel epigenetic mark 5-hydroxymethylcytosine (5hmC) is present in dorsal root ganglia (DRG) neurons and glia, and its levels increase following nerve injury. Furthermore, we show that the 5hmC-generating Ten-eleven translocation 1–3 (TET1–3) proteins are expressed in a cell-type specific manner in the DRG, with Tet3 displaying differential upregulation after injury, suggesting a potential role in neuropathic pain.

Keywords

Chronic pain; epigenetics; neuropathic pain

Introduction

Acute noxious insults, such as those caused by surgery or trauma, frequently lead to chronic neuropathic pain for a significant portion of the population (Kehlet et al. 2006; Cohen and Mao 2014). Although the mechanisms that underlie this acute-to-chronic transition are still incompletely understood, epigenetic processes, such as histone modifications, DNA methylation, and chromatin remodeling, have emerged as important determinants in the pathogenesis of chronic pain (Denk and McMahon 2012; Alvarado et al. 2013; Denk et al. 2013; Descalzi et al. 2015; Laumet et al. 2015). One novel epi-genetic mark that has been intensely studied in the central nervous system (CNS) is 5-hydroxymethylcytosine (5hmC),

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which is produced by hydroxylation of 5-methylcytosine (5mC) through the action of the Ten-eleven translocation hydroxylases (TET1–3) (Pastor et al. 2013). 5hmC has been implicated in many neuronal processes, including learning, memory, synaptic transmission, and disease states such as cocaine addiction (Szulwach et al. 2011; Kaas et al. 2013; Feng et al. 2015). Genome-wide studies have revealed that 5hmC correlates positively with gene expression in neurons, in contrast to its progenitor, 5mC, which generally acts to silence gene expression (Xu et al. 2011; Denk and McMahon 2012; Colquitt et al. 2013).

While much is now known about 5hmC in the brain, neither the existence nor the function of 5hmC in the somatosensory neurons of the dorsal root ganglia (DRG), which subserve sensations of touch, temperature, and pain, has been demonstrated. In this study, we report that 5hmC is widely distributed in the neurons and glia of the DRG, and that TET1–3 are expressed in this tissue in a cell-type specific manner. Moreover, we discovered that 5hmC is dynamically regulated in an animal model of nerve-injury-induced neuropathic pain, in parallel with an upregulation of *Tet3*.

Results

To determine whether and to what extent 5hmC is present in the DRG, we used a 5hmC-specific antibody for immunohisto-chemical analysis. We observed a 5hmC signal in the nuclear compartment of nearly all primary sensory neurons of the DRG, without any preference for a subclass of neuron, as evidenced by colocalization with Nissl staining (Figure 1(A)), isolectin B4 (IB4) (Figure 1(B)), and neurofilament 200 (NF200) (Figure 1(C)). We also observed 5hmC in non-neuronal cells, namely, Satellite cells and Schwann cells, which are identified by the markers glutamine synthetase (GS) and S100b, respectively (Figure 1(D, E)).

Having established that 5hmC is widely expressed in the DRG, we asked whether 5hmC levels are altered in response to nerve injury using the spared nerve injury (SNI) model, which recapitulates many of the clinical features of chronic, neuropathic pain (Bourquin et al. 2006). Paw withdrawal thresholds were measured using an electronic von Frey apparatus. Beginning 3 days after injury, withdrawal thresholds measured in the sural territory of the ipsilateral hindpaw decreased significantly, reaching a peak reduction on post-operative day (POD) 7, which is consistent with prior studies of the SNI model in mice (Bourquin et al. 2006). Accordingly, we chose the POD7 time point to quantitate 5hmC levels using an enzyme-linked immunosorbant assay (ELISA) specific for 5hmC. We observed a significant increase in 5hmC levels in the ipsilateral lumbar DRGs (L3–L5) from SNI mice compared to those of sham controls (Figure 2(B); unpaired *t*-test, $p = 0.017$, $n = 9–10$).

5hmC is generated by the TET proteins via the hydroxylation of 5mC (Pastor et al. 2013). In light of our findings, we surmised that the TET proteins must be present in the DRG and that they might be dynamically regulated by nerve injury, as has been shown in pathological contexts in the CNS where 5hmC has been implicated (Hamidi et al. 2015). Indeed, we found that the mRNA for *Tet1–3* is expressed in DRG tissue in naive animals, with *Tet2* having the highest relative abundance, followed by lower and roughly equivalent levels of

Tet1 and *Tet3* expression (Figure 3(A)). The specificity of the polymerase chain reaction (PCR) was confirmed by agarose gel electrophoresis (Figure 3(B)).

By immunofluorescence microscopy, we observed TET1–3-immunoreactivity (IR) in DRG sections using specific antibodies (Figure 4). TET1-IR was seen in some neuronal profiles, with the signal found predominantly in the nucleus, but also with some weak signal in the cytoplasm of some cells. Non-neuronal cells did not appear to possess TET1 (Figure 4(A–C)). TET2 was strongly and widely expressed, appearing in the nuclear compartment of DRG neurons, as well as in nuclei of non-neuronal cells, most likely Satellite and Schwann cells (Figure 4(D–F)). TET3, on the other hand, showed strong and distinct expression in the nuclear compartment of a subset of DRG neurons, but was absent from non-neuronal cells (Figure 4(G–I)). To further define the pattern of TET3 expression, we analyzed the size distribution of TET3-IR, and also performed colocalization analysis with peripherin, which marks small, unmyelinated neurons. We found that TET3 predominates in small- to medium-sized DRG neurons (Figure 5(B)), comprising 46% ($\pm 0.4\%$) of all DRG neurons. Consistent with this predominance in small- and medium-sized neurons, we also found that 90% ($\pm 0.3\%$) of TET3-positive neurons colocalize with peripherin (Figure 5(A)). Thus, TET3 expression is restricted to neurons in the DRG, and shows a preference for small- to medium-sized, peripherin-positive DRG neurons, which likely represent nociceptors (Lagerström et al. 2011).

Given our prior finding that nerve injury increases 5hmC levels in DRG tissue, we hypothesized that expression levels of one or more of the *Tet* proteins might be regulated by injury. To determine whether nerve injury alters *Tet* levels, we assayed mRNA expression in whole lumbar DRG tissue in SNI and sham animals by quantitative reverse transcription polymerase chain reaction (qRT-PCR). We found that *Tet3* is significantly increased in SNI over sham at POD7, while *Tet1* and *Tet2* remain unchanged at the mRNA level (Figure 6(C)). Taken together, these results suggest that *Tet3* may be the predominant driver of 5hmC upregulation after nerve injury.

Discussion

Epigenetic mechanisms are increasingly appreciated to contribute to the pathogenesis of chronic pain (Denk and McMahon 2012; Denk et al. 2013; Alvarado et al. 2015; Laumet et al. 2015). In this study, we report that the epigenetic mark, 5hmC, is present abundantly in nearly all primary sensory neurons and glial cells of the DRG in adult mice. This is, to the best of our knowledge, the first report of 5hmC in the somatosensory system. The widespread presence of 5hmC in the DRG suggests a potentially important role for 5hmC in this tissue in both normal and pathological conditions, such as neuropathic pain.

Previous studies have demonstrated that 5hmC correlates positively with gene expression in neurons of the CNS and in olfactory neurons (Khare et al. 2012; Colquitt et al. 2013; Perera et al. 2015), and that some neurological disease states show dynamic regulation of 5hmC (Feng et al. 2015). Microarray and RNA sequencing (RNA-Seq) experiments in animal models of neuropathic pain have shown that hundreds of genes are dynamically upregulated by injury and that such changes contribute to the development of neuropathic pain (Costigan

et al. 2002; Scholz and Woolf 2007; LaCroix-Fralish et al. 2011). Accordingly, we hypothesized that an increase in 5hmC levels might occur after nerve injury, thus creating a permissive epigenetic state for increased gene expression. Indeed, we found that in the SNI model, 5hmC is globally increased in the affected lumbar DRGs in injured subjects, but not in sham controls. Moreover, this increase correlated with the time point of peak mechanical hypersensitivity, suggesting that 5hmC may contribute to the regulation of pain-related genes in the SNI model. For example, genes such as *Serpina3n* and *Csf1* have been shown to increase dramatically in DRG neurons after injury and to affect pain behaviors in this setting (King et al. 2011; Guan et al. 2015). Based on our results, we would predict that such genes undergo *de novo* 5-hydroxymethylation in gene regulatory regions such as promoters and enhancers, as well as within gene bodies themselves, leading to increased gene expression. To answer such questions, future studies using next-generation sequencing methodologies such as genome-wide hMedIP and RNA-Seq are currently underway in order to elucidate the precise relationships between 5hmC and specific genes that contribute to pain in the setting of injury.

5hmC is generated through the oxidation of 5mC by the TET enzymes (TET1–3) (Pastor et al. 2013). Consistent with the presence of 5hmC in the DRG, we identified all three TET members in this tissue at both the mRNA and protein levels. Interestingly, the TET proteins showed a cell-type specific expression pattern, suggesting differential functions of each TET. The ubiquitous and strong expression of TET2 points to a general “housekeeping” role for this enzyme, likely regulating common and critical genes in both neurons and glia. In contrast, the neuronally restricted expression of TET1 and TET3 suggests that these TET members participate in the regulation of neuron-specific genes. TET3 shows the most striking specificity, with its expression predominating in small- and medium-sized, peripherin-positive neurons, most of which are presumed to be nociceptors (Basbaum et al. 2009; Shields et al. 2012; Le Pichon and Chesler 2014). Thus, TET3 is likely to play a unique role in modulating the expression of pain-related genes such as *Trpv1*, *Trpa1*, and *Nav1.7* in nociceptors. That TET3 may play an especially important role in neuropathic pain is supported by our finding that *Tet3* alone is upregulated in injured DRG in the SNI model. It should be noted, however, that this finding does not preclude the participation of the other TET proteins in the setting of nerve injury, since mechanisms such as post-translational modification could enhance the enzymatic activities of TET1 and TET2 without affecting their production. Future studies using pharmacological antagonists, conditional knockout animals, or RNAi-mediated knockdown in DRG neurons will be needed to decipher the specific contributions of TET1–3 to neuropathic pain development.

A greater understanding of 5hmC and TET proteins in pain conditions could lead to new avenues for therapeutic intervention. Given the multitude of genes regulated by nerve injury, it would be therapeutically advantageous to target a core mechanism, such as DNA 5-hydroxymethylation, that controls the totality of aberrant expression changes, rather than targeting a single gene. Indeed, the efficacy of this approach was exemplified in a recent report wherein the inhibition of the histone-modifier G9a normalized the expression of more than 600 genes that are altered by nerve injury (Laumet et al. 2015). Blocking injury-induced 5hmC alterations through TET inhibition could function in a similar manner to normalize maladaptive gene expression changes caused by surgery or trauma.

Methods and materials

Animals and surgery

All the animal procedures were approved by the Institutional Animal Care and Use Committee of Duke University. Animal experiments were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

Adult male C57BL/6J (8–12 weeks) mice purchased from Jackson Laboratories (Bar Harbor, ME) or adult male CD1 mice (8–12 weeks) purchased from Charles River (Skokie, IL) were used for this study. After arrival from the supplier, the mice were housed in the Duke animal facility with an inverted 12 hour light–dark cycle.

The SNI model of neuropathic pain was performed as previously described (Bourquin et al. 2006). Briefly, mice were anesthetized under 2% isoflurane. An incision was made near the lower thigh region, and the tibial, common peroneal, and sural nerves were exposed. The tibial and common peroneal nerves were ligated using 6/0 silk, and then transected using small iris scissors. Care was taken to avoid touching or stretching the sural nerve. The muscle was closely approximated and the skin incision was closed using 9mm wound clips. Sham surgery was performed in the same manner but without the nerve ligation and transection. Animals were returned to their home cages following surgery and monitored.

Behavior

Animals were habituated to the testing environment in individual Plexiglas mouse runs for at least 2 days before testing. On testing days, the mice were placed in individual mouse runs and mechanical thresholds were determined using the MouseMet electronic von Frey system (Topcat Metrology, Ely, Cambridgeshire, UK). For each mouse at each time point, three measurements were taken on the left (ipsilateral) hind-paw in the lateral portion (sural territory), and an average measurement was calculated. Consecutive measurements were spanned by an interval of at least 5 min. Pressure was applied through a soft-tipped probe at a force-rise rate of 1 g/s. The force that elicited paw withdrawal was determined using the MouseMet software. For data analysis, the raw paw withdrawal threshold values were log transformed, in accordance with Weber's Law. Behavior testing was performed by the same experimenter on all days, and due to the apparent difference between injured and uninjured mice, blinding was not possible.

Quantitative 5hmC enzyme-linked immunosorbant assay (ELISA)

Ipsilateral DRG (L3–L5) from SNI ($n = 10$) and sham ($n = 9$) mice were rapidly collected and snap frozen at -80°C . Genomic DNA was then isolated using the DNeasy Blood and Tissue Kit (Qiagen, Germantown, MD) according to the manufacturer's recommendations. DNA was concentrated using ethanol precipitation and quantitated using the Nanodrop 2000 (Thermo Fisher Scientific, Waltham, MA). The Quest 5hmC DNA ELISA Kit (Zymo Research, Irvine, CA) was used according to the manufacturer's recommendations to measure 5hmC levels. Each sample was assayed using 100 ng of DNA per reaction well. Absorbance values were detected using the xMark microplate reader (Bio-Rad, Hercules, CA). 5hmC % values were interpolated from a standard curve.

Immunofluorescence

Animals were deeply anesthetized with isoflurane and transcardially perfused with 4% paraformaldehyde. After perfusion, lumbar DRG (L3–L5) were removed and postfixed in the same fixative overnight at 4°C. Then, the tissues were cryo-preserved in 30% sucrose/phosphate-buffered saline (PBS) solution for at least 24 h. DRG sections were cut at 12 µm in a cryostat and stored at –20 °C until further processing. For immunostaining, tissues were blocked in a solution containing 1% bovine serum albumin (BSA) and 0.4% Triton-X 100 for 1 h at room temperature (RT). After blocking, the sections were then incubated overnight with primary antibodies diluted in 1% BSA with 0.2% Triton-X 100 at 4°C. After washing 3 times in PBS for 5 min at RT, sections were incubated with the appropriate secondary antibody for 1 h at RT followed by three washes in PBS for 5 min. Before mounting, some sections were counterstained with DAPI and fluorescent Nissl stain (NeuroTrace 640/660, Thermo Fisher Scientific). Slides were then mounted in Mowiol medium (Sigma, St. Louis, MO). For immunofluorescence for 5hmC, an antigen retrieval step was performed before blocking. Sections were first rehydrated with PBS and then incubated with 0.4% Triton-X in PBS for 15 min at RT. Then, 2 N HCl was applied for 15 min, followed by neutralization with 100mM Tris (pH 8) for 10 min. Subsequently, the sections were carried to the block step, as described above. Primary antibodies were used at the following dilutions: rabbit anti-5hmC (1:500, Active Motif, Carlsbad, CA, USA, 39770), mouse anti-NF200 (1:500, Sigma, N0142), rat anti-Tet1 (1:100, Active Motif, 61741), rabbit anti-Tet2 (1:1000, Millipore, Billerica, MA, ABE364), rat anti-Tet3 (1:500, Active Motif, 61743), mouse anti-GS (1:200, Millipore, MAB302), mouse anti-S100b (1:500, Sigma, S2657), chicken anti-peripherin (1:1000, Aves Labs, Tigard, OR, USA, Per). Secondary antibodies were as follows: Cy3-conjugated anti-Rabbit Cy3 (1:500, Jackson ImmunoResearch, West Grove, PA), Alexa 488-conjugated anti-Rat (1:500, Thermo Fisher Scientific), Alexa 594-conjugated anti-Chicken (1:500, Thermo Fisher Scientific), Alexa 488-conjugated anti-Mouse (1:500 Thermo Fisher Scientific). For some sections, Alexa 568-isolectin GS-IB4 from *Griffonia simplicifolia* (Thermo Fisher Scientific) was used at a 1:1000 dilution during secondary antibody incubation. Fluorescence was detected using an epifluorescence microscope (Nikon Eclipse NiE). Images were taken at 20× and 40× magnification. For quantification, all images were taken using the same acquisition settings. Four to six sections were selected from four animals. Each first section was selected randomly, and each subsequent section was 60 µm apart, in order to avoid double-counting. Images were analyzed using Adobe Photoshop CC.

Quantitative real-time reverse transcription PCR (qRT-PCR)

One week after injury, ipsilateral DRG (L3–L5) from SNI ($n = 5$) and sham ($n = 5$) mice were rapidly collected and snap frozen at –80 °C. The DRG were homogenized in 350 µl of TRI reagent (Zymo Research) using bead homogenization (BeadBug, Benchmark, Scientific, Edison, NJ). The lysate was applied to a QIAshredder column (Qiagen) to further ensure complete homogenization. RNA was then extracted using the Direct-zol RNA Miniprep Kit (Zymo Research) according to the manufacturer's recommendations. RNA concentration was quantitated using the Nanodrop 2000 (Thermo Fisher Scientific). A total of 400 ng of RNA from each sample was reverse-transcribed using the QuantiTect Reverse Transcription Kit (Qiagen). qRT-PCR was performed using the 2× KAPA Sybr Fast qPCR

mix (Kapa Biosystems, Wilmington, MA) on the CFX96 Real-time RT-PCR system (Bio-Rad, Hercules, CA) using gene-specific primers. Primer sequences used in this study were as follows: GAPDH-Fw: 5'-AGGTCGGTGTGA ACGGATTTG-3'. GAPDH-Rev: 5'-GGGGTCGTTGATGGCAACA-3'. Tet1-Fw: 5'-ACATCCCA CAGACCGAAGA-3'. Tet1-Rev: 5'-CAGCCGTTGAAATACATGCTC-3. Tet2-Fw: 5'-GCCAGAAGCAA GAAACCAAG-3'. Tet2-Rev: 5'-GCAATGACAGTAGCCAGGTT-3'. Tet3-Fw: 5'-GAGTTCCCTACC TGCGATTG-3'. Tet3-Rev: 5'-CCTTTTCTCCATACCGATCCTC-3'. Primers for Tet1-3 were predesigned assays (PrimeTime) purchased from Integrated DNA Technologies (Coralville, IA) that are exon spanning and with efficiency >95%. Assay numbers: Tet1 (Mm.PT.58.43326803), Tet2 (Mm.PT.58. 30089849), Tet3(Mm.PT. 58.11954119).

For all amplification reactions, the same amount of reverse transcription product was used. A total of 200 nM of forward and reverse primers were used per reaction, in a total volume of 10 μ l. The thermal cycling conditions were as follows: 3 min of polymerase activation at 95 °C, 45 cycles of 10 s of denaturation at 95 °C, and 30 s of annealing and extension at 60 °C, followed by a DNA melting curve for the determination of amplicon specificity. For the comparison of SNI to sham mRNA, the expression level of mRNA was first normalized to the expression of GAPDH mRNA using the 2^{-C_t} method, and then SNI was normalized to sham (Schmittgen and Livak 2008). For relative abundance of *Tet1-3* mRNA in naive samples, Tet expression levels were normalized to GAPDH mRNA from the same samples.

Statistical analysis

All data were expressed as the mean $\pm$ SEM (standard error of the mean). Behavioral analysis was tested using a two-way analysis of variance (ANOVA) with a *post hoc* Sidak multiple comparison test. Gene expression analysis was tested using either two-way ANOVA with *post hoc* Sidak multiple comparison test or an unpaired Student *t*-test when appropriate. All analyses were performed in GraphPad Prism 6.0 (Graphpad, San Diego, CA). The criterion for statistical significance was $p < 0.05$.

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Figure 1.

5hmC is expressed in DRG neurons, Satellite cells, and Schwann cells. Representative immunofluorescence images of DRG sections showing 5hmC colocalizing with: (A) fluorescent Nissl stain; (B) isolectin B4 (IB4); (C) neurofilament 200 (NF200); (D) glutamine synthetase (GS); and (E) S100b. Scale bars: (A) = 100 μm; (B, C) = 50 μm; (D, E) = 20 μm.

Figure 2.

5hmC levels are upregulated in ipsilateral lumbar DRG 1 week after SNI. (A) Mechanical hypersensitivity develops after SNI, reaching a maximum on post-operative day (POD) 7, as reflected by a reduced paw withdrawal threshold (PWT). *** $p < 0.001$, **** $p < 0.0001$, two-way ANOVA and *post hoc* Sidak multiple comparison test. Data are expressed as mean $\pm$ SEM. SNI (circle), $n = 9$; sham (square), $n = 7$. On POD7, ipsilateral DRGs (L3-L5) were collected and 5hmC levels were quantitated by a 5hmC-specific ELISA. 5hmC levels, as expressed as the percentage of cytosine residues that are 5hmC (% 5hmC), were significantly higher in the SNI group compared to sham controls. * $p < 0.05$, unpaired Student *t*-test. Data are expressed as mean $\pm$ SEM. SNI, $n = 10$; sham, $n = 9$.

Figure 3.

Tet1–3 mRNA is expressed in DRG tissue in naive mice. (A) The relative abundance of mRNA for *Tet1–3* from DRG (L3–L5) in naive adult mice was determined by qRT-PCR. *Tet2* is expressed most highly in the DRG while *Tet1* and *Tet3* are expressed at lower levels. Data were normalized to GAPDH and expressed as mean $\pm$ SEM, $n = 5$. (B) Following qRT-PCR, the reactions were run on an agarose gel to confirm the specificity of the reactions. bp: base pair.

Figure 4.

Tet1–3 show distinct expression patterns in DRG sections by immunofluorescence. Immunofluorescence microscopy on lumbar DRG sections from naive adult mice. Representative images for each *Tet* protein are shown individually, and merged with Nissl stain to demonstrate neuronal colocalization. Insets are magnified regions, as indicated by the white box. *Tet1* and *Tet3* are expressed in DRG neurons only, while *Tet2* is expressed broadly in neuronal and non-neuronal cells. White arrows (H) indicate non-neuronal cells that are *Tet2*-positive, most likely representing satellite cells. Scale bars: (Left and Middle Column) = 100 μm ; (Right Column)=50 μm .

Figure 5.

Tet3 predominates in small- to medium-sized DRG neurons and colocalizes extensively with peripherin. (A) Representative immunofluorescence images of DRG neurons stained for *Tet3* and peripherin. Note the broad overlap of *Tet3* with peripherin in the merge composite. Scale bar = 25 μm . (B) Size-distribution histogram of *Tet3*-positive DRG neurons (left, 669 cells, 4 sections per mouse, $n = 5$ mice) and all DRG neurons (right, 1455 cells, 4 sections per mouse, $n = 4$ mice).

Figure 6.

Tet3 expression increases in ipsilateral DRG 1 week after SNI, but not *Tet1* or *Tet2*. One week after SNI, *Tet3* transcript levels were significantly increased in ipsilateral DRGs (L3–L5), but *Tet1* and *Tet2* expression levels remained unchanged. Transcript levels were first normalized to GAPDH as a reference gene, and then to sham for comparison to SNI. All data are mean $\pm$ SEM ($n = 5$, SNI and sham). ** $p < 0.01$, two-way ANOVA with *post hoc* Sidak multiple comparison test.