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Research paper

Abnormal DNA methylation in the lumbar spinal cord following chronic constriction injury in rats

Ying Wang, Zhi-Ping Lin, Hui-Zhe Zheng, Shuang Zhang, Zong-Luan Zhang, Yan Chen, Yi-Sheng You, Ming-Hua Yang*

Department of Anesthesiology, Fujian Provincial Cancer Hospital, Teaching Hospital of Fujian Medical University, Fuzhou 350014, China

HIGHLIGHTS

- The role of abnormal DNA methylation in the lumbar spinal cord in neuropathic pain was explored in this study.
- DNMT3a, DNMT3b and MeCP2 expression increased, MBD2 expression decreased, and DNMT1, MBD1 and MBD3 expression hardly changed in the lumbar spinal cord in CCI rats on day 14 after surgery.
- GAD 67 expression decreased, and GAD 1 promoter methylation increased in the lumbar spinal cord in CCI rats on day 14 after surgery.

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ABSTRACT

Pathogenesis of neuropathic pain is complex and not clearly understood. Glutamate decarboxylase 67 (GAD 67) is a key synthetic enzyme for the main inhibitory transmitter gamma-aminobutyric acid (GABA), and diminishes in the spinal dorsal horn in rats following chronic constriction injury (CCI). GAD 67 is coded by gene GAD 1. DNA methylation can regulate the expression of GAD 67 by regulating the methylation of GAD 1 promoter in the psychotic brain. DNA methylation is primarily mediated by DNA methyltransferases (DNMTs) and methyl-DNA binding domain proteins (MBDs). In this study, in order to discover whether DNA methylation regulates GAD 67 expression in the spinal cord in CCI rats and is involved in neuropathic pain, we examined mRNA levels of DNMTs, MBDs and GAD 67 with real-time reverse transcriptase-polymerase chain reaction (qRT-PCR), and methylation of GAD 1 promoter with Pyromark CpG Assays in the lumbar spinal cord in CCI rats on day 14 after surgery. Our results showed that DNMT3a, DNMT3b and methyl-CpG binding protein 2 (MeCP2) expression increased, MBD2 expression decreased, and DNMT1, MBD1 and MBD3 expression hardly changed in the lumbar spinal cord in CCI rats on day 14 after surgery. GAD 67 expression decreased, and methylation of GAD 1 promoter increased in the lumbar spinal cord in CCI rats on day 14 after surgery. These results indicate that decreased GAD 67 may be associated with increased GAD 1 promoter methylation, which may be mediated by DNMT3a, DNMT3b, MeCP2 and MBD2 in CCI rats. These indicate that abnormal DNA methylation may be highly involved in CCI-induced neuropathic pain.

1. Introduction

Neuropathic pain is a chronic pain, which is caused by primary or secondary damage or dysfunction of the nervous system, and is characterized by hyperalgesia, allodynia and spontaneous pain [18]. The pathogenesis of neuropathic pain is complex and not clearly understood. The transcription and expression level of pain-associated genes are highly involved in the generation and maintenance of neuropathic pain [25]. Decreased gamma-aminobutyric acid (GABA)-ergic inhibition in the spinal dorsal horn plays an important role in neuropathic pain [4,28]. Glutamate decarboxylase 67 (GAD 67) is a key synthetic enzyme for the main inhibitory transmitter GABA, and diminishes in the spinal dorsal horn in rats following chronic constriction injury (CCI) [19,29].

Epigenetic mechanisms that induce heritable changes in gene expression without causing alterations in the DNA sequence can regulate the transcription and expression of pro- or antinociceptive genes [5,8], and may play an important role in neuropathic pain [3,27]. DNA methylation is one of the earliest and most characteristic epigenetic mechanisms in mammals, and is a major contributor to the stability of gene expression [12]. DNA methylation is catalyzed by DNA methyltransferases (DNMTs), which

* Corresponding author.
E-mail address: yangminghua0591@sina.com (M.-H. Yang).

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transfer a methyl group from *S*-adenosyl-L-methionine to cytosine bases of cytosine–phosphate–guanine (CpG) in DNA [9]. The CpG dinucleotides tend to cluster in regions called CpG islands, defined as regions of more than 200 bases with a G + C content of at least 50% and a ratio of observed to statistically expected CpG frequencies of at least 0.6 [23]. The methylation of CpG islands in the gene promoter plays an important role in regulating gene expression. Increased methylation of CpG islands in the gene promoter can downregulate gene expression, and decreased methylation of CpG islands in the gene promoter can upregulate gene expression [26]. There are two functionally different classes of DNMTs—maintenance methyltransferases DNMT1 and *de novo* methyltransferases DNMT3a and DNMT3b [16]. Methylated DNA relies on interfering with transcription factors directly and mainly via methyl-CpG-binding proteins (MBDs) indirectly to inhibit gene transcription [2,20]. Methyl-CpG binding protein 2 (MeCP2) and MBD 1–3 are the most studied MBDs in the central nervous system (CNS) [16].

GAD 67 is encoded by gene GAD 1. Recent evidences indicate that DNA methylation can regulate the expression of GAD 67 by regulating the methylation of GAD 1 promoter in the psychotic brain [6,10]. And there is a CpG island in rat GAD 1 promoter by scanning the rat GAD 1 promoter region (GenBank accession number AF110132) and recent rat GAD 1 sequence from UCSC Genome Browser on Rat Nov. 2004 (Baylor 3.4/rn4) Assembly. In this study, in order to discover whether DNA methylation regulates GAD 67 expression in the spinal cord in CCI rats and is involved in neuropathic pain, we examined mRNA levels of DNMTs, MBDs and GAD 67, and methylation of GAD 1 promoter in the lumbar spinal cord in CCI rats on day 14 after surgery.

2. Materials and methods

2.1. Animal surgeries and neuropathic pain measurements

This study was performed in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals. The Animal Care Committee of the Fujian Provincial Cancer Hospital in China approved all the procedures. Sixteen male Sprague-Dawley (SD) rats, weighing 220–250 g, provided by the animal experiment center of Fujian Medical University were divided into CCI rats ($n = 8$) and sham operation rats ($n = 8$). Under deep anesthesia induced by intraperitoneal injection of 10% chloral hydrate (300–350 mg/kg), a modified CCI to the sciatic nerve was performed on the left side according to Bennett and Xie [1]. The trunk of the sciatic nerve was freed before it branched. 4–0 chromic catgut was mildly ligated in the trunk with appropriate strength, which made the epineuria slightly compressed and relative muscles gently tremoring. Under a dissecting microscope, the surface vessel of the sciatic nerve was found mildly pressured, but the blood flow was not interrupted. Four ligations were made in the sciatic nerve trunk with an interval of 1 mm. A sham operation just exposed the nerve but did not ligate it. Ipsilateral mechanical allodynia and thermal hyperalgesia of all rats were monitored using an 2390 Electronic von-Frey Anesthesiometer (IITC Life Science, USA) with the adaption of a 0.8 mm rigid tip and Hargreaves Tes7370 (Ugo Basile, Italy) respectively as previously reported [30]. The endpoints of the two tests were characterized with the removal of the paw followed by definite flinching movements. After paw withdrawal, the intensity was recorded automatically. Each rat was measured five times, with an interval of 5 min. Mechanical withdrawal threshold (MWT) and thermal withdrawal latency (TWL) were averaged by removing the maximum and minimum values. The MWT and TWL of each rat were measured before, 3, 5, 7, 10 and 14 days after CCI surgery. All the behavioral testing was done by observers unaware of animal treatments. On day 14 after CCI surgery, all rats were killed under deep anesthesia induced by intraperitoneal injection of 10% chloral hydrate (300–350 mg/kg), and their lumbar spinal cords were freshly dissected.

Table 1
Primer sequences for quantitative real-time reverse transcriptase–polymerase chain reaction.

Gene	Primer	Sequence (5′–3′)
DNMT1	Forward	CAGATGTTCCATGCACACT
	Reverse	TGTGGATGTAGGAAAGTTGCA
DNMT3a	Forward	TTCAGCAAAGTGAGGACCAT
	Reverse	GGACAGGGAAGCCAAACA
DNMT3b	Forward	GCCTCAAACCCAACAACA
	Reverse	AGAAGCGGCAAAGTCAAT
MBD1	Forward	TGCCTGCGGATTGTGGAG
	Reverse	GGCGTTTGGTCGCTGATG
MBD2	Forward	ACCTGGGAAATGCTGTTG
	Reverse	TGTCTAATTGGCAATGTTGTG
MBD3	Forward	GCTTCCCTTCTGCCTTGC
	Reverse	TGGATGCCTGCCTCTTGC
MeCP2	Forward	CGCGAAAGCTTAAACAGAGGA
	Reverse	TGCAATCAATTCTACTTTAGAGCGA
GAD67	Forward	GCGGGAGCGGATCCTAATA
	Reverse	TGGTGCATCCATGGGCTAC
β-actin	Forward	TTACTGCCCTGGCTCCTA
	Reverse	ACTCATCGTACTCCTGCTTG

2.2. Real-time quantitative reverse transcriptase–polymerase chain reaction (qRT-PCR) analysis

Total RNA was extracted using Trizol Reagent (Invitrogen, USA). cDNA templates were synthesized by reverse transcription using M-MLV First- Strand Synthesis System for qRT-PCR (Invitrogen, USA) and saved at −20°C. qRT-PCR was performed using StepOnePlus™ Real-Time PCR System (Applied Biosystems, USA) and All-in-One™ qPCR Mix (GeneCopoeia, USA). β-actin was used as an internal standard, and $2^{-\Delta\Delta CT}$ values were used as the relative expression level of target genes. Primers were produced by Shanghai Bio-Engineering Company in China and are listed in Table 1.

2.3. Pyromark CpG Assays

There is one CpG island in the GAD 1 promoter region (GenBank accession number AF110132). According to the rat GAD 1 promoter region (GenBank accession number AF110132) and recent rat GAD 1 sequence from UCSC Genome Browser on Rat Nov. 2004 (Baylor 3.4/rn4) Assembly, the region (chr3:52789201–52789242) in the CpG island in GAD 1 promoter was chosen for Pyromark CpG Assays to quantify site-specific methylation. Pyromark CpG Assays were performed as previously reported [15]. Genomic DNA extraction was carried out with the Cwbio TissueGen DNA Kit (Cwbio, China). 1 μg freshly extracted DNA was used for the bisulfite treatment with the EpiTect Bisulfite Kit (Qiagen, German). Primers for pyrosequencing were designed using Pyrosequencing Assay Design Software v 1.0.6 (Qiagen, German). GAD 67 PCR primer: forward: 5-GTTAGAGGTAGTTAGATATTTGTAAAGG-3, reverse: 5-biotin-CCTTACTTACAAAATCCCTAATCC-3; length: 203 bp. GAD 67 sequencing primer: 5-TATTTGTAAAGGAGTTTTAG-3. PCR was performed using a 9700 PCR system (Applied Biosystems, USA) and the HotStarTaq DNA polymerase kit (TakaRa, Japan). The sequencing samples were prepared with the Vacuum Prep Workstation (Biotage AB, Sweden). Pyrosequencing was performed using the PyroMark Q96 ID (Giagen, German) and the PyroMark Gold Q96