

ORIGINAL ARTICLE

DNA Methylation Status of Tyrosine Hydroxylase Gene in the Locus Ceruleus of Rats during Morphine Abstinence

Farideh Jalali Mashayekhi

Department of Biochemistry and Genetics, School of Medicine, Arak University of Medical Sciences, Arak, Iran

ABSTRACT

Background: Repeated use of addictive drugs, alters gene expression. The epigenetic mechanisms are implicated in stimulant-induced gene expression and addictive behaviors. The expression of tyrosine hydroxylase (TH) gene is known to be regulated in the locus ceruleus (LC) during morphine exposure. Increased synthesis of dopamine and norepinephrine is believed to contribute to withdrawal. We investigated the correlation between regulation of TH expression and the methylation level of the TH promoter in chronic morphine treatment and withdrawal.

Materials and Methods: In this experimental study, morphine dependence was induced in male rats by rising doses of morphine (10 to 50 mg/kg, i. p) twice a day for 11 days, and then at least three rats per group. DNA was extracted and after its modification, promoter methylation of TH gene in the samples was analyzed.

Results: MS-PCR analysis at the TH promoter (-75/+111) showed no difference between methylation status in morphine abstinence and chronic morphine-treated groups.

Conclusion: In our experiment, there was no relationship between methylation status and TH gene expression at different time points after cessation of chronic morphine administration.

Key Words: DNA methylation, Morphine, Rats, Tyrosine 3-monooxygenase

➤ How to cite this paper:

Jalali Mashayekhi F. DNA Methylation Status of Tyrosine Hydroxylase Gene in the Locus Ceruleus of Rats during Morphine Abstinence. Journal of Iranian Clinical Research. 2015; 1(1): 11-15.

INTRODUCTION

Morphine and other opiates are highly abused substances that apply their effects via G-protein-coupled μ -opioid receptors [1]. The consequences of repeated opiate exposure include increased drug craving, tolerance to opioid analgesia, and withdrawal syndrome [2, 3].

There is some evidence representing that repeated morphine administration, as well as abstinence from morphine can induce changes in gene expression in the brain, which cause addiction [4-6].

Up-regulation of tyrosine hydroxylase (TH), the rate-limiting enzyme in the biosynthesis of catecholamines, is suggested as one of the most consistent neuroadaptations induced by chronic morphine treatment and withdrawal in the ventral tegmental area (VTA) and locus ceruleus (LC) [7, 8]. This adaptation is characterized by increased norepinephrine biosynthesis in the LC after precipitated withdrawal in these regions. It was found that norepinephrine-release

contributes to behavioral changes such as physical dependence [7]. LC, the major brain noradrenergic nucleus, is involved in physical dependence and withdrawal [9].

Alterations in gene expression in the key regions of the brain after abusing some drugs could be induced by various mechanisms comprising of gene transcription, RNA processing, and mRNA translation [10]. Regulation of gene transcription is an essential mechanism by which morphine can cause addiction [4]. Epigenetic mechanisms such as histone modification and DNA methylation are mediated-regulators of gene expression [11, 12]. DNA methylation takes place after DNA synthesis by the enzymatic transfer of a methyl group from the methyl donor, S-adenosylmethionine, to the carbon-5 position of cytosine (Cs). Cs is usually located at 5' to guanosines (Gs) and differentially methylated in human genome [13].

DNA methylation frequently correlates with transcriptional silencing of the associated genes [14]. DNA methylation within the TH gene is species-specific. In the human genome, the CpG islands include the promoter, the first exon, and part of the first intron of the TH gene [15]. There is a scarcity of information regarding the epigenetic regulation of TH gene expression by the abused substances.

In our previous study, we noted a more than threefold increase in the mRNA level of TH gene in the LC but not in the VTA during forced abstinence from morphine. In addition, we demonstrated involvement of H3 acetylation in the regulation of TH gene expression in the LC of rats during morphine withdrawal.

In the present study, we investigated the relationship between TH gene expression and methylation of the TH gene after cessation of chronic morphine treatment.

MATERIALS AND METHODS

In this experimental study, we analyzed the methylation pattern of the TH gene by applying methylation-specific polymerase chain reaction (MS-PCR) method [16]. These findings could provide fundamentally new insight into regulation of gene transcription in the brain by morphine.

Animals

Six groups of adult male Sprague-Dawley rats (at least three rats per group) weighing 230-270 g were used in this study at controlled temperature of 22±2°C. The rats were kept under a 12-h light/dark cycle, and they had free access to water and food. The conduct of animal care and treatment in this study was approved by the Ethics Committee of Shiraz University of Medical Sciences, Shiraz, Iran.

Drug and Treatment

Animals in the three test groups were injected intraperitoneally with morphine sulfate (Temad Company, Iran) diluted with saline in ascending doses twice a day for 11 days. Briefly, we administered morphine as follows: days 1 and 2: 10 mg/kg, days 3 and 4: 20 mg/kg, days 5 and 6: 30 mg/kg, days 7 and 8: 40 mg/kg, days 9 and 10: 50 mg/kg. In the morning of day 11, the rats received a single dose of morphine (50 mg/kg) [5, 17]. Afterwards, the rats were decapitated at three time points of 2 h, 24 h, and 7 days after receiving the last dose. The control group was treated with saline following the same procedure as mentioned above.

The brains were immediately removed and

VTA and LC were dissected according to visual anatomical landmarks and the Atlas of Paxinos and Watson [18]. The tissues were immediately frozen in liquid nitrogen and stored at -70°C until use.

DNA isolation and methylation specific PCR (MS-PCR)

In MS-PCR assay, genomic DNA was subjected to bisulfite modification, consequently, unmethylated Cs was converted to uracil. The modified DNA was used as a template in PCR with primers specific for methylated versus unmethylated DNA [19]. The first genomic DNA was extracted from VTA and LC samples using the standard phenol-chloroform method. After DNA extraction, bisulfite treatment was performed according to the standard protocol. In brief, DNA was denatured by 0.3 M NaOH treatment, bisulfite solution was added for overnight treatment, and the samples were heated at 55°C and treated in agarose blocks. After desalting, desulfonation, and neutralization, the treatment was completed. The samples were precipitated by ethanol and prepared for PCR amplification. Bisulfite-treated DNA was stored at -80°C up to the time it was needed.

Primer sequence was designed with Methyl Primer Express version 1 (ABI). The sequences of primers used for amplification of the promoter region of each TH gene were methylated primer (forward: 5'-GTTAGTTTGGTTTTTAAGAGGTCGT-3' and reverse: ATAACAACATATCATCCTCAC CGTA-3') and unmethylated primer (forward: 5'-GTTAGTTTGGTTTTTAAGAGGTTGT-3' and reverse: 5'-ATAACAACATATCATCCTCACACCATA-3'). PCR mixture included 5 µl 10× reaction buffer, 3 µl MgCl₂ (50 mM), 1 µl dNTP (10 mM), 2 µl each primer (10 mM), and 2 µl bisulfite-modified DNA in a final volume of 50 µl.

Reactions were started at the heat of 95°C for 5 min, after which 1.25 units of taq polymerase were added. Cycle condition was denaturation at 94°C for 45 s, annealing at 54°C for 45 s with an extension at 72°C for 45 s (for 35 cycles) and amplification length of 186 bp. PCR products were electrophoresed on agarose gel (2.0% w/v), stained with ethidium bromide, and visualized under UV illumination.

RESULTS

Methylation Analysis of the TH Gene Promoter in the LC by MS-PCR

To investigate the correlation between methylation status and the up-regulation of TH gene expression in the LC, the methylation level of the TH was analyzed using MS-PCR. It is worth mentioning that in the previous study, using real-time quantitative reverse transcription PCR

(qRT-PCR), we assayed the TH gene expression in the LC and VTA of the rats chronically treated with morphine or saline. Afterwards, we sacrificed them at three time points of 2 h (chronic morphine treatment), 24 h, and 7 days (morphine withdrawal) after the last morphine injection.

Our results showed that TH gene expression level in LC significantly increased 24 h after the last injection as compared to 2 h and 7 days after the last injection. Notably, at the VTA tissue, chronic morphine treatment and withdrawal did not cause any significant alterations in the levels of TH transcription at any of the time points.

In the present study, the methylation status of TH gene promoter was evaluated by amplifying a 186-bp fragment (-75/+111) in the LC. No difference was observed in the methylation status of TH gene promoter 24 h after the last injection in the LC in comparison with 2 h and 7 days after the last injection, as well as the control group.

DISCUSSION

In this experimental study, we observed no correlations between methylation status and TH gene expression at different time points after cessation of chronic morphine administration.

There are numerous mechanisms by which repeated exposure to drug abuse can alter gene expression in the brain [20, 21]. According to the current views, regulation of gene expression is one of the mechanisms by which these drugs can induce relatively long-term changes in the brain to cause a state of addiction [21-23].

There has been a major movement in laboratory and clinical research to understand the role of epigenetic in neurobiology, especially the neurobiology of learning and memory, drug addiction, and cognitive disorders. These studies suggest that stable gene expression changes in neurons are mediated, in part, by epigenetic mechanisms [23, 24].

TH gene is regulated in the LC and VTA by chronic morphine and its withdrawal [8]. The explanation of the mechanism of TH gene regulation is one of the key issues in the field of neurology. TH catalyzes the rate-limiting step for the biosynthesis of the catecholamine, dopamine, norepinephrine, and epinephrine [25, 26]. Therefore, TH is related to various neuropsychiatric disorders such as Parkinson's disease [27], bipolar disorders [28], and schizophrenia [29]. Only few in-vivo studies have investigated the epigenetic mechanisms of

TH gene expression during the last decade. Therefore, to examine whether morphine administration affects DNA methylation, and thereby, TH expression, in this study we analyzed methylation pattern of TH gene using MS-PCR in the LC of rats.

Under our experimental conditions, the status of DNA methylation at TH promoter did not distinguish between chronic morphine treated and control group animals. Plentiful evidence indicated an association between the character of DNA methylation, the chromatin structure, and transcriptional gene activity.

However there is a scarcity of studies investigating the role of DNA methylation in drug addiction. But, it seems the methylation status of CpG dinucleotides located in or near regulatory elements, can affect gene expression. Analysis of overall methylation level in cell lines using the bisulfite sequencing method showed that DNA demethylation treatment with 5-azacytidine upregulated TH expression [15]. It can be concluded that lisdextroamphetamine (a psychostimulant) increases the TH gene transcriptional activity in rats' hypothalamus. Moreover, TH upregulation is associated with CpG demethylation in CpG site in some regulatory elements of the TH gene [30]. Another study in human neural stem cells and dopaminergic neuron-like cells indicated that CpG methylation correlated with decreased TH gene expression and inhibition of DNA methylation with 5-azacytidine restored TH expression [25]. Subsequently, these findings suggested that DNA methylation is an important mechanism in regulation of TH transcription.

CONCLUSION

In conclusion, although demethylating agents upregulate the expression of the TH gene, we found no correlation between the methylation level of CpG dinucleotides of TH promoter in the region of interest and its transcription. These types of studies provide insight into the molecular mechanisms by which morphine produces neural and behavioral plasticity. Such studies might also lead to a development of the theories for the molecular basis of relapse of the behavior in addicted patients after years or even decades of abstinence and reveals mechanisms for the treatment of morphine addiction.

ACKNOWLEDGEMENTS

This paper was extracted from a PhD dissertation by Farideh Jalali Mashayekhi and was supported by a grant (grant no.: 4269) from Deputy of Research of Shiraz University of

Medical Sciences. We appreciate the cooperation of Dr Nader Tanideh from the Stem Cell and Transgenic Technology Research Center, Shiraz University of Medical Sciences, Shiraz, Iran.

REFERENCES

- Kieffer BL, Gavériaux-Ruff C. Exploring the opioid system by gene knockout. *Progress in neurobiology*. 2002;66(5):285-306.
- Kreek MJ. Drug Addictions. *Annals of the New York Academy of Sciences*. 2001;937(1):27-49.
- Nishikawa K, Tanobe K, Hinohara H, Okamoto T, Saito S, Goto F. [Molecular mechanism of morphine tolerance and biological approaches to resolve tolerance]. *Masui The Japanese journal of anesthesiology*. 2004;53(5):502-7.
- McClung CA, Nestler EJ, Zachariou V. Regulation of gene expression by chronic morphine and morphine withdrawal in the locus ceruleus and ventral tegmental area. *The Journal of neuroscience*. 2005;25(25):6005-15.
- Ammon S, Mayer P, Riechert U, Tischmeyer H, Höllt V. Microarray analysis of genes expressed in the frontal cortex of rats chronically treated with morphine and after naloxone precipitated withdrawal. *Molecular brain research*. 2003;112(1):113-25.
- Nestler EJ. Molecular mechanisms of drug addiction. *Neuropharmacology*. 2004;47:24-32.
- Nakai T, Hayashi M, Ichihara K, Wakabayashi H, Hoshi K. Noradrenaline release in rat locus coeruleus is regulated by both opioid and α 2-adrenoceptors. *Pharmacological research*. 2002;45(5):407-12.
- Boudny VA, Gold SJ, Messer CJ, Chen J, Son JH, Joh TH, et al. Regulation of tyrosine hydroxylase promoter activity by chronic morphine in TH9. 0-LacZ transgenic mice. *The Journal of neuroscience*. 1998;18(23):9989-95.
- Van Bockstaele E, Reyes B, Valentino R. The locus coeruleus: a key nucleus where stress and opioids intersect to mediate vulnerability to opiate abuse. *Brain research*. 2010;1314:162-74.
- Nestler EJ, Hyman SE, Malenka RC. *Molecular Basis of Neuropharmacology*. New York: McGraw-Hill; 2001.
- Li E. Chromatin modification and epigenetic reprogramming in mammalian development. *Nature Reviews Genetics*. 2002;3(9):662-73.
- Martinowich K, Hattori D, Wu H, Fouse S, He F, Hu Y, et al. DNA methylation-related chromatin remodeling in activity-dependent BDNF gene regulation. *Science*. 2003;302(5646):890-3.
- Azhikina T, Sverdlov E. Study of tissue-specific CpG methylation of DNA in extended genomic loci. *Biochemistry (Moscow)*. 2005;70(5):596-603.
- Reik W. Stability and flexibility of epigenetic gene regulation in mammalian development. *Nature*. 2007;447(7143):425-32.
- Arányi T, Faucheux BA, Khalfallah O, Vodjdani G, Biguet NF, Mallet J, et al. The tissue-specific methylation of the human Tyrosine Hydroxylase gene reveals new regulatory elements in the first exon. *Journal of neurochemistry*. 2005;94(1):129-39.
- Sulewska A, Niklinska W, Kozłowski M, Minarowski L, Naumnik W, Niklinski J, et al. Detection of DNA methylation in eucaryotic cells. *Folia Histochemica et Cytobiologica*. 2008;45(4):315-4.
- Mashayekhi FJ, Rasti M, Rahvar M, Mokarram P, Namavar MR, Owji AA. Expression levels of the BDNF gene and histone modifications around its promoters in the ventral tegmental area and locus ceruleus of rats during forced abstinence from morphine. *Neurochemical research*. 2012;37(7):1517-23.
- Levenson VV. DNA methylation as a universal biomarker. *Expert Review of Molecular Diagnostics*. 2010;10(4):481-8.
- Paxinos G, Watson C. *The Rat Brain in Stereotaxic*. San Diego: Academic Press; 1998.
- Nestler EJ. *Molecular neurobiology of addiction*. *The American journal on addictions*. 2001;10(3):201-17.
- Robison AJ, Nestler EJ. Transcriptional and epigenetic mechanisms of addiction. *Nature reviews neuroscience*. 2011;12(11):623-37.
- Spijker S, Houtzager SW, de Gunst MC, de Boer WP, Schoffemeer AN, Smit AB. Morphine exposure and abstinence define specific stages of gene expression in the rat nucleus accumbens. *The FASEB journal*. 2004;18(7):848-50.
- Tsankova N, Renthal W, Kumar A, Nestler EJ. Epigenetic regulation in psychiatric disorders. *Nature reviews neuroscience*. 2007;8(5):355-67.
- Renthal W, Nestler EJ. Epigenetic mechanisms in drug addiction. *Trends in molecular medicine*. 2008;14(8):341-50.
- Yang JW, Choi EY, Park MJ, Lee MA. Expression of tyrosine hydroxylase is epigenetically regulated in neural stem cells. *Biochemical and biophysical research communications*. 2011;414(4):712-8.
- Nakashima A, Hayashi N, Kaneko Y, Mori K, Sabban E, Nagatsu T, et al. Role of N-terminus of tyrosine hydroxylase in the biosynthesis of catecholamines. *Journal of neural transmission*. 2009;116(11):1355-62.
- Liu D, Jin L, Wang H, Zhao H, Zhao C, Duan C, et al. Silencing α -synuclein gene expression enhances tyrosine hydroxylase activity in MN9D cells. *Neurochemical research*. 2008;33(7):1401-9.
- Gos T, Krell D, Bielau H, Brisch R, Trübner K, Steiner J, et al. Tyrosine hydroxylase immunoreactivity in the locus coeruleus is elevated in violent suicidal depressive patients. *European archives of psychiatry and clinical*

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

- neuroscience. 2008;258(8):513-20.
29. Hu J, Chan LF, Souza RP, Tampakeras M, Kennedy JL, Zai C, et al. The role of tyrosine hydroxylase gene variants in suicide attempt in schizophrenia. *Neuroscience letters*. 2014;559:39-43.
30. Vakhitova I, Sadovnikov S, Iamidanov R, Seredenin S. [Cytosine demethylation in the tyrosine hydroxylase gene promoter in the hypothalamus cells of the rat brain under the action of an aminoadamantane derivative Ladasten]. *Genetika*. 2006;42(7):968-75.