Biochemical and Biophysical Research Communications 502 (2018) 375–381

Contents lists available at ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Mechanisms involved in epigenetic down-regulation of *Gfap* under maternal hypothyroidism

Praveen Kumar [a], Nachiket M. Godbole [b], Chandra P. Chaturvedi [c], Ravi S. Singh [a], Nelson George [d], Aditya Upadhyay [a], B. Anjum [a], Madan M. Godbole [a, **], Rohit A. Sinha [d, *]

[a] Department of Molecular Medicine & Biotechnology, Sanjay Gandhi Postgraduate Institute of Medical Science, Lucknow, 226014, India
[b] Department of Microbiology, Sanjay Gandhi Postgraduate Institute of Medical Science, Lucknow, 226014, India
[c] Stem Cell Research Facility, Department of Hematology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, 226014, India
[d] Department of Endocrinology, Sanjay Gandhi Postgraduate Institute of Medical Sciences, Lucknow, 226014, India

ARTICLE INFO

Article history:
Received 21 May 2018
Accepted 26 May 2018

Keywords:
GFAP
Maternal hypothyroidism
Neocortex
Astrocytes
Gliogenesis
Epigenetics

ABSTRACT

Thyroid hormones (TH) of maternal origin are crucial regulator of mammalian brain development during embryonic period. Although maternal TH deficiency during the critical periods of embryonic neo-cortical development often results in irreversible clinical outcomes, the fundamental basis of these abnormalities at a molecular level is still obscure. One of the key developmental process affected by maternal TH insufficiency is the delay in astrocyte maturation. Glial fibrillary acidic protein (*Gfap*) is a predominant cell marker of mature astrocyte and is regulated by TH status. Inspite, of being a TH responsive gene during neocortical development the mechanistic basis of *Gfap* transcriptional regulation by TH has remained elusive. In this study using rat model of maternal hypothyroidism, we provide evidence for an epigenetic silencing of *Gfap* under TH insufficiency and its recovery upon TH supplementation. Our results demonstrate increased DNA methylation coupled with decreased histone acetylation at the *Gfap* promoter leading to suppression of *Gfap* expression under maternal hypothyroidism. In concordance, we also observed a significant increase in histone deacetylase (HDAC) activity in neocortex of TH deficient embryos. Collectively, these results provide novel insight into the role of TH regulated epigenetic mechanisms, including DNA methylation, and histone modifications, which are critically important in mediating precise temporal neural gene regulation.

1. Introduction

Astrocytes play an important role in the maintenance, development, disease processes, and injury responses of the brain [1]. In the developing brain, astrocytes facilitate the formation of complex neocortical circuitries involving a complex process of synaptogenesis, maturation, and synaptic pruning [2]. The differentiation and maturation of astrocytes in the rodent neocortex coincides with he period of extensive synapse formation and maturation starting from the late embryonic period to the 2–3 postnatal week [3]. Astrocytes are derived from a common neural precursor radial glia, and their transition to mature astrocytes is marked with the appearance of Glial fibrillary acidic protein (*Gfap*). *Gfap* transcriptional regulation has been studied extensively because of its association with astrocyte development [4].

Maternal thyroid hormones (TH), are essential for the embryonic neurodevelopmental process before the onset of the fetal thyroid function (FTF) [5]. One of the neuronal process, affected by the deficiency of maternal TH, is the maturation of astrocytes [6,7]. Coincidently, maternal hypothyroidism also results in defective neocortical synaptogenesis [8] which itself relies on timely maturation of astrocytes. TH have long been known to regulate *Gfap* expression in developing brain [6,7,9–11].

Experiments in rodents have shown that TH affects the development of astroglia in forebrain and hippocampus, accelerating the transition from vimentin-positive to *Gfap*-positive cells in both the above brain regions [12]. Furthermore, TH treatment induces cortical astrocytes which had flatten morphology *in vitro* to become process-bearing cells followed by an increase in the *Gfap* content in midbrain and cerebral hemisphere astrocytes [13]. Although

* Corresponding author.
** Corresponding author.
E-mail addresses: madangodbole@yahoo.co.in (M.M. Godbole), anthony.rohit@gmail.com (R.A. Sinha).

https://doi.org/10.1016/j.bbrc.2018.05.173
0006-291X/

putative thyroid hormone response element (TRE) have been identified in the *Gfap* gene promoter [14], its functional relevance remains unclear. Therefore, even though TH responsiveness of *Gfap* is well established, the molecular basis of its gene regulation by TH is still elusive.

Genomic plasticity required for developmental dynamics in response to hormonal and environmental cues is achieved via epigenetic changes on gene promoters [15]. These changes, involving alteration in promoter DNA methylation and histone modification, are known to regulate *Gfap* transcription [16]. In this study, we for the first time provide evidence of an epigenetic control of *Gfap* promoter activity in maternal TH dependent manner. Our results show that maternal hypothyroidism significantly reduces neo-cortical *Gfap* expression, which correlates with its increased promoter DNA methylation and decreased histone acetylation. These results shed light on the importance of TH mediated epigenetic programming as a means of dynamic regulation of *Gfap* gene expression during brain development.

2. Material & methods

2.1. Animal model of maternal hypothyroidism

To induce hypothyroidism, 2-mercapto-1-methylimidazole (MMI; 0.025% w/v; Sigma St. Louis, MO) was given to the pregnant Sprague Dawley (SD) rats in drinking water from embryonic day 6 (E6) and continued until the animals were sacrificed and neocortices from embryos were harvested at different developmental stages on days 14, 16 and 18 respectively from euthyroid and hypothyroid groups. For reversibility group studies, hypothyroid dams on MMI regimen were injected with a single daily dose (1.5 μg/100 gm body weight) of T4 (Thyroxine, Sigma St. Louis, MO) three days prior to E14, E16 and E18 respectively. All animal procedures performed were approved by the institutional animal ethics committee CPCSEA, New Delhi (IACUC No: A-03:PGI/EP/IAEC/13/11.02.2011).

2.2. Thyroid hormone estimation

To validate the animal model and reversibility with TH, total circulating T3 (TT3) and T4 (TT4) levels were measured with a Coat-A-Count Kit (DPC, Los Angeles, CA), by solid phase radioimmunoassay wherein 125I-labelled T4 or 125I-labelled T3 competes for a fixed time with T4 or T3 respectively in sample for antibody binding sites.

2.3. HDAC activity assay

Total HDAC activity was assessed in neocortical tissue under euthyroid and hypothyroid conditions. Homogenates (25 μg of total protein) from neocortices were incubated with Fluor-de-Lys substrate in triplicates for 30 min at 37 °C to initiate the HDAC reaction. Fluor-de-Lys Developer was then added and the mixture was incubated for another 10 min at room temperature. Fluorescence intensity was measured by fluorometer Synergy XT with an excitation wavelength of 360 nm and an emission wavelength of 460 nm. HDAC activity was calculated according to manufacturer's instructions (Enzo Life Sciences, NY 11735, USA).

2.4. Western blotting

Cortical tissue samples were lysed using RIPA lysis buffer and immunoblotting was performed as per manufacturer's guidelines. Image acquisition was done using ChemiDoc (Bio-Rad ChemiDoc™ MP System, 1708280). Densitometry analysis was performed using ImageJ software (NIH, Bethesda, MD, USA). Antibodies used were from Cell Signaling Technology, USA: Histone Deacetylase (HDAC) Antibody Sampler Kit #9928, SirT1 (1F3) Mouse mAb #8469, β-Actin Antibody #4967.

2.5. RNA isolation and qRT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, Waltham, Massachusetts, USA) Complementary DNA was synthesised from 2 μg of total RNA using High Capacity cDNA Reverse Transcription Kit (Invitrogen Corp., Carlsbad, CA, USA) according to manufacturer's instructions. qRT-PCR was performed to measure the gene expression levels for Gfap, Mecp, Mbd1, Mbd2, Mbd3 and Mbd4 and GAPDH as internal control. Primer sequence used are: Gfap; Forward - 5′-ACCTCGGCACCCTGA GGCAG-3′, Reverse -5′-CCAG-CACTCAACCTTCCTC-3′ Mecp2; Forward-5′-GTCGCTCTGCTGGAAA GAT-3′, Reverse 5′-TGGGCTTCTTCGGTGGTT C-3′, Mbd1; Forward-5′-CAGCAGTCACAACCTTCCTG-3′ Reverse 5′-GGTGCCAATCCCTCCTACT-3′, Mbd2; Forward- 5′-GTCGGCGGCCCAGGAGTAATGAT-3′ Reverse 5′GACTCGCT CTTCCTGTTTCCT-3′, Mbd3; Forward-5′-CTGAA-CACTGCACTGCCGTA-3′ Reverse-5′-GTTTCTTCTCCCAGAAAAGCTG-3′, Mbd4; Forward-5′-CCTACCGGATCTTTT GTCT CA-3′ Reverse -5′-GATTTTCCCAAAGCCAGCAT-3'.

2.6. Methylation-specific PCR (MSP)

Cortical tissues (50 mg) from each study group at all different stages (E14, E16 and E18) were collected. DNA was isolated as per manufacturer's instructions (Qiagen). Tissues were digested for overnight at 55 °C in water bath, next day DNA was purified by using columns provided with the kit. Purity of the DNA was measured by NanoDrop (Thermo, ND 1000) at 260 nm. We used 1 μg of genomic DNA for the bisulfite conversion in all study groups of rats at all developmental stages. Methylation analysis was performed using EpiTect® Bisulfite kit (Qiagen). Methylation-specific Real Time quantitative PCR was performed with methylated and unmethylated primers of target genes. Sequences of the Unmethylated (Un) and Methylated (Me) sets of primers used for MSP are given below. These primers were designed to cover *Gfap* promoter region 1000bp upstream of transcription start site using Methyl Primer Express Software v1.0 (Applied Biosystems). The sequence of the primers used are: Gfap (Un): Forward -5′-GTTTTTATGTTTGATAGGTAAGTGA-3' & Reverse- 5′-CA AAACCTTTATTAAAAAACAAA-3′, and Gfap (Me): Forward -5′-GTTTTTATGTTTG ATAGGTAAGCGA-3' & Reverse- 5′-ATCAAAACCTTTATTAAAAAACGAA-3'.

2.7. Chromatin immunoprecipitation (ChIP)

Histone acetylation ChIP was performed on embryonic neocortical tissues (100 mg) using native ChIP Protocol [17] with slight modifications. The tissues were minced, lysed, and digested with MNase (1 Unit MNase for 5–10 μg of DNA) followed by hydroxyapatite purification Chromatin samples were precleared and incubated with antibodies against histone H3 acetylated (Anti-Histone H3 (acetyl K9) antibody - ChIP Grade (ab10812); Abcam), histone H4 pan-acetylated at lysines 5, 8, 12, and 16 (Pan-H4ac; number 06–598; Millipore), or normal rabbit IgG (sc2027; Santa Cruz Biotechnology) overnight at 4 °C. Subsequently, the DNA associated with modified histones was purified and the analysis of the DNA was done by Real Time quantitative PCR on ABI 7500 (Applied Biosystems) using SYBER Green Master Mix. The primers used for qPCR covered the *Gfap* promoter region from −200 to −400 bp upstream of the transcription start site. The sequence of the primers used are: Forward-5′GCCCTCTCTTGACCCATTTAC3′ and Reverse-5′AAGGTCACT GTACCCAGAGT3'.