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Differential epigenetic regulation of *BDNF* and *NT-3* genes by trichostatin A and 5-aza-2′-deoxycytidine in Neuro-2a cells

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ABSTRACT

To understand epigenetic regulation of neurotrophins in Neuro-2a mouse neuroblastoma cells, we investigated the alteration of CpG methylation of brain-derived neurotrophic factor (*BDNF*) promoter I and neurotrophin-3 (*NT-3*) promoter IB and that of histone modification in Neuro-2a cells. Bisulfite genomic sequencing showed that the CpG sites of *BDNF* promoter I were methylated in non-treated Neuro-2a cells and demethylated following 5-aza-2′-deoxycytidine (5-aza-dC) treatment. In contrast, methylation status of the *NT-3* promoter IB did not change by 5-aza-dC treatment in Neuro-2a cells. Furthermore, we demonstrated that *BDNF* exon I–IX mRNA was induced by trichostatin A (TSA) treatment. However, *NT-3* exon IB–II mRNA was not induced by TSA treatment. Chromatin immunoprecipitation assays showed that the levels of acetylated histones H3 and H4 on *BDNF* promoter I were increased by TSA. These results demonstrate that DNA methylation and/or histone modification regulate *BDNF* gene expression, but do not regulate *NT-3* gene expression in Neuro-2a cells.

1. Introduction

Brain-derived neurotrophic factor (BDNF) is a member of the neurotrophin family that includes nerve growth factor (NGF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) [1,2]. BDNF plays an important role in neuronal survival and differentiation, extension of neuritis, and synaptic plasticity in the central nervous systems [3,4]. Aberration of the *BDNF* gene expression results in various neurological disorders, such as depression, Alzheimer's, Parkinson's and Huntington diseases [5–8].

The *BDNF* gene has eight 5′-untranslated exons and one coding exon, exon IX (Fig. 1A) [9,10]. By alternative splicing, each untranslated exon is spliced to exon IX. *BDNF* gene expression is regulated by multiple activity-dependent and tissue-specific promoters located upstream of each 5′-untranslated exon [11,12]. The *NT-3* gene consists of at least two untranslated exons, exons IA and IB, and one protein coding exon, exon II (Fig. 1B) [13,14]. Promoters are located upstream of exons IA and IB, and *NT-3* alternative transcripts are generated in various tissues. For example, we previously reported that *NT-3* exon IB–II transcript was predominantly expressed in neurons [15]. Therefore, elucidation of tissue type-specific *BDNF* and *NT-3* genes expressions would contribute to the understanding of the regulation mechanisms controlling gene expression in neurons.

Previous studies have demonstrated that the *BDNF* gene expression is regulated by several transcriptional factors, such as cAMP-response element binding protein (CREB) and upstream stimulatory factors 1/2 (USF1/2), which are associated with *BDNF* promoters I and IV [16–18]. Additionally, calcium-responsive transcription factor (CaRF) plays an important role in *BDNF* transcriptional activation by binding to *BDNF* promoter IV [19]. However, it is still unclear how the transcription activities of *BDNF* promoters I and IV are differentially controlled. On the other hand, we previously demonstrated that two GC-boxes of the *NT-3* promoter IB recruited transcription factors, Sp3 and Sp4, in neurons [15]. This result suggests that different transcription regulation mechanisms control transcription of *BDNF* and *NT-3* genes.

It is reported that epigenetic regulations, such as DNA methylation and histone modification, play a crucial role in tissue type-specific and activity-dependent regulation of *BDNF* gene expression [10,20,21]. Moreover, it is reported that expression of *BDNF* exon I–IX mRNA, an alternatively spliced form containing exon I, is induced by 5-aza-2′-deoxycytidine (5-aza-dC), a DNA methyltransferase inhibitor, in Neuro-2a neuroblastoma cells [10]. Another study shows that Sp3 function as activator when Sp3 is bound to nonmethylated CpG sites of the mouse delta-opioid receptor (*mDOR*) promoter, whereas Sp3 repress *mDOR* promoter

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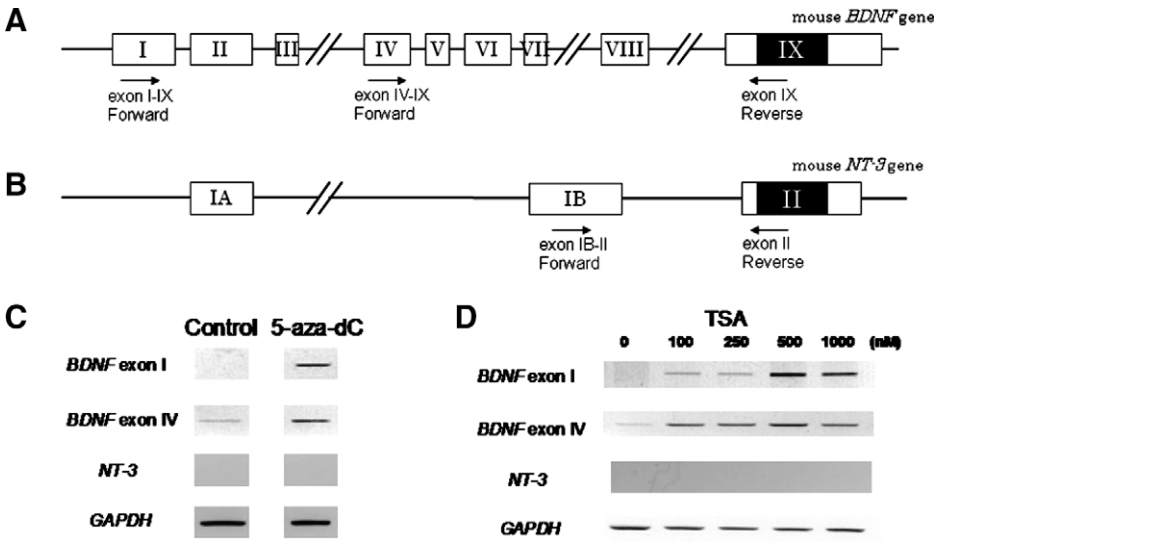

Fig. 1. Expression levels of *BDNF* and *NT-3* genes after 5-aza-dC and TSA treatment. (A) Mouse *BDNF* gene structure as described by Aid et al. [10]. Each of the eight untranslated exons is spliced to give a common 3′-protein coding exon, exon IX. (B) Mouse *NT-3* gene structure as described by Kendall et al. [14]. Exons are shown as boxes and introns are shown as lines. Open boxes represent untranslated exons. The filled box indicates the protein coding region. (C) Neuro-2a cells were treated with 3 μM 5-aza-dC or PBS (control) for 2 days. *BDNF* exon I and *BDNF* exon IV indicate transcripts containing exons I and IV, respectively. (D) Neuro-2a cells were treated with TSA (100, 250, 500 or 1000 nM) or DMSO (control) for 24 h. The expression levels were measured by RT-PCR.

activity as repressor when Sp3 is bound to methylated CpG sites of *mDOR* promoter [22]. Because Sp3 binds to *NT-3* promoter IB containing the CpG sites, the *NT-3* promoter IB activity may be controlled by DNA methylation. Therefore, in this study, we analyzed the DNA methylation status of *BDNF* promoters I, IV and *NT-3* promoter IB in Neuro-2a to clarify epigenetic regulation of *BDNF* and *NT-3* genes.

2. Materials and methods

2.1. Cell culture and drug treatment

Mouse neuroblastoma Neuro-2a cells (American Type Culture Collection) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% fetal bovine serum (FBS, Invitrogen), 50 U/ml penicillin, and 50 μg/ml streptomycin (Invitrogen) at 37 °C in a humidified 5% CO_2 incubator. Cells were treated with trichostatin A (TSA, Merck) (0, 100, 250, 500 and 1000 nM) for 24 h or 3 μM 5-aza-dC (Sigma) for 2 days; 5-aza-dC was replaced with fresh 5-aza-dC at each 24 h.

2.2. RT-PCR

Total cellular RNA was isolated using ISOGEN (Nippongene). The isolated RNA was treated with RNase-free DNase (Invitrogen) to degrade contaminated genomic DNA. One microgram of total cellular RNA was reverse transcribed with oligo(dT) primers (Invitrogen) and M-MLV reverse transcriptase (Invitrogen) in a 20 μl of total reaction volume following the manufacture's instructions. Subsequently, PCR was performed using HotStarTaq Plus Master Mix Kit (QIAGEN) containing 1 μl of cDNA in a 20 μl of the total volume. Thirty-five amplification cycles (45 s at 95 °C; 45 s at 60 °C; 1 min at 72 °C) were conducted for *BDNF* exons I–IX and IV–IX and *NT-3* exon IB–II, and 25 identical cycles for the glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*) gene. PCR primer sequences were: *BDNF* exons I–IX: 5′-GTGTGACCTGAG CAGTGGGCAAAGGA-3′ and 5′-GAAGTGTACAAGTCCGCGTCCTTA-3′, *BDNF* exons IV–IX: 5′-CTC TGCCTAGATCAAATGGAGCTTC-3′ and 5′-GAAGTGTACAAGTCCGCGT CCTTA, *NT-3* exon IB–II: 5′-GCCCGGC GCAACTTTCTTCCC-3′ and 5′-G GGGCGAATTGTAGCGTCTCT-3′, *GAPDH*: 5′-GCCCAGAACATCATCCC TGC-3′ and 5′-GCCTCTCTT GCTCAGTGTCC-3′.

2.3. Bisulfite genomic sequencing

Genomic DNA was isolated using Wizard® Genomic DNA Purification System (Promega). Bisulfite modification of genomic DNA was performed following a published procedure [23]. One microgram of bisulfite-treated genomic DNA was then subjected to PCR experiments. PCR amplification composed of 30 cycles (30 s at 95 °C; 1 min at 54 °C; 1 min at 72 °C) after the initial Taq activation step (10 min at 95 °C). PCR primers used for initial amplification were: 5′-GTTGGAGATTTTAGTTATGGTGG-3′ and 5′-TTACCCACTAC TCAAATCACACC-3′ for mouse *BDNF* promoter I, 5′-GTGAATTTGTTAG GATTGGAAGTGAAAATA-3′ and 5′-CTCTTACTATATATTTCCCCTTCTC TTC-3′ for mouse *BDNF* promoter IV, 5′-GGGA AGGTTAAAAGAGGG-GATAATG-3′ and 5′-ggcatctagaAAAACCTTCAACTCTAAATCCC-3′ for mouse *NT-3* promoter IB (Lower cases of primer sequence indicate adaptor sequences for restriction enzymes.). Nested PCR (for mouse *BDNF* promoters I, IV) and semi-nested PCR (for mouse *NT-3* promoter IB) were performed using 2 μl of the initial reaction mixture with conditions similar to those described above. PCR primers used in the nested and semi-nested PCR were 5′-cactaagcttTTATGGTGGG GGAGGGGTA-3′ and 5′-ggcatctagaCTACTCAAATCACACCTAAAACTC TAA-3′ for mouse *BDNF* promoter I, 5′-cactaagcttGGATTGGAAGTGA AAATATTTATAAAGTATG-3′ and 5′-ggcatctagaCTAAACAAAAAC-TAAAAAATTTCATACTAACTC-3′ for mouse *BDNF* promoter IV, 5′-cactaagcttTTTTT TTTGAAGTGGATTAGGAG-3′ and 5′-ggcatctagaAAAAC CTTCAACTCTAAATCCC-3′ for mouse *NT-3* promoter IB. PCR products were digested with HindIII/XbaI and the fragments were cloned into HindIII/XbaI sites of the plasmid vector pUC19. Thirteen independent plasmid clones were selected and their nucleotide sequences were determined.

2.4. The Chromatin immunoprecipitation assays

The Chromatin immunoprecipitation (ChIP) assays were performed by using ChIP-IT™ express kit (ACTIVE MOTIF). Briefly, Neuro-2a cells were treated with 500 nM TSA or DMSO (Control)