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MiRNA-181d Is Involved in CREB1 Expression in PC12 Cells

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Abstract

MicroRNAs are involved in regulation of the central nervous system (CNS) development, and miR-181d highly expressed in mature neurons. CREB is many signal pathways converged point in hippocampal neurons, and it plays a crucial role in learning and memory. In this study, we detected the negative relationship between CREB1 protein and miR-181d expression in lewis rat hippocampus development. And the bioinformatic analysis showed that, CREB1 mRNA contains complementary sequence to the miR-181d seed region. Then we further demonstrated that miR-181d controls the expression level of CREB1 gene in PC12 cells by luciferase assay and western blot. Taken together, our data demonstrated that CREB1 mRNA is the target gene of miR-181d, and conformed CREB1 protein expression was regulated by miR-181d in PC12 cells.

Keywords

miR-181d, CREB1, PC12 Cells

1. Introduction

The microRNAs (miRNAs) are a group of small noncoding RNAs that are single stranded chains consisting of 19 - 25 nucleotides (~22 nucleotides). They are transcribed by RNA polymerase II or III in the nucleus [1] [2]. During transcription, the primary transcripts (pri-miRNA) are capped, polyadenylated, and then cleaved by the Drosha ribonuclease III enzymes to form ~70 nucleotide stem-loop precursor miRNA [3] [4], which is then transported to the cytoplasm

by exportin 5 [5], and further processed into mature miRNAs by a RNase III enzyme Dicer [6]. More and more studies show miRNAs play an important role in gene regulations and the basic regulation mechanism is that miRNAs imperfectly bind to the 3'-untranslated region (3'-UTR) of the target mRNAs leading to either translational repression or target mRNA cleavage [7] [8].

CREB, the cAMP response element binding protein, is a 43-kDa nuclear transcription factor belonging to the CREB/ATF family that regulates the transcription of the downstream genes [9]. It is one of the common nuclear targets of the extracellular signal-regulated kinase (ERK) pathway as well as the PI 3-kinase signaling pathway [10]. There is a large amount of evidence to suggest that CREB plays a significant role in memory formation, stabilization and retention [11] [12]. CREB controls the transcriptional responses of neurons to many extracellular stimuli and also participates in synaptic plasticity and memory consolidation [13] [14]. Hippocampal CREB gene transfer increases CREB expression and significantly improves the learning and memory function in aging rats [15]. During the aging process CREB activity is decreased resulting in reduced learning and memory function; on the other hand, memory function can be improved by enhancing CREB protein levels [15]. PC12 cell line is a model of neuronal tissue derived from a transplantable rat adrenal phaeochromocytoma. It responds reversibly to nerve growth factor by induction of neuronal phenotype. Pugazhenthi's study shows stimuli mediated phosphorylation and transcriptional activation of CREB in PC12 cells [16].

MiR-181 family, including miR-181d, miR-181a, miR-181b and miR-181c, is one of the identified miRNAs that widely exists in vertebrate cells. MiR-181d was found highly expressed in adult mouse brain tissues [17] [18]. And miR-181d, showing different expression profiles at different stages of human neurodevelopment, was observed highly expressed in mature human neurons [19]. These studies strongly suggest that miR-181d is closely related to brain cell development and differentiation. In addition, the expression of CREB delays the arrival of cognitive and memory dysfunction with aging. In this study, we investigated is it possible that miRNA-181d acted as a negative regulator for CREB1 expression and participated in regulation of CREB1 expression.

2. Materials and Methods

2.1. Animals

Newborn Lewis wild type rats (postnatal day, PD < 2 days; n = 9) and adult Lewis wild type rats (9 - 11 months old; n = 9) were used in the study. The rats were maintained and bred under standard conditions. The investigation conforms to the guide for the care and use of laboratory animals published by the U.S. National Institutes of Health (NIH Publication No. 85-23, revised 1996). Each rat was treated briefly with CO_2 before decapitation. The brain was quickly removed and placed on a chilled glass plate on ice. Fresh hippocampi were then dissected from the brain according to the procedures described by Glowinski *et al.* [20].

Hippocampal tissue samples were stored at −70˚C until the day of assay.

2.2. Cell Culture

Rat pheochromocytoma (PC12) cells were maintained in Dulbecco's Modified Eagle's Medium (DMEM) (HG/DMEM, Invitrogen, Ontario, Canada) containing 10% fetal bovine serum (FBS) (HyClone, Logan, UT), 5% heat inactivated horse serum (HyClone, Logan, UT), 100 mg/ml streptomycin, and 100 micro-units/ml penicillin at 37˚C. HEK 293 cells were cultured in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin at 37˚C.

2.3. Bioinformatics Analysis

The mature sequence of miR-181d (5'-AACAUUCAUUGUUGUCGGUGGGU-3') was retrieved from the miRBase Sequence Database (http://www.mirbase.org/), and mRNA 3'UTRs of CREB1 from human and rat were aligned with miR-181d sequence using the ClustalW program (http://www.ebi.ac.uk/Tools/msa/clustalw2/). The miR-181d gene targets were predicted from the MicroCosm Targets Version 5 (http://www.ebi.ac.uk/enright-srv/microcosm/cgi-bin/targets/v5/search.pl), and Target Scan version 5.1 (www.targetscan.org) was used to scan for seed matches between the miR-181d seed region and the predicted gene.

2.4. Vector Construction and Transfection

Total RNA was extracted using Trizol reagent (Invitrogen). cDNA was synthesised using the PrimeScript RT Reagent Kit (TaKaRa, Tokyo, Japan), and a 622 bp fragment of the rat CREB1 mRNA 3'UTR (corresponding to nt 4303 - 4924 of the Entrez PubMed transcript FQ211925) was PCR amplified from CREB1 full length cDNA. The PCR products were cloned into pGL3-control luciferase reporter vector (Promega) via an XbaI (TaKaRa, Tokyo, Japan) restriction site, immediately downstream of the luciferase gene, named pGL3C-CREB1 3'UTR and pGL3C-CREB1 3'UTR mutant. The primers selected areas the following:

CREB1 3'UTR-wt-up: 5'-GCTCTAGAGCCGTAGAAAGAAGAAAGAAT-3';
CREB1 3'UTR-wt-dn: 5'-TGCTCTAGATGGCAATCAACACTTCTTCAT-3';
CREB1 3'UTR-mu-up: 5'-TGCGGACGTCCACAACCGCTTCCACTT-3';
CREB1 3'UTR-mu-dn: 5'-GGACGTCCGCAGTTTAACGCGAAAGCAG-3'.

The miR-181d mimics, negative control, and miR-181d inhibitor were purchased from RiboBio (RiboBio Co. Ltd., Guangdong, China). Transfection of microRNA or microRNA inhibitor was performed using the X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's instruction.

2.5. Western Blot Analysis

PC12 cells (1.5×10^5) were seeded in 6-well plates and allowed to settle for 24 h. miR-181d mimics (50 nM, 100 nM) or miR-181d inhibitor (100 nM, 200 nM) were transfected into cells using the X-tremeGENE HP DNA Transfection Reagent (Roche). Cells were collected 24 h after transfection. Hippocampal and cells were homogenized in RIPA buffer (25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1%

NP-40, 1% Sodium deoxycholate and 0.1% SDS) containing complete EDTA-free protease inhibitor (Roche). Total proteins were determined by colorimetric method using BSA protein assay reagent (Pierce, Rockford, IL, USA). 60 μg proteins were electrophoresed through 12% polyacrylamide gel, transferred onto polyvinylidene fluoride membrane at 200 mA for 120 min. After blocked with 5% non-fat milk in TBS/0.1% Tween-20 (TBST), the membranes were incubated overnight at 4˚C with primary antibodies: rabbit monoclonal antibody (mAb) anti-CREB1 (#9197, Cell Signaling Technology Inc., Danvers, MA), followed by the corresponding horseradish peroxidase-conjugated secondary antibody for 1 hr at room temperature.

Bands were detected using Super Signal Femto chemiluminescent reagent (Pierce) and quantified using the Chemi-doc gel quantification system (Bio-Rad). All data were normalized to mouse β-actin.

2.6. RNA Isolation and Quantitative RT-PCR Detection

Total RNA was isolated using TRIzol reagent (Invitrogen) according to the manufacturer's instructions, and converted into cDNA using the PrimeScript RT reagent kit (TaKaRa, Tokyo, Japan). Detection of the mature form of miR-181d was performed using Quantitect SYBR Green PCR Kit (TaKaRa, Tokyo, Japan) and quantitative RT-PCR Primer Sets (Ribobio, Guangzhou, China) with the U6 small nuclear RNA as an internal control.

2.7. Luciferase Assay

HEK 293 cells (1×10^4) were seeded in triplicates in 96-well plates and allowed to settle for 24 h. 100 ng of luciferase reporter plasmids of pGL3C-CREB1 3'UTR (wt/mu), or the control-luciferase plasmid, or miR-181d mimics (100 ng), or miR-181d inhibitor (200 ng), plus 1 ng of pRL-TK renilla plasmid (Promega, Madison, WI), were transfected into cells using the X-tremeGENE HP DNA Transfection Reagent (Roche) according to the manufacturer's recommendation. Luciferase and renilla signals were measured 48 h after transfection using the Dual Luciferase Reporter Assay Kit (Promega, Madison, WI) according to the protocol provided by the manufacturer. Three independent experiments were performed and the data are presented as means ± SD.

2.8. Data Analysis

All data were analyzed by Prism (PrismGraphPad Software). Data are presented as mean ± SEM. Differences between groups were calculated using the Student's t-test. P Values less than 0.05 were defined as statistically significant.

3. Results

3.1. miR-181d Expression *in Vivo*

To examine possible expression of miRNA-181d *in vivo*, we analyzed the miRNA expression profile by miRNA microarray in hippocampus and found that

miR-181d expression was much lower in adult Lewis wild type rat then in the newborn (data not shown). With quantitative RT-PCR we confirmed the presence of miR-181d (Figure 1). These data suggest that the expression of miR-181d was decreased during growth and development.

3.2. CREB1 Protein Expression *in Vivo*

CREB1 expression was evaluated by western blot. As seen in Figure 2, Western blot analysis of materials extracted from hippocampus revealed that CREB1 protein levels were significantly higher in adult Lewis wild type rats compared with the newborns (5.43 ± 0.11 vs 1.00 ± 0.19, P < 0.001).

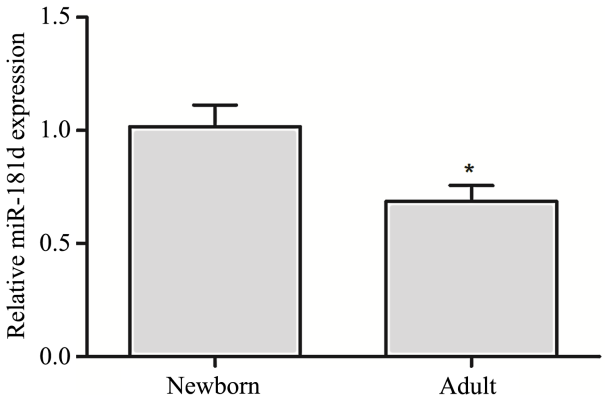

Figure 1. Hippocampal miR-181d expression levels with Q-PCR. miR-181d expression was detected with quantitative RT-PCR in hippocampus. U6 small nuclear RNA was regarded as an endogenous normalize, and the relative miR-181d expression level is shown. Values are mean ± SEM run in triplicates, *, P < 0.05. (Newborn: PD < 2 days; Adult: 9 - 11 months old)

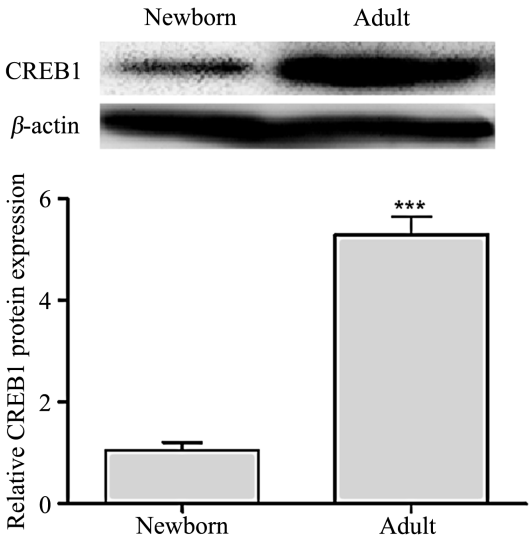

Figure 2. Hippocampal CREB1 protein levels. CREB1 protein expression was analyzed by western blot. β-actin signal was used to normalize the data. Values are mean ± SEM run in triplicates, ***, P < 0.001. A representative blot is shown. (Newborn: PD < 2 days; Adult: 9 - 11 months old)

3.3. Identification of miR-181d Target Gene in PC12 Cells

Since CREB1 plays a key role in learning and memory and CREB controls the transcriptional responses of neurons to various extracellular stimulations and miR-181d is highly expressed in mature neurons and rich in the adult brain tissue (26 - 27), there could be a close regulation relationships between miR-181d and CREB1. First we used Target Scan to screen the candidate miRNAs and observed the presence of about 90 miRNAs, including miR-181d. Then we retrieved the predicted 783 target genes of miR-181d from the miRBase target database and confirmed CREB1 is one of them. By manually aligning the mRNA 3'UTR and the miR-181d seed region, we found that they are highly matched, as shown in Figure 3.

We investigated whether miR-181d could affect the identified gene target through interaction with the mRNA 3'UTR. In HEK 293 cells, co-transfection with miR-181d mimics could repress the luciferase activity generated by luciferase vectors containing the mRNA 3'UTR of CREB1 of rat (Figure 4), evidencing direct binding between the miRNA sequence and the CREB1 gene. In comparison, the negative control miRNA, or scramble miRNA, did not cause any significant reduction of luciferase activity (Figure 4). Similarly, miR-181d mimic co-transfection with pGL3C-CREB1 3'UTR mutant did not reduce the luciferase activity obviously (Figure 4).

3.4. CREB1 Protein Was Inhibited with miR-181d Mimics

To evaluate the repression of CREB1 by miR-181d, we used transfection to

Position 4356-4363 of CREB1 3' UTR mo-miR-181d	5' ... ACGCUUACUGAUUAGUGAAUGUA ... 3' UGGGUGGCUGUUGUUACUUACAA	8mer	-0.247	0.003	0.014	0.265	0.033	0.074	>-0.03	1	1.664	0.52

Figure 3. Sequence alignment between miR-181d seed region and the seed matches on CREB1 mRNA 3'UTR region. The analyses were performed using the miRBase target database. The line indicates conserved seed match (A-T, C-G) in rat.

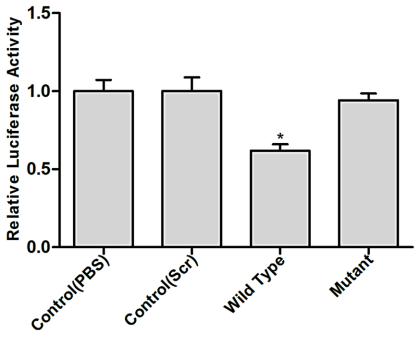

Figure 4. miR-181d directly binds and represses CREB1 mRNA through 3'UTR. pGL3-control luciferase vector containing the mRNA 3'UTR of CREB1 gene of rat origin and the mutant were co-transfected with the indicated amount of miR-181d mimic in HEK293 cells, and luciferase activity was analyzed 48 h post-transfection. The structurally unrelated scramble miRNA served as negtive control. Values are mean ± SEM run in triplicates, *, $P < 0.05$. (Wild Type: pGL3C-CREB1 3'UTR; Mutant: pGL3C-CREB1 3'UTR mutant)

over-express miR-181d (50 nM, 100 nM) in PC12 cells. As expected, CREB1 expression was repressed in miRNA transfected cultures (0.73 ± 0.11 vs 1.00 ± 0.07, $P < 0.05$; 0.24 ± 0.03 vs 1.00 ± 0.07, $P < 0.05$) (Figure 5). The densitometry values from the acquired images provide quantitative indices for this repression effect, plotted as histograms of miRNA expression in relationship to the repressed level of CREB1 in transfected cells.

3.5. CREB1 Protein Expression Was Upregulated with miR-181d Inhibitor

As shown in Figure 6, transfection with miR-181d inhibitor (100 nM, 200 nM) in PC12 cells resulted in miR-181d knock down and marked up-regulation of CREB1 product (1.67 ± 0.09 vs 1.00 ± 0.21, $P < 0.05$; 1.46 ± 0.14 vs 1.00 ± 0.21, $P < 0.05$), presumably because of a decrease in miR-181d mediated CREB1 mRNA inhibition or degradation.

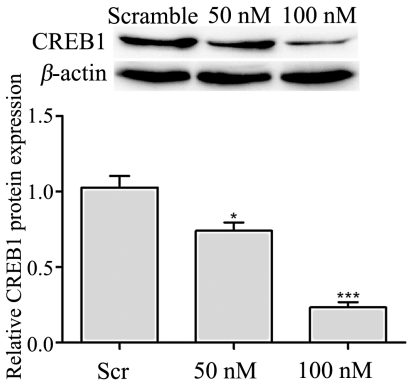

Figure 5. CREB1 protein expression was inhibited by miR-181d mimics. CREB1 protein expression were examined in PC12 cells 24 h after transfection with miR-181d mimics (50 nM, 100 nM). CREB1 protein level was was analyzed by western blot. β-actin signal was used to normalize the data. Values are mean ± SEM run in triplicates, *, $P < 0.05$. ***, $P < 0.001$. A representative blot is shown.

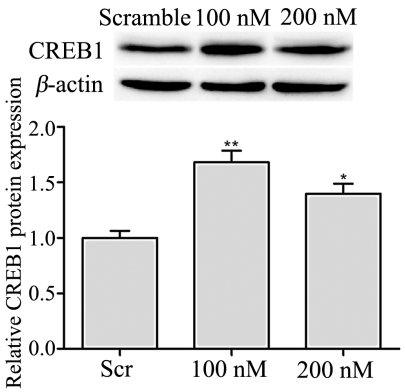

Figure 6. CREB1 protein expression was upregulated with miR-181d inhibitor. CREB1 protein expression were examined in PC12 cells 24 h after transfection with miR-181d inhibitor (100 nM, 200 nM). CREB1 protein level was analyzed by western blot. β-actin signal was used to normalize the data. Values are mean ± SEM run in triplicates, *, $P < 0.05$. A representative blot is shown.

4. Discussion

MiRNA is prevalent in multi-cellular organisms, and has considerable numbers, for about 2% of the entire genome [21] [22]. Bioinformatics analysis prompted near the 30 percent of human genes was controlled by the miRNAs [23]. MiRNA is involved in regulation of the central nervous system (CNS) development, making the central nervous system development and differentiation in the correct timing and spatial order, at the same time, miRNA is also involved in the maintenance of variety different nerve cells in the normal morphology. MiRNA expression has conservative, short-term and tissue-specific features in CNS [24]. miR-181d was highly expressed in mature neurons and adult mouse brains [17] [19]. CREB is many signal pathways converged point in hippocampal neurons, and it plays a crucial role in learning and memory [13] [14] [25]. MiRNA-181d expression in PC12 neuronal cell lines has not been studied and its role in regulation of CREB1 remains to be determined. In this study, we successfully confirmed CREB1 mRNA 3'UTR is direct target of miR-181d, implicating a role for miR-181d in regulation of the transcription factor CREB1 expression in PC12 cells.

In this report, we first examined CREB1 and miR-181d expression *in vivo* (**Figure 1** and **Figure 2**). Western blot analysis revealed that CREB1 protein levels were significantly higher in hippocampus of adult lewis wild type rats compared with the newborns (**Figure 2**). While miRNA microarray and quantitative RT-PCR results (**Figure 1**) showed that miR-181d expression was obviously lower in hippocampus of adult lewis wild type rats compared with the newborns. These results indicate that miR-181d expression was negatively correlated with CREB1 expression level during the hippocampal growth and development. These results suggest the possibility that the higher miR-181d expression inhibit CREB1 protein expression in hippocampus of newborn lewis wild type rats.

In order to confirm this proposition, we simply advance to validate the possible relationship between miR-181d and CREB1 in vitro. Then, we examined whether miR-181d can bind and affect the predicted target mRNA through CREB1 mRNA 3'UTR interactions. Accordingly the mRNA 3'UTR of CREB1 was cloned into a reporter vector, downstream of a firefly luciferase cDNA. As seen in **Figure 4**, in HEK 293 cells, the vector pGL3C-CREB1 3'UTR containing the 3'UTR of CREB1 mRNA displayed clearly light emission reduction upon co-transfection with miR-181d mimics. On the other hand, in the groups of scramble miRNA and miR-181d mimics co-transfection with pGL3C-CREB1 3'UTR mutant, the luciferase activity didn't reduce obviously, indicating the presence of a direct binding site for miR-181d.

When transfected with miR-181d inhibitor and miR-181d mimics in PC12 cells, western blot analysis of CREB1 protein levels demonstrated that CREB1 was induced upon miR-181d repression and inhibited with over-express miR-181d in PC12 cells (**Figure 5** and **Figure 6**), further supporting an effect of miR-181d regulation on CREB1 gene.

In summary, this study demonstrated that miR-181d can inhibit expression of CREB1 by specifically targeting its mRNA, and confirmed the repressed expression of miR-181d may be involved in regulation of CREB1 expression in PC12 cells, which could be a suitable candidate for neurodegenerative diseases.

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Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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