Neuroscience Letters 553 (2013) 153–158

Contents lists available at ScienceDirect

Neuroscience Letters

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

MicroRNA profiling and the role of microRNA-132 in neurodegeneration using a rat model

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HIGHLIGHTS

- miRs expression profiling shown 20 miRs deregulated in affected rats.
- miR-132 level was increased in affected rats.
- Nurr1 protein level was decreased in mesencephalic neurons of affected rats.
- BDNF serum and mesencephalic brain tissue levels were decreased in affected rats.

ARTICLE INFO

Article history:
Received 20 May 2013
Received in revised form 19 July 2013
Accepted 2 August 2013

Keywords:
MicroRNA
MicroRNA-132
Nurr1
BDNF
Neurodegeneration
Rat model

ABSTRACT

MicroRNAs (miRs) are endogenous small RNAs that regulate gene expression at the post-transcriptional level by mediating mRNA degradation or transcriptional inhibition. MiRs were implicated in the pathogenesis of numerous neurodegenerative diseases, including Parkinson's disease (PD). In this study we analyzed the possible role of miRs in the neurodegenerative process in a spontaneous autosomal recessive rat model for neurodegeneration developed in our laboratory. To investigate the role of miRs in the etiology of PD, we conducted miR expression profiling using microarrays. We found 20 miRs that are deregulated in affected rats and many of these are implicated in neurodegenerative disease, including PD. In this study we were particularly interested in the expression of miR-132, a miR that has been reported to be highly expressed in neurons, and to have a potential role in neurodegenerative diseases. We found a significant increase in miR-132 in affected rats by microarray and the result was confirmed by qPCR. Next we analyzed one of the known downstream targets of miR-132, nuclear receptor related 1 protein (Nurr1), which is essential in neurogenesis of midbrain dopaminergic neurons. Western blot analysis and immunohistochemistry revealed a significant decrease in Nurr1 protein expression in the mesencephalic neurons. Finally, we found a significant decrease in both serum and mesencephalon brain tissue of brain-derived neurotrophic factor (BDNF), which is known to be a direct target of Nurr1. Taken together, our findings suggest that miR-132 can regulate Nurr1 levels and might influence the development and function of midbrain dopaminergic neurons.

1. Introduction

Parkinson's disease (PD) is an age-related and the second most common neurodegenerative disorder after Alzheimer disease, affecting 1–2% of the population over 60 years old with no cure [8]. This disease is characterized by motor symptoms such as resting tremor, slowness of initial movement, rigidity and general postural instability. The neurodegenerative process in PD affects the midbrain dopamine projections from the substantia nigra with disruption of dopamine transmission in the motor loop of the basal ganglia [33,37].

A spontaneous autosomal recessive rat animal model for neurodegeneration was developed in our laboratory [33]. These rats demonstrated progressive increases in α-synuclein (α-syn) in the brain mesencephalon, followed by loss of the dopamine terminal in the basal ganglia and motor impairments. Decreased dopamine and overexpression of α-syn in the brain mesencephalon provide a naturally occurring animal model of PD and other synucleinopathies.

MicroRNAs (miRs) are non-coding RNAs identified as post-transcriptional regulators of gene expression with relevant roles in physiological and pathological aspects of the central nervous system [4,13]. Several studies have described a potential role of miRs in the development of PD [14,15,19]. Recent studies have shown that miR-132 overexpression in embryonic stem cells reduces the

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http://dx.doi.org/10.1016/j.neulet.2013.08.001

differentiation of dopaminergic neurons and directly down-regulates nuclear receptor related protein 1(Nurr1) expression and identified Nurr1 as a transcription factor essential for the survival and/or maintenance of mesencephalon dopaminergic neurons [39].

Nurr1 is a member of the nuclear receptor superfamily and is highly expressed in the developing and in adult ventral midbrain [6]. Nurr 1 is required for the acquisition and maintenance of the dopaminergic phenotype in nigrostriatal neurons [28]. A decline in Nurr1 expression correlates with loss of tyrosine hydroxylase [5,29]. Recently, Nurr1 mutant mice were recognized as a potential model for PD [5,18]. It was demonstrated that a decrease in Nurr1 gene expression is followed by a decrease in brain-derived neurotrophic factor (BDNF) mRNA and protein levels and identified *Bdnf* as a putative Nurr-1-regulated gene [35].

BDNF has emerged as a major regulator of neuronal survival. BDNF is a potent dopaminergic neurotrophin and its decrease is reported in the substantia nigra of PD patients, especially in the pars compacta [17,24,40] and in serum of PD patients [30].

Here we analyzed the possible role of miRs in the neurodegenerative process in a spontaneous autosomal recessive rat model for neurodegeneration developed in our laboratory. We particularly focused on deregulation of miR-132 and the proteins Nurr1, a known downstream target of miR-132, and BDNF. Our findings suggest that miRs can regulate important targets that can influence the function of midbrain dopaminergic neurons and can be potential therapeutic targets for the neurodegenerative diseases.

2. Materials and methods

2.1. Animals

Brains from Berlin-Druckrey IV (BD-IV) affected and from age-matched control (BD-IV littermate) rats were used. The background of this rat colony was previously described [33]. All animal procedures were performed according to the protocol approved by the Texas A&M University Institutional Animal Care and Use Committee.

2.2. Tissue processing and collection

For western blot (WB), enzyme-linked immunosorbent assay (ELISA), miR microarray and quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR) analysis rats were sacrificed by decapitation and mesencephalon brain areas, were immediately removed after death and placed on dry ice. Brains from BD-IV affected rats ($n = 3$) and from control BD-IV rats ($n = 3$) at 25 days post natal (dpn) were used for WB and were used at 10, 22 and 24 dpn for ELISA. For immunohistochemistry (IHC), BD-IV affected ($n = 3$) and control ($n = 3$) rats at 25 dpn under anesthesia (ketamine hydrochloride, 1.5 mL/kg, 75 mg/kg [Phoenix Scientific, Inc., St. Joseph, MO, USA] and Xylazine, 0.5 mL/kg, 10 mg/kg [Bayer AG, Pittsburgh, PA, USA]) were perfused via the left ventricle with 2% paraformaldehyde (PFA) solution (Electron Microscopy Science, Hatsfield, PA, USA) in phosphate buffered saline (PBS, pH 7.2 at 4 °C) using a peristaltic pump. For miR microarray and qPCR analyses total RNA was extracted using an SV RNA extraction kit (Promega, Madison, WI, USA). RNA was quantified by absorbance at 260 nm.

2.3. Immunohistochemistry

IHC was carried out as previously described [22]. Anti-Nurr1 antibody was purchased from Abcam (Cambridge, MA, USA). Following the primary antibody reaction, sections were washed and incubated with anti-mouse (1:200 dilution) IgG (Vector Laboratories, Burlingame, CA, USA). Sections that were not incubated with primary antibody served as negative controls.

2.4. Western blot

WB was carried out as previously described [22]. Nuclear extracts were prepared from the mesencephalon brain area from affected and control rats as previously described [21]. The membranes were incubated with anti-Nurr1 (Abcam) and β-actin antibody from Sigma–Aldrich Chemical Co. (St. Louis, MO, USA). Peroxidase-labeled anti-mouse secondary antibodies (1:5000) were purchased from Kirkegaard and Perry Laboratories (Gaithersburg, MD, USA).

2.5. ELISA

The levels of BDNF were measured in serum samples and mesencephalon brain tissues from affected ($n = 3$) and control ($n = 3$) rats. Serum and tissue levels of BDNF were measured by BDNF sandwich ELISA, using a commercial kit according to the manufacturer instructions (Millipore).

2.6. MicroRNA microarray analysis

MiR microarray analysis was performed on BD-IV affected rats ($n = 3$) and control ($n = 3$) littermates at 25 dpn using miRNA chips containing 700 mature rat miRs (Chip ID miRRat 19.0 version, LC Sciences, Houston, TX, USA), as described in the literature [10,12,13]. Data were analyzed by first subtracting the background and then normalizing the signals using a LOWESS filter (Locally-weighted Regression) [2]. For two color experiments, the ratio of the two sets of detected signals (log 2 transformed, balanced) and p-values of the t-test were calculated.

2.7. Quantitative reverse transcriptase-polymerase chain reaction (qRT-PCR)

qRT-PCR was performed to verify the miR-132 levels in BV-IV affected ($n = 3$) and control ($n = 3$) rats at 25 dpn using TaqMan microRNA assays (Applied Biosystems) by LC Sciences (LC Sciences, Houston, TX). All samples were analyzed in duplicate.

2.8. Statistical analysis

For microarray data the normalization was carried out using a cyclic LOWESS (Locally-weighted Regression) method [26]. A t-test was performed between "control" and "affected" sample groups. miRs with p-values below a critical p-value (typically 0.01) were selected for cluster analysis. The clustering was done using hierarchical method and was performed with average linkage and Euclidean distance metric [9]. Densitometry analysis of WB bands was performed on a Macintosh computer using the public domain NIH Image program (developed at the US National Institutes of Health and available on the Internet at http://rsb.info.nih.gov/nih-image). All data are presented as mean ± SD and P-values were calculated using Student's unpaired t-test. Statistical significance was taken at $P < 0.05$.

3. Results

3.1. miRNA expression profiling and their role in neurodegeneration

To investigate the role of miRs in neurodegeneration in our animal model, we conducted miR expression profiling in the brain of

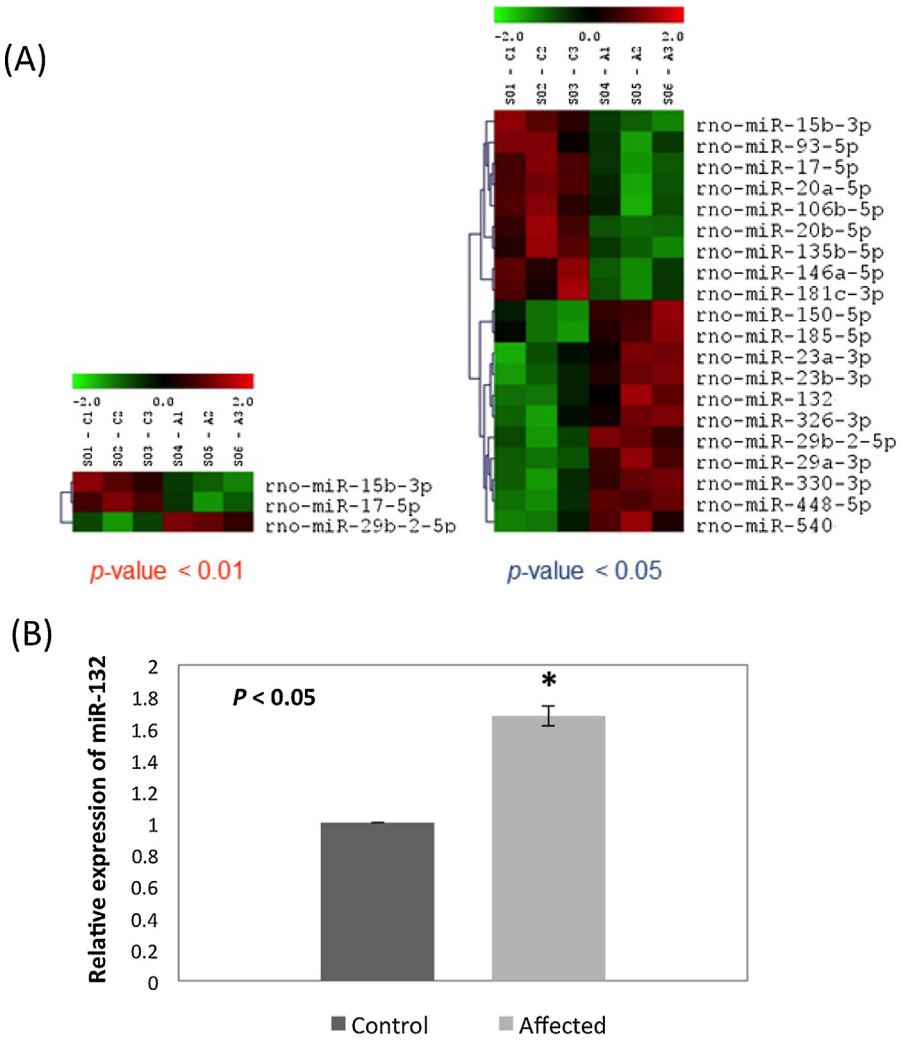

Fig. 1. (A) Heatmap of miR expression profile in the brain of BD-IV affected ($n = 3$) and control ($n = 3$) rats at 25 dpn. The green color denotes down-regulated expression and the red color denotes up-regulated expression level of miRs. (B) miR-132 expression levels. MiR-132 levels were determined in affected ($n = 3$) and control ($n = 3$) BD-IV rats by qPCR. Data are presented as the means ± standard deviation from three independent experiments ($^*P < 0.05$).

affected and control BD-IV rats using microarray analysis. We analyzed 720 different miRs among which three miRs (miR 15b-3p, miR-17-5p and miR 29b-5p) had a p-value < 0.01 and 17 miRs had a p-value < 0.05 (Fig. 1A).

Several miRs were found to be deregulated in our animal model. We found significant down-regulation of miR-17-5p, miR-20a, miR-93, miR-106 in the affected rats compared with controls (Table 1). Studies showed that inhibition of miR-17-5p, miR-20a, miR-93, miR-106, which share extensive sequence similarity, leads to G1 checkpoint due to the accumulation of DNA double strand breaks, resulting from premature temporal accumulation of the E2F transcription factor [6,27,34]. These miRs were found to regulate adult neural stem/progenitor cell proliferation and neuronal differentiation [3,11]. Two other important miRs found to be up-regulated in affected rats were miR-29a and miR-29b. Up-regulation of miR-29b was found to promote neuronal cell death by inhibiting Bcl-2-like protein 2 (Bcl2L2) and induced myeloid leukemia cell differentiation protein (Mcl-1), an anti-apoptotic Bcl-2 family member [25,31,38] and are also implicated in apoptosis and involved in mitochondrial deregulation [1]. These may be of interest in our model and need to be further explored, as we have found a loss of mitochondrial membrane potential, decrease in Complex 1 enzymatic activity and oxidative stress in affected rats compared with controls (unpublished data).

Table 1
miRs involved in neurodegeneration.

miRAN	Functions of miRs	References
miR-17-5p, miR-20a, miR-93 miR-106	Key regulator of G1/S phase cell cycle arrest	Cloonan et al. [6]
	Involved in regulation and differentiation of embryonic stem cells development	Pickering et al. [27] and Brett et al. [3]
	Regulates adult neural stem/progenitor cell proliferation and neuronal differentiation	Foshay and Gallicano (2009)
miR-29b	Regulation of apoptosis	Mott et al. [25]
	Loss of mitochondrial membrane potential	Shi et al. (2012)
	Role in dementia by targeting progranulin	Jiao et al. (2010)
miR-29a	Target mitochondrial voltage dependent anion channel	Bargaje et al. [1]
miR-132	Involved in Parkinson, Alzheimer and Schizophrenia	Miller et al. [23], Wanet et al. [36]
	Regulates the differentiation of dopamine neurons	Yang et al. [39]

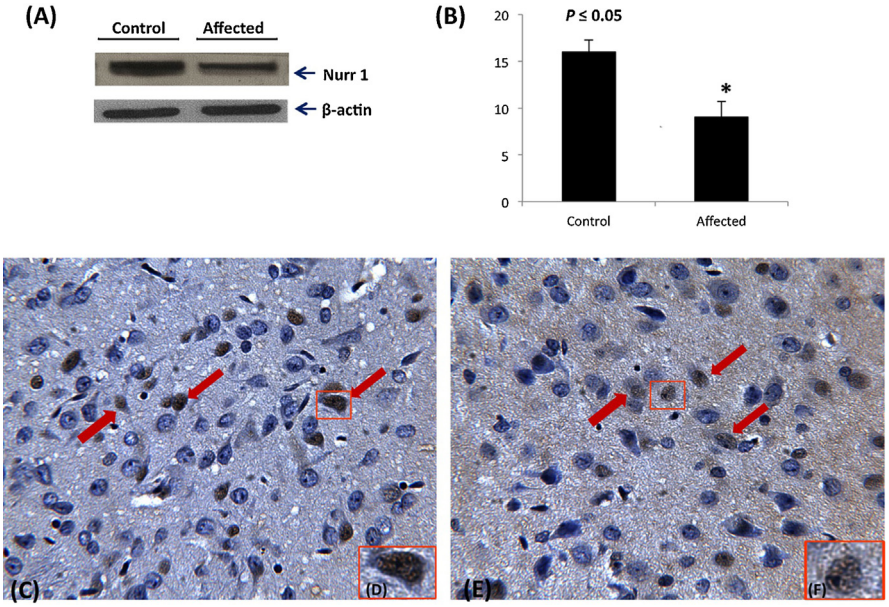

Fig. 2. Brain Nurr-1 protein levels. (A) Western blot analysis showing decreased Nurr1 protein levels in the brain of affected rats. (B) Densitometric analysis of Nurr1 protein bands showing significant decrease in Nurr1 protein expression in the affected compared to control rats. Results represent the mean ± standard deviation for three independent experiments. Asterisks indicate statistically significant differences ($P \leq 0.05$). β-Actin served as a loading control. (C–F) Immunohistochemistry showing decreased Nurr1 protein expression in the nucleus of mesencephalic neurons of affected BD-IV rats (E, F) (red arrows) at 25 dpn when compared with age-matched control littermates (C, D) (red arrows). No staining was found in the negative control sections.

Finally, miR-132 was found to be up-regulated in our animal model. miR-132 was found to be necessary for the proper development, maturation and function of neurons and whose deregulation was associated with several neurological disorders including PD, Alzheimer's and Schizophrenia [23,36]. MiR-132 was recently designated as a "NeurimmiR", a class of miRNAs regulating both neuronal and immune function [32]. miR-132 is an important molecule regulating embryonic stem cell differentiation into dopaminergic neurons by directly targeting Nurr1 gene expression [39].

3.2. qPCR analysis of miR-132

To confirm the up-regulation of miR-132 microarray result we performed qPCR analysis for miR-132. The results confirm the up-regulation of miR-132 in the brain of BD-IV affected rats at 25 dpn (Fig. 1B).

3.3. Nurr1 is down-regulated in affected rats

Considering that Nurr1 was recently found to be down-regulated by miR-132 [39], and miR-132 is found to be up-regulated in our affected rats, we analyzed the levels of Nurr1 protein in the nuclear extracts of mesencephalic neurons of affected and control rats at 25 dpn by WB and IHC. WB showed a significant decrease of Nurr1 protein expression in the affected rats when compared with control littermates (Fig. 2A and B). IHC analysis showed a significant loss of Nurr1 staining in the nuclei of affected mesencephalic neurons in affected rats (Fig. 2E and F) when compared with control animals (Fig. 2C and D).

3.4. BDNF serum and brain tissue levels are down-regulated in affected rats

RNA interference experiments showed that decreased *Nurr1* expression is followed by tyrosine hydroxylase and BDNF mRNA and protein down-regulation [35]. Here we evaluated the expression of BDNF in both serum and mesencephalon brain tissue samples. We found a significant down-regulation in BDNF both in serum samples and the mesencephalon of affected rats at 24 dpn (Fig. 3).

4. Discussion

Our data identified several miRs that are deregulated in our animal model for neurodegeneration. These miRs were also found to be either up- or down-regulated in human neurodegenerative diseases such as PD, Alzheimer's and Schizophrenia [23,36]. One miR that was down-regulated in the affected rats was miR-17-5p, which was found to be the key regulator of the G1/S phase cell cycle transition [6] and along with its family members, miR-20a, miR-93 and miR-106, which are all found to be down-regulated in our affected rats, function to control the differentiation of stem cells [11]. It was also demonstrated a 2-fold increase in the E2F transcription factor upon inhibition of miR-17-5p and its family members [16,27]. These findings suggest that cell-cycle regulators may be effective targets for therapeutic strategies aimed at slowing or halting neurodegeneration in PD.

Next, we analyzed the expression of miR-132 and its downstream target. Our miR microarray results and qPCR analysis showed a significant up-regulation of miR-132 in affected rats. Previous studies showed that miR-132 regulates the differentiation of dopaminergic neurons by directly targeting Nurr1 expression [39]. Nurr1 is one of the most important transcription factors in determining dopaminergic neuron development and differentiation [39]. Our results showed a significant decrease in Nurr1 protein levels in the brain mesencephalon of affected rats. Previous studies also showed that Nurr1 is essential for induction of the mesencephalic dopamine phenotype and for the survival and/or maintenance of these neurons [20]. The decline in Nurr 1 expression was correlated with the loss of tyrosine hydroxylase [5]. We previously reported a significant loss of tyrosine hydroxylase in affected rats by western blot [33]. Moreover, it was found that decreased Nurr1 protein expression was related to α-syn aggregation in the substantia nigra in PD [5]. A recent study also described Nurr1

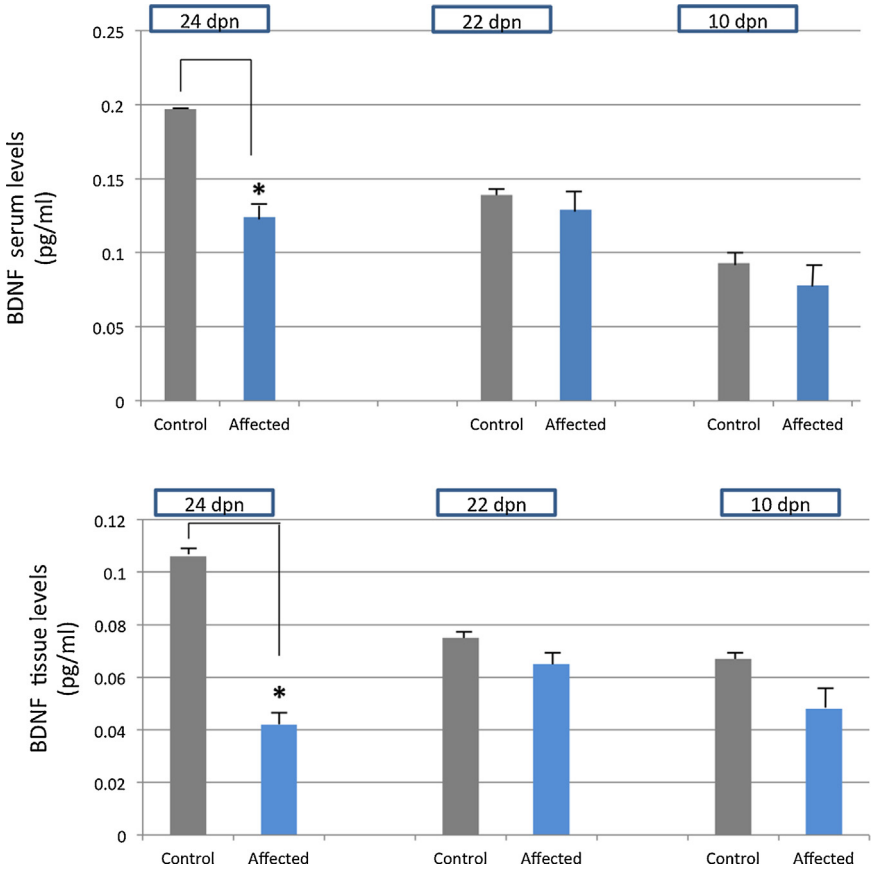

Fig. 3. BDNF serum and brain tissue levels. ELISA shows a significant decrease in the BDNF levels in both serum and mesencephalon samples of 24 dpn BD IV affected rats when compared with age-matched control rats. Results represent the mean ± standard deviation for three independent experiments. All samples were run in duplicate. Asterisks indicate statistically significant differences (*$P < 0.05$).

as a key player in the cellular defense against α-syn toxicity and highlighted Nurr1 as a promising new target for neuroprotective therapy [7]. The studies regarding Nurr1 and α-syn are particularly relevant for our study as we have previously reported a significant increase in α-syn protein and mRNA expression in the mesencephalon of affected rats and also an increased oligomerization of α-syn [33]. Considering the importance of Nurr1 in neuroprotection, the development of drugs designed to activate Nurr1 function may hold particular promise.

Finally, our results showed a significant decrease in BDNF levels both in serum and in mesencephalon brain areas of affected BD-IV rats at 25 dpn but not at earlier time points. BDNF is a neurotrophin known to exert trophic effects on many neuronal types including midbrain dopaminergic neurons [35]. The decrease in BDNF levels in our affected rats can be a consequence of both α-syn overexpression and decreased Nurr1 transcription factor. It was demonstrated by Yuan et al. that overexpression of α-syn downregulates BDNF expression [40]. Therefore, it is highly likely that BDNF down-regulation might play an important role in the pathogenesis of PD.

In conclusion, we found up-regulation of miR-132 and a significant decrease of Nurr1 and BDNF levels in the mesencephalon of affected rats. We will focus our future studies on increasing Nurr1 levels in affected rats by decreasing miR-132 expression using RNA interferance in order to study the role of Nurr1 in our rat model. It is of particular interest in our model to determine whether the overexpression of miR-132 is associated with alpha-synclein accumulation and aggregation and whether the increase in miR-132 expression is the cause or the effect of the accumulation and aggregation of alpha-synuclein. It is possible that dopaminergic neurons, which respond to global stress due to accumulation of alpha-synuclein, activate miR-132, thereby shutting down Nurr1 and decreases BDNF, a major regulator of neuronal survival.

These data highlight miR-132 as promising potential biomarker and target for neuroprotective therapy in PD. The developing of drugs designed to decrease miR-132 activity may provide a novel strategy for PD and for other synucleinopathies.

Acknowledgments

This work was supported by a grant from the Michael J. Fox Foundation for Parkinson's Research, RRIA 2010 (Rapid Response Innovation Award).

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