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DOWNREGULATION OF MIR-124 IN MPTP-TREATED MOUSE MODEL OF PARKINSON'S DISEASE AND MPP IODIDE-TREATED MN9D CELLS MODULATES THE EXPRESSION OF THE CALPAIN/CDK5 PATHWAY PROTEINS

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Abstract—Parkinson's disease (PD) is a debilitating neurodegenerative disorder causing severe motor disabilities resulting from the loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) region of the midbrain. MicroRNAs (miRNAs) are small, non-coding RNAs which play a major role in several cellular processes in health and disease by regulating gene expression post-transcriptionally. Aberrant miRNA expression has been detected in post-mortem human PD brain samples, *in vitro* and *in vivo* PD models. However, none of the studies have focused on the role of the brain-abundant miR-124 in PD. In this study, we have evaluated the expression changes of miR-124 in the SN of the 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP)-induced PD mouse model. MiRNA expression analysis by qPCR revealed a decrease in the expression of brain-enriched miR-124 in the SN of MPTP-treated mice as compared to controls. Further, *in vitro* study revealed a decrease in the expression of miR-124 in MN9D dopaminergic neurons treated with MPP iodide. The expression of calpains 1 and 2 which is modulated by miR-124 was increased in the SNc of MPTP-treated mice as observed at different time points after treatment and in the MN9D dopaminergic neurons treated with MPP iodide leading to increased expression of the p35 cleavage product, p25 and cyclin-dependent kinase 5 (cdk5). Calpain-p25-mediated increase in cdk5 expression leading to dopaminergic neuronal death has been demonstrated in human PD and MPTP-PD models. Increased expression of calpain 1/cdk5 pathway proteins was observed in anti-miR-124-transfected MN9D cells in our studies. Knockdown of miR-124 led to increased production of reactive oxygen species (ROS) and hydrogen peroxide (H_2O_2) both known to increase oxidative stress. Further, experiments with miR-124 target protector sequences specific to calpain 1 revealed an interaction of miR-124 with calpain 1. Overexpression of miR-124 after MPP iodide treatment on MN9D cells was found to attenuate the expression of the calpain 1/p25/cdk5 proteins while improving cell survival. These results suggest that miR-124 acts to modulate the expression of calpain/cdk5 pathway proteins in the dopaminergic neurons. A better understanding of the mechanisms controlling the expression of miR-124 will aid in targeting miR-124 for better treatment strategies for PD.

Key words: Parkinson's disease, microRNA, miR-124, MPTP-induced mouse model of Parkinson's disease, MN9D cells.

INTRODUCTION

Parkinson's disease (PD) is an incapacitating neurodegenerative disorder characterized by severe motor symptoms like tremor, muscle rigidity, paucity of voluntary movements and postural instability (Lang and Lozano, 1998). The pathological hallmarks of PD are the specific loss of dopaminergic neurons in the substantia nigra pars compacta (SNc) and the formation of intracytoplasmic Lewy bodies that are mainly composed of fibrillar α-synuclein (Braak et al., 2003). Although several PD genes have been identified to date (Abou-Sleiman et al., 2006), the pathogenic process of PD is still not fully understood. The neurotoxin, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP) replicates most of the biochemical and pathological alterations in PD, including the loss of the dopaminergic neurons of the SNc in several mammalian species and has thus been widely used to generate animal models to investigate the molecular mechanisms involved in human PD and new treatment strategies (Przedborski et al., 2001). Current therapies available for PD provide a symptomatic relief rather than inhibiting the progression of the disease. Owing to the inadequacy of these drugs to be a cure for PD and their associated severe side effects on prolonged usage (Meissner et al., 2011), consistent research efforts are focused on the search for better neuroprotective strategies. There has been growing evidence of genetic and epigenetic factor involvement in PD (Habibi et al., 2011; Coppede, 2012) and an enhanced understanding of these

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Abbreviations: cdk5, cyclin-dependent kinase 5; DAT, dopamine transporter; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; H_2O_2, hydrogen peroxide; miRNA, microRNA; MPP, methyl phenyl pyridinium; MPTP, 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine; PBS, phosphate-buffered saline; PD, Parkinson's disease; Prx, peroxiredoxin; RNS, reactive nitrogen species; ROS, reactive oxygen species; RT-PCR, real time-polymerase chain reaction; SDS, sodium dodecyl sulfate; SN, substantia nigra; SNc, substantia nigra pars compacta; TH, tyrosine hydroxylase.

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factors in PD development and progression can contribute immensely to the development of effective and improved treatment strategies.

MicroRNAs (miRNAs) are small (~20–24 nucleotides long), non-coding RNAs derived from ~70 nucleotide hairpin pre-miRNA's, constituting about 1–2% of the genes in worms, flies and mammals (Bartel, 2009; Ebert and Sharp, 2012). They suppress mRNA expression via translation inhibition, degradation or deadenylation of mRNA (Jackson and Standart, 2007; Pillai et al., 2007). Their role in many fundamental biological processes like embryonic development, cell proliferation and differentiation is well known (Ambros, 2004; Wienholds et al., 2005; Wurdinger et al., 2008; Gangaraju and Lin, 2009). Further, the deregulated expression of specific miRNAs has been implicated in cancers, heart disorders and neurodegenerative diseases (Volinia et al., 2006; Lukiw, 2007; Johnson et al., 2008; Ha, 2011; Skalsky and Cullen, 2011). They have an immensely large regulatory potential owing to the fact that each miRNA can target multiple mRNAs (Sassen et al., 2008). MiRNAs are promising therapeutic targets and tools in several major diseases owing to their small size and ease of delivery (Harraz et al., 2011). With increasing evidence of abnormal miRNA expression in the pathogenesis of PD (Kim et al., 2007; Miñones-Moyano et al., 2011; Mouradian, 2012), alterations in specific miRNAs will provide important insights into molecular mechanisms of the disease, and could help in generating novel targets for therapeutic intervention (Junn and Mouradian, 2012).

In this context, there have not been many published data in the literature showing the changes in the expression of miRNA in the MPTP-induced PD animal models. Hence, our study was focused on identifying specific miRNAs that are significantly altered and their role in inducing gene expression changes that occur in the MPTP-induced PD mouse model. A qPCR miRNA expression analysis of the substantia nigra (SN) of MPTP-induced PD mice revealed significant changes in the expression levels of miRNA-124.

MiR-124 is a brain-enriched miRNA (He and Hannon, 2004), shown to play a role in neuronal differentiation during the development of the central nervous system and in adult neurogenesis (Lim et al., 2005; Makeyev et al., 2007; Yu et al., 2008; Cheng et al., 2009). Plasma concentration of miR-124 has been suggested to be a promising candidate biomarker for cerebral infarction (Weng et al., 2011). Downregulation of miRNA-124 has been implicated in the progression of medulloblastoma and glioblastoma (Pierson et al., 2008; Skalsky and Cullen, 2011). The autophagy-lysosomal pathway has been shown to be significantly altered in several neurodegenerative diseases including PD (Pan et al., 2008; Wong and Cuervo, 2010). MiR-124 has been predicted to target 52 genes of the autophagy-lysosomal pathway by a systems biology-based computational analysis (Jegga et al., 2011), implicating a role for miR-124 in the function of the pathway. Furthermore, a negotiating role between the nervous and immune systems has also been proposed for miR-124 (Soreq and Wolf, 2011). With such varied roles predicted for miR-124 and owing to its abundance in the brain, evaluating its role in PD will provide important insights into how it regulates the pathogenic processes in PD. The calpains are calcium-activated non-lysosomal proteases, demonstrated to be involved in the dopaminergic neuron loss in PD acting through the cyclin-dependent kinase 5 (cdk5) pathway (Crocker et al., 2003; Vosler et al., 2008) and are known to be modulated by miRNA-124 (Jegga et al., 2011); yet, the involvement of miRNA-124 in the processes leading to their activation in MPTP-induced PD models has not been established. Hence, our study was aimed at investigating the role of miRNA-124 in modulating the expression of the calpain/cdk5 pathway in MPTP-induced mouse model of PD and MPP iodide-treated MN9D dopaminergic neurons.

In the present study, we found that miR-124 expression was decreased in the SN of the MPTP-induced PD mouse model and that the loss of miR-124 in the dopaminergic neurons contributes, in part, to the increase in expression of the calpain/cdk5 pathway proteins by interacting with the calpain 1 mRNA. It was also found that overexpression of miR-124 after MPP+ insult diminishes the expression of calpain 1/p25/cdk5 proteins while improving cell viability compared to the negative control transfected MPP+-treated group.

EXPERIMENTAL PROCEDURES

Animals and treatment

Eight- to ten-week-old male C57BL/6J mice (weighing 20–25 g) were used for the MPTP treatment. All animal experiments were conducted according to protocols approved by the Institutional Animal Care and Use Committee, National University of Singapore. Mice were given four injections of MPTP–HCl (Sigma, Saint Louis, Missouri, USA.) at 2-h intervals (total dosage—72 mg/kg of body weight equally distributed over four injections) (Jackson-Lewis and Przedborski, 2007). Control animals were injected an equal volume of 0.9% sterile saline. Animals were sacrificed 1, 3, 5, 7, 10 days after the last MPTP injection. The SN was dissected bilaterally and total RNA was isolated using the miRNeasy mini kit (Qiagen, Hilden, Germany) for real time-polymerase chain reaction (RT-PCR) analysis.

Cell culture and MPP iodide treatment

MN9D cells (obtained from Drs. Alfred Heller and Lisa Won, University of Chicago) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS) (Choi et al., 1991). Cells were differentiated using 1.5 mM sodium butyrate in 10% FBS + DMEM for 5 days before being subjected to further experiments. Differentiated cells were treated with 100 and 200 μM MPP (methyl phenyl pyridinium) iodide (Sigma Aldrich, Saint Louis, Missouri, USA) for 24 h to mimic the MPTP-induced *in vivo* model based on Chee et al. (2005). Cells were plated on poly-L-lysine-coated cover slips and differentiated for immunofluorescence studies and *in situ* hybridization.

Cresyl Violet (Nissl) staining

Brain sections of thickness 20 μm cut using the cryostat were immersed sequentially in 100%, 75% and 50%