miRNA-mediated regulation of neurodegeneration in Parkinson's disease models

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BY

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I dedicate my labor of love, dedication, perseverance & sacrifices

my *doctoral thesis*

to the people without whom it would have been immaterial

Maa & Papa

IICB
आइ. आइ. सी. बी.

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TO WHOM IT MAY CONCERN

This is to certify that the thesis entitled "**miRNA mediated regulation of neurodegeneration in Parkinson's disease models**" submitted by Smt. **Pallabi Bhattacharyya** who got her name registered on 28.02.2018 for the award of Ph. D. (Science) degree of Jadavpur University, is absolutely based upon her own work under the supervision of Dr. Subhas C. Biswas and that neither this thesis nor any part of it has been submitted for either any degree / diploma or any other academic award anywhere before.

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Acknowledgements

My PhD is a story of my metamorphosis.
It is a story of coming of age- of a young academically-motivated dreamy-eyed girl, to becoming a pragmatic scholar- a student of science. She is still dreamy-eyed, but with her feet more rooted to the ground. It's a story of hardships and perseverance, late nights and no weekends, joy of new findings and no results, appreciations/awards and dejections, finding friends in least expected situations and feeling let-down from known companions, of innumerable conferences and travels, collaborations and associations, of numerous gains and a few losses. Actually, only gains. What I have gained is a life-time worth of experiences, worth every bit of this time.

Every story has a helms-man, for me, it has been my supervisor, Dr. Subhas C. Biswas. Before joining a PhD, I was inundated with stories from my peers and seniors from university about how PhD scholars have no freedom of thought, that they are mere trainees, with no voice of their own. For me, it has not been any farther from this myth. My supervisor has given me complete freedom in every step of my PhD journey. From designing of hypothesis to changing projects, from ordering of reagents to executing the experiments, Sir has never said "no" to me for anything. I particularly remember the time when Sir was on a sabbatical to the United States and I was devising my project; he would patiently listen to me every week, encouraging my thought process-no matter how naïve, and correct me with his experience and wisdom. He has been on my side when I was going through a major crisis in lab, even if that meant reprimanding one of his most favorite senior students. There have been innumerable instances when I have felt dejected, demotivated, perhaps even depressed, but I could easily confide in him and he has always calmed me down and pulled me back up on my feet with his comforting words. He gave me complete liberty to attend as many conferences or training workshops I wanted, no matter if they were directly related to my project or something I wanted to get trained in for my future research goals. "Go ahead" is what Sir would always tell me; two words, that both define and sum up my equation with my supervisor.

PhD is a long journey with different companions at every stage. I joined this lab along with three colleagues- Subhalakshmi, Akash and Anoy. I met some of my supervisor's first batch of PhD students, my seniors, Nandini di, Rumana di, Pampa di, Priyankar da, Suraiya di and my batch-mate yet lab senior, Hrishita. It has been a bitter-sweet memory with all of them but one person, who has been a constant companion all throughout, is Anoy. He has been my source of pure joy and unadulterated laughter in lab. We have shared food and each other's idiosyncrasies- I have suffered his sarcasm and he has endured my mood-swings! Anoy is a guy with an earst-while temper of a lion (true to his sun-sign, Leo!) but a heart full of emotions and warmth. He is very witty and really smart, just needs to take himself a tad bit more seriously, at times. My PhD journey would not have been half as fun without him. I would also like to thank Rebecca di, who, although was a part of our lab for a brief period, helped me immensely with my *in vivo* experiments.

Towards the end of my tenure, we moved labs. A new room, with a bunch of new juniors, all very enthusiastic and friendly. Sukanya, my junior from college (St. Xavier's) and now my lab-mate, is one person who is a queen of multi-tasking. This girl is super productive and juggles so many projects together so efficiently- it's worth admiring for sure. Soumita is my Scorpio buddy. A cheerful, effervescent girl, trying to charter her way through a completely new project in lab on her own. She definitely reminds me of my own days. I call Naqiya, the youngest of the lot, my daughter! She is absolutely adorable and has always been very helpful. Diptesh is way mature for his age. We have shared deep discussions on a variety of topics while waiting for our samples to incubate on Friday nights. Last but not the least, Kusumika di. She is undoubtedly the expert of Western blots in lab. She has been my go-to person for everything- from project discussions to career confusions to emotional analysis, she has always patiently lent ear to everything I have had to say. The recently joined juniors like Angshuman and Ananya are all very bright. Our lab has also hosted some wonderful trainees like Abdul Jalil Shah (Zain), Swati, Bhanupriya, Vandana, Manisheeta, Arnab, Ashish and Chayan, who all became a part of my journey in their own ways. My last few years

with all of them have comprised the most memorable part of my PhD tenure and I wish everyone all the very best for their future.

I have met some wonderful colleagues outside my own lab as well. I am particularly thankful to Dr. Suvendra Nath Bhattacharyya, Dr. Partha Chakraborty, Dr. Debabarata Biswas and Dr. Siddhartha Roy's labs for always helping me out whenever I have needed access to anything. Most particularly, Sinjini, Sambit and Anirban for welcoming me in their lab and being the most helpful. However, my PhD research journey would be incomplete without thanking my dear friend and colleague, Dushyant. He has helped me in ways that is beyond the realm of words. He has been my constant supporter in every scientific endeavour during this tenure. Sometimes, science alone is enough for two people to bond so well. My friend Kamran, for sharing laughter and funny wisdom. And lastly, I would like to extend my thanks to Dr. Krishnananda Chattopadhyay who is also a part of my PhD evaluation committee, Prof. P.K. Sarkar for his guidance, Dr. Arun Bandopadhyay, Director of CSIR-IICB for all kinds of institutional support and CSIR (Delhi) for providing me with the prestigious Shyama Prasad Mukherjee Fellowship to carry forward my PhD research.

There have also been a few people beyond my institute who have been instrumental in my PhD journey. First and foremost, Prof. Atanu Biswas (Bangur Institute of Neurosciences), who extended his support and collaboration on the clinical aspect of my thesis. During my course-work at Jadavpur University, I made friends with Arathi and Rahul, whose friendships I still cherish. In 2016, I met Touseef Ahmad Sheikh at a conference in NBRC who completely changed the way I looked at life. Arumoy, my old friend, who would always provide me with necessary informations and was a wonderful host during my training at a workshop at NCBS. Finally, Prof Pankaj Seth (NBRC), Prof. Shubha Tole (TIFR), Prof. Gaiti Hassan (NCBS) and Dr. Shane A. Liddelow (NYU) are some of the extraordinary neuroscientists who I have had the privilege to interact with, in person, and who have inspired me tremendously to be in this path of science and research.

This PhD journey would be meaningless without family support. I would like to dedicate this career milestone to my paternal grandparents- Dadu and Thamma. I still remember Thamma's little anecdotes of wisdom everytime I went to see her off before my exams in my school days. I hope both Thamma and Dadu, wherever they are now, are being proud of their granddaughter's achievements. My maternal grandparents- Ammi and Buduk. Ammi would sit with me for hours as I learned my history chapters and I would wait for Buduk to come every weekend to teach me English and Maths. They would always be the first people to know whenever I topped my class throughout school. I wish Ammi and Buduk were here to witness this momentous part of my life. Buduk, I miss you every single day. I would also like to thank my driver, Nitai mama, who relentlessly drove me to lab at all the odd hours, weekdays and weekends alike, and waited on me for hours on end while I ran my experiments.

I don't know how to thank the two people who have been the sole reasons for all that I am. *My Maa and Papa.* My desire for PhD was sown as I grew up idolizing my father, Dr. Ashish Bhattacharyya, and witnessing how he achieved his own PhD degree, acing against all odds. How can I possibly enumerate my mother's contributions for me; she is behind everything that I have been or aspire to be in life. Amidst all the struggles and sacrifices, tears and smiles, sweat and blood, the one desire that has pulled me through, is the desire to see my Maa and Papa happy, to hear them say," Pallabi, were are so proud of you."

My PhD has been a labour of love, patience, perseverance, dedication and ambition. It has witnessed a global pandemic and survived the test of time. It has truly been a blessing of the Lord and perhaps an indication of destiny. This PhD has been a journey of a life-time. But it is only the first step. The journey of science has just begun.

-Pallabi Bhattacharyya

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Abstract

Thesis title: **Micro-RNA mediated regulation of neurodegeneration in Parkinson's disease models**

Submitted by: **Pallabi Bhattacharyya**

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Abstract

microRNAs (miRNAs/miRs) are non-coding small RNAs that are important for post-transcriptional gene regulation and maybe released from the parent cell packaged inside certain membrane-bound extracellular microvesicles called exosomes. For my Ph.D. thesis, I have focused on brain-enriched miRNAs- miR-128 (neuron-enriched) and miR-23a (astrocyte-enriched) and studied their role in Parkinson's disease (PD) pathogenesis. miR-128 is a neuron-enriched miRNA which was observed to be down-regulated in the cellular models of PD under 6-OHDA treatment. miR-128 prevented activation of the transcription factor FoXO3a and could regulate the expression of the downstream targets of FoXO3a- the pro-apoptotic proteins PUMA and FasL. By down-regulating these proteins, miR-128 could successfully shut-down both the intrinsic and extrinsic pathways of apoptosis. Additionally, miR-128 could prevent mitochondrial superoxide generation. Furthermore, miR-128 overexpression inhibited 6-OHDA induced neurite shortening and promoted neurite formation. Finally, miR-128 overexpression improved synaptic health by up-regulating the expressions of synaptic proteins synaptophysin and PSD-95. On the other hand, miR-23a is an astrocyte-enriched miRNA that was downregulated in astrocytes upon Rotenone treatment. Interestingly, Rotenone treatment led to Ca^{2+} surge in the astrocytes, followed by activation of the $Ca^{2+/}$CaM dependent protein phosphatase, Calcineurin. Calcineurin was also found to co-express in the reactive astrocytes of acute MPTP models of PD. Further downstream of Calcineurin pathway, miR-23a

underwent release from the astrocytes via exosomes. This exosomal miR-23a proved to be neuroprotective in different neuronal models of PD. 3'UTR cloning revealed that miR-23a can directly bind to and down-regulate the expression of pro-apoptotic PUMA in the neuronal cells, thereby preventing induction of 6-OHDA mediated neuronal apoptosis. Finally, both miR-128 and miR-23a was found to be differentially expressed in the exosomes derived from the PD patient plasma. This data was further validated from human sRNA-Seq data obtained from miRNA expression and exosome repositories like DIANA-miTED and EVAtlas respectively. Thus, brain-enriched miRNAs- miR-128 (neuron-enriched) and miR-23a (astrocyte-enriched) proved to play significant roles in the pathogenesis of PD and showed potential to be important biomarkers and therapeutic targets for the detection, diagnosis and cure of PD.

Abbreviations

1-Methyl-4-phenylpyridinium MPP+

1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine MPTP

3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide MTT

3'UTR 3´untranslated region

6-OHDA 6-Hydroxydopamine

ACM Astrocyte conditioned medium

ATRA all-trans Retinoic Acid

BSA Bovine serum albumin

CaN Calcineurin

CNS Central nervous system

CSF Cerebrospinal fluid

DIV Days *in vitro*

DMEM Dulbecco's modified eagle medium

DMSO Dimethyl sulfoxide

DN Dopaminergic neuron

E18 Embryonic day 18

EV Extracellular vesicle

FasL Fas ligand

FBS Fetal Bovine serum

FoxO3a Forkhead box class O 3a

GFAP Glial Fibrillary Acidic Protein

HRP Horse radish peroxidase

HS Horse serum

LB Lewy body

LN Lewy neurites

LP Lewy pathology

miRNAs/miRs microRNAs

ncRNA Non-coding RNA

NDD Neurodegenerative disorder

NFDM Non-fat dry milk

NGF Nerve Growth Factor

PBS Phosphate buffer saline

PC12 Pheochromocytoma 12

PD Parkinson's disease

PDL Poly D-Lysine

PFA Paraformaldehyde

PUMA p53 upregulated modulator of apoptosis

qRT-PCR Quantitative real-time polymerase chain reaction

SDS Sodium dodecyl sulfate

SDS-PAGE SDS-Polyacrylamide gel electrophoresis

SNPc Substantia nigra pars compacta

sRNA-Seq Small RNA sequencing

TBS Tris buffered saline

TBST Tris buffered saline Tween 20

Chapter 1:

Introduction & Objectives

Introduction:

Parkinson's disease (PD) is one of the most predominant neurodegenerative disorders (NDDs) and motor disorders in the world affecting more than 10 million people across the globe. However, even after 200 years of its first incidence, the available medications for PD are still mostly symptomatic, with side-effects further deteriorating the quality of life. Additionally, although a lot of protein and genetic biomarkers have been studied for the early detection of PD, they have mostly failed to successfully diagnose the disease. Thus, for my PhD thesis, I decided to focus my study on certain non-coding regulatory RNAs called miRNAs that are known to regulate gene expression at the post-transcriptional stage, with precise spatial and temporal control, and maybe preferentially expressed in the brain tissue. The main aim of my PhD thesis was to determine how certain brain-associated miRNAs, either neuron-enriched or astrocyte-enriched, regulate the molecular pathways involved in PD pathogenesis and progression. We also wanted to check whether these brain-specific miRNAs could be detected in the circulating exosomes derived from PD patient blood (plasma) samples, with the view of identifying some potential target biomarkers for PD detection and diagnosis. With that goal in mind, I have completed three projects for my PhD thesis; the objectives for each project are summarized below:

Objectives:

Part 1: To study the role of neuron-enriched miR-128 in apoptosis, neurite formation and synaptic health during PD pathogenesis

1. To study the expression pattern of neuron-enriched miR-128 in *in vitro* models of PD.
2. To determine the role of miR-128 on neuronal health- is it neuro-protective or neuro-detrimental?
3. To determine the role of miR-128 on neuronal apoptosis pathways.

4. To study the effect of miR-128 on neuronal morphology and neurite formation.

5. To study the role of miR-128 on synaptic integrity and/or synaptic protein expressions.

Part 2: To study the interplay of Calcineurin and astrocyte-enriched miR-23a in astrocyte-neuron cross-talk and its effect on neuronal health during PD pathogenesis.

1. To check the expression of Calcineurin within astrocytes in models of PD (*in vitro* and *in vivo*).

2. To determine the expression pattern of astrocyte enriched miR-23a in astrocytes.

3. To determine the role, if any, of Calcineurin signaling on miR-23a expression.

4. To determine the effect of miR-23a on neuronal health during PD pathogenesis.

Part 3: To determine the expression pattern of miR-128 and miR-23a in the exosomes derived from PD patient samples

1. To determine the presence or absence of miR-128 and miR-23a in secretory extracellular vesicles across body fluids.

2. To determine the expression pattern of miR-128 and miR-23a in human PD patient brain tissue.

3. To detect the expression of miR-128 and miR-23a in the circulating exosomes derived from the blood samples of a local cohort of PD patients and compare it with age-matched controls.

Chapter 2:

Review of Literature

2.1 History of Parkinson's disease (PD)

PD is the second most prevalent age-related neurodegenerative disorder (NDD) in the world after AD, affecting about 1% of the world population above the age of 65 (Beal 2001). PD was first described by clinician Dr. James Parkinson in his article, *An Essay on the Shaking Palsy* in 1817 (Obeso et al. 2017). The term '*Parkinson's disease*' was however, coined much later, by another clinical neurologist Dr. Jean-Martin Charcot in 1888. PD is primarily a motor disorder with characteristic motor dysfunctions. The cardinal pathology of clinical PD involves degeneration and death of dopaminergic neurons (DN) in the substantia nigra pars compacta (SNPc) of the mid-brain and the presence of intracytoplasmic inclusions known as Lewy bodies (LB) in the brain-stem (Brundin et al. 2008). Clinical PD is mostly detected by neuroimaging techniques like Magnetic Resonance Imaging (MRI), Positron Emission Tomography (PET) and Single Photon Emission Computed Tomography (SPECT). So far, there are no cures for PD and the treatments are mostly symptomatic that involve administration of Levodopa/L-DOPA (a biosynthetic precursor of dopamine), dopamine agonists, catechol-O-methyl transferase (COMT) inhibitors, monoamine oxidase B (MAO-B) inhibitors, amantadine and anti-cholinergic molecules or immunomodulatory therapies. Alternatively, neurosurgical techniques like Deep Brain Stimulation (DBS) may also be used where a neurostimulator is surgically implanted in the brain such that it is able to deliver electrical stimulation to target areas that control movement, or block the abnormal nerve signals that cause tremor, rigidity and movement dysfunctions (Lozano et al. 2019).

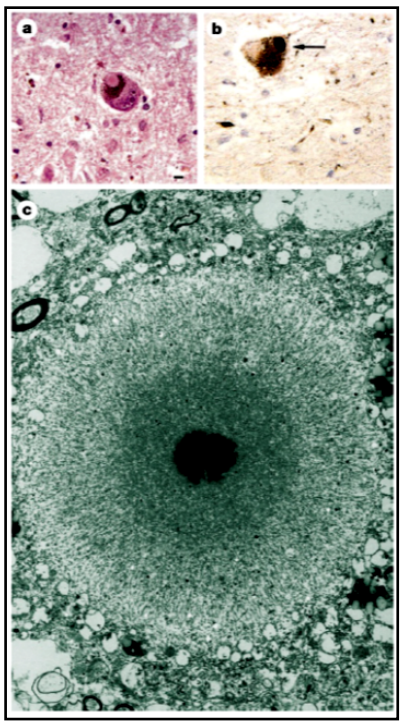


Figure 1: LBs are small spherical inclusions a) LBs can be stained with hematoxylin/eosin. **b)** LBs contain the protein α-synuclein. **c** | LBs consist of a dense granular core surrounded by a halo of radiating filaments as seen in the electron micrograph (Beal 2001).

2.2 Clinical symptoms of PD

2.2.1 Motor symptoms

PD is associated with a range of clinical features for its diagnosis. The most characteristic feature are motor deficits like hallmark *bradykinesia* (abnormal slowness of movement), *rigidity* (stiffness and increased tone of muscles), *resting tremor* (produced while the body is at rest) and posture or gait instability (*camptocormia*), all or most of which are usually manifested unilaterally or asymmetrically. The motor features predominantly stem from the loss of DN in the mid-brain and

thus, the current symptomatic therapies for PD focus on mainly dopamine replacement strategies. With the advancement of the disease and increment in doses of levodopa, development of motor complications is observed (Moustafa et al. 2016). These are characterized by wearing off periods and off periods, during which bradykinesia and rigidity reappear, an absence of on periods, and development of involuntary dyskinesia is also observed.

2.2.2 Non-motor symptoms

Many of the motor symptoms of PD can be preceded, sometimes for several years, by onset of non-motor symptoms. Some of these symptoms like hyposmia, rapid eye movement (REM) sleep behavior disorder (RBD), depression and constipation can appear several years before the onset of actual disease pathology (Schapira, Chaudhuri, and Jenner 2017). On the other hand, global cognitive decline, particularly executive dysfunction and loss of working memory, may arise much later as the disease progresses, often observed in patients over 70 years of age regardless of the age of disease onset. None of these symptoms, however, is specific for PD, but the presence of combinations of these symptoms and correlation with preclinical loss of DN as observed by advanced imaging techniques, can improve sensitivity and specificity of the prediction and diagnosis of pre-symptomatic to symptomatic PD.

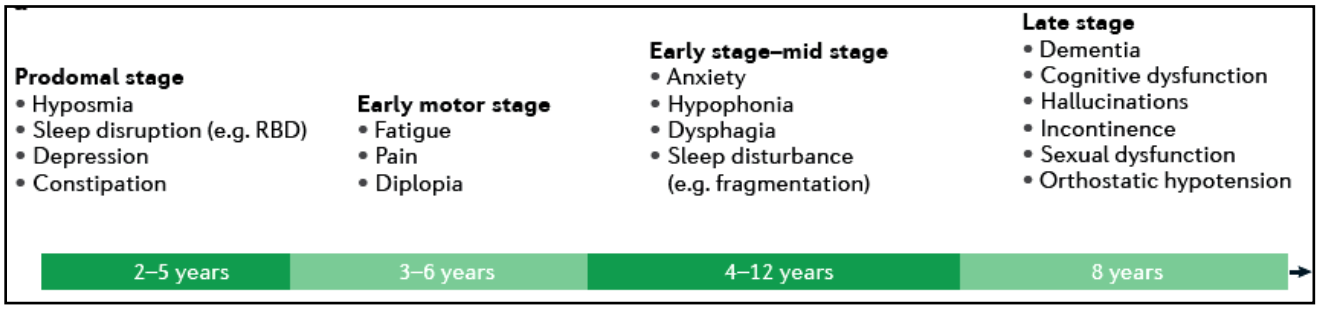


Figure 2: Time courses of the onset of the non-motor features of PD. A schematic representation of the tentative timeline by which the non-motor features of PD may appear (Schapira, Chaudhuri, and Jenner 2017).

2.3 Pathophysiology of PD

Clinical PD is described by a set of inclusion and exclusion criteria set by the Movement Disorders Society (MDS) which are discussed below.

2.3.1 Death of dopaminergic neurons

The most consistent characteristic neuropathological feature found in all patients with clinical PD is degeneration of the DN in the SNPc. However, DN loss also occurs in many other parkinsonian neurodegenerative conditions, making it a necessary neuropathology, but not entirely specific for PD. The pattern of dopaminergic neuron loss in the SNPc region is distinctive for PD, with the most severe loss occurring in the ventrolateral region while those in the nearby ventral tegmental area remain almost entirely intact.

2.3.2 α-synuclein and Lewy bodies

The second diagnostic marker of idiopathic PD (not familial PD usually) is the presence of misfolded protein in the form of *Lewy bodies* (LBs) in the brain stem. The affected brain regions exhibit characteristic intracytoplasmic inclusions of LBs which are present in the neuronal cell bodies and Lewy neurites (LNs) which are present in the neuronal processes. LBs are small (5–25 μm) spherical inclusions consisting of a dense granular core surrounded by a halo of radiating

filaments. These inclusion bodies are particularly rich in aggregated α-synuclein, a protein normally enriched in nerve terminals and involved in synaptic functions (Obeso et al. 2017). During PD as well as normal aging, levels of natively folded α-synuclein are found to increase in the cytoplasm of nigral neurons. In the LBs however, α-synuclein is misfolded, post-translationally modified (nitrosylation, phosphorylation) and ubiquitinylated. Although the roles of LBs and LNs in PD are unclear, the proportion of nigral neurons containing LBs is relatively constant (~3–4%) and independent of the stage of PD. Hypothesis suggests that neurons in different regions of brain vary in their propensity for to protein misfolding such that the pattern of LB formation reveals the susceptibility to α-synuclein misfolding. Regardless of the different pathogenic pathways that might lead to the development of LBs, the α-synuclein inclusions constitute an excellent indicator for the advancement of PD (Surmeier, Obeso, and Halliday 2017).

2.3.3 Possible underlying mechanisms

Several interesting hypotheses have been put forward to explain the propagation of PD pathology which has been discussed below. These molecular events may not be mutually exclusive and it is possible that two or more of such mechanisms co-operate to generate characteristic PD pathology.

Inflammation: Inflammation is a well-established phenomenon in the PD brain. Microglia are known to be activated in PD with increased levels of pro-inflammatory mediators such as tumour necrosis factor-α, interleukin-1β (IL1-β), IL6, inducible nitric oxide synthase and cyclooxygenase 2, being found in the striatum and SNPc. These inflammatory mediators may promote α-synuclein aggregation. For example, IL1-β treatment raises neuronal levels of α-synuclein while activated

macrophages increase α-synuclein nitrosylation in neural cells leading to its aggregation (Q. Wang, Liu, and Zhou 2015).

Oxidative stress and excitotoxity: Oxidative stress is a major player in PD pathogenesis as significant evidences from post-mortem studies implicate oxidative stress in the pathology of the PD. Reports show that DN might be particularly vulnerable to oxidative damage which can lead to protein modifications (nitrosylation) along with enhanced protein misfolding and aggregation including α-synuclein (Blesa et al. 2015). Another possible trigger of oxidative stress is excitotoxicity resulting from excessive extracellular glutamate in PD brain. Chronic exposure to low-grade excitotoxicity might trigger oxidative stress inside the DN and may initiate α-synuclein changes (J. Wang et al. 2020).

Loss of neurotrophic support*:* DNs are dependent on trophic stimuli to maintain a normal internal milieu. Reports suggest that the basal ganglia of PD patients express reduced levels of neurotrophic factors like glial cell-derived neurotrophic factor (GDNF) and brain-derived neurotrophic factor (BDNF) (Sun et al. 2005). It is possible that, in the absence of appropriate levels of growth factors, the neurons fail to maintain their homeostasis which hinders their antioxidant defenses and inhibits the molecular machineries required for refolding or targeted degradation of misfolded α-synuclein.

Environmental factor: Epidemiological research indicates that environmental conditions such as rural living, pesticide use, well-water consumption and certain occupations like mining and welding are associated with increased risk of PD. More males are affected than females, in a ratio of 1.5:1.0. Interestingly, protective effects have been shown for cigarette smoking, alcohol and caffeine intake

but how these agents may influence disease risk is not clear. Although environmental factors contribute to parkinsonism, no specific causative agent has yet been identified. Mitochondrial and proteasomal toxins have been associated with atypical parkinsonism, but the evidence is debatable (Farrer 2006). Nevertheless, environmental effects play an important role in the pathogenesis of PD.

2.4 Braak stages for Parkinson disease

Braak and group have argued that the distribution of Lewy pathology (LP) develops over time and could be staged from well-defined starting points. In the Braak scheme, the generation of LP is broken down into six stages aligned with clinical PD symptoms. As per this theory, PD has been proposed to begin in the gastrointestinal and olfactory systems, after which it spreads along axonal pathways, advances to the brainstem through the vagal nerve and eventually reaches the SNPc and the neocortex. However, only about 50% people with clinical PD have a distribution of LP consistent with the Braak staging model. Thus, the biggest limitation lies in the fact that without a validated biomarker for longitudinal tracking of PD from pre-symptomatic to symptomatic stages, it is difficult to confirm the Braak staging of PD (Surmeier, Obeso, and Halliday 2017).

2.5 Familial PD and associated genetic mutations

Familial PD comprises of about 10% of clinical PD cases. It is associated with multiple mutations in predominantly 5 genes as observed in families with a Mendelian pattern of PD inheritance. Dominantly inherited mutations include SNCA (α-synuclein) and LRRK2 (Leucine-rich repeat

kinase 2) while the recessive mutations include *PINK1* (PTEN-induced putative kinase 1), *PARK2* (which encodes parkin) and *PARK7* (which encodes DJ1 protein) (Farrer 2006).

2.5.1 Dominantly inherited mutations

α-Synuclein: One of the first genetic mutations identified to be associated with PD were pathogenic missense mutations in the gene *SNCA* that encodes α-synuclein. The *SNCA* 209G>A (Ala53Thr) mutation was identified through genome-wide linkage studies in several families with autosomal dominant parkinsonism which was followed by identification of two additional missense mutations *SNCA* 88G>C (Ala30Pro) and 188G>A (Glu46Lys). α-synuclein wild-type protein is a potent inhibitor of phospholipase D2 which is functionally involved in signal transduction, membrane vesicle trafficking and cytoskeletal dynamics. It is also a competitive inhibitor of tyrosine hydroxylase, the enzyme involved in tyrosine to L-DOPA biosynthesis. Wild-type α-synuclein forms an amphipathic, α-helical domain when the protein associates with lipids (Golovko et al. 2009). In the mutant variety, the affinity of α-synuclein for lipids is reduced, the cytoplasmic pool of the protein might increase along with an enhanced propensity to take on a β-sheet conformation. Consequently, α-synuclein might self-assemble into oligomers forming pore-like structures before conforming into higher molecular weight fibrils. Additionally, α-synuclein is a major component of LB and LN pathology in both familial and sporadic PD. Cases of clinical PD with *SNCA* mutations have an early onset of PD motor signs and non-motor symptoms like dementia; LP in their brains is more widespread than in non-genetic cases and extends to the cerebral cortex. These patients have a broader distribution of neuronal death not only in the SNPc but also in the locus coeruleus and hippocampus, which is not commonly observed in patients with non-genetic PD (Liu et al. 2021).

Leucine-rich repeat kinase 2: *LRRK2* mutations are the most common autosomal-dominant mutations implicated in familial PD. The *LRRK2* 6099G>A (Gly2019Ser) mutation is most frequent in Caucasians, causing 0.5–2.0% of sporadic PD and 5% of familial parkinsonism. However, in Ashkenazi Jews and North African Arab populations this mutation might lead to 18–30% of PD cases. *LRRK2* is expressed in most brain regions including the SNPc, caudate nucleus or the putamen, and the LRRK2 protein is involved in multiple functions, including substrate binding, protein phosphorylation and protein-protein interactions. Most *post mortem* cases of LRRK2 parkinsonism show typical LB disease although some cases might have a tau-positive pathology without LBs (D. Dickson, personal communication) while some others show only nigral degeneration with neither LBs nor neurofibrillary tangles. Thus, it is unclear how LRRK2 substitutions result in PD neuropathology (Rui et al. 2018).

2.5.2 Recessively inherited mutations

The recessive forms of parkinsonism are relatively rare, loss-of-function mutations that result in an early age of onset (typically, at less than 40 years of age) and a slow progression. These forms of parkinsonism are characterized by a more selective loss of DN than sporadic, late-onset PD.

Parkin: Mutations in the parkin gene (*PARK2*) were originally discovered in Japanese families which showed autosomal recessive, juvenile PD. Parkin functions as an E3-ligase, conjugating ubiquitin to proteins and targeting them for degradation by the proteasomal system. Community-based studies indicate that about 50% of familial PD cases with early onset (<45 years) and a significant proportion of sporadic cases are caused by *PARK2* mutations. Importantly, patients possessing homozygous exonic deletions that leads to complete loss of parkin expression show

selective loss of DNs in the SNPc and locus ceruleus, without LB or neurofibrillary tangle formation. By contrast, parkin overexpression in experimental models has shown to be neuroprotective, suggesting that its upregulation might be one route to therapy (Dawson and Dawson 2010). Additionally, parkin being a component of the ubiquitin–proteasome system strengthens the hypothesis that proteasome dysfunction and associated protein aggregation is central to PD pathology.

PTEN-induced kinase 1: PINK1 is a ubiquitously expressed protein that encodes a mitochondrial targeting motif and a highly conserved protein kinase domain, similar to the $Ca^{2+/}$Calmodulin family of serine–threonine kinases. Mutations in *PINK1* have functional effects on protein stability, localization and kinase activity. Reports suggest that wild-type PINK1 in cell culture can protect neurons from mitochondrial dysfunction and apoptosis (Morais et al. 2009). However, the neuropathology caused by *PINK1* mutations is yet to be deciphered.

DJ1: mutations are rare and causes <1% of early-onset parkinsonism. DJ1 is a mitochondrial protein and is a member of the ThiJ/PfpI family of molecular chaperones known to be induced during oxidative stress. Interestingly, in experimental models, reduced DJ1 expression results in susceptibility to oxidative stress and proteasome inhibition and shows selective sensitivity to environmental toxins like paraquat and rotenone. These chemicals have been implicated in development of sporadic PD, suggesting that cross-talk between genetic and environmental factors might contribute to disease susceptibility (Repici and Giorgini 2019).

2.6 Experimental models of PD

For *in vitro* and *in vivo* studies, there are three commonly used neurotoxic models of PD using 6-hydroxydopamine, rotenone and MPTP along with transgenic mouse models of PD. These models are discussed below:

6-hydroxydopamine (6-OHDA): is a dopamine analog and the first neurotoxin used to model PD. Upon injecting into the SNPc, 6-OHDA accumulates in DN, leading to the selective death of these neurons through generation of free radicals. The toxic effect of 6-OHDA may be observed in cell culture models, rodent models, as well as primates. In rodents, the extent of dopamine depletion can then be evaluated by examining rotatory behavior of treated rodents in response to amphetamine and apomorphine. 6-OHDA lesions, however, do not result in LB formation in the SNPc. The major advantage of this model is the quantifiable motor deficit (rotation) (Hernandez-Baltazar, Zavala-Flores, and Villanueva-Olivo 2017).

Rotenone: a commonly used insecticide, affirms the hypothesis that pesticides and similar environmental toxins may be involved in the pathogenesis of PD. Rotenone is known to be a high-affinity specific inhibitor of mitochondrial complex I, involved in oxidative phosphorylation, deficits of which has been observed in the SNPc of PD patients (Greenamyre, Betarbet, and Sherer 2003). The rotenone injected rats are reported to develop a progressive degeneration of nigrostriatal neurons with associated loss of tyrosine hydroxylase, dopamine transporter and vesicular monoamine transporter immunoreactivity. Additionally, the nigral neurons show cytoplasmic inclusions similar to LBs. The rats also exhibit PD-like motor dysfunctions namely, bradykinesia, postural instability, unsteady gait along with tremor. Rotenone model is remarkable as it shows that

an inhibitor of complex I of the electron transport chain, which otherwise acts uniformly throughout the brain, has the ability to cause selective degeneration of nigrostriatal neurons. Although there is variability in the susceptibility of individual rats to this neurotoxin, rotenone-based model meets most of the criteria needed for an excellent animal model of PD (Cannon et al. 2009).

α-synuclein mouse models. Mice models lacking the α-synuclein gene remain viable, show normal brain structure with no deficits in dopamine cell bodies or terminals and report no parkinsonian phenotype. The α-synuclein deficit mice, however, show a mild reduction in striatal dopamine (18%) and an attenuation of dopamine-dependent locomotor response to amphetamine. On the other hand, mice over-expressing wild-type α-synuclein are shown to develop a progressive accumulation of α-synuclein and ubiquitin-immunoreactive inclusions in the SNPc but the fibrillar aggregates that are characteristic of LBs are not reported. There is no loss of DN within the SNPc, but a loss of dopamine terminals is observed. They also showed impairment on rotarod testing to some extent. This model therefore produces only some aspects of the pathology of dementia with LB, but fails to significantly replicate PD (Gómez-Benito et al. 2020).

MPTP model: The best-characterized model of PD so far, is developed by using the neurotoxin 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine (MPTP). MPTP was first discovered accidentally in 1982 when a group of drug addicts in California developed sub-acute onset of severe parkinsonism upon self-administration of a synthetic heroin analogue that was contaminated by a byproduct i.e. MPTP, during manufacture. MPTP administration has ever since been used to model PD in both mice and primates. MPTP is a highly lipophilic molecule that readily crosses the blood–brain barrier. It is converted into its active metabolite, 1-methyl-4-phenylpyridinium (MPP+) by

monoamine oxidase B. MPP+ is taken up by the plasma-membrane dopamine transporter and inside the cells, MPP+ is concentrated in the mitochondria where it inhibits complex I of the electron transport chain. This reduces ATP generation and increases free-radical production. Interestingly, MPTP toxicity in primates can replicate all the clinical signs of PD, including tremor, rigidity, akinesia and postural instability (Meredith and Rademacher 2011). Additionally, primates treated with MPTP show an excellent response to L-DOPA (3,4-dihydroxyphenylalanine) and dopamine receptor agonists. The main difficulty with MPTP model of PD, however, is that it is an acute or sub-acute process, whereas PD is essentially a slowly progressive disorder known to evolve over years. Additionally, MPTP model lacks LB formation. To overcome this, chronic administration of MPTP in primates using several dosing regimens has also been tried which has shown to develop parkinsonian syndromes slowly with selective depletion of DN in SNPc and production of LB like inclusions (Mat Taib and Mustapha 2020).

2.7 Apoptosis in PD

A number of NDDs are characterized by neuronal death, including PD. The most well-documented pathway of neuronal death is programmed cell death or apoptosis. Apoptosis involves a conserved sequence of biochemical and morphological changes, triggered by a number of cell death signals that include- *lack of neurotrophic factors*, over-activation of excitatory glutamate receptors (*excitotoxicity*), increased *oxidative stress* during which free radicals (superoxide anion radicals, hydroxyl radicals) damage cellular lipids, proteins and nucleic acids, age-associated *metabolic stress* and *environmental stress,* all or most of which are also implicated in PD (Mattson 2000).

2.7.1 Mediators of apoptotic death

Caspases: The main mediators of apoptosis are the cysteine proteases called 'caspases', ubiquitously present in almost every animal cell. Caspases exist as *procaspases* that may become activated in response to cell death signals, triggering cleavage of their substrates at specific aspartic acid residues. Mammalian caspases may be divided into *initiators* (procaspases-2, -8, -9 and -10) and *effectors* (procaspases-3, -6 and -7). The initiators are among the first set of caspases to become activated in the cell death cascade. Upon activation, initiator caspases cleave effector procaspases into their active forms, thereby irreversibly leading to cell death (Vila and Przedborski 2003).

Bcl2 family of proteins: The B-cell lymphoma-2 (Bcl2) family of proteins comprises members that have either anti-apoptotic such as Bcl2 and Bcl-xL or pro-apoptotic such as Bcl- 2-associated X protein (BAX) and Bcl-2-antagonist/killer (BAK) functions during cell death. These multi-domain Bcl2 members possess up to four Bcl2-homology domains i.e. BH domains 1 to 4. There are also certain BH3-only proteins that share sequence homology only with the BH3 domain, which include BH3 interacting domain death agonist (BID), BIM (also known as BCL2L11), PUMA (also known as BBC3) and Bcl-2-associated agonist of cell death (BAD). These BH3-only proteins mainly act as intracellular death ligands (Vila and Przedborski 2003).

2.7.2 Apoptotic death cascades

The death receptor (or extrinsic) apoptotic pathway: The extrinsic pathway is triggered by activation of cell-surface death receptors such as Fas/CD95 and tumor necrosis factor receptor 1 (TNFR1). Death receptor activation in turn, is initiated by their binding with specific ligands called *death activators*, viz. the Fas ligand binds to Fas receptor, and TNFα binds to TNFR1. Next, the

intracellular ‘death domains’ on these receptors associate with the adaptor proteins which contain ‘death effector domains’. For example, Fas associates with FADD (Fas-associated death domain protein), and TNFR1 associates with FADD and TRADD (TNFR-associated protein with death domain). The adaptor protein then recruits procaspase-8, leading to its activation. Activated caspase-8 then activates other downstream caspases, either directly or indirectly, through cleavage of Bid. Additionally, procaspase-8 may also be activated through the intrinsic pathway (Hongmei 2012).

The mitochondrial (or intrinsic) apoptotic pathway: This pathway may be triggered through a death receptor-independent stimulus by inducing translocation of pro-apoptotic molecules (Bax) to the mitochondria, with the subsequent release of mitochondrial apoptotic messengers (cytochrome *c*) to the cytosol. Upon release from mitochondria, cytochrome *c* interacts with two other cytosolic protein factors-Apaf1 and procaspase 9, to form a multimeric Apaf1–cytochrome *c* complex which eventually activates caspase-3. Another mitochondrial inter-membrane protein that is released into the cytosol on induction of apoptosis is Smac/Diablo. In the cytosol, Smac/Diablo interacts with certain proteins called *inhibitors of apoptosis* (IAPs), causing the IAPs to relieve their inhibitory effect on initiators (caspase-9) and effectors (caspase-3). However, the release of AIF (apoptosis-inducing factor) and endonuclease G from the mitochondrial intermembrane space does not lead to caspase activation. AIF and endonuclease G may translocate from the mitochondria to the nucleus wherein it leads to caspase-independent large-scale DNA fragmentation. On the other hand, endoplasmic reticulum (ER) associated stress may also result in apoptosis through activation of caspase-12 which cleaves caspase-9. Pro-apoptotic proteins Bax and Bak operate at the ER to maintain calcium homeostasis and regulate ER-dependent apoptosis (Elmore 2007).

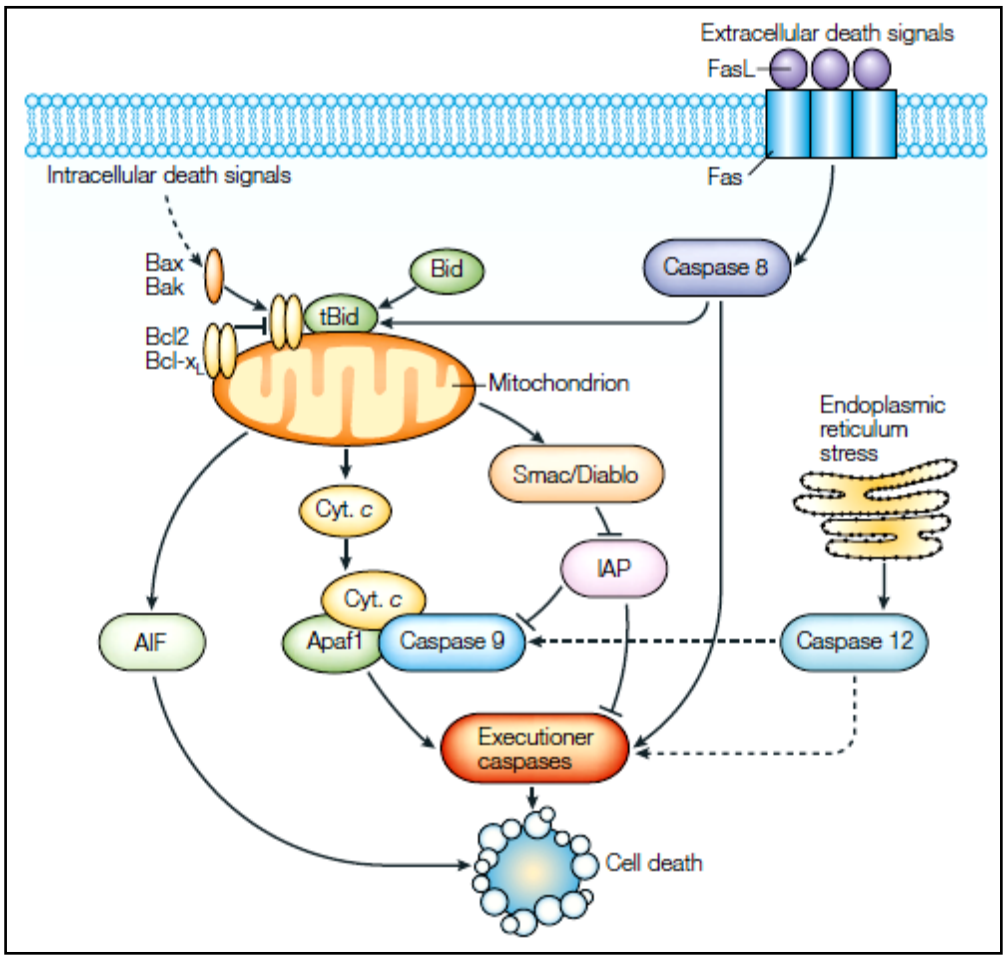
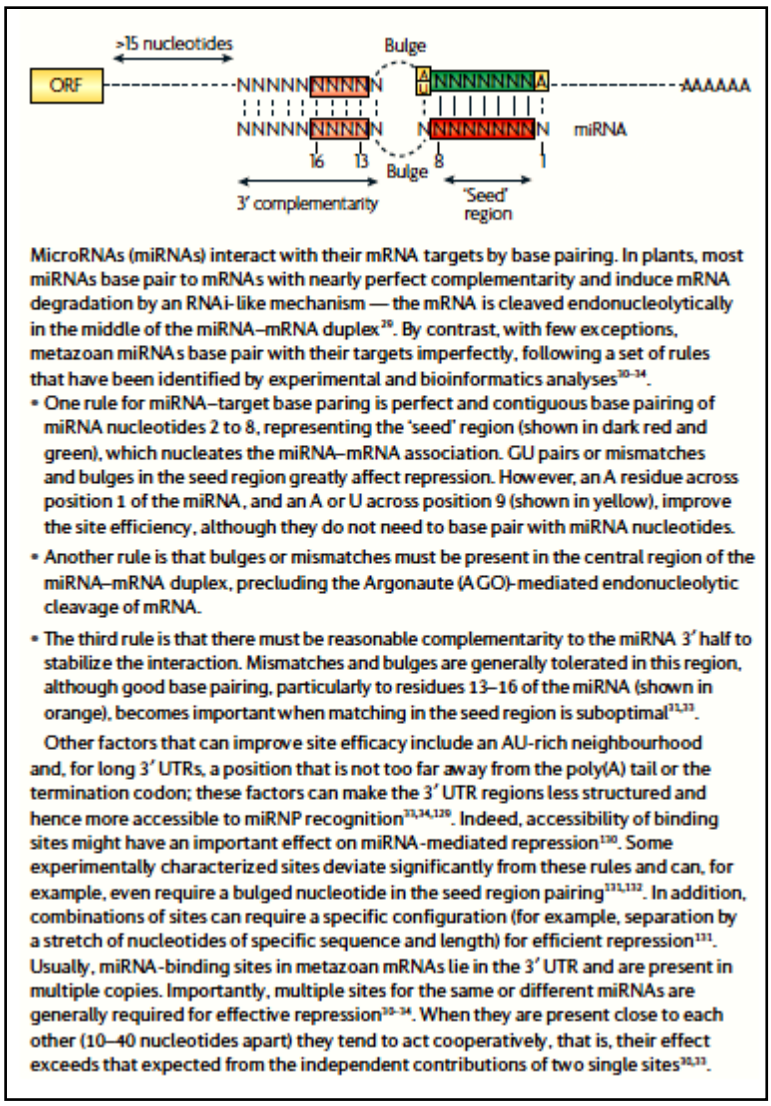


Figure 3: Mechanisms of apoptotic cell death (Taylor, Cullen, and Martin 2008)

2.8 Role of microRNAs in PD

microRNAs (miRNAs/miRs) are a class of small regulatory RNAs that have been identified in a range of tissues including the brain. These miRNAs are 18–25 nucleotides long and are generated from long polymerase II-transcribed RNA by a series of cleavage events mediated by protein complexes formed by Drosha and Dicer. miRNAs guide the RNA-induced silencing complex (RISC) to the 3' or 5' UTR of mRNAs that have a target sequence complementary to that of the miRNA. This miRNA-mRNA complementarity however, is not absolute. The pairing involves

nucleotides 2–7 of the miRNA known as the *seed sequence*. In most cases, the mRNA targeted by RISC will be either translationally silenced or destabilized and degraded. In both cases, this results in a decrease in protein production with consequences that depend on the function of the target mRNA (Filipowicz, Bhattacharyya, and Sonenberg 2008). In the nervous system, for instance, these consequences may include effects on neurogenesis, neuronal survival or apoptosis and dendritic spine formation, among others. The most significant feature of miRNAs is their ability to control entire networks of genes in neurons and neural cells under different physiological conditions or pathogenic stimuli. Moreover, there are a group of miRNAs that are brain-enriched and neuron-specific or glia-specific (astrocytes, microglia) that is crucial for synaptic plasticity and cell death or cell survival (Prada et al. 2018).



MicroRNAs (miRNAs) interact with their mRNA targets by base pairing. In plants, most miRNAs base pair to mRNAs with nearly perfect complementarity and induce mRNA degradation by an RNAi-like mechanism — the mRNA is cleaved endonucleolytically in the middle of the miRNA–mRNA duplex[29]. By contrast, with few exceptions, metazoan miRNAs base pair with their targets imperfectly, following a set of rules that have been identified by experimental and bioinformatics analyses[30–34].

- One rule for miRNA–target base paring is perfect and contiguous base pairing of miRNA nucleotides 2 to 8, representing the 'seed' region (shown in dark red and green), which nucleates the miRNA–mRNA association. GU pairs or mismatches and bulges in the seed region greatly affect repression. However, an A residue across position 1 of the miRNA, and an A or U across position 9 (shown in yellow), improve the site efficiency, although they do not need to base pair with miRNA nucleotides.
- Another rule is that bulges or mismatches must be present in the central region of the miRNA–mRNA duplex, precluding the Argonaute (AGO)-mediated endonucleolytic cleavage of mRNA.
- The third rule is that there must be reasonable complementarity to the miRNA 3′ half to stabilize the interaction. Mismatches and bulges are generally tolerated in this region, although good base pairing, particularly to residues 13–16 of the miRNA (shown in orange), becomes important when matching in the seed region is suboptimal[31,33].

Other factors that can improve site efficacy include an AU-rich neighbourhood and, for long 3′ UTRs, a position that is not too far away from the poly(A) tail or the termination codon; these factors can make the 3′ UTR regions less structured and hence more accessible to miRNP recognition[33,34,129]. Indeed, accessibility of binding sites might have an important effect on miRNA-mediated repression[130]. Some experimentally characterized sites deviate significantly from these rules and can, for example, even require a bulged nucleotide in the seed region pairing[131,132]. In addition, combinations of sites can require a specific configuration (for example, separation by a stretch of nucleotides of specific sequence and length) for efficient repression[131]. Usually, miRNA-binding sites in metazoan mRNAs lie in the 3′ UTR and are present in multiple copies. Importantly, multiple sites for the same or different miRNAs are generally required for effective repression[30–34]. When they are present close to each other (10–40 nucleotides apart) they tend to act cooperatively, that is, their effect exceeds that expected from the independent contributions of two single sites[30,33].

Figure 4: Principles of miRNA-target mRNA interaction (Filipowicz, Bhattacharyya, and Sonenberg 2008)

2.8.1 Canonical miRNA biogenesis

Animal miRNAs are encoded as individual genes (monocistronic), as gene clusters (polycistronic) or in introns of host genes (intronic). In the nucleus, miRNA genes are transcribed by RNA polymerase II which generates primary miRNA (pri-miRNA) transcripts containing hairpin loops and 5′ and 3′ flanking sequences. The pri- miRNA is cleaved at the stem of the hairpin by a

microprocessor complex consisting of a DiGeorge critical region 8 (DGCR8) dimer and Drosha. As a consequence, a precursor miRNA (pre- miRNA) is generated with a characteristic 3′ hydroxyl group (OH), overhangs of 2 nucleotides and a 5′phosphate. Next, Exportin 5 (Exp5) binds to the pre- miRNAs and facilitates its export into the cytoplasm, where it is cleaved by Dicer at the region within the stem close to the terminal loop liberating a miRNA duplex intermediate. Now, Dicer, *trans*- activation-responsive RNA- binding protein (TRBP) and an Argonaute (AGO) protein assemble to form a loading complex whereby one miRNA strand (guide strand) is preferentially transferred to the AGO protein, resulting in the formation of the RNA- induced silencing complex (RISC) (Treiber, Treiber, and Meister 2019).

2.8.2 Mechanism of miRNA function

Upon loading of mature miRNAs into the RNA- induced silencing complex (RISC), the miRNA guides RISC to complementary sequences located mainly in the 3′untranslated region (3'UTR) of its target mRNAs. This may induce deadenylation and decay of target. Alternatively, it can lead to repression of translation initiation at either the cap-recognition stage or at the 60S subunit joining stage. These mRNAs, thus repressed by deadenylation or at the translation-initiation stage, are then moved to P-bodies (discrete cytoplasmic granules of eukaryotic cells) for either degradation or storage. Repression may also occur at post-initiation phases of translation, as a consequence of either slowed elongation or ribosome 'drop-off'. Alternatively, proteolytic cleavage of nascent polypeptides may also lead to miRNA-induced repression of target protein production (Filipowicz, Bhattacharyya, and Sonenberg 2008).

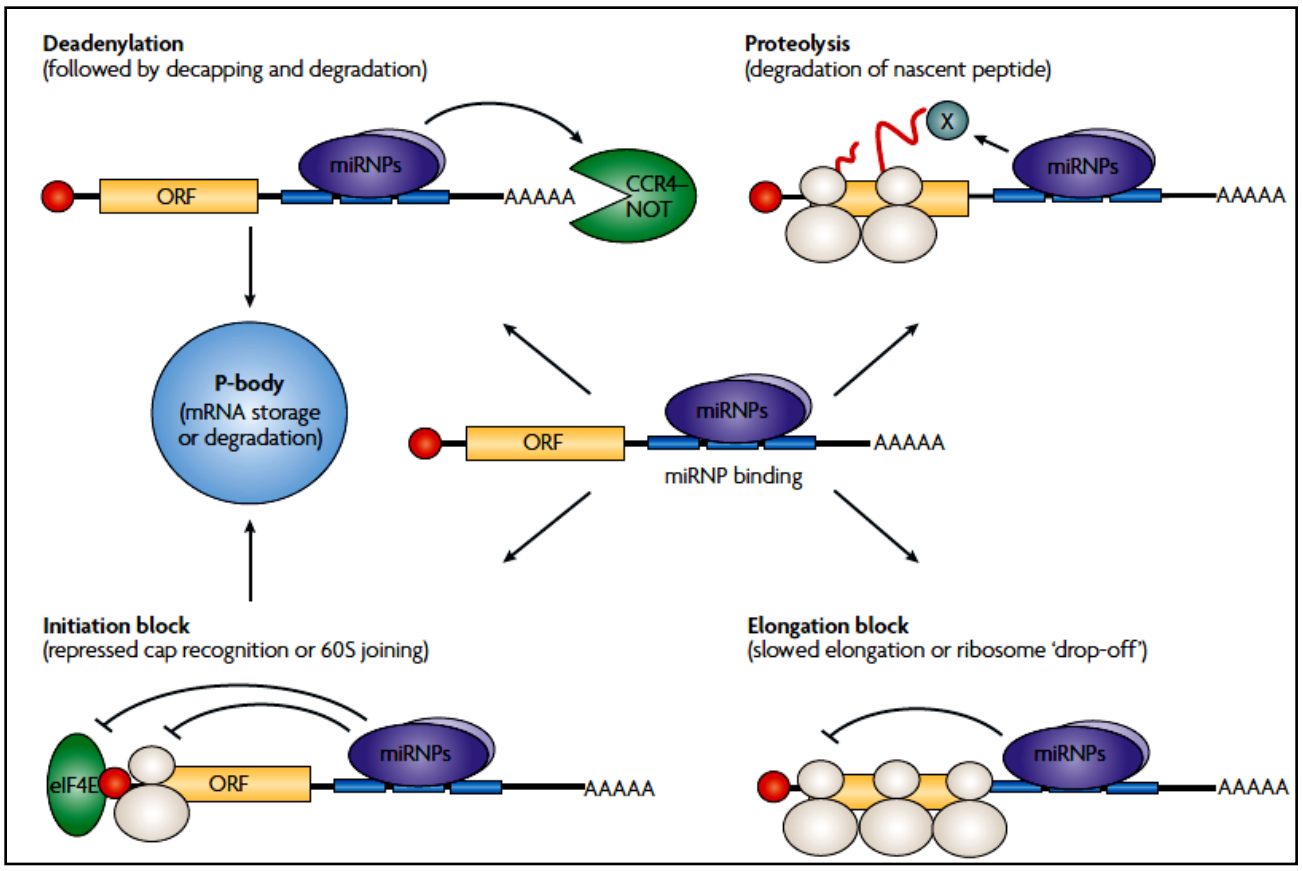


Figure 5: Mechanisms of miRNA-mediated gene repression in animals (Filipowicz, Bhattacharyya, and Sonenberg 2008)

2.8.3 miRNAs as therapeutics for PD

The most robust feature of miRNA mediated regulation is that a single miRNA may have multiple mRNA targets, potentially affecting different pathways, whereas a single mRNA maybe regulated by multiple miRNAs under a given condition. Currently, various miRNA-based strategies are being explored as a therapeutic for PD. miRNA mimics and anti-miRNAs or antago-miRs may be synthesized as potential therapeutic tools in a number of pathological conditions, including PD. *miRNA mimics* are double-stranded RNA molecules that are synthesized to match the mRNA target sequence in order to restore the miRNA activity; while on the other hand, *antago-miRs* are single-

stranded RNA molecules, designed to target the miRNAs to be inhibited. Both mimics and antago-miRs are capable of being administered to various tissue types without the need of a delivery vehicle or strategy. However, the main road-block in using miRNAs to treat NDDs is the ability to cross the blood-brain-barrier (BBB). One of the strategies that may be used to deliver miRNAs into the mammalian brain is through viral vectors, such as the recombinant adeno-associated viruses (rAAV) or lentiviruses. Among the non-viral vector methods, lipid-based carriers (liposomes) may be used. Another powerful way to deliver miRNAs into the brain is represented by the *exosomes*, which can easily cross the BBB, have reduced immunogenicity and show no cellular toxicity. In context to brain disorders, antisense inhibitor against miR-10b developed by Regulus Therapeutics is in preclinical trial for glioblastoma (Li et al. 2014).

2.9 Exosomes as carriers of miRNAs

Exosomes: Extracellular vesicles (EVs) are membrane-bound vesicles that are released from parent cells into the extracellular space to carry cellular cargo that do not readily cross the plasma membrane barrier. Such cargo includes membrane proteins, cytosolic proteins, genetic material like mRNAs as well as non-coding RNAs like miRNAs. EVs may be categorized into microvesicles (100 nm to 1 μm in diameter) and exosomes (30–100 nm in diameter), on the basis of their subcellular origin- the plasma membrane or multivesicular bodies (MVBs) respectively. Apoptotic cells also release vesicles (0.8–5.0 μm in diameter) called apoptotic bodies. However, apoptotic bodies are engulfed by phagocytic cells immediately upon their release into the extracellular milieu and thus probably do not have a role in intercellular communication in the nervous system (Budnik, Ruiz-Cañada, and Wendler 2016).

Mode of formation: MVBs, the source of exosomes, can form via the endocytic or the secretory pathway inside the cell. During this process, the vesicles which originate by endocytosis at the plasma membrane or those which are generated by the Golgi complex, fuse with the limiting membrane of the endosomal compartment and bud inwardly into the lumen of the endosome. Secretory MVBs fuse with the plasma membrane and shed exosomes into the extracellular space. In contrast to exosomes, microvesicles form by the outward budding of the plasma membrane, whereby microvesicles are released after the bud pinches off from the cell surface. On the other hand, an apoptotic cell sheds apoptotic bodies, which bud off from the plasma membrane. Tetraspanins (CD63 and CD81), the ESCRT (endosomal sorting complex required for transport) proteins, ALG2-interacting protein X (ALIX) and tumor susceptibility gene 101 (TSG101) protein are frequently used as identifying markers for exosomes (Turchinovich, Weiz, and Burwinkel 2012).

Exosomes as shuttles for therapeutic intervention: Among the EVs, *exosomes* are the most significantly studied modes of cell-to-cell communication and in the brain, they are the major mediators of neuron-glia (astrocytes, microglia) cross-talk. Depending on their cargo content, exosomes may show both neuro-detrimental and neuro-protective functions in brain pathologies. Several features make exosomes potentially powerful shuttles for the delivery of therapeutic agents to the brain (Prada et al. 2018). Exosomes can cross the BBB, possess low immunogenicity and may be loaded with therapeutic miRNAs targeted for specific cell types. Additionally, exosomes may constitute non-invasive biomarkers for the diagnosis and the progression of PD, as they are readily detectable in almost all bodily fluids.

2.10 Role of astrocytes in PD

Neuroglia: Neuroglia are non-neuronal cells in the nervous system that occupy almost half of the brain space and outnumber the neurons by 10 to 1. There are two major classes of glia in the central nervous system (CNS): the macroglia, consisting of astrocytes and oligodendrocytes, and the microglia, which are macrophage-like cells of the brain. While both astrocytes and oligodendrocytes develop from neuroepithelial cells, the origin of microglia remains debatable, with majority favoring a hematopoietic origin (Ferreira and Romero-Ramos 2018).

Physiological function: Glial cells, particularly astrocytes are involved in some major aspects of neural function. Under normal physiological conditions, astrocytes are responsible for maintaining the homeostatic environment for proper neuronal functioning. Astrocytes are known to store energy, buffer pH, balance ion concentrations (e.g. K+) and recycle neurotransmitters (e.g. glutamate) after neuronal excitation. Astrocytes are also critical components of the neurovascular unit- they ensheath the microvessels via specialized endfoot processes, thereby maintaining the integrity of the BBB. Additionally, astrocytes form synaptic communication with neurons by enwrapping synaptic terminals, thereby forming the *tripartite synapse*, one of the hallmarks of neuronal identity (Verkhratsky and Nedergaard 2018). Morphologically, astrocytes are in close association with neurons, have extensive contacts with capillaries and are interconnected with other astrocytes by gap junctions. Astrocytes, therefore, are the mediators for signaling between neurons-astrocytes, astrocytes-astrocytes and neurons-capillaries (Hasel and Liddelow 2021).

Pathological role: For decades, astrocytes were considered as non-excitable support cells of the brain or 'brain glue'. In recent times, however, it has been observed that during CNS pathology, astrocytes often take on a different morphology, in other terms, become 'reactive'. During *astrocyte activation* or *astrocyte reactivity*, the common morphological changes include- hypertrophy of astrocyte somata and processes with a concomitant increase in the expression of the glial fibrillary acidic protein (GFAP). Besides activated microglia, astrocyte reactivation is another hallmark of neuroinflammation and is a widely documented feature of acute CNS injuries as well as NDDs. Additionally, under pathological conditions, astrocytes are known to play divergent and complex roles- they act as a defense system by producing trophic factors and anti-inflammatory cytokines (e.g. TIMP1, ICAM-1) (Saha et al. 2020; Guha et al. 2022) for neuronal survival or may even mediate neuronal degeneration by releasing pro-inflammatory cytokines (e.g., interleukin 1β (IL-1β), tumor necrosis factor α (TNFα), and interferon γ, under increased levels of stress. Thus, astrocyte reactivation and/or dysfunction are major consequences as well as contributors of neuropathology during various NDDs (Liddelow and Barres 2017).

Astrocytic Ca^{2+} signaling: Unlike neurons, although astrocytes are not electrically excitable, they still display a complex network of intracellular Ca^{2+} signalling that is temporally and spatially regulated either at a single astrocyte level or across inter-astrocytic networks. The physiological significance of these dynamic changes in astrocytic Ca^{2+} levels however, still remains largely unclear. Astrocytic intracellular Ca^{2+} signals are vital for the optimal functioning of the CNS and maybe considered the astrocytic counterparts of neuronal membrane potential dynamics like action potentials (Bazargani and Attwell 2016). However, compared to neuronal

excitability, the properties, underlying mechanisms and physiological or pathophysiological significance of astrocytic Ca^{2+} signals are far less understood till date. Hence, decoding astrocytic Ca^{2+} signalling is a current challenge in understanding the molecular and cellular aspects of astrocyte physiology in context to neuropathologies.

2.11 $Ca^{2+/}$Calcineurin signaling in astrocytes

Calcineurin (CaN): is a $Ca^{2+/}$Calmodulin ($Ca^{2+/}$CaM)-dependent protein phosphatase expressed in almost all mammalian tissues, and is found at especially high levels in the brain. Within the cell, CaN is ubiquitously present throughout the cytosol and the nucleus. The CaN holoenzyme contains a 60 KDa catalytic subunit (CaN A) and a 19 KDa regulatory subunit (CaN B). CaN A contains the catalytic core and binding sites for CaN B and $Ca^{2+/}$CaM. An autoinhibitory domain is present near the C terminus that suppresses the catalytic activity of CaN A when Ca^{2+} levels in the cells are low. The CaN B subunit contains four Ca^{2+-}binding sites and is physically bound to CaN A at resting Ca^{2+} levels. During rapid intracellular Ca^{2+} fluctuations, allosteric interactions occur between CaN B, $Ca^{2+/}$CaM, and the autoinhibitory domain, leading to activation of CaN (Sompol and Norris 2018).

Astrocytic CaN and neuroinflammation: In the mid-1980s to early 1990s it was believed that high levels of CaN are only expressed in the neurons, with little to no expression reported in the glial cells. Recently however, Christopher M. Norris and his group have identified CaN expression in primary astrocytes and astrocytic cells of intact brain tissue, notably during AD associated neuroinflammation. A significantly high number of CaN positive astrocytes have been reported in the hippocampus of postmortem AD brain tissue, particularly in the immediate

vicinity of extracellular Aβ deposits. Moreover, CaN was found to be critical for triggering astrocyte phenotype switching (i.e., reactivate vs. non-reactivate) and neuroinflammatory signaling inherent to astrocytes during neural degeneration and dysfunction (Norris 2005). Interestingly, CaN pathways are capable of both driving and resolving neuroinflammatory signaling in astrocytes. Factors that activate astrocytes (cytokine, Aβ peptides) stimulate CaN activation as well. CaN in turn, directly dephosphorylates and activates the transcription factors NFAT and/or NFκB. NFATs and NFκB translocate to the nucleus, and drive the expression of numerous pro-inflammatory cytokines involved in the generation and maintenance of neuroinflammation (Abdul et al. 2010). CaN may also dephosphorylate and activate FOXO3 transcription factors, which, in synergy with NFκB, may contribute to immune/inflammatory signaling in astrocytes. On the other hand, activation of IGF-1 receptors in astrocytes also leads to activation of CaN, but this suppresses its interaction with FOXO3 while simultaneous activating PPARγ and NFκB. This leads to reduction or resolution of neuroinflammation. Thus, the clear connection between astrocytes and neuro-immune signaling indicates a strong linkage between astrocytic CaN and neuroinflammation which is an inherent characteristic of most acute and chronic NDDS, including PD (Furman and Norris 2014).

Chapter 3:

Materials & Methods

3.1 Materials

Table S1:

miRNA assay	**Catalog number (Ambion)**
hsa-miR-128a	4427975 Assay ID: 002216
U6	4427975 Assay ID: 001973
miR-128 mimic	MC11746
Negative control mimic	4464058
hsa-miR-23a	4427975
miR-23a mimic	MC10644

Table S2:

mRNA primers	**Sequence**
PUMA forward primer	5'ACGACCTCAACGCACAGTACGA3'
PUMA reverse primer	5'CCTAATTGGGCTCCATCTCGGG3'
FasL forward primer	5'GGTTCTGGTTGCCTTGGTAGGA3'
FasL reverse primer	5'CTGTGTGCATCTGGCTGGTAGA3'
GAPDH forward primer	5'TCAACAGCAACTCCCACTCTT3'
GAPDH reverse primer	5'ACCCTGTTGCTGTAGCCGTAT3'

Table S3:

Primary antibodies	Catalog number
p-FoxO3a (Ser253)	Cell Signaling Technology (9466)
FoxO3a	Cell Signaling Technology (2497)
PUMA	Novus Biologicals (NBP1-76639)
FasL	Santa Cruz Biotechnology (sc-6237)
PSD-95	Santa Cruz Biotechnology (sc-32290)
Synaptophysin	Novus Biologicals (NBP2-25170)
Calcineurin A	Cell Signaling Technology (2614)
β-Actin	Sigma-Aldrich (A3854)

3.2 Methods:

3.2.1 Human blood sample collection and plasma separation

Blood samples were collected from PD patients (age>45 years) and respective age-matched controls in EDTA-vials (BD Biosciences). Plasma was separated from the blood samples by centrifugation at 2000*g for 10 minutes at 4^0C. The upper yellow plasma layer was separated and further centrifuged at 3000*g for 15 minutes at 4^0C. The clear supernatant (plasma) was used for further downstream processing.

3.2.2 Exosome isolation by miRCURY kit

Exosomes were separated from the human plasma samples by miRCURY Exosome Serum/Plasma kit (Qiagen) as per manufacturer's protocol. Briefly, plasma samples were incubated with Thrombin at room temperature for 5 minutes and then centrifuged at 10,000*g for 5 minutes. The supernatant was collected and Precipitation Buffer was added to it. The sample was allowed to incubate overnight at 4^0C. It was then centrifuged at 500*g for 5 minutes at 20^0C. Resuspension buffer was added to the pellet and this purified exosome sample was used for further downstream processing. Alternatively, the pellet was resuspended in respective cell culture growth medium and added to recipient cells.

3.3.3 Extracellular vesicle isolation by ultracentrifugation

The astrocyte conditioned medium (ACM) were subjected to extracellular vesicle (EV) isolation (Ghoshal, Bertrand, and Bhattacharyya 2021). Briefly, the ACM was centrifuged at 2000 g for 15 min to remove all cell debris. Next, the supernatant was collected and centrifuged at 10,000 g for 30 min. The supernatant thus obtained was further passed through a 0.22μm filter unit. This was followed by ultracentrifugation of the supernatant at 100,000 g for 90 min. After ultracentrifugation, the pellet was resuspended in respective cell culture growth medium and added to recipient cells.

3.2.4 Nano-partice tracking analysis (NTA) of exosomes

Purified exosomes were 10-fold diluted in 1X PBS and subjected to nano-particle tracker analysis (NTA) (NanoSight NS300) as per manufacturer's guidelines.

3.2.5 Human small-RNA Seq datasets

For human small RNA-Seq (sRNA-Seq) datasets, repository resources from Diana: miTED (http://www.microrna.gr/mited) (Kavakiotis et al. 2022) and EVAtlas (http://bioinfo.life.hust.edu.cn/EVAtlas) (Liu et al. 2022) were used.

3.2.6 RNA isolation and quantitative real-time PCR (qRT-PCR)

RNA isolation was done using TRIzol Reagent (Thermo Fisher) as per phenol-chloroform based total RNA extraction method, following manufacturer's protocol. For miRNA analysis, cDNA was prepared using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher) using manufacturer's protocol, followed by qRT-PCR using TaqMan Universal PCR Master Mix (Thermo Fisher) and specific miRNA assay reagents (Thermo Fisher) as enlisted in Table S1. For mRNA analysis, cDNA was prepared using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio) using manufacturer's protocol, followed by qRT-PCR using SYBR Green PCR Master Mix (Thermo Fisher). The primers used for mRNA qRT-PCR based analysis are enlisted in Table S2. U6 snRNA and GAPDH mRNA were used as endogenous controls in miRNA and mRNA qRT-PCR analysis respectively. All qRT-PCR reactions were done in StepOnePlus Real-Time PCR System (Thermo Fisher). Comparative C_T Method ($\Delta\Delta C_T$ Method) was used for the qRT-PCR data analysis.

3.2.7 miRNA target prediction

TargetScan (http://www.targetscan.org/), Pictar (http://pictar.mdc-berlin.de), and microRNA.org-Targets and Expression (http://www.microrna.org/ microrna/home.do) databases were used for prediction of potential miRNA binding sites on 3′UTR of transcripts.

3.2.8 Primary culture of astrocytes

Primary astrocytes were cultured following the protocol described earlier (Garwood et al. 2011; Saha and Biswas 2015). Briefly, 0-1 day old Sprague Dawley rat pups were taken and the whole brain was dissected out while carefully removing the meninges. Cortex was isolated and cut into small pieces. These cortical tissue pieces were further minced and subjected to trypsinization for 30 min at 37^0C. Trypsinized brain tissue was triturated in DMEM medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum/FBS (Gibco) and passed through a nylon mesh to avoid clumps. The single cell suspension thus obtained, was added onto PDL (working concentration 0.1 mg/ml) coated plates and incubated for 2–3 min for preferential sticking of neurons. Next, the unattached cells were collected and harvested by centrifugation at 500g for 5mins. Cells were resuspended in freshly prepared medium (DMEM+ 10%FBS) and seeded at a density of 1.2 million/35 mm plate or 0.4 million/well of a 24-well plate. Cells were maintained for 14 DIV with medium change given every other day.

3.2.9 Primary culture of dopaminergic neurons

Primary dopaminergic neurons (DN) were cultured from the midbrain of E18 rat embryo in neurobasal medium supplemented with B27 for 14 days (Weinert et al. 2015).

3.2.10 Human SH-SY5Y cell line maintenance and differentiation

The human neuroblastoma cell line SH-SY5Y (NCCS, Pune) was maintained in DMEM medium supplemented with 10% FBS and differentiated for 5-7 days in the same medium supplemented with all-trans retinoic acid/ATRA (10μM) (Sigma).

3.2.11 Rat PC12 cell line maintenance and differentiation

The rat pheochromocytoma (PC12) cell line was maintained in DMEM medium supplemented with 10% heat-inactivated horse serum/HS (Gibco) and 5% FBS. It was differentiated in DMEM medium supplemented with 1% HS and nerve growth factor/NGF−β (50ng/mL) (Sigma) for 5-7 days.

3.2.12 Human 1321N1 cell line maintenance

The human astrocytoma cell line 1321N1 was maintained in DMEM+ 10% FBS.

3.2.13 6-OHDA treatment

6-OHDA or 6-hydroxydopamine (Sigma) was dissolved in DMEM medium to prepare a stock concentration of 10mM. It was added to the cells *in vitro* at a final concentration of 100μM or at indicated concentrations for respective time-points as mentioned in the experiments.

3.2.14 Rotenone treatment

Rotenone (Sigma) was dissolved in DMSO to prepare a stock concentration of 1.25mM. It was further diluted in DMEM medium to prepare a sub-stock at 10μM concentration and added to the cells at a final concentration of 200nM.

3.2.15 MPP+ treatment

1-Methyl-4-phenylpyridinium (MPP+) was dissolved in DMEM medium to prepare a stock concentration of 10mM and added to cells at a final concentration of 500μM.

3.2.16 Acute mouse model of MPTP

10-week-old C57Bl6 mice (n = 4 per group) were divided into two different groups consisting of (i) a control group treated with saline alone; (ii) a group treated with MPTP alone. MPTP at a dose of 20 mg/kg free base was administered intraperitoneally four times a day every 2 h. All animals were sacrificed on the 7th day after MPTP treatment (Kaidery et al. 2013; Thomas et al. 2012; L'Episcopo et al. 2011).

3.2.17 Immunohistochemistry

The C57Bl6 mice- both wild-type and MPTP treated, were anesthetized with sodium pentobarbital, transcardially perfused with 0.9% saline followed by 4% paraformaldehyde in 0.1 M PBS (pH 7.4). Brains were dissected, taken out, post-fixed in 4% paraformaldehyde for 24 h and cryopreserved in 30% sucrose/PBS for 48 h. The snap-frozen brains were coronally sectioned at 40 mm thickness using a cryostat (Thermo Shandon, Pittsburg, PA, USA). Coronal sections were collected in PBS and processed free-floating. Sections from substantia nigra (SNPc) region were washed in PBS and endogenous peroxidase was quenched by incubation with 3% H2O2 in 10% methanol solution for 10 min. Sections were then washed in PBS and blocked in 10% normal goat serum (NGS) and 0.1% Triton X-100 in PBS for 1 h at room temperature (RT). To assess GFAP co-localization with Calcineurin, double immunofluorescence labeling was performed. Sections were incubated with rabbit polyclonal anti-CN A antibody (1:50) and mouse monoclonal anti-GFAP antibody (1:50) in 2% NGS containing 0.1% Triton X-100 at 4^0C overnight. Next, sections were washed in PBS and incubated in Alexa Fluor conjugated secondary antibodies in PBS containing 10% NGS, 0.1% Triton X-100 for 1 h at RT. Sections were washed in PBS three times and mounted on gelatin coated glass slides using mounting media (Prolong Gold Antifade, Invitrogen). For microscopic

analysis, fluorescence was visualized using a confocal microscope (Zeiss). The corrected total cell fluorescence (CTCF) was calculated by including integrated density of staining, area of the cell, and the background fluorescence of different experimental conditions. CTCF = Integrated density − (area of selected cell × mean fluorescence of background readings).

3.2.18 miRNA mimic transfection

SH-SY5Y or PC12 cells were plated and primed for 2 days in the presence of ATRA or NGF-β respectively. Transfection with miRNA mimic and negative control mimic (NCM) (Ambion) (Table S1) was done on the second day of priming in Opti-MEM medium (Thermo Fisher) using Lipofectamine RNAiMAX Reagent (Invitrogen), following manufacturer's protocol. Six hours post-transfection, Opti-MEM medium was replaced with the respective priming medium. Cells were further maintained for 48 hours and then treated with 6-OHDA on the fifth day of priming.

3.2.19 GW4869 treatment

GW4869 (Calbiochem) was dissolved in DMSO to prepare a stock solution of 5 mM. It was then diluted in culture medium to achieve final concentration of 10 μM.

3.2.20 VIVIT transfection

GFP-VIVIT plasmid (Addgene) was transfected in 1321N1 cells using Lipofectamine 3000 (Invitrogen), following manufacturer's protocol.

3.2.21 Plasmid isolation

Cells were either transformed in DH5-α or Stbl3 cells and plated on Luria agar medium with appropriate selection. The clones formed colonies on the plate. A single colony was taken and inoculated in 50ml Luria broth. After overnight incubation at 37^0C and 200rpm, plasmids were isolated from the broth using Hi-pure plasmid mini-preparation kit (Qiagen) followed by plasmid midi-prep kit (Qiagen), as per manufacturer's protocol.

3.2.22 3'UTR cloning

To produce reporter plasmids containing 3′UTR of mouse PUMA, sequences were PCR-amplified, digested, gel purified, ligated, and cloned into pmirGLO vector (Promega), downstream of the luciferase coding region, digested with XbaI and SalI restriction enzymes. The following primers (Sabirzhanov et al. 2020) were used to amplify 3'UTR of mouse PUMA:

Forward primer 5'**TCTAGA**GTGCCTACACCCGCCCGG3'

Reverse primer 5'**GTCGAC**CACTGTTCAATCTGATTTTATTGAAAAGGA3'

3.2.23 Luciferase assay

PC12 cells were seeded in 96-well plates. For control samples, cells were co-transfected with 150 ng pmirGLO reporter plasmid without any insert (Promega) along with 50 nM NCM. For test samples, cells were transfected with 150 ng reporter plasmid along with 50 nM miR-23a mimic. Lipofectamine 2000 and RNAiMAX (Invitrogen) were used for transfection of plasmid DNA and miRNA mimics, respectively. 24 hours after transfection, cells were analyzed for luciferase activity using the Dual-Glo Luciferase Assay System (Promega) as per the manufacturer's protocol using a multimode plate reader (Perkin Elmer). Normalized firefly luciferase activity (firefly luciferase

activity/Renilla luciferase activity) for each construct was compared to that of the pmirGLOVector no-insert control.

3.2.24 Flow cytometry

Cells were analysed by flow cytometry using LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher), using manufacturer's protocol. Briefly, cells *in vitro* were trypsinized and a cell suspension was prepared in culture medium. Green fluorescent dye Calcein-AM (staining live cells) and red fluorescent dye Ethidium homodimer-1 (staining dead cells) was added to the cell suspension and incubated at room temperature for 15-20 minutes in dark. Flow cytometric analysis was done with excitation at 488nm using BD LSRFortessa.

3.2.25 Live-cell imaging for mitochondrial superoxide detection

Cells *in vitro* were incubated with MitoSOX Red reagent (Thermo Fisher) prepared in HBSS buffer ($Ca^{2+,}$ $Mg^{2+,}$ no phenol red) for 10 minutes in dark in the humidified cell culture incubator (37^0C, 5% CO_2). Cells were washed with warm buffer and imaged under fluorescent microscope (Leica) under 20X objectives with excitation at 510nm and emission at 580nm.

3.2.26 Subcellular fractionation

Subcellular fractionation was done as described earlier (Sanphui and Biswas 2013). Briefly, the cells were collected and resuspended in buffer A (20 mM HEPES, 40 mM Kcl, 1 mM DTT, 0.2 mM sodium orthovanadate, 2 mM MgCl2, 10% glycerol, 0.1 mM EDTA, 0.1 mM EGTA, 0.25 M sucrose, protease inhibitors). Cells were then lysed with 20–30 strokes in a homogenizer. Lysates were then centrifuged for 10 min at 200 g in a microcentrifuge. The supernatant thus obtained is the

cytosolic fraction. The pellet contains the crude nuclear fraction and unbroken cells. The pellet was further washed twice in buffer B (0.5% ethylhexadecyl dimethyl ammonium bromide, 0.28% glacial acetic acid, 0.5% Triton X-100, 2 mM MgCl2, 2.2 mM NaCl, 0.1× PBS). Buffer B lyses cell membrane leaving intact the nuclear membrane. The lysate was centrifuged at 425 g for 10 min and the supernatant was discarded. The pellet was washed twice in ice-cold PBS and resuspended in 10 mM HEPES, 500 mM Nacl, 1% TritonX, 10% glycerol, 1mMNaF, and 1 mM Sodium orthovanadate and sonicated three times for 10 s each. This is the nuclear fraction. All the steps were performed at 4°C.

3.2.27 Immunoblotting

Cultured cells *in vitro* were lysed in RIPA buffer (Thermo Scientific) containing proteasome inhibitor cocktail (Clontech) and protein samples (25-50µg) from whole cell lysates were resolved by SDS-PAGE. Gels were transferred on PVDF membranes (GE Healthcare) at 100V for 1-2 hours at 4^0C. Membranes were blocked in 5% non-fat dry milk or 5% bovine serum albumin (BSA) for 1 hour at room temperature. Primary antibodies were diluted in the blocking solution (Table S3) and incubated overnight at 4^0C. HRP-conjugated secondary antibodies diluted in blocking solution were used against the respective primary antibodies and incubated at room temperature for 1 hour. Detection of protein bands was carried out by using Clarity Max Western ECL substrate (Bio-Rad) as per manufacturer's protocol. Images of all the blots were taken using iBright Imaging system (Thermo Fisher).

3.2.28 Immunoprecipitation

The cells were washed with PBS and lysed in lysis buffer as described previously. For immunoprecipitation, 3 µg anti-FoxO3a antibody was incubated with 25 µl of protein Agarose beads for 2 hr in 4°C under shaking condition. The agarose-conjugated FoxO3a antibodies were collected and incubated with cell lysates containing equal amounts of protein, overnight at 4°C under shaking condition. The antigen–antibody complexes were then isolated and boiled in sample buffer for 5 min. The agarose beads were collected and the supernatant was subjected to immunoblotting using anti-acetylated lysine (Abcam) as primary antibody.

3.2.29 Chromatin Immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were done using ChIP assay kit (Millipore) following the manufacturer's protocol. Rabbit polyclonal anti-FoxO3a antibody was used to immunoprecipitate the protein-DNA complexes. The primers used for PCR amplification of the rat FasL promoter were as follows:

Forward primer: 5’ GGGAGTCTCGGTAAAAGTCATT 3’

Reverse primer: 5’ ACTAACAGGGCTATACCCCCAT 3’

PCR products were analyzed on a 1.5% agarose gel and visualized by staining with ethidium bromide. Direct binding of FoxO3a with the FasL gene was also quantified by qRT-PCR.

3.2.30 Immunocytochemistry

Immunocytochemistry was performed following the methods described previously (Saha and Biswas 2015). Briefly, cells cultured on PDL-coated glass cover slips were fixed with freshly made 4% PFA in PBS for 10 min at RT followed by two washes with PBS. The fixed cells were

permeabilized and blocked with 3% goat serum, 0.3% Triton X-100 in PBS for 1–2 h. Cells were then incubated with GFAP (1:50, Sigma-Aldrich, St. Louis, MO, USA) antibody overnight at 4 °C. Next, they were incubated with secondary antibodies Alexafluor 546/488 for 1–2 h at room temperature. Hoechst 33342 (Molecular Probes, Invitrogen, MA, USA) was added at a concentration of 2 μg/ml in PBS for 30 min at room temperature, washed carefully and mounted in Prolong Gold Anti-fade reagent (Invitrogen) for long-term storage.

3.2.31 Neurite length measurement

Primed PC12 cells were observed under bright field microscope using 20X objectives. Neurite length was measured using the NeuronJ plugin of Fiji software (https://imagescience.org/meijering/software/neuronj/manual/) (Popko et al. 2009).

3.2.32 Calcineurin assay

1321N1 cells were plated in 96-well plates and lysed on ice in lysis buffer containing protease inhibitors. Calcineurin activity assay was performed using Calcineurin Cellular Activity Assay Kit (Calbiochem) using manufacturer's protocol. Briefly, the RII phosphopeptide substrate is supplied in this kit which is a peptide substrate for calcineurin. The detection of free phosphate released is based on the malachite green assay and is detected colorimetrically at A_{620}nm. Relative CaN activity was plotted as compared to control (Moon, Hong, and Park 2021).

3.2.33 Intracellular Ca^{2+} measurement by Fluo-AM

Fluo-4AM, a calcium indicator, was dissolved in DMSO to prepare a stock solution of 1mM which was further dissolved in DMEM medium to a final concentration of 2μM. Intracellular Ca^{2+} levels

were measured using Fluo-4AM (Invitrogen) following manufacturer's protocol. Briefly, the cells were incubated with the Fluo-AM solution for 30mins at 37^0C in dark. Fluorometric readings were obtained at Ex/Em of 494/506 nm in a multi-plate reader (Thermo Scientific).

3.2.34 Caspase-8 fluorimetric assay

Caspase-8 activity was determined using CaspaTag Caspase-8 In Situ Assay Kit (Merck Millipore) following manufacturer's protocol. Briefly, fluorochrome inhibitors of caspases (FLICA reagent) was used which produces a green signal indicating the amount of active caspase-8 inside the cells. End-point reading was obtained in a multi-plate reader (Thermo Scientific) using excitation at 490nm and emission at 520nm.

3.2.35 Caspase-9 colorimetric assay

Caspase-9 activity was determined using Caspase-9 Colorimetric Activity Assay Kit (Merck Millipore) following manufacturer's protocol. Spectrophotometric detection of the chromophore product p-nitroaniline was done at 405nm in a multi-plate reader (Thermo Scientific). Fold change in the caspase-9 activity in the cells was calculated from the obtained O.D.

3.2.36 Caspase-3 colorimetric assay

Caspase-3 activity was determined using Caspase-3 colorimetric Assay Kit (Sigma-Aldrich) following manufacturer's protocol. Briefly, the hydrolysis of the peptide substrate by active caspase-3 resulted in the release of the chromophore product p-nitroaniline which was detected at 405nm using a multi-plate reader (Thermo Scientific). From the obtained readings, fold change in the caspase-3 activity was calculated.

3.2.37 Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL)

TUNEL assay was performed according to the manufacturer'sprotocol (Clontech, ApoAlert DNA Fragmentation kit, Takara). Briefly, adherent cells grown on glass coverslips were fixed with freshly pre-pared 4% PFA for 15 min. Following this they were washed with PBS twice. Cells were then permeabilized with pre-chilled 0.1% Triton-X in 0.1% Sodium Citrate solution for 5 min on ice. Next, the cells were washed twice with PBS. The samples were then equilibrated in equilibration buffer at RT for 10 min. The samples were incubated in a solution containing the nucleotide mix and Tdt enzyme for 1 h at 37^0C in dark. The reaction was stopped by 2X SSC buffer provided in the kit at RT for 15 mins. Samples were rinsed with PBS. Following this, they were stained with Hoechst solution for 30 min at 37^0C and then washed with PBS before mounting with Prolong Gold Antifade with DAPI for microscopic analysis. For quantitative analysis in cells, 4–5 equal window images were taken from different regions of the coverslip at 63 × magnification, following which total number of cells in each field (Hoechst stained) and total number of TUNEL positive cells in each field (fluorescein+ Hoechst+) were counted and percentage of TUNEL positive cells were calculated by dividing the TUNEL positive cell count with the total number of cells multiplied by 100.

3.2.38 Cell viability by Trypan blue staining

A 0.4% solution of trypan blue was prepared in PBS (pH 7.4). Next, 0.1 mL of trypan blue stock solution was added to to 0.1 mL of cells. Cells were loaded on a hemacytometer and examined immediately under a bright-field microscope. The number of blue staining cells were counted as well as the number of total cells. Cell viability was calculated as follows:

% viable cells = [1.00 – (Number of blue cells ÷ Number of total cells)] × 100

3.2.39 Cell viability by intact nuclei counting method

Survival assay of cultured neurons was performed by a method that has been routinely used to assess viability of neuronal cells (Rukenstein et al., 1991; Troy et al., 2002; Sanphui et al., 2013). Cells were lysed in a detergent containing buffer that dissolves cell membrane but not nuclear membrane. The intact nuclei were then counted on a hemocytometer.

3.2.40 Cell survival by MTT assay

MTT powder was dissolved in culture medium and added to cells *in vitro* at a final concentration of 0.5mg/ml. Cells were then incubated for 3-4 hours in the humidified cell culture incubator (37^0C, 5% CO_2) until purple formazan crystals were visible. The culture medium was carefully discarded and replaced with DMSO to dissolve the formazan crystals in an orbital shaker for 15-20 minutes. Optical density (O.D.) was measured at 570nm.

Schematic representation

All the schematic diagrams were prepared using BioRender.com.

Statistical analysis

All graphs, statistical analyses and image analyses were done using GraphPad Prism 9.0, ImageJ and Fiji softwares. Student's *t*-test was performed as unpaired, two-tailed sets of arrays. One-way ANOVA was performed followed by Tukey's multiple comparison for data sets of more than two groups. P-value <0.05 was considered significant and depicted as * while p-value<0.01 was depicted as **. All the experiments were conducted at-least 3 times. Error bars indicate mean +/- SEM.

Chapter 4:

Results & Discussion

Part I

Neuron-enriched miR-128 maintains synaptic integrity, improves neurite formation and prevents against neuronal apoptosis via the FOXO3a/PUMA/FasL axis in models of PD

4.1.1 Introduction:

More than 200 years have passed since the first incidence of PD (Obeso et al. 2017) but till this day, there is a lack in proper understanding of the disease mechanism and pathogenesis which reflects in the fact that the current treatments available for the disease are mostly symptomatic and no suitable biomarkers are available for its early detection and diagnosis yet. To address the issue, this study focuses on the brain-enriched non-coding small RNAs called microRNAs (miRNAs) that regulate post-transcriptional gene expression and may be released into the circulating body fluids packaged into membrane-bound extracellular micro-vesicles called exosomes. Over the years, miRNAs have been implicated in different pathologies. Interestingly, there are certain miRNAs which are brain-enriched and are preferentially expressed in different types of brain cells like neurons or glial cells making these miRNAs very important regulatory candidates in the pathogenesis of brain-related disorders like PD.

miR-128, is one such miRNA, which is highly enriched in the brain tissue (He et al. 2012, Shao et al. 2010), even more abundant in the neurons than the other cell types in the brain (Smirnova et al. 2005, Liu and Xu 2011). In context to motor diseases, miR-128 first came in prominence when Tan et al. (Tan et al. 2013) identified that miR-128 deficiency causes motor dysfunction and seizure induced death in epileptic mouse models while ectopic expression of miR-128 could restore excitability in post-natal dopamine responsive (D1) neurons. Since then, the relevance of miR-128 in motor function and diseases as a *motomiR* has been explored (Hawley et al. 2017) but so far, only a few reports have come out implicating the relevance of miR-128 in PD. For instance, (Zhou et al. 2018) reported that miR-128 can directly regulate AXIN1 and protect dopaminergic neurons from apoptosis in PD models. While on the other hand, (Zhang et al. 2020) showed that HIF-

1α/microRNA-128-3p axis is neuro-protective via the *Axin1*-mediated Wnt/β-catenin signaling pathway in PD models. Certain reports have detected the presence of miR-128 in the CSF of PD patients as well (van den Berg et al. 2020)**.** However, a detailed mechanistic study of the involvement of miR-128 in the apoptotic pathways leading to neuronal death associated with PD still remained unexplored.

In this study, we have identified the brain-enriched miR-128 to be significantly reduced in the neuronal cells in *in vitro* models of PD and determined its comprehensive role in the pathways leading to neuronal apoptosis and maintenance of synaptic integrity, which reveals important implications of miR-128 in pathogenesis and progression of PD.

4.1.2 Results:

miR-128 expression is decreased in neuronal cells upon 6-OHDA treatment

Our first objective was to investigate the mechanistic role of miR-128 in PD pathogenesis and progression. Since primary culture of dopaminergic neurons from rodent brains have low purity, we used standard *in vitro* models of PD for our experiments, using cell lines like human SH-SY5Y and rat PC12 cells which have reported to show dopaminergic properties upon priming with ATRA and NGF-β respectively (Falkenburger, Saridaki, and Dinter 2016) and treated these cells with the neurotoxin 6-OHDA.

Firstly, we treated primed SH-SY5Y cells with varying doses of 6-OHDA and chose the D_{50} dose of 100μM for our further experiments (Fig.1A). Next, we treated the above mentioned cells with

100 µM dose of 6-OHDA for varying time points and checked for the expression of miR-128 by qRT-PCR. Interestingly, there was a significant decrease in the expression of miR-128 with increasing time point of 6-OHDA treatment (Fig.1B). In order to confirm that this is not a cell-line specific phenomenon, we repeated the experiment in primed rat PC12 cells with 100µM dose of 6-OHDA (Sanphui, Kumar Das, and Biswas 2020) and found a similar decrease in miR-128 expression with increasing time points of treatment (Fig.1C). This proves that miR-128 expression decreases in the *in vitro* models of PD as well. Depending on these results, for our subsequent experiments henceforth, we decided to choose 100µM 6-OHDA in the treatment window of 4h-16h on either cell lines.

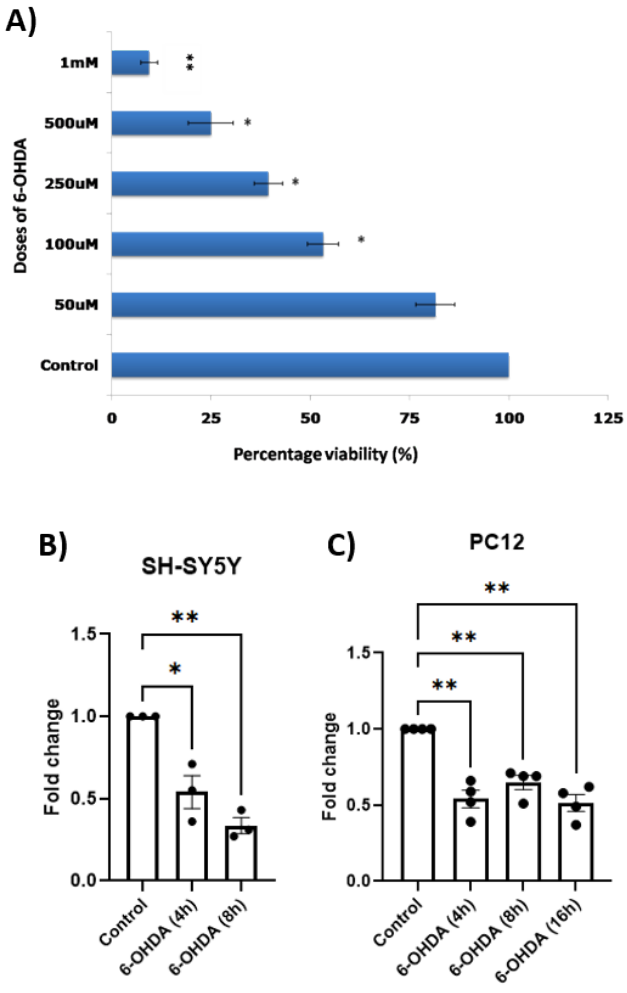


Figure. 1: Intracellular reduction of miR-128 in cellular models of PD: A) Human SH-SY5Y cells were primed with 10μM ATRA for 5 days and were then treated with varying doses (50μM, 100μM, 250μM, 500μM and 1mM) of 6-OHDA for 24h respectively. MTT assay was done after 24h and percentage viability of cells was calculated with respect to untreated (control) cells. B) Primed SH-SY5Y cells were treated with 100μM dose of 6-OHDA for 4h and 8h respectively and miR-128 expression was checked by qRT-PCR with respect to control. C) Rat PC12 cells were primed with 100ng NGF-β for 5 days and were then treated with 100μM dose of 6-OHDA for 4h, 8h and 16h respectively and miR-128 expression was checked by qRT-PCR with respect to control. U6 expression was used as endogenous control for normalization in both (B) and (C). Asterisks (*) indicate significant difference with respect to control, unless otherwise indicated in the figure by straight line between two given conditions. All experiments have been done in triplicate. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

miR-128 can prevent neuronal death and improve mitochondrial health

Since miR-128 expression was found to decrease in our PD models, we wanted to check whether miR-128 has an overall protective or detrimental role on neurons. We performed flow cytometry-based live/dead cell assay using green-fluorescent calcein-AM dye that indicates intracellular esterase activity in live cells and red-fluorescent ethidium homodimer-1 that indicates loss of plasma membrane integrity in dying or dead cells. We used either untreated SH-SY5Y cells (control) or treated cells with 6-OHDA for 16h or pre-transfected cells with miR-128 mimic or negative control mimic (NCM) for 48h before treatment with 6-OHDA. Next, the cells were incubated with calcein-AM and ethidium homodimer-1 dyes respectively and the percentage of live and dead cells was determined by flow cytometry. There was a significant decrease in live cells

(Q4) and increase in dying or dead cells (Q2+Q1) with 6-OHDA treatment which was significantly reversed in cells pre-transfected with miR-128 mimic (Fig. 2A). The fold change in the total percentage of dying and dead cells (Q2+Q1) with respect to control is expressed in Fig.2B. These results indicate that miR-128 can protect cells against neuronal death as determined through the PD models.

Now, mitochondrial deterioration is a well-known event during PD-related neurodegeneration and thus we decided to check the effect of miR-128 on mitochondrial health next. Since mitochondrial deterioration is a relatively early event during neuronal apoptosis, we treated SH-SY5Y cells with 6-OHDA for 4h. To test our hypothesis, we also pre-transfected the cells with either miR-128 mimic or NCM and then treated the cells with 6-OHDA post 48h of transfection. We performed MitoSOX assay for detection of mitochondrial superoxide production in live cells under fluorescent microscope. As compared to the control, there was an increase in superoxide production indicated by increase in red fluorescence in the 6-OHDA treated cells which was significantly reduced in the cells pre-transfected with miR-128 mimic. No such reduction in fluorescence was observed in the cells transfected with the NCM (Fig.2C). An arbitrary unit (A.U.) of red fluorescence was assigned as threshold such that cells with equal or higher fluorescence intensity were considered MitoSox positive and percentage of MitoSox positive cells were calculated (Fig.2D). Thus, miR-128 showed an overall protective effect on the neurons whereby it not only improved mitochondrial health but also protected the neurons against 6-OHDA mediated cell death.

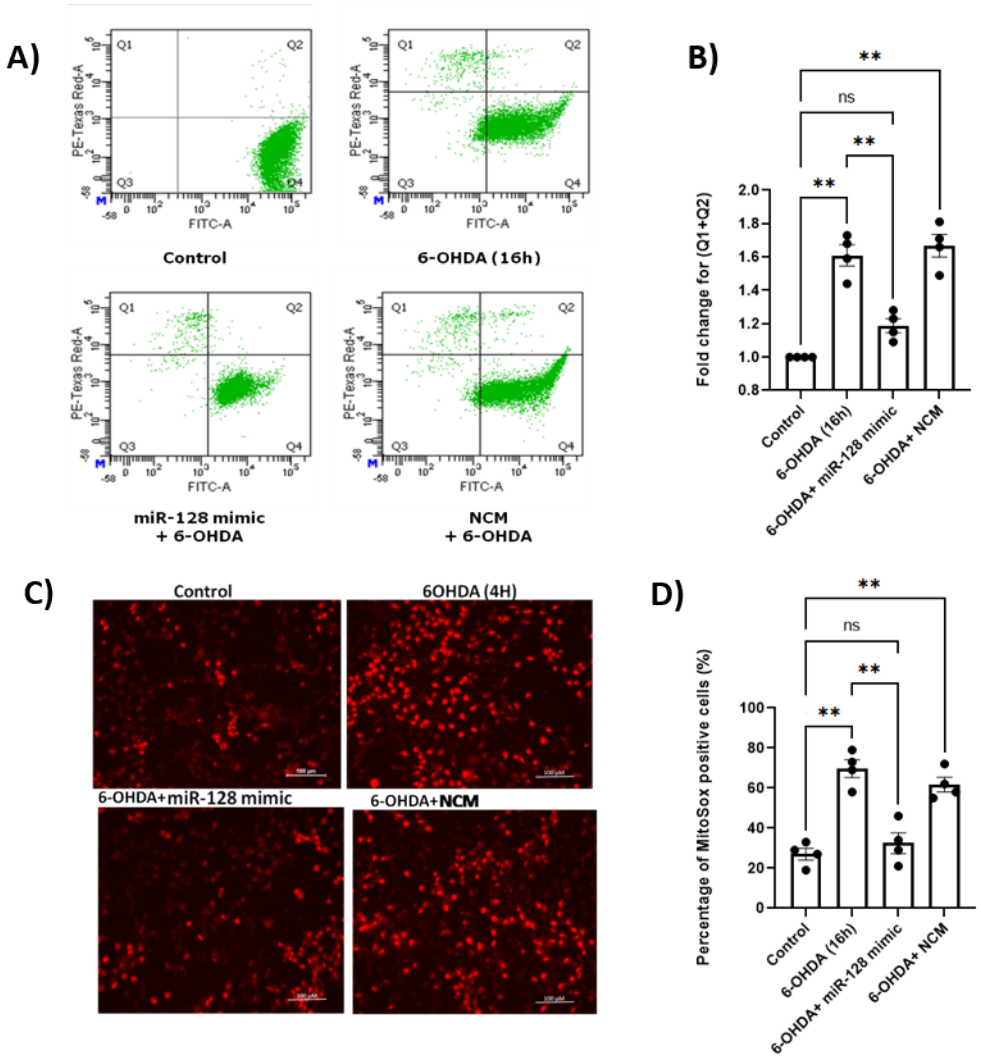


Figure 2: miR-128 overexpression protects against neuronal death and mitochondrial superoxide production: A) Primed SH-SY5Y cells were either untreated (control) or treated with 100µM 6-OHDA for 16h or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100µM) for 16h. The cells were then incubated with calcein-AM and ethidium homodimer-1 dyes respectively and the percentage of live and dead cells were determined by flow cytometry. Q4 indicates live cells while (Q2+Q1) represent total dying or dead cells respectively. B) Graphical representation of the fold change of dying and dead cells (Q1+Q2) with respect to control, as determined in (A). C) Primed SH-SY5Y cells were either untreated (control) or treated

with 100μM 6-OHDA for 4h or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100μM) for 4h. The cells were incubated with MitoSOX dye and visualized under fluorescence microscope (20X). D) Percentage of MitoSox positive cells as calculated from (C). Asterisks (*) indicate significant difference with respect to control, unless otherwise indicated in the figure by straight line between two given conditions. All experiments have been done in triplicate. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

miR-128 can regulate activation of the transcription factor FOXO3a

Keeping our above-mentioned results in mind, we decided to explore the involvement of miR-128 in the molecular pathways leading to cell death in PD i.e apoptosis. Previously we have shown that in AD models, apoptotic death in neurons is induced by activation of the transcription factor FOXO3a (Akhter, Sanphui, and Biswas 2014). We have reported activation of FOXO3a in PC12 cells under 6-OHDA treatment as well (Sanphui, Kumar Das, and Biswas 2020). Thus, we investigated whether the neuroprotective role of miR-128 is via regulation of FOXO3a activation. Activation of the transcription factor FoxO3a leads to its translocation from the cytosol to the nucleus wherein FoxO3a undergoes certain post-translational modifications (PTMs) that determine its retention in the cytoplasm or translocation to the nucleus (as reviewed by (Nho 2014)). In its deactivated form, one of the PTMs that retains FOXO3a in the cytoplasm is its phosphorylation at Ser 253 position where as upon activation, it translocates from the cytosol to the nucleus in a dephosphorylated state at Ser 253. Thus, a reduction of its phosphorylated form at Ser 253 indicates activation of FoxO3a. We used untreated SH-SY5Y cells (control) or treated the cells with 6-OHDA for 4h or pre-transfected the cells with either miR-128 mimic or NCM for 48h before treatment with 6-OHDA and subjected the protein samples to immunoblotting against p-FOXO3a

(Ser 253) andtotal FOXO3a. There was a marked reduction in the p-FOXO3a (Ser 253) levels in the 6-OHDA treated samples, indicating FOXO3a activation, which was significantly reversed in miR-128 mimic transfected cells, while the total FOXO3a levels remained relatively unchanged throughout (Fig. 3A and 3B). This indicates that miR-128 protects against neuronal death via preventing activation of FOXO3a.

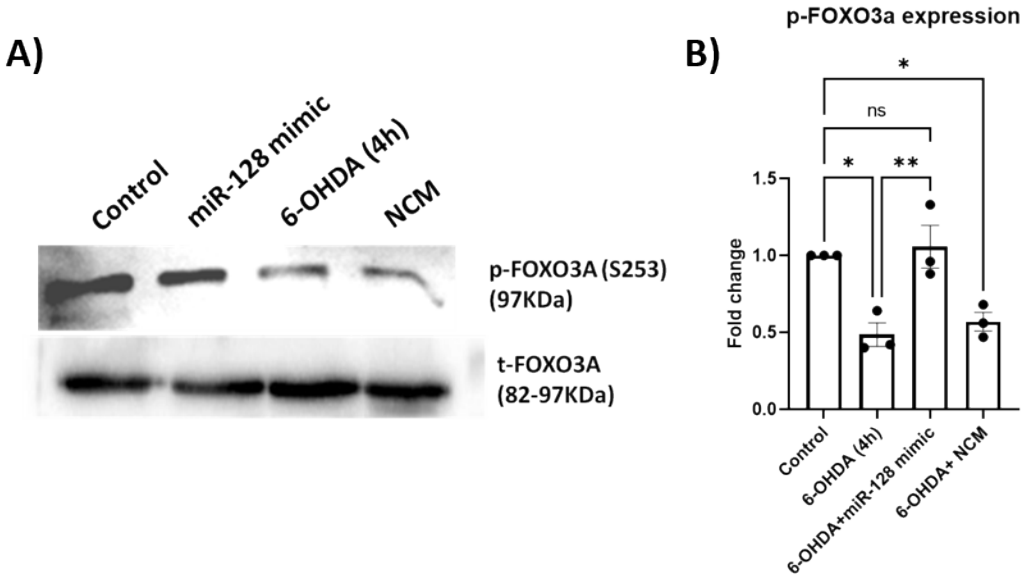


Figure. 3: miR-23a prevents 6-OHDA mediated activation of FoXO3a: Primed SH-SY5Y cells were either untreated (control) or treated with 100μM 6-OHDA for indicated time-points or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100 μM) for the respective time-points, in all the experiments. A) Total protein lysates were subjected to immunoblotting against p-FOXO3a (Ser253) and total FOXO3a. B) Graphical representation of the immunoblots as represented in (A). Normalization was done against total FOXO3a. Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. All experiments were done at least in triplicate. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

miR-128 can prevent the intrinsic pathway of apoptosis by regulating PUMA

It has been reported that the transcription factor FOXO3a can induce expression of pro-apoptotic proteins such as PUMA and FasL in response to various apoptotic stimuli (Nho 2014). Now, apoptosis maybe induced by two major signaling cascades that is, via the extrinsic and intrinsic pathways. One of the proteins implicated in the early stages of intrinsic and extrinsic pathways of apoptosis are PUMA and FasL respectively (Elmore 2007). Since our previous experiments indicated that miR-128 can regulate FOXO3a activation, we decided to check whether miR-128 can regulate the induction of apoptotic pathways- intrinsic or extrinsic or both, downstream of FOXO3a.

We treated SH-SY5Y cells with 6-OHDA or pre-transfected the cells with either miR-128 mimic or NCM for 48h before treatment with 6-OHDA and checked the expression of PUMA mRNAs by qRT-PCR. We observed a marked increase of PUMA mRNA expressions by 8h of 6-OHDA treatment which was significantly reduced by pre-transfection with miR-128 mimic (Fig. 4C). To further validate our observations, we collected protein samples from the same experimental set and performed immunoblotting against PUMA which showed similar reduction in the expression of PUMA (Fig. 4A and 4B) upon miR-128 over-expression.

One of the decisive and irreversible steps in the apoptotic pathways is activation of downstream caspases (Fan et al. 2005). To further validate that miR-128 can regulate the intrinsic apoptotic pathway, we decided to check for the effect of miR-128 on the activation of caspase-9. For the colorimetic assay of caspase-9 activity, we lysed the cells and incubated the cytosolic extract with caspase-9 substrate. The product is a chromophore which was detected at 405nm. We observed an

increase in the caspase-9 activity with 6-OHDA treatment which was subsequently reduced by overexpression of miR-128 (Fig. 4D). Thus, our results showed that the role of miR-128 can regulate both the PUMA-mediated intrinsic pathways of apoptosis.

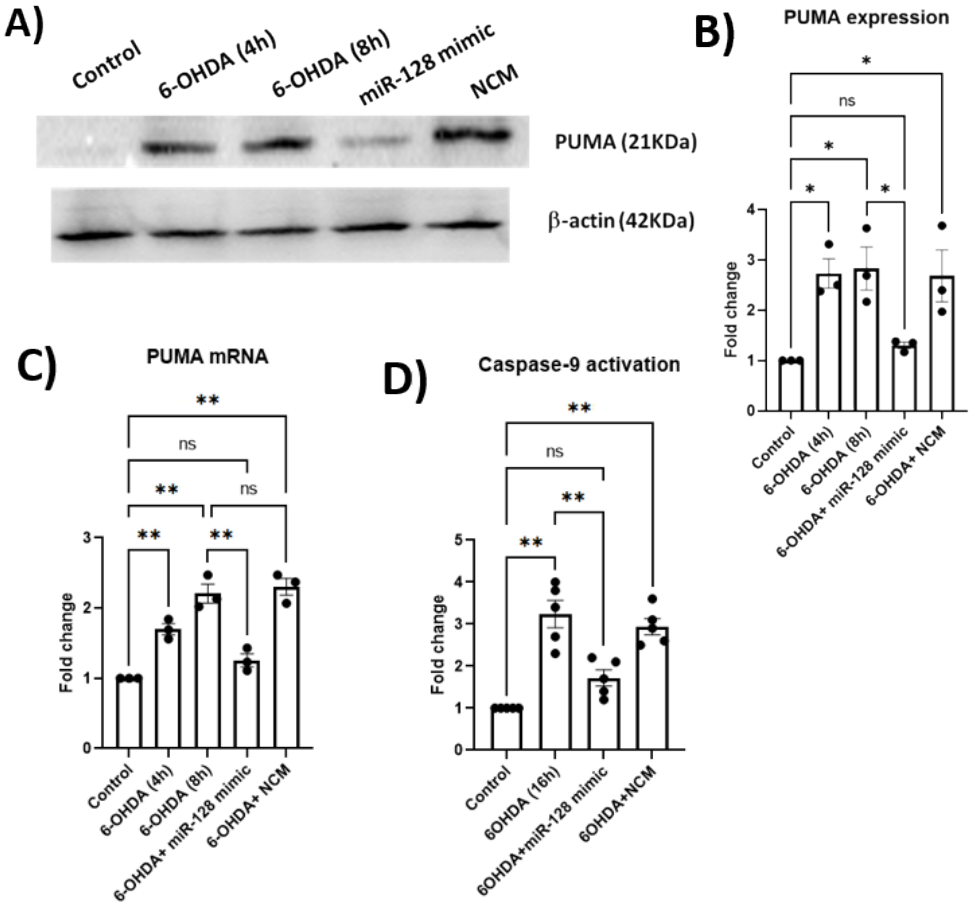


Figure. 4: miR-23a prevents PUMA-mediated intrinsic pathway of apoptosis: Primed SH-SY5Y cells were either untreated (control) or treated with 100μM 6-OHDA for indicated time-points or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100 μM) for the respective time-points, in all the experiments. A) Total protein lysates were subjected to immunoblotting against PUMA. β-actin protein expression was used as endogenous control for normalization. B) Graphical representations of protein expressions of PUMA as represented in (A). C) Total RNA was isolated from the cells and mRNA levels of PUMA was

measured by qRT-PCR. GAPDH expression was used as endogenous control for normalization. D) Cell lysate was incubated with the chromophore labeled substrate (LEHD-*p*NA) and O.D. was measured at 405nm. Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. All experiments were done at least in triplicate. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$

miR-128 can prevent the extrinsic pathway of apoptosis by regulating FasL

Next, we treated SH-SY5Y cells with 6-OHDA or pre-transfected the cells with either miR-128 mimic or NCM for 48h before treatment with 6-OHDA and checked the expression of FasL mRNAs by qRT-PCR. We observed a marked increase of FasL mRNA expressions by 8h of 6-OHDA treatment which was significantly reduced by pre-transfection with miR-128 mimic (Fig. 5C). We also collected protein samples from the same experimental set and performed immunoblotting against FasL which showed similar reduction in the expression of FasL protein (Fig. 5A and 5B) upon miR-128 over-expression.

To further validate that miR-128 can regulate the extrinsic apoptotic pathway, we checked for the effect of miR-128 on the activation of caspase-8 through a fluorescence-based assay of caspase-8 activity. For this, we incubated the cells with a cell-permeable fluorochrome containing dye that detects the amount of active caspase-8 inside the cells in real time and gives out a green fluorescent signal. We detected an increase in the caspase-8 activity upon 6-OHDA treatment which was significantly reduced by pre-treatment with miR-128 mimic (Fig. 5D). Now activation of both caspases- 9 and -8 eventually lead to the irreversible downstream activation of caspase-3, which conclusively leads to apoptosis (Fan et al. 2005). So, we performed caspase-3 activity assay by

detecting the chromophore product at 405nm which indicated that miR-128 overexpression could attenuate 6-OHDA mediated activation of caspase-3 as well (Fig.5E). Thus, our results so far suggest that the role of miR-128 is crucial to apoptotic pathways- it can not only regulate the PUMA-mediated intrinsic apoptotic pathway but also regulate FasL-mediated extrinsic pathway of apoptosis, downstream of FOXO3a.

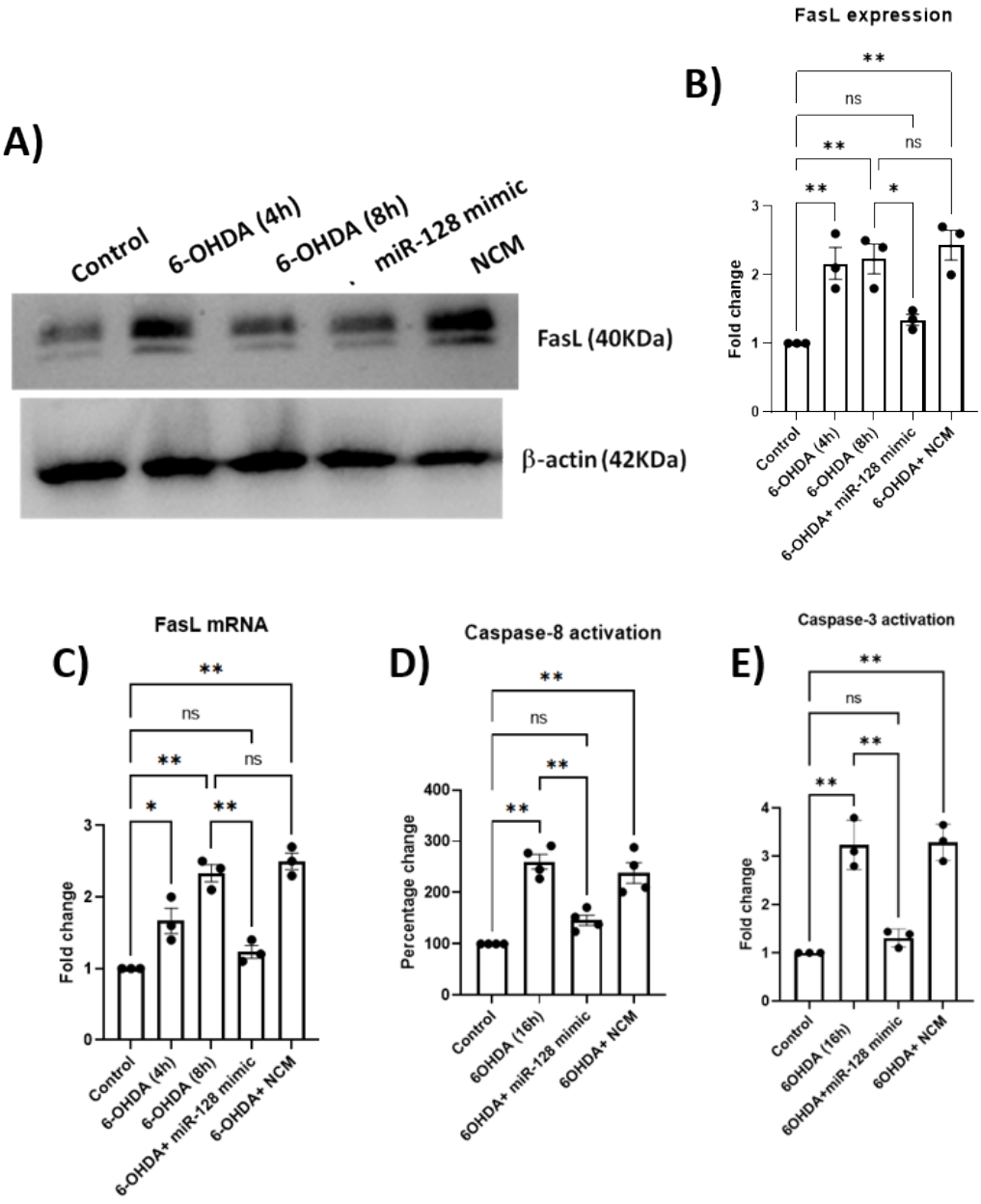


Figure. 5: miR-23a prevents PUMA-mediated intrinsic pathway of apoptosis: Primed SH-SY5Y cells were either untreated (control) or treated with 100μM 6-OHDA for indicated time-points or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100 μM) for the respective time-points, in all the experiments. A) Total protein lysates were subjected to immunoblotting against FasL. β-actin protein expression was used as endogenous control for normalization. B) Graphical representations of protein expressions of FasL as represented in (A). C) Total RNA was isolated from the cells and mRNA levels of FasL was measured by qRT-PCR. GAPDH expression was used as endogenous control for normalization. D) Cells were incubated with FLICA reagent and fluorescence end point reading was detected at E_x=490nm and E_m=520nm. E) Cell lysate was incubated with the chromophore labeled substrate (LEHD-*p*NA) and O.D. was measured at 405nm. Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. All experiments were done at least in triplicate. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

miR-128 can improve neurite length formation

Since miR-128 is a neuron-enriched miRNA known to be highly localized at the neurites as previously mentioned, we decided to check for the effect of miR-128 on neurite length formation and overall morphology of the neurons. We treated primed PC12 cells with 6-OHDA for 16h or pre-transfected the cells with miR-128 mimic or NCM, 48h before treatment, and compared their morphology with respect to control under bright-field microscope. We found that as compared to the control, there was a prominent reduction in neurite length and rounding off of primed cells under 6-OHDA treatment. However, this reduction in neurite length was significantly reversed by

miR-128 overexpression as well as the overall morphology of the primed neuronal cells was also maintained, as shown in Fig. 6A. We also checked the effect of miR-128 over-expression (without 6-OHDA treatment) on the morphology of the neuronal cells and interestingly, we did find a significant increase in neurite lengths as compared to control. This proved that miR-128 has a role in maintaining proper neurite lengths. Neurite length/cell (μm) was measured by Neuron J plugin of Fiji software (Fig. 6B).

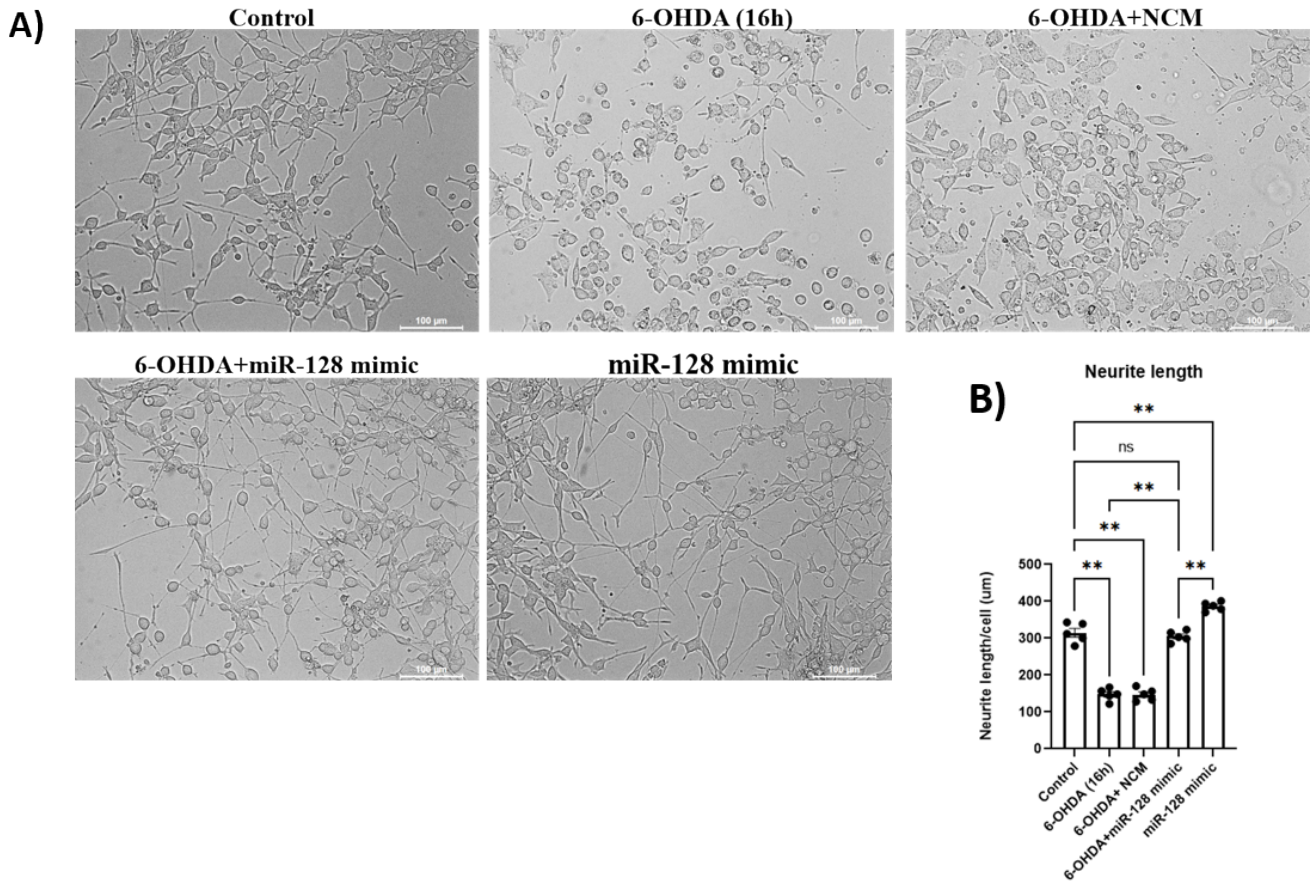


Figure. 6: miR-23a overexpression improves neurite formation: A) Primed PC12 cells were either untreated (control) or treated with 6-OHDA (100μM) for 16h or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100μM) for 16h. The cells were

observed under bright-field microscope (20X). B) Neurite length (μM) per 50 cells per field was calculated using Neuron J plugin of Fiji software. Asterisks (*) indicate significant difference and 'ns' indicates non-significant difference with respect to control, unless otherwise indicated in the figures. All experiments were done in triplicate. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

miR-128 can improve synaptic integrity by up-regulating synaptic protein expression

Next, we decided to check whether miR-128 has any effect on synaptic integrity. We treated SH-SY5Y cells with 6-OHDA for 16h or pre-transfected the cells with either miR-128 mimic or NCM for 48h before treatment with 6-OHDA and checked the expression of the pre-synaptic protein synaptophysin and the post-synaptic protein PSD-95 by immunoblotting. We observed a decrease in the expression of both synaptophysin (Fig.7A and 7B) and PSD-95 (Fig.7C and 7D) by 16h of 6-OHDA treatment but interestingly, this decrease was attenuated by overexpression of miR-128. Therefore, our results indicate that miR-128 can maintain the overall neuronal morphology and prevent neurite length shortening as well as keep the synaptic integrity intact by maintaining the expression of pre- and post-synaptic proteins like synaptophysin and PSD-95 respectively.

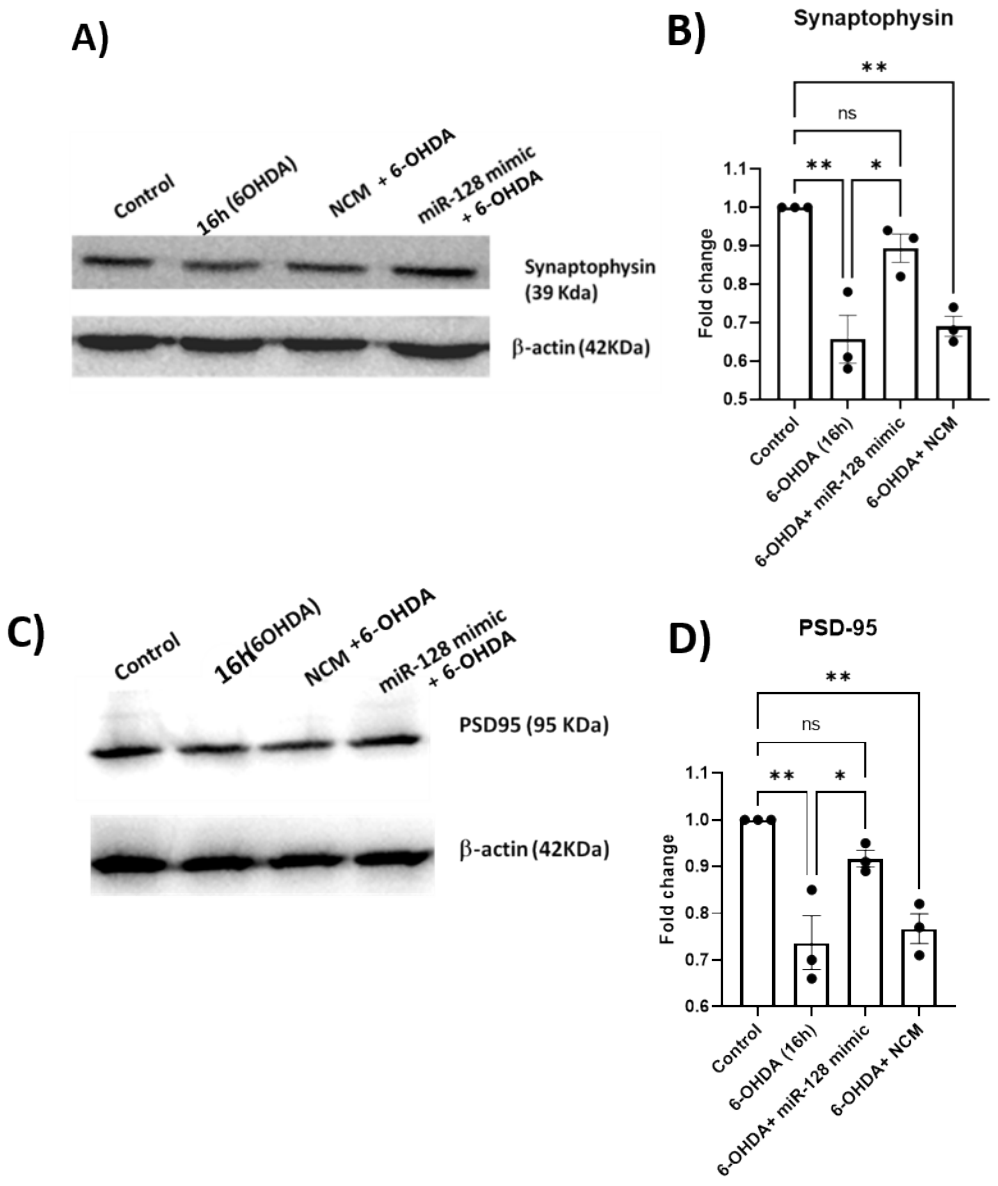


Figure. 7: miR-23a regulates pre- and post-synaptic protein expression: A) and C) Primed SH-SY5Y cells were either untreated (control) or treated with 100μM 6-OHDA for 8h or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100μM) for 8h. Total protein lysates were subjected to immunoblotting against synaptophysin (A) and PSD-95 (C). β-actin protein expression was used as endogenous control for normalization in both cases. B) and D) Graphical representations of protein expressions of synaptophysin (A) and PSD-95 (B) as

normalized with respect to β-actin expression. Asterisks (*) indicate significant difference and 'ns' indicates non-significant difference with respect to control, unless otherwise indicated in the figures. All experiments were done in triplicate. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

4.1.3 Discussion:

In this study, we investigated the role of miR-128 in PD, which is regarded as a *motomiR* due to its significant implications in motor diseases (Hawley et al. 2017). Our results showcase a comprehensive mechanistic function of miR-128 in the pathways leading to neurodegeneration in PD., We identified miR-128 to have neuroprotective functions- it can prevent both the intrinsic and extrinsic pathways of apoptosis by preventing activation of FOXO3a upstream. It is worth mentioning here that although miR-128 has been predominantly reported to be neuroprotective in nature (Shi et al. 2021,Fang et al. 2019, Mao et al. 2017, Geng et al. 2018), it is also shown to promote neuronal degeneration under certain conditions (Geng et al. 2018),(Adlakha and Saini 2013).

Using standard *in vitro* models of PD, our fluorescent imaging and flow cytometry-based experiments respectively showed that miR-128 over-expression could attenuate the production of mitochondrial superoxide radicals as well as prevent cells from 6-OHDA mediated death. Previously we had reported that 6-OHDA can induce neuronal apoptosis via activation of the transcription factor FoxO3a (Sanphui, Kumar Das, and Biswas 2020). Keeping that in mind, we explored the involvement of miR-128 in apoptotic pathways and its effect on FoxO3a activation. Upon immunoblotting against p-FoxO3a (Ser 253), we found a sharp decrease in the p-FoxO3a

levels during 6-OHDA treatment which was significantly reversed upon over-expression of miR-128, thereby indicating that miR-128 can regulate the activation of FoxO3a. FoxO3a has been reported to induce apoptosis in various cell death models via both the intrinsic and extrinsic pathways of apoptosis, as mentioned above. One of the means by which the extrinsic pathway of apoptosis maybe induced is by binding of Fas ligand (FasL) to the FADD receptor protein, leading to irreversible downstream activation of caspase-8. On the other hand, during induction of the intrinsic apoptotic pathway, one of the genes to be up-regulated in the early stages is the pro-apoptotic protein PUMA further leading to downstream activation of caspase-9. However, both the extrinsic and intrinsic pathways ultimately lead to irreversible activation of caspase-3 eventually leading to apoptotic cell death (Elmore 2007). Interestingly, miR-128 over-expression lead to downregulation of both FasL and PUMA expression at the mRNA as well as protein levels which was otherwise found to increase upon treatment with 6-OHDA. Furthermore, while 6-OHDA treatment lead to an increase in the irreversible downstream activation of caspases-8, -9 and -3, miR-128 over-expression could significantly attenuate the activation of these caspases. Thus, our results clearly indicated a mechanism by which miR-128 over-expression could inhibit neuronal death through both the PUMA-mediated intrinsic and FasL-mediated extrinsic pathways of apoptosis via regulating the upstream activation of the transcription factor FoxO3a.

miR-128 is a neuron-enriched miRNA which is reported to be highly distributed in the neurites and at the synapse (Kumar et al. 2020, Franzoni et al. 2015). This prompted us to check the effect of miR-128 on overall neuronal morphology and synaptic health. Interestingly, miR-128 over-expression could help in the maintenance of neurite lengths which was otherwise reduced significantly by 6-OHDA treatment. Additionally, immunoblotting against synaptic proteins

synaptophysin and PSD95 showed that while 6-OHDA leads to a downregulation of these proteins, miR-128 could increase the expression of both the pre- and post-synaptic proteins respectively, to a significant extent. However, it is not clear how miR-128 could attenuate the 6-OHDA-mediated decrease in the levels of these synaptic proteins. In our models of study, we were not able to find a direct molecular target of miR-128 or an altered downstream signaling pathway through which miR-128 could regulate the expression of the above-mentioned synaptic proteins and thus it requires further investigation.

Overall, we can conclude that we have identified the brain-enriched neuron-specific miR-128 to possess important regulatory functions in the pathogenesis and progression of PD.

Part II:

Exosomal release of miR-23a down-stream of Ca^{2+}/Calcineurin pathway regulates astrocyte-neuron cross-talk and protects against PUMA-mediated neuronal apoptosis in models of Parkinson's disease

4.2.1 Introduction:

Astrocyte dysfunction is implicated in a number of NDDs associated with neuroinflammation, including PD (Booth, Hirst, and Wade-Martins 2017). During neuroinflammation, astrocytes undergo a morphological and metabolic change called 'reactivation' (Escartin et al. 2021). One of the important intracellular mechanisms implicated in this phenotypic switching of 'reactive' astrocytes during injury, neuroinflammation and neurodegeneration is the $Ca^{2+/}$calmodulin-dependent phosphatase calcineurin (CaN) (Furman and Norris 2014). CaN is rapidly proteolyzed and irreversibly activated following a surge in Ca^{2+} levels during neural damage (Abdul et al. 2009). Many reactive astrocytes in the AD brain tissue and AD mouse models show high levels of calcineurin (CN) expressions (Norris 2005) and could be linked to synapse loss, altered plasticity, neuronal viability/death, and impaired cognitive responses (Wu et al. 2010). However, the role of CaN in PD is not studied yet. Moreover, in cardiomyocytes, hypertrophic reactions are also reported to be critically dependant on calcineurin activation (Molkentin et al. 1998). In one such study, it was shown that in cardiac myocytes, CaN activation causes up-regulation of miR-23a expression (Lin et al. 2009). Interestingly, in the brain tissue, miR-23a is found to be astrocyte-enriched possessing predominantly anti-apoptotic functions (Li et al. 2020, Zhao et al. 2014). Is CaN activated in astrocytes during PD? What is the status of astrocytic miR-23a expression in PD? Are CaN pathway and miR-23a expressions linked in context to PD? That is what we decided to find out, in the *in vitro* and *in vivo* models as well as patient samples of PD.

4.2.2 Results:

Calcineurin is activated in astrocytes in MPTP mouse models of PD

Firstly, we decided to check whether or not CaN is expressed in the astrocytes in context to PD, by using animal models of the disease. We used acute MPTP mouse models (described in Methods section) which has been previously reported to express reactive astrocytes (Thomas et al. 2012; L'Episcopo et al. 2011). After 7 days of MPTP treatment, the animals were perfused, sacrificed and the SNPc region was cryo-sectioned from the brain tissue. Immunohistochemistry was performed on the tissue sections from both MPTP and wild-type (WT) mice. The sections were co-immunostained for GFAP and CaN A respectively to observe the expression of CaN A in GFAP+ astrocytes under confocal microscope (20X). In the WT mice, reduced expression of CaNA was observed overall, with very few reactive astrocytes. However, in the MPTP mice, the overall tissue expression of CaN A was significantly increased along-with increased number of reactive astrocytes. Interestingly, co-expression (yellow) of CaN A (red) was found within GFAP+ astrocytes (green) in the SNPc regions of MPTP mice while no such co-localization was observed in the WT mice (Fig. 1A). CTCF values of GFAP and CaN expression intensities were plotted (Fig. 1B, 1C). Percentage of GFAP+ cells was calculated on the basis of total Hoechst + cells (Fig. 1D). Percentage of both GFAP+ and CaNA+ cells i.e. colocalized cells (yellow) was calculated compared to total Hoechst+ cells (Fig.1E). Overall, our findings revealed that CaN is expressed in the reactive astrocytes in MPTP models of PD.

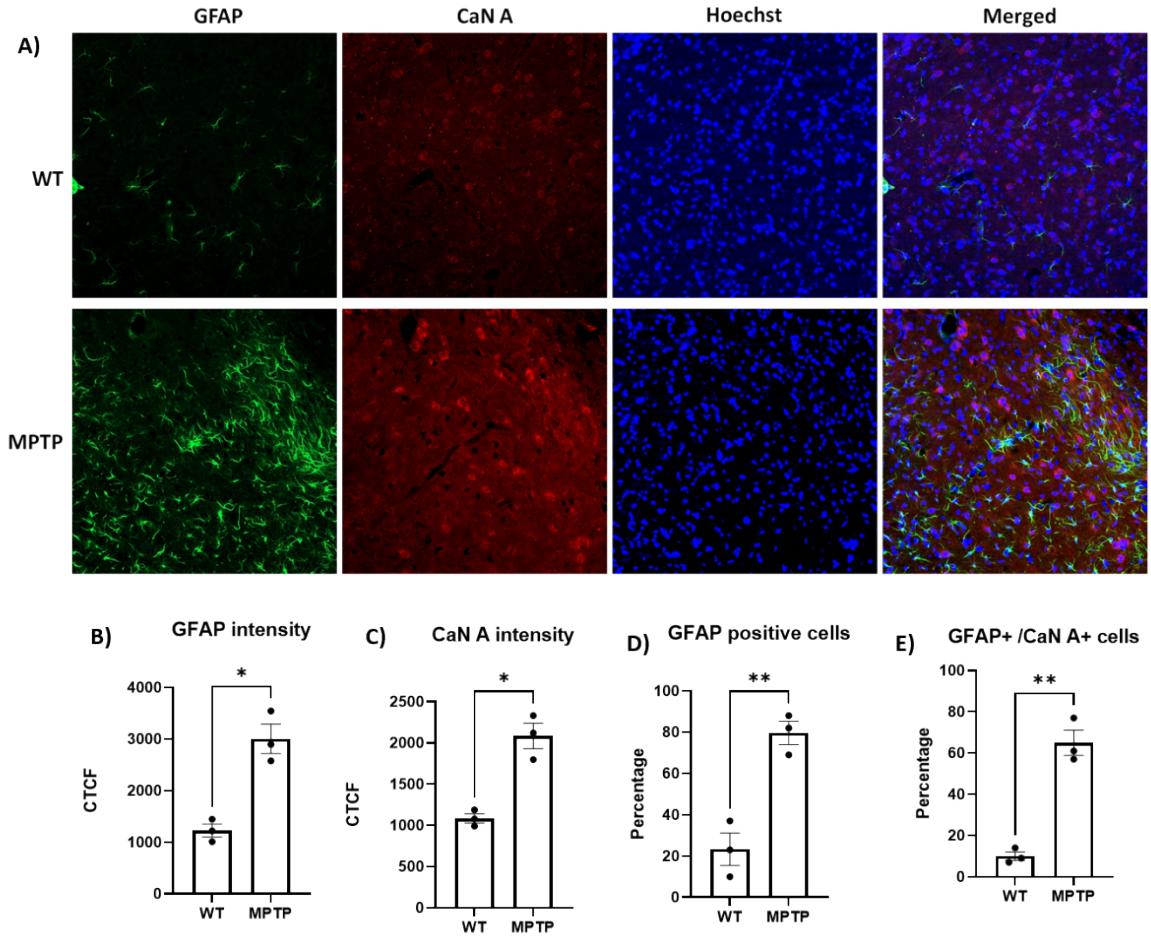


Figure1: Calcineurin is expressed in the reactive astrocytes of MPTP mice: A) Immunohistochemistry of SNPc sections from WT and MPTP treated (acute) mice. Left to right – First panel shows GFAP+ positive cells (green), second panel shows CaN A expression (red), third panel shows nuclei stained by Hoechst (blue) and the fourth panel shows the merged images where co-localization of GFAP and Can A expression is indicated by yellow. All images were taken at 20X magnification under confocal microscope. B) and C) Fluorescence intensities were measured by using ImageJ software. Bar diagrams represent corresponding CTCF values of the respective groups. D) and E) Percentage of GFAP+ cells (green) and GFAP+/CaNA+ cells (yellow) were calculated compared to total Hoechst+ cells (blue). Results are plotted as Mean ± S.E.M where *p < 0.05 (three images per animal and n = 3 per group).

Rotenone activates Ca^{2+}/Calcineurin in astrocytes

Next, we wanted to explore the consequences of astrocytic CaN expression in PD pathogenesis and progression. To do so, we decided to check if CaN is expressed in its catalytically activate form within the astrocytes in standard *in vitro* models of PD. We used Rotenone, a common neurotoxin (Innos and Hickey 2021), to treat our astrocyte cultures since Rotenone is a plasma membrane permeable molecule which can easily enter both astrocytes and neurons without the need of any specialized receptor. We treated human astrocytoma cell line 1321N1 with varying doses of Rotenone for 24 hours and determined the IC_{50} dose of 200nM by MTT assay (Fig. 2A). Next, we treated the 1321N1 cells with 200nM Rotenone for varying time-points and checked the expression of CaN A by immunoblotting against its N-terminal (CST-antibody), which detects both the full-length (59 KDa) and truncated (activated) versions (45 KDa) of CaN A. Interestingly, we found more than 2-fold increase in the levels of both full-length (Fig. 2C) and around 2-fold increase in the levels of activated CaN (Fig. 2D) from 4hrs through 8hrs of Rotenone treatment (Fig. 2B). To confirm the activation of CaN at the enzymatic level, we did a CaN activation assay, which also showed a similar increase in the activation of CaN by 4hrs and 6hrs of Rotenone treatment (Fig. 2E). Now, CaN is a Ca^{2+}-activated protein phosphatase and its maximal activity is achieved when Ca^{2+} is bound to its catalytic subunit. Thus, we decided to check the Ca^{2+} levels in the astrocytes upon Rotenone treatment as well. We treated the cells with the cell permeable calcium indicator dye Fluo-4AM, and observed its fluorescence at 488nm. There was a significantly increased fluorescence at 4hrs and 6hrs of Rotenone treatment, following which there was a slight dip at 8hrs (Fig. 2F). Nevertheless, our results show both calcium surge and CaN activation in the astrocytic cells at early time-points of Rotenone treatment, indicating activation of the Ca^{2+}/CaN pathway in cellular models of PD.

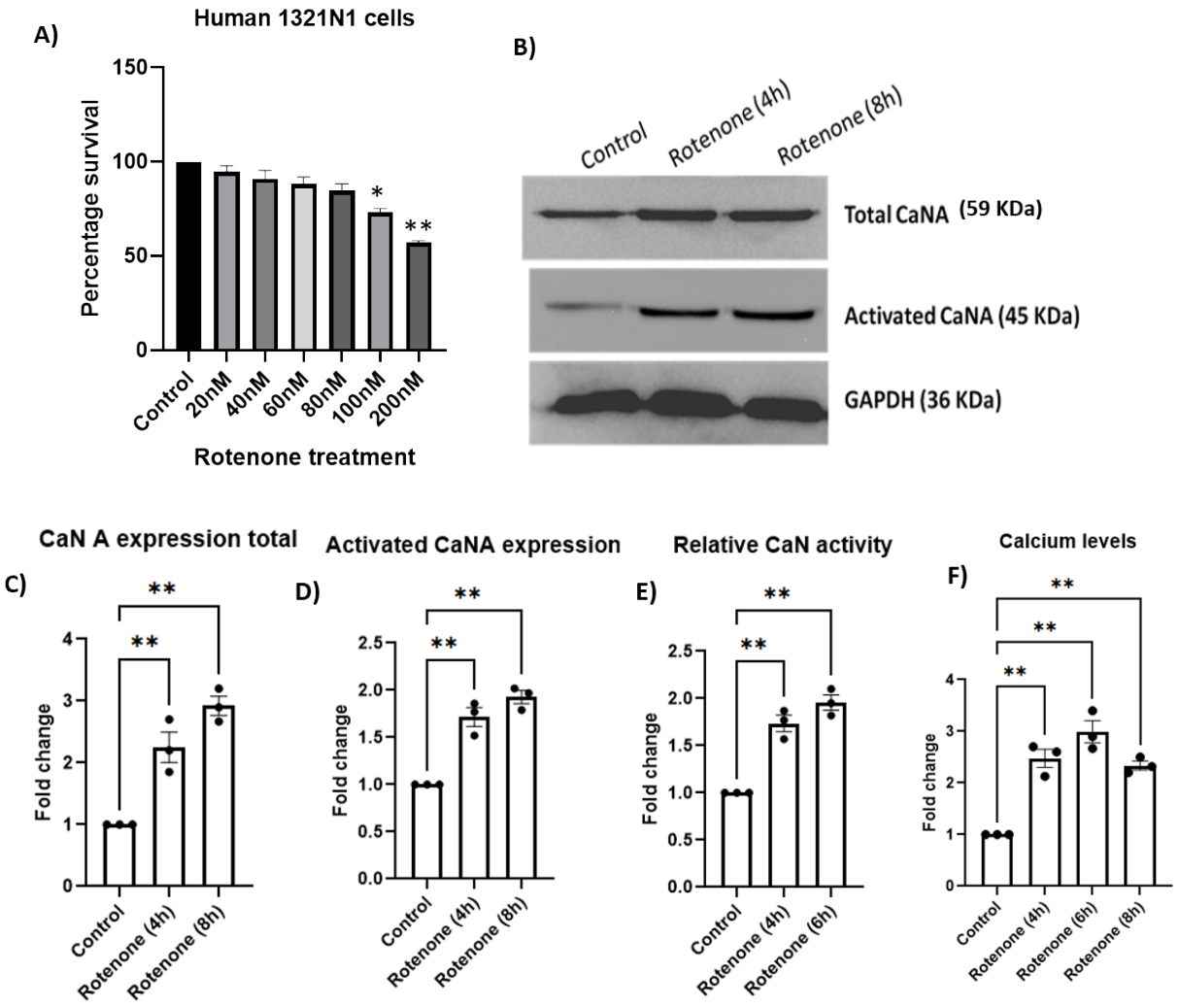


Figure 2: Rotenone-induced activation of Ca^{2+}/CaN pathway in astrocytes: A) Human astrocytic 1321N1 cells were treated with Rotenone (20nM, 40nM, 60nM, 100nM and 200nM) for 24h respectively. MTT assay was done after 24h and percentage viability of cells was calculated with respect to untreated (control) cells; (n=4). B) 1321N1 cells were either untreated (control) or treated with Rotenone (200nM) for indicated time-points. Total protein lysates were subjected to immunoblotting for CaN A against its N-terminal. Graphical representation of the total CaN A levels (C) and activated CaN A levels (D) respectively, as represented in (B). Normalization was done against GAPDH; (n=3). E) Control and Rotenone treated cells (4hrs, 6hrs) were subjected to

enzymatic assay of CaN. The cell lysates were incubated with RII phosphopeptide substrate and colorimetric detection of the free phosphate released was done at 620nm. Results are plotted as relative fold change with respect to control; (n=3). F) 1321N1 cells were incubated with Fluo-4AM (2μM) for 30mins at 37^0C and fluorometric readings were obtained at Ex/Em of 494/506 nm; (n=3). Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

Astrocyte-enriched miR-23a expression is decreased upon Rotenone treatment

Since our *in vivo* and *in vitro* results in models of PD strongly indicated the activation of CaN in astrocytes, we decided to explore the implications of Ca^{2+}/CaN activation in the pathogenesis of PD. Through literature survey, we came across a paper (Lin et al. 2009) that showed that CaN pathway can directly upregulate the expression of a particular miRNA, miR-23a, in cardiomyocytes during cardiac hypertrophy. Now, interestingly enough, in the brain, miR-23a is an astrocyte-enriched miRNA (Zhao et al. 2014) that has also been found to be differentially expressed in the serum of AD patients (Galimberti et al. 2014). However, the mechanistic implications of miR-23a in PD, if any, have not been reported till date. Thus, we decided to check the level of miR-23a expression in our cellular models of PD.

We treated the 1321N1 cells with Rotenone (200nM) and interestingly, we found a significant decrease in the expression of miR-23a by 4hrs and 8hrs of treatment, which is the time-frame where CaN was found to be activated as well. However, this observation was in stark contrast to that reported by Lin et al (2009), as mentioned above, where activation of CaN pathway led to increased levels of miR-23a expression in cardiomyocytes. Thus, we decided to validate our observation in

the primary astrocyte culture. Cortical astrocyte cells were cultured from rodent brain (14 DIV) and treated with Rotenone (100nM) (Rathinam et al. 2012) for 4hrs and 8hrs respectively. Interestingly, we observed a similar decrease of miR-23a expression with increasing time-points of Rotenone treatment in the primary astrocyte culture as well. Thus, our results showcased that although Rotenone causes activation of CaN, on the other hand, it leads to decreased expression of miR-23a in astrocytes.

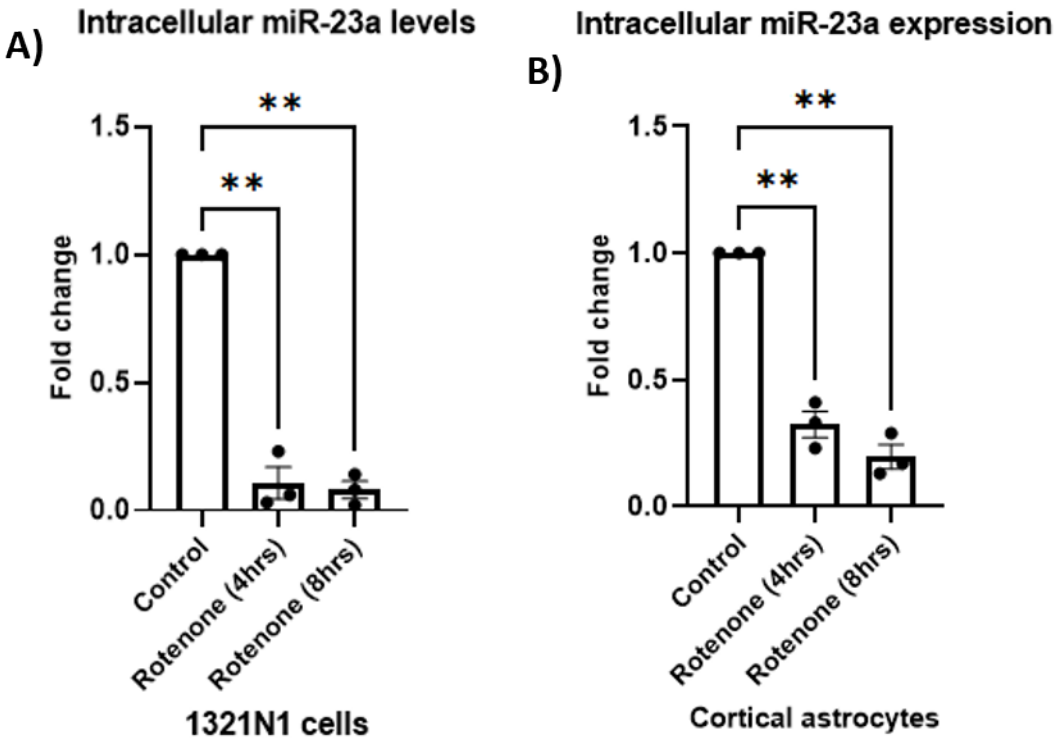


Figure 3: Intra-astrocytic expression of miR-23a is decreased upon Rotenone treatment: Human 1321N1 cells (A) and primary rat cortical astrocytes (B) were treated with Rotenone in a concentration of 200nM and 100nM respectively, for indicated time-points, or were in untreated condition (control). Intracellular miR-23a expression was checked by qRT-PCR with respect to control. U6 expression was used as endogenous control for normalization in both; (n=3 for each).

Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

Rotenone treatment causes release of miR-23a from astrocytes via exosomes

Although miR-23a is reported to be a downstream target of CaN pathway, our previous results showed that CaN activation did not in fact, increase the expression of miR-23a in the astrocytes. To address this contradiction, we hypothesized- do the astrocytes release the miR-23a in the extracellular milieu? Now, astrocytes are reported to release their intracellular cargo via extracellular microvesicles, namely, exosomes (Venturini et al. 2019). So we decided to check whether miR-23a might be released from the astrocytes through exosomes. To answer that, we treated the 1321N1 cells with Rotenone and collected the astrocyte conditioned medium (ACM) at 0hrs (control), 4hrs and 8hrs of treatment. Next, we isolated the exosomes from the ACM by miRCURY exosome isolation kit for cell/urine/CSF (Qiagen) and checked the expression of miR-23a in the total exosomal RNA. Surprisingly, there was a significant increase in the exosomal miR-23a expression levels by 4hrs and 8hrs of Rotenone treatment with respect to control, which corroborated exactly with the time-frame of decrease in the intra-astrocytic levels of miR-23a. Thus, our results suggested that the Rotenone-induced decrease in miR-23a expressions within the astrocytes was due to a concomitant release of miR-23a by astrocytes through exosomes.

Next, to further validate the exosomal release of miR-23a, we co-treated 1321N1 cells with GW4869 (10μM), an exosome inhibitor (Chen et al. 2020; Mukherjee et al. 2016), and Rotenone for 8hrs. Next, we checked for the levels of miR-23a within the astrocytes as well as in the

exosomes collected from the ACM. In the ACM samples, there was a significant decrease in the exosomal miR-23a levels by 8hrs of Rotenone co-treatment with GW4869. However, converse was found inside the astrocytes, where by 8hrs of Rotenone co-treatment with GW4869, a significant increase in the intra-astrocytic levels of miR-23a was observed.

Thus, our results clearly indicated that while Rotenone treatment resulted in CaN activation in the astrocytes, the concomitant reduction in miR-23a levels was in fact, due to the exosomal release of miR-23a by the astrocytes.

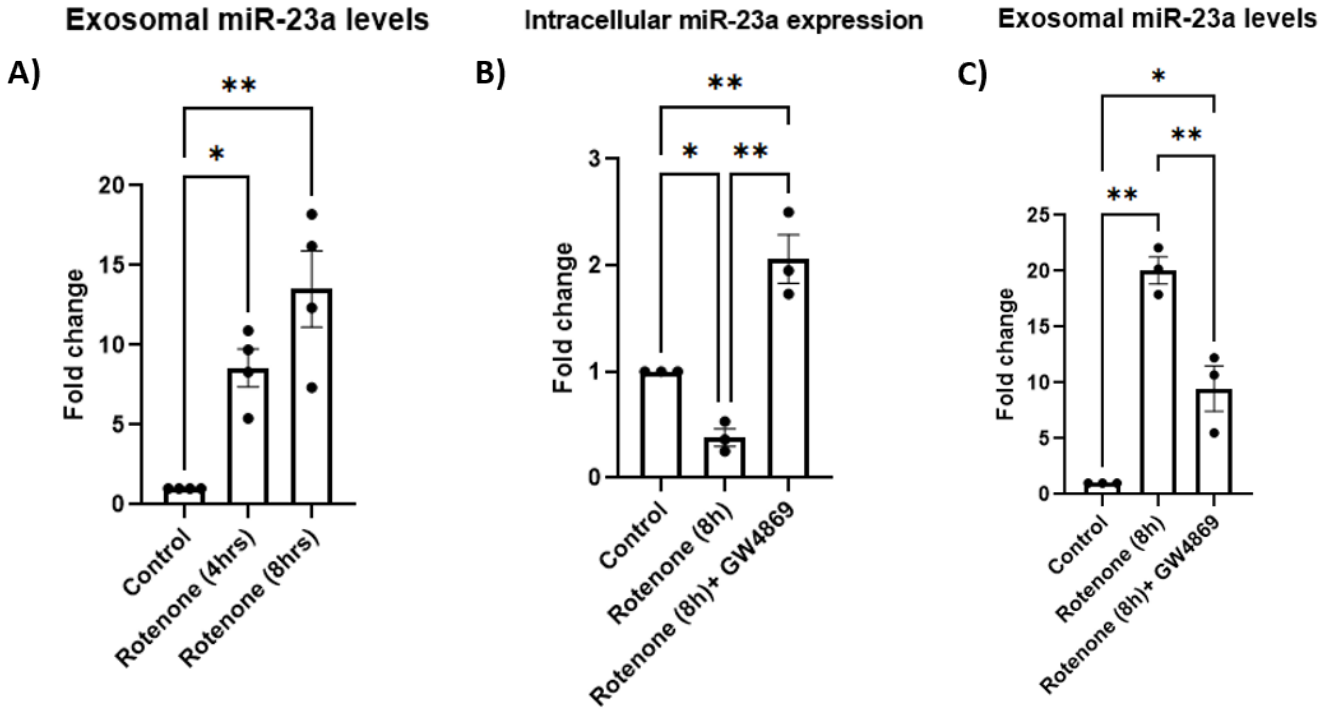


Figure 4: miR-23a is released from the astrocytes via exosomes: A) 1321N1 cells were either untreated (control) or treated with Rotenone for 4hrs and 8hrs respectively and ACM was collected. Exosome was isolated from the ACM and total exosomal RNA was isolated. miR-23a levels were detected by qRT-PCR and normalized with U6 used as endogenous control; (n=4). B and C)

1321N1 cells were either untreated (control), or treated with Rotenone (8hrs) or co-treated with GW486 (10μM) and Rotenone for 8hrs. Intracellular miR-23a expression levels (B) and expression of miR-23a in exosomes collected from ACM (C) were detected by qRT-PCR and normalized with U6 as endogenous control; (n=3 for each). Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

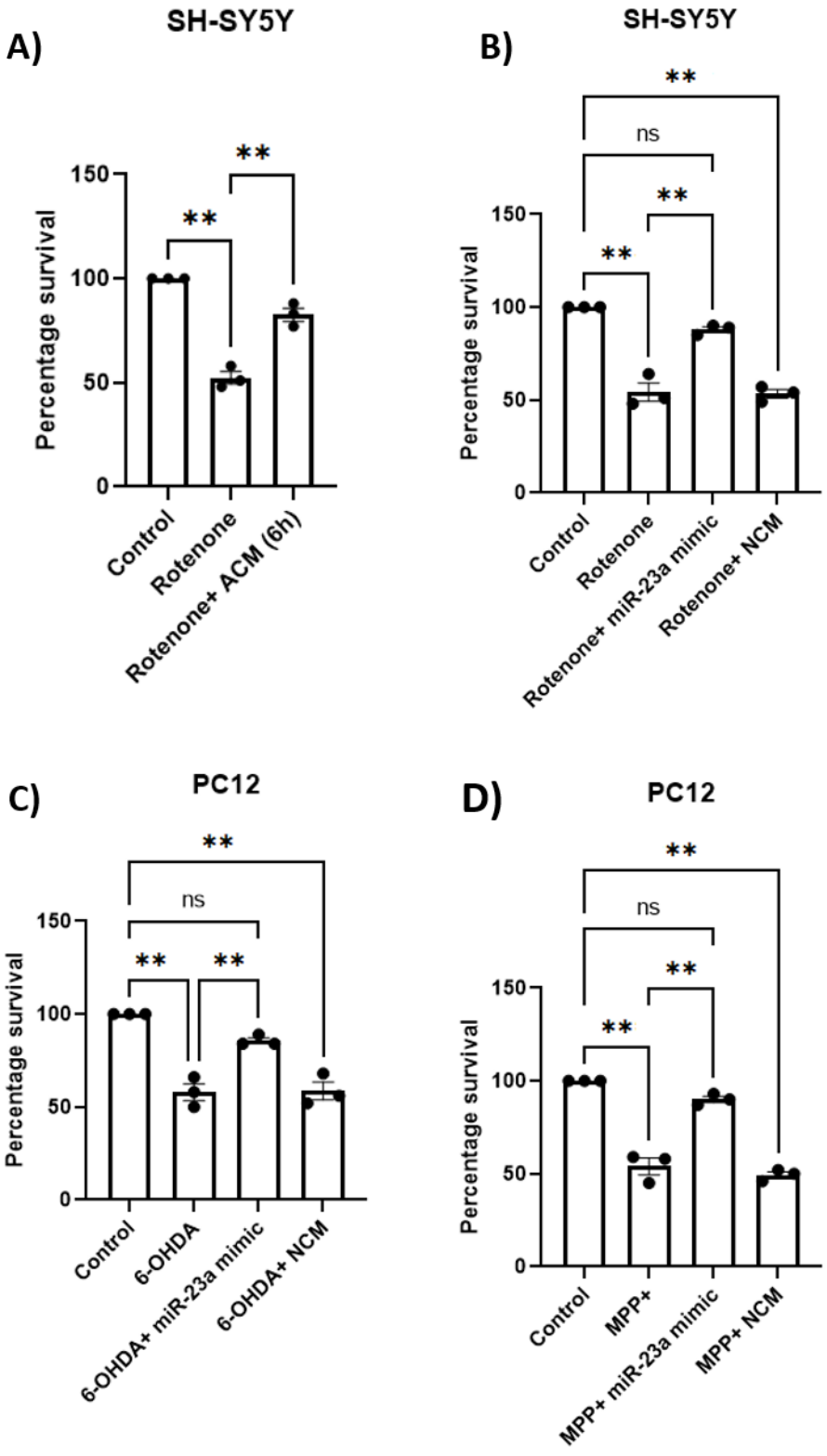
Exosomal miR-23a derived from astrocytes are neuro-protective

Next, we decided to check the consequence of this exosomal release of miR-23a by astrocytes. It is already known that astrocytes can show both neuro-protective (Saha et al. 2020) and neuro-detrimental (Guttenplan et al. 2021) roles under stress conditions. On the other hand, exosomes are reported to be important mediators of cell-to-cell communication, including astrocyte-neuron cross-talk (Venturini et al. 2019). Thus, we hypothesized- what could be the effect of astrocytic release of miR-23a on neuronal cells? Does it have an overall protective or destructive effect on the neurons?

To answer that, we treated the 1321N1 cells with Rotenone for 6hrs and collected the ACM. Exosomes were isolated from the ACM (as mentioned previously), the exosome pellets were resuspended in culture medium (Ghoshal, Bertrand, and Bhattacharyya 2021) and added to SH-SY5Y cells. These SH-SY5Y cells were then treated with Rotenone for 24hrs at an IC_{50} dose of 200nM (Newhouse 2004). Next, MTT assay was performed to determine the percentage of live cells. It was observed that upon treating the cells with exosomes, the percentage of live cells was significantly increased as compared to cells treated with no added ACM (Rotenone only) (Fig. 5A).

Although these experiments showed that exosomes derived from ACM are neuro-protective, it did not specifically confirm that this neuro-protective effect was exerted by miR-23a. The cargo inside the exosomes is not exclusive for miR-23a and may contain various other factors that could exert the neuro-protective effect. Previous reports from our lab have already shown in AD models that ACM at 6hrs was neuro-protective due to presence of cytokines like ICAM-1 (Guha et al. 2022) and TIMP-1 (Saha et al. 2020). Thus, to confirm that the neuro-protection provided in our experimental set up was at least to some extent due to miR-23a, we pre-transfected SH-SY5Y cells with miR-23a mimic (over-expression constructs) or negative control mimic (NCM) for 48hrs before treating them with Rotenone for 18hrs. This was followed by MTT assay which showed that miR-23a over-expression could provide significant protection against Rotenone-induced neurotoxicity (Fig. 5B).

In order to determine that it is not a cell line or neurotoxin specific phenomenon, we repeated the experiment on 6-OHDA treated (100μM) and MPP+ treated (500μM) primed PC12 cells. MTT assay post 18hrs of either treatment revealed similar observations, where over-expression of miR-23a by miR-23a mimics could protect the primed neuronal PC12 cells from 6-OHDA (Fig. 5C) and MPP+ (Fig. 5D) induced cell deaths respectively. Thus, our results suggested that the astrocytic release of miR-23a via exosomes at early time-points of stress (8hrs) has a possible neuro-protective function.


A)
SH-SY5Y
Percentage survival
150
100
50
0
**
**
Control
Rotenone
Rotenone+ ACM (6h)
B)
SH-SY5Y
Percentage survival
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Control
Rotenone
Rotenone+ miR-23a mimic
Rotenone+ NCM
C)
PC12
Percentage survival
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Control
6-OHDA
6-OHDA+ miR-23a mimic
6-OHDA+ NCM
D)
PC12
Percentage survival
**
ns
**
**
Control
MPP+
MPP+ miR-23a mimic
MPP+ NCM

Figure 5: Exosomal miR-23a is neuro-protective: A) SH-SY5Y cells were either untreated (control) or treated with Rotenone only (24hrs) or co-treated with exosomes collected from ACM (6hrs post Rotenone treatment) and Rotenone for 24hrs. MTT assay was performed and percentage of cell survival was calculated with respect to control; (n=3). B) SH-SY5Y cells were either untreated (control) or treated with Rotenone for 18h or pre-transfected with either miR-23a mimic or NCM for 48h before treatment with Rotenone for 18h. C) Primed PC12 cells were either untreated (control) or treated with 6-OHDA for 18h or pre-transfected with either miR-23a mimic or NCM for 48h before treatment with 6-OHDA for 18h. D) Primed PC12 cells were either untreated (control) or treated with MPP+ for 18h or pre-transfected with either miR-23a mimic or NCM for 48h before treatment with MPP+ for 18h. In all cases (B-D), MTT assay was performed and percentage of cell survival was calculated with respect to control; (n=3 for each). Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

Pro-apoptotic PUMA is a direct target of miR-23a

Next, we decided to check the molecular mechanism by which miR-23a exerted this neuro-protective effect. We have previously reported that 6-OHDA (100μM) can induce apoptosis in primed PC12 cells by induction of the pro-apoptotic molecule PUMA (Sanphui, Kumar Das, and Biswas 2020). Thus, we decided to explore if over-expression of miR-23a has any effect on PUMA expression. Interestingly, transfection of miR-23a mimic proved to reduce PUMA expression at both the transcript (Fig. 6A) as well as protein levels (Fig. 6B, 6C), which was otherwise found to increase by 4hrs of 6-OHDA treatment on primed PC12 cells.

Next, we wanted to check if PUMA is a direct target of miR-23a. miRNA target prediction tool TargetScan and literature survey revealed that there is a direct binding site of miR-23a at PUMA 3'UTR. The 3'UTR of PUMA was cloned in pmirGLO Dual-Luciferase miRNA Target Expression Vector (Promega) which was followed by luciferase assay. For the experiment, PC12 cells were co-transfected with: miR-23a mimic and pmirGLO reporter plasmid containing PUMA 3'UTR or miR-23a mimic and pmirGLO empty vector or NCM and pmirGLO empty vector or NCM and pmirGLO reporter plasmid containing PUMA 3'UTR. 24hrs following transfection, firefly (FL) and renilla luciferase (RL) signals were measured using the Dual-Luciferase Reporter Assay kit (Promega) and relative luciferase activities were determined by normalizing FL to the RL activity. It was observed that as compared to the control set i.e. pmirGLO empty vector co-transfected with NCM, there was a significant reduction in the luciferase activity of the experimental set i.e. pmirGLO with PUMA 3'UTR co-transfected with miR-23a mimic, indicating direct binding of miR-23a to the 3'UTR of PUMA (Fig. 6D).

Finally, since PUMA is a pro-apoptotic protein, we wanted to check whether miR-23a can inhibit induction of the apoptotic pathway by regulating expression of PUMA. Activation of caspase-3 is an irreversible step in the induction of apoptotic cell death. We performed a colorimetric caspase-3 activity assay which showed that miR-23a over-expression by miR-23a mimics can attenuate 6-OHDA mediated activation of caspase-3 in neuronal PC12 cells (Fig. E). Thus, overall, our results prove that miR-23a exerts its neuroprotective effect by directly down-regulating the expression of the pro-apoptotic protein PUMA and preventing apoptotic neuronal death.

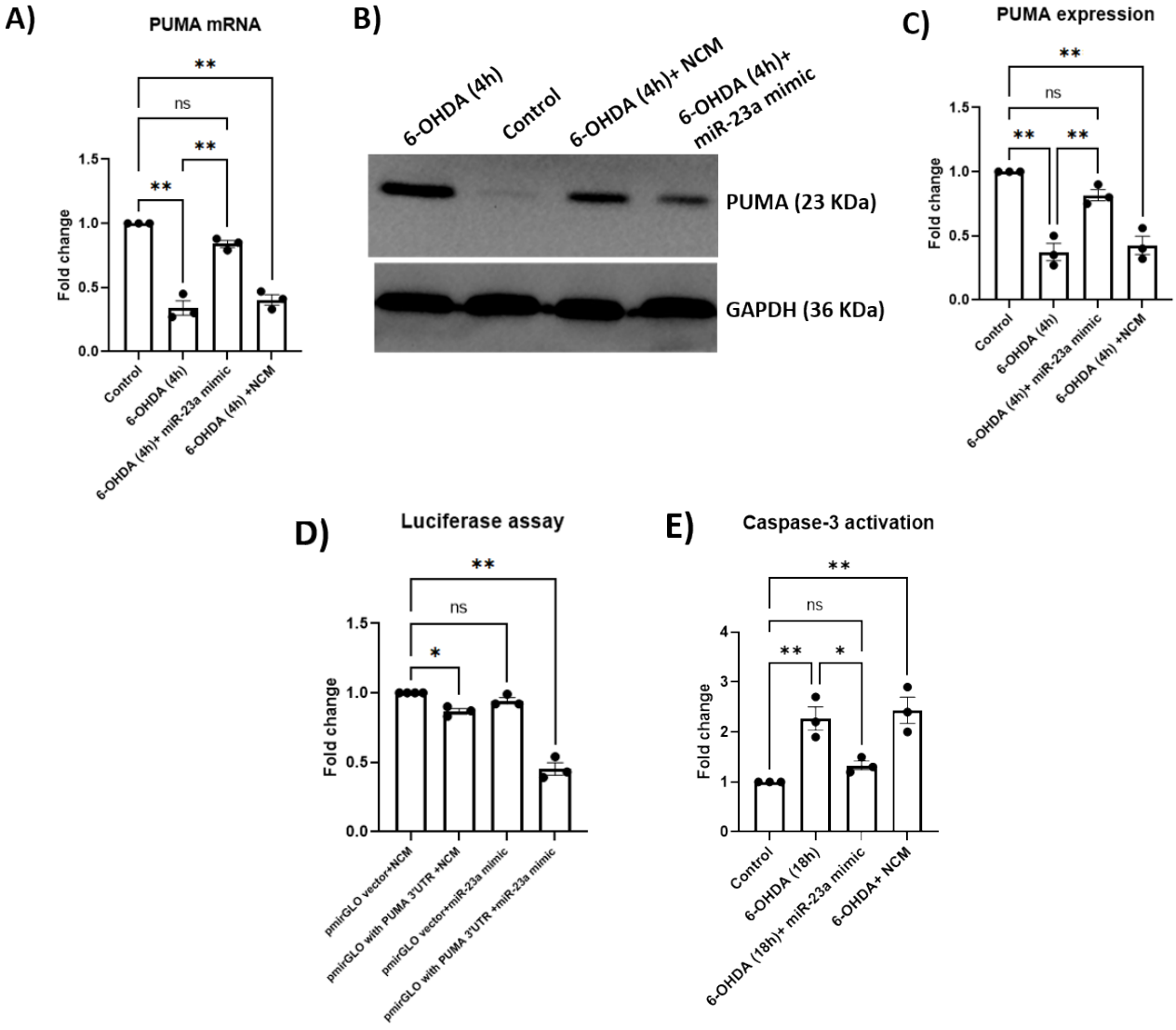

Figure 6: miR-23a can bind directly to pro-apoptotic protein PUMA: A-C and E) Primed PC12 cells were either untreated (control) or treated with 100μM 6-OHDA for 4hrs or pre-transfected with either miR-23a mimic or NCM for 48h before treatment with 6-OHDA (100 μM) for the indicated time-point, in all the experiments. For (A) total RNA was isolated from the cells and mRNA levels of PUMA was measured by qRT-PCR. GAPDH expression was used as endogenous control for normalization; (n=3). For (B) total protein lysates were subjected to immunoblotting against PUMA. β-actin protein expression was used as endogenous control for normalization. (C) is

the graphical representations of protein expressions of PUMA as represented in (B); (n=3). D) Primed PC12 cells were co-transfected with: miR-23a mimic and pmirGLO reporter plasmid containing PUMA 3'UTR, miR-23an mimic and pmirGLO empty vector, NCM and pmirGLO empty vector, NCM and pmirGLO reporter plasmid containing PUMA 3'UTR respectively. 24hrs post-transfection luciferase assay was done. FL signal was normalized to RL signal. Fold change for each experimental set was determined with respect to control (pmirGLO vector + NCM) taken as 1; (n=3). E) Cell lysate was incubated with the chromophore labeled substrate (LEHD-*p*NA) and O.D. was measured at 405nm; (n=3). Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. Data shown as mean ± SEM with *$p<0.05$ and **$p<0.01$.

4.2.3 Discussion:

Thus, our results showcase for the first time, activation of CaN in the astrocytes of PD models. We found CaN expression to co-localize with reactive astrocytes in the brain tissue of MPTP mouse models. We also found Ca^{2+}/CaN activation in the human astrocytoma cell line 1321N1 upon Rotenone treatment. Simultaneously, a down-regulation of astrocyte-enriched miR-23a was observed intracellularly in both 1321N1 cells as well as primary astrocyte cells derived from rodent brain cortex. This down-regulation of miR-23a was due to CaN mediated release of miR-23a from the atrocytes via exosomes. When transferred to the neurons, miR-23a could directly bind to the 3'UTR of the pro-apoptotic molecule PUMA and down-regulate its expression, thereby showcasing neuro-protective functions in various models of PD Thus, miR-23a proved to be instrumental in astrocyte-neuron cross-talk during PD.

Reactive astrocytes, as implicated in various NDDs and neuroinflammatory conditions are associated with hypertrophy of astrocyte somata and processes along with an increase in the expression of the glial fibrillary acidic protein (GFAP). Although weakly expressed within astrocytes in healthy adult brain, CaN is reported to express at high levels in reactive astrocytes associated with aging (Norris 2005), acute injury (Goździk et al. 2019), and AD pathology (Norris 2005; 2005). In this study, we found CaN to co-express in the reactive astrocytes as well as found an increased expression of GFAP in the astrocytes where CaN was expressed in acute MPTP mice models.

There are contrasting reports on CaN having protective or detrimental effect on the astrocytes. For instance, one report used immunosuppressant inhibitors of calcineurin and found that they protected astrocytes from apoptosis (Matsuda et al. 2002), while another report found the reverse effect-calcineurin inhibitors increased apoptosis of reactive astrocytes (Pyrzynska et al. 2001). Interestingly, in this study we found activation of CaN pathway to cause a decrease in expression of a particular astrocyte-enriched miR-23a (Zhao et al. 2014). This result however, is in contrast to that reported by Zhao et al. where they report that transcription factor NFATc3 downstream of CaN activation, directly binds to the promoter of miR-23a and causes its upregulation in cardiomyocytes. However, we could explain the decrease in intra-astrocytic miR-23a expression since miR-23a was found to be released from the astrocytes through exosomes upon Rotenone treatment. This is in accordance with another report (Hudson et al. 2014; Fry 2014) that shows miR-23a is released through exosomes downstream of CaN-NFAT pathway during muscle atrophy.

Now, miR-23a is found to show pro-apoptotic roles in cardiomyocytes (Lin et al. 2009), airway epithelial cells (Jin et al. 2019) and human granulosa cells (Nie et al. 2015), while it shows anti-apoptotic functions in neurons during TBI (Li et al. 2020). In this study, we found miR-23a to be neuro-protective against various neurotoxin generated stress such as MPP+, 6-OHDA and Rotenone. Additionally, miR-23a was found to directly bind to the 3'UTR of PUMA, thereby down-regulating its expression, and preventing 6-OHDA mediated induction of neuronal apoptosis.

Thus, our results showcase for the first time that CaN pathway is activated in the astrocytes in PD models, wherein this activated CaN causes release of miR-23a from the astrocytes through exosomes, which mediates astrocyte-neuron cross-talk and protects neurons against PUMA-induced neuronal apoptosis, during PD pathogenesis.

Part III:

Determination of neuron-enriched miR-128 and astrocyte-enriched miR-23a expression profiles in human PD patient samples

4.3.1 Introduction:

For a long time, miRNAs have been considered potential targets as biomarkers in a number of diseases like cancers, liver disorders, infections and neuropathologies like glioblastoma, AD, HD and PD. The biggest advantage of miRNAs as a candidate biomarker is that they are readily detectable in the body fluids like blood (plasma, serum), saliva, urine and even CSF, and show differential expression profiles. It is worth noting here that miRNAs maybe released by the relevant cells or tissue into the circulatory system via two major ways- either as a ribonucleoprotein complex associated with Argonaute proteins, or through membrane bound extracellular vesicles (EVs) like apoptotic bodies, shedding vesicles, and exosomes (Turchinovich, Weiz, and Burwinkel 2012)**.** Among these, exosomes (40–160 nm diameter) (Kalluri and LeBleu 2020), are the most commonly identified microvesicles to carry miRNAs in the body fluid. Exosomes are usually released actively by the respective cells or tissues as a consequence of an altered cellular signalling cascade, thereby reflecting the altered intracellular state during disease pathogenesis and progression. Reports have suggested that cells can modulate selective packaging of miRNAs into exosomes and actively secrete them which maybe further taken up by the neighbouring recipient cells, thus becoming important means of cell to cell communication (Valadi et al. 2007), (Desrochers, Antonyak, and Cerione 2016). Additionally, exosomes show remarkable ability to cross the blood-brain-barrier (BBB) (Haney et al. 2015) and are also impermeable to RNases, keeping the circulating miRNA remarkably stable inside them (Koga et al. 2011). Additionally, exosomal miRNAs can be detected reliably at low concentrations making them useful for disease diagnosis by relatively economical, less time-consuming and less labour-intensive detection methods as qRT-PCR. All these factors allow exosome-derived brain-enriched miRNAs, as opposed to other ubiquitously circulating cell-free miRNAs, to more accurately represent the signature changes associated with specific brain-

associated disorders like PD and become more relevant in the proper understanding of the actual disease pathogenesis. Each disease has its unique pathological characteristics, including the miRNA content and the type of brain cells they are secreted from, all of which makes the exosomal brain-enriched miRNAs, like miR-128 and miR-23a, serve as signatures for differential diagnosis of neurodegenerative diseases like PD.

4.3.2 Results:

miR-128 and miR-23a are released in the circulating extracellular vesicles of human body fluids

First, we decided to check whether miR-128 and miR-23a are at all released inside the EVs of the human circulatory system. For that, we used EVAtlas, which is a latest comprehensive database for non-coding RNA expression in human extracellular vesicles obtained from high-throughput human sRNA-Seq data (C. J. Liu et al. 2022). The EVAtlas repository is curated from 2030 small RNA-seq datasets for human EVs, including 1506 sEV, showcasing comprehensive expression profiles and patterns of ncRNAs in EVs, with the view of aiding biomarker discovery. Our search in the EVAtlas repository confirmed the presence of both miR-128 and miR-23a in the circulating EVs, including the ones secreted in the body fluids. It revealed a higher abundance of miR-128 in the total small extracellular vesicles (sEV) derived from plasma in comparison with other body fluids (Fig.1A), while the expression of miR-23a was comparable across different body fluids (Fig.1B).

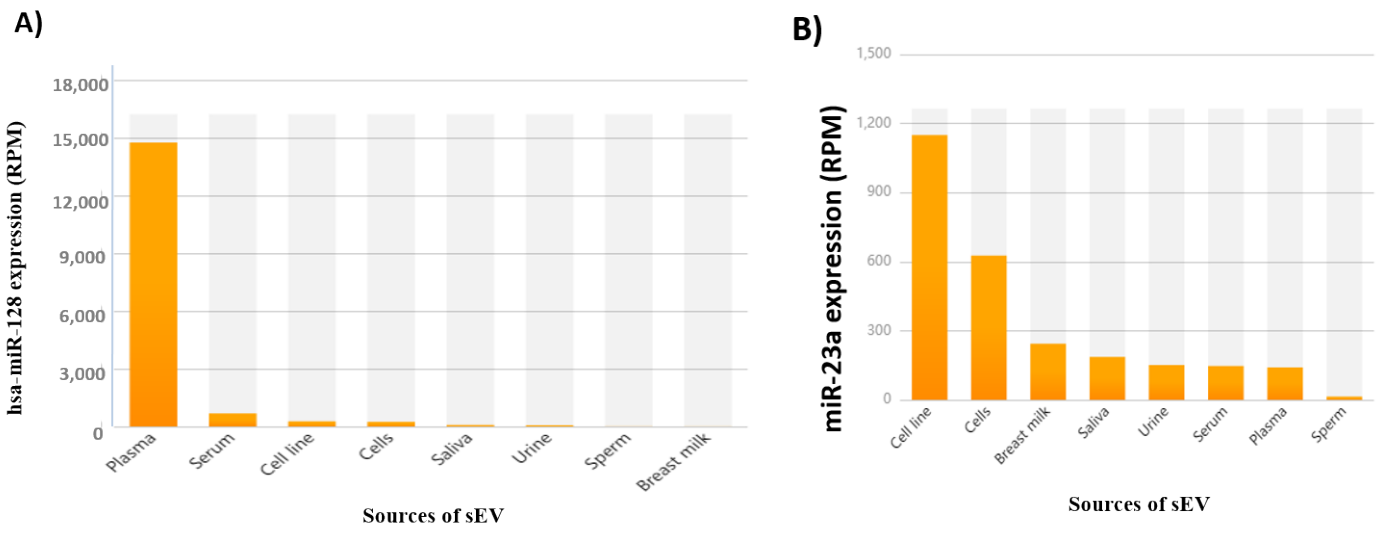

Figure 1: miRNA expression in circulating body fluids: miR-128 (A) and miR-23a (B) expression in the sEV derived from different extracellular sources in the EVAtlas database. miRNA expression is plotted in RPM values.

Small RNA-Seq database-dependent determination of miR-128 and miR-23a expression in PD brain tissue

Having confirmed its presence in the circulating EVs, we next decided to check the expression pattern of miR-128 in PD patient brain. To assess that, we used the recently developed DIANA-miTED, which is the latest miRNA tissue expression atlas derived from human datasets (Kavakiotis et al. 2022). Briefly, DIANA-miTED is the largest comprehensive collection of miRNA expression values derived by analyzing 15,183 raw human small RNA-Seq (sRNA-Seq) datasets obtained from the NCBI-Sequence Read Archive (NCBI-SRA) and The Cancer Genome Atlas (TCGA) respectively. Thus, it helps to derive the abundance estimates of a given miRNA amongst various pathological conditions. The results are represented in reads per million (RPM) which indicates high abundance (> 100 RPM), medium abundance (10–100 RPM) or low abundance (1–10 RPM).

We checked the relevance of miR-128 in PD and compared it with another predominant motor disorder, Huntington's disease (HD) as well as a non-motor disorder, medulloblastoma (MB). We put our query as "hsa-miR-128-3p" in "brain tissue" against "Parkinson's disease" as well as "Huntington's disease" and "medulloblastoma". Interestingly, we found that the expression values of miR-128 were in the range 114,500-246,300 for HD; for PD in the range 99,200-155,400 and for MB in the range 8440-14,140 where all the values are expressed in read counts. Thus, while the expression of miR-128 in the PD patient brain tissue was significantly higher than that of MB, it was comparable or lower than that of HD. (Fig.2A)**.**

Next, we checked the relevance of miR-23a in PD and compared it with HD as well as a non-motor astrocyte-associated disorder, astrocytoma (AC). We put our query as "hsa-miR-23a-3p" in "brain tissue" against "Parkinson's disease" as well as "Huntington's disease" and "astrocytoma". Interestingly, we found that the expression values of miR-23a were in the range 1863-3484 for HD; for PD in the range 1626.5-2066 and for AC in the range 5171-12,150 where all the values are expressed in read counts. Thus, the expression of miR-23a in the PD patient brain tissue was significantly lower than that of both HD and MB (Fig.2B)**.**

Thus, our human sRNA-Seq database dependent results clearly indicated that both neuron-specific miR-128 and astrocyte-specific miR-23a have a strong relevance in the pathophysiology of PD.

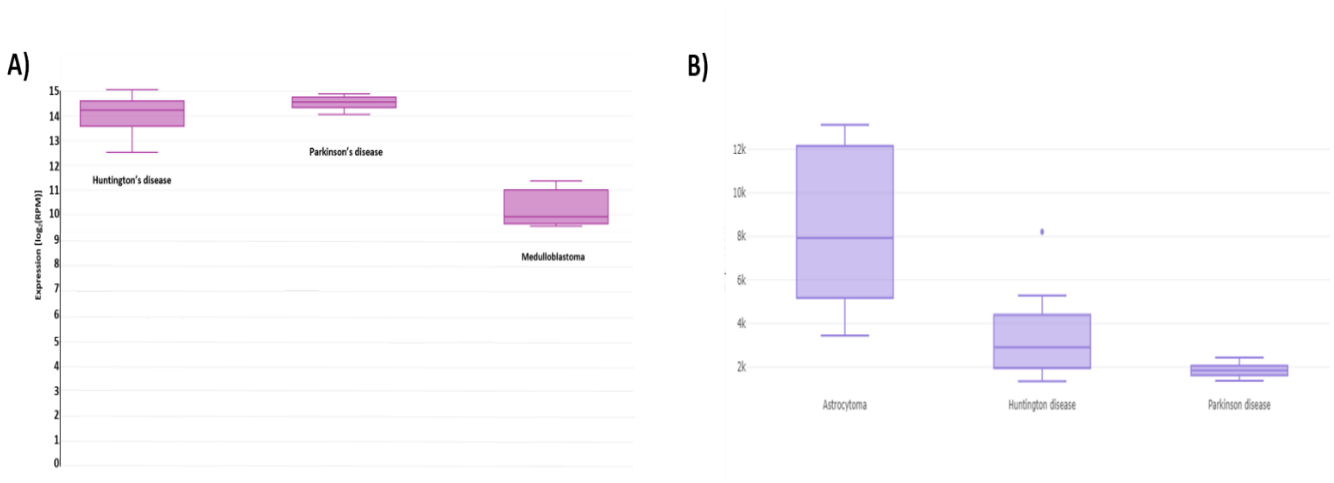

Figure 2: Human small RNA-Seq derived bioinformatics-based determination of the relevance of miR-128 and miR-23a in PD: Bioinformatic tool called DIANA- miTED was used. A) Query was put as "hsa-miR-128-3p" in "brain tissue" against "Parkinson's disease", "Huntington's disease" and "medulloblastoma". Abundance distribution of miR-128 in indicated diseases is expressed as $\log_2$(RPM). B) Query was put as "hsa-miR-23a-3p" in "brain tissue" against "Parkinson's disease", "Huntington's disease" and "astrocytoma". Abundance distribution of miR-23a in indicated diseases is expressed as $\log_2$(RPM).

Neuron-specific miR-128 expression is decreased in the exosomes derived from PD patient plasma

Since our bioinformatics-based results suggested a strong implication of miR-128 in PD incidence, we decided to check the expression levels of miR-128 in the exosomes derived from PD patient plasma samples of a local cohort. We collected blood samples from PD patients (age 40 and above) and respective age-matched controls (with no history of any neurological disorder) and separated plasma from them by centrifugation. We collected intact exosomes by centrifugation using miRCURY exosome isolation kit from serum/plasma (López-Pérez et al. 2021) and further isolated

the total RNA contained in them. From this exosomal RNA, we checked for the expression of miR-128 using qRT-PCR. Interestingly, in comparison to age-matched controls, we found a significant decrease in the expression of exosomal miR-128 collected from PD patient plasma samples as represented in Fig. 3A. We further subjected the intact isolated exosomes to nano-particle tracking analysis (NTA) to confirm the size of the exosomes, as indicated in Fig.3B. Interestingly, there was no significant difference in the number of exosomes isolated from PD patient plasma in comparison to control (Fig.3C), which indicated that the difference in the level of exosomal miR-128 between the PD patient and control samples is due to a decrease in the expression of miR-128 and not due to any change in the quantity of exosomes released. Thus, our initial results showed that there is a significant reduction in the levels of the brain-enriched miR-128, as identified in the exosomes circulating in the blood of PD patients

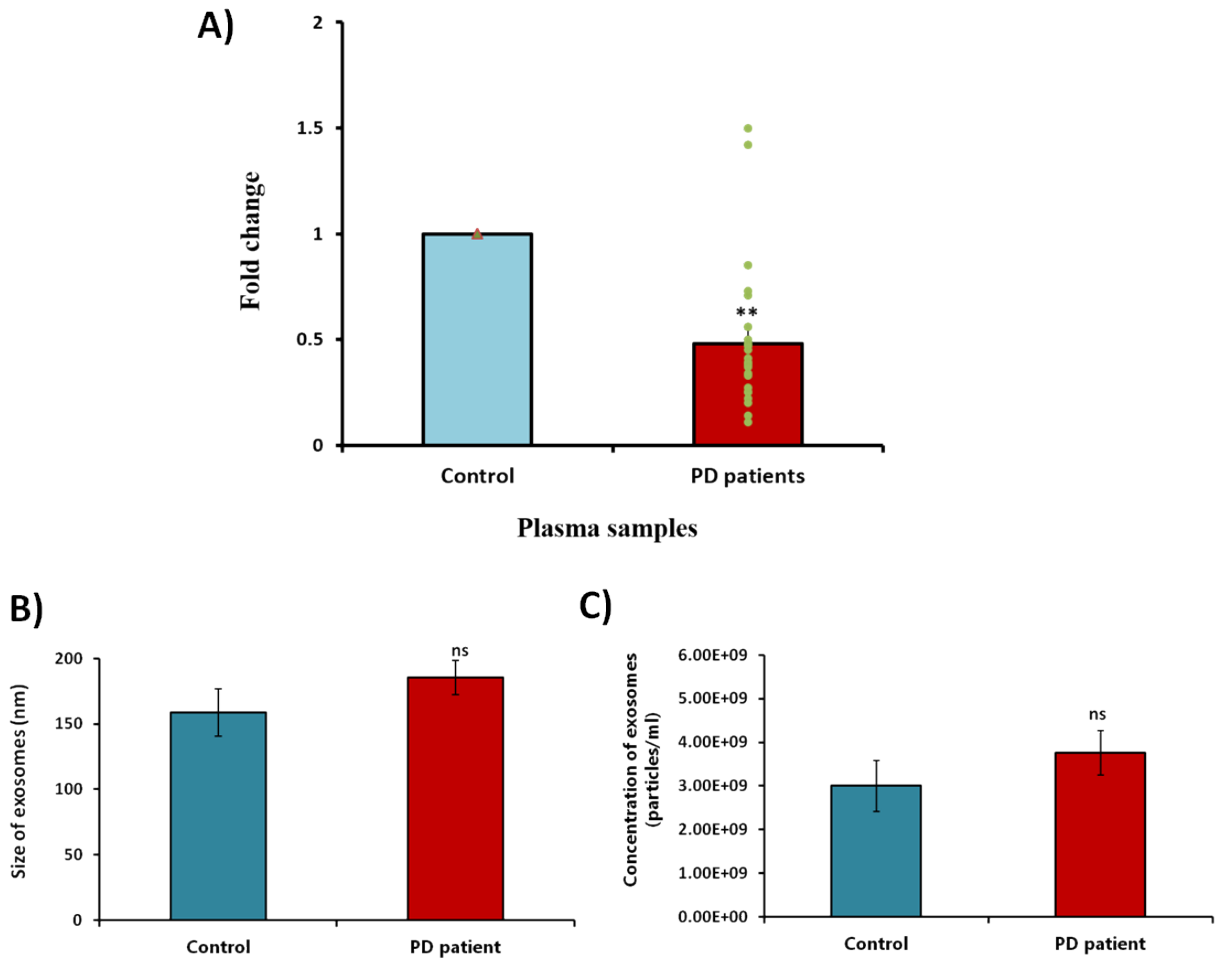

Figure 3: miR-128 expression decreases in the human PD patient plasma-derived exosomes: A) miR-128 expression in the exosomes derived from PD patient (age >40) plasma samples (n=25) as compared to age-matched controls (n=20). U6 expression was used as endogenous control for normalization. B) NTA analysis of the size of exosomes isolated from control (n=5) and PD patient plasma (n=5). C) NTA analysis of the number of isolated exosomes from control (n=5) and PD patient plasma (n=5). Asterisks (*) indicate significant difference and ns indicates non-significant difference with respect to control. Data shown as mean ± SEM with *p<0.05 and **p<0.01.

Astrocyte-specific miR-23a expression is increased in the exosomes derived from PD patient plasma

In the same PD patient and control subject pool, we determined the expression of miR-23a in the exosomes derived from the blood (plasma), as described above. In comparison to age-matched controls, we found a significant increase in the expression of exosomal miR-23a collected from PD patient plasma samples as represented in Fig. 4. Thus this data corroborated and confirmed our previous findings where there was an intra-astrocytic decrease in miR-23a expression, in the in vitro models of PD.

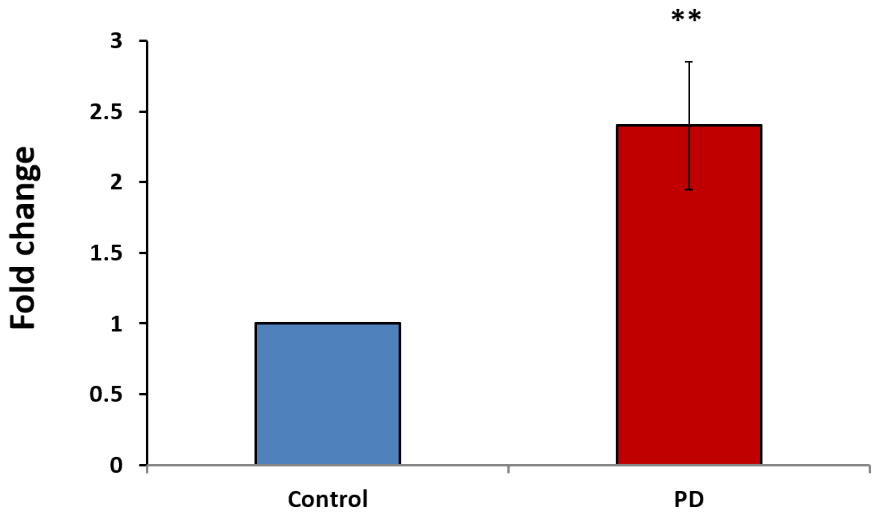

Figure 4: miR-23a expression increases in the human PD patient plasma-derived exosomes: A) miR-23a expression in the exosomes derived from PD patient (age >40) plasma samples (n=25) as compared to age-matched controls (n=20). U6 expression was used as endogenous control for normalization. Asterisks (*) indicate significant difference and ns indicates non-significant difference with respect to control. Data shown as mean ± SEM with *p<0.05 and **p<0.01.

4.3.3 Discussion:

We identified miR-128 and miR-23a in the exosomes derived from the plasma of PD patient blood samples which depicted a significant decrease and increase in the levels of miR-128 and miR23a respectively, in comparison to age-matched controls. Repository of tissue-expression databases generated from human small RNA-Seq datasets showed a higher abundance of miR-128 and lower abundance of miR-23a in PD patient brain-tissue, confirming our observations. Earlier reports have shown the presence of certain miRNAs inside the circulating exosomes of PD patients (Cao et al. 2017),(Gui et al. 2015),(Yao Y.F. et al., n.d.), but to our knowledge, an extensive study on exosome-derived brain-enriched miRNAs, like miR-128 and miR-23a, from the plasma of the PD patients has not been done till date. Since miR-128 is a neuron-enriched and miR-23a is an astrocyte-enriched miRNA, these results suggest that any variation in the levels of miR-128 and miR-23a in the exosomes derived from PD patient plasma- is most likely caused by intracellular changes in the neurons and astrocytes respectively, in the course of PD pathogenesis and progression. Thus, ours is a novel approach in understanding the actual pathogenesis and progression of PD, since we believe, active release of brain-enriched miRNAs through the exosomes is a more appropriate reflection of the dysregulated cellular states and actual disease mechanism involved in brain-related disorders in contrast to certain membrane vesicle-free ubiquitous miRNAs that could be in circulation simply as a consequence of passive release of cytosolic components following cell death derived from any tissue (Gurung et al. 2021). This provides us advantage to correlate the circulating levels of specific neuronal or glial miRNAs like miR-128 and miR-23a respectively, with the disease incidence and progression as well as track the response to treatments. However, our data needs to be validated in large datasets to prove its candidacy as a potential biomarker for PD. Exosome-based miRNA delivery possesses minimal

toxicity with high bio-compatibility, they can evade immunogenicity and also show a remarkable ability to overcome the BBB barrier (Banks et al. 2020), all of which indicates that exosome-based biomarkers might represent an important non-invasive source for detecting potential miRNA as biomarkers of neurodegenerative disease like PD.

Chapter 5:

Conclusion & Summary

Overall, in my PhD thesis, I have identified certain brain-enriched miRNAs, namely, the neuron-enriched miR-128 and the astrocyte-enriched miR-23a and characterized their mechanistic function in the pathogenesis and progression of PD.

miR-128 is a brain- and neuron-enriched miRNA that was first reported to regulate motor dysfunctions related to epilepsy by Tan et al. in 2013. So we decided to check for the relevance of miR-128 in PD by using human small RNA-Seq datasets curated from the miRNA tissue-expression repository DIANA-miTED. Interestingly, we found a reduced expression of miR-128 in PD brain tissue compared to other motor diseases like Huntington's disease. miR-128 was also found abundantly in the exosomes derived from different body fluids as reported in the human exosome database EVAtlas. Next, we checked for the expression of miR-128 in the exosomes derived from the PD patient blood samples. Interestingly, there was a significant decrease in the expression of exosomal miR-128 in the PD patient plasma as compared to age-matched controls. Having found evidence of dysregulated expression of miR-128 in PD patient samples, we decided to check for the mechanistic role of miR-128 in PD pathogenesis in standard *in vitro* models of PD. We treated primed human SH-SY5Y and rat PC12 cells with 6-OHDA (neurotoxin) which showed a decrease in the miR-128 expression with increasing time-points of treatment. Further, fluorescent imaging and flow cytometry-based studies revealed that miR-128 over-expression could protect against mitochondrial superoxide formation as well as reduce the percentage of neuronal death from 6-OHDA treatment. Since earlier reports from our lab showed that 6-OHDA can induce neuronal apoptosis via activation of the transcription factor FoxO3a, we decided to check the effect of miR-128 on apoptosis via FoxO3a activation. We found that 6-OHDA-mediated FoxO3a activation was significantly reversed upon over-expression of miR-128. Interestingly, miR-128 over-expression

also lead to down-regulation of pro-apoptotic proteins like FasL and PUMA and attenuated the 6-OHDA mediated downstream activation of caspases-8, -9 and -3, thereby showing that miR-128 can regulate both the intrinsic and extrinsic pathways of apoptosis. Furthermore, miR-128 over-expression could not only improve neurite length formation but also increase the expressions of synaptic proteins, Synaptophysin and PSD-95 which was reduced during 6-OHDA treatment. Thus, we identified the neuron-enriched miR-128 to be characteristically reduced in the PD patient plasma-derived exosomes and determined for the first time, its comprehensive role in neuronal apoptosis and maintenance of synaptic integrity via the FoXO3a/FasL/ PUMA axis in context to PD.

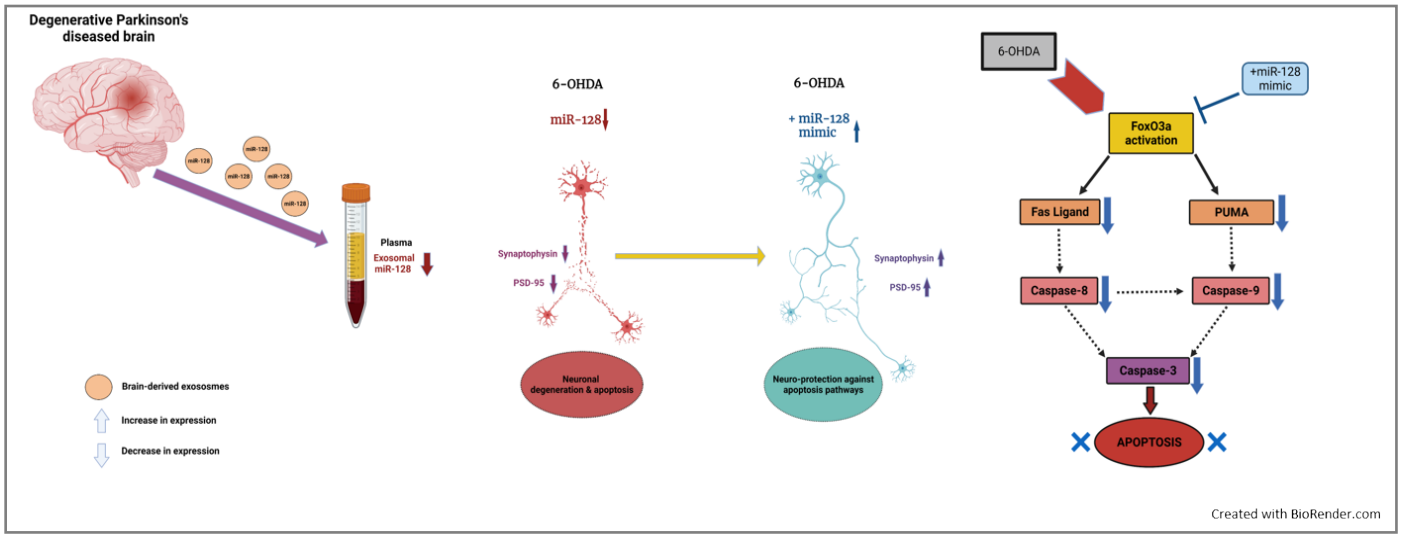

Figure 1: Schematic representation of the role of miR-128 in PD pathogenesis

Astrocyte dysfunction is well implicated in various neurodegenerative disorders like PD and Alzheimer's disease (AD). In recent times, Christopher M. Norris and his group have shown that Ca^{2+}/Calcineurin (Ca^{2+}/CaN) signaling may induce astrocyte dysfunction in AD. However, very little is known regarding its implications in PD. In order to check that, we used MPTP mouse models of PD and identified activation of CaN in the astrocytes by immunohistochemistry. Next,

activation of Ca^{2+}/CaN pathway was also confirmed in the human astrocytic cell line 1321N1 treated with Rotenone (neurotoxin). Further, in both 1321N1 and primary astrocyte cultures from rat brain, activation of Ca^{2+}/CaN led to an intracellular decrease in the expression of miR-23a, an astrocyte-enriched miRNA, reported to be a downstream target of activated CaN pathway. This decrease in the intra-astrocytic miR-23a levels was a result of a concomitant release of miR-23a from the astrocytes via exosomes. The astrocytic miR-23a proved to be neuro-protective via downregulating the pro-apoptotic PUMA in neuronal cells. We cloned the 3'UTR of PUMA and performed luciferase assay which showed a direct binding of miR-23a to the PUMA 3'UTR. Thus, miR-23a was instrumental in regulating astrocyte-neuron cross-talk in context to PD. Next, DIANA-miTED derived analysis revealed a lower expression of miR-23a in PD brain-tissue as compared to HD or astrocytoma (AC), confirming our *in vitro* results. Finally, miR-23a expression was found to characteristically increase in the exosomes isolated from PD patient plasma, thereby indicating a vital role of the astrocyte-enriched miR-23a in PD pathogenesis.

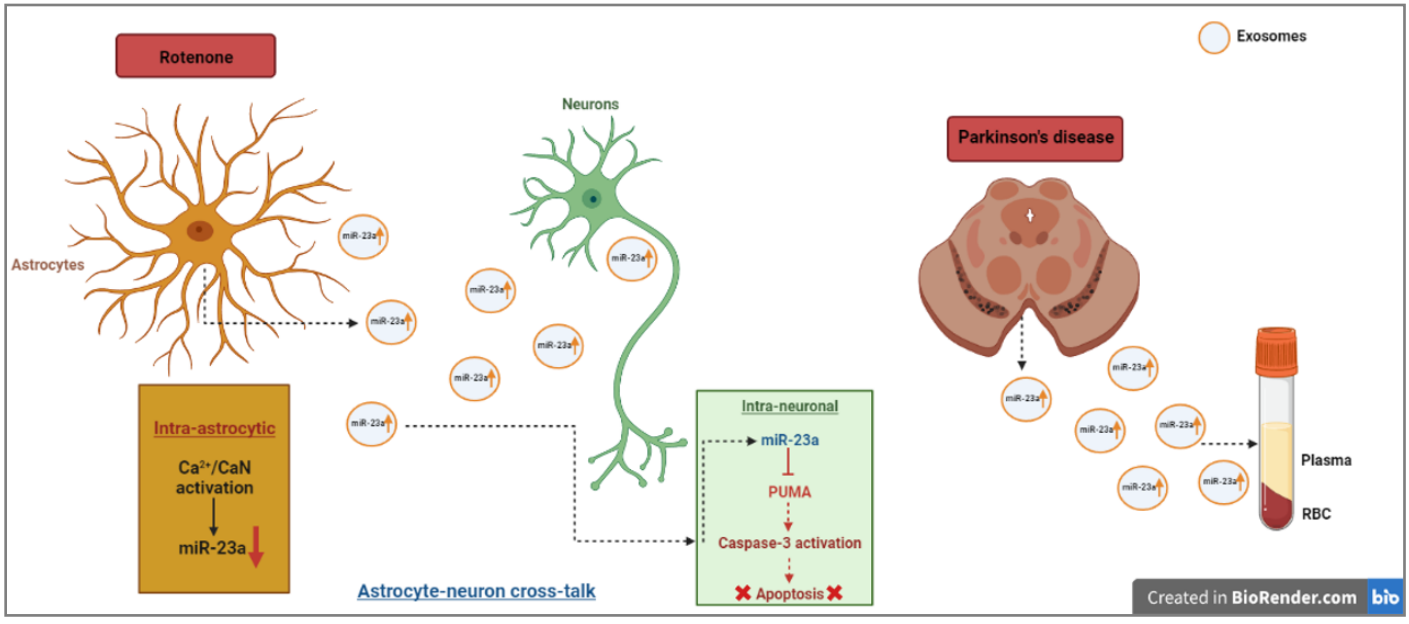

Figure 2: Schematic representation of the role of miR-23a in PD pathogenesis

Thus, through my Ph.D. projects, I have identified two such brain-enriched miRNAs- miR-128 (neuron-enriched) and miR-23a (astrocyte-enriched) and explored in depth, the molecular mechanisms by which they regulate neurodegeneration in various models of PD. Additionally, these miRNAs were found to be characteristically altered in the exosomes obtained from PD patient plasma, suggesting their potential as candidate PD biomarkers.

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Appendix I: List of publications

1. Bhattacharyya P, Biswas A, Biswas SC*. (2022). Brain-enriched miR-128 that maintains synaptic integrity and protects against 6-OHDA induced neuronal apoptosis via FoXO3a/PUMA/FasL axis is also characteristically reduced in exosomes of Parkinson's patient plasma. Frontiers in Cellular Neuroscience (under revision).

2. Bhattacharyya P, Biswas A, Biswas SC*. Exosome-derived miR-128 is decreased in Parkinson's patient plasma that maintains synaptic integrity and protects against both intrinsic and extrinsic pathways of neuronal apoptosis in models of Parkinson's disease. 19 July 2022, PREPRINT (Version 1) available at Research Square [**doi:**10.21203/rs.3.rs-1838421/v1]

3. Bhattacharyya P, Biswas A, Biswas SC*. (2022). Exosome mediated release of miR-23a from the astrocytes down-stream of $Ca^{2+/}$Calcineurin pathway protects the neurons against PUMA-mediated apoptotic death in models of PD. (Manuscript submitted).

4. Bhattacharyya P, Biswas SC*. (2021). Ca^{2+}/Calcineurin-mediated release of exosomal miR-23a regulates astrocyte-neuron cross-talk and protects against neuroinflammation and neurodegeneration. AAIC Neuroscience Next 2021 abstracts. Alzheimer's & Dementia. Vol. 17, Issue S2 (e058226) [**doi:**10.1002/alz.058226].

5. Bhattacharyya P, Biswas SC*. (2020).
   $Ca^{2+/}$Calcineurin mediated release of exosomal miR-23a has a role in astrocyte dysfunction and ne urodegeneration in Parkinson's disease model. 2020 ASCB Annual Meeting abstracts. Molecular Biology of the Cell. Vol. 31, Issue. 26 (abstract #P587) [**doi:**10.1091/mbc.E20-10-0665].

6. Bhattacharyya P, Biswas SC*. (2020). Small Non-coding RNAs: Do They Encode Answers for Controlling SARS-CoV-2 in the Future? Front Microbiol. Sep 18; 11:571553 [**doi:**10.3389/fmicb.2020.571553].

7. Bhattacharyya P. (2015). Poly (ADP-Ribose) Polymerase-1 causes mitochondrial damage and neuron death mediated by Bnip3, J Neurosci. 2014 Nov 26; 34(48):15975-87. Ann Neurosci. Jul; 22(3):180 [**doi**:10.5214/ans.0972.7531.220309].

Appendix II: List of conferences & workshops attended

CONFERENCE PRESENTATIONS:

1. *Ca^{2+}/Calcineurin mediated release of astrocytic miR-23a regulates neuron-glia cross-talk and is increased in the exosomes derived from PD patient plasma*
   - Accepted for **poster presentation** at **ISFTD 2022**, November 2-5, 2022, Paris & Lille, France.

2. *Exosomal miR-128 expression is decreased in the Parkinson's patient plasma which maintains synaptic integrity and protects the neurons against both intrinsic and extrinsic pathways of apoptosis*
   - **Oral presentation** at the **Indian Academy of Neurosciences: Kolkata Chapter 2022**, August 26, 2022, India.

3. *Exosomal miR-128 is decreased in the Parkinson's patient plasma that maintains synaptic integrity and protects the neurons against both intrinsic and extrinsic pathways of apoptosis*
   - **Poster presentation** at the **EMBO India Lecture Course 2022** 'Functional nucleic acids: recent landscapes and therapeutic applications', 16-19 August, 2022, India.

4. *Exosomal miR-128 is altered in the Parkinson's patient plasma and protects the neurons against apoptosis and maintains synaptic integrity in Parkinson's disease models*
   - **Poster presentation** at the **FENS Forum 2022,** July 9-13, 2022, Paris, France.

5. *miR-128 protects neuronal cells from both extrinsic and intrinsic pathways of apoptosis and maintains synaptic integrity in Parkinson's Disease model.*
   - **Oral presentation** under the 'Young Talent Forum' at the **NeuroUpdate** conference, November 28, 2021, India.

6. *Ca^{2+}/Calcineurin-mediated release of exosomal miR-23a regulates astrocyte-neuron cross-talk and protects against neuroinflammation and neurodegeneration.*
   - **Poster presentation** (virtual) at **AAIC Neuroscience Next**, Oct. 12-13, 2021.

7. *Ca^{2+}/calcineurin mediated release of exosomal miR-23a has a role in astrocyte dysfunction and neurodegeneration in Parkinson's disease model.*

   - **Poster presentation** (online) at the **Cell Bio Virtual 2020**, an online **ASCB-EMBO Meeting**, 2-16 December, 2020.

8. *The Role of Ca^{2+}/Calcineurin in astrocyte dysfunction and neurodegeneration via exosomally released miR-23a in Parkinson's disease model*

   - **Poster presentation** at the **37th Annual Conference of Indian Academy of Neurosciences:** Neuron to Behavior, co-sponsored by the **International Society for Neurochemistry (ISN)**, 18-21 November 2019, India.

9. *'Transcription factor FOXO3a regulates pro-apoptotic Fas ligand via microRNA 21 in models of Parkinson's disease'*

   - **Oral presentation** at **Neurocon: International conference** on growth, senescence and mortality of neurons: from traditional medicine to cutting edge technology, 19-22 January 2017, India.

8. *'Transcription factor FOXO3a regulates Fas ligand via microRNA 21 in a cellular model of Parkinson's disease'*

- **Poster presentation** at the **34th Annual meeting of Indian Academy of Neurosciences**: Molecules to Mind, 19-21 October 2016, India.

WORKSHOPS ATTENDED:

1. Accepted for **CAJAL Advanced Neuroscience Training** on **Neuroepigenetics: writing, reading and erasing the epigenome,** 21 November- 9 December 2022, Bordeaux School of Neuroscience, Bordeaux, France.

2. **EMBL Course: Genome Engineering: CRISPR/Cas from Cells to Mice**, Virtual, 27 September - 4 October 2021.

3. **CRISPR-Cas9 based genome editing: 3rd hands-on international worshop** organized by CSIR- Institute of Genomics and Integrative Biology, India, April 08-12, 2019.

4. **Bioscopy 2019: Hands-on microscopy (Confocal) workshop organized by the Indian Biophysical Society (IBS),** March 17-20, 2019 at IISER- Kolkata, co-organized by C-CAMP (Bangalore), RRI (Bangalore) and IACS (Kolkata), India.

5. **EMBO workshop: Molecular Neuroscience: From Genes To Circuit In Health And Disease,** February 4-7, 2019 at National Center for Biological Sciences (NCBS), Bangalore, India.
   Poster presentation entitled **"**Role of Calcineurin in regulating astrocyte integrity and neurodegeneration via exosomally released miR-23a in Parkinson's disease model"

6. **Brainmodes: A multimodal Brain: Spatiotemporal Network Mechanisms And Models,** December 11-14, 2017 at National Brain Research Center, India**.**

7. **ADBS hands-on workshop: Reprogramming Human Somatic Cells to hiPSC,** November 6-11, 2017 at InStem, NCBS, India.

Appendix III: List of awards & honors

1. **First Prize** for **oral presentation** at the **Indian Academy of Neurosciences: Kolkata Chapter 2022** symposium (August 26, 2022), India.
2. **EMBO Travel Award** for attending the EMBO India Lecture Course 2022: Functional Nucleic Acids (August 16-19, 2022), India.
3. **First Prize** for **oral presentation** under the 'Young Talent Forum' at the NeuroUpdate conference (November 28, 2021), India.
4. **EMBL Corporate Partnership Programme Registration Fee Waiver** for the EMBL Course: Genome Engineering: CRISPR/Cas from Cells to Mice (27 September - 4 October, 2021).
5. **Ravindra & Lalita Nath Travel Fellowship** by XXXVII Annual Conference of Indian Academy of Neurosciences, co-sponsored by the International Society for Neurochemistry (2019).
6. **CSIR-Shyama Prasad Mukherjee Fellowship,** for qualifying the CSIR-NET-JRF exam in Biology with an **All India Rank 5** (December 2013).

Exosomal brain-enriched miR-128 is characteristically reduced in the Parkinson's patient plasma that maintains synaptic integrity and protects against neuronal apoptosis via the FoxO3a/PUMA/FasL axis

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Conflict of interest statement

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest

Author contribution statement

PB conceived and designed the study and SCB supervised the project. PB performed bioinformatics, biochemical, molecular and cellular experiments. AB performed the clinical assessment of patients and collection of blood. PB and SCB analyzed the data and contributed to writing the manuscript. All authors read and approved the final submitted manuscript.

Keywords

MiR-128, Parkinson's disease, Brain-enriched miRNAs, Neuron-enriched miRNAs, Apoptosis, Fas - ligand, PUMA (p53 upregulated modulator of apoptosis), FoxO3a

Abstract

Word count: 250

Parkinson's disease (PD) is a progressive neurodegenerative disorder associated with death of dopaminergic neurons. Unfortunately, no effective cures or diagnostic biomarkers for its detection are available yet. To address this, the present study focuses on brain-enriched small non-coding regulatory RNAs called microRNAs (miRNAs) that are released into the circulation packaged inside small extracellular vesicles called exosomes. We collected blood samples from a PD patient cohort and isolated exosomes from the plasma. qPCR-based detection revealed a particular neuron-enriched miR-128 to be significantly decreased in the patient-derived exosomes. Interestingly, a concomitant decreased expression of miR-128 was observed in the cellular models of PD. Fluorescent live cell imaging and flow-cytometry revealed that over-expression of miR-128 can prevent 6-OHDA-mediated mitochondrial superoxide production and induction of neuronal death respectively. This neuroprotective effect was found to be induced by miR-128-mediated inhibition of FoxO3a activation, a transcription factor involved in apoptosis. miR-128 over-expression also resulted in down-regulation of pro-apoptotic FoxO3a targets- FasL and PUMA, at both transcript and protein levels. Further downstream, miR-128 over-expression inhibited activation of caspases-8, -9 and -3, preventing both the intrinsic and extrinsic pathways of apoptosis. Additionally, over-expression of miR-128 prevented down-regulation of synaptic proteins- Synaptophysin and PSD-95 and attenuated neurite shortening, thereby maintaining overall neuronal integrity. Thus, our study depicts the intracellular mechanistic function of neuroprotective miR-128 and its implications as a biomarker in the circulating exosomes detectable in PD patient blood. The characterization of such exosomal brain-enriched miRNAs could be the key to effective diagnosis of degenerative brain disorders like PD.

Contribution to the field

Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder affecting around 10 million people worldwide. Unfortunately, no effective cure or diagnostic biomarkers for PD have been identified till date. Our study aims at targeting this scientific lacuna by focusing on certain small non-coding regulatory RNAs called microRNAs (miRNAs) that are released into the circulating body fluids packaged inside small extracellular vesicles called exosomes. These exosomal miRNAs may be detected in blood, saliva, urine and CSF, making them important targets as biomarkers for brain diseases, including PD. miR-128, is a highly brain-enriched miRNA which is specifically more abundant in the neurons. In this study, we have identified miR-128 to be characteristically reduced in the exosomes derived from the PD patient plasma and determined its integral role in the pathways leading to neuronal apoptosis and maintenance of synaptic integrity in cellular models of PD. Our results indicate that identification and characterization of such brain-enriched exosome-derived miRNA signatures are essential for understanding PD pathogenesis and are more appropriate candidates as PD biomarkers, in contrast to the ubiquitously expressed cell-free miRNAs that could be in circulation as a consequence of passive lysis of cells derived from any tissue.

Ethics statements

Studies involving animal subjects

Generated Statement: No animal studies are presented in this manuscript.

Studies involving human subjects

Generated Statement: The studies involving human participants were reviewed and approved by Human Ethics Committee of CSIR-IICB. The patients/participants provided their written informed consent to participate in this study.

Inclusion of identifiable human data

Generated Statement: No potentially identifiable human images or data is presented in this study.

Data availability statement

Generated Statement: The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

Exosomal brain-enriched miR-128 is characteristically reduced in the Parkinson's patient plasma that maintains synaptic integrity and protects against neuronal apoptosis via the FoxO3a/PUMA/FasL axis

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Running Title: Exosomal miR-128 in PD pathogenesis

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Total figures: 4

Abstract

Parkinson's disease (PD) is a progressive neurodegenerative disorder associated with death of dopaminergic neurons. Unfortunately, no effective cures or diagnostic biomarkers for its detection are available yet. To address this, the present study focuses on brain-enriched small non-coding regulatory RNAs called microRNAs (miRNAs) that are released into the circulation packaged inside small extracellular vesicles called exosomes. We collected blood samples from a PD patient cohort and isolated exosomes from the plasma. qPCR-based detection revealed a particular neuron-enriched miR-128 to be significantly decreased in the patient-derived exosomes. Interestingly, a concomitant decreased expression of miR-128 was observed in the cellular models of PD. Fluorescent live cell imaging and flow-cytometry revealed that over-expression of miR-128 can prevent 6-OHDA-mediated mitochondrial superoxide production and induction of neuronal death respectively. This neuroprotective effect was found to be induced by miR-128-mediated inhibition of FoxO3a activation, a transcription factor involved in apoptosis. miR-128 over-expression also resulted in down-regulation of pro-apoptotic FoxO3a targets- FasL and PUMA, at both transcript and protein levels. Further downstream, miR-128 over-expression inhibited activation of caspases-8, -9 and -3, preventing both the intrinsic and extrinsic pathways of apoptosis. Additionally, over-expression of miR-128 prevented down-regulation of synaptic proteins- Synaptophysin and PSD-95 and attenuated neurite shortening, thereby maintaining overall neuronal integrity. Thus, our study depicts the intracellular mechanistic function of neuroprotective miR-128 and its implications as a biomarker in the circulating exosomes detectable in PD patient blood. The characterization of such exosomal brain-enriched miRNAs could be the key to effective diagnosis of degenerative brain disorders like PD.

Keywords: Parkinson's disease, brain-enriched miRNAs, neuron-enriched miRNAs, miR-128, exosome, PUMA, FasL, FoxO3a.

Introduction

Parkinson's disease (PD), a progressive age-related neurodegenerative disorder, is mediated by the loss of dopaminergic neurons in the mid-brain (Bekris et al. 2010; Lin and Farrer 2014). It is primarily characterized by motor dysfunctions namely muscle rigidity, bradykinesia, postural instability and tremor although non-motor manifestations like sleep disorder, fatigue, depression, gastrointestinal or sexual dysfunctions are also not uncommon (Bloem et al. 2021). Incidentally, more than 200 years have passed since the first identification of PD by James Parkinson in 1817 (Obeso et al. 2017), but unfortunately, any effective cure or diagnostic biomarker for its detection are not available till this day. Our present study aims at targeting this scientific lacuna by focusing on a category of small non-coding RNAs called microRNAs (miRNAs) that are released more into the circulatory system by the brain tissue during pathogenesis of PD.

miRNAs (20-23 nucleotides) are important regulators of gene expression, typically at the post-transcriptional level (Bhattacharyya and Biswas 2020; Filipowicz et al. 2008). Interestingly, miRNA expression, itself, is temporally and spatially controlled, with some miRNAs known to be expressed ubiquitously, while certain others are expressed preferentially in specific cell or tissue types. Incidentally, there are specific miRNAs which are brain-enriched and are preferentially expressed in particular brain cells like neurons or glia, suggesting their important regulatory functions in the pathogenesis of brain-related disorders like PD. Additionally, miRNAs may be detected extracellularly, in circulating body fluids such as blood, saliva, urine and CSF, making them important targets as biomarkers for brain diseases, including PD. There has been a plethora of work identifying a wide range of differentially expressed circulating miRNAs associated with PD, but none of them have been successful as a biomarker so far. One of the biggest caveats of such studies, we believe, is the *source* of miRNAs under study. While the circulating miRNAs may show a dysregulated pattern, these ubiquitously expressed miRNAs could be derived from any tissue or cell type and may not be specific to the particular tissue under consideration (in this case, brain). This makes reproducibility a major problem, as the miRNA profile may vary from subject to subject. Secondly, the *type* of cell-free miRNAs to be identified also needs careful consideration. It is worth noting here that miRNAs maybe released by the parental cells or tissue in two major ways- either as a ribonucleoprotein complex associated with Argonaute proteins, or encapsulated within membrane-bound extracellular vesicles (EVs) like apoptotic bodies, shedding vesicles and exosomes (Turchinovich et al. 2012)**.** Among these, exosomes are the most commonly identified small extracellular vesicles (sEV) to carry miRNAs in the circulating body fluids.

Exosomes (40-160 nm diameter), are most commonly released by the cells as means of secreting cargo (miRNAs, in this case) (Kalluri and LeBleu 2020). These exosomes may be uptaken by the neighboring cells, thereby facilitating cell-to-cell communication (Desrochers et al. 2016; Valadi et al. 2007). Additionally, exosomes are released by the parental cells or tissue in an energy-dependent manner, through specific signaling cascades that maybe dysregulated during disease pathogenesis (Turchinovich et al. 2012). Thus, the exosomal miRNA profile would better reflect the altered intracellular states under diseased conditions, in contrast to the cell-free miRNAs that maybe released as a passive by-product of cell lysis, not particularly indicative of the diseased state

(Gurung et al. 2021). Further, exosomes are specifically important in context to brain diseases as they are known to cross the blood-brain-barrier effectively (Haney et al. 2015).

Taking into consideration the above factors, we have hypothesized that identification and characterization of specific brain-enriched exosome-derived miRNA signatures are essential for understanding PD pathogenesis and could be more appropriate candidates as PD biomarkers. Through extensive literature review, we zeroed down on a particular neuron-enriched miRNA, miR-128 (He et al. 2012; Liu and Xu 2011; Shao et al. 2010; Smirnova et al. 2005), which seemed to be an interesting candidate for our study on PD. miR-128 first came into prominence when Chan Lek Tan et al. (Tan et al. 2013) identified that miR-128 deficiency can cause motor dysfunction and seizure- induced death in epileptic mouse models. On the other hand, ectopic expression of miR-128 was found to restore excitability in post-natal dopamine responsive (D1) neurons. Since then, the significance of miR-128 in different motor neuron function and diseases as a *motomiR* has been explored (Hawley et al. 2017). Interestingly, a recent report has detected the presence of miR-128 in the CSF of PD patients as well (van den Berg et al. 2020), although the mechanistic implications of miR-128 in PD pathogenesis, if any, is still largely unexplored.

In the present study, we identified the expression of miR-128 to be characteristically reduced in the circulating exosomes derived from the blood (plasma) samples of a PD patient cohort. miR-128 being a neuron-enriched miRNA, we decided to corroborate whether the altered extracellular expression pattern of the miRNA is also associated with dysregulated intracellular diseased states in cellular models of PD. We found that miR-128 can prevent 6-OHDA-mediated neuronal death through both the intrinsic and extrinsic pathways of apoptosis via regulating the activation of the transcription factor, FoxO3a. miR-128 was also instrumental in maintaining neurites and synaptic integrity, showing neuroprotective functions overall. Therefore, our results indicate that brain-enriched exosomal miR-128 could be a potential candidate biomarker for PD.

Materials & Methods

Human blood sample collection and plasma separation

Blood samples were collected at the Bangur Institute of Neurosciences (BIN), Kolkata, India, from PD patients (age>45 years) and respective age-matched controls, in EDTA-vials (BD Biosciences). The upper plasma layer was separated from the blood samples by centrifugation at 2000*g for 10 minutes at 4^0C.

Exosome isolation

Exosomes were separated from the human plasma samples by miRCURY Exosome Serum/Plasma kit (Qiagen) as per manufacturer's protocol. Briefly, plasma samples were incubated with Thrombin at room temperature for 5 minutes and then centrifuged at 10,000*g for 5 minutes. The supernatant was collected and Precipitation Buffer was added to it. The sample was allowed to incubate overnight at 4^0C. It was then centrifuged at 500*g for 5 minutes at 20^0C. Resuspension buffer was added to the pellet and this purified exosome sample was used for further downstream processing.

Nano-partice tracking analysis (NTA) of exosomes:
Purified exosomes were 10-fold diluted in 1X PBS and subjected to nano-particle tracker analysis (NTA) (NanoSight NS300) as per manufacturer's guidelines.

RNA isolation and quantitative real-time PCR (qRT-PCR)
RNA isolation was done using TRIzol Reagent (Thermo Fisher) as per phenol-chloroform based total RNA extraction method. For miRNA analysis, cDNA was prepared using TaqMan MicroRNA Reverse Transcription Kit (Thermo Fisher) using manufacturer's protocol, followed by qRT-PCR using TaqMan Universal PCR Master Mix (Thermo Fisher) and specific miRNA assay reagents (Thermo Fisher) as enlisted in Supplementary Table ST1. For mRNA analysis, cDNA was prepared using PrimeScript 1st strand cDNA Synthesis Kit (Takara Bio) using manufacturer's protocol, followed by qRT-PCR using SYBR Green PCR Master Mix (Thermo Fisher). The primers used for mRNA qRT-PCR based analysis are enlisted in Supplementary Table ST2. U6 snRNA and GAPDH mRNA were used as endogenous controls in miRNA and mRNA qRT-PCR analysis respectively. All qRT-PCR reactions were done in StepOnePlus Real-Time PCR System (Thermo Fisher). Comparative C_T Method ($\Delta\Delta C_T$ Method) was used for the qRT-PCR data analysis.

miRNA mimic transfection
SH-SY5Y or PC12 cells were plated and primed for 2 days in the presence of ATRA or NGF-β respectively. Transfection with miRNA mimic and negative control mimic (Ambion) (Supplementary Table ST1) was done on the second day of priming in Opti-MEM medium (Thermo Fisher) using Lipofectamine RNAiMAX Reagent (Invitrogen), following manufacturer's protocol. Six hours post-transfection, Opti-MEM medium was replaced with the respective priming medium. Cells were further maintained for 48 hours and then treated with 6-OHDA on the fifth day of priming.

Flow cytometry
Cells were analysed by flow cytometry using LIVE/DEAD Viability/Cytotoxicity Kit for mammalian cells (Thermo Fisher), using manufacturer's protocol. Briefly, cells *in vitro* were trypsinized and a cell suspension was prepared in culture medium. Calcein-AM and ethidium homodimer-1 was added to the cell suspension and incubated at room temperature for 15-20 minutes in dark. Flow cytometric analysis was done with excitation at 488nm using BD LSRFortessa.

Live-cell imaging for mitochondrial superoxide detection
Cells *in vitro* were incubated with MitoSOX Red reagent (Thermo Fisher) prepared in HBSS buffer ($Ca^{2+,}$ $Mg^{2+,}$ no phenol red) for 10 minutes in dark in the humidified cell culture incubator (37^0C, 5% CO_2). Cells were washed with warm buffer and imaged under fluorescent microscope (Leica) under 20X objectives with excitation at 510nm and emission at 580nm.

Immunoblotting
Cultured cells *in vitro* were lysed in RIPA buffer (Thermo Scientific) and protein samples (25-50μg) from whole cell lysates were resolved by SDS-PAGE. Gels were transferred on PVDF membranes (GE Healthcare) at 100V for 1-2 hours at 4^0C. Membranes were blocked in 5% non-fat dry milk or 5% bovine serum albumin (BSA) for 1 hour at room temperature. Primary antibodies were diluted in the blocking solution (Supplementary Table ST3) and incubated overnight at 4^0C.

HRP-conjugated secondary antibodies diluted in blocking solution were used against the respective primary antibodies and incubated at room temperature for 1 hour. Detection of protein bands was carried out by using Clarity Max Western ECL substrate (Bio-Rad) as per manufacturer's protocol. Images of all the blots were taken using iBright Imaging system (Thermo Fisher).

Neurite length measurement

Primed PC12 cells were observed under bright field microscope using 20X objectives. Neurite length was measured using the NeuronJ plugin of Fiji software.

Schematic representation

All the schematic diagrams were prepared using BioRender.com.

Statistical analysis

All graphs, statistical analyses and image analyses were done using GraphPad Prism 9.0, Microsoft Excel, ImageJ and Fiji softwares. Student's *t-test* was performed as paired or unpaired, two-tailed sets, as required, to determine the p-value. P-value <0.05 was considered significant and depicted as * while p-value<0.01 was depicted as **. All the experiments were conducted at-least 3 times. Error bars indicate mean +/- SEM.

Results

miR-128 expression is decreased in the exosomes derived from PD patient plasma

We collected blood samples from PD patients (age 40 and above) and respective age-matched controls (with no history of neurological disorder) of a local cohort and separated plasma by centrifugation. Next, intact exosomes were collected using miRCURY exosome isolation kit from serum/plasma (López-Pérez et al. 2021) and the intra-exosomal total RNA was isolated. qRT-PCR based detection of the expression of miR-128 revealed a significant decrease in the expression of exosomal miR-128 obtained from PD patient subsets as compared to controls, as represented in Fig. 1A. We further subjected the intact isolated exosomes to nano-particle tracking analysis (NTA) to confirm the size of the exosomes, as indicated in Fig.1B. Interestingly, there was no significant difference in the number of exosomes isolated from PD patient plasma in comparison to control (Fig.1C). This indicated that the difference in the level of exosomal miR-128 between the PD patient and control samples is due to a decrease in the expression of miR-128 and not due to any change in the quantity of exosomes released. Thus, our initial results showed that there is a significant reduction in the levels of the brain-enriched miR-128, as identified in the exosomes circulating in the blood of PD patients.

We further used a recently developed bioinformatics tool called DIANA-miTED (Kavakiotis et al. 2022), to determine the abundance of miR-128 in brain tissues of PD patients. Briefly, DIANA-miTED is a comprehensive database of miRNA expression values derived from human small RNA-Seq (sRNA-Seq) datasets obtained from the NCBI-Sequence Read Archive and The Cancer Genome Atlas respectively. Thus, it gives abundance estimates of a given miRNA amongst various pathological conditions. We checked the abundance of miR-128 in PD and compared it with another

predominant motor disorder, Huntington's disease (HD) as well as a non-motor disorder, medulloblastoma (MB). Interestingly, we found a higher abundance of miR-128 in PD brain tissues as compared to HD and MB (Supplementary Fig.S1A). Next, we determined the prevalence of miR-128 in circulating sEV through another tool called EVAtlas (Liu et al. 2022) which is a comprehensive database for non-coding RNA expression in sEV obtained through human sRNA-Seq datasets. It showed a higher abundance of miR-128 in the total sEV derived from the plasma as compared to other body fluids (Supplementary Fig.S1B). Thus overall, our results clearly indicated that miR-128 has a strong relevance in the pathophysiology of PD.

miR-128 prevents neuronal death and inhibits mitochondrial superoxide production in cellular models of PD

miR-128 being a neuron-enriched miRNA, we next investigated if there is any intra-neuronal mechanistic significance of this reduced levels of miR-128 in context to PD. Since rodent dopaminergic neuron cultures have low purity, we used standard cellular models of PD, using human SH-SY5Y and rat PC12 cell lines which have reported to show dopaminergic neuronal properties upon priming with ATRA and NGF-β respectively (Falkenburger et al. 2016). We treated the cells with the neurotoxin 6-OHDA, a dopamine analog, that is up-taken by the dopamine transporters and is known to selectively damage the dopaminergic neurons (catecholaminergic) in the mid-brain (Simola et al. 2007).

First, we treated primed SH-SY5Y cells with varying doses of 6-OHDA and identified the D_{50} dose of 100μM by MTT assay (Fig.2A). Next, we treated the cells with 100 μM dose of 6-OHDA for varying time points and checked the expression of miR-128 by qRT-PCR. Interestingly, there was a significant decrease in the expression of miR-128 by 4h and 8h of 6-OHDA treatment (Fig.2B). To confirm that this result is not a cell-line specific, we repeated the experiment in primed PC12 cells with 100μM dose of 6-OHDA (Sanphui et al. 2020) and found a similar decrease in miR-128 expression with increasing time-points of treatment (Fig.2C). This proved that 6-OHDA treatment results in a decrease in miR-128 expression in the neuronal models of PD. Depending on these results, for our subsequent experiments, we decided to choose 100μM of 6-OHDA in the treatment window of 4h-16h on SH-SY5Y or PC12 cell lines. In all cases, we used four experimental conditions: untreated cells (control), cells treated with 6-OHDA for indicated time-points, cells pre-transfected with miR-128 mimic (over-expression constructs) or negative control mimic (NCM) for 48h before treatment with 6-OHDA.

Firstly, we investigated whether miR-128 has an overall protective or detrimental role on neurons. We performed a flow cytometry-based live/dead cell assay. SH-SY5Y cells were treated with 6-OHDA for 16h.The cells were then incubated with the green fluorescent dye calcein-AM (staining live cells) and the red fluorescent dye ethidium homodimer-1 (staining dead cells) and the percentage of live/dead cells was determined by flow cytometry. There was a significant decrease in live cells (Q4) and increase in total dying or dead cells (Q2+Q1) with 6-OHDA treatment which was significantly reversed in cells pre-transfected with miR-128 mimic (Fig. 2D). The change in the total percentage of dying and dead cells (Q2+Q1) is expressed in Fig.2E. These results indicate that miR-128 can protect against neuronal death.

Next, we decided to check the effect of miR-128 on mitochondrial health. Mitochondrial deterioration being a relatively early event preceding neuronal death, we treated SH-SY5Y cells

with 6-OHDA for 4h. We performed MitoSOX assay for detection of mitochondrial superoxide production in live cells under fluorescent microscope. As compared to the control, there was an increase in superoxide production indicated by increase in red fluorescence in the 6-OHDA treated cells which was significantly reduced in the cells pre-transfected with miR-128 mimic. No such reduction in fluorescence was observed in the cells transfected with the NCM (Fig.2F). An arbitrary unit (A.U.) of red fluorescence was assigned as threshold such that cells with equal or higher fluorescence intensity were considered MitoSox positive and percentage of MitoSox positive cells were calculated (Fig.2G). Thus, our results indicated that miR-128 has an overall neuroprotective function whereby it not only improved mitochondrial health but also protected against 6-OHDA mediated cell death.

miR-128 prevents both intrinsic and extrinsic pathways of apoptosis by regulating activation of FoxO3a

Apoptosis being one of the major pathways of neuronal death in PD, we explored the involvement of miR-128 in the apoptotic pathways. We have previously shown in AD models that activation of the transcription factor FoxO3a is a major inducer of apoptotic death in neurons (Akhter et al. 2014). We have also reported activation of FoxO3a in PC12 cells under 6-OHDA treatment (Sanphui et al. 2020). Thus, we investigated whether miR-128 exhibited its neuroprotective effect via regulating activation of FoxO3a. FoxO3a activation involves its translocation from the cytosol to the nucleus which is triggered by specific post-translational modifications (Nho 2014). In the deactivated state, FoxO3a is phosphorylated at Ser 253 position and is retained in the cytoplasm. Dephosphorylation of FoxO3a at Ser 253 triggers activation signals whereby FoxO3a is translocated to the nucleus. Thus, a reduction of its phosphorylated form at Ser 253 indicates activation of FoxO3a. We treated SH-SY5Y cells with 6-OHDA for 4h and subjected the protein samples to immunoblotting against p-FoxO3a (Ser 253) and total FoxO3a. In the 6-OHDA treated samples, there was a marked reduction in the p-FoxO3a (Ser 253) levels, indicating FoxO3a activation which was significantly reversed in miR-128 mimic transfected cells. The total FoxO3a levels remained relatively unchanged throughout (Fig. 3A and 3B). This indicated that miR-128 can protect against neuronal death via preventing activation of the transcription factor FoxO3a.

Two major signaling cascades- the extrinsic and intrinsic pathways of apoptosis, involve up-regulation of pro-apoptotic proteins FasL and PUMA respectively, at the initial stages of apoptotic induction (Elmore 2007). FoxO3a has been reported to induce expression of both PUMA and FasL in response to various apoptotic stimuli (Nho 2014). Since miR-128 was found to regulate FoxO3a activation, we decided to check whether miR-128 can regulate the induction of apoptotic pathways downstream of FoxO3a. We treated SH-SY5Y cells with 6-OHDA for 4h and 8h respectively and checked the expression of PUMA and FasL mRNAs by qRT-PCR. A marked increase of both PUMA and FasL transcript levels was observed by 4h of 6-OHDA treatment which was significantly reduced by pre-transfection with miR-128 mimic (Fig. 3C and 3D). These results were further validated when immunoblotting showed similar reduction in the expression of PUMA (Fig. 3E and 3F) and FasL (Fig. 3G and 3H) upon miR-128 over-expression.

To further validate the involvement of miR-128 in apoptotic pathways, we checked the effect of miR-128 on the irreversible activation of downstream caspases. Intrinsic and extrinsic pathways of apoptosis involves activation of caspases- 9 and -8 respectively, both of which lead to the activation

of caspase-3, thus eventually resulting in apoptosis (Fan et al. 2005). By using colorimetic assay, we observed an increase in the caspase-9 activity with 6-OHDA treatment which was subsequently reduced by over-expression of miR-128 (Fig. 3I). Next, we performed a fluorescence-based assay for caspase-8 activity and detected an increase in the caspase activity upon 6-OHDA treatment which was significantly reduced by pre-treatment with miR-128 mimic (Fig. 3J). Lastly, we performed a colorimetric caspase-3 activity assay which indicated that miR-128 over-expression could attenuate 6-OHDA mediated activation of caspase-3 as well (Fig.3K). Thus, our results comprehensively indicate that miR-128 prevents both the apoptotic pathways- PUMA-mediated intrinsic and FasL-mediated extrinsic, by inhibiting the upstream activation of FoxO3a.

miR-128 prevents neurite shortening and maintain pre- and post-synaptic protein expressions

miR-128 being enriched at the neurites and synaptic vesicles (Franzoni et al. 2015; Kumar and Reddy 2020), we decided to check the effect of miR-128 on neurite and synaptic integrity respectively. We treated primed PC12 cells with 6-OHDA for 16h and observed their morphology under bright-field microscope. As compared to the control, a marked reduction in neurite lengths under 6-OHDA treatment was observed. However, this shortening of neurite lengths was significantly reversed by miR-128 over-expression whereby the overall morphology of the primed neuronal cells was maintained (Fig. 4A). Neurite length/cell (μm) was measured by Neuron J plugin of Fiji software (Fig. 4B).

Next, we investigated whether miR-128 has any effect on synaptic protein expressions. We treated SH-SY5Y cells with 6-OHDA for 16h and checked the expression of the synaptic proteins synaptophysin and PSD-95 by immunoblotting. By 16h of 6-OHDA treatment we observed a significant decrease in the levels of both synaptophysin (Fig.4C and 4D) and PSD-95 (Fig.4E and 4F). Interestingly, this decrease in synaptic protein expression was attenuated by over-expression of miR-128. Thus, our results showcase that miR-128 can not only maintain the overall neuronal morphology by preventing neurite shortening, but also keep the synaptic integrity intact by maintaining the expression of pre- and post-synaptic proteins like synaptophysin and PSD-95 respectively.

Discussion

In the present study, we investigated the role of a particular neuron-enriched miRNA, miR-128, in PD pathogenesis. Our study identifies miR-128 to be significantly decreased in the exosomes derived from the PD patient plasma as compared to age-matched control subsets. This characteristic decrease in the circulating exosomal miR-128 expression was further corroborated with a similar reduction in miR-128 expression in the standard cellular models of PD. We also depicted detailed mechanistic implications of this altered miR-128 expression in the pathogenesis of PD.

So far, only a handful of reports have come out suggesting the role of miR-128 in PD. For instance, miR-128 was reported to regulate AXIN1 and protect dopaminergic neurons from apoptosis in models of PD (Zhou et al. 2018). Another report showed that HIF-1α/mRNA-128-3p axis is neuroprotective via the *Axin1*-mediated Wnt/β-catenin signaling pathway in PD models (Zhang et al. 2020). It is worth mentioning here that although miR-128 has been predominantly reported to be

neuroprotective in nature (Shi et al. 2021, Fang et al. 2019, Mao et al. 2017), it is also shown to promote neuronal degeneration under certain conditions (Geng et al. 2018, Adlakha and Saini 2013). However, a detailed mechanistic study on the involvement of miR-128 in the apoptotic pathways of neuronal death associated with PD was still largely unexplored.

This study showcases a comprehensive mechanistic function of miR-128 in regulating the molecular pathways leading to neurodegeneration in PD. In cellular models of PD, miR-128 was shown to have neuroprotective functions whereby it prevented 6-OHDA induced neuronal apoptosis via regulating the activation of the transcription factor FoxO3a. Within the nucleus, FoxO3a is instrumental in activating apoptotic pathways, under various conditions of cellular stress (Nho 2014). Apoptosis, a major cell death mechanism, maybe induced through two major pathways- the intrinsic and the extrinsic pathways (Elmore 2007). One of the pro-apoptotic proteins found to be up-regulated in the early stages of intrinsic and extrinsic apoptotic pathways is PUMA and FasL respectively, both being direct targets of activated FoxO3a. Interestingly, via regulating FoxO3a activation, miR-128 was found to regulate the expressions of both PUMA and FasL. Additionally, miR-128 could prevent the activation of caspases-8, -9 and -3, eventually shutting down both the intrinsic and extrinsic pathways of apoptosis.

Being a neurodegenerative disorder, PD is also associated with synaptic dysregulation preceding neuronal death. Additionally, miR-128 has been detected in synaptic vesicles and has been found to be enriched at the neurites (Franzoni et al. 2015; Kumar and Reddy 2020). Interestingly enough, our results depicted that miR-128 over-expression can not only inhibit 6-OHDA induced neurite shortening but also prevent down-regulation of pre- synaptic terminal protein Synaptophysin and post-synaptic density protein PSD-95. However, we were unable to find a direct molecular target through which miR-128 could regulate the expression of the above-mentioned synaptic proteins, in our models of study. Additionally, mitochondrial dysfunction is known to be associated with both the sporadic and familial forms of PD and various interventions targeting mitochondrial impairment and superoxide production have been reported in the past (Gao et al. 2022; Luo et al. 2015). Interestingly, our study revealed that miR-128 over-expression could be an important strategy in controlling mitochondrial superoxide production during PD pathogenesis.

In the past, there have been reports on the presence of a wide range of miRNAs in the circulating exosomes of PD patient cohorts (Cao et al. 2017, Gui et al. 2015, Yao Y.F. et al.). But to our knowledge, a detailed and targeted study on dysregulated brain-enriched miRNAs, like miR-128, obtained from the exosomes of PD patients plasma has not been done till date. Althoughit needs validation in large and varied patient pool to test its candidacy as a potential biomarker for PD, our study cohesively proves the importance of miR-128 in PD pathogenesis and detection at both the extracelluar and intra-neuronal states.

Conclusion

Overall, through our results obtained from human patient samples and cellular models, we suggest that the altered expression of circulating exosomal miR-128 could be an important target in the pathogenesis and detection of PD. Exosome-based miRNA delivery poses minimal toxicity, can evade immunogenicity and shows a remarkable ability to overcome the blood-brain-barrier (Banks

et al. 2020) all of which indicates that administration of exosomes with target miRNAs may become a promising next-generation miRNA-based therapy for treating incurable brain disorders like PD.

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An online non-peer-reviewed version of the manuscript is available at the pre-print repository, RESEARCH SQUARE (doi.org/10.21203/rs.3.rs-1838421/v1).

Abbreviations

Parkinson's disease (PD), microRNA (miRNA, miR), all-trans retinoic acid (ATRA), nerve growth factor- beta (NGF-β), negative control mimic (NCM), 6-hydroxydopamine (6-OHDA), post-translational modifications (PTMs), blood brain barrier (BBB), Nanoparticle tracking analysis (NTA).

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material, further inquiries can be directed to the corresponding authors.

Ethics statement

All human studies were carried out in accordance with the guidelines formulated by the Institutional Human Ethics Committee (IHEC).

Author contributions

PB conceived and designed the study and SCB supervised the project. PB performed bioinformatics, biochemical, molecular and cellular experiments. AB performed the clinical assessment of patients and collection of blood. PB and SCB analyzed the data and contributed to writing the manuscript. All authors read and approved the final submitted manuscript.

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Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be constructed as a potential conflict of interest.

Supplementary Material

Supplementary methods
Supplementary figures: S1A, S1B
Supplementary tables: ST1, ST2, ST3

Figure legends

Fig. 1: miR-128 expression decreases in the human PD patient plasma-derived exosomes: A) miR-128 expression in the exosomes derived from PD patient (age >40) plasma samples (n=25) as compared to age-matched controls (n=20). U6 expression was used as endogenous control for normalization. B) NTA analysis of the size of exosomes isolated from control (n=5) and PD patient plasma (n=5). C) NTA analysis of the number of isolated exosomes from control (n=10) and PD patient plasma (n=10). Asterisks (*) indicate significant difference and ns indicates non-significant difference with respect to control. Data shown as mean ± SEM with *p<0.05 and **p<0.01.

Fig. 2: miR-128 decreases mitochondrial superoxide production and prevents death of neuronal cells *in vitro*: A) Human SH-SY5Y cells were primed with 10μM ATRA for 5 days and were then treated with varying doses (50μM, 100μM, 250μM, 500μM and 1mM) of 6-OHDA for 24h respectively. MTT assay was done after 24h and percentage viability of cells was calculated with respect to untreated (control) cells. B) Primed SH-SY5Y cells were treated with 100μM dose of 6-OHDA for 4h and 8h respectively and miR-128 expression was checked by qRT-PCR with respect to control. C) Rat PC12 cells were primed with 100ng NGF-β for 5 days and were then treated with 100μM dose of 6-OHDA for 4h, 8h and 16h respectively and miR-128 expression was checked by qRT-PCR with respect to control. U6 expression was used as endogenous control for normalization in both (B) and (C). D) Primed SH-SY5Y cells were either untreated (control) or treated with 100μM 6-OHDA for 16h or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100μM) for 16h. The cells were then incubated with calcein-AM and ethidium homodimer-1 dyes respectively and the percentage of live and dead cells were determined by flow cytometry. Q4 indicates live cells while (Q2+Q1) represent total dying or dead cells respectively. E) Graphical representation of the percentage of dying and dead cells (Q1+Q2) as determined in (D). F) Primed SH-SY5Y cells were either untreated (control) or treated with 100μM 6-OHDA for 4h or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100μM) for 4h. The cells were incubated with MitoSOX dye and visualized under fluorescence microscope (20X). G) Percentage of MitoSox positive cells as calculated from (F). Asterisks (*) indicate significant difference with respect to control, unless otherwise indicated in the figure by straight line between two given conditions. All experiments have been done in triplicate. Data shown as mean ± SEM with *p<0.05 and **p<0.01.

Fig. 3. miR-128 regulates activation of FOXO3a and prevents neuronal death via both intrinsic and extrinsic apoptotic pathways *in vitro*: Primed SH-SY5Y cells were either untreated (control) or treated with 100μM 6-OHDA for indicated time-points or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100 μM) for the respective time-points, in all the experiments. A) Total protein lysates were subjected to immunoblotting against p-FOXO3a (Ser253) and total FOXO3a. B) Graphical representation of the immunoblots as represented in (A). Normalization was done against total FOXO3a. C) and D) Total RNA was isolated from the cells and mRNA levels of PUMA and FasL was measured by qRT-PCR. GAPDH expression was used as endogenous control for normalization. E) and G) Total protein lysates were subjected to immunoblotting against PUMA and FasL respectively. β-actin protein expression was used as

endogenous control for normalization in both cases. F) and H) Graphical representations of protein expressions of PUMA and FasL as represented in (E) and (G) respectively. I) Cell lysate was incubated with the chromophore labeled substrate (LEHD-*p*NA) and O.D. was measured at 405nm. J) Cells were incubated with FLICA reagent and fluorescence end point reading was detected at E_x=490nm and E_m=520nm. K) Cell lysate was incubated with the chromophore labeled substrate (LEHD-*p*NA) and O.D. was measured at 405nm. Asterisks indicate significant difference and 'ns' indicates non-significant difference with respect to control, if not otherwise indicated in the figure. All experiments were done at least in triplicate. Data shown as mean ± SEM with *p<0.05 and **p<0.01

Fig. 4: **miR-128 improves neurite length and maintains pre- and post-synaptic protein expression *in vitro*:** A) Primed PC12 cells were either untreated (control) or treated with 6-OHDA (100μM) for 16h or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100μM) for 16h. The cells were observed under bright-field microscope (20X). B) Neurite length (μM) per 50 cells per field was calculated using Neuron J plugin of Fiji software. C) and E) Primed SH-SY5Y cells were either untreated (control) or treated with 100μM 6-OHDA for 8h or pre-transfected with miR-128 mimic or NCM for 48h before treatment with 6-OHDA (100μM) for 8h. Total protein lysates were subjected to immunoblotting against synaptophysin (C) and PSD-95 (E). β-actin protein expression was used as endogenous control for normalization in both cases. D) and F) Graphical representations of protein expressions of synaptophysin (C) and PSD-95 (E) as normalized with respect to β-actin expression. Asterisks (*) indicate significant difference and 'ns' indicates non-significant difference with respect to control, unless otherwise indicated in the figures. All experiments were done in triplicate. Data shown as mean ± SEM with *p<0.05 and **p<0.01.

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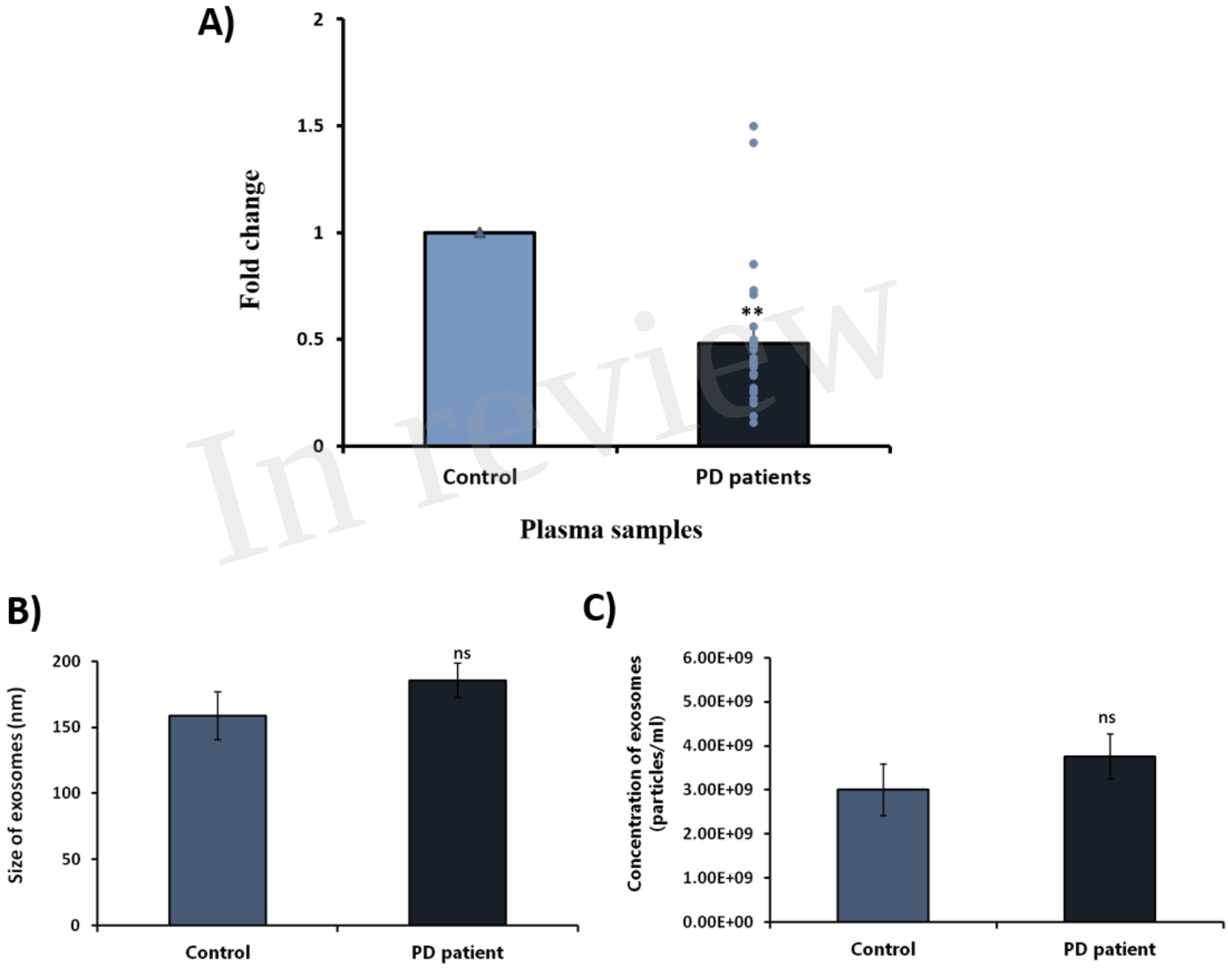
Figure 1.TIFF



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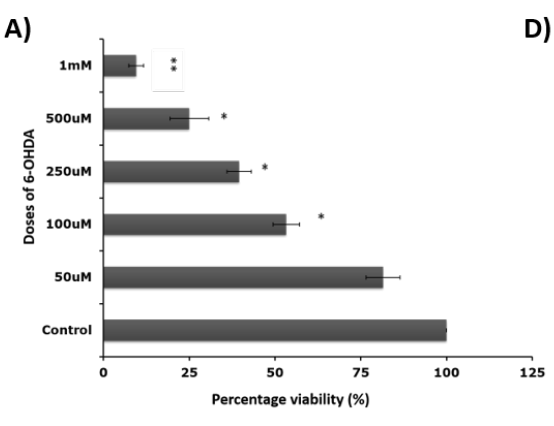

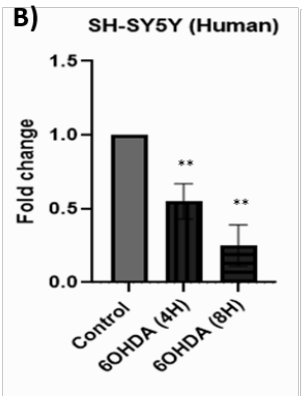

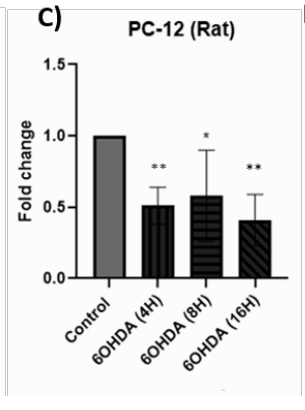

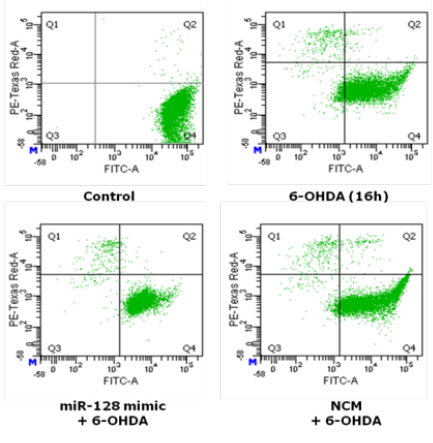

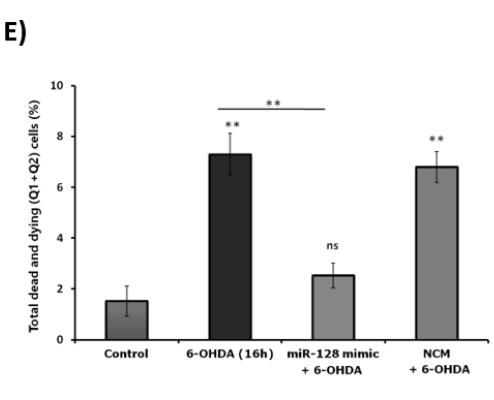

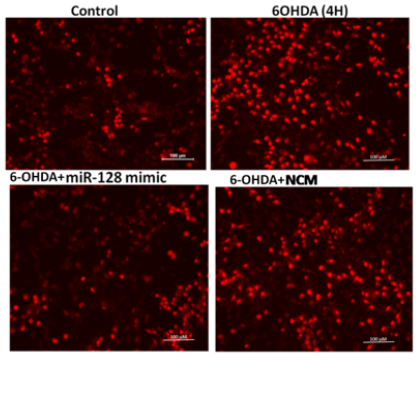

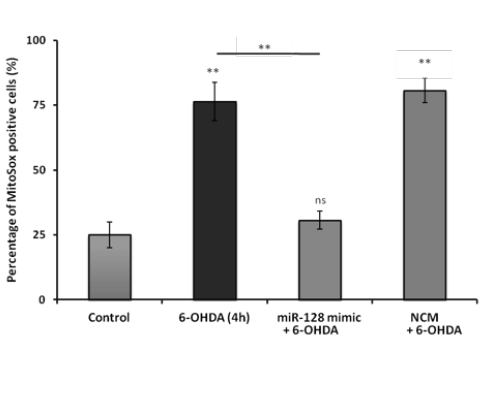

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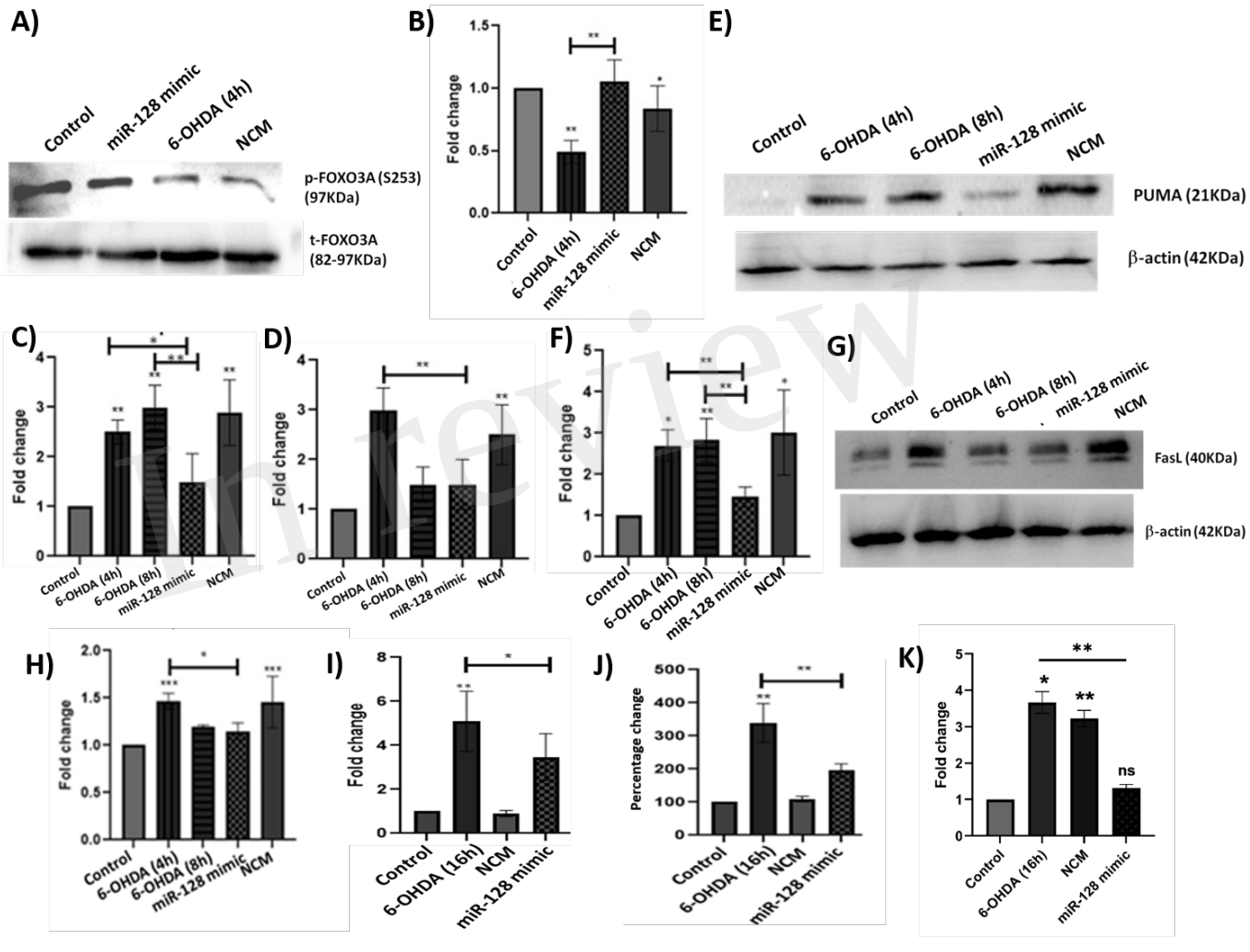
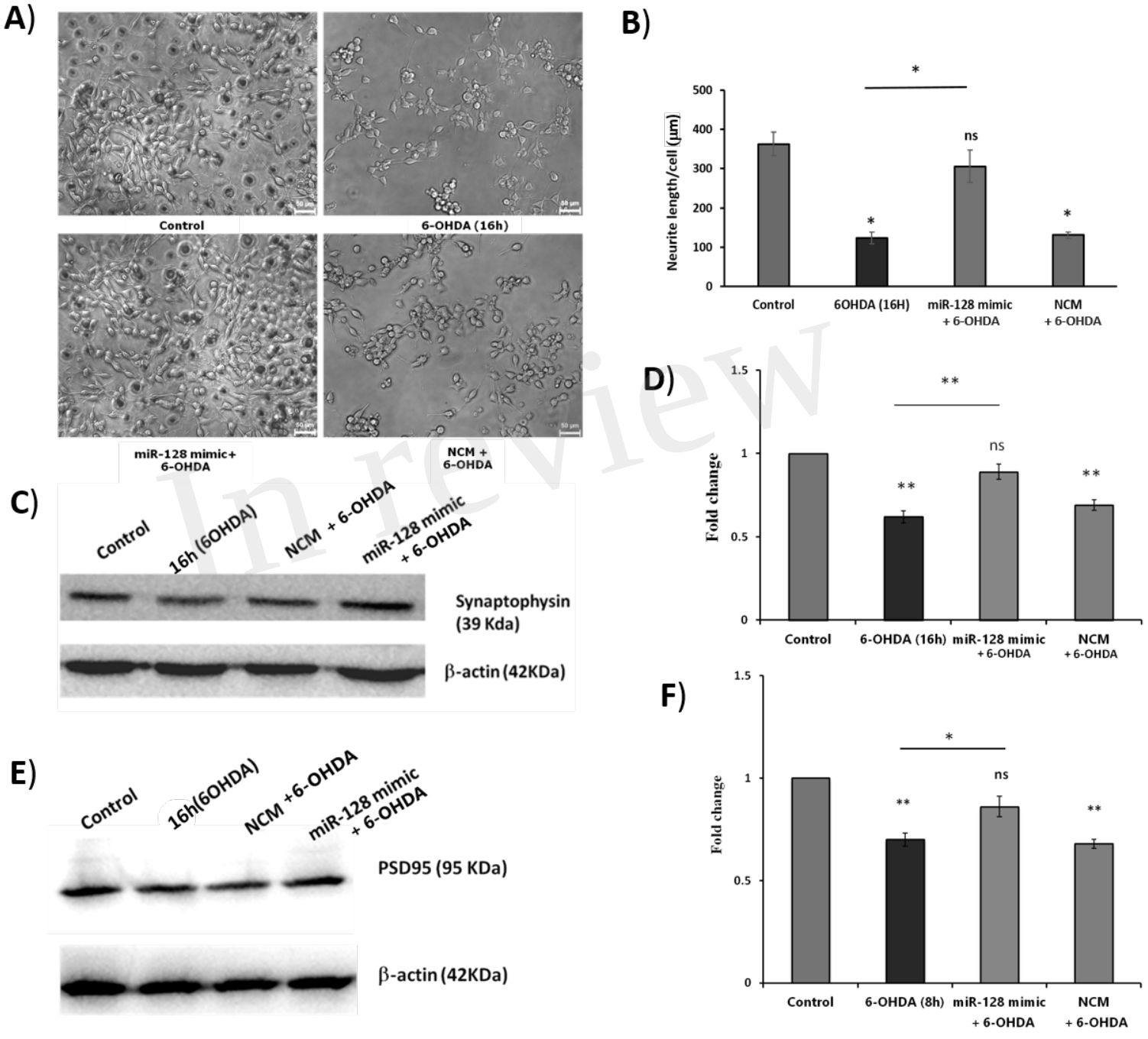


miR-128 mimic and NCM denote miR-128 mimic+6-OHDA and NCM+6-OHDA respectively for indicated time-points.

Figure 4.TIFF



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Small Non-coding RNAs: Do They Encode Answers for Controlling SARS-CoV-2 in the Future?

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SARS-CoV-2 (severe acute respiratory syndrome coronavirus 2) is a novel coronavirus responsible for the current COVID-19 (coronavirus disease 2019) pandemic, which has hit the world since December 2019. It has spread to about 216 countries worldwide, affecting more than 21.7 million people so far. Although clinical trials of a number of promising antiviral drugs and vaccines against COVID-19 are underway, it is hard to predict how successful these drug- or vaccine-based therapeutics are eventually going to be in combating COVID-19 because most of such therapeutic strategies have failed against human coronaviruses such as SARS-CoV and MERS-CoV (Middle East respiratory syndrome coronavirus) responsible for similar pandemics in the past. In that context, we would like to bring to scientific attention another group of endogenous regulatory molecules, the small non-coding RNAs, especially the microRNAs, which are found to regulate critical cellular pathways in a number of disease conditions, including RNA viral infections. This review will focus on understanding the effect of altered microRNA expression during coronavirus-mediated infections and how it may provide clues for further exploring the pathogenesis of SARS-CoV-2, with a view of developing RNAi-based therapeutics and biomarkers against COVID-19.

Keywords: COVID-19, coronaviruses, RNA viruses, microRNAs, non-coding RNAs, MERS-CoV, SARS-CoV-2, SARS-CoV

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INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) or human coronavirus 2019 (HCoV-19) is a novel coronavirus (CoV), first discovered in Wuhan, China, in December, 2019. It is an exceptionally infectious virus that has since then spread to more than 216 countries across the world causing a form of acute pulmonary respiratory disorder named COVID-19 (coronavirus disease 2019) and was declared a pandemic by the World Health Organization (WHO) on March 11, 2020. As of August 19, 2020, SARS-CoV-2 has affected about 21.7 million people with a mortality rate of around 3.5% (World Health Organization, 2020).

Severe acute respiratory syndrome coronavirus 2 is essentially a beta-CoV possessing a positive-sense single-stranded RNA genome with a 5′ cap, a 3′ poly-A tail, and 5′- and 3′ UTRs (untranslated regions; Chen and Zhong, 2020). It is the seventh CoV known to infect humans, which includes the likes of SARS-CoV, Middle East respiratory syndrome coronavirus (MERS-CoV), HCoV-OC43, HCoV-229E, HCoV-NL63, and HCoV-HKU1 (Centers for Disease Control and Prevention, 2020). However, the origin of SARS-CoV-2 is presumed to be from bats. Andersen et al. (2020) have reported that SARS-CoV-2 has about 96% sequence similarity to RaTG13, a CoV isolated from the bat *Rhinolophus affinis*. On the other

hand, cryo-EM data have shown that structurally, SARS-CoV-2 has significant similarity to the SARS-CoV (Wrapp et al., 2020). Additionally, both SARS-CoV and SARS-CoV-2 utilize the same molecule, angiotensin-converting enzyme 2 as a receptor for entry into the host cells (Samavati and Uhal, 2020). Like its predecessors SARS-CoV and MERS-CoV, to date, no vaccine or drug has proven to be successfully effective against the deadly HCoV-19 virus. Although a number of promising therapeutic candidates are in advanced stages of clinical trials, it is too early to predict their outcome.

Generally, CoVs are known to encode 16 non-structural proteins (nsps), mainly related to their RNA synthesis (Perlman and Netland, 2009). However, a unique feature of CoVs is the fact that nsp14 of CoVs codes for a special 3′–5′ exoribonuclease called ExoN. ExoN is found to be essential for RNA replication fidelity by regulating a complex RNA-dependent RNA proofreading machinery, in a manner distinct from the viral RNA–dependent RNA polymerase (RdRp), a mechanism uncommon and unprecedented in other RNA viruses (Minskaia et al., 2006). Perhaps this ExoN-mediated enhanced fidelity gives the CoVs advantage over genetic bottleneck events and prevents accumulation of deleterious mutations beyond tolerance limit, which could even lead to reduced virulence (Denison et al., 2011). This aids in persistence of infection by increasing the stability of the virus. Recently, SARS-CoV-2 nsp14 gene has shown about 95% sequence identity (Shannon et al., 2020) and 98.7% structural similarity (Gordon et al., 2020) with that of SARS-CoV. It would be interesting to check if SARS-CoV-2 possesses a functional ExoN and, if so, whether the ExoN activity has any effect on the infectivity, viability, and persistence of SARS-CoV-2 in the host. ExoN may also provide cues for understanding the viral replication, which is important for understanding the pathogenesis of the virus.

In order to successfully develop a therapeutic target for SARS-CoV-2, it is very important to understand the viral replication mechanism as well as host–virus interaction and how those may be regulated inside the host. While the development of protein-based vaccines or drug targets is still underway, it may be important to shift the focus to another group of endogenous regulatory molecules—the small non-coding RNAs (ncRNAs) such as microRNAs (miRNAs or miRs), which are known to be instrumental in regulating gene expression under various infectious disease conditions and have shown significant potential as therapeutic targets. In this review, we will be discussing the importance of miRNA-mediated gene regulation in RNA virus infections and their prospect as a therapeutic target in the ongoing SARS-CoV-2–mediated pandemic, COVID-19.

RNA VIRUSES, INCLUDING CoVs, CAN ENCODE miRNA-LIKE SMALL REGULATORY RNAs

MicroRNAs, about 18–22 nucleotides long, are a class of ncRNAs that regulate gene expression under various stress, infection, or disease conditions, typically by binding to the 3′ UTR of the target mRNA by virtue of which they may either repress its translation or cause deadenylation and decay of the target mRNA (Filipowicz et al., 2008). Viruses, especially DNA viruses, are known to encode miRNAs and generally utilize the host cellular miRNA machinery for doing so (Kincaid and Sullivan, 2012). The existence of miRNAs in RNA viruses, however, was initially controversial, because they were believed to not encode miRNAs in order to avoid excision of their genomes during excision of the precursor miRNAs by the miRNA processing machinery (Varble and tenOever, 2011). Particularly, the cytoplasmic RNA viruses were thought to not express miRNAs as they may not get access to the nuclear enzyme Drosha needed for miRNA processing. However, Shi et al. (2014) showed for the first time that hepatitis A virus, a cytoplasmic RNA virus, can encode in its coding region, not one, but two novel functional miRNAs, namely, hav-miR-1-5p, and hav-miR-2-5p. This indicated that cytoplasmic RNA viruses too may express small regulatory RNAs such as miRNAs. However, miRNAs of RNA viruses do not seem to possess the canonical stem-loop structure present normally, and as such, their biogenesis and mechanism of function are not very clear (Varble and tenOever, 2011; Mishra et al., 2020). Nonetheless, significant research has been focused on identifying such altered ncRNAs during various cytoplasmic RNA virus–mediated infections, including those by the CoVs. Peng et al. (2011) had initially conducted next-generation sequencing of lung samples from each of SARS-CoV and influenza virus–infected mice strains and had shown differential expression of a number of small ncRNAs—including miRNAs, small nucleolar RNAs or snoRNAs, and non-annotated novel small RNAs—in the host cells when infected by each of the viruses. Morales et al. (2017) too performed deep sequencing of RNA from the lungs of SARS-CoV–infected mice and found three types of small viral RNAs (svRNAs), about 18–22 nucleotides long, much like miRNAs, being produced, depending on the degree of viral replication. These svRNAs were seen to modulate the host response to viral infection by regulating the production of certain proinflammatory cytokines (viz. CCL2, interleukin 6, and CXCL10).

Thus, these studies were the stepping stones that paved the way for further exploring how miRNAs may be instrumental in regulating the host–virus dynamics during RNA virus–mediated infections, particularly in the case of HCoV-mediated diseases.

SIGNIFICANT FINDINGS ON miRNA-BASED GENE REGULATION DURING HCoV INFECTION, INCLUDING THAT OF SARS-CoV-2

In SARS-CoV-2 infection, much like SARS-CoV, the main cause of death is ARDS or acute respiratory distress syndrome, which results from an uncontrolled systemic inflammatory response in the host in the form of a deadly antiviral "cytokine storm" (Huang et al., 2020). As the host immune response is triggered, it may lead to differential expression of both viral and host

miRNAs, as mentioned previously, which in turn, may have various repercussions. Viral miRNAs may regulate different stages of their own life cycle; they may either induce or inhibit the expression of host miRNAs involved in host antiviral response pathways or regulate the turnover and function of host mRNAs related to the same. Host miRNAs, on the other hand, may regulate viral replication and may even regulate or induce the proviral host factors (Bruscella et al., 2017). Thus, the altered miRNA expression may be in favor of or against the existence and replication of the virus inside the host, depending on the host–virus dynamics during viral infection.

MicroRNAs have been reported to affect the mechanism of pathogenesis in a number of respiratory RNA viral infections. For example, in influenza virus (H1N1), miR-323, miR-491 and miR-654 cause a decrease in the expression of viral genes in the infected host by inhibiting viral replication via degradation of the transcript for a viral RNA polymerase catalytic subunit (Song et al., 2010; Izzard and Stambas, 2015; Leon-Icaza et al., 2019), whereas in Rhino viruses, miR-23b leads to downregulation of the transmembrane lipoprotein receptors used by the virus to infect host cells (Ouda et al., 2011; Tahamtan et al., 2016). However, when it comes to CoV-mediated diseases, miRNA-based gene regulation is still a relatively new and upcoming area of research. Nonetheless, there are some promising findings with respect to CoV-mediated diseases in animals. For example, miR-221-5p is seen to directly target the 3′ UTR of genomic RNA of the porcine epidemic diarrhea virus to inhibit its replication and lead to activation of the nuclear factor (NF-kβ)–mediated signaling cascade (Zheng H. et al., 2018). For HCoVs as well, some very interesting and important observations have been made, including those for SARS-CoV-2, which are highlighted as below and summarized in **Table 1**:

1. *HCoV nucleocapsid protein (N) can directly bind to small regulatory RNAs and modulate antiviral immune response in host*:

N protein of CoVs is an RNA-binding protein that binds to the genomic RNA of the virus to form a helical capsid and plays an important role in viral replication (Wurm et al., 2001; Zúñiga et al., 2007). Lai et al. (2014) had an unexpected observation wherein they showed that upon HCoV-OC43 infection, N protein could bind to miR-9 and lead to host inflammatory response by NF-kβ activation. miR-9 is known to negatively regulate NFKB1, involved in the NF-kβ pathway (Bazzoni et al., 2009), and under stimulus and/or OC43 infection, expression of both miR-9 and NFKB1 increases. But Lai et al. observed that miR-9 cannot interact with NFKB1 as it is bound by the N protein, which eventually leads to prolonged activation of the NF-kβ pathway.

Interestingly, Cui et al. (2015) noted that coronaviruses can also utilize the N protein as a viral suppressor of RNA silencing (VSR) to overcome the antiviral response elicited by host RNAi mechanism. They showed that the coronavirus N protein could sequester siRNAs and other double-stranded RNAs, thereby preventing Dicer-mediated processing of these RNA molecules. In that way, the virus could facilitate its replication by suppressing the host RNAi-mediated antiviral response against the infection.

Thus, a viral genomic RNA-binding protein was found to be capable of binding to host miRNAs and siRNAs and modulate its inflammatory response. Because the nucleocapsid N protein is found in other HCoVs as well, it could be an important mechanism for these viruses to modulate the host immune response and may be an important area of focus while designing therapeutic antiviral strategies. It would be interesting to explore whether any such N protein-miRNA–mediated interaction is elicited in the form of host response mechanism during SARS-CoV-2 infection.

2. *Endonuclease APE1 can cleave miRNAs and other RNA components of SARS-CoV*:

APE1 is an apurinic/apyrimidinic endonuclease 1, but interestingly, Kim et al. (2010) showed that APE1 has RNA cleaving properties as well. In the absence of divalent metal ions, APE1 was seen to cleave mRNAs (c-myc, CD44), miRNAs (miR-10b, miR-21), and three RNA components of SARS-CoV, namely, Orf1b, Orf3, and spike. Thus, APE1 can potentially cleave any

TABLE 1 | List of important miRNAs involved in some HCoV infections.

Human coronavirus	Dysregulated miRNAs	Target molecules/pathway	References
HCoV-OC43 (N protein)	miR-9	Host NF-kβ pathway (activation)	Lai et al., 2014
SARS-CoV	[miRNAs-17*, -574-5p, and -214] (upregulation) miR-223 (downregulation) miR-98 (downregulation)	Viral replication (inhibition) Viral nucleocapsid (N) protein Viral spike (S) protein	Mallick et al., 2009
SARS-CoV-2	[miR-1307-3p miR-3613-5p] [hsa-let-7a, hsa-miR-101, hsa-miR-125a-5p, hsa-miR-126, hsa-miR-222, hsa-miR-23b, hsa-miR-378, hsa-miR-380-5 hsa-miR-98]	Viral 3′ UTR *Host protein targets*: Interferon β (IFN-β), ATP synthase F1 subunit β (ATP5B), Erb-B2 receptor tyrosine kinase 2 (ERBB2) *Viral protein targets*: Polymerase basic protein 2 (PB2) Polymerase acidic protein (PA) Non-structural protein (NS1) Nucleoprotein (NP) major capsid protein (VP1)]	Chen and Zhong, 2020; Sardar et al., 2020

RNA *in vitro*. It would be interesting to see if this RNA-cleaving property of APE1 may be further explored as a tool to target specific regulatory RNAs implicated in viral infections, including that of SARS-CoV-2.

3. *SARS-CoV exploits the miRNA machinery of bronchoalveolar stem cells (BASCs) for persistent infection*:

Bronchoalveolar stem cells are one of the major cell types infected by SARS-CoV (Mallick et al., 2009; Leon-Icaza et al., 2019). Upon SARS-CoV infection, Mallick et al. observed that miRNAs-17*, -574-5p, and -214 were upregulated in BASCs, which the virus utilized to suppress its own replication and bypass elimination by the host immune system. On the other hand, miR-223 and miR-98, which target nucleocapsid (N) and spike (S) proteins, respectively, were found to be downregulated, eventually leading to activation of proinflammatory cytokines during the viral infection.

4. *Computational predictions of altered miRNAs related to SARS-CoV-2 infection*:

Most of the information regarding dysregulated miRNAs during SARS-CoV-2 infection has been based on computational predictions so far. Nonetheless, these findings seem quite promising and are worth to be noted. For example, miR-1307-3p and miR-3613-5p are reported to be downregulated in lung cancer patients (Zheng Y. et al., 2018; Song et al., 2019). Interestingly, software-based predictions have shown that these miRNAs have significant binding probability to the 3′ UTR of SARS-CoV-2 as well. Moreover, tyrosine kinase inhibitors used to treat lung cancer patients was also found to upregulate miR-1307-3p (Chen and Zhong, 2020). Sardar et al. (2020) too, using *in silico* methods, have identified nine miRNAs, namely, hsa-let-7a, hsa-miR-101, hsa-miR-125a-5p, hsa-miR-126, hsa-miR-222, hsa-miR-23b, hsa-miR-378, hsa-miR-380-5, and hsa-miR-98, unique to only SARS-CoV-2 infection. The genes predicted to be targeted by these miRNAs were enlisted as interferon β (IFN-β), ATP synthase F1 subunit β (ATP5B), Erb-B2 receptor tyrosine kinase 2 (ERBB2), and some viral (influenza A and enterovirus) proteins such as polymerase basic protein 2 (PB2), Polymerase acidic protein (PA), non-structural protein (NS1), nucleoprotein (NP), and major capsid protein (VP1). These interesting findings, however, are subject to further experimental validations in *in vitro* and *in vivo* models of SARS-CoV-2. Important *in silico* comparative studies of different miRNAs dysregulated in a number of CoV infections including SARS-CoV-2 have also been done by Demirci and Adan (2020), Fulzele et al. (2020) and Identify et al. (2020)).

These observations are just the tip of the iceberg, and there is a lot of scope for exploring how miRNAs might regulate the pathogenesis of SARS-CoV-2. To begin with, it is important to perform deep sequencing to get a clear picture of the entire RNA repertoire of the virus, with special focus on the small ncRNAs. It would also be interesting to explore if SARS-CoV-2 encodes its own viral miRNAs and, if so, how those may affect the host immune response against the virus. The biogenesis and processing pathway of viral miRNAs may too withhold important information for designing future therapeutic strategies. As mentioned previously, cytoplasmic RNA viruses usually produce miRNAs in a non-canonical miRNA biogenesis pathway, probably in a Drosha- or Dicer-independent manner (Usme-Ciro et al., 2013). A type of short introns called mirtrons may be responsible for miRNA processing in a Drosha-independent pathway. Mirtrons are processed by spliceosome to produce pre-miRNAs, which are then exported via exportin-5 from the nucleus, subsequently processed by Dicer and loaded to the RISC complex for initiation of gene silencing process (Ruby et al., 2007). Alternatively, miRNA processing may also occur in a Dicer-independent manner via Simtrons (splicing-independent mirtron-like miRNAs) through a pathway that includes Drosha but excludes DGCR8, exportin-5, Dicer, and AGO-2, which are otherwise crucial for miRNA biogenesis in the canonical pathway (Havens et al., 2012). Recently, Drosha-dependent miRNA processing has been observed in certain cytoplasmic RNA viruses where, during infection, Drosha cannot get phosphorylated and, in the absence of such nuclear localization signal, the unphosphorylated Drosha is redistributed to cytoplasm where it induces non-canonical miRNA biogenesis (Shapiro et al., 2012). However, miRNA biogenesis and processing in the case of SARS-CoV-2 are unknown until now and needs to be explored. If indeed identified, they may prove to be important factors in the prognosis of SARS-CoV-2 as the factors involved in the non-canonical miRNA processing pathway of the virus are majorly different from those involved in the canonical miRNA biogenesis pathway of the host and as such can become important for specifically targeting the viral miRNAs while designing therapeutics. It may also help in bypassing viral suppression of RNAi, which mainly targets factors of the canonical miRNA processing pathway and thus prevent viral resistance against RNAi (Leonard and Schaffer, 2006).

Thus, study of regulatory ncRNAs like miRNAs demands serious research attention as it may withhold critical cues for understanding viral disease pathogenesis, how the host immune response mechanisms may be modified during viral infections, and whether it has any effect on the replication or persistence of the virus inside the host. It may be an especially important point to ponder with the view of developing successful therapeutics against SARS-CoV-2, whose infectivity has encompassed the world population.

RNAi-BASED ANTIVIRAL THERAPIES TARGETED AT miRNA-MEDIATED GENE REGULATION SHOW CLINICAL POTENTIAL

Since the last decade or so, research has been focused on either overexpressing or downregulating miRNAs by using miRNA mimics and miRNA antagomirs (antisense miRNAs), respectively, as therapeutics against various diseases (Hanna et al., 2019). To enhance their stability, these miRNA therapeutics are usually designed in the form of locked nucleic acids or LNAs, which are chemically modified RNAs with reduced reactivity.

The clinical potential of these miRNA-targeted therapeutics may be assessed from the fact that many have shown considerable promise in clinical trials for a number of diseases. For example, MRG-201/Remlarsen (LNA RNA mimic for miR-29b) is in phase II clinical trial against fibrotic diseases, whereas MRG-106/Cobomarsen (LNA antagomir against miR-155) is in clinical trials (phase II) for treating T-cell lymphomas (Bonneau et al., 2019). In recent times, miRNA inhibitors have also shown immense prospect as an antiviral therapeutic. For example, hepatitis C virus (HCV), a positive-sense single-stranded RNA virus, is known to utilize liver-specific miR-122 to positively regulate its own copy number (Jopling et al., 2005; Cox and Sullivan, 2014). Antisense inhibitor of miR-122 has proved to be effective against HCV, and currently, Santaris Pharma/Roche has developed the same under the trade name Miravirsen, which has completed phase II clinical trials in 2017 (Sarnow and Sagan, 2016). Research has also been focused on targeting viral miRNAs, instead of host miRNAs in treating viral infection–based diseases as it offers more specificity. In the case of Epstein-Barr virus, for example, antagomirs against viral miR-BART7-3p have shown positive results in mice models (Cai et al., 2015; Wang et al., 2019).

LNA-based strategy of treating antiviral infections offers various advantages, besides being highly stable. First, LNAs usually show high target specificity and lower off-target effects compared to other RNAi-based strategies. Second, they are not known to interfere with other therapeutics and may in fact be used in a combinatorial manner for increased efficacy of treatment.

DELIVERY STRATEGIES FOR SUCCESSFUL RNAi-BASED THERAPEUTICS AGAINST COVID-19

An effective RNAi-based therapeutics targeting miRNAs also requires a suitable and efficient delivery system. *In vivo* delivery of chemically modified miRNA or anti-miRNA oligonucleotides may be obtained via two main carrier systems: viral vectors and non-viral carriers (Baumann and Winkler, 2014; Fu et al., 2019).

Viral vectors are vectors with a viral backbone, modified to transfer gene of interest to the target organs/tissues with high efficiency for long-term gene expression/repression. The commonly used viral vectors for miRNA delivery are as follows:

1. *Adenoviral vectors* (*AdVs*), derived from the Adenoviridae family, may be used for miRNA delivery across various tissues and organs. For example, in the case of hepatitis B virus, delivery of pri-miR-122/5, pri-miR-31/5, and pri-miR-31/5-8-9 by AdVs led to short-term inhibition of viral replication in the liver tissue (Mowa et al., 2014). However, these vectors are found to elicit strong immune responses in the host, which largely restricts its use as a gene transfer vehicle.
2. *Adeno-associated viral vectors* are derived from the Parvoviridae family and lead to sustained gene expression *in vivo* (Miyazaki et al., 2012).
3. *Retroviral vectors* (*RVs*) are RNA viral vectors that, when they enter the host cells, produce double-stranded DNA by the reverse transcriptase enzyme and integrate into the host genome, thus offering sustained expression of the gene of interest. RVs are used commonly to deliver miRNAs (Ramanujam et al., 2016).
4. *Lentiviral vectors* (*LVs*) too are RNA virus vectors, and like RVs, they too integrate into the host genome, thus offering persistent expression of the inserted gene. For example, miR-133b was delivered by LVs to ameliorate spinal cord injury in mice (Theis et al., 2017).

Although the viral vectors are usually modified for reduced immunogenicity, they may still show significant antigenic reactions and cytotoxicity in some cases and are thus nowadays replaced with non-viral vectors. Some non-viral carriers commonly implicated and reported to be used in delivery of miRNA based therapeutics are as follows:

1. *Inorganic material–based systems*: They include gold nanoparticles, which when modified by attaching thiol or amino groups to their surface become suitable for transferring miRNAs *in vivo* (Jia et al., 2017).
2. *Lipid-based nanovectors*: Cationic lipids are amphiphilic molecules possessing both hydrophilic (head) and hydrophobic (tail) parts, suitable for delivering oligonucleotides *in vivo*. They may be conjugated with non-ionic hydrophilic polymers such as polyethylene glycol (PEG) for increased delivery efficiency and stability within the tissue/organ (Endo-Takahashi et al., 2015).
3. *Cell-derived membrane vesicles*: Extracellular vesicles (EVs) are membrane-bound vesicles released by the cells, usually for cell-to-cell communication. Depending on size, EVs may be broadly classified into exosomes (40–120 nm), microvesicles (100–1,000 nm), and apoptotic bodies (50–5,000 nm). These EVs have the advantage of low cytotoxicity, negligible immunogenicity, and high delivery efficacy and thus show great potential for delivery of miRNA based therapeutics (Zhou et al., 2018).
4. *Three-dimensional (3D) scaffold–based systems*: 3D biomaterial scaffolds such as hydrogels and electrospun fibers when conjugated with PEG have proven to lead to stabilized and sustained RNAi delivery (Nguyen et al., 2018).

However, the greatest advantage of RNAi in respiratory diseases such as COVID-19 is the fact that administration of miRNAs directly to the lung tissue may be achieved by inhalation. Oligonucleotides are water-soluble and may be aerosolized to penetrate both upper and lower respiratory tracts. For example, live attenuated viral vaccine expressing miR-93 when administered intranasally could successfully confer protection against viral infections (DeVincenzo, 2012; Li et al., 2015; Leon-Icaza et al., 2019). In another scenario, intranasal administration of siRNA (ALN-RSV01) against respiratory syncytial virus (RSV) nucleocapsid protein has been successful in phase II clinical trials against RSV-induced respiratory disease (Dyawanapelly et al., 2014).

Thus, depending on the nature of the RNAi-based therapeutics, any of the previously mentioned delivery mechanisms may be utilized for effective RNAi-based therapy against COVID-19.

SIGNIFICANCE OF RNAi AS A THERAPEUTIC STRATEGY TO TREAT SARS-CoV-2–MEDIATED INFECTION

Designing an appropriate therapeutic against SARS-CoV-2 needs to consider certain factors. First, SARS-CoV-2 is reported to be evolving into various mutant subtypes and strains (Mercatelli and Giorgi, 2020; Osório and Correia-Neves, 2020; Tang et al., 2020). Second, so far, SARS-CoV-2 is known to cause acute infections, lasting generally for about 2 to 4 weeks. To address these concerns, miRNA-based therapies may be useful as miRNAs can evolve fairly fast and target new mRNA transcripts, which give them a certain functional flexibility. Additionally, instead of targeting a single gene, a combinatorial RNAi targeting multiple conserved genes commonly implicated across a broad range of SARS-CoV-2 strains may be more effective in combating the disease and may prevent viral escape or development of viral resistance against RNAi. Combinatorial RNAi has also shown potential against other acute viral infections such as influenza A (Estrin et al., 2018). However, critical considerations must be made to minimize cytotoxicity and off-target effects of RNAi while designing such therapeutic strategies. Second, RNAi appears to be more appropriate in targeting acute infections where consecutive administration of synthetic oligonucleotides only a limited number of times may prove to be sufficient in suppressing the infection, as compared to persistent infections where a stable expression system is necessary to elicit a prolonged effect of the therapeutic (Ketzinel-Gilad et al., 2006). Moreover, RNAi strategy may be designed to offer a transient or reversible effect which may be advantageous since long-term silencing of certain genes may prove to be toxic to the cell. Most importantly, most acute infections induce a "cytokine storm" as previously mentioned, leading to activation of the host immune responses to combat the viral infection. In such a scenario, modulation of antiviral genes within a short span of time as offered by the miRNA machinery may not only enhance the host immune response but also provide a robust and quick means of eliminating the virus, which may be especially important in a pandemic situation.

FUTURE PROSPECTS OF REGULATORY ncRNAs IN COMBATING COVID-19

From the above discussion, it is quite evident that regulatory ncRNAs, particularly miRNAs, are emerging as important players in regulating virus-mediated infection and subsequent disease conditions. miRNA-mediated gene regulation is unique, because a single miRNA may have multiple mRNA targets, or a single mRNA may be targeted by multiple miRNAs. This pleotropic nature of miRNAs makes it a suitable therapeutic candidate. Additionally, because miRNAs have an endogenous origin and are processed utilizing the host cellular machinery, there is less fear of eliciting immunogenic reaction against them.

However, recent reports suggest that CRISPR-Cas13–based gene editing may be a potential alternative therapeutic strategy against viral infections including that by SARS-CoV-2 (Freije et al., 2019). Particularly, Abbott et al. (2020) developed a CRISPR-Cas13d–based intervention strategy named PAC-MAN (prophylactic antiviral CRISPR in human cells) to target conserved viral regions across different strains of each of SARS-CoV-2 and H1N1 viruses, with the view of developing a pan-coronavirus inhibition therapeutic strategy. Although promising, these CRISPR-based strategies need to cross multiple checkpoints like minimizing off-target effects, devising effective *in vivo* delivery methods, and assessment of probable immunogenic and cytotoxic effects must be done before being considered as suitable clinical intervention options, which might take a good few years. Thus, in the rising emergency of COVID-19, RNAi seems to be more suitable as it is a comparatively time-, labor-, and cost-effective therapeutic strategy.

To add to that, miRNAs, including viral miRNAs are clinically relevant as biomarkers because they may be secreted in body fluids such as blood, saliva, urine, etc. For example, viral miR-VP-3p can be detected in the sera of Ebola virus–infected patients (Chen et al., 2016), whereas miR-US4-1 is found in the sera of patients with hepatitis B virus receiving IFN-a treatment (Pan et al., 2016). The clinicaltrials.gov database lists completed clinical studies for miRNA biomarkers, which even includes advanced (phase IV) clinical trials.

In the past, no vaccine or drug has been proven effective against HCoVs, including the likes of SARS-CoV or MERS-CoV, which too were responsible for pandemic respiratory diseases in 2002 and 2012, respectively. Although clinical trials of potential vaccines and drug targets are ongoing against COVID-19, it is too early to predict how useful they are eventually going to be in eliminating SARS-CoV-2, which is reported to be mutating and evolving at a considerable rate. In such a scenario, identification of altered or dysregulated miRNAs during SARS-CoV-2 infection may prove instrumental in designing RNAi-based therapeutics for combating COVID-19 and may open up new avenues for developing biomarkers against this deadly pandemic in the future.

AUTHOR CONTRIBUTIONS

PB conceived and wrote the manuscript. SB supervised and reviewed the article. Both authors contributed to the article and approved the submitted version.

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Ca^{2+}/Calcineurin-mediated release of exosomal miR-23a regulates astrocyte-neuron cross-talk and protects against neuroinflammation and neurodegeneration

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Abstract

Background: Astrocyte dysfunction and associated neuroinflammation is well implicated in various neurodegenerative disorders like Alzheimer's (AD) and Parkinson's diseases (PD). Importantly, Ca^{2+}/Calcineurin signaling is reported to induce astrocyte dysfunction in AD (1) but very little is known regarding PD. So, our objective was to study the role of Ca^{2+}/Calcineurin signaling in astrocyte-neuron cross-talk during PD-related neuroinflammation which is unreported till date.

Method: 1.Mammalian cell culture 2.Immunoblotting. 3.qRT-PCR. 4.Exosome isolation. 5.Intracellular Ca^{2+} measurement. 6.Transfection (miRNA mimics). 7. 3'UTR cloning. 8.Luciferase assay.

Result: We treated human astrocytic 1321N1 cells with Rotenone, a neuro-toxin known to induce PD-associated neuroinflammation (2). At the dose of Rotenone which caused about 40% death in 24 hrs, simultaneous activation of the Ca^{2+}-dependent protein phosphatase Calcineurin and intracellular surge in the Ca^{2+} levels were also observed. Now, miR-23a is an anti-apoptotic miRNA and Calcineurin/NFAT-mediated upregulation of miR-23a has been reported in cardiac hypertrophy (3). Surprisingly, in our study, Rotenone caused a Calcineurin-mediated decrease of intracellular miR-23a levels in astrocytes. Now this decrease, we found, was due to a concomitant release of miR-23a via exosomes. Can this anti-apoptotic miR-23a released by astrocytes protect the neurons? To answer this, we treated neuronal SH-SY5Y cells with Rotenone and found that the pro-apoptotic protein PUMA was upregulated although the miR-23a levels remained unchanged. Interestingly, over-expression of miR-23a caused attenuation of the Rotenone-mediated death in neurons. PUMA being a predicted target of miR-23a, we cloned the 3'UTR of PUMA and subsequent luciferase assay revealed that indeed PUMA is a direct target of miR-23a under our experimental conditions.

Conclusion: Our results suggest a novel mechanism by which Ca^{2+}/Calcineurin-mediated release of miR-23a through exosomes regulates astrocyte-neuron cross-talk and plays a probable role in protection against neuroinflammation and neurodegeneration.

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growth and guidance and are testing mutants in genes that are upregulated after injury to determine their functional role in axon guidance. Funding: NINDS NRSA F32NS103219

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Ca^{2+}/calcineurin mediated release of exosomal miR-23a has a role in astrocyte dysfunction and neurodegeneration in Parkinson's disease model

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Rationale: Parkinson's disease (PD) is the second most prevalent neurodegenerative disorder affecting mainly the midbrain dopaminergic neurons (mDN). Astrocyte dysfunction is well implicated in a number of neurodegenerative diseases, including PD. Now, Ca^{2+}/Calcineurin signaling is reported to induce astrocyte dysfunction in Alzheimer's disease (1) but very little is known in context to PD. So, our objective was to study the role of Ca^{2+}/Calcineurin signaling in astrocyte-neuron cross-talk during PD which is unreported so far. **Methods:** 1. Mammalian cell culture. 2. Rotenone treatment. 3. MTT assay. 4. Immunoblotting. 5. qRT-PCR. 6. Exosome isolation. 7. Intracellular Ca^{2+} measurement. 8. Transfection. 9. 3'UTR cloning. **Results:** As a cellular model of PD, we chose the human astrocytic 1321N1 cell line and treated the cells with varying doses of Rotenone, eventually choosing a dose which showed 40% death in 24 hrs. A simultaneous activation of the Ca^{2+}-dependent protein phosphatase, Calcineurin and an intracellular surge in the Ca^{2+} levels were also observed at this dose. Now, miR-23a is an anti-apoptotic miRNA and Calcineurin/NFAT-mediated upregulation of miR-23a has been reported in cardiac hypertrophy (2). Surprisingly, in our study, Rotenone treatment caused a Calcineurin-mediated decrease of intracellular miR-23a levels in astrocytes. Now this decrease, we observed, was due to a concomitant release of miR-23a via exosomes. So the next question we asked was whether the astrocytes release this anti-apoptotic miR-23a via exosomes to protect the neurons. To answer this query, we treated neurons with Rotenone and found that the pro-apoptotic protein PUMA was upregulated in those neurons. However, the neurons showed no change in the miR-23a levels upon Rotenone treatment. Interestingly, overexpression of miR-23a showed attenuation of the Rotenone mediated death in neurons. Since PUMA is a predicted target of miR-23a, we have cloned the 3'UTR of PUMA to check by luciferase assay if miR-23a can directly bind to it, in our study. **Conclusion:** Our results so far, suggest a novel mechanism by which astrocytes cause Ca^{2+}/Calcineurin mediated release of miR-23a through exosomes which, in turn, play a probable role in protection of Rotenone induced death of mDN in PD. **Acknowledgement:** We thank Prof. Sharmistha Banerjee (Hyderabad University) for providing the 1321N1 cell line and to CSIR, GoI, for funding the project. The authors declare no conflict of interest. **References:** 1. Sompol P. et al. Calcineurin/NFAT Signaling in Activated Astrocytes Drives Network Hyperexcitability in Aβ-Bearing Mice. J Neurosci. 2017 Jun 21;37(25):6132-6148. 2. Lin Z. et al. miR-23a functions downstream of NFATc3 to regulate cardiac hypertrophy. Proc Natl Acad Sci U S A. 2009 Jul 21;106(29):12103-8.

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The interaction of tau protein with filamin A contributes to its accumulation in neuronal cells

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In Alzheimer disease (AD), tau, an axonal microtubule associated protein, becomes hyperphosphorylated, accumulates and aggregates in the somatodendritic compartment. The

Poly (ADP-Ribose) Polymerase-1 causes mitochondrial damage and neuron death mediated by *Bnip3, J Neurosci. 2014 Nov 26;34(48):15975-87*

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Background

Hypoxia/ischemia is one of the major causes of mitochondrial dysfunction and neuronal cell death. So far, it has been reported that the DNA damage repair enzyme Poly (ADP-Ribose) Polymerase-1 (PARP1) gets activated during hypoxia/ischemia, leading to mitochondrial membrane permeability transition and caspase independent neuronal death mediated by nuclear translocation of the mitochondrial proapoptotic factor AIF. On the other hand, Bnip3, a proapoptotic BH3-only protein of the Bcl-2 family, is also found to be upregulated during hypoxia/ischemia, resulting in mitochondrial permeability transition and neuronal death in a manner similar to that of PARP1. This led the authors to hypothesize that the pathway of neuronal cell death induced by PARP1 and Bnip3 may be linked. Also, it is reported that PARP1 mediated NAD^+ depletion leads to inhibition of SIRT1,[1] a NAD^+ dependent class III histone deacetylase enzyme which targets many transcription factors responsible for maintaining mitochondrial integrity including the FoxO (Fork-head box O) family of proteins. Thus, they also hypothesized that PARP1 mediated inhibition of SIRT1 may lead to derepression of FoxO3a resulting in upregulation of Bnip3 under hypoxic conditions.

Study Design

All the experiments were done in primary neuronal culture from 16th day embryonic mouse cortices. Hypoxia was induced by a mixture of 5%CO_2/95%N_2 in a humidified hypoxic chamber at 37^{0}C. 50 μM of MNNG, a DNA alkylator, was used for 30 min to activate PARP1. As negative controls, cells were either treated with the PARP1 inhibitor PJ34 or were taken from $Parp1^{-/-}$ and $Bnip3^{-/-}$ mice.

The authors used fluorescence microscopy for quantifying intracellular propidium iodide (red) and calcein fluorescence (green) as indicators for dead and live cells respectively and demonstrated that hypoxia induces neuronal cell death which occurs via activation of PARP1 and Bnip3. During normoxic conditions, MNNG mediated activation of PARP1 leads to upregulation of Bnip3 which in turn leads to mitochondrial membrane potential loss as measured by the fluorescent dye JC-1 under confocal microscope. Bnip3 dependent nuclear localization of mitochondrial AIF was also observed under such conditions which could be mitigated by exogenous addition of NAD^+. Hyperactivation of PARP1 during hypoxia was found to cause a decline in the NAD^+ levels in the neuronal cells which in turn lead to inhibition of Sirtuin 1 (SIRT1). Immunoprecipitation followed by immunoblotting revealed that SIRT1 interacts directly with FoxO3a at a baseline level. Also, during hypoxia induced PARP1 activation, increased FoxO3a acetylation and nuclear translocation of FoxO3a was observed. Further, by chromation immunoprecipitation followed by real time-PCR, increased binding of FoxO3a was observed at the upstream promoter region of Bnip3 during PARP-1 dependent hypoxia. SIRT1 silencing by lentiviral shRNA treatment during normoxic conditions in the absence of PARP1 activation caused Bnip3 upregulation, enhanced FoxO3a acetylation and increased binding of FoxO3a to the Bnip3 upstream promoter. FoxO3a silencing during hypoxia leads to decreased Bnip3 transcription- validating the role of FoxO3a as a transcription factor in the expression of Bnip3, decreased loss of mitochondrial membrane potential and enhanced neuronal survival.

Implication

The authors have demonstrated that under hypoxic conditions, PARP1 activation leads to NAD+ depletion which in turn leads to inhibition of SIRT1 causing hyperacetylation and nuclear translocation of FoxO3a. FoxO3a drives the expression of Bnip3 which leads to mitochondrial membrane potential loss and AIF release ultimately resulting in neuronal death.[2] Thus, Bnip3 is the mediator in PARP1 induced mitochondrial integrity loss and neuronal cell death during hypoxia. Interestingly, the authors here have shown for the first time that NAD+ depletion has a direct effect on inhibition of SIRT-1, which is a master regulator of genes like FoxO3a and Bnip3 responsible for maintaining mitochondrial integrity and function. Thus, by replacing NAD+ exogenously, PARP-1 mediated neuronal cell death during hypoxia maybe inhibited.[3]

The authors, however, have not shown the pathway leading to neuronal death downstream of Bnip3. Also, the role of AIF in Bnip3 mediated neuronal death needs to be explored further.

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