Identification of neurotoxin regulated microRNAs and study their role in neurodegeneration

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By

Tanisha Singh
MSc. Biotechnology

Under the Supervision of

Dr Sanjay Yadav
Scientist
Developmental Toxicology Division
Systems Toxicology and Health Risk Assessment Group
CSIR-Indian Institute of Toxicology Research
Lucknow- 226 001, India

Department of Biochemistry
School of Dental Sciences
Babu Banarasi Das University
Lucknow- 226 028, India

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Dedicated to my loving family for their eternal love and support

CERTIFICATE OF THE SUPERVISOR

This is to certify that the thesis, entitled **"Identification of neurotoxin regulated microRNAs and study their role in neurodegeneration"** submitted by **Ms Tanisha Singh** for the award of Degree of Doctor Philosophy by Babu Banarasi Das University, Lucknow is a record of authentic work carried out by her under my supervision. To the best of my knowledge, the matter embodied in this thesis is the original work of the candidate and has not been submitted elsewhere for the award of any other degree or diploma.

Dr Sanjay Yadav

Scientist

CSIR-Indian Institute of Toxicology Research

31, Vishvigyan Bhawan, Post box-80, MG Road

Lucknow-226001, India

DECLARATION BY THE CANDIDATE

I, hereby, declare that the work presented in this thesis, entitled **"Identification of neurotoxin regulated microRNAs and study their role in neurodegeneration"** in fulfilment of the requirements for the award of Degree of Doctor of Philosophy of Babu Banarasi Das University, Lucknow is an authentic record of my own research work carried out under the supervision of **Dr Sanjay Yadav,** Scientist, at **CSIR-Indian Institute of Toxicology Research, Lucknow**.

I also declare that the work embodied in the present thesis is my original work and has not been submitted by me for any other Degree or Diploma of any university or institution.

Date: Tanisha Singh

PhD candidate

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Tanisha Singh

LIST OF CONTENTS

I: ABBREVIATIONS

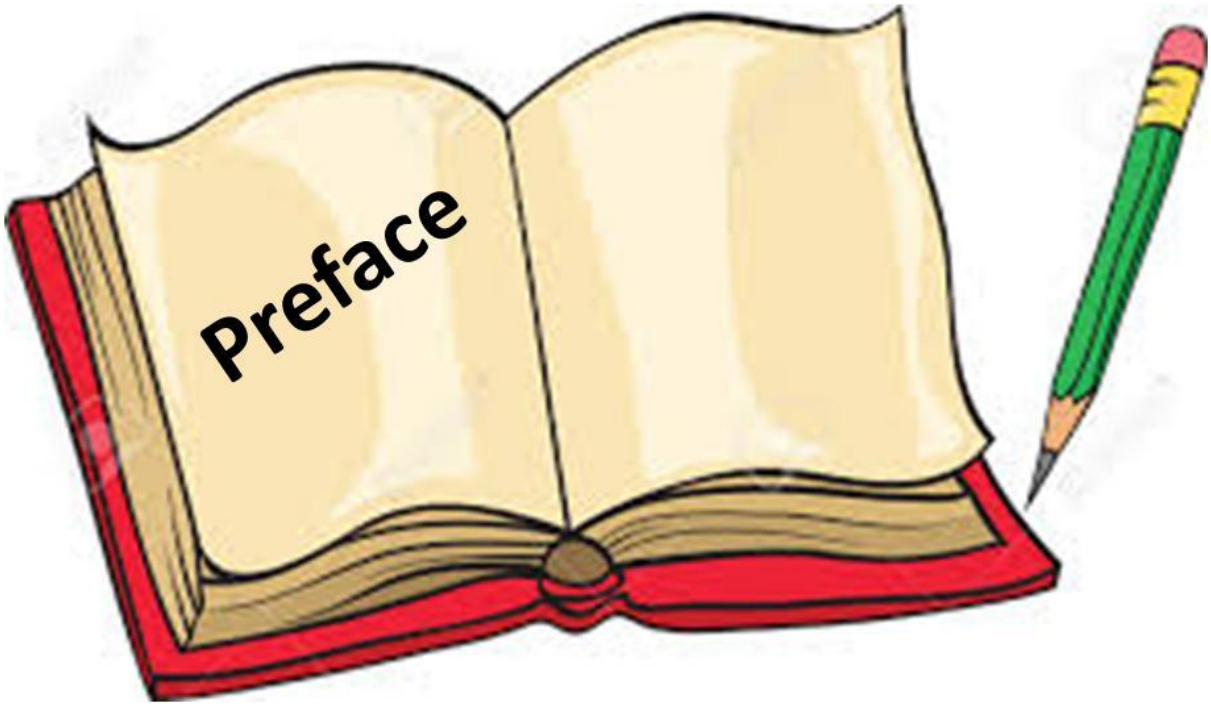
Aβ	Amyloid Beta
Ab/Am	Antibiotic/Antimycotic
AD	Alzheimer's Disease
Ago	Argonaute
ALS	Amyotrophic Lateral Sclerosis
APP	Amyloid Precursor Protein
BSA	Bovine Serum Albumin
cDNA	complementary Deoxyribonucleic Acid
CNS	Central Nervous System
Ct	Cycle threshold
DAPI	4,6-diamidino-2-phenylindole
DF	Differentiated
DCX	Doublecortin
DGCR8	DiGeorge Syndrome Critical Region 8
DTT	Dithiothreitol
dNTP	Deoxyribonucleotide triphosphate
ETS	Electron transport system
FBS	Fetal Bovine Serum
FITC	Fluorescein isothiocyanate
GD	Gestation day
GO	Gene ontology

HD	Huntington's Disease
HS	Horse Serum
IDV	Integrated density value
IGF	Insulin Growth Factor
MB	Maneb
miR	Mature microRNAs
miRNA	microRNA
mM	Milimolar
MMP	Mitochondrial membrane potential
µM	Micromolar
µL	Microlitre
mRNA	Messenger Ribonucleic Acid
MTT	3-(4, 5-Dimethylthiazol-2-yl)-2, 5 diphenyl tetrazolium bromide
NFL-H	Neurofilament- Heavy
NFL-L	Neurofilament- Light
NFL-M /NEFM	Neurofilament- Medium
NGF	Nerve Growth Factor
NTC	Non targeting control
NeuroG2	Neurogenin 2
PBS	Phosphate Buffer Saline
PC12	Pheochromocytoma cell line

PLL	Poly-L-Lysine
PD	Parkinson's Disease
Pre-miRNA	Precursor microRNA
Pri-miRNA	Primary microRNAs
PSD95	Post synaptic density protein 95
PVDF	Poly Vinyldene Fluoride
PCR	Polymerase chain reaction
PQ	Paraquat
ROS	Reactive oxygen species
RT-PCR	Reverse transcriptase-polymerase chain reaction
RQ	Relative quantification
RT	Reverse transcription
SDS	Sodium dodecyl sulphate polyacrylamide Gel electrophoresis
siRNA	Small interfering RNA
SLA	Spontaneous locomotor activity
SNCA	Alpha Synuclein
snRNA	Small Nuclear RNA
SYP	Synaptophysin
SYN1	Synapsin1
TBST	Tris Buffer Saline with Triton X 100
TEMED	N, N, N', N'- Tetramethylethylenediamine
TFs	Transcription factors

TLDA	Taqman low density array
TRIS	Tris[hydroxymethyl]aminomethane
UTR	Untranslated Region
W	Week

II. DETAILS OF FIGURES


Preface

Worldwide, neurodegeneration associated diseases are increasing due to increased lifespan and exposure of several pesticides and heavy metals, which have capability to cross blood-brain barrier and induce neurodegeneration. Neurodegeneration is induced by interplay of multiple factors including environmental exposure, genetic predisposition and increased aging. Degeneration of neurons is observed in several diseases like Parkinson disease, Alzheimer disease, Huntingtin disease and Amyotropic lateral sclerosis which affect a large number of people worldwide. Misfolding of proteins and their aggregation along with generation of reactive oxidative species are common cellular events to all neurodegenerative disorders; however molecular events and mechanism responsible for initiation of neurodegeneration is still unknown. In spite of several studies carried out for exploring neurodegenerative disorders, these diseases are still incurable. Moreover, less detection of these diseases poses major problems in the treatment of these diseases. For this, studies are required to identify the regulatory molecules involved in neurodegeneration and develop early biomarker of these diseases.

Mammalian brain development can be divided in prenatal and postnatal phases, which involves separate period of neurogenesis, neuronal apoptosis, synaptogenesis and synaptic pruning. Successful development of mature and fully functional brain requires coordination in expression of mRNA, proteins and regulatory RNA molecules. In mammalian nervous system, developing brain involves diverse cycles of proliferation, apoptosis, maturation and reorganization of synapses. Each and every function of brain is regulated by expression of several genes in sequential and cell specific manner. In neurons, gene expression is highly complicated which is regulated

at transcriptional, post-transcriptional and translational levels. MicroRNAs (miRNAs) are most characterized and studied small regulatory RNA molecules with potential to regulate around 60-80% of protein coding genes at post-transcriptional level. In present study, attempts were made to validate role of Dicer processed mature miRNAs in neuronal development and identify the set of miRNAs deregulated by exposure of sodium arsenite or PQ+MB in developing neurons & developing brain. Attempts were also made to compare the regulation of "brain specific miRNAs" between differentiated neurons and adult brain for developing a cellular model of developmental neurotoxicity for miRNA studies. Dicer, a type III ribonuclease is essential for generating mature miRNAs. Interestingly, when Dicer was knocked out in mice, they did not survive through postnatal development which proves that expression of miRNAs is essential for development. Previous studies from our lab have shown that knocking down Dicer promotes senescence, when neuronal cells are exposed with neuronal differentiation inducing chemicals and neurotoxicants. Global miRNA profiling of developing neurons and NSCs have identified dramatic changes in expression of global miRNA profile, which also supports role of miRNAs in brain development. Earlier studies have also shown that knocking-out Dicer selectively in neurons results in apoptosis and loss of neuronal integrity which leads to neurodegeneration.

Brain is the most indispensable organ which plays a vital role in controlling and balancing the key functions of a body. Its development carries a complex network of phases. Studies delineated that in case of humans, major brain development takes place before 5th year, but at adolescence stage, brain develops and pursue various

processes including synapse formation and maturation. MiRNAs play a crucial role in all the phases of brain development. Neuronal degeneration takes place due to various reasons including genetic factors or environmental factors (exposure of neurotoxicants). Neurotoxicants are known to affect the structure and functions of neuronal cells. Developing brain is more vulnerable towards toxicants, easily inclined towards neurodegeneration. Several studies explored that exposure of any environmental toxicant during postnatal brain development impairs the proper functioning of brain which results in neurotoxicity and neurodegeneration. In response to which, neuronal gene expression can be changed adversely and these responsive changes are regulated by specific miRNAs. So, to understand the molecular pathways or mechanisms behind neurotoxicants induced neurodegeneration, regulation of miRNAs needs to be studied.

In order to identify the miRNAs deregulated by neurotoxicants, rats of different age group (pre-adolescent, adolescent, post-adolescent and young adults) are exposed with sodium arsenite or PQ+MB for 7 consecutive days. Exposure of sodium arsenite or PQ+MB (known neurotoxicants) have produced maximum alterations in expression of "brain specific miRNAs" at postnatal 6^{th} week, which is most dynamically regulated brain developmental stage and corresponds to adolescent period of humans. Surprisingly, adult rats have shown more changes in miRNA expression than pre and post-adolescent aged rats. Volcano and box plot analysis of "brain specific miRNAs" expression and neurobehavioral assays has shown 6^{th} postnatal week as most vulnerable postnatal age of brain development for neurotoxicants exposure. Sodium arsenite or PQ+MB exposed adolescent rats identified miR-29 family whose role is

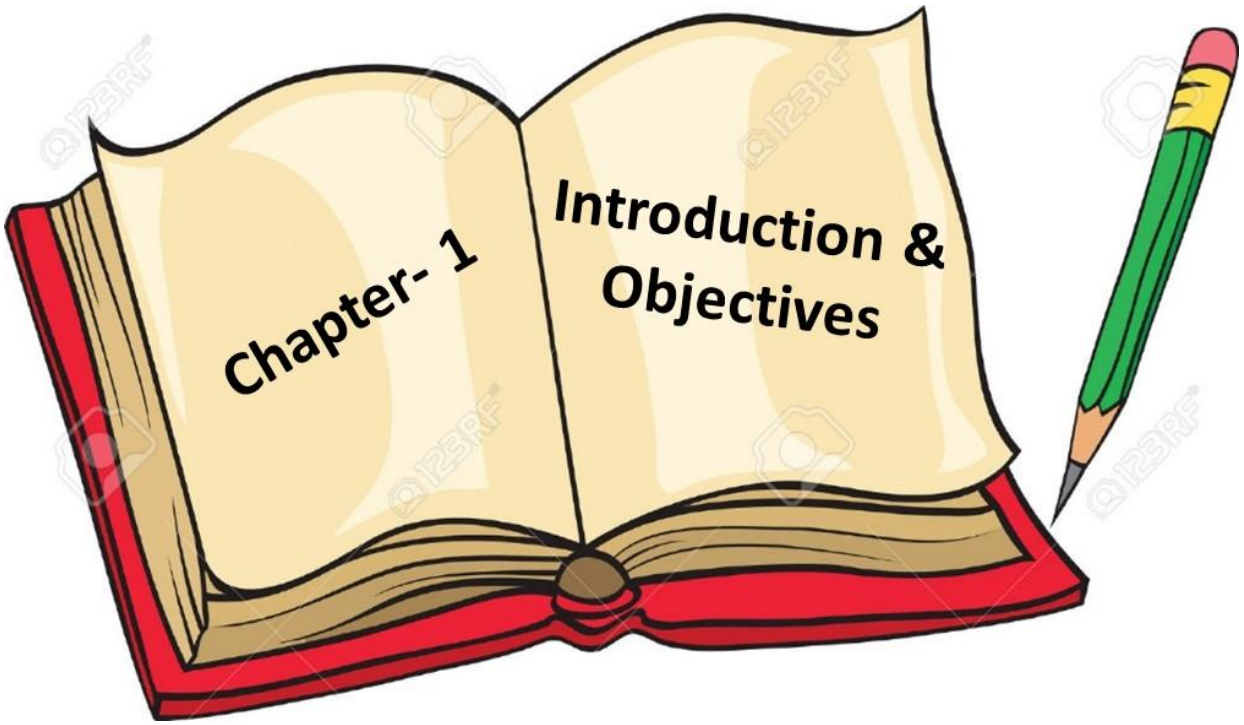
associated with various neurodegenerative diseases. Moreover, exposure of sodium arsenite or PQ+MB deregulated the protein levels of various neuronal and synaptic markers in adolescent brain which further suggests that 6week time point is more susceptible towards neurotoxicants exposure. Furthermore, sodium arsenite or PQ+MB hamper the normal brain development by down-regulating the expression of neuronal and synaptic markers at adult stage.

PC12, a pheochromocytoma of rat adrenal medulla is most widely used cellular model of neuronal differentiation. PC12 cells provide us a highly extrapolative and innovative tool to understand the mechanisms of neuronal development. Cells were allowed to differentiate (8 days) under the influence of NGF and expression (mRNA and protein) studies were carried out for selected markers of neurogenesis at different time point of exposure (0, 2, 4, 8 days) which confirms its suitability as a neuronal model. Our earlier studies identified miR-200, miR-221/222 and miR-34 families as majorly up-regulated families in NGF induced differentiated PC12 cells and elucidated the role of miR-200 family in neuronal differentiation. In present study, role of miR-34 family in neuronal differentiation was demonstrated. Present study has identified P53 as mediator of nerve growth factor (NGF) induced miR-34a up-regulated expression in differentiating PC12 cells. Further, miR-34 is identified as brain enriched miRNA family, which up-regulates with neuronal maturation and brain ageing while co-operative regulation of P53 and miR-34a helps arrests cells in G1 phase, which helps to maintain the mature neurons in differentiated stage.

In order to identify the miRNAs deregulated by sodium arsenite or paraquat in mature neurons, differentiated PC12 cells were exposed with non-cytotoxic dosages of sodium arsenite or paraquat (5μM and 50μM). Exposure of non-cytotoxic dosages of sodium arsenite (5μM) and paraquat (50μM) to PC12 cells induced reactive oxygen species (ROS) generation, altered mitochondrial membrane potential (MMP) and induced apoptosis. In continuation, these neurotoxicants deregulated various synaptic and neuronal markers in NGF induced differentiated PC12 cells. Furthermore, exposure of sodium arsenite or paraquat down-regulated the expression of miR-34 family which was found to be up-regulated in neuronal development and implicated its role in neurodegeneration.

In adult rats (12week aged), exposure of paraquat maximally up-regulated the expression of miR-674* and miR-511 while sodium arsenite produced maximum up-regulation in miR-674* and miR-150. Interestingly, expression of miR-150 was also significantly up-regulated by exposure of sodium arsenite (8.87 folds) or paraquat (3.84 folds) in differentiated PC12 cells. The identification of miR-150 in sodium arsenite exposed young adult rat brain and mature neuron (NGF induced differentiated PC12) indicates its crucial role in neurodegeneration. Further, studies have identified neurofilament (NEFM) as target of miR-150 which is crucial for neurocytoskeletal structure. NEFM is required for maintaining strength and axonal calibre of neuron. Loss of function studies with miR-150 has shown that miR-150 protects neuronal cells from degeneration.

In conclusion, present study demonstrated that expression of mature miRNAs is essential for neural development and set of specific miRNAs are deregulated by exposure of different neurotoxicants. Adolescent brain is most vulnerable period of postnatal rat brain development and expression of miR-150 can be targeted for development of biomarker of neurodegeneration.


Chapter- 1
Introduction &
Objectives

CHAPTER 1: INTRODUCTION

Age-related diseases like neurodegenerative disorders (NDDs) are coming up as major health concern in society due to accumulating exposure of pesticides/environmental chemicals and increased life span of humans. According to World health organization (WHO) prediction, NDDs will beat cancer and become the second most debilitating and fatal disease after cardiovascular disorders by 2040 (1). NDDs exemplify the progressive loss in structure and function of neuronal cells resulting loss of neuronal cells (2). NDDs are heterogeneous in nature and results from culmination of numerous genetic and environmental factors. Examples of NDDs are Parkinson's, Alzheimer's, Huntington's, Amyotrophic lateral sclerosis, differentiated by the site of affected region and effects, which are quite specific to each disease. Exposure of environmental chemicals leads to abnormal molecular events including mitochondrial dysfunction, oxidative stress, neuroinflammation, protein misfolding, DNA damage, aberrant RNA processing which collectively or individually induces neurodegeneration (2). Other factors like gender, endocrine conditions, strokes, tumours, depression, infections, metabolic and immune conditions also help in the development of neurodegenerative disorders. However, actual reason for the development and pathogenesis of NDDs is not known, consequently, no cure is available for these diseases, which is a major issue for neurobiologists. Process of brain development is very fascinating, which starts from proliferation of progenitor cells, and migrates and differentiated to produce mature and functional neurons (3). Brain development involves various different separate phases of neuronal

proliferation, differentiation, apoptosis, synaptogenesis and pruning (4). Each phase engaged the molecular changes at transcriptional, post transcriptional and post translational level. Proteins are considered as the main working unit of cells, perform most of the cellular functions. Genes having protein coding capability are transcribed into messenger RNAs (mRNAs). Amount of mRNAs in cells are directly correlated to levels of corresponding protein, until the microRNAs (miRNAs) are not discovered. Emergence of small regulatory RNA or miRNAs added new paradigm in biological research. Since their discovery, miRNAs regulate genes and their functions in almost every cell types. Brain cells are highly transcriptionally active and studies have demonstrated them as the most important organ for miRNA mediated gene regulation (5). Among the various tissues, brain is identified to express maximum number of unique miRNAs which suggests their significance in development and degeneration of brain (6,7). Each and every step of brain development is tightly regulated at transcriptional level and requires a specific network or system of gene regulatory mechanisms to work in co-ordination. Earlier published studies from our lab and elsewhere have reported that miRNAs are the crucial players of brain development and its processes like proliferation, differentiation and apoptosis of neurons (8-10). It can be assumed that any alterations in expression of miRNAs in developing or adult brain can results in brain degeneration.

MiRNAs are a class of small, non coding RNA molecules, which are present in majority of the organisms. Structurally, miRNAs are short RNA sequences expressed from longer transcripts encoded in animals, plant and virus genomes (11,12). MiRNAs regulate the expression of protein coding mRNAs by binding in sequence

specific manner mostly at 3'UTR. Precursor miRNAs are processed by an endoribonuclease of RNase III family member known as Dicer. Dicer processing produces mature miRNAs and moreover, dicer also helps in loading of mature miRNAs at target sites of miRNAs (13). Various knockout studies of Dicer genes from our lab and elsewhere have shown that miRNAs are essential for neuronal development (8,14). Studies from our lab have also elucidated that knocking down Dicer gene, impairs neuronal differentiation and lead cells towards senescence (14). Knockout Dicer, specifically in the cerebral cortex region of brain, slimming the cortical walls as well as reduces the progenitor numbers, along with unusual neuronal differentiation (15). Additionally specific deletion of Dicer in post-mitotic dopaminergic neurons has resulted in progressive loss of dopaminergic neurons by apoptosis in the mid-brain which implies towards neurodegeneration (16). Studies reported that deletion of astroglial dicer results in non-cell-autonomous neuronal dysfunction and degeneration (17). Numerous studies has been revealed regarding the noteworthy role of Dicer or miRNAs in brain development and maturation ,as dicer deletion results apoptosis, as well as loss of integrity of neurons which leads to neurodegeneration (18). MiRNAs regulate the expression of target genes by binding to complementary sites in their transcripts to cause translational repression or transcript degradation. Dynamic regulation of miRNAs during different stages of brain development has indicated towards their regulatory role in neurogenesis, proliferation, differentiation, apoptosis, maturation and neurodegeneration.

Brain is the most sophisticated organ which plays an imperative role in controlling and balancing the key functions of a body. Heimberg et al. have suggested that

miRNAs are largely responsible for morphological complexity of the CNS (19). They evaluated the evolution of miRNA families and that so of new structural complexity particularly apparent in the CNS. Many miRNAs have been identified to be highly abundant in certain regions of the brain which regulates various crucial events. These crucial events include proliferation, migration, differentiation, synaptogenesis, apoptosis, gliogenesis and myelination (20). In last two decades, these crucial events of postnatal brain development are well characterized and compared among different mammalian model organisms including humans (20). In rats, postnatal week 2-4 corresponds to pre-adolescence period of humans and well characterized to phase of neuronal proliferation or postnatal neurogenesis. Postnatal 5-7 week rat brain corresponds to human's adolescent period, known for excessive synaptic and receptor formation. Further, studies has shown that brain of 8-10 postnatal week corresponds to post adolescence period of human brain, which is well known for synaptic pruning and synaptic maturation. Brain matures at postnatal week 12 and considered as adult brain of humans. Therefore, we used the rats of postnatal 3 week (Pre-adolescence) 6 week (Adolescence), 9 week (Post-adolescence) and 12 week (Adult) age to study the effect of sodium arsenite and Paraquat+ Maneb on expression of brain specific miRNAs at major events of postnatal brain development (4,21,22).

Neuronal differentiation is one of the most important event of brain development which results in generation of mature and functional neurons (14,20,23). Cell based models of neuronal differentiation have provided an opportunity to investigate the role of individual genes or proteins in brain development. One of the most commonly used cellular model for neuronal development is PC12 cells, as these cells can

willinglyrecapitulate the key events of neuronal development like neuronal differentiation, which includes neurites network formation and amplification and summarize the major brain development mechanisms (8,23,24). Naive PC12 cells looks like immature adrenal chromaffin cells that are round and polygonal in shape and lack any anyneurite like structures. The exposure of nerve growth factor (NGF) results to differentiation in generation of new neurites, which functionally resembles the mature neurons and displays properties of mature sympathetic neurons such as electrical excitability, secretion of neurotransmitters (dopamine, noradrenalin and acetylcholine), and expression of neuronal specific genes i.e. Neurofilament, MAP-2 and β-III-tubulin (25-27). NGF protein or neurotrophic factor which helps in the regulation of growth, maintenance, proliferation, and survival of neurons. Thus, NGF was used to promote neuronal differentiation in PC12 cells (28). PC12 cells are majorly used in the morphological evaluation of cell differentiation, mechanisms of toxicity involved in apoptosis, disturbance in neurotransmitter synthesis and oxidative stress. Earlier studies from our lab have identified significant up-regulation in the expression of 22 miRNAs in differentiated PC12 cells. Among these miRNA families, miR-221/222, miR-200 and miR-34 are the majorly up-regulated in compare to naive PC12 cells (8). Apart from these, role of miR-200 has also been elucidated inneurotoxicity of cypermethrin, a pyrethroids insecticide (29). Neurotoxicity can result from exposure to substances such as metals, solvents, pesticides, etc., which leads to adverse effects on the nervous system. Unfavourable changes in structure/function of nervous system due to any neurotoxicant profounds consequences of neurological, behavioural and related body functions and processes.

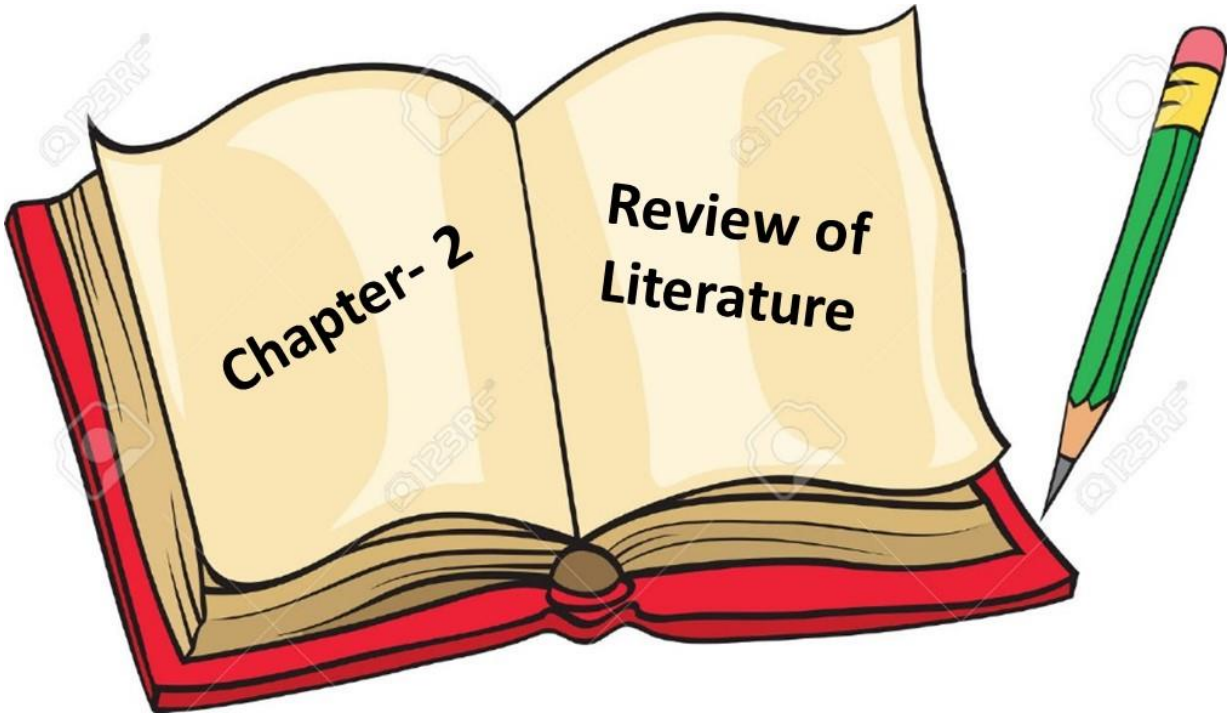
It is extensively believed that environmental constituent such as metals, pollutants, pesticides and somewhere lifestyle plays a crucial role in brain health either direct or in an indirect manner. This toxin disrupts/kill the cells during development of brain and hampers neuronal functions as well as mechanisms. For instance, the environment to which a fetus is exposed during the gestational tenure plays a noteworthy role in the future health of an individual as developing brain is more vulnerable to environmental insults. Unfavorable and adverse environmental conditions during prenatal and postnatal period disturb the homeostasis and increase the risk of neurodegenerative diseases (NDDs). In response to exogenous stress, including environmental chemicals, gene expression can be changed adversely and these responsive changes are regulated by specific miRNAs.Most of toxic compounds affects the critical stages of brain development which commonly known as developmental neurotoxicants. Exposure of these developmental neurotoxicants during developing period or postnatal development period may be responsible to a variety of neurological or NDDs, such as Parkinson's or Alzheimer's diseases. The class of these developmental neurotoxicants possess a variety of pesticides, insecticides as well as herbicides. Paraquat (PQ) is one of the most widely used herbicide in developing countries and a renowned developmental neurotoxicant. Paraquat, chemically known as 1'-dimethyl-4,4'-bipyridinium, induces oxidative stress caused by extreme generation of reactive oxygen species (ROS) via redox cycling reactions and results in neuronal cell death (30). Studies have been reported that PQ cause deleterious effect to dopaminergic neurons in substantial nigra in parallel with accumulation of α-synuclein which resembles PD like symptoms (31). Another pesticide, Sodium arsenite which is a

known carcinogen, now evolved as a developmental neurotoxicant as it crosses the placenta and modulates the neuronal system by altering neurotransmitters function (32). Numerous studies has been identified that it disrupts the neurocytoskeletal system and affecting the memory and learning features (33,34). The eventual role of miRNAs in neuronal differentiation, neuronal proliferation and neuronal apoptosis has been discussed in various individual studies. Also, there are few reports available in the literature identifying the role of these neurotoxicants inneurodegeneration. Reports have been suggested that any alterations in expression of miRNAs by neurotoxicants can modulate developing brain and leads neurotoxicity and degeneration. The present study was commenced to understand the cellular and molecular mechanisms in case of PQ and Sodium arsenite induced neurodegeneration as no study has been carried out to understand the cellular and molecular mechanisms of miRNA involved in neurotoxicants induced neurodegeneration. Thus, our study was proposed to develop a responsive*in vitro* model system as well as *in vivo* to study the expression and responsiveness of genes (miRNAs and mRNAs) involved in damage under the effect of PQ and Sodium arsenite using NGF induced differentiating PC12 cells and rat model.

To fulfil the hypothesis, study has been carried out under three objectives:

- ✓ To study the effect of neurodegeneration inducing pesticides, Paraquat + maneb (PQ+MB) or arsenic (As) (Sodium arsenite) on regulation of brain specific miRNAs in rats aged 3 week or 6 week or 12 week.

- ✓ To study the effect of PQ and As on regulation of brain specific miRNAs in cellular model of neuron development. Nerve growth factor (NGF) differentiated PC12 cells will be used in this study as cellular model for neuronal differentiation. Studies will also be carried out to compare regulation of miRNAs between cellular model (NGF differentiated PC12 cells) and animal model (developing rat brain) and attempts will be made to identify the similarity between both models.
- ✓ To understand the role of neurotoxin regulated miRNAs in neurotoxin induced neurodegeneration and identify their target genes and possible pathways involved in regulating neurodegeneration.


Chapter- 2
Review of Literature

CHAPTER 2. REVIEW OF LITERATURE

Neurodegenerative diseases (NDDs) are among the biggest challenges and the most severe health problems that have to be faced by the people across the world, in bright light of increasing population age. NDDs are incurable and progressive conditions which involves continuous degeneration and death of nerve cells. Neurodegeneration is induced by interplay of multiple factors including environmental exposure, genetic predisposition and increased aging. Imbalance in oxidative status is one of the common features of neurodegenerative diseases. Deregulation in several proteins related to mitochondrial functions or antioxidant system leads to oxidative imbalance in cells. In spite of several thousand studies carried out on NDDs, proper mechanism behind these is remained unclear. Unfortunately, because of which, development of potential biomarkers as well as new therapeutic targets and agents still remains difficult. Till date, studies are undergoing in revealing the possible strategy in preventing and curing these NDDs at genetic level as well as at environmental level.

2.1 Epigenetic mechanisms

The word "Epigenetic" was firstly given by Conrad Waddington (35) and defines in a classic way "the heritable change in cell phenotype that is independent of changes in genomic DNA sequence". But now adays, modern interpretation suggests that "study of changes in gene function that are mitotically and/or meiotically heritable and that do not entail a change in DNA sequence." Increasing interest in the subject of epigenetics which is one of the most quickly expanding fields in biomedical research has been accompanied by several breakthroughs such as finding new histone variants

and modifications, the discovery of the CpG and most of all possibility of genome-wide analysis of epigenetic marks, epigenomic analysis at single nucleotide resolution (36).

Current advances have evolved our understanding of epigenetic mechanisms and the range of molecular interactions and cellular functions that are linked to these processes. The appreciation of the role of epigenetics and its importance in human disease was first discussed in cancer biology and later extended to neurodevelopment as well as neurodegenerative diseases (37,38). Epigenetic regulations include DNA methylation, Histone modifications and RNA based mechanisms (37). RNA based mechanisms involves the pathways related to non coding RNA molecules. In general, non-coding RNA (ncRNAs) function to regulate gene expression at the transcriptional and post-transcriptional level. A ncRNA is a functional RNA molecule that is transcribed from DNA but not translated into proteins include miRNA, siRNA, piRNA and lncRNA.

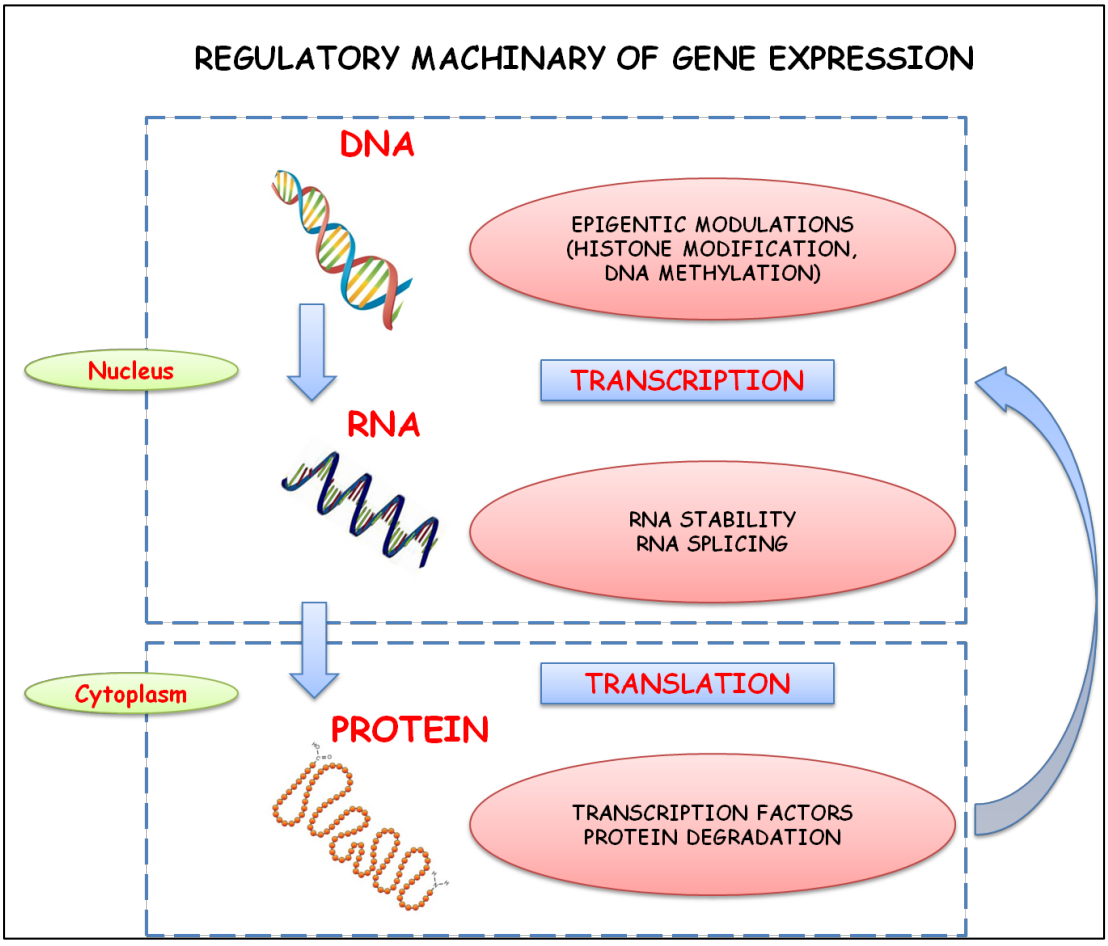


Fig 2.1: Regulatory mechanism of gene expression

2.2 Post-transcriptional factors

Studies and attempts are undergoing to explain the pathogenic mechanism related to gene expression and regulation. Studies revealed that the human genome sequencing shown approx 95% of cellular RNAs is non-coding in nature which includes miRNAs (39). Fortunately, miRNAs emerged as novel key players for the NDDs because of their presence and expression in one of the most vital organ of a physiological system known as Brain (9). MiRNAs controls the normal CNS development and function and have a key role in the onset and progression of various NDDs which prove to be a

pioneer in the research of neuroscience. The participation of miRNAs in NDDs provides a target for novel neuroprotective therapies due to high sensitivity, reproducibility and specificity in compared to protein as therapies (40).

2.2.1 MiRNAs and its biogenesis

MiRNAs are a class of non-coding RNAs is found in plants, animals, and in a variety of organisms to regulate gene expression. These regulatory RNA molecules were first reported in *Caenorhabditis elegans* to regulate developmental timing. MiRNAs have been shown to regulate most of the cellular events including proliferation, differentiation, migration, and apoptosis (41). Till date more than 2500 miRNAs have been reported in humans and stored in miRNAs repository. MiRNAs are small, single stranded, approx 22nt in length, regulatory RNA molecules implicated in the regulation of gene expression that bind posttranscriptionally with the 3'-untranslated region of target mRNAs (42). MiRNAs bind to complementary sequences at 3′UTR of target genes and hinders gene expression. MiRNAs are transcribed by RNA polymerase II and initial transcripts are known as primary-miRNA (pri-miRNA) either from inter or intragenic region. Pri-miRNA is folded into a stem loop structure which is cleaved by a complex of riboendonuclease enzyme, Drosha and DGCR8 protein into a 70-80nt hairpin structure called precursor-miRNA (pre-miRNA). The hairpin loop structure (pre-miRNA) formed in nucleus is transported to cytoplasm via Ran-GTP dependent mechanism by exportin-5, where it gets cleaved by RNase III-enzyme named Dicer and TAR RNA- binding protein 2 (TRBP), which creates a short RNA duplex of 22 nt. The newly born short RNA duplex binds with a protein called

Argonaute forming a complex named miRNA-induced silencing complex (miRISC complex). Out of two strands of duplex, one is passenger strand or miRNA* and the other one is guide strand or mature (usually dominant and functional) miRNA. RISC controls gene expression by binding through complementarity to the 3′UTR of target mRNAs leading to degradation of mRNAs, when there is sufficient complementary or translational repression of protein expression (43). Overall miRNAs provide an extra level of regulation in protein synthesis, which is dependent on gene sequence. In consequence, miRNAs provide a complex network of regulation that result in fine tuning, sturdiness and complexity of the transcriptome and proteome (44).

The significance of miRNAs has enhanced so far, due to its involvement in the majority of the biological and developmental processes. Brain is enriched with miRNAs and expression of each miRNA changes according to the brain area. These brain specific miRNAs regulates various processes from neuroregeneration to neurodegeneration. The functional unit of brain, neuron, requires a tight control in several gene expression pathways. Deregulation in any one of the pathways leads to abnormal expression of genes and proteins which results in the inability of neurons to function properly. These abnormal proteins get accumulated and behave as a waste product which results in neurotoxicity, malfunction of neurons or ultimately neuronal cell death.

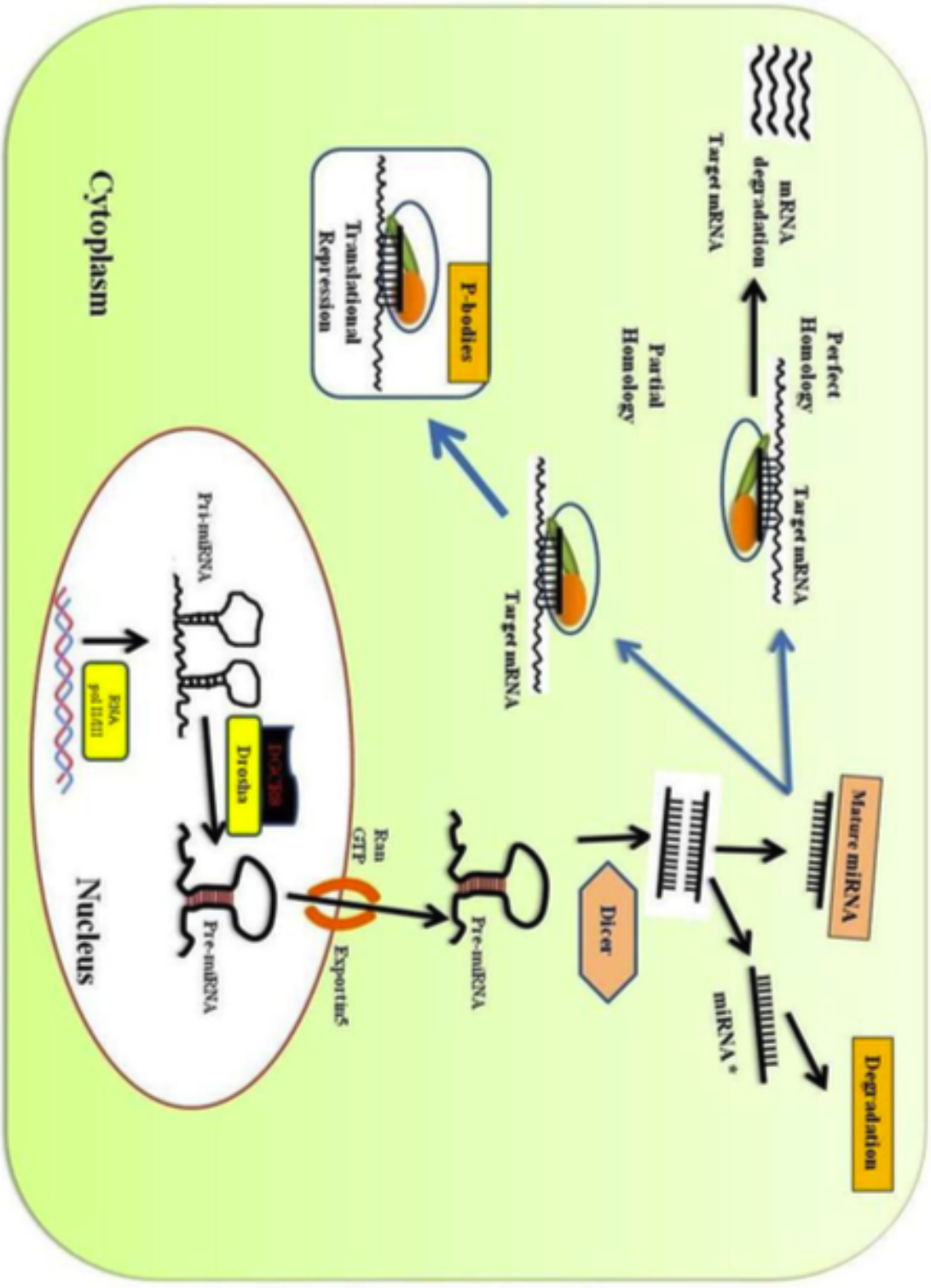


Fig 2.2: MicroRNAs Biogenesis and Mode of action

2.3 Neurodegenerative diseases

The nervous system is a highly multifaceted and complex organ that is responsible for a variety of tasks including receiving and processing sensory information and in controlling the highly complex behaviours required for proper survival, even in case of phylogenetically lower organisms. Physiological and anatomical differences in between higher species (e.g. mammals) and lower species are properly evident. These differences clearly related with behavioural complexity and overall functioning. This complexity of nervous system for normal function can leave the system prone towards a variety of insults or exposures. In reference to environmental exposures; age, sex, route of exposure, dose, and genetic profile, have also a major influence on the level of effects, specificity and the phenotypic consequences on an individual (45)**.** Any environmental exposure or insult develops behavioural as well as anatomical abnormality carried out under acute or chronically.

Selected degeneration of neurons in brain causes several diseases commonly known as Neurodegenerative disorders or diseases (NDDs). In particular, neurodegeneration is a broad term, which mostly affects the structure and function of neurons in brain. A family of NDDs consists of many members (Parkinson's, Alzheimer's, Huntington's, Amyotrophic lateral sclerosis) whose nature depends on the affected region and neuron types, which are quite specific to each disease. Possible aspects including mutated genes and environmental factors consists toxicants, solvents, metals, pesticides, etc leads to various abnormal molecular events including mitochondrial dysfunction, axonal transport deficits, oxidative stress, calcium deregulation

,neuroinflammation, neuron–glial interactions, protein misfolding, DNA damage, aberrant RNA processing which concludes neurodegeneration. Other possible causes include gender, poor education, endocrine conditions, stroke, hypertension, diabetes, smoking, head trauma, depression, infection, tumors, vitamin deficiencies, immune and metabolic conditions, and chemical exposure. Among all the possible factors, accumulation of unfolded or misfolded proteins in brain has been reported to be a major mechanism that triggers neurodegeneration. Because the pathogenesis of many of these diseases remains unknown, the role of environmental factors needs to be considered. Among the major NDDs, Parkinson's disease (PD), Alzheimer's disease (AD), Amyotrophic lateral sclerosis and Huntington's disease are well known in affecting large number of population worldwide.

2.3.1 Alzheimer's Disease

Alzheimer disease (AD) ranked the first, NDD, characterized by neuronal loss and inflammation, and represents the most frequent form of dementia found in elderly people affecting learning, language, memory and behaviour (46). Studies have established that some mutations in genes of amyloid precursor protein (APP) and Presenilins can also cause early onset of AD. Later, the accumulation of these abnormal protein aggregates in the form of plaques and tangles in brain occurs which begin 10–20 years prior to significant cognitive and behavioural symptoms is a remarkable feature of AD (47). Amyloid-β (Aβ) results from the sequential cleavage of the amyloid precursor protein (APP) by β-site APP-cleaving enzyme 1(BACE1) and the γ-secretase complex (48). The exact pathological mechanisms underlying or

causing AD are currently unknown and unrevealed. Various clinical and research evidences shown that abnormal or aberrant regulation of miRNA-dependent gene expression is closely linked with molecular events responsible for Aβ production, neuroinflammatory tangles (NFT) formation, and neuronal cell death.

Distinguished and prominent role of miRNAs in development and progression of AD have been confirmed in various studies. In 2007, Lukiw *et al* firstly provided an evidence for the role of miRNAs in AD pathology (49). Regulation of individual miR-132 or miR-132/212 cluster in AD pathogenesis has been reported in many studies makes it an essential player (50-54). Deficiency of miR-132/212 has been investigated in mice which impairs the normal tau mechanisms and leads towards its aggregation (50). In addition, recent study defined the mechanism behind the association of cluster miR-132/212 and AD pathology. They revealed that loss of miR-132/212 increases the level of nitric oxide, followed by disturbing the S-nitrosylation (SNO) balance and induces tau pathology, may contribute to the pathogenesis of AD and other tauopathies (53). Recently, studies were carried out on human cortical brain tissues and examined the consistent down-regulation of miR-132/212 cluster and implicated its crucial role towards AD pathology (52). Interestingly, studies of Hebert *et al*. (2008) reported the crucial role of miR-29 in the development of AD. Using neuronal cellular models, they have shown that expression of miR-29 is inversely correlated with BACE1 expression which results in Aβ production abnormally (55). Recent study suggested the role of miR-29a as a biomarker for AD used as a cell free CSF (56). Studies provide an insight into AD pathological mechanisms and revealed miR-34 family as a therapeutic approach

(57,58). Using transgenic AD model and AD patients, down-regulation of miR-34c was identified whose inhibition upregulates the VAMP2 expression and salvaged synaptic failure as well as learning and memory deficits caused by Aβ toxicity (57). Sarkar *et al* (2016) identified the role of miR-34a in targeting genes associated to synaptic plasticity and energy metabolism in case of AD brains (58). Recent studies of Li *et al*. (2016) have shown that miR-302 regulate the neurotoxicity induced by amyloid-β through Akt pathway regulation (59). In 2016, Kim *et al* identified the crucial role of miR-186 in AD pathogenesis. They reported that inhibition of miR-186 suppresses the BACE1 expression in aged brain, which is the prominent risk of causing AD (60). In SH-SY5Y cells, up-regulation of miR-146a, a known inflammatory regulator miRNA, is reported which inhibits LRP2 expression followed by increasing cellular apoptosis and suggested its major role in regulating AKL/LRP2 pathway in AD (61). Recently, studies of Li *et al* (2016) identified the expression of miR-613 in AD by regulating BDNF (62). Using mouse model, protective role of miR-200c was identified in AD pathology induced by ER stress (63).

2.3.2: Parkinson disease

Parkinson's disease (PD) is a progressive NDD, affects a large number of people worldwide characterized by bradykinesia, rigidity, tremor, shaking followed by degeneration of dopaminergic neurons in the substantial nigra pars compacta. However, as the disease develops, the pathology spreads to affect other regions of brain like amygdala, higher cortical regions and cingulate gyrus, which finally results to dementia and psychosis. Two important genes which are known to associated with

PD are SNCA and LRRK2. SNCA (Alpha synuclein) are localized in the presynaptic terminals in association with plasma membrane and its expression is widely present in the adult brain, particularly the neocortex, hippocampus, and substantia nigra (64). LRRK2 is a member of the leucine-rich repeat kinase family and is present mainly in the cytoplasm, but also associated or linked with the mitochondrial outer membrane. It is largely expressed in the brain, with the highest levels of expression in the hippocampus and striatum (65,66). Though PD is a multigenic and fatal disease but various efforts have been made in the direction of the therapeutic approach using miRNAs, and the results are encouraging, even if far from clinical implementation. Recent studies of Yang *et al* (2016) exemplify that overexpression of miR-22 shown a neuroprotective effects on the 6-OHDA (a known neurotoxicant, PD inducer) induced PC12 cells by targeting TRPM7 (67). Further, studies have identified down-regulation of miR-144-3p in MPTP induced SH-SY5Y cells while its up-regulation increased the targeted genes involved in maintaining mitochondrial function, including peroxisome proliferator-activated receptor γ coactivator-1α (PGC-1α), nuclear respiratory factor 1 (NRF-1) and mitochondrial transcription factor A (TFAM). Its overexpression also inhibits its target gene β-Amyloid Precursor Protein (68). Neuroinflammation in case of neurodegenerative diseases acts as a marker for diagnosing the effects of neuronal damage. Recent study identified the crucial role and regulation of miR-155 on mediating the inflammatory response in PD (69). Studies have also demonstrated ectopic expression of miR-7, reduced level of SNCA significantly in cultured neuronal cells, whereas knocking down of miR-7 increased the expression of SNCA significantly (70). Another miRNA, miR-153 has been predicted to target and bind to

3'UTR of SNCA in between 462–468 bases, which is a conserved sequence among all the vertebrate species. MiR-153 is highly expressed in brain, and reported to regulate expression of SNCA negatively (71). In 3'UTR region of SNCA, multiplicity of miRNAs seed sequences has been observed, therefore miR-7 and miR-153 have shown an additive effect in the regulation of SNCA level (70). Significant studies of Chaudhuri *et al* (2016) reported the contribution of miR-7 in mitochondrial dysfunction which is one of the contributor of PD (72). They identified VDAC1 as a target of miR-7, and suggested to be a novel biomarker which protects neuronal cells undergoes mitochondrial abnormality (72). MiRNA profiling carried out in PD and control tissues of brain reported the alterations in various miRNAs including MiR-198, -135b, -485-5p, and -548d were considered the best candidates implicated their role in molecular mechanisms of PD (73). Significant role of miRNAs has also been demonstrated by profiling the expression pattern in PD patients. By profiling expression of miRNAs in PD patients, it has been identified that level of miR-133b has significantly deregulated (74). Moreover, it has also been shown that a feedback loop mechanism takes place between Pitx3 and miR-133b, which represents a link between miRNAs and neurodegenerative disorders (74). Nevertheless, miR-133b has been reported as a negative regulator of differentiation of dopaminergic neurons, as its ectopic over expression in primary rat embryonic midbrain cultures resulted in decreased number of dopaminergic neurons, whereas its knockdown has shown the opposite effect (74). Furthermore, another miRNAs expression profiling study in PD brains has shown the significant decrease in the expression of miR-34b and miR-34c in brain regions including amygdala, frontal cortex, substantia nigra and cerebellum

(75). Additionally, *in-vitro* studies were also suggested the role of miR-34b and miR-34c in pathogenesis of PD. Depletion of miR-34b or miR-34c was observed in differentiated SH-SY5Y cells that results in cell viability reduction as well as cellular mitochondrial function abnormality (75). Later, studies were also revealed that down-regulation or inhibition of miR-34b and miR-34c enhances α-synuclein expression in PD (76).

2.3.3: Huntington's disease

Huntington's disease (HD) is an autosomal dominant neurodegenerative disease caused due to increase in CAG repeats beyond 36 at the exon1 of the gene huntingtin (HTT). The symptoms behind HD include mental disorders, psychopathological dysfunctions as well as motor and cognitive abnormalities. It is also characterized by the loss of long projections of neurons and synapse in the cortex and striatum region of brain. Among the various dysfunctions in biological processes in case of HD, transcription deregulation due to abnormalities in actions of transcription factors has been considered to be one of the important pathological conditions. Several pathways are targeted and interacted by miRNAs during the development of HD and its progression. Numerous studies have been cited till date about the role and regulation of miRNAs in HD. Studies of Fu MH *et al* (2015) revealed the therapeutic potential of miR-196a in HD using bioinformatics approach (77) which was previously reported as a terminator of pathological phenotypes of HD in different models (78). Recently, miR-196a has emerged as a key player in HD pathogenesis that may help to understand the regulatory mechanism behind HD as it ameliorates cytotoxicity and cellular phenotype in

transgenic huntington's disease monkey neural cells (79). In recent times, the protective role of miR-22 has been identified in HD where its decreased expression suggests its anti-apoptotic and anti-neurodegenerative role in AD also. Moreover, miR-22 targets 3ÚTR of histone deacetylase 4, REST corepressor 1 and Rgs2, three major genes as implicated in the development of HD (80). Ectopic expression of miR-22 has shown to inhibit Htt171-82Q, a fragment of mutated huntingtin, mediated degeneration in cortical and striatal neurons (80). Another miRNA, miR-214 has been reported in HD cell model which targets mitofusin2 (MFN2), where it's up-regulation altered mitochondrial morphology and deregulated cell cycle. Later, it was suggested that its inhibition could be a possible target of intervention in HD pathogenesis (80). One of the brain specific miRNA, miR-124 has been investigated in lowering the progression of HD by inducing neurogenesis in the striatum of brain (81). Compiled studies of next generation sequencing profiling carried out on human HD and control brain tissues identified various miRNAs linked to neuronal differentiation, neurite outgrowth, cell death and survival. Out of which miR-10b-5p and miR-30a-5p were informed to target BDNF which is known to associated with neuronal functions (82). Furthermore, regulation of miR-10b-5p expression in HD pathogenicity was reported in context with age as well as onset and extent of striatal association (83). This miR-10b-5p can be considered as a biomarker for the detection and progression of HD (83). MiR-34b is also reported as putative plasma based biomarker for HD as miR-34b significantly elevated mutated huntingtin (mHTT) cultured neurons (84).

2.3.4: Amyotrophic lateral sclerosis:

Amyotrophic lateral sclerosis (ALS) is a most common and severe type of NDD leads to motor neuron disease affecting worldwide causing death within 3-5 years from its diagnosis .Mutations occurring in almost twenty genes associated with familial forms of ALS have provided insights into the mechanisms proceeds to motor neuron death. ALS is characterized by loss of motor neurons, muscle atrophy, deterioration of target muscles, and results paralysis. ALS occurs under 2 forms, either familial ALS (FALS) (~10% of cases) or sporadic ALS (SALS) (~90% of cases). Indeed, it affects a large number of people but due to its little understanding of mechanisms there is presently no cure has explored. Regulation of miRNAs during the development of ALS is well accepted and recent studies provide an inclusive support for the role of miRNAs in the disease development. Report of Williams *et al*. (2009) reported the key role of miR-206 in the development of ALS in a mouse model. They reported that mice which were genetically deficient for miR-206 formed normal neuromuscular synapses during development, while miR-206 deficiency in the ALS mouse model increases the disease progression (85). Studies of Koval *et al*. (2013) revealed the regulation of miRNAs in rodent model of ALS and reported significant alteration in 12 miRNAs. Out of 12 identified miRNAs (miR-17, miR-19b, miR-20a, miR-24-2, miR-106, miR-142-3p, miR-142-5p, miR-146a, miR-146b, miR-155, miR-223 and miR-338-3p) six were tested and confirmed in human ALS tissues. Specifically, miR-155 was up-regulated 2-fold in human as well as 5-folds in mice spinal cords samples. Moreover, injection of anti-miR-155 extended survival by 10 days and disease duration by 15 days in SOD1G93A mice (38%) (86,87). Recent studies of De Andrade *et al* (2016)

identified 11 miRNAs differentially expressed in skeletal muscle and plasma of patients with ALS and identified MiR-424 and miR-206 are potential prognostic markers for ALS. Freischmidt A *et al* identified miR-1234-3p and miR-1825 in sera of sporadic ALS patients and reported as a signature role in ALS development (88). Research is going on to identify novel miRNAs which will behaves like a prognostic as well as diagnostic biomarkers for these NDDs.

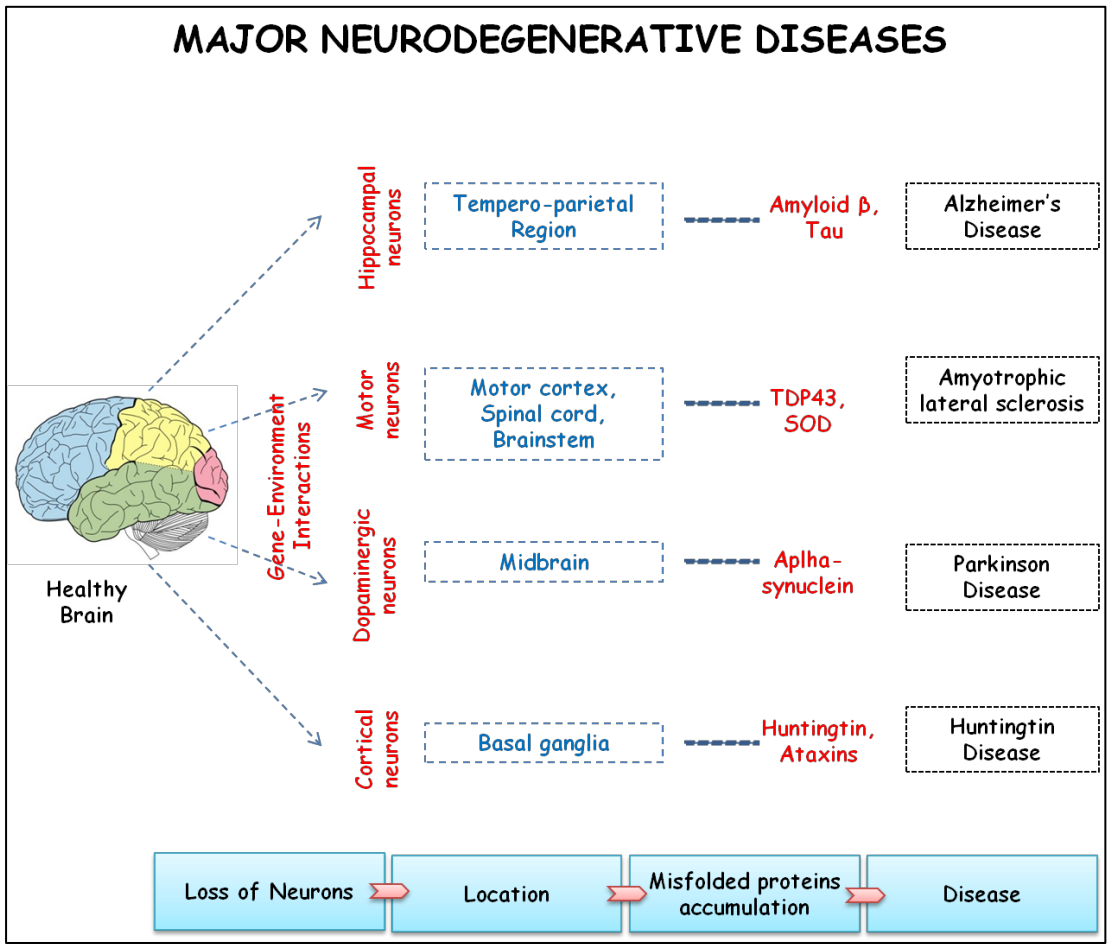


Fig 2.3: Major neurodegenerative diseases (NDDs)

2.4 MiRNAs and Neurotoxicity

The word 'Neurotoxicity' refers to an abnormal activity of the brain in response to toxic agents, whether they are natural/man made. Neurotoxicity can result from exposure to substances such as metals, solvents, pesticides, etc., which leads to adverse effects on the nervous system (89). Unfavourable changes in structure/function of nervous system due to any neurotoxicant profounds consequences of neurological, behavioural and related body functions and processes. It is extensively believed that environmental constituent such as metals, pollutants, pesticides and somewhere lifestyle plays a crucial role in brain health either direct or in an indirect manner (90). There are various studies which have proven that environmental chemicals and toxicants induced changes in miRNA expression.

2.4.1 Metals induced neurotoxicity and the role of miRNAs

Nowadays, metals are emerged as environmental contaminants. Numerous metals are vital in certain physiological processes while some of the environmental metals have been associated with a vast number of diseases, such as cancer, cardiovascular diseases, and neurological disorders. The use of metals increases the rate of oxidative stress as well as various immune disorders (89). It is widely accepted that the aetiological role of metals depends on the genetic background and the amount along with the duration of exposure. As the role of miRNAs in all aspects of biological processes has been reported so far, evidence is increasing day by day that certainly suggest an important role of miRNAs in toxicology, which could provide a relation between environmental influences and gene expression (91). Various studies have also

linked altered miRNA expression with exposure to metals including lead, cadmium, aluminium which results in a number of diseases like cancer, metabolic disorders and NDDs. Lead, a known developmental neurotoxin, damages the developing CNS, by disturbing the functional blood brain barrier leads to apoptosis (programmed cell death), affecting neurotransmitter storage and release and varying neurotransmitter receptors, mitochondria, second messengers and both astroglia and oligodendroglia (92). Lead enhances the lipid peroxidation, mitochondrial dysfunction, oxidative stress which increases the rate of neuronal cell death resulting NDDs. As a result of toxic exposure, alterations in gene expression along with varied regulation of miRNAs were found in many cellular as well as in animal models of lead neurotoxicity (93). Studies of Jun An *et al*, 2014 reported various miRNAs (miR-204, miR-211, miR-448, miR-449a, miR-34b, and miR-34c, miR-494) in chronic lead exposed rat hippocampus. These miRNAs are known to play a key role in neural injury and neurodegeneration, axonal and synaptic functions, neural development and regeneration (94). Recent study shed light on miR-203, which acts as a potential microRNA in ameliorating blood–cerebrospinal fluid barrier (BCB) function by mediating Tricellin protein. Tricellin (TRIC) protein is expressed in choroidal epithelial cells, which gets reduced by lead exposure. Another neurotoxicant, methylmercurychloride (MeHgCl) widely used as fungicide, causes adverse physiological and developmental processes (95). Study of Pallocca *et al* 2013, evaluated the role of miRNA as a useful tool for *in vitro* developmental neurotoxicity (DNT) using methyl mercury chloride (MeHgCl) (95). They exposed neuronal cultures to MeHgCl and identified various miRNAs (miR-302b, miR-367, miR-372,

miR-196b and miR-141) that are known to be involved in the regulation of developmental processes or mechanisms and cellular stress response mechanisms. They also investigated the possible effect of MeHgCl exposure on signaling pathways involved in axon guidance and learning and memory processes in the brain (96). Using Human *In Vitro* Model for Neural Development, S. Nerini-Molteni *et al* in 2012, employing miRNA-profiling for Pathway Analysis. Using differentiated pluoripotent stem cells as a model, exposed with MeHgCl, alteration of 12 miRNAs were found including miR-15a, miR-526b*, miR-488, miR-429, miR-146a, miR-183, miR-339-5p, miR-9*, miR-15a*, miR-185, miR-572, miR-378*. Fascinatingly, they also suggest a possible connection between MeHgCl and the ubiquitin-proteasome protein degradation pathway which is a remarkable feature of NDDs. It is accepted that any imbalance between metal-ion homeostasis directly leads to pathogenesis of NDDs including AD (97). Studies of Wang *et al* in 2013, reported deregulation of various miRNAs (miR-203a, miR-199*, miR-16a, miR-16c, and miR-25) due to copper toxicity in olfactory system of zebrafish. This study also reported that numerous miRNAs (let-7, miR-7a, miR-128, and miR-138) which takes part in neurogenesis gets altered due to copper mediated toxicity. A number of studies have also been accounted regarding aluminum and iron sulphates in neurotoxicity by damaging cultured human brain cells. Even a nanomolar concentration of these sulphates affects brain by inducing ROS which results in cellular apoptosis. Expression of miRNAs (miR-9, miR-125b and miR-128) gets up-regulated in Al/Fe sulphates mediated toxicity in cultured neuronal cells. These miRNAs have also been accounted and up regulated in AD which shown a relation between these metal

sulphates and appearance of NDDs (49). Growing evidences has verified that various environmental chemicals induce neurotoxicity and alters miRNA expression but lots of studies are required in relation to gene expression as well as pathways.

2.4.2 Pesticides induced neurotoxicity and role of miRNAs

There is a rising alarm regarding the extensive use of pesticides and their probable impacts on public health. Pesticides are substances widely used for preventing, destroying, repelling or mitigating pests, insects, rodents, weeds, and a host of other unwanted organisms (98). These pesticides are organophosphates, carbamates, pyrethroids and organochlorine in nature which directly/indirectly target nervous tissue and damages CNS. Various studies have been reported regarding mechanism of these pesticides, insecticides, herbicides which are commonly used worldwide including rotenone, cypermethrin, MPTP, paraquat and maneb, 6-OHDA, etc. Most of these destroy neuronal cells via mitochondrial dysfunction, oxidative stress, imbalance in intracellular calcium balance and endoplasmic reticulum (ER) stress. In 1984, hypothesis came that pesticide exposures may be related to PD development, a second major NDD (99). It was taken into consideration after the report, that intravenous injection of 1-methyl l-4phenyl-1, 2, 3, 6-tetrahydropyridine (MPTP), a byproduct in the synthesis of heroin (morphine), developed a PD like syndrome includes selectively damage dopaminergic neurons in the substantia nigra in brain. Since then, environmental factors/contaminants with parallel and similar toxicological profiles have recieved consideration and expected as potential risk factors for NDDs. In 2009, Junn *et al* reported the down regulation of miR-7 in MPTP-induced cultured cells as a

neurotoxin model by increasing alpha-synuclein expression (100). They also provide a mechanism by which the levels of alpha-synuclein are regulated in neurons, which is a pathological feature in case of PD. They too suggested the possibility of miR-7 as a therapeutic target for PD and other alpha-synucleinopathies. Alpha Synuclein (SNCA) is the primary structural component of Lewy bodies, suggesting that its abnormal accumulation results in sporadic PD. Another study of Choi *et al* in 2014, provide a novel mechanism in which they found miR-7 targets RelA and provide a shield against 1-Methyl-4-Phenylpyridinium-induced cell death and suggest miR-7 as a therapeutic target for PD (101). Kanagaraj N *et al* in 2014 firstly reported the role of the brain-abundant miR-124 in PD. They have shown that downregulation of miR-124 in two PD models including, MPTP-exposed mouse model and MPP iodide-treated MN9D cells amends the expression of proteins in calpain/cdk5 pathway (102). Recently, Wang *et al* indicated the role of brain enriched miRNA in relation to PD. They elucidated that miR-124 regulates apoptosis and autophagy in MPTP model of PD by targeting BIM (Pro-apoptotic marker) using SH-SY5Y cells as *in vitro* model (103). Further, Kim *et al* in 2014 reported the contribution of miR-126 in Parkinson's disease as it dysregulates the insulin-like growth factor/phosphoinositide 3-kinase signalling (104). Study also revealed that blockage to miR-126 function, increased IGF-1 trophism and providing neuroprotection to 6-OHDA neurotoxicity. 6-OHDA, another neurotoxicant results in a massive destruction of nigrostriatal dopaminergic neurons, and a model which is mainly used to investigate motor and biochemical dysfunctions in PD. Impairment in redox balance by paraquat (PQ) is well recognized but the role and regulation of miRNAs have not been more elucidated. Paraquat is

known to inhibit complex-I of electron transport chain (ETC) in mitochondria, induces ROS followed by cell death. Paraquat is alone or in combination of maneb (MB), a fungicide generates NDDs like symptoms when exposed to *in vivo* models. Studies of Zhou *et al* in 2013, implicated that miR-195 targets ADP-ribosylation factor-like protein 2(ARL2) and provokes apoptosis in human neural progenitor cells (NPCs) derived from embryonic stem cells (105). They worked on paraquat, and found that it induced cell death through apoptosis pathway along with an increment in miR-195 while a reduction in ARL2 expression in hESC-NPCs, demonstrating the probable involvement of miR-195 and ARL2 in paraquat-induced NPC apoptosis. Studies of Narasimhan *et al* supported that overexpression of miRNAs majorly affects the Nrf2 (antioxidant promoting factor) and associated glutathione-redox balance in SH-SY5Y dopaminergic neurons (106). They further reported in 2014, that hydrogen peroxide responsive miRNA-153 targets Nrf2/ARE cryoprotection in paraquat induced dopaminergic neurotoxicity using SH-SY5Y as a cellular model. Their findings suggested a significant role in case of oxidant mediated miR153-Nrf2/ARE pathway interaction in paraquat neurotoxicity.

2.4.3 Exogenous stressors/factors induced neurotoxicity and role of miRNAs

With the advancement in society, lifestyle and health habits of people rising a lot of NDDs day by day. Use of drugs like alcohol, nicotine, morphine, coccaine etc. is familiar with people worldwide. Alcohol is a known teratogen which passes through placenta and easily reached to the fetus and has a potential to erupt many diseases. Alcoholism, is a complex disorder which damages the structural, functional and behavioural characteristics of the brain, but the precise molecular mechanisms

underlying ethanol addiction remains indefinable. Various findings shown that small non-coding RNAs (miRNAs) pathway and signalling contributes to complex behavioural disorders including drug addiction. Diverse reports have suggested the involvement of miRNAs in alcoholic disorders, including FASD, and other NDDs. Various studies has been done so far from our lab and elsewhere reported the neurotoxicity of ethanol and alteration in gene expression. Sathyan *et al* in 2007, investigated the role of miRNAs in fetal mouse cerebral cortex-derived culture model, where they found a down regulation of miR-21, miR-335, miR-9, and miR-153, upon exposure to ethanol (107). A pioneer study of Yuanlin Qi *et al* in 2014, recognized the role of miR-29b in ethanol-induced neuronal cell death in developing cerebellum through Sp1/Rax/PKR using mice model (108). Study of Pietrzykowski AZ *et al* in 2008, demarcated the regulation of calcium- and voltage-activated potassium channel (BK channel) whose splice variant stability provided by miR-9 underlies neuroadaptation to alcohol (109). Another study of Wang *et al* in 2008, indicated towards an important role of miRNAs in development and associated birth defects (110). They reported the up-regulation of miR-10a, miR-10b, miR-9, miR-145, miR-30a-3p and miR-152 and down-regulation of miR-200a, miR-496, miR-296, miR-30e-5p, miR-362, miR-339, miR-29c and miR-154 in prenatally ethanol exposed mice brain. These marked miRNAs play a role in fetal teratogenesis and mental retardation. It is well recognized that under chronic alcoholism, remarkable features including brain shrinkage and cognitive defects occurs due to neuronal cell death. To explore the role of miRNAs in neuronal apoptosis, we published a study in 2011, identified two major miRNAs including miR-497 which mediates through BCL2 (mitochondrial

pathway) and the other one is miR-302b which mediates through CCND2 (non-mitochondria pathway) (10). A study in 2012 by Tal TL *et al*, identified miR-9/9* and miR-153c which controls the neurobehavioral development and functions using zebrafish as a model (111). Study of Bahi and Dreyer in 2013; shed a light on the linkage between striatal miR-124a and BDNF signaling which have crucial roles in alcohol consumption (112). Report of Li, J *et al* in 2013, identified miR-382 as a critical novel gene of alcohol addiction and indicated towards its role as novel therapeutic targets for alcoholism (113). Apart from degenerative disorders, alcohol-induced neuroinflammation which is mediated and regulated by pro-inflammatory cytokines and chemokines including TNFα, MCP1,IL-1β etc. Studies of Lippai Di *et al* in 2013 have suggested that chronic alcohol-induced microRNA-155 which contributes to neuroinflammation in a TLR4-dependent manner in mice (114). An additional study on miRNAs in neuroinflammation was reported by Zhang *et al* in 2014, which investigated that miR-339-5p inhibits alcohol-induced brain inflammation via regulating NF-κB pathway (115). Recently, study of Zhang *et al* 2015, suggested that prenatal ethanol exposure amends adult hippocampal VGLUT2 expression with associateed changes in miR-467b-5p levels, promoter DNA methylation and H3K4 trimethylation (116). Another class of drugs, or psychostimulants like cocaine, morphine, nicotine persuades strong neuroadaptive changes through a surplus of gene regulatory mechanisms leading to addiction. A study in 2009 by Chandrasekar V1 and Dreyer JL has delineated a set of miRNAs (let-7d, miR-181a and miR-124) which plays a key role in cocaine-responsive plasticity genes (117). Hollander *et al* in 2010, defined the role of miR-212 (striatal miRNA) in

controlling cocaine intake through CREB signaling (118). Further, they published a report in 2010 on methyl CpG binding protein 2 (MeCP2) which play a role in controlling BDNF expression and cocaine intake through homeostatic interactions with microRNA-212. Eipper-Mains JE *et al* in 2011, identified miR-8 family, which is recognized for its role in cancer, is also highly enriched miRNA and cocaine regulated at striatal synapses and confirmed cocaine-mediated alterations in protein expression for numerous predicted synaptic target genes (119). In 2013, Zhao *et al* explained the role of miR-let-7d which has already been reported in various neurological disorders regulates the TLX/microRNA-9 cascade to manage and control neural cell fate and neurogenesis (120). MiRNAs appears as an important player in drug abuse but their expression profile in neurological disorders of cocaine abuse has not been well characterized yet. Chen *et al* in 2013 explored the changes in miRNAs expression in rat hippocampus subsequent repeated cocaine exposure and identified various miRNAs including miR-133b, miR-134, miR-181c, miR-191, miR-22, miR-26b, miR-382, miR-409-3p and miR-504 which were thought to be used as therapeutic target for drug abuse (121). Numerous studies are still undergoing to demarcate the role of miRNAs in drug abuse and addiction which causes neurological disorders. There is a well established relationship between alcohol consumption and tobacco use and more studies are undergoing to understand the mechanistic pathways between them. Nicotine mediated neurotoxicity is well established and suggested that chronic use of nicotine leads to addiction, which alters neuronal functions and behaviors. Study of Huang and Li in 2009, describes the process where nicotine regulates Dynamin1 expression via miR-140* (122). It has been reported by Lippi *et al* in 2011 that

exposure to nicotine or cocaine upregulates the expression of miR-29a/b in multiple brain regions as well as in primary hippocampal neurons using mouse model (123). A set of opoid drugs, such as morphine used as analgesics all over the world. Chronic use of these drugs results in unwanted effects such as drug tolerance, opoid dependence and addiction as well as reduces the size of the dopaminergic neurons. Study of Sanchez-Simon FM *et al* in 2010, demonstrated that morphine regulates miR-133b:Pitx3 duo to increase Pitx3 expression in immature hippocampal neurons, therefore promoting neurotoxicity in neuronal differentiation using zebrafish as a model (124).

2.5 Revealing the connection between brain aging and neurodegenerative diseases

Ageing is a complex biological process that is an essential aspect of every living individual. Steady or regular loss of function in multiple organs is positively related with time. Apart from organs, stem cells, which are believed to replace damaged cells within the tissues and organs, long-term or chronic exposure to any harmful environmental factors or toxicants results in deleterious or damaged cells or implicate towards senescence or ageing. So it is believed that any disturbance between homeostatic stability among the replacement and damage mechanism, organ dysfunctions occur which is a major characteristic of ageing. As ageing continues the prevalence of chronic conditions such as neurological disorders and cancer rises within individuals. Aging is major risk factor in NDDs, but limited information is known about cellular pathways that intercede age-associated brain degeneration. Thus,

it is more important to understand the mechanisms of ageing which will help to overcome, or attenuate, the influence of this phenomenon on individuals. The leading studies of epigenetic in case of ageing is a promising field which critically involves the mechanisms or pathways that affect gene expressions regulating the occurrence of ageing. Possible factors such as DNA methylation, histone modification and noncoding RNAs has known to contribute the broad variety of phenotypes of ageing. Among them all, major studies focus on miRNAs, and their impact on facets of organismal aging as well as cellular senescence.

Brain aging provokes various cellular and functional changes in the brain and neurons that somewhat compensated by adaptive neuroprotective processes. Precise and characteristic age-associated degenerative changes critically includes abnormal and dysfunctional axons and neurites, lower level in neurotransmitter system or network, and presence of abnormal structures called amyloid plaques around blood vessels in brain. Studies reported a decade ago that a small number of these changes are similar to changes observed in neurological diseases, with an exception of neuronal loss (125). Among the most of the neurological disorders, AD was considered the most eminent neurodegenerative disorder as dementia occurred in approx 80% of people in developed countries (126). Dementia, vascular dementia along with dementia with lewy bodies is affecting a major population in the world. Various studies reported that miRNAs play a key role in both aging and neurological disorders. It has been reported earlier that lack of Dicer, miRNA processing enzyme,is associated with cerebellar degeneration and development of ataxia *in vivo*. Recent findings explored an important role of miRNAs in age-associated neurodegeneration. Published study of

Sethi&Lukiw, 2009 identified that miR-9, miR-125b and miR-146 get increased in temporal lobes neocortexes and hippocampal regions of Alzheimer's patients (127). Earlier study of Lukiw *et al*, stated that miR-9 and miR-128 get significantly up-regulated compared to age-matched healthy adults (128). MiR-29 is one of the brain enriched miRNA and known to play critical roles in neurodevelopment, neurodegeneration as well as in aging. Study in 2008, He´bert *et al* revealed the importance of miR-29, as removal of miR-29a/b-1 cluster is associated with sporadic AD (55). This extraordinary study investigated the probable regulators of the BACE1/β-secretase which are crucial mediators in Alzheimer's. These BACE1 or Amyloid precursor protein (APP) may possibly contribute to Aβ in case of sporadic AD. MiR-29a and 29-b1 are involved in the controlling BACE1 expression are found to be down-regulated in Alzheimer's brains and shown high BACE1 levels. Further studies of He'bert *et al* identified miR-20a, miR-17-5p and miR106b as regulators of Alzheimer's Amyloid precursor protein (55,129). Along with the key role in Alzheimer's, this miR-29 cluster has important roles in the brain that relevant to aging. Decrease in miR-29 expression is found in diseased brains while increase in miR-29 expression levels in other tissues during aging and characterized the role of miR-29 in regulating cellular senescence (130). Another major miRNA known as miR-34 plays a number of roles in various processes including cancer, neuronal differentiation, neuronal apoptosis, and aging as well as in neurological disorders. Published studies of miR-34 revealed that its level changes during aging in mice, human or drosophila (131,132). Recent studies have shown that various miRNAs are up- or down-regulated during mammalian aging. Study comprised the co-relation of

calorie restriction and miR-34a,a member of miR-34 family, where reduction of miR-34a , miR-30e and miR-181a levels promotes higher level of BCl2 in hippocampus and cortex region in calorie restricted aged mice (133). MiR-34c only, is abundant in hippocampal regions of brain and its expression is further up-regulated by aging as well as in AD patients (132). Limited clues suggested that the miR-34 family controls age-related metabolic pathways. MiR-34 has many targets and functions which may differ and implies towards aging process and diseases. Studies undergone in Alzheimer's patients brain expressed a downregulation of miR-107 and believed to be involved in accelerating disease progression through regulation of BACE1 (134), which further validated in neocortex of Alzheimer's disease brain by comparing the plaques counts (135). A follow-up study of Wang *et al* in 2010, identified that miR-107 has another important target progranulin which is crucial in case of neurodegeneration (136). Progranulin is reported to promote neural growth and regulate inflammatory responses (137). Interestingly, progranulin deficiency is co-relatedwith frontotemporal dementia (FTD), a foremost early onset age-dependent NDD (137). Consequently, in FTD decreased levels of miR-107 may be beneficial to restore progranulin levels. A breakthrough finding indicates that miR-29b also regulates progranulin levels negatively (138).

2.6 Known neurotoxicants which triggers neurodegeneration.

Till date, literature has provided substantial evidences that metals, pollutants, insecticides, pesticides, herbicides affects brain health either directly or indirectly. The use of pesticides is to control useless pests such as insects, fungi, weeds, and rodents.

Majority of the pesticides are commonly used but not greatly selective, which induce toxicity in nontarget species, including humans and critically affects their central nervous system. The class of pesticides includes the metallic salts, organophosphates, pyrethroids, carbonates, organochlorines, and other chemical compounds. These pesticides/ insecticides/ herbicides are well known to interfere in neurotransmission systems, whose disturbance cause neurotoxic effects that could be results lethal. Most of the pesticides are widely used worldwide including Rotenone, MPTP, Paraquat, 6-OHDA etc and share common features like inducing oxidative stress, mitochondrial dysfunction, α-synuclein fibrillization and neuronal cell loss by damaging nervous system.

2.6.1 Paraquat and maneb

Paraquat (PQ) is one of the most widely used herbicide. Though, it is banned in developed countries like USA and Europe but still being used in developing countries. Its non-selective and rapid-active nature easily damages the green tissue which comes in contact which makes it a relevant herbicide. In 1960s, PQ came into existence after the discovery of neurotoxicant, MPP+ (an active metabolite of MPTP) under the name of Cyperquat, whose structure is quite similar to PQ (139). PQ belongs to the chemical class of bipyridyl (also 2 called bipyridylium) quaternary ammonium herbicides characterized by two covalently 3 linked methylpyridine rings (140). Exposure of PQ for shorter duration leads to lung toxicity while significant damage to brain occurs after exposing to lethal dose . The cellular toxicity of PQ is associated with redox cycle, in which acceptance of electron from an appropriate donor with

subsequent reduction of O_2 to produce superoxide while generating a parent compound (141,142). A class of enzymes known as cellular diphorases play an essential role in PQ regulating redox cycle by transferring electrons from NAD(P)H to small molecules called PQ. These PQ monocation free radical is then further oxidised in the presence of O_2 producing the superoxide radicals. The generation of these free radicals cause deleterious and damaging effects to cells by inducing and promoting reactive oxygen species (ROS majorly, hydrogen peroxide ;H2O2, and hydroxyl radicals; $HO^{\cdot}$) (143). The presence of ROS in an abnormal amount leads to neuronal stress and ultimately neuronal death.

The mechanisms behind PQ neurotoxicity and neurodegeneration are not due to because of single cause, it's a multifactorial process which hampers the development of CNS. Prolong PQ exposure is associated with PD, second most neurodegenerative disease by stimulating many factors. The contribution of mitochondria in the mechanism of ROS production by PQ has explored which was firstly reported that PQ may generate ROS by accepting electrons from complex I of the respiratory chain (143). It has been demonstrated that PQ can be taken up intact, respiring brain mitochondria which represent a major cellular source in PQ induced ROS production (144). An indirect excitotoxic mechanism in response to PQ has also been proposed in literature. It has been shown that PQ stimulates glutamate efflux from neurons which results in calcium influx through non-NMDA receptor channels which activate neuronal NOS, may contribute to the formation of peroxynitrites and participate in the redox cycling process (145). Reports has suggested that PQ reduces the number of dopaminergic neurons in rat organotypic midbrain cultures in a concentration-

dependent manner, which is further prevented by inhibitors of NMDA, NOS, and caspases (145). These studies emphasizes that several mechanisms are responsible for linking PQ-induced oxidative stress with ultimate neuronal cell demise.

Studies using animal models suggested that PQ behaves as a relevant model in causing neurodegeneration. It has been shown that, dopaminergic (DAergic) neurons in the substantial nigra and striatum are more sensitive to PQ, while other subpopulations of neurons remain unaffected (146). PQ exposure alters the normal locomotion process by reducing the motor activity and striatal DAergic nerve fibers in mice after receiving booster PQ injections (147). In addition, evidence are there to support PQs status in behaving as parkinsonian toxicant where upregulation and aggregation of α-synuclein within substantial nigra pars compacta (SNPC) neurons in PQ treated mice (148).

Paraquat (PQ) along with maneb (MB), a manganese ethylene bis-dithiocarbamate represents a more relevant mixture for the aetiology of PD by altering the nigrostriatal dopaminergic system (149). In support of PQ+MB mixture, studies were carried out which indicates that exposure to PQ with or without MB induces neurodegeneration occur via an early inflammatory response in young adult animals (150). Another study reported that MB potentiates PQ neurotoxicity by a proposing a combination of divergent mechanisms, which possibly involves alkylation by MB and oxidation by PQ (151). Interesting findings indicated that exposure to pesticides during postnatal period can generates permanent and progressive lesions in the nigrostriatal dopaminergic system, which suggests that developmental exposure to neurotoxicants may be involved in the promotion of neurodegenerative disorders or altering the

normal aging process (152). Later, Effect of PQ+MB was studied on adult hippocampal neurogenesis using 2 models which suggested that this combination alters the transcriptional regulation of neurogenesis related genes. This study identified specific groups of genes that are reactive to each stressor, and revealing a novel function for Fox transcription factors in PD (153). Recently, studies demonstrated that PQ+MB mixture induces toxicity in hippocampus in early life of rats, and provide a foundation for further investigation of mechanism involved and methods that can be taken to avoid PQ+MB neurotoxicity (154).

Studies responded *in vitro* or using cellular models also supports that PQ undergoes neurotoxicity via various factors including producing ROS, lipid peroxidation, mitochondrial dysfunction, etc and leads to cell death. Evidence has supported that in PC12 cells (Rat Pheochromocytoma) PQ causes cellular lipid peroxidation, which leads to cell death and implicated in assessing the risk of PD (155). PQ induces neuronal cell death by promoting oxidative stress in differentiated SHSY-5Y cells (Human neuroblastoma) as an *in vitro* model ,but pre-exposure of water-soluble CoQ10 can prevent oxidative stress and neuronal damage induced by PQ which shed a light for preventing NDDs caused by environmental toxicants (156). Recently, studies carried out in PC12 cells identified that PQ toxicity is due to mitochondrial membrane permeability (MMP) which is a crucial process in inducing neurodegeneration. In addition PQ toxicity is through redox cycle which may serially result in increased outer mitochondrial apoptosis-induced channels (MAC) and inner mitochondrial permeability transition pores (mPTP) MMP and suggested that MMP could be implicated as a therapeutic target in treating NDDs like PD (157).

Targeted studies regarding PQ's mechanism of neurotoxicity have well-established the role of ROS and oxidative stress in a variety of systems including *in vitro* and *in vivo*. Though, with the initiation of PQ as a potential neurotoxicant, unique and specific cellular targets have been identified in the brain. Further studies are obligatory to resolve the contributions of these targets in neurodegeneration. The advantages of using PQ as a Parkinson's model come from its widespread usage as a pesticide and its utter presence in the environment. Moreover the chronic exposure provides a better representation of pathology linked with the disease, especially the presence of α-synuclein and lewy body (LB-like inclusions). Another compound which have been in use for than 50 years on crops are the dithiocarbamates and known to link with neuronal behaviour abnormalities due to disturbance in dopaminergic systems (158). Maneb (MB) belongs to the dithiocarbamate (DTC) family of fungicides is an organomanganese in nature whose chronic exposure leads to Parkinson like symptoms in humans (159). Similarly studies were reported that maneb administration leads to motor deficit in mouse models. The potent fungicidal ingredient in MB is manganese ethylene-bis-dithiocarbamate (Mn-EBDC), which is neurotoxic in nature (160,161). Assumptions has been made that maneb has an ability to cross the blood brain barrier (BBB) based on studies showing neurochemical and behavioral changes following exposure, while no specific transport mechanism has been identified (152). While Mn-EBDC is relatively stable in nature, but it may breakdown to manganese and EBDC, both of which behaves as an potential neurotoxicant. Manganese is known to cause damaging effects to brain, and its chronic exposure is linked to PD-like symptoms (162,163). It has been reported that direct injection of Mn-EBDC to lateral ventricles

in the rat brain produces selective DAergic cell degeneration and extensive striatal DA efflux, implicating a direct role of this compound in inducing NDDs. In isolated rat brain mitochondria, Mn-EBDC preferentially inhibits mitochondrial complex III, leading to ROS production and mitochondrial dysfunction (164). Furthermore, administration of nontoxic doses of maneb in primary mesencephalic cultures decreases the ATP levels, inhibit NADH linked respiration, and cause impairment of normal mitochondrial function (165). However, substantial evidence has come using animal models utilizing co-administration of DTCs such as maneb and other parkinsonian environmental toxicants including paraquat.

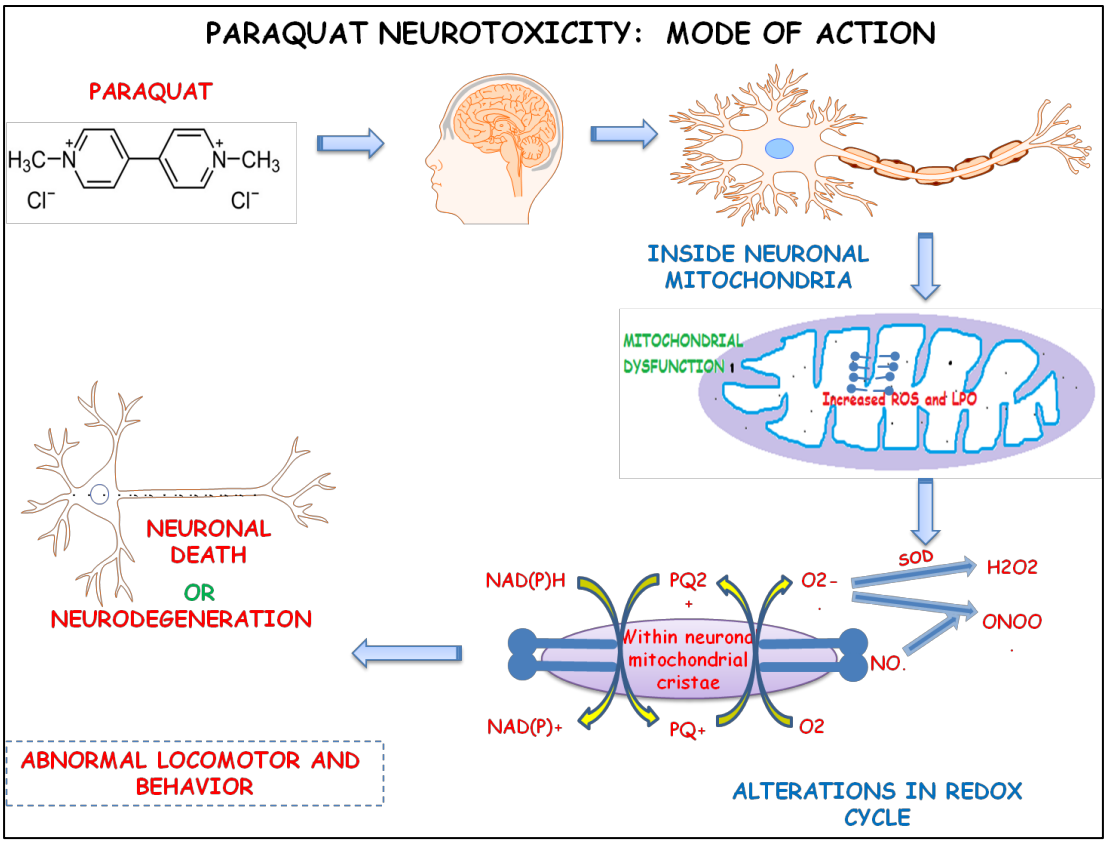


Fig 2.4: Mode of action of Paraquat

2.6.2 Sodium arsenite

In today's era, exposure of metals results in the emergence of various fatal diseases. Out of many hazardous metals, arsenic, a renowned metalloid found in environment from natural and anthropogenic sources and exists in inorganic as well as organic forms (166). Humans can be exposed to arsenic via air and food but major exposure through contaminated drinking water, especially in countries like India, Bangladesh, China, and some Central and South America (166). It has been reported that chronic arsenicosis occurs due to drinking arsenic contaminated water affects more than 200 million people worldwide, with approximately 38 million residing in the Indo-Bangladesh region (167-169). Arsenic exposure is associated with many disorders including cancer, peripheral neuropathy, peripheral vascular disorders like Blackfoot disease and alterations in CNS (170,171). Various mechanisms related to arsenic neurotoxicity were hypothesized, out of which oxidative stress and neurocytoskeletal damage are the most accepted (172,173).

Sodium arsenite, a sodium salt of arsenous acid, is an inorganic compound primarily used as pesticide (174). It's the major inorganic compound found in groundwater used widely for drinking or cooking purposes. In brief, arsenic metabolism includes methylation reactions where both arsenates and arsenites undergo methylation and producing monomethylarsonic (MMA) and dimethylarsinic acids (DMA) (175). These reactions use S-adenosyl-methionine (SAM) as a cofactor and catalyzed by methyl-transferases and release methylated arsenicals from the body (175). Post-exposure arsenic is known to induce impaired cognition, dementia and motor malfunctions

leading NDDs (176). Relevantly, to support this, various independent studies were carried out that represents inorganic arsenic may lead to neuronal impairment or death (177). Decade ago, it has been reported that arsenic crosses the placenta and reach to foetus after exposure to mother (178,179). Once access to neonate, it crosses blood brain barrier (BBB) and affects the nervous system development (180-183). Studies have been postulated that arsenite causes neural tube defects, prosencephalic hypoplasia, pharyngeal arch defects and lethality (183,184). Use of various *in vivo* and *in vitro* models helped in elucidating the mechanisms behind arsenic neurotoxicity. Studies have reported that gestational exposure of sodium arsenite decreased the body weight of newborn pups (183). They also suggested that it also affects the motor reflex, learning as well as memory functions results in apoptosis in pups (177,183). Sodium arsenite appears to have a toxic affect on neurotransmission systems. Decrease in acetylcholinestrase (AChE) in axonal fraction of spinal cord or brain regions including cerebellum, brainstem and hypothalamus were reported after sodium arsenite exposure to rats (185,186). In addition, studies were also shown that sodium arsenite exposure leads to insufficient 3,4-dihydroxyphenylacetic acid (DOPAC) in striatum and dopamine in hypothalamus regions of brain (187,188). Additionally, arsenic has been found to produce decreased levels of different neurotransmitters, such as DA, norepinephrine (NE), epinephrine (EPN), serotonin (5-HT), 3,4-dihydroxyphenylacetic acid (DOPAC) and homovanillic acid (HVA) in the corpus striatum and hippocampus of rats exposed to sodium arsenite (189). Sodium arsenite has also been reported to lowers the level of glutamate (GLU) expression levels in the cerebral cortex and hippocampus (190).

Numerous reports have elucidated that sodium arsenite induces demyelination or cytoskeletal defects in neuronal axons known to cause peripheral neuropathy which results in reducing the nerve conduction velocity (NCV) (45,191). Arsenic promotes demyelination due to reduction in basic myelin protein at the arginine residue through protein methylase I which is important post-transcriptional process in contribution to myelin integrity (192). Arsenic contribution towards neuropathy were also confirmed by increasing lipid peroxidation and reducing myelination in nerves which affects neural transmission in CNS (193). Neurocytoskeletal proteins are a bendable framework for cells that enables communication between cell parts along with their functions. Myelinated neurons comprised the nerve proteins called neurofilaments (NF-H, NF-M, NF-L). Neurofilaments regulates the axoskeleton and functionally maintain neuronal caliber. These proteins also play a role in intracellular transport to axons and dendrites and commonly used as a biomarker of neuronal damage. Studies have explored that arsenic alters the expression of neurofilaments in neuronal cells, decreases their transport to axonal points while increasing perikaryal expression (164). Studies have been investigated that transient (165). Studies of Rao *et al* (2003), identified that neurofilament middle molecular mass subunit carboxyl-terminal tail domains is crucial for the radial growth and cytoskeletal architecture of axons but not for regulating neurofilament transport rate.

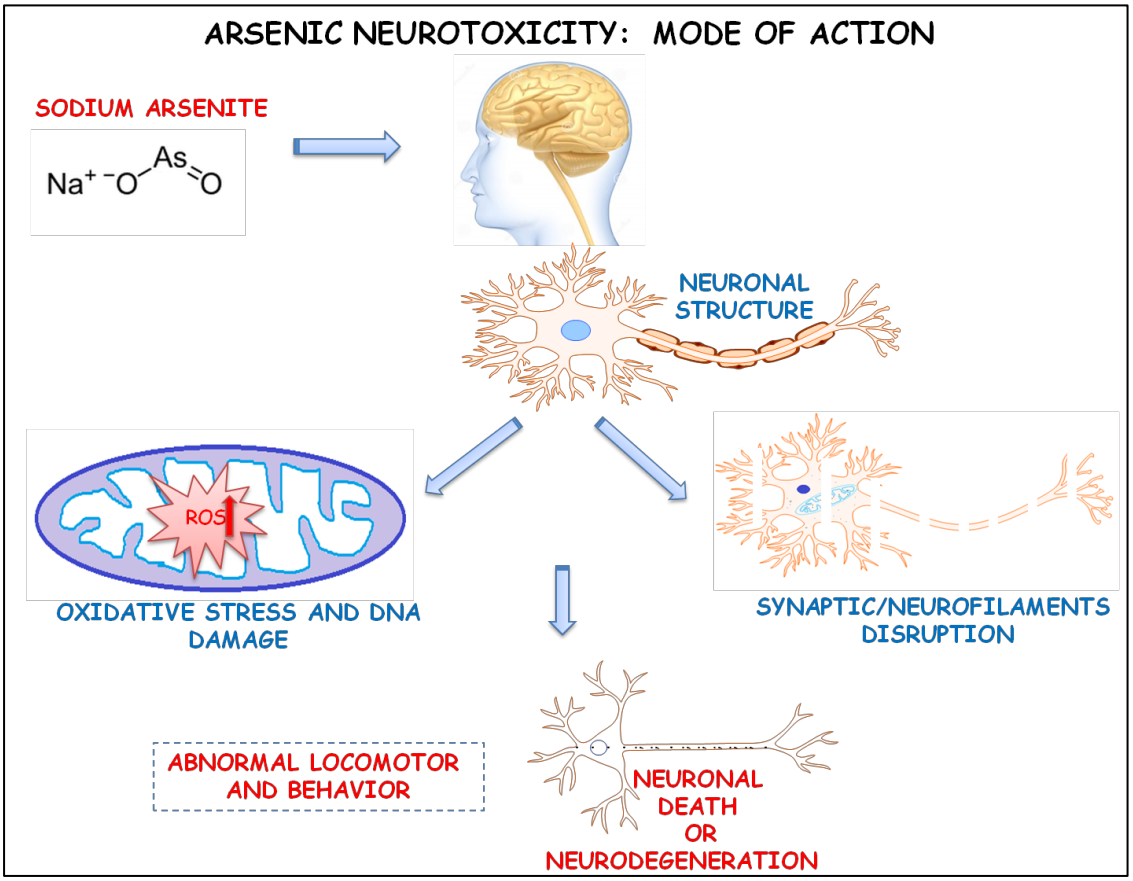


Fig 2.5: Mode of action of Sodium arsenite

2.7 Common mechanisms of Neurotoxicant-Induced Neurodegeneration in Neurodegenerative Diseases(NDDs)

Though, the cause of most of the NDDs is still unknown, but the pathogenic processes and mechanisms behind these diseases are much understood. In fact, major mechanisms are shared between almost all NDDs including blood brain barrier(BBB) disruption, protein aggregation, oxidative stress, and mitochondrial dysfunction. Neurotoxicants have a capability to either initiate or potentiate these processes, which ultimately leading to neurodegeneration.

2.7.1 Blood brain barrier (BBB) disruption

Retaining constancy of the internal biochemical environment is of major importance and requisite for brain. The function of BBB and blood-CSF barriers is to regulate transport in and out of the CNS as well as in maintaining ion concentrations in the CNS (194). Molecular movement or transportation across biological membranes (BBB) may occur through diffusion, pinocytosis, carrier-mediated transport, and transcellular transport (194,195). Specialized endothelial cells in brain capillaries are the site of BBB, forming continuous tight junctions that create the specialized barrier. Neurotoxicants that are highly lipid soluble in nature may easily move across biological membranes and thus cross the BBB by simple diffusion, gaining access to the brain. Neurotoxicants having similar chemical structures to endogenous molecules may also enter the brain by this process or mechanism. For example, the dopaminergic neurotoxicant paraquat has a similar structure to MPTP, which is highly lipophilic and able to cross biological membranes. However, In case of human body, paraquat is considered to be in a charged state, a polar molecule that is unable to cross membranes. Paraquat is thought to gain access to the brain but due to its structural similarity to amino acids, its entering become easier through a neutral amino acid carrier (196). It is certainly conceivable that BBB dysfunction as a primary pathogenic event, or in the early stages of disease, could permit both endogenous molecules and environmental toxicants to enter the brain that couldn't be possible in normal circumstances.

2.7.2 Oxidative Stress

The action of major neurotoxicants in promoting neuronal death is due to oxidative stress. Oxidative stress is due to because of many reactive species for available antioxidants, where oxidative damage is the bimolecular damage caused by attack of reactive species upon the constituents in living organisms (196). Enormous amount of studies have reported that these NDDs shown signs of oxidative damage and modifications in major biomolecules such as DNA, proteins, and lipids. Lipid peroxidation, nitrotyrosine, reactive carbonyls, and nucleic acid oxidation are increased in vulnerable neurons of AD patients compared with control, regardless of whether individual neurons contain AD pathology (197,198). Thus, signs of oxidative damage lead other pathological events in AD and are an early event iron content in the substantia nigra (which is thought to be a contributor to oxidative stress), superoxide dismutase (involved in cellular response to oxidative stress), lipid peroxidation (a marker for oxidative damage to lipids), protein oxidation, and DNA oxidation in compare to age-matched controls (199-201).

The major neurotoxicant-based models of PD, including MPTP, 6-OHDA, paraquat, and rotenone, are all thought to produce oxidative stress and ultimately lesion dopamine neurons through oxidative stress, although the primary mechanisms of action differ significantly. Paraquat is thought to affect dopamine neurons through redox cycling, and 6-OHDA likely auto-oxidizes after selective uptake into dopamine neurons through the dopamine transporter (202). Given that paraquat and rotenone are

thought to act through oxidative stress and have been identified as environmental risk factors for PD, it is very likely that oxidative stress is a common downstream mechanism by which neurotoxicants can damage the highly susceptible nigral dopamine neurons. Ultimately, any toxicant capable of entering dopamine neurons, either by diffusion or through carrier-mediated transport (commonly the dopamine transporter) and eliciting oxidative stress, could potentially be a risk factor for PD. Antioxidant therapies also represents a major research focus, even though clinical trials utilizing antioxidants have been largely unsuccessful.

2.7.3 Protein aggregation

Cytoplasmic protein aggregation is a pathological feature observed in many NDDs. Thus, the stimulation of abnormal protein processing leading to aggregation is a major mechanism by which environmental neurotoxicants are able to elicit neurodegeneration. The unique mechanistic subject about NDDs is protein aggregation and accumulation. Though each disease generates different characteristic protein and involves different neurons. In case of PD e.g., the major pathological characteristic is the presence of cytoplasmic inclusions in nigral dopaminergic neurons known as lewy bodies, in which majorly aggregated is α-synuclein (203). The critically aggregated tau protein in neurofibrillary tangles, the pathological hallmark of AD known as tautopathies (204). In case of ALS, the tendency of SOD1 to aggregate may be more important in pathophysiology than alterations in dismutase activity (205). A well-known example is the pesticide rotenone, which was the first neurotoxicant-based model to accurately reproduce the aggregation of α-synuclein

characteristic of PD (206). The rotenone model has been found to replicate and reproduce several pathways in neurodegeneration, including mitochondrial dysfunction, oxidative stress, and proteasomal dysfunction/protein aggregation (206).

2.7.4 Mitochondrial Dysfunction

Another major feature of neurodegenerative diseases is mitochondrial dysfunction. Mitochondrial dysfunction is characterized by a loss of efficiency in the mechanism of electron transport chain and reduction in the synthesis of high-energy molecules, such as ATP, is a resemblance of age-related disorders. Reports have indicated that it may be the prime event in which environmental toxicants may induce neurodegeneration and results in multiple neurodegenerative diseases. Reports have shown that mitochondrial dysfunction is most prominent and early event in case of Alzheimer's disease (AD) (207). In continuation, studies have revealed that mitochondrial accumulation of Aβ occurs in AD patients as well as in transgenic mouse models (208,209). This accumulation has been suggested that it affects mitochondrial import and membrane potential, ROS production, as well as the integrity of mitochondrial DNA (207). The occurrence of Parkinson disease (PD) is majorly due to systemic mitochondrial complex I inhibition (210,211). Two well established models of PD, MPTP and Rotenone, acts through complex I inhibition of electron transport chain in mitochondria. MPTP selectively inhibits complex-I in case of catecholamine neurons, whereas rotenone is known to inhibit activity in all cells, creating a selective lesion in nigral dopamine neurons (202). Diverse environmental toxicants has known to affect mitochondrial activity and able to produce lesions in substantial nigra which is a

characteristic feature of PD. In ALS, the role of mitochondria is quite interesting. SOD1 protein, which is found to accumulate in the intermembrane space, matrix, and outer membrane of mitochondria (212,213). Enhanced mutant SOD1 mitochondrial localization caused mitochondrial pathology which further accelerated the disease has been found in transgenic animal models (214). This aggregated SOD1 has confined to mitochondria which cause multiple alterations in mitochondrial function, including impaired respiration, redox homeostasis, depleted ATP production, initiation of apoptotic cascade and altered calcium homeostasis (214). So, the role of environmental neurotoxins in perturbing the mitochondrial mechanisms is critically occurred in case of major neurodegenerative diseases.

2.7.5 Disruption of neurocytoskeletal integrity

Neuronal cells are having unique architecture having distinct morphology with branching of axon and dendrites. These cells having specialized structures closely related to function like electrical signalling, synaptic transmission etc. To ensure that information is transmitted properly, the neuron has a unique cytoskeleton organization and contains several specialized proteins like microfilaments, microtubules and intermediate filaments. Neurofilaments (NFs) are intermediate filaments with a diameter of 10nm and found abundantly in axons. These are known for providing promising strength, growth of axons and transmission of impulse (215,216). These filaments are highly expressed in neurons and categorized into their subunits on the basis of molecular weight. These are NF-L (Light chain~60kda), NF-M (Medium chain~160kda) and NF-H (Heavy chain~205kda). In case of NDDs, alterations in

neurofilaments takes place which includes disturbed NFs synthesis, distorted axonal structure, abnormal accumulation of NFs and their proteolysis. In relation to AD and ALS, NF-L level was decreased at transcriptional level which is also reported in case of PD (217-221). Region of NEFM gene coding for the rod domain 2B of NF-M has been identified with point mutation in an individual suffering with PD (Lavedan C., Buchholtz S., Nussbaum R. L., Albin R. L. andPolymeropoulos M. H. (2002)). A mutation in the human neurofilament-medium gene in PD that suggests a role for the cytoskeleton in neuronal degeneration (57–61). It is already reported that mutations in this similar region of neurofilament proteins might cause peripheral motor nerve axonal loss in NEFL and central dopaminergic loss in NEFM (221). Additionally, In AD, abnormal accumulation of these proteins results in aggregated filaments and leads to neurofibrillary tangles and plaques which are its hallmarks. Hyperphosphorylation of NFM has also been reported in AD amyloid plaques which signifies its critical role in neurodegenerative disorders.

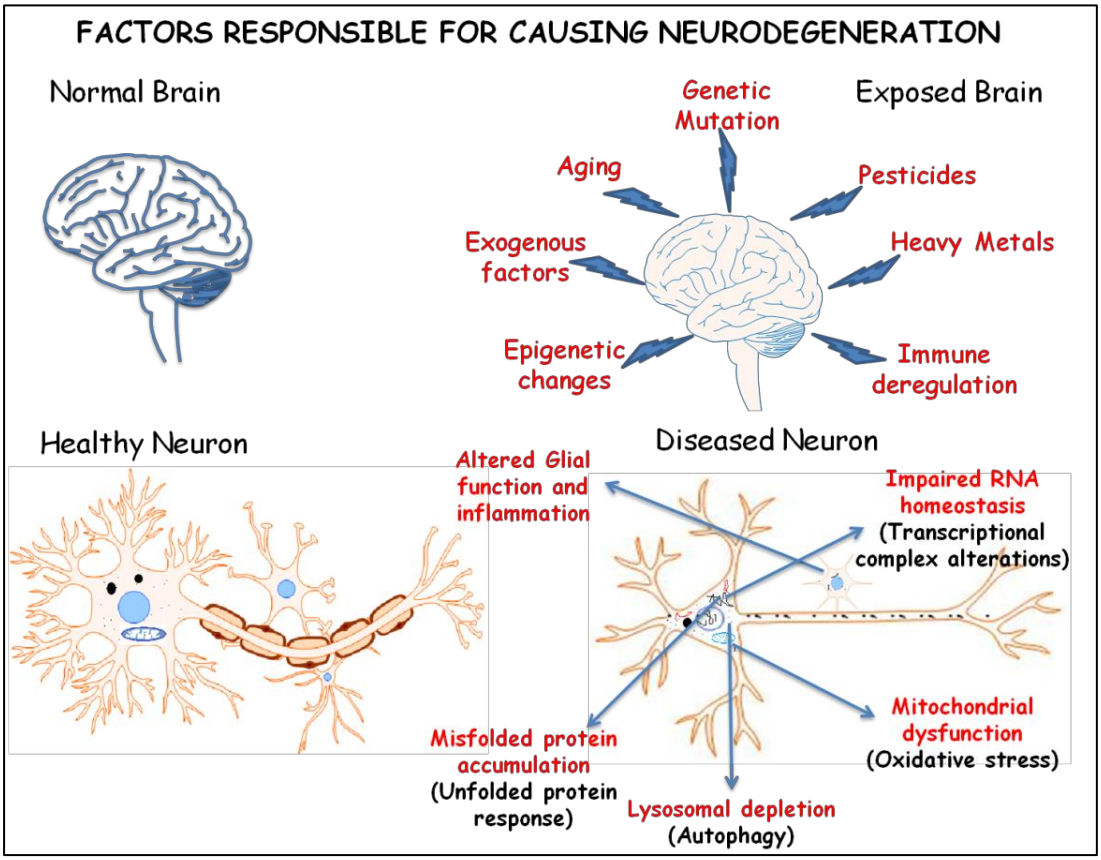
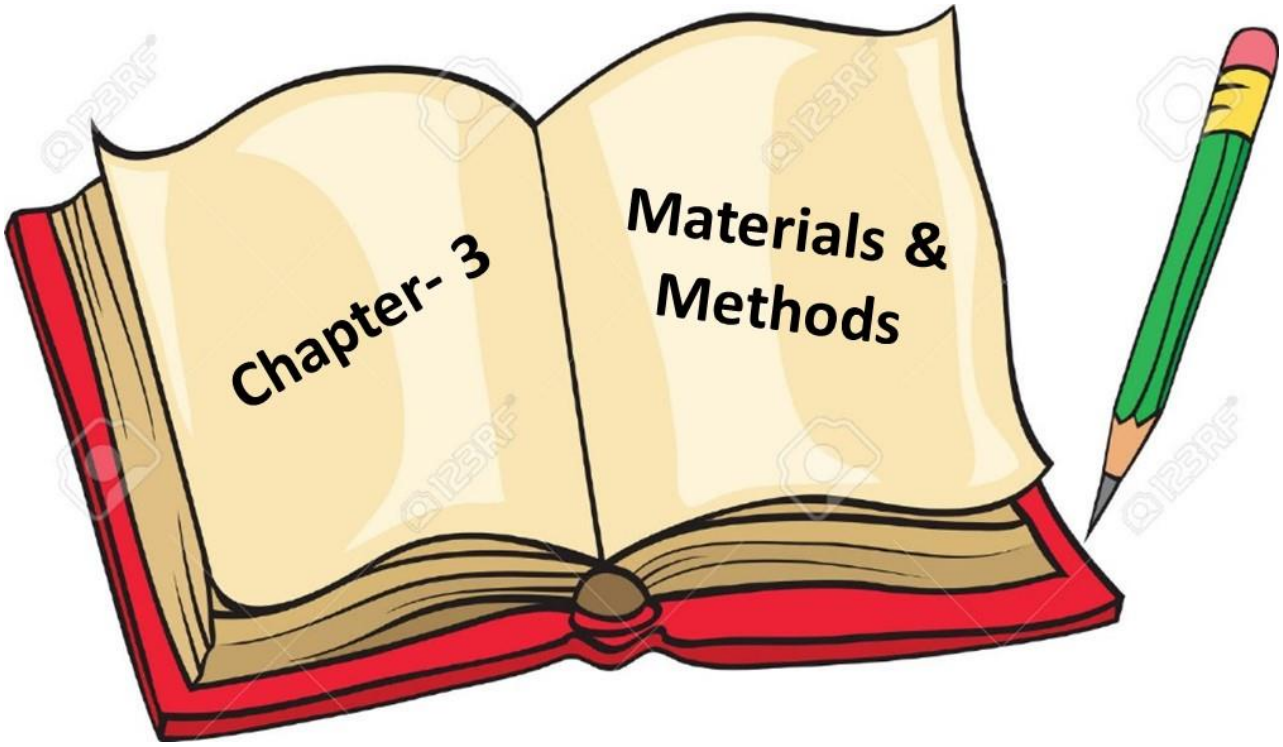


Fig 2.6: Possible factors responsible for neurodegeneration


Chapter- 3
Materials &
Methods

CHAPTER 3. MATERIAL AND METHODS

3.1 Chemicals and Reagents:

PC12 cells were procured from Sigma-Aldrich. Culture medium RPMI-1640, fetal bovine serum, horse Serum and antibiotic-antimycotic solutions were procured from Life technologies, Carlsbad, CA, USA. Paraquat (Cat#-36128), Maneb (Cat#-R8875), Sodium arsenite(S7400), were procured from Sigma-Aldrich, Saint Louis, MO, USA. Transfection reagent DharmaFECT1 (T200102), siRNA, against Dicer (ON-TARGET plus SMART pool Dicer siRNA) and Non Target Control (NTC) were procured from Dharmacon, Pittsburgh, PA, USA. Mimics and inhibitors of miRNAs were obtained from Applied Biosystems, Foster City, CA, USA. Primers for real time assay were purchased from Integrated DNA Technologies. Antibody against β-Actin (A5441) was procured from Sigma-Aldrich, Saint Louis, MO, USA. Antibodies for βIII-tubulin (ab18207), Neurofilament medium (NFL-M) (ab64300), Neurogenin 2 (NeuroG2) (ab109236), Doublecortin (DCX) (ab77450), Post synaptic density protein 95 (PSD95) (ab76115), Synaptophysin (SYP) (ab32127), and Synapsin 1 (SYN1) (ab18814) were procured from Abcam, Cambridge, MA, USA. Antibody against GAPDH was procured from Santacrutz biotechnology USA. Primary antibodies for Anti-P53 (134100) was procured from Thermo Fisher Scientific, USA. Infra Red labelled secondary antibodies were obtained from LI-COR Biosciences, Lincoln City, NE, USA. TaqMan Low-Density Array (TLDA), individual miRNA assays, reverse transcription (RT) kit, pre-amplification and Taqman universal master mixture, mirVana and other reagents including universal SYBR mix required for real-time

PCR, were procured from Applied Biosystems, Foster USA. All other regular chemicals were purchased from Sigma-Aldrich. MTT (3-(4, 5-Dimethylthiazol-2-yl)-2, 5 diphenyltetrazolium bromide, a tetrazole) was procured from SRL, India, AlamarBlue reagent was obtained from Invitrogen, USA. Apoptosis assays were performed with the FITC- Annexin V apoptosis detection kit of BD Pharmingen. For non-targeting control (NTC) transfections, ON TARGET plus control siRNA, mimic negative control and hairpin inhibitor negative control were used as per requirement. Propidium Iodide (P4170), CP-31398 (PZ0115) and CelLytic-M cell lysis reagent (C2978) were procured from Sigma-Aldrich, Saint Louis, MO, USA. Culture wares and plastic wares were procured from Nunc, Denmark and Corning, NY. Autoclaved Milli-Q water was used in all the experiments.

3.2 Animals:

Albino Wistar rats were obtained from the animal house facility of CSIR-Indian Institute of Toxicology Research, Lucknow. All the animals were maintained on a commercial pellet diet and water *ad libitum* in a temperature controlled room with a 12/12 h light/dark cycle and cared for in accordance to the policy laid down by Animal Care Committee of CSIR- Indian Institute of Toxicology Research. The animal experimentation was approved by the ethical committee of the CSIR-Indian Institute of Toxicology Research, Lucknow.

3.2.1 Animal Exposure:

Male wistar rats of 3 week, 6 week, 9 week and 12 week ages were exposed with paraquat+maneb(PQ+MB) or sodium arsenite and expression of “Brain specific miRNAs” were identified. All aged groups were administered with PQ+MB (10+30

mg/kg b.w intraparitonially) or sodium arsenite(20 mg/kg b.w orally) along with their vehicle control for 7 consecutive days. All groups were sacrificed by cervical dislocation and whole brain were dissected out and used for the study of alterations in brain specific miRNAs expression by PQ+MB or sodium arsenite as well as for immunoblotting and real time PCR studies.

3.2.2 Neurobehavioral studies:

Spontaneous locomotor activity (SLA) was performed using Actimot to study the locomotor deficits induced by paraquat+maneb or sodium arsenitein pre-adolescent (3 week), adolescent (6 week) post-adolescent (9 week) and young adult (12 week) rats. All the aged group rats (3Week, 6 Week, 9 Week and 12 Week) treated with paraquat+maneb or sodium arsenite along with their vehicle controls were monitored for spontaneous locomotor activity to measure the locomotory impairments. Six animals from each group were randomly chosen and were monitored individually in the activity monitoring cage. After acclimatization of 1minute the motor activity was recorded in terms of total distance travelled, moving time, resting time, numbers of rearings and stereotypic count for 5 min.

3.3 Cell culture, differentiation, immunocytochemistry, calcein dye and senescence studies:

3.3.1 Cell culture:

PC12 cells procured from Sigma and were grown in RPMI-1640 supplemented with 5 % fetal bovine serum, 10% horse serum, 0.2% sodium bicarbonate and 1% antibiotic and antimycotic solution and were kept in 5% CO2 - 95% atmosphere with high

humidity at 37 ^{o}C. Culture medium was replaced with fresh medium at every alternate day and cells were passaged at 70% confluency.

3.3.2 Neuronal differentiation:

Before differentiation, cells were seeded on poly-L-lysine (0.01% solution) coated surface for 10 minutes followed by washing of 3 minutes each. Whenever required, cells were counted by countess automated cell counter using Trypan blue dye. Before counting, equal volume of Trypan blue dye (0.4% w/v) was added in cells and mixture was immediately loaded to cell counting chamber slides and cells were counted and seeded for differentiation. For full differentiation, PC12 cells were exposed with 50 ng/ml nerve growth factor (NGF) for 8 days, and after every two days fresh NGF was added in differentiating PC12 cells. Cells were kept in 5% CO2 - 95% atmosphere with high humidity at 37 ^{o}C. For quantifying neuronal differentiation, neurite length and number was measured with phase contrast microscope using NisElement BR software of Nikon.

3.3.3. Immunocytochemistry studies:

For immunocytochemistry, PC12 cells were seeded in PLL coated chamber slides (20,000.00/well of 4 well slides) and exposed with NGF for five days. After washing with PBS, cells were fixed with 4% paraformaldehyde. After 20 minutes of fixation at room temperature, they were again washed with PBS and exposed to 0.5% H_2O_2 in methanol for 1 hour. After washing with PBS, cells were incubated in blocking solution containing 0.2% triton X-100 in 0.1% bovine serum albumin for 1 hour. After blocking, cells were incubated with primary antibody (1:200 dilution for 2 hours at room temperature) followed by washing with PBS and transferred to secondary

antibody (1:500 dilution for 2 hours at room temperature) labelled with Alexa fluor-488. After final washing with PBS, 4,6-diamidino-2-phenylindole (DAPI (nuclear stain)) with antifade was added on cells and images were taken using Nikon fluorescent microscope.

3.3.4 Calcein AM dye studies:

Calcein AM is a cell-permeant dye that can be used to determine neuronal cell health. In live cells, the nonfluorescent calcein AM is converted to a green-fluorescent calcein after acetoxymethyl ester hydrolysis by intracellular esterases. In brief, differentiated PC12 cells were exposed to paraquat and sodium arsenitefollowed by washing with PBS. Cells were incubated with Calcein AM dye for 30 minutes and images were taken using fluorescence microscope.

3.3.5. Senescence studies:

Cell senescence was assayed by cytochemical staining of senescence associated β-galactosidase using Senescence β-Galactosidase Staining Kit (Cat #9860) of Cell Signaling Technology, USA, as per manufacturer protocol. Briefly, cells were fixed in fixative solution provided in kit for 10-15 minute at room temperature at gentle shaking followed by 3 washes with phosphate buffer saline (PBS). After washings, cells were stained with staining solution of β-galactosidase and incubated overnight in dry CO_2 incubator at 37°C. After incubation, cells were monitored and images were captured after the development of blue colour. All the experiments were done in triplicate, and one of the representative results is shown.

3.4 Identification of non-cytotoxic dose of paraquat and sodium arsenite:

Non-cytotoxic dose of paraquat and sodium arsenite for PC12 cells were identified by exposing them to different concentrations (1-300) μM of paraquat or sodium arsenite for 72 hours(h) by MTT assay(3-(4,5-dimethylthiazol-2-yl)-2,5diphenyltetrazolium bromide, a yellow tetrazole) and Alamar Blue assays. Paraquat or sodium arsenite was dissolved in autoclaved water and cells were exposed with different concentrations of (1-300) μM of paraquat or sodium arsenite. PC12 cells (10,000 in number) were seeded in PLL pre-coated 96-well tissue culture plates and incubated for 24h prior to experiment. The medium was aspirated and different concentrations of paraquat and sodium arsenite were added in fresh medium. Further, cells were allowed to incubate and MTT and Alamar blue assay was performed at 72 hrs. In, Alamar blue assay, reagent was added in 1:10 ratio and were incubated at 37°C for 2 hours followed by incubation, fluorescence were measured at excitation of 560 nm and emission of 590nm. The values were then compared with control sets, run parallel under identical conditions without the test compound .The results were expressed in percent control with control as 100%. In case of MTT, Tetrazolium (10μl/well containing 100μl of cell suspension; 5mg/ml of stock in PBS) salt was added 4h prior to completion of incubation periods. Then, the reaction mixture was carefully taken out and l00μl of DMSO was added to each well. After 10 min, the colour was read at 550 nm using micro plate reader. The untreated control was also run simultaneously under the identical conditions & served as control (100% viability).

3.5 Flow cytometric studies:

flow cytometry studies were done using BD influx.

3.5.1 Reactive Oxygen Species (ROS) Estimation:

Reactive oxygen species (ROS) formation was measured using 2', 7'dichlorodihydrofluorescein diacetate (DCFDA), a non-fluorescent dye which is converted into highly fluorescent (2', 7'-dichlorofluorescein) molecule in presence of ROS. In brief, PC12 cells were exposed with paraquat and sodium arsenite for 72 hours and incubated at 37ºC. After incubation, cells were taken and suspended in phosphate buffer saline containing 20 µM DCFDA in the dark at 37ºC for 30min. After 30 minutes the cells were pelleted and re-suspended in PBS and analysed by flow cytometry.

3.5.2 Mitochondrial membrane potential (MMP):

Changes in MMP were studied using JC-1, a cationic dye, which shows potential dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green to red. In brief, Paraquat or sodium arsenite exposed PC12 cells were washed and incubated in PBS containing 10µM JC-1 in the dark at 37ºC for 20 minutes. After 20 min, cells were pelleted and re-suspended in PBS and analysed by flow cytometry.

3.5.3 Apoptosis assay:

Cell death or apoptosis in PC12 cells was measured by means of FITC labelled Annexin-V. Cells were exposed to paraquat and sodium arsenite for 72 hours, After completion of treatment period the cells were taken, washed twice with PBS and suspended in 100µl of binding buffer. 2µl of annexin-V was added and the cells were incubated in the dark for 15min. Subsequently, 2µl of propidium iodide (PI) was added and cell death was measured using flow cytometry .

3.5.4 Cell cycle analysis:

Cell cycle assay was performed using propidium iodide (PI) uptake method. In Brief, transfected or NGF exposed cells were harvested from the culture plate, washed thrice with phosphate buffer saline (PBS) and suspended in 70% ethanol and incubated in -20°C for 30 minutes. After fixation, cells were pelleted and washed with PBS for 3 times. Finally, cells were suspended in resuspension buffer containing RNAseA (10mg/ml) and PI (1mg/mL) for 30 minutes. After incubation cell cycle was assayed using flow cytometer.

3.6 Transfection studies:

All the transfections were performed using DharmaFECT1 transfection reagent by using following protocol. 5 μM siRNA solution was prepared in 1X siRNA dilution buffer and diluted 10 times with serum free- medium. In a separate tube DharmaFECT transfection reagent was also diluted to obtain final volume according to cells type and number in serum-free medium. The contents of both tubes were gently mixed separately by pipetting up and down and incubated at room temperature for 5 minutes. Contents of both tubes were mixed to get 20 μl final volumes and incubated at room temperature for 20 minutes. After incubation 80 μL of antibiotic-free complete medium was mixed to get final volume of 100 μL transfection medium with final siRNA concentration of 25 nM. Cell were transfected with this transfection medium and after 24 hours of transfection medium was replaced with fresh complete culture medium.

3.7: RNA isolation and Real time PCR:

Total RNA containing small RNAs were isolated by using mirVana miRNA isolation kit as described by manufacturer. Briefly, tissues or cells were homogenized in lysis

binding buffer in 1:10 ratio. A clear homogenate was formed after homogenization, in which homogenate additive solution was added in 1/10 ratio of the total volume and incubated on ice for 10 min. After the incubation in homogenate additive, equal volume of acid:phenol:chloroform mixture was mixed and tube was centrifugated at 15000 g for 30 minute. Three layers were formed, upper aqueous layer was separated and mixed with 1.25 volume of absolute ethanol. The solution was passed by columns provided with the kit and washed two times with wash solutions after that total RNA was eluted in pre heated elution buffer by centrifugation. RNA concentration was determined by spectrophotometer followed by cDNA preparation. Transcriptional changes were studied using quantitative Real Time PCR. Briefly, total RNA was isolated from both exposed and unexposed control sets using miRVana kit. Total RNA (1000 ng) was reverse transcribed into cDNA by High Capacity microRNAs reverse transcription kit of Applied Biosytems USA. Real Time PCR for expression of miRNAs was performed by using Taqman Chemistry, while SYBR Green chemistry was used for all other genes. Real time reactions were performed in triplicate for each samples. The miRNAs primers were procured from Applied biosystems. U6 was used as internal control to normalize ct values in miRNA expression assays. Expressional changes are expressed in relative quantification (RQ). The details of primers used with Sybr Green chemistry are as follows:

HPRT (Rat)

FP:-5'-GGTGAAAAGGACCTCTCGAAG-3'

RP:5'-GCTTTTCCACTTTCGCTGATG-3';

NeuroG2 (Rat)

FP:5'-TGCTCAGTTCCAATTCCACC-3'

RP:5'-AGTCACCTGCTTCTGCTTC-3';

DCX (Rat)

FP:5'-CAGTCAGCTCTCAACACCTAAG-3'

RP:5'-CATCTTTCACATGGAATCGCC-3';

βIII-tubulin (RAT)

FP:5'-GGCCTTTGGACACCTATTCAG-3'

RP:5'-TCTCACATTCTTTCCTCACGAC-3'

NFL-M (Rat)

FP: 5'-CCACAACCACGACCTCAG-3'

RP:5'-CGATGTCCAGAGCCATCTTG-3'

PSD95 (Rat)

FP:5'-CAAGAAATACCGCTACCAAGATG-3'

RP:5'-CCCTCTGTTCCATTCACCTG-3'

SYP (Rat)

FP: 5'-AGTGCCCTCAACATCGAAG-3'

RP:5'-GCCACGGTGACAAAGAATTC-3'

SYN1 (Rat)

FP:5'-CCTGTAGTTGTGAAGATGGGC-3'

RP:5'-AGTGGCATATGTCTTAGTCAGTG-3'

P53 (Rat):

FP:5'-GCACAAACACGAACCTCAAAG-3',

RP: 5'-TCCGACTATACCACTATCCACTAC-3'

3.8: MiRNAs expression profiling using "Brain Specific array":

In our earlier studies (manuscript under preparation), brain specific array has been developed which contains 96 miRNAs, which are highly regulated in developing rat brain or differentiating neurons or miRNAs which are expressed constitutively at high levels in adult rat brain. In brief, customized brain specific Taqman Low Density miRNAs Arrays (Brain specific miRNA araay), were used for brain specific miRNAs

profiling. Primers of 96 brain specific miRNAs, identified by our previous studies were present along with endogenous controls and negative control. For brain specific miRNA expression profiling in developing brain, total RNA including small RNAs from whole brain of 3week, 6 week, 9 week and 12 week exposed with sodium arsenite or paraquat+maneb or control were used along with differentiated neurons using NGF exposed PC12 cells. For TLDA, the total RNA, which also contained small RNAs were isolated using the mirVana miRNA isolation kit as described by the manufacturer. Reverse Transcription (RT) reaction was carried out using 350 ng of total RNA with customized RT primers pool which are predefined primer pools which contain stem-looped RT primers for mature miRNAs. RT was performed with the high capacity TaqMan miRNA Reverse Transcription Kit of Applied biosystems, as described by the manufacturer protocol. In brief, the reaction mixture of RT reactions, contained 1X RT primers pool, 2.6mM dNTPs with multiscribe reverse transcriptase (75 units), 1X buffer, 3mM MgCl2, 2 units of RNAse inhibitor and 350 ng total RNA with the final volume of 7.5µl. RT reactions were performed in thermal cycler at 40 cycles of 16^{o}C for 2 min, 42^{o}C for 1 min and 5^{0o}C for 1 second followed by denaturation of reaction mixture at 85^{o}C. After RT, pre-amplification was carried out by means of Taqman preamplification master mixture and preamplification primers. For preamplification, 12.5µl of preamp master mixture was mixed with 2.5µl preamp primers and 2.5 µl reverse transcription product with total volume of 25µl. Temperature conditions for thermal cycling of preamplification reaction were 10 minutes at 95^{o}C, 2 minutes at 55^{o}C, 2 minutes at 72^{o}C and 12 cycles of 15 seconds at 95^{o}C and 4 minutes at 60^{o}C. Finally, the preamp products were incubated for 10 min.

at 99.9°C. Before real time PCR, pre-amplification products were diluted by 4 folds with 0.1 X Tris-EDTA buffers. For TLDA, 450µl of the Taqman universal PCR master mixture was mixed with 9µl of the diluted pre-amplification product and make up the volume 900 µl by nuclease free water. In each port of TLDA plate, 100µl from the above mentioned reaction mixture was added in TLDA plates and after sealing and spinning, the plates were loaded and run on a thermal cycler for 2 minute at 50°C, 10 minutes for 94.5°C and 40 cycles of 97.0°C and 59.7°C for 30 seconds and 1 minute respectively. Relative quantification was done by means of -ΔΔCt method considering the levels of mammalian U6 snRNA as endogenous and control for each time point as a reference sample. TLDA plates were run in duplicates for all the samples. The detail of Brain specific miRNAs array are as follows:

miR-434-3p	miR-200c	miR-149	miR-219	miR-29a#-	miR-29b	miR-302b	miR-20a#	miR-144	miR-298	miR-1937c	let-7e
miR-125b-3p	miR-204	miR-139-5p	miR-221	miR-29b-2#	miR-338-3p	miR-339-3p	miR-20a	miR-200b	miR-490	miR-1960	let-7i#
miR-29c	miR-34a	miR-141	miR-222	miR-338-5P	miR-219-2-3p	miR-344-3p	miR-212	miR-200c	miR-499	miR-19b	miR-7b
miR-335-3p	miR-34b-3p	miR-146b#	miR-483#	miR-194	miR-29c#-	miR-708#	-miR-217	miR-153	miR-511	miR-30b	miR-9
miR-674	miR-34c	miR-150	miR-487b	miR-199a-3p	miR-343	miR-93#	miR-218	miR-24	miR-218-2#-	miR-30c	miR-7a
miR-106a	miR-374#	miR-17	miR-503	miR-200a	miR-349	miR-125b-5p	miR-363	miR-26a	miR-33a#	miR-342-5p	ath-miR-159a
miR-132	miR-384-5p	miR-182	miR-542-5p	miR-664	miR-206	miR-126-3p	miR-449b	miR-291a-3p	miR-543	miR-344	U6 snRNA
miR-200b	miR-598-5p	miR-191	miR-214#	miR-29a	miR-296-3p	miR-138	miR-136#	miR-292-3p	miR-1937b	miR-20b-3p	U-87

3.9: Cells and Tissue lysates preparation and Immunoblotting studies:

Whole brain was used to isolate protein for immunoblotting studies. Whole brains were dissected out and washed with chilled 1X PBS to remove any blood. Tissue were minced on ice and homogenized in Radio immune precipitation assay (RIPA) buffer. In general, 500 µl RIPA buffer containing appropriate amount of protease inhibitor cocktail and Dithretiol (DTT), was used for every 10 mg of tissue. Tissue homogenate was thoroughly incubated on ice for 30 minute and vortexed occasionally. After the incubation homogenate was centrifugated at 10,000 g for 20 minutes at 4°C. Clear supernatant was transferred to a fresh microfuge and pellet was discarded. Protein concentration of lysates was determined bicinchoninic acid (BCA) protein estimation assay. Samples were freezed at -80°C for longer use. For cultured cells, cells were lysed in CellLytic M cell lyses reagent of Sigma, supplemented with a protease inhibitor cocktail and DTT. For Immunoblotting, protein samples after quantification with BCA method were denatured in Laemlli buffer for 10 minute at 94ºC. Equal amount of all protein samples were subjected to SDS– PAGE (5% acrylamide stacking gel and 10 or 15 % acrylamide separating gel). After electrophoresis, proteins were transferred to a low fluorescence polyvinylidene fluoride (PVDF) membrane procured from Millipore. The membrane was blocked with 5% skimmed milk prepared in Tris Buffer Saline Tween 20 (TBST) at room temperature for 45 minutes followed by incubation in primary antibodies (1:1000 dilutions) at 4ºC overnight with gentle shaking. For detection of target proteins, InfraRed-labeled secondary antibodies (1:10000) used, and membranes were scanned using LI-COR, ODYSSEY Clx system. Densitometry for immunoblots was performed using Image J software.

3.10 In silico analysis:

TargetScan web portal (http://www.targetscan.org) was used for scanning 3'untranslated region (3'-UTR) of different genes and identification of probable miRNA sites. Biological processes targeted by miRNAs were identified using the GO process analysis tool of MetaCore platform of Thomson Reuters, London, UK, a data mining and pathway analysis platform. Potential target genes of miRNAs were identified from TargetScan portal and fetched on the GO process analysis tool, which arranges the biological processes according to their significance, showing the most significance on top of the list.

3.11: Volcano plot analysis:

Volcano plot is a kind of scatter-plot, which is used to study the alterations in large replicative data sets. Volcano plots are plotted between significance (pValue) versus fold change at Y-axes and X-axes, respectively. Volcano plots are common to identify the changes experiments such as proteomics, genomics and metabolomics, where thousands of replicative data points between two conditions. Volcano plots are used to identify most meaningful changes quickly. Volcano plots used combined statistical significance calculated from analysis of variance (ANOVA) model with the change in magnitude, which enabled visual identification data points that displayed large magnitudinal alterations, which are statistically significant. In our experiments, volcano plot of miRNA expression in developing brains are plotted between fold changes at log2 scale versus its significance value (pValue*) calculated from Student-t test. Two black vertical lines in the centre of the volcano plots represent ±2-fold

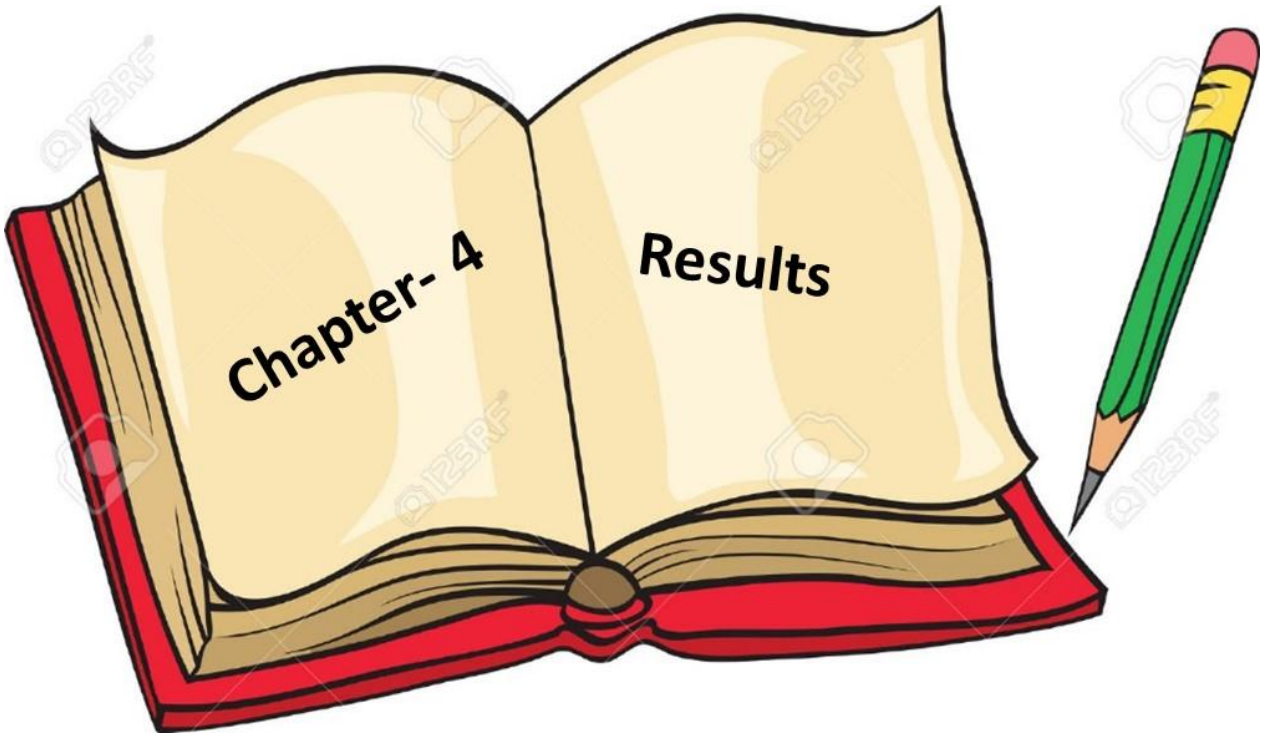
change boundary in gene expression on each side. The single horizontal blue line inside the boxes of volcano plots represents the threshold significance value (0.05).

3.12: Box plot analysis:

The box plot displays the distribution of threshold cycle (Ct) values for each sample, making it easy to view the variation in total studied transcriptome in values among biological groups. For each box in the plot: The solid box shows the range of the middle 50% of the Ct values for the target or the sample. The horizontal black bar shows the median Ct value. The black circle shows the mean Ct value. The ends of the vertical lines (or "whiskers") show the maximum and minimum Ct values, unless outliers are present. Mild outliers are displayed as open circles and represent samples or targets with Ct values up to 1.5 X the inter-quartile region (IQR). The IQR is the difference between the 3rd quartile and the 1st quartile. There is one circle for each Ct in this range. Extreme outliers are displayed as open triangles and represent samples or targets with Ct values up to 3.0 X the IQR. There is one triangle for each Ct in this range.

3.13: Statistical analysis:

All the experiments were carried out in triplicate, except TLDA arrays, which were run in duplicate. The student's t-test was employed to calculate the statistical significance. $p < 0.05$ in student's t-test was considered as significant.


Chapter- 4
Results

CHAPTER 4: RESULTS

4.1 Effect of sodium arsenite or paraquat + maneb (PQ+MB) exposure on locomotor behaviour during different stages of post-natal brain development:

Before proceeding towards identification of miRNAs, behaviour of neurotoxicants exposed developing rats was studied. Spontaneous locomotor activity test was performed to study the alterations in the locomotor behavior of pre-adolescent (3week age), adolescent, (6week age), post-adolescent (9week age) and young adult (12week age) rats exposed with sodium arsenite or PQ+MB. Spontaneous locomotor activity test has shown that sodium arsenite or PQ+MB induced significant locomotory deficit in adolescent rats (6week). However, changes were also observed in young adults rats (12week) exposed with sodium arsenite or PQ+MB when compared with their age matched control groups. However, sodium arsenite or PQ+MB did not induced any significant alterations in locomotor behaviour of pre-adolescent and post- adolescent aged rats (Fig 4.1). Spontaneous locomotor activity experiment in adolescent rats have shown that time resting is significantly increased, while total distance, rearing, time moving and stereotypic count is significantly decreased in sodium arsenite or PQ+MB exposed rats (Fig 4.1).

4.2. Effect of sodium arsenite exposure on the expression of "brain specific miRNAs" in developing rats:

Regulation of "brain specific miRNAs" by sodium arsenite in pre-adolescent, adolescent, post-adolescent and young adult aged rats was studied by "brain specific miRNA array". Considering 2 fold as minimum change with pValue*≤0.05, volcano plot analysis of "brain specific miRNAs" has shown that sodium arsenite induces

alterations (up or down regulation) in expression of 19 miRNAs (1 up-regulated and 18 down-regulated), 23 miRNAs (17 up-regulated and 6 down-regulated), 10 miRNAs (all up-regulated), 16 miRNAs (3 up-regulated and 13 down regulated) in whole brain of pre-adolescent, adolescent, post-adolescent, and young adult rats respectively (Fig.4.2).

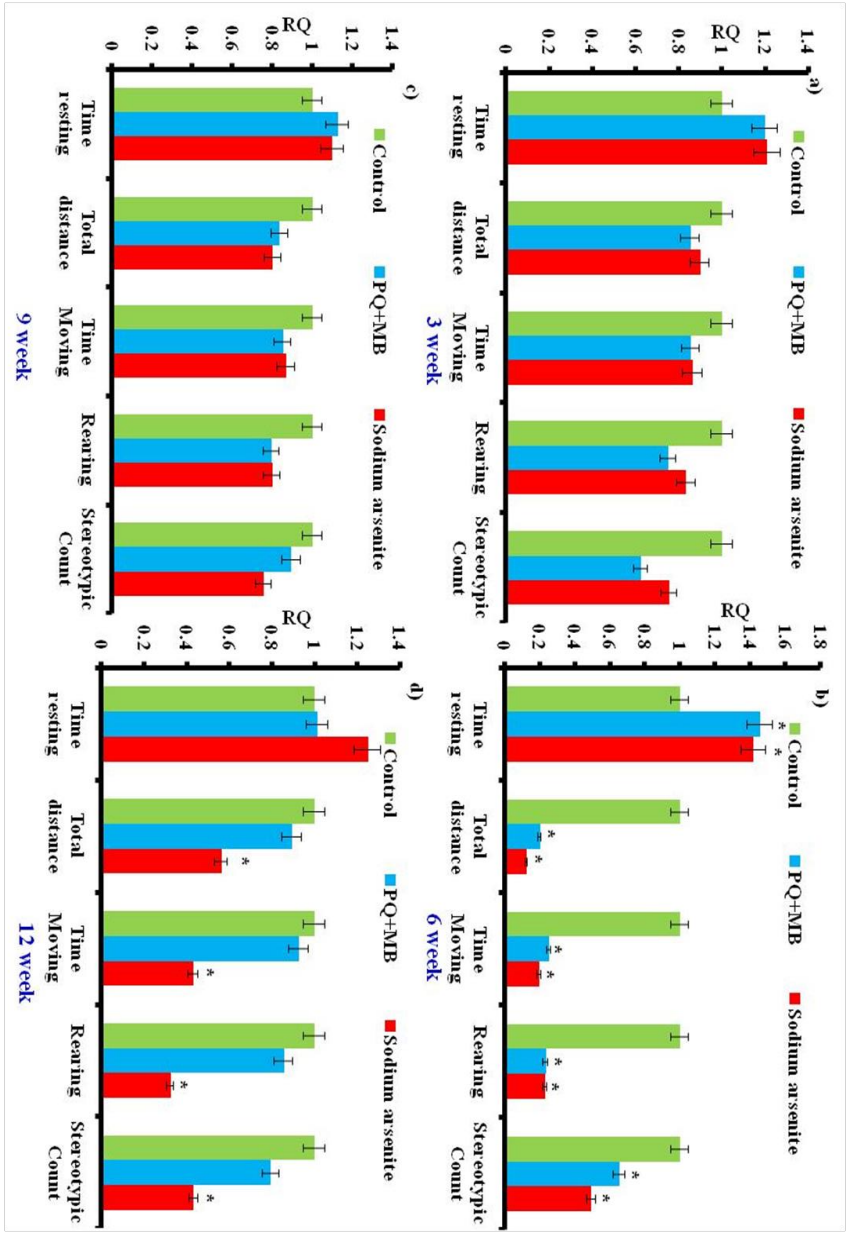


Fig 4.1: Effect of sodium arsenite or paraquat + maneb (PQ+MB) on locomotor behavior during rat brain development: Effect of sodium arsenite or PQ+ MB exposure was measured on locomotor behavior by spontaneous locomotor activity test in 3 week (a) ,6 week (b) , 9 week (c) and 12 week (d) aged rats.

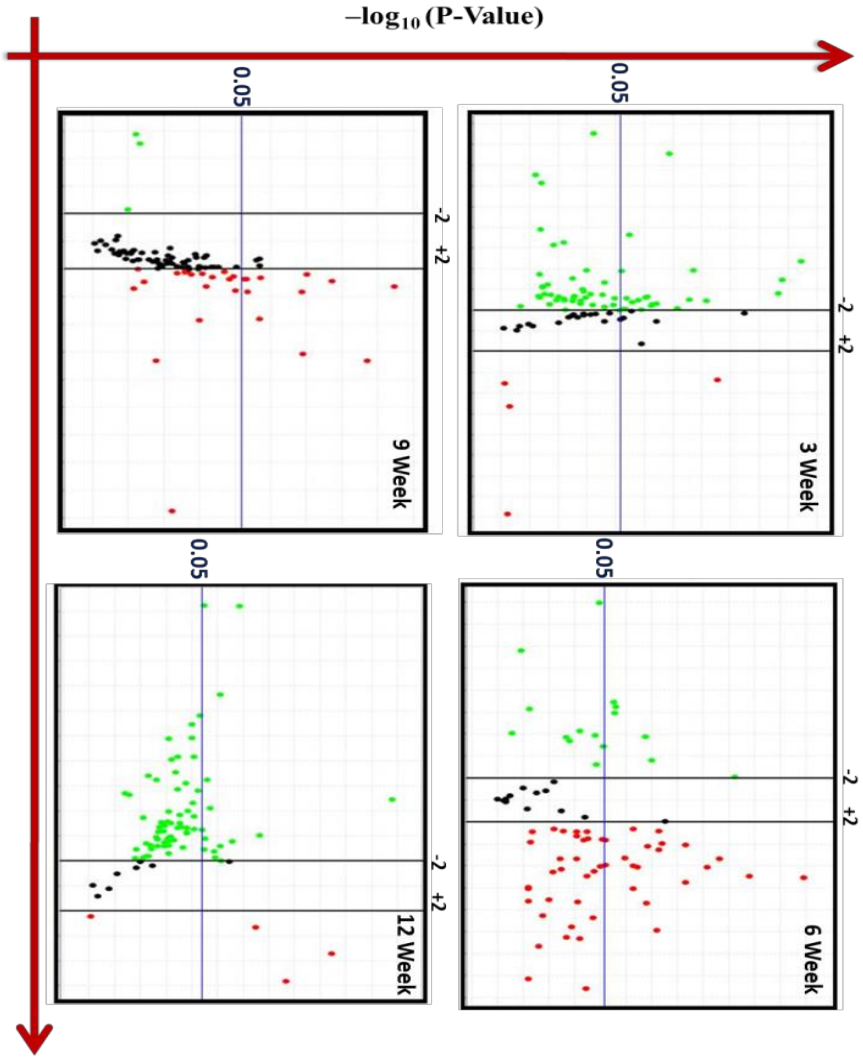


Fig 4.2: Effect of sodium arsenite on the expression of "brain specific miRNAs" in developing rat brain. Volcano plot analysis of "brain specific miRNA" profile of 3 week, 6 week, 9 week, and 12 week aged rat's brain exposed with sodium arsenite (Endogenous control; U6 snRNA, pValue<0.05, Fold Change boundary 2 folds)

4.3. Box plot analysis of "brain specific miRNAs" regulated by sodium arsenite:

Box plot analysis of "brain specific miRNAs" was performed to study the overall brain specific miRNA transcription pattern in sodium arsenite exposed developing rat's brain. Substantial decrease was observed in mean Ct of brain specific miRNAs in brain of 6week old rats, which were exposed with sodium arsenite (Fig 4.3). Decrease in mean Ct indicates the increase in overall transcription of brain specific miRNAs as Ct values are inversely proportional of transcriptional level. Interestingly, box plot

analysis has shown increase in mean Ct of brain specific miRNAs in young adult rat's brain exposed with sodium arsenite, which suggests decrease in overall transcription of brain specific miRNAs, Similar to volcano plot analysis. Moreover, no significant differences were observed in overall transcription of brain specific miRNAs in pre-adolescent (3week), and post-adolescent (9week) brain exposed with sodium arsenite (Fig 4.3).

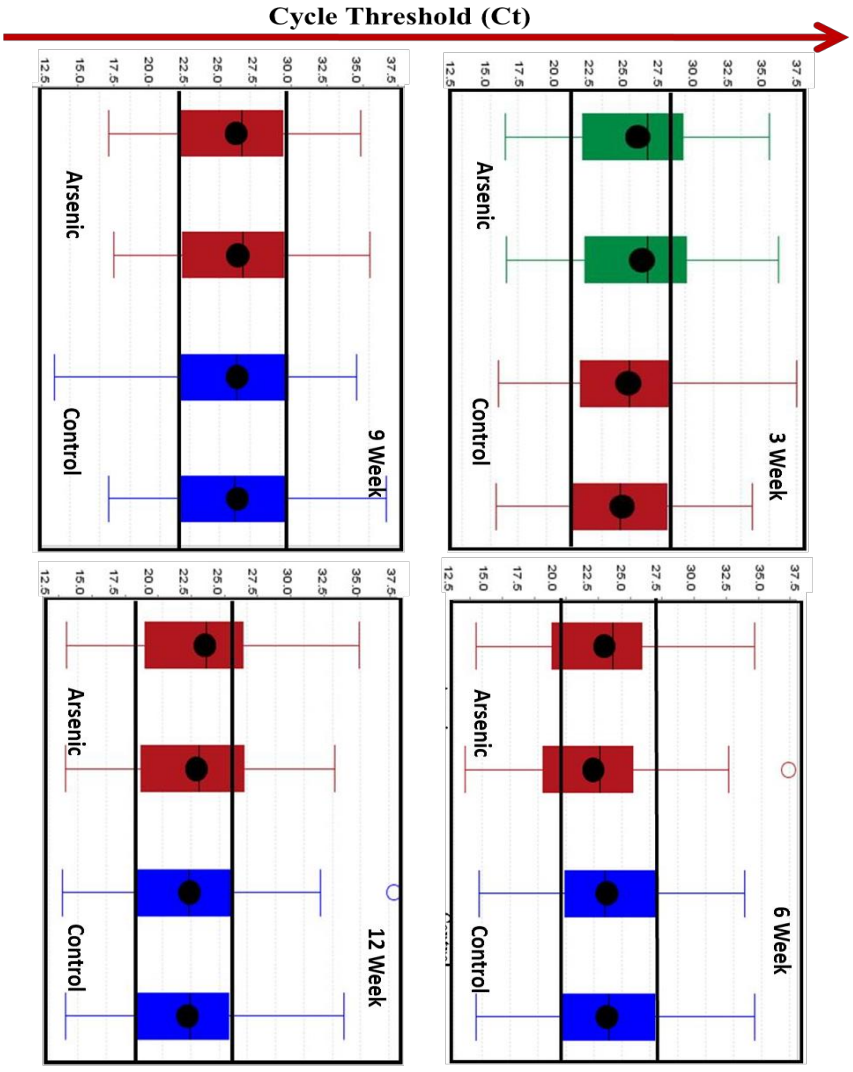


Fig 4.3: Box plots analysis of "brain specific miRNAs" regulated by sodium arsenite in developing rat brain. Box plot analysis was performed to identify the total transcriptome regulation of brain specific miRNAs in brain of 3 week, 6 week, 9 week and 12 week aged rats. (Ct: Threshold cycle).

4.4 Regulation of "brain specific miRNAs" by sodium arsenite in 3week aged rats:

Customized "brain specific miRNA array" was used to study the effect of sodium arsenite on regulation of miRNAs in 3week aged rat's brain. Sodium arsenite exposure (20mg/kg b.wt orally for 7consecutive days) administered orally significantly up-regulated expression of Let-7i*(5.14 folds), while expression of miR-374* was significantly down-regulated (25 folds) in 3week aged rat's brain. Moreover, expression of miR-217, miR-17, miR-499, miR-212, miR-191, miR-29c*, miR-19b, miR-339-3p, miR-664, miR-219-2-3p, miR-125b, miR-298, miR-487b, miR-182, miR-200b, miR-200a, and miR-429 were found to be significantly down-regulated by sodium arsenite exposure in 3 week aged rat's brain (≥2 folds and pValue*≤0.05) (Fig 4.4).

4.5 Regulation of "brain specific miRNAs" by sodium arsenite in 6week aged rats:

Customized "brain specific miRNA array" was used to study the effect of sodium arsenite on regulation of miRNAs in 6week aged rat's brain. Exposure of sodium arsenite exposure (20mg/kg b.wt orally for 7 consecutive days) in 6week old rats induced dramatic alterations in expression of "brain specific miRNAs". Exposure of sodium arsenite altered maximum changes in 6week aged rats (adolescent stage). Exposure of sodium arsenite significantly up-regulated the expression of 23 miRNAs, particularly miR-29c* (61 folds) and miR-29a (25 folds), while significantly down-regulated the expression of miR-200 family; miR-200c (21 folds), miR-200a (18 folds) and miR-200b (15 folds). Out of 23 miRNAs altered by sodium arsenite

exposure in 6 week aged rat's brain, expression of miR-136*, miR-219-3p, miR-664, miR-138, miR-449, miR-150, miR-338-5p, miR-7e, miR-222, miR-139-5p, miR-132, miR-191, miR-218, miR-1937c, and miR-33a* were also found to be up-regulated while miR-19b, miR-17 and miR-296-3p were significantly down-regulated (≥2 folds and pValue*≤0.05) (Fig 4.5).

4.6 Regulation of "brain specific miRNAs" by sodium arsenite in 9week aged rats:

Effect of sodium arsenite (20mg/kg b.wt orally for 7 consecutive days) on expression/regulation of brain specific miRNAs was also studied in 9 week aged rat's brain. Sodium arsenite has shown maximum increase in expression of miR-429 (20 folds). In addition, expression of miR- 200a, miR-200b, miR-483*, miR-182, miR-511, miR-344-3p, miR-292-3p and miR-34b-3p and miR-29b (>2 folds pValue*≤0.05), was also up-regulated by sodium arsenite in 9 week aged rat's brain. Interestingly, no miRNAs was found down-regulated by sodium arsenite in 9 week aged rat's brain (Fig 4.6).

4.7 Regulation of "brain specific miRNAs" by sodium arsenite in 12week aged rats:

Customized "brain specific miRNA array" was used to study the effect of sodium arsenite on regulation of "brain specific miRNAs" in 12week aged rat's brain. Sodium arsenite exposure (20mg/kg b.wt orally for 7 consecutive days) significantly up-regulated expression of miR-674*(14.1 folds), miR-150 (7.06 folds) and miR-511(6.56 folds), while expression of miR-194, miR-17, miR-1937c, miR-126, miR-199a-3p, miR-219, miR-490, miR-93*, miR-7i*,miR-182, miR-141, miR-191 and

miR-30b were significantly down-regulated by sodium arsenite in 12 week aged rat's brain. Maximum down-regulation was observed in miR-30b (-35 folds) in sodium arsenite exposed 12week rat brain (≥2folds and pValue* ≤0.05) (Fig.4.7).

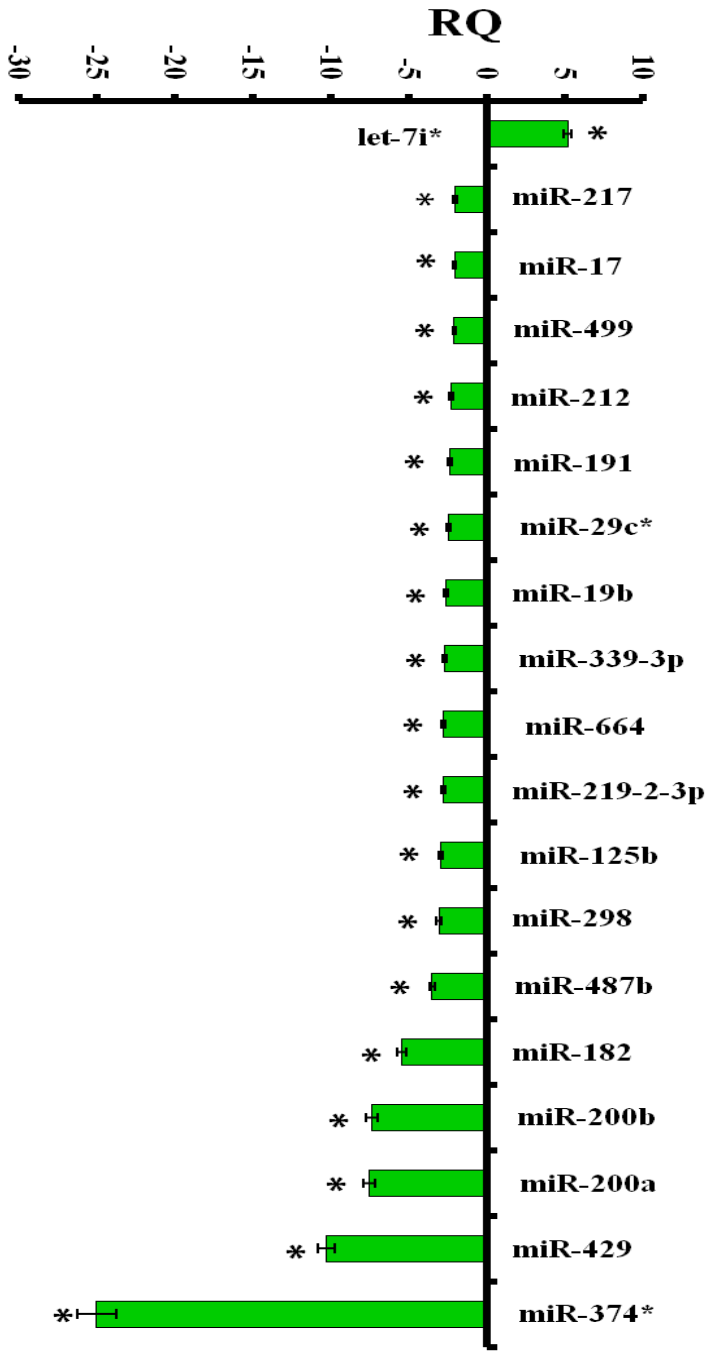


Fig 4. 4: Effect of sodium arsenite on expression of "brain specific miRNAs" in 3week aged rat's brain. Sodium arsenite up-regulation as well as down- regulation in the expression of miRNAs at 3rd postnatal week (RQ: Relative quantification, pValue* <0.05).

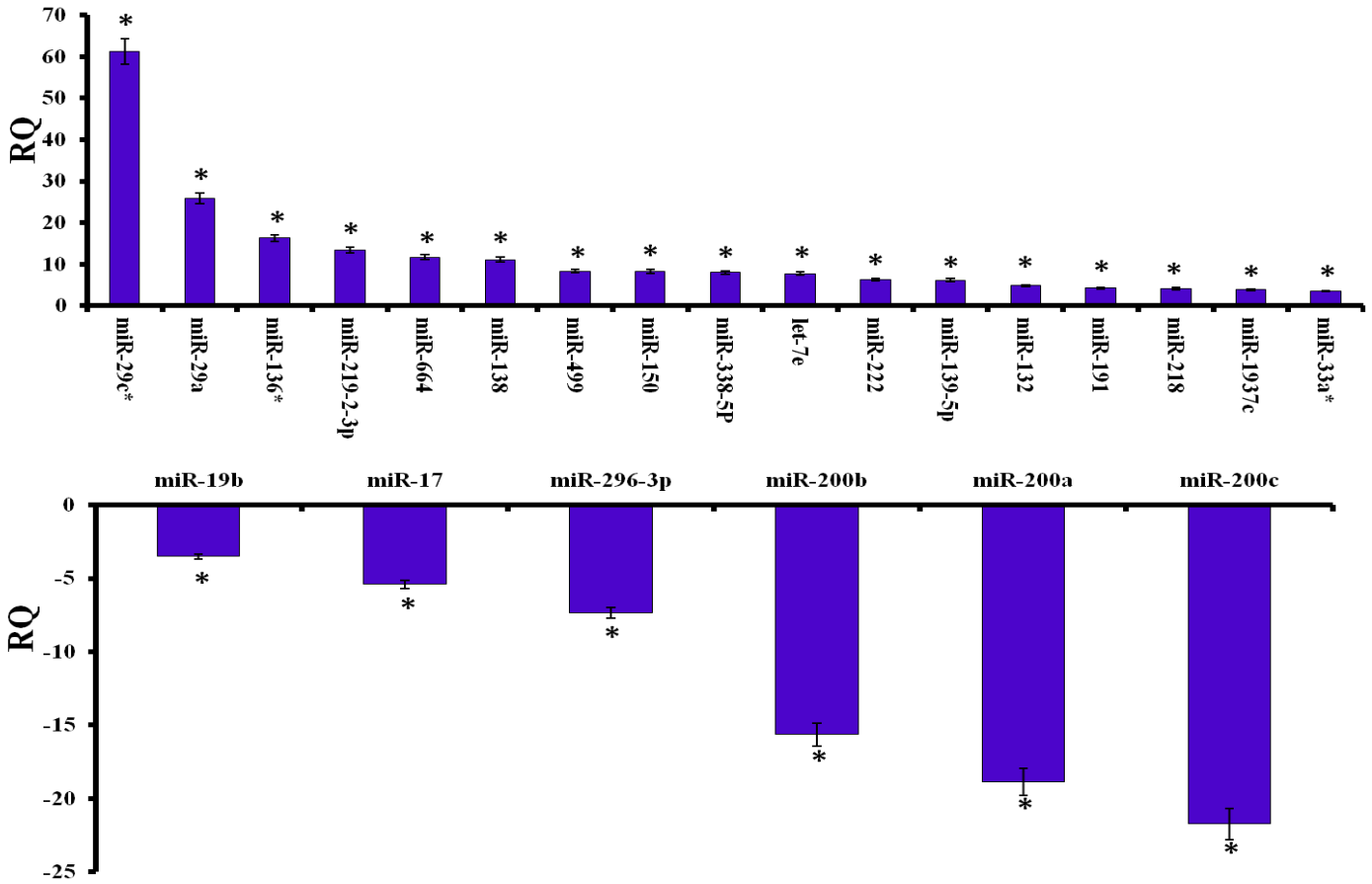


Fig 4. 5: Effect of sodium arsenite on expression of "brain specific miRNAs" in 6 week aged rat's brain. Sodium arsenite up-regulation as well as down –regulation in the expression of miRNAs at 6th postnatal week , (RQ: Relative quantification, pValue* <0.05).

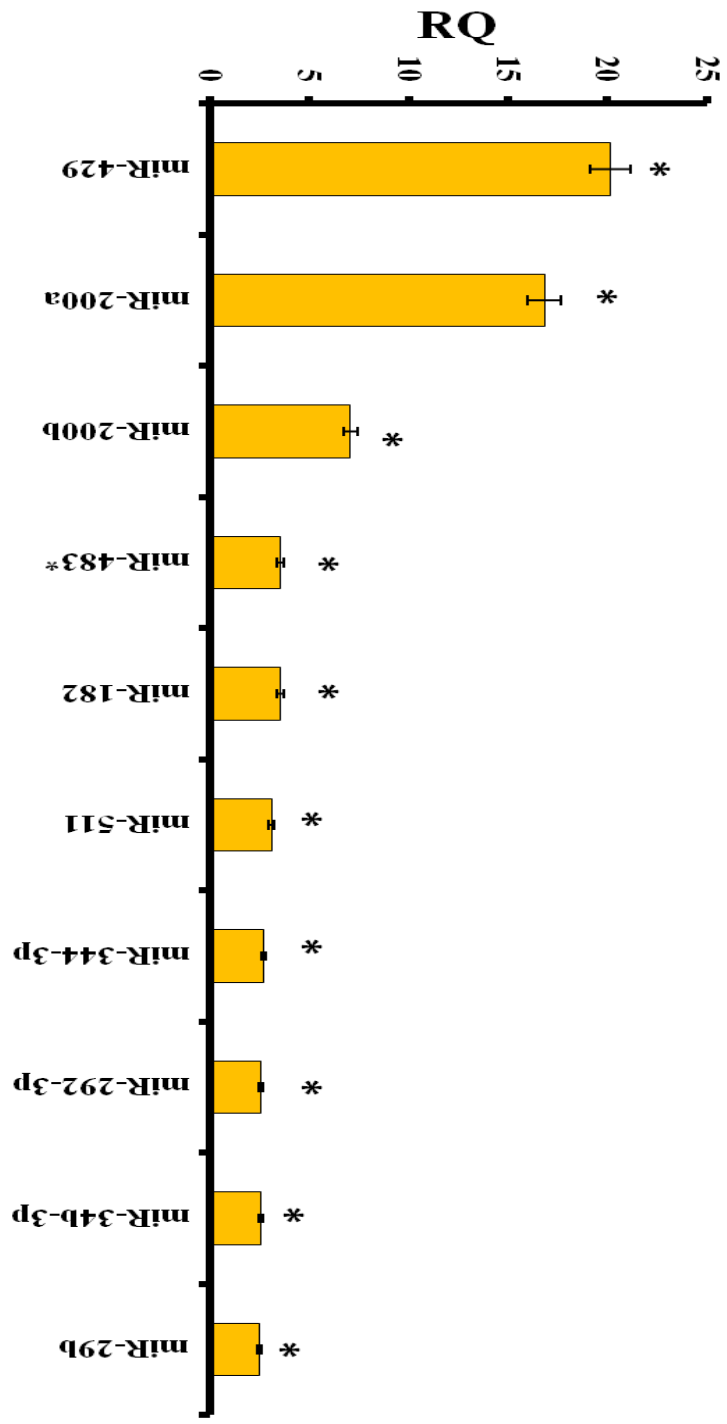


Fig 4. 6: Effect of sodium arsenite on expression of "brain specific miRNAs" in 9 week aged rat's brain. Sodium arsenite induced up-regulation in expression of miRNAs at 9th postnatal week (RQ: Relative quantification, pValue* <0.05).

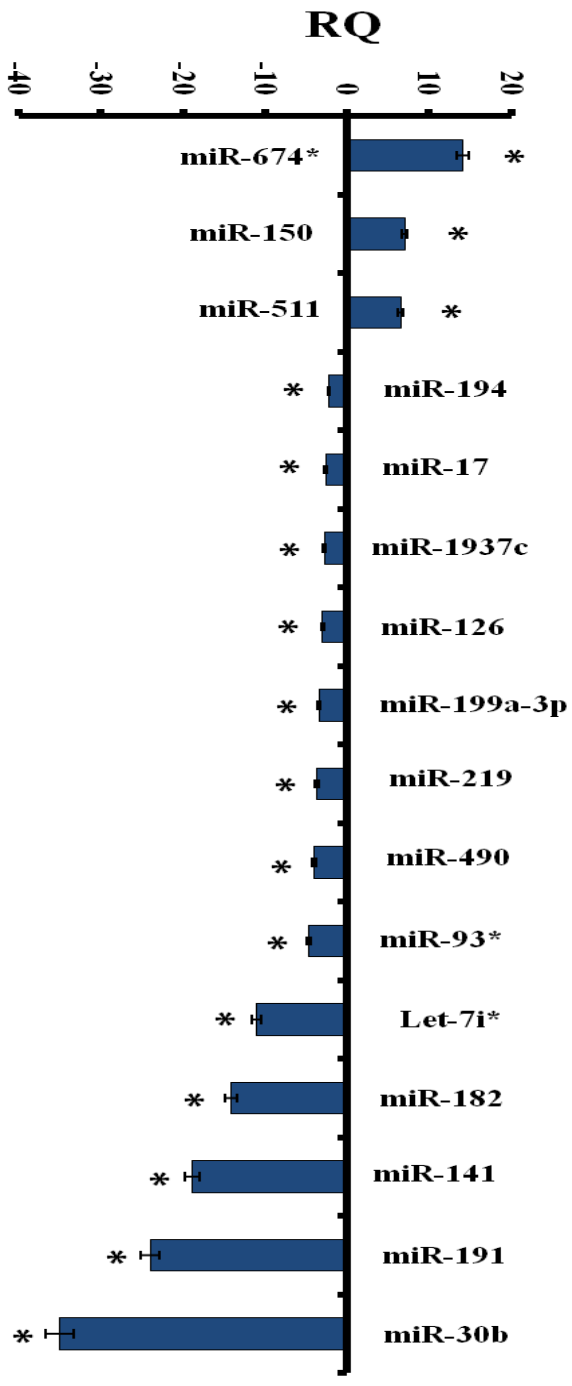


Fig 4.7: Effect of sodium arsenite on expression of "brain specific miRNAs" in 12week aged rat's brain. Sodium arsenite up-regulation as well as down-regulation in the expression of miRNAs at 12th postnatal week (RQ: Relative quantification, pValue* <0.05).

4.8 Effect of paraquat+maneb (PQ+MB) exposure on the expression of "brain specific miRNAs" in developing rat's brain:

Considering 2 fold as minimum change with pValue ≤0.05, volcano plot analysis of "brain specific miRNAs" has shown that PQ+MB induced alterations in expression of 27 miRNAs (1up-regulated and 26 down-regulated), 41 miRNAs (9up-regulated and 32down-regulated), 8 miRNAs (7 up-regulated and 1down-regulated) and 25 miRNAs (19up-regulated and 6down-regulated) in pre-adolescent, adolescent, post-adolescent, and young adult rats respectively (Fig 4.8).

4.9 Box plot analysis of "brain specific miRNAs" regulated by paraquat + maneb (PQ+MB):

Box plot analysis of "brain specific miRNAs" was performed to study the overall brain specific miRNA transcription pattern in PQ+MB exposed developing rat's brain. Substantial decrease was observed in mean Ct of brain specific miRNAs in brain of 6week old rats, which were exposed with PQ+MB (Fig 4.9). Decrease in mean Ct indicates the increase in overall transcription of brain specific miRNAs as Ct values are inversely proportional of transcriptional level. Interestingly, box plot analysis has shown increase in mean Ct of brain specific miRNAs in young adult rat's brain exposed with PQ+MB, which suggests decrease in overall transcription of brain specific miRNAs, similar to volcano plot analysis. Moreover, no significant differences were observed in overall transcription of brain specific miRNAs in pre-adolescent (3week), and post-adolescent (9week) brain exposed with PQ+MB (Fig 4.9).

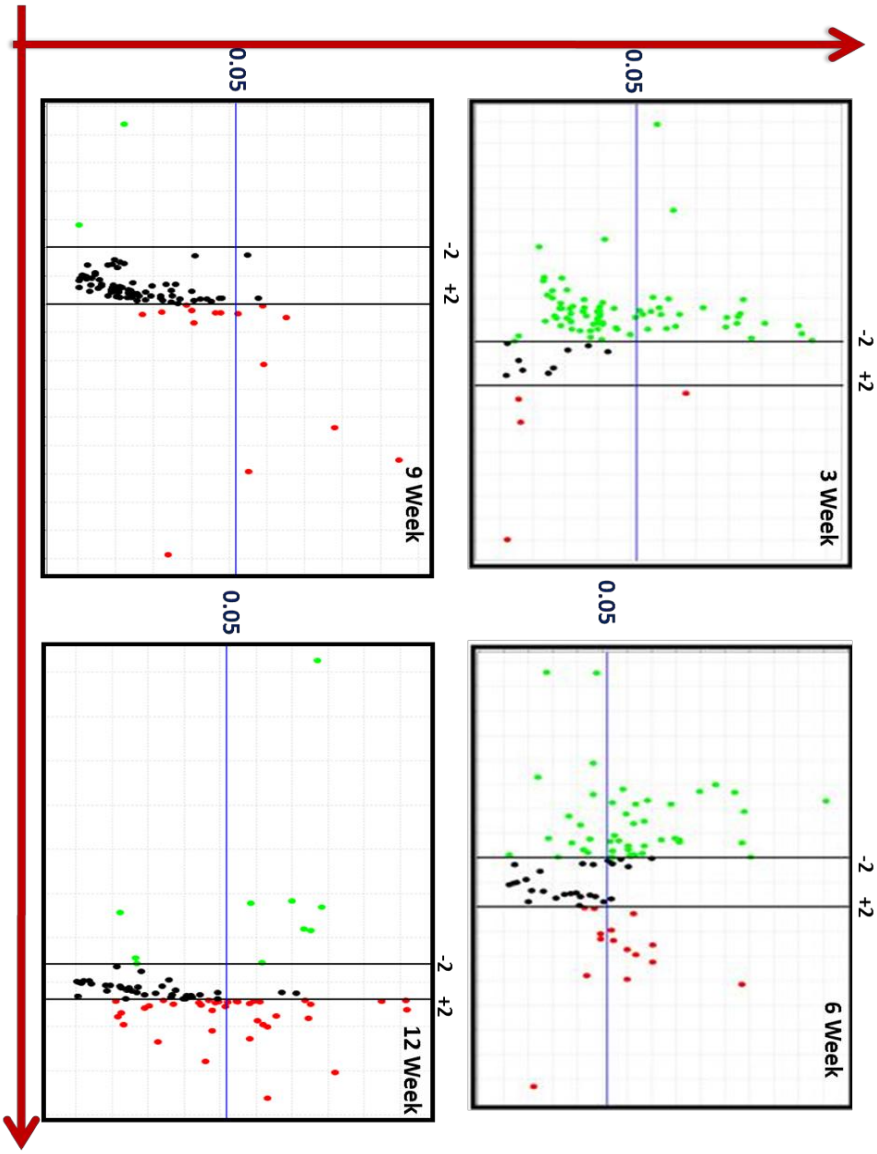


Fig 4. 8: Effect of PQ+MB on the expression of brain specific miRNAs in developing brain. Volcano plot analysis of brain specific miRNA profile of 3 week, 6 week, 9 week, and 12 week aged rat's brain exposed with PQ+MB (Endogenous control; U6 snRNA, pValue<0.05, Fold Change boundary 2 folds)

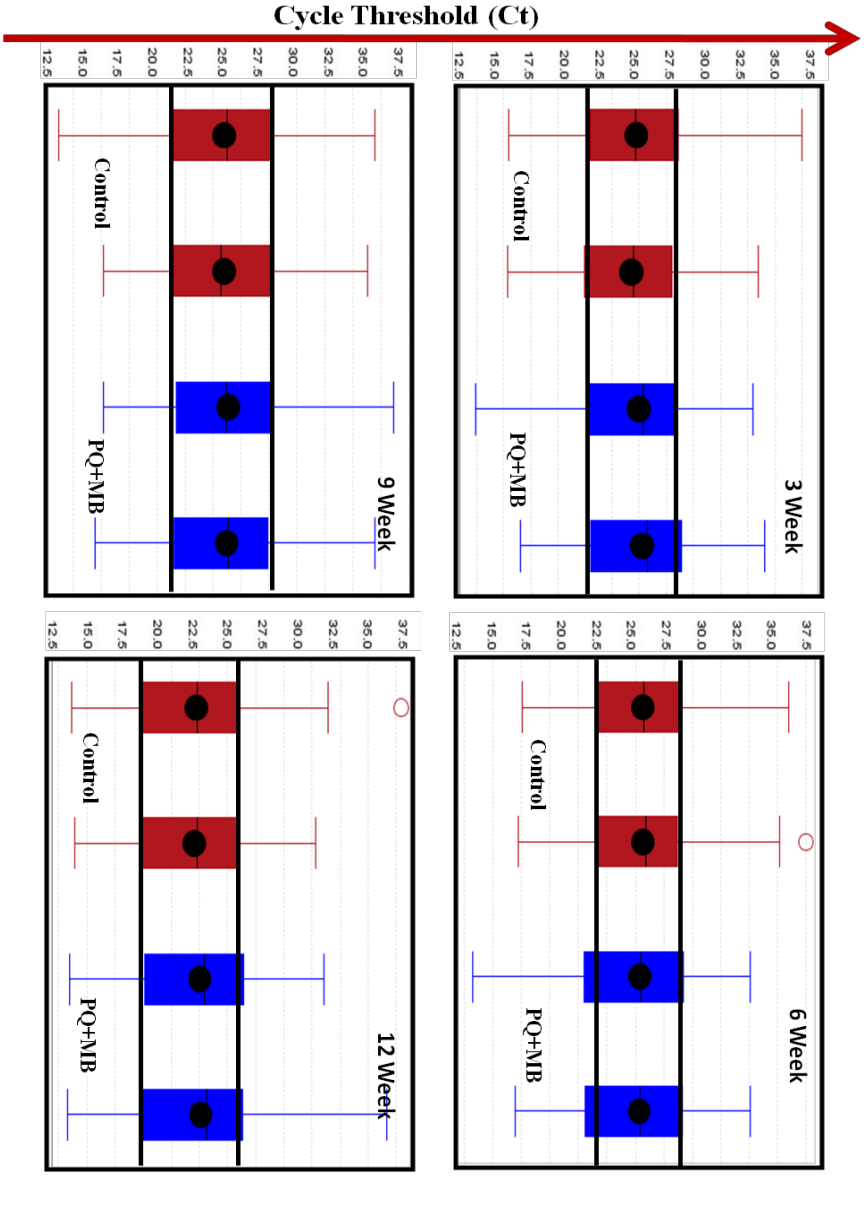


Fig 4.9: Box plots analysis of "brain specific miRNAs" regulated by PQ+MB in developing rat brain. Box plot analysis was performed to identify the total transcriptome regulation of brain specific miRNAs in brain of 3 week , 6 week , 9 week and 12 week aged rats. (Ct: Threshold cycle).

4.10 Regulation of "brain specific miRNAs" by paraquat + maneb (PQ+MB) in 3 week aged rats:

Customized "brain specific miRNA array" was used to study the effect of PQ+MB on regulation of miRNAs in 3week aged rat's brain. Exposure of PQ+MB (Paraquat -10 mg/kg and Maneb- 30 mg/kg b.wt intraperitoneally for 7 consecutive days) induced maximum expression in Let-7i* (2.54 fold) while expression of miR-191 (20 folds) was significantly down-regulated by PQ+MB exposure in 3week aged rat's brain. Moreover, expression of miR-38-3p, miR-153, miR-136*, miR-219, miR-338-3p, miR-7b, miR-194, miR-218, miR-29a*, miR-221, miR-199a-3p, miR-146b*, miR-125b, miR-17, miR-19b, miR-29b-2*, miR-296-3p, miR-93*, miR-125b-1*, miR-298, miR-214*, miR-342-5p, miR-20b-3p, and miR-126 was found to be down-regulated by PQ+MB exposure in 3week aged rat's brain (≥2 folds and pValue* ≤0.05) (Fig 4.10)

4.11 Regulation of "brain specific miRNAs" by paraquat + maneb (PQ+MB) in 6 week aged rats:

Customized "brain specific miRNA array" was used to study the effect of PQ+MB on regulation of brain specific miRNAs in 6 week aged rat's brain. Exposure of PQ+MB (Paraquat -10 mg/kg and Maneb- 30 mg/kg b.wt intraperitoneally for 7 consecutive days) in 6 week old rats induced dramatic alterations in expression of "brain specific miRNAs (Fig.4.11). Exposure of PQ+MB affects maximum changes in 6week aged rats (adolescent stage). PQ+MB exposure significantly up-regulated expression of 9 miRNAs and down-regulated the expression of 32 miRNAs. Out of 41 up-regulated miRNAs by PQ+MB exposure in 6 week aged rat's brain, miR-218-2*and miR-29c

(15 folds) was significantly up-regulated and miR-217 (16 folds) was found to be maximally down regulated. Out of total studied miRNAs, miR-29b, miR-212, miR-29a*, miR-338-3p, miR-150, miR-219, and miR-139-5p were up-regulated by PQ+MB exposure in 6 week aged rats brain while miR-200b, miR-349, miR-221, miR-292-3p, miR-194, miR-93*, miR-33a*, miR-141, miR-434-3p, miR-34b-3p, miR-449b, miR-200c, Let-7e, miR-483*, miR-125b, miR-343, miR-34c, miR-200a, miR-200b, miR-182, miR-335*, miR-9, miR-199a-3p, miR-214*, miR-543, miR-342-5p, miR-17, miR-106a, miR-19b, miR-296-3p, and miR-503 were found to be significantly down-regulated (Fig 4.11).

4.12 Regulation of "brain specific miRNAs" by paraquat + maneb (PQ+MB) in 9 week aged rats:

Customized "brain specific miRNA array" was used to study the effect of PQ+MB on regulation of brain specific miRNAs in 9week aged rat's brain. Exposure of PQ+MB exposure (Paraquat -10 mg/kg and Maneb- 30 mg/kg b.wt intraperitoneally for 7 consecutive days) significantly up-regulated the expression of miR-200a, miR-200b, miR-182, miR-141, miR-200c, and let7e (>2 folds pValue*≤0.05) in 9 week aged rat's brain (Fig.4.12). Moreover, expression of miR-429 (120 folds) was found to be maximally up-regulated by PQ+MB exposure in 9 week aged rat's brain. Broadly, PQ+MB up-regulated the expression of members of miR-200 family (miR-200a, miR-200b and miR-429) in 9 week aged rats (Fig.4.12).

4.13 Regulation of "brain specific miRNAs" by paraquat + maneb (PQ+MB) in 12 week aged rats:

Customized "brain specific miRNA array" was used to study the effect of PQ+MB on regulation of "brain specific miRNAs" in 12week aged rat's brain. Exposure of PQ+MB (Paraquat -10 mg/kg and Maneb- 30 mg/kg b.wt intraperitoneally for 7 consecutive days) significantly up-regulated the expression of 19up-regulated and 6down-regulated miRNAs including miR-674*, miR-511, miR-503, miR-194, miR-499, miR-136*, miR-29c, miR-33a*, miR-206, miR-218-2*, miR-338-3p, miR-29b, miR-1937c, miR-106a, miR-219, miR-29c*, miR-126, miR-17 and miR-138 while expression of miR-141, miR-146b*, miR-200c, miR-200b and miR-182 were significantly down-regulated (>2 folds pValue*≤0.05) in 12 week aged rat's brain (Fig 4.13). Out of identified up-regulated miRNAs, miR-674* was maximally up-regulated (42 folds). Moreover, expression of miR-200a (66 folds) was found to be maximally down-regulated by PQ+MB exposure in 12 week aged rat brain (Fig 4.13).

4.14. Expression of neuronal and synaptic markers during postnatal brain development at transcriptional or translational level.

We studied the modulation in the expression of synaptic and neuronal markers during postnatal brain development at pre-adolescent (3week), adolescent (6week), post-adolescent (9week) and young adult (12week) aged rat's brain brain by exposure of sodium arsenite (20mg/kg b.wt orally for 7consecutive days) or PQ+MB (Paraquat -10 mg/kg and Maneb- 30 mg/kg b.wt intraperitoneally for 7 consecutive days). Expression of neuronal and synaptic markers, Neurogenin 2 (NeuroG2) and

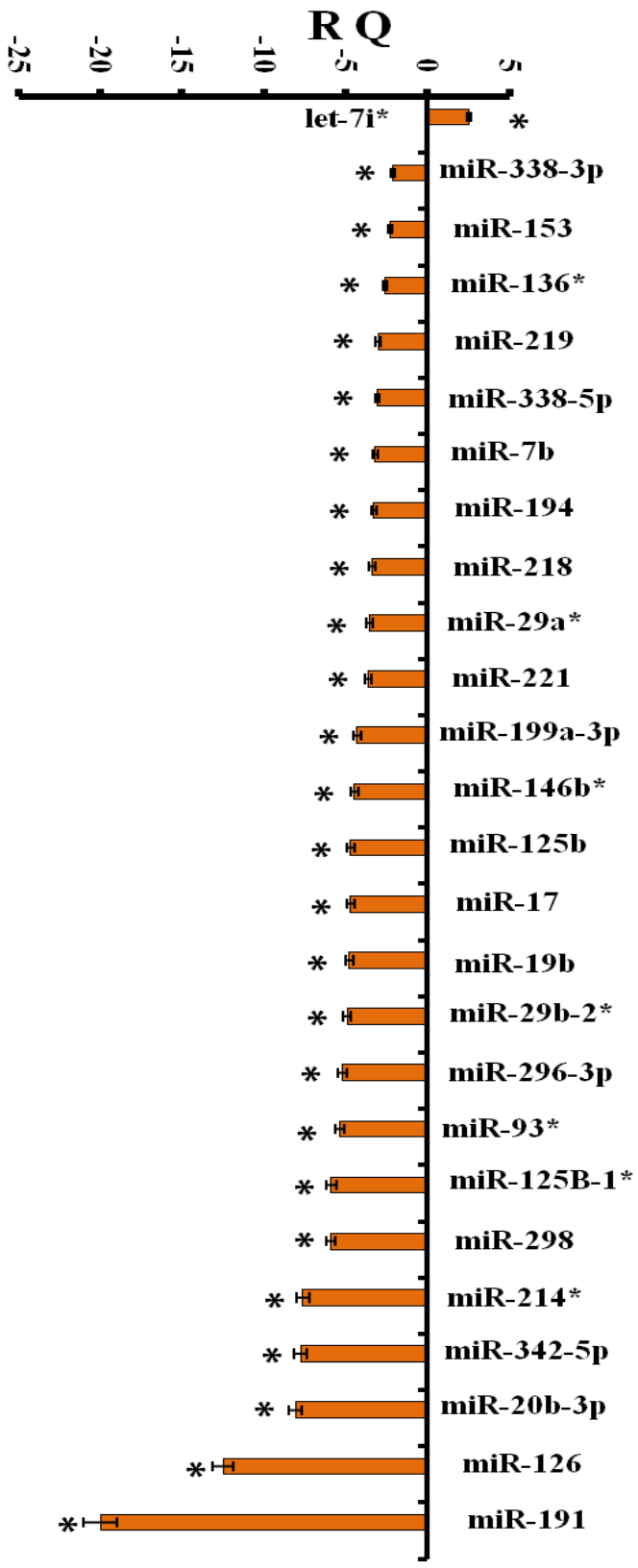


Fig 4.10: Effect of PQ+MB on expression of brain specific miRNAs in 3 week aged rat's brain .PQ+MB induced up-regulation or down-regulation in expression of miRNAs at 3rd postnatal week (RQ: Relative quantification, pValue* <0.05).

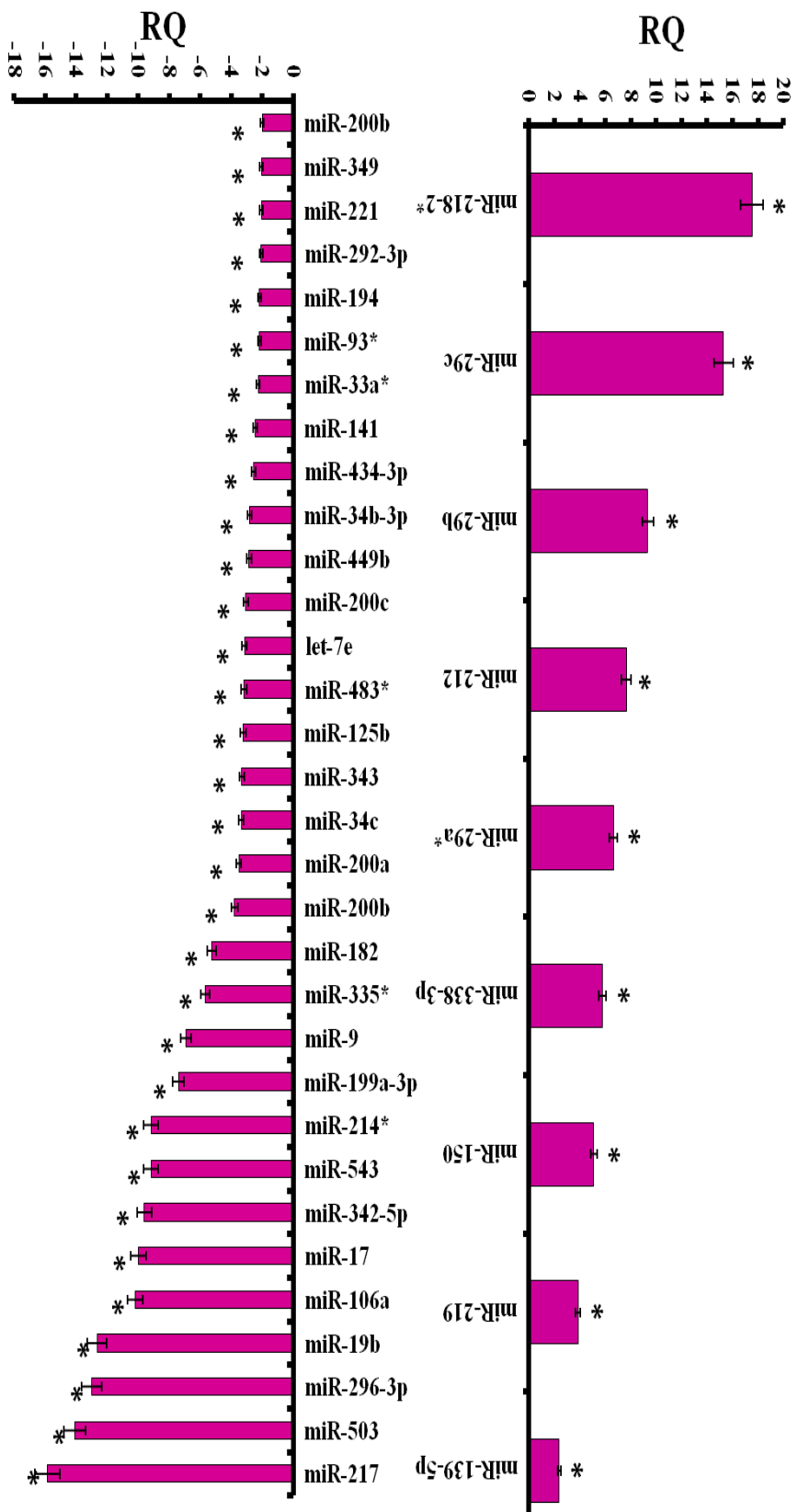


Fig 4.11: Effect of PQ+MB on expression of brain specific miRNAs in 6 week aged rat's brain. PQ+MB up-regulation and down-regulation in expression of miRNAs at 6th postnatal week (RQ: Relative quantification, pValue* <0.05).

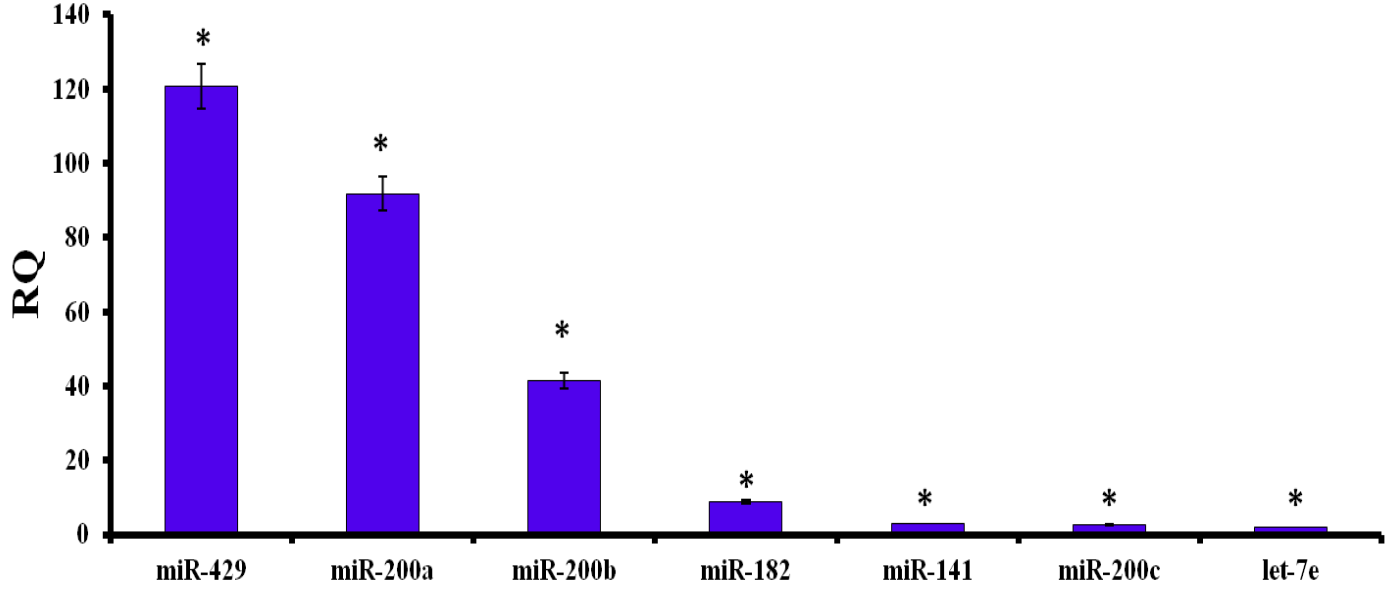


Fig 4.12: Effect of PQ+MB on expression of brain specific miRNAs in 9 week aged rat's brain. PQ+MB induced up-regulation in expression of miRNAs at 9th postnatal week (RQ: Relative quantification, pValue* <0.05).

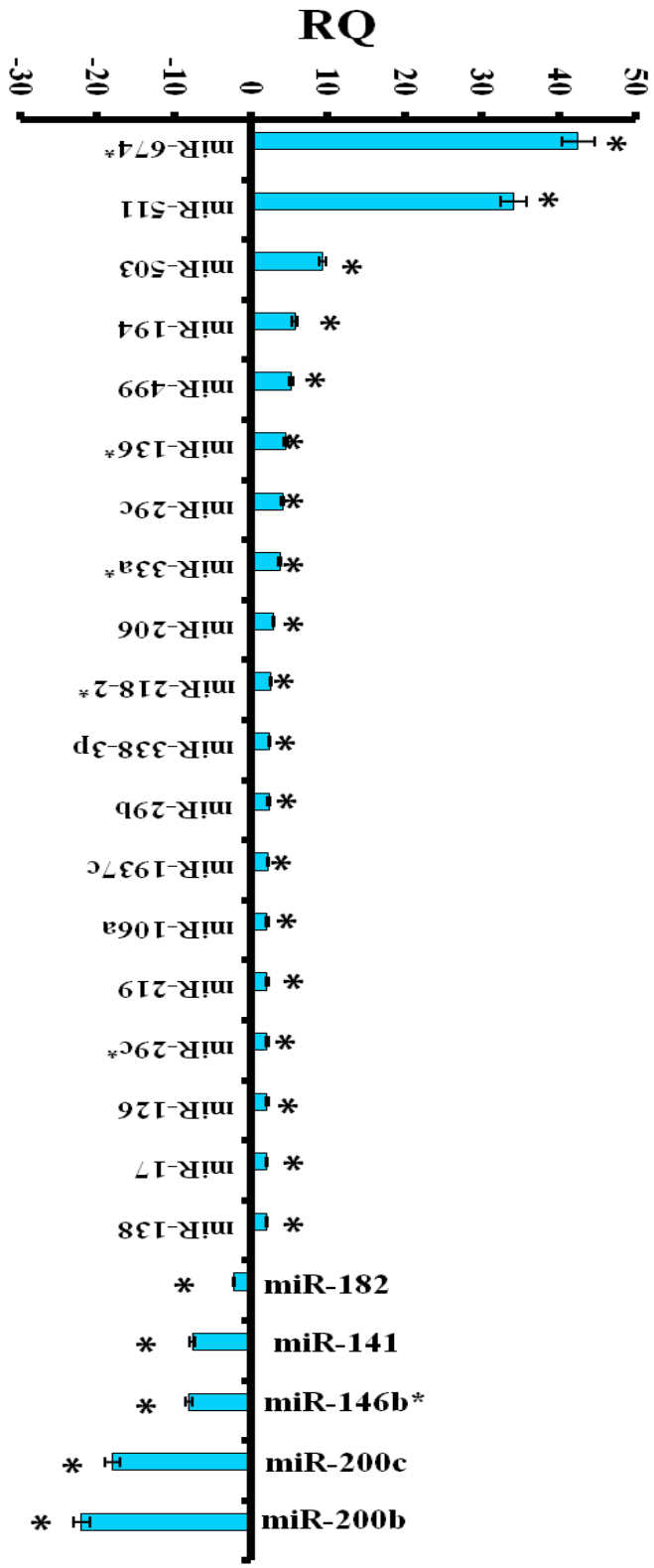


Fig 4.13: Effect of PQ+MB on expression of brain specific miRNAs in 12week aged rat's brain. PQ+MB induced up-regulation and down-regulation the expression of miRNAs at 12th postnatal week (RQ: Relative quantification, pValue* <0.05).

Doublecortin (DCX), Neurofilament medium (NFL-M/NEFM), βIII-Tubulin, Post Synaptic Density 95 (PSD95), Synaptophysin (SYP) and Synapsin 1 (SYN1) was studied by immunoblotting and real time PCR (qPCR) in control as well as neurotoxicants (sodium arsenite and paraquat+maneb(PQ+MB)) exposed developing rat brain. Expression of neuronal and synaptic markers have shown that exposure of sodium arsenite or PQ+MB significantly down-regulated the expression of NeuroG2 and DCX, NFL-M/NEFM, βIII-Tubulin, PSD95, SYP and SYN1 at 6 week (adolescent stage) (Fig 4.16-4.17) in compare to 3 week (pre-adolescent) (Fig 4.14-4.15) and 9 week (post-adolescent) aged rats at transcriptional as well as at translational level (Fig 4.18-4.19). Expression of these neuronal and synaptic markers, NeuroG2 and DCX), NFL-M/NEFM, βIII-Tubulin, PSD-95, SYP and SYN1 was also found to be down-regulated at 12week (young adult) stage at transcriptional as well as at translational level (Fig 4.20-4.21).

4.15 Regulation of neuronal and synaptic markers by sodium arsenite or paraquat+maneb (PQ+MB) during different stages of postnatal rat brain development:

Regulation of neuronal and synaptic markers by the exposure of sodium arsenite or PQ+MB in pre-adolescent (3 week), adolescent (6 week), post-adolescent (9 week) and young adult (12 week) rats was studied by immunoblotting and real time PCR. Immunoblotting and real time PCR studies has shown that sodium arsenite exposure (20mg/kg b.wt for 7 consecutive days) did not induced significant alterations in expression of PSD95, βIII-Tubulin, NFL-M/NEFM, DCX, NeuroG2 and SYN1 at

protein and mRNA level while it altered the expression of SYP at protein as well as at mRNA level in brain of 3week aged rats when compared to their matched controls. PQ+MB (Paraquat -10 mg/kg and Maneb- 30 mg/kg b.wt for 7 consecutive days) did not induced significant alterations in expression of SYP, PSD95, βIII-Tubulin, NFL-M/NEFM, DCX, NeuroG2 and SYN1 at protein as well as mRNA level in brain of 3 week aged rats (Fig 4.14-4.15). Immunoblotting and Real time PCR studies has shown that expression of immature neuronal markers, DCX, and NeuroG2, mature neuronal markers, NFL-M/NEFM and βIII-Tubulin, and synaptic markers PSD95, SYP and SYN1 were down-regulated in 6 week aged rat's brain exposed with sodium arsenite or PQ+MB when compared to their matched controls (Fig 4.16-4.17). Moreover, changes were more prominent in sodium arsenite exposed rats in compare to PQ+MB at mRNA and at protein level (Fig 4.16-4.17).

Similarly, effect of sodium arsenite or PQ+MB was also studied on expression of neuronal and synaptic markers at 9week and 12week aged rat's brain. Immunoblotting studies has shown alterations in expression of PSD95 in sodium arsenite exposed 9week aged rat's brain (Fig 4.18-4.19) while changes were identified in SYP expression in PQ+MB exposed 9week aged rat brain. Moreover, sodium arsenite induced alterations in SYN1 and βIII-Tubulin expression whereas PQ+MB dysregulated the expression of βIII-Tubulin at mRNA level in 9week aged rat's brain. Furthermore, in 12week aged rats, immunoblotting studies has shown that exposure of sodium arsenite induced changes in the expression of βIII-Tubulin, PSD95, NFL-M/NEFM, and SYN1 while PQ+MB dyregulated the expression of NeuroG2, βIII-Tubulin, PSD95,SYN1 and DCX at protein level when compared to their matched

controls. Relative quantification studies identified the downregulation in SYP, βIII-Tubulin, NeuroG2, SYN1 and NFL-M/NEFM expression in sodium arsenite exposed 12week aged rats, whereas PQ+MB altered the expression of βIII-Tubulin, NeuroG2 and NFL-M/NEFM at mRNA level in 12 week aged rat's brain (Fig 4.20-4.21).

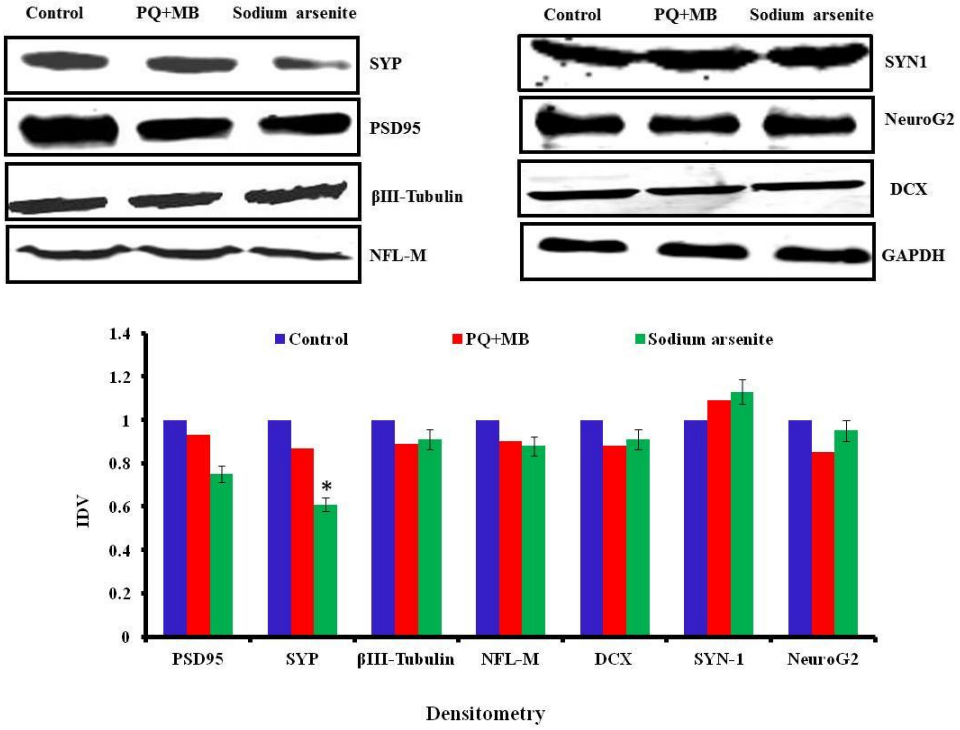


Fig 4.14: Effect of sodium arsenite or PQ+MB on expression of neuronal and synaptic markers at protein level in 3week aged rat's brain. Western blots and densitometry of SYP, PSD95, βIII-Tubulin, DCX, NeuroG2, SYN1 and NFL-M/NEFM in whole brain lysates exposed with sodium arsenite or PQ+MB in 3 week aged rat's brain (IDV: Integrated Density Value pValue* <0.05, densitometry of western blots was performed using Image J software).

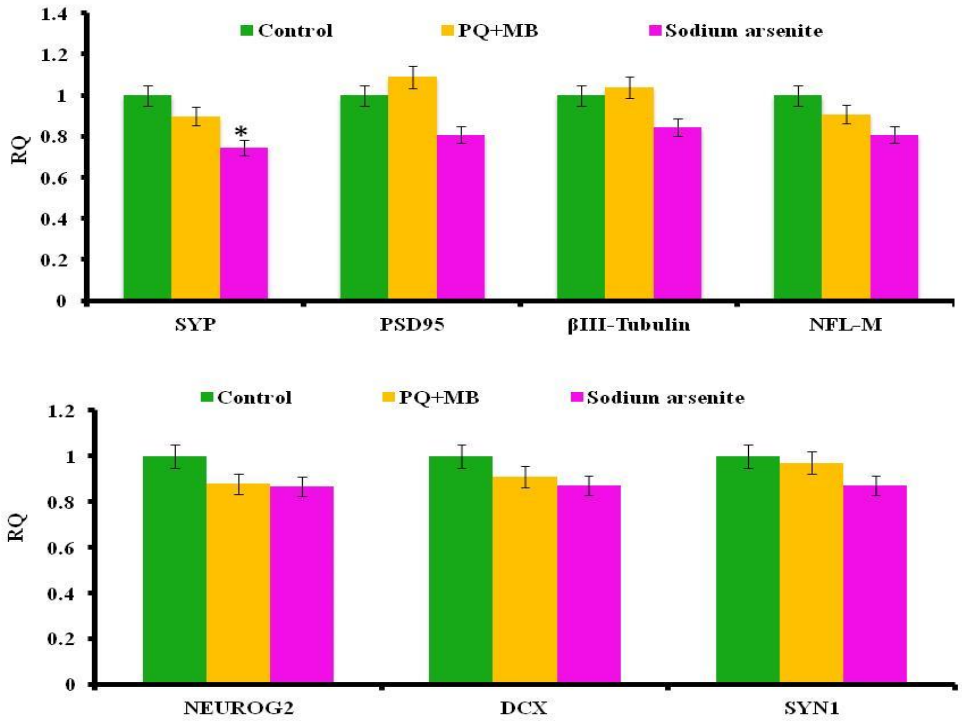


Fig 4.15: Effect of sodium arsenite or PQ+MB on expression neuronal and synaptic markers at mRNA level in 3 week aged rat's brain. Real Time PCR of SYP, PSD95, βIII-Tubulin, NFL-M/NEFM, DCX, and SYN1and NeuroG2 in RNA isolated from whole brains exposed with sodium arsenite or PQ+MB (RQ: Relative quantification).

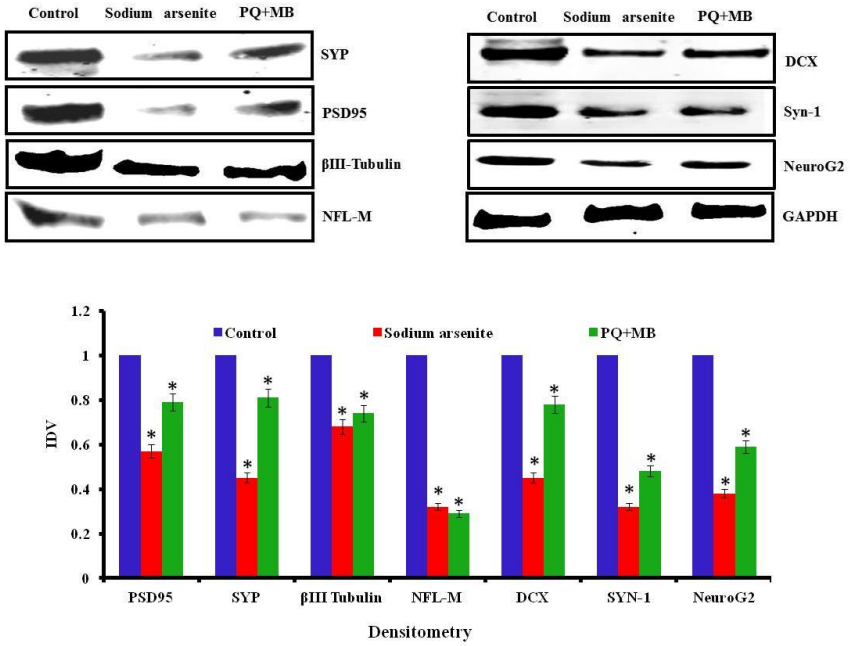


Fig 4.16: Effect of sodium arsenite or PQ+MB on expression neuronal and synaptic markers at protein level in 6week aged rat's brain. Western blots and densitometry of SYP, PSD95, βIII-Tubulin, NFL-M/NEFM, DCX, NeuroG2, and SYN1 in whole brain lysates exposed with sodium arsenite or PQ+MB in 6 week aged rat's brain (IDV: Integrated Density Value pValue* <0.05, densitometry of western blots was performed using Image J software).

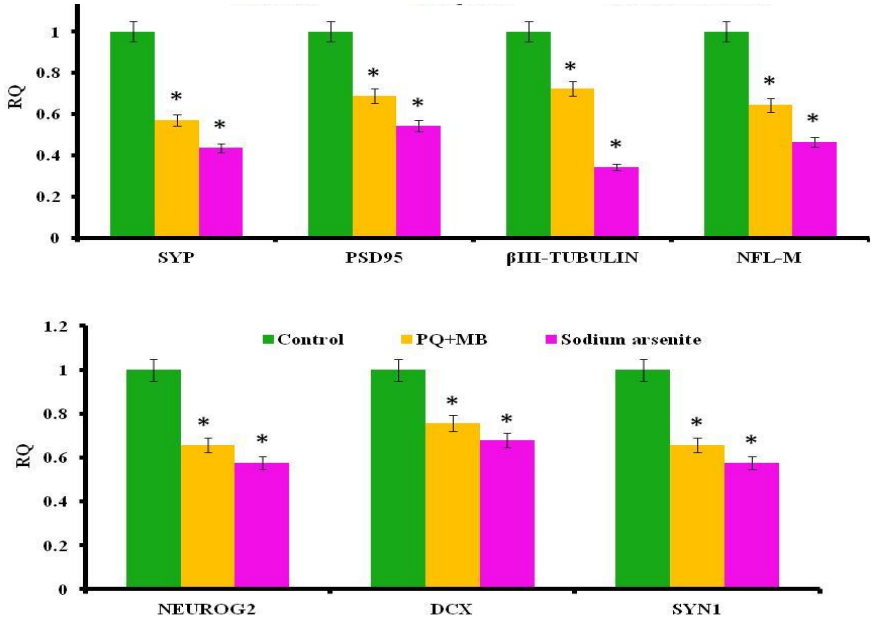


Fig. 4.17: Effect of sodium arsenite or PQ+MB on expression neuronal and synaptic markers at mRNA level in 6 week aged rat's brain. Real Time PCR of SYP, PSD95, βIII-Tubulin, NFL-M/NEFM, SYN1, DCX and NeuroG2 in RNA isolated from whole brains exposed with sodium arsenite or PQ+MB (RQ: Relative quantification).

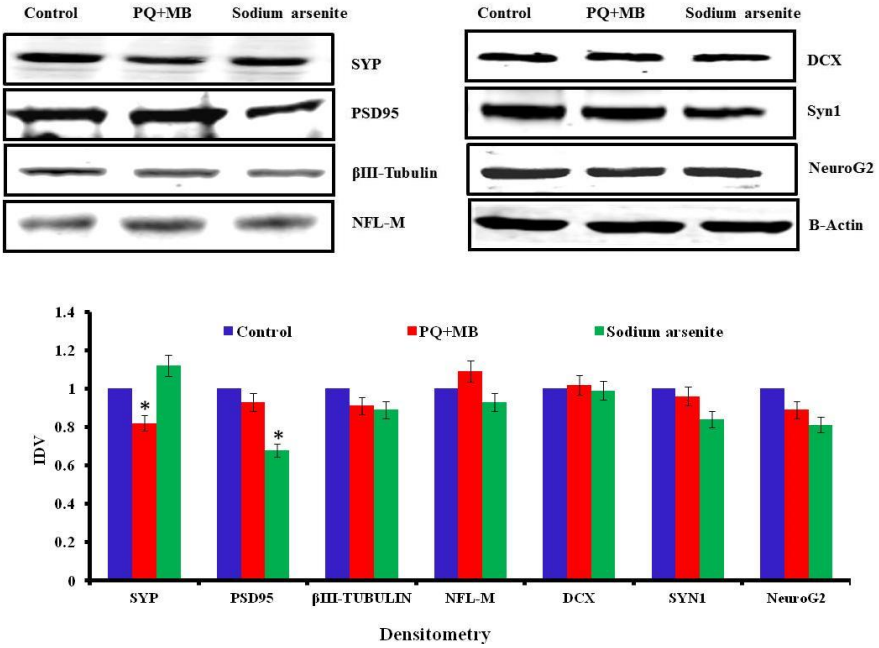


Fig. 4.18: Effect of sodium arsenite or PQ+MB on expression neuronal and synaptic markers at protein level in 9 week aged rat's brain. Western blots and densitometry of SYP, PSD95, βIII-Tubulin, NeuroG2, DCX, SYN1 and NFL-M/NEFM in whole brain lysates exposed with sodium arsenite or PQ+MB in 9week aged rat's brain (a&b). (IDV: Integrated Density Value pValue* <0.05, densitometry of western blots was performed using Image J software).

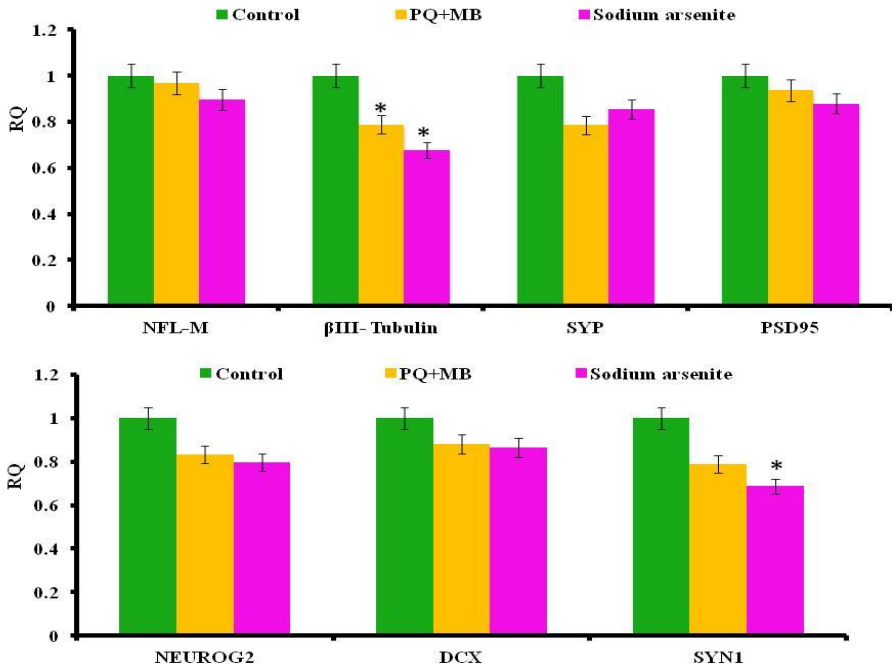


Fig. 4.19: Effect of sodium arsenite or PQ+MB on expression neuronal and synaptic markers at mRNA level in 9 week aged rat's brain. Real Time PCR of SYP, PSD95, βIII-Tubulin, NeuroG2, DCX, SYN1 and NFL-M/NEFM in RNA isolated from whole brains exposed with sodium arsenite or PQ+MB . (RQ: Relative quantification).

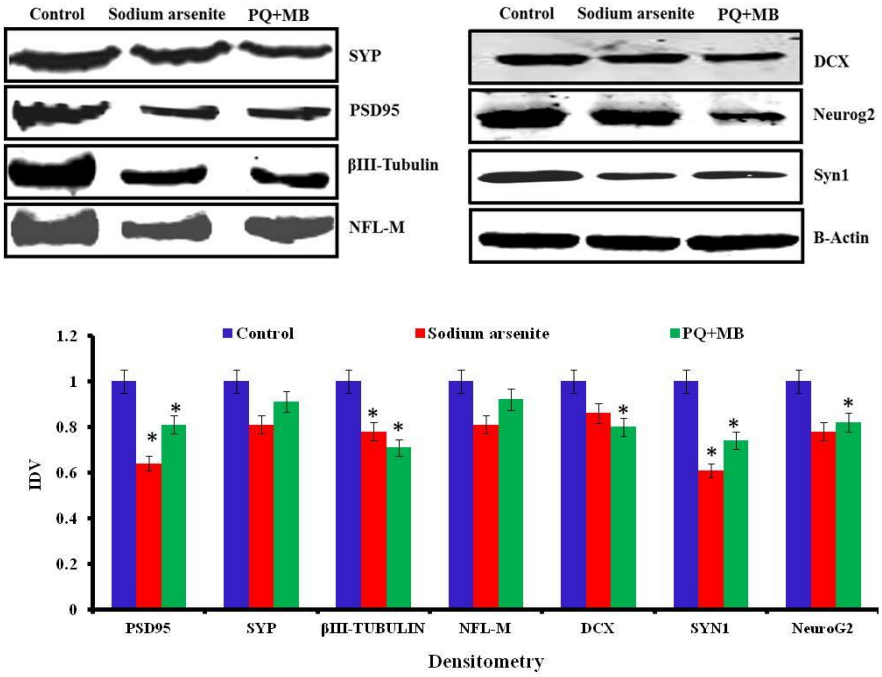


Fig. 4.20: Effect of sodium arsenite or PQ+MB on expression of neuronal and synaptic markers at Protein level in12 week aged rat's brain. Western blots and densitometry of SYP, PSD95, βIII-Tubulin, DCX, NeuroG2, SYN1 and NFL-M/NEFM in whole brain lysates exposed with Sodium Arsenite or PQ+MB in 12 week aged rat's brain. (IDV: Integrated Density Value pValue* <0.05, densitometry of western blots was performed using Image J software).

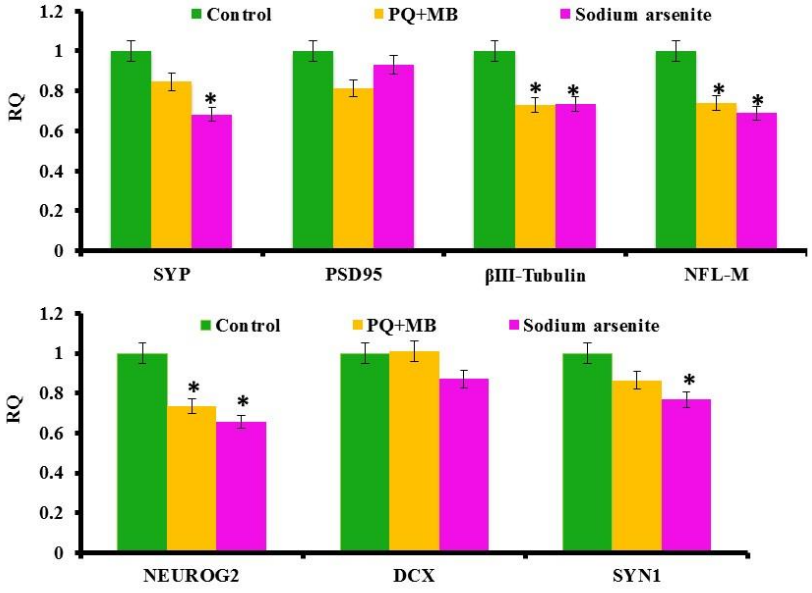


Fig. 4.21: Effect of sodium arsenite or PQ+MB on expression of neuronal and synaptic markers at mRNA level in 12 week aged rat's brain. Real Time PCR of SYP, PSD95, DCX, NFL-M/NEFM, SYN1 and NeuroG2 in RNA isolated from whole brains exposed with sodium arsenite or PQ+MB. (RQ: Relative quantification).

4.16 *In silico* pathway analysis of targets of up or down regulated miRNAs by sodium arsenite or paraquat + maneb (PQ+MB).

To identify the possible biological pathways targeted by sodium arsenite or PQ+MB exposure in 3week, 6week, 9week and 12week aged rats; *in silico* target based pathway analysis was performed using GeneGO web portal. All the potential targets of up-regulated and down-regulated miRNAs by sodium arsenite or PQ+MB in 3 week, 6 week, 9 week and 12 week aged rats were identified using TargetScan web portal and fetched on GO process analysis option of MetaCore platform.

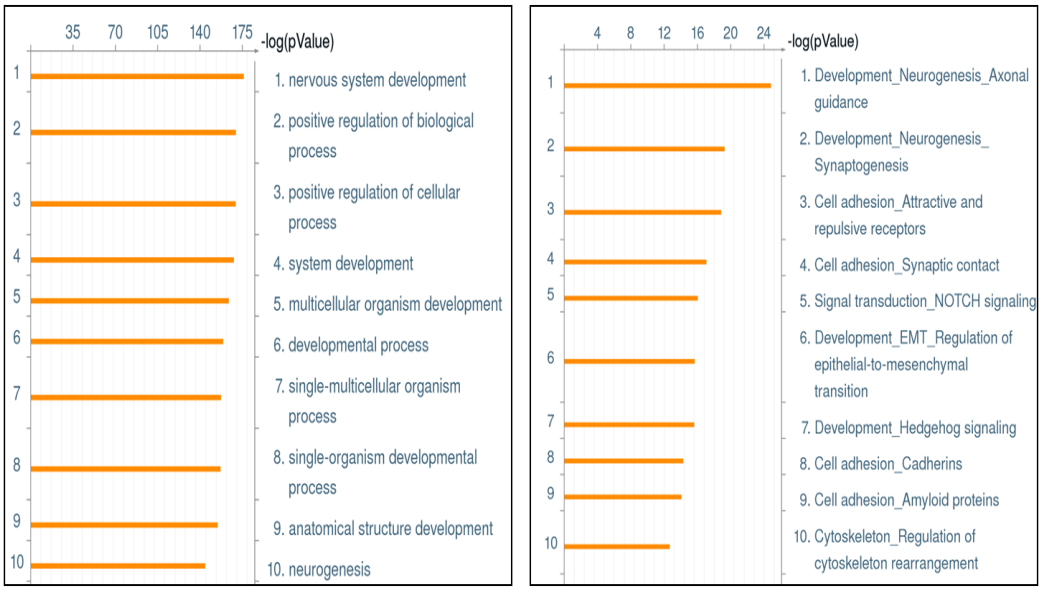
In sodium arsenite exposed 3 week aged rats, *in silico* mapping has shown nervous system development, positive regulation of cellular process, system development, neurogenesis or axonal guidance as top five most significant targeted biological pathways by the targets of miRNAs up or down-regulated by exposure of sodium arsenite or PQ+MB (Fig.4.22-4.23)

In 6 week aged rats, *in silico* mapping has shown development, nervous system development, positive regultion of biological process, cell adhesion synaptic contact and neurogenesis or synaptogenesis as top five most significant targeted biological pathways by the targets of miRNAs up or down-regulated by exposure of sodium arsenite or PQ+MB (Fig 4.24-4.25.).

In 9 week aged rats, *in silico* mapping has shown single organism process, cellular component organization or biogenesis, neurogenesis, neuron projection guidance and axon guidance as top five most significant targeted biological pathways by the targets

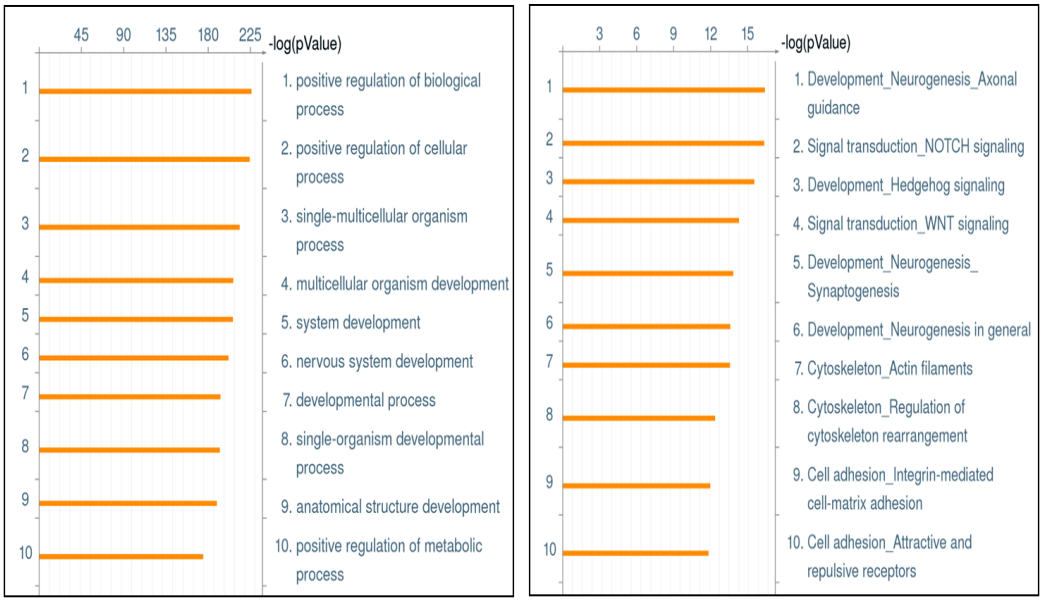
of miRNAs up or down-regulated by exposure of sodium arsenite or PQ+MB (Fig.4.26-4.27)

In 12week aged rats, *in silico* mapping has shown regulation in nervous system development, positive regulation of cellular process, system development, cell adhesion-attractive or repulsive receptors, cell ad as top five most significant targeted biological pathways by the targets of miRNAs up or down regulated by exposure of sodium arsenite or PQ+MB (Fig.4.28-4.29)



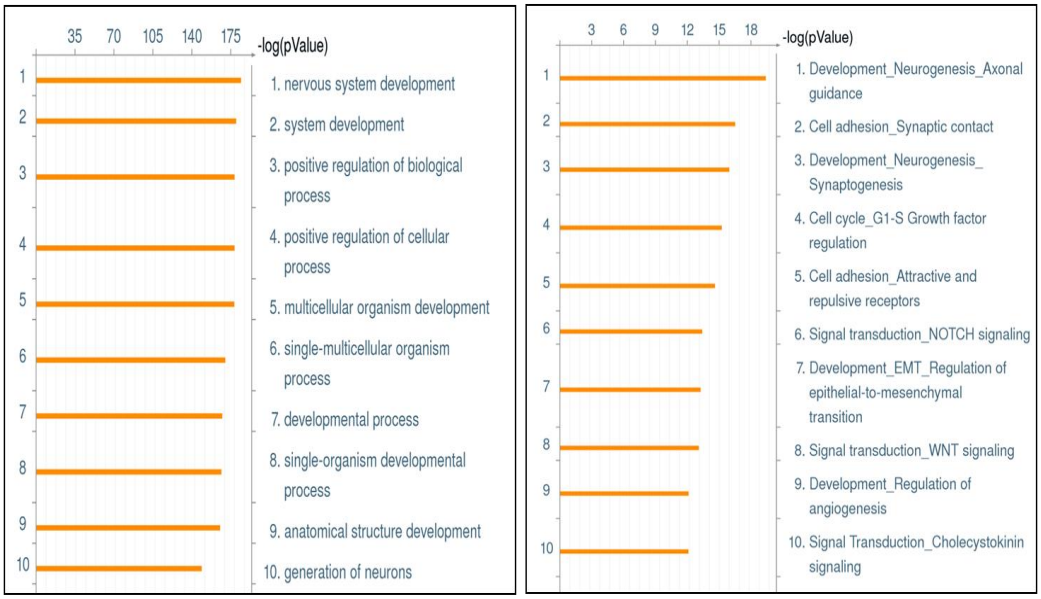
a) GO Process b) Process network

Fig 4.22: *In silico* pathway analysis of miRNAs up or down regulated by sodium arsenite exposure in 3week aged rat's brain. Most significant top 10 biological processes targeted by up or down regulated miRNA by sodium arsenite in 3week aged rat's brain identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.



a) GO Process b) Process network

Fig 4.23: *In silico* pathway analysis of miRNAs up or down regulated by PQ+MB exposure in 3week aged rat's brain. Most significant top 10 biological processes targeted by up or down regulated miRNA by PQ+MB in 3 week aged rat's brain identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.

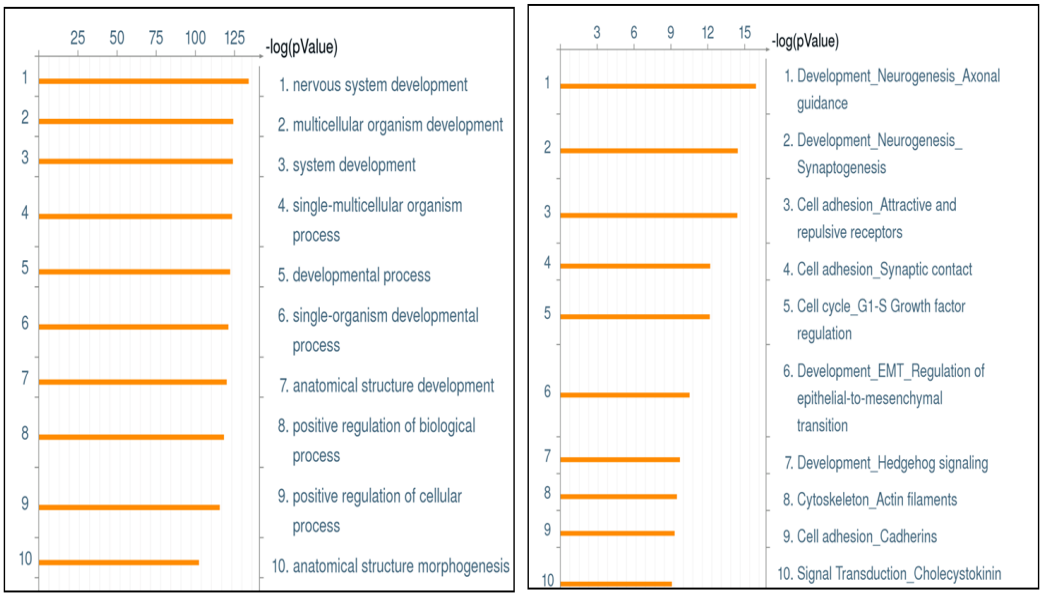


a) GO Process b) Process network

Fig.4.24: *In silico* pathway analysis of miRNAs up or down regulated by sodium arsenite exposure in 6 week aged rat's brain. Most significant top 10 biological processes targeted by up or down regulated miRNA by sodium arsenite in 6 week aged rat's brain identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.

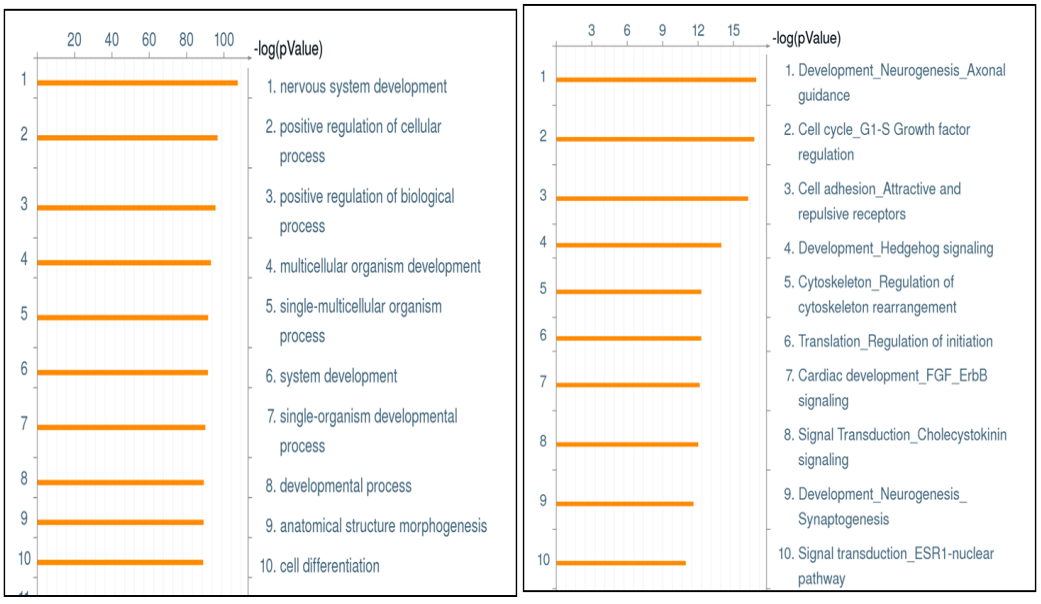
a) GO Process b) Process network

Fig.4.25: *In silico* pathway analysis of miRNAs up or down regulated by PQ+MB exposure in 6 week aged rat's brain. Most significant top 10 biological processes targeted by up or down regulated miRNA by PQ+MB in 6 week aged rat's brain identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.



a) GO Process b) Process network

Fig.4.26: *In silico* pathway analysis of miRNAs up or down regulated by sodium arsenite exposure in 9 week aged rat's brain. Most significant top 10 biological processes targeted by up or down regulated miRNA by Sodium Arsenite in 9 week aged rat's brain identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.

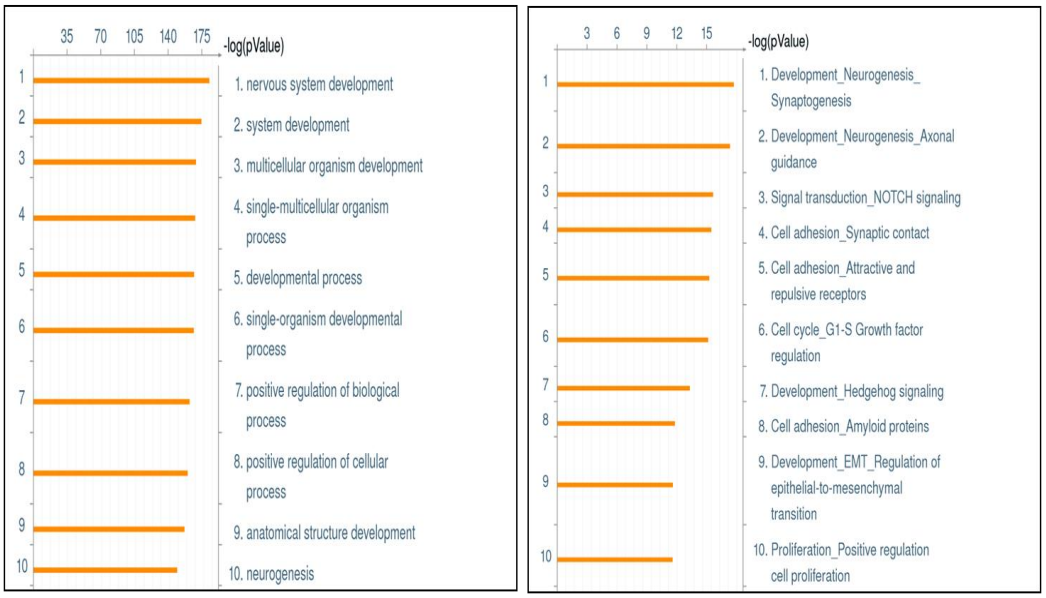


a) GO Process b) Process network

Fig.4.27: *In silico* pathway analysis of miRNAs up or down regulated by PQ+MB exposure in 9 week aged rat's brain. Most significant top 10 biological processes targeted by up or down regulated miRNA by PQ+MB in 9 week aged rat's brain identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.

a) GO Process b) Process network

Fig.4.28: *In silico* pathway analysis of miRNAs up or down regulated by sodium arsenite exposure in 12 week aged rat's brain. Most significant top 10 biological processes targeted by up or down regulated miRNA by Sodium Arsenite in 12 week aged rat's brain identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.



a) GO Process b) Process network

Fig.4.29: *In silico* pathway analysis of miRNAs up or down regulated by PQ+MB exposure in 12 week aged rat's brain. Most significant top 10 biological processes targeted by up or down regulated miRNA by PQ+MB in 12 week aged rat's brain identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.

4.17 Development of cellular model of neural development for mechanistic studies involving miRNAs.

PC12 cells are the clonal cells originated from a transplantable rat pheochromocytoma and exhibits the phenotypic properties coupled with pheochromocytomas and adrenal chromaffin cells. An imperative feature of PC12 cells is that they are known to counters the nerve growth factor (NGF) with a dramatic change in phenotype and acquire a number of properties which are the characteristics of sympathetic neurons. NGF-exposed PC12 cells cease proliferation, extend neurites, and become electrically excitable in nature.

4.18 Neurites outgrowth analysis

PC12 cells plated on multiwall dishes coated with poly-l-lysine (PLL; 0.01%) were exposed to NGF (50ng/ml) for 8 days. PC12 cells and neurites outgrowth quantification was performed to evaluate the neuritogenesis in cells. Cell morphology was observed and percentage of neurite-bearing PC12 cells was measured at time dependent manner by phase contrast microscopy. NGF significantly increases neurite outgrowth (12.58±1.11, 24.01±1.22, and 27.59±0.65 μm) in most of the cells (14.00±1.05, 22±1.25 and 26±1.32) after 2, 4 and 8 days of NGF exposure (Fig 4.30).

Morphological studies revealed that exposure of NGF (50ng/ml) to PLL adhered PC12 cells induced differentiation in PC12 cells. Immunocytochemical studies with anti-βIII-Tubulin has confirmed formation of new neurites by day 4 and formation of fully mature neuron by day 8 with fully developed dendrites and interconnections between the neurons (Fig 4.30).

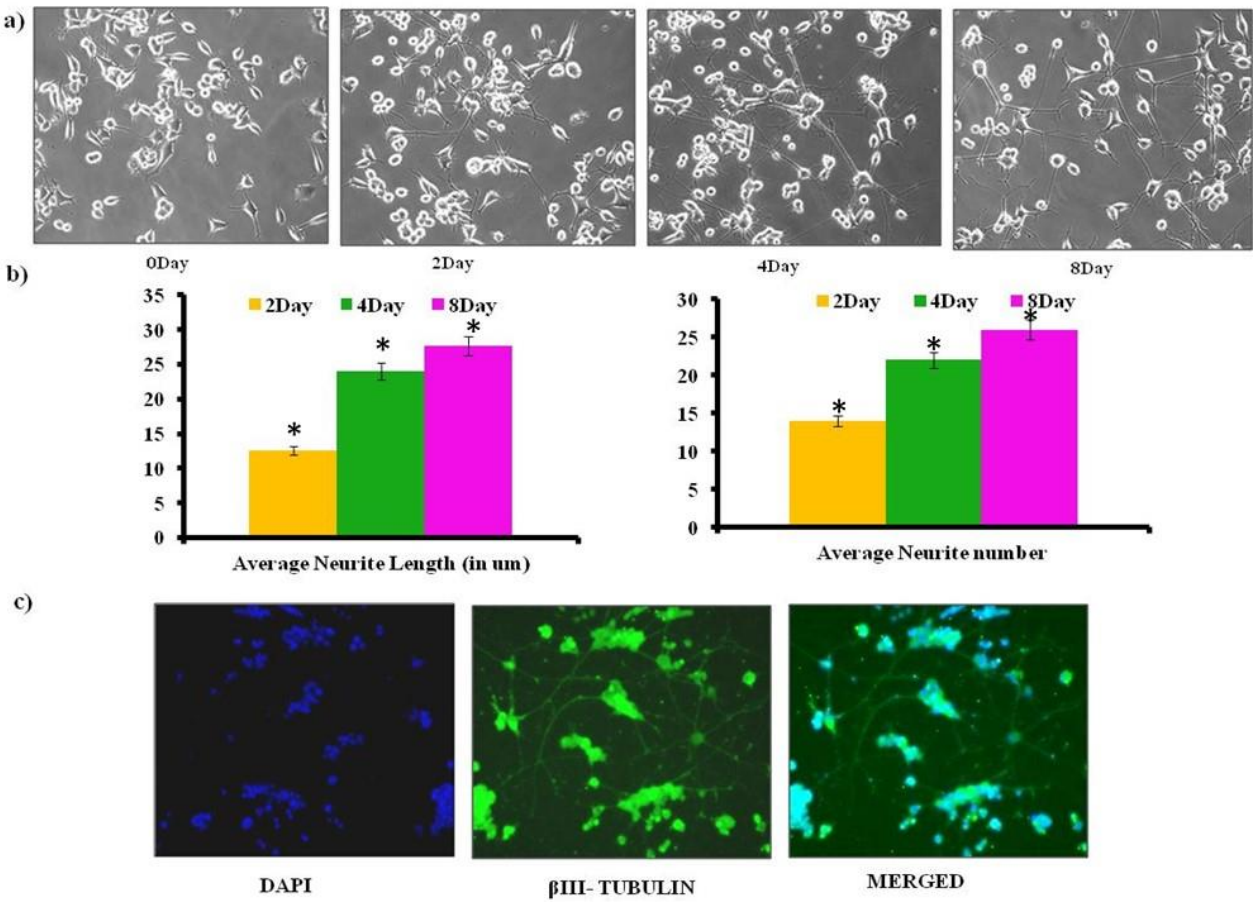


Fig 4.30. Exposure of NGF induced neuronal differentiation in PC12 cells: Phase contrast images (20x/40x) of NGF induced differentiation ofPC12 cells captured at 0 Day, 2 Day, 4 Day or 8 Day(a).Average neurite length and number in NGF induced differentiated PC12 cells at 0 Day, 2 Day, 4 Day and 8 Day (b). Immunocytochemical analysis of βIII-Tubulin expression in 8 Day NGF induced differentiated PC12 cells (c). Average neurite length and number were measured using Neuron J/ Image J software by analysing 10 independent images of each group. Images were captured using NIKON fluorescence inverted microscope.

4.19 Expression of neuronal and synaptic markers in NGF induced differentiated PC12 cells.

Expression of mRNAs and proteins of markers of neuronal development was studied in NGF induced differentiated PC12 cells at different stages of neuronal development. Western blot and real time PCR studies have shown a significant time dependent increase in the expression of NFL-M/NEFM, βIII-Tubulin, SYP, PSD95, NeuroG2, DCX and SYN1 at protein and mRNA level during differentiation of PC12 cells (Fig 4.31&4.32). Relative quantification studies of studied has shown that expression of NFL-M/NEFM (5.98,8.2,12.3), βIIITubulin (3.9,5.19,10.75), SYP (5.11,7.91,10.3), PSD95 (5.6,6.87,10.05), NeuroG2 (4.32,6.34,7.76), DCX (5.11,6.45,8.32), and SYN1 (5.31,7.98,10.21) significantly up-regulated in differentiated PC12 cells in time dependent manner (2day, 4day and 8day) as compare to undifferentiated PC12cells at mRNA level (Fig. 4.32)

4.20 Dicer knock-down impaired PC12 cells differentiation and increased senescence.

For studying the role of miRNAs in neuronal differentiation, Dicer (miRNA maturation enzyme) was knock-down in PC12 cells. Downregulation of Dicer was confirmed by immunoblotting and qPCR, which has shown significant downregulation in mRNA expression and protein levels of Dicer gene (Fig.4.33). Effect of Dicer knock-down in differentiating PC12 cells was studied on initiation of senescence using β-galactosidase staining, which is a well-established marker of senescence. Exposure to NGF for 5 days substantially increased staining of β-

galactosidase at pH 6 in Dicer knock-down cells, which confirms presence of senescent cells (Fig 4.33).

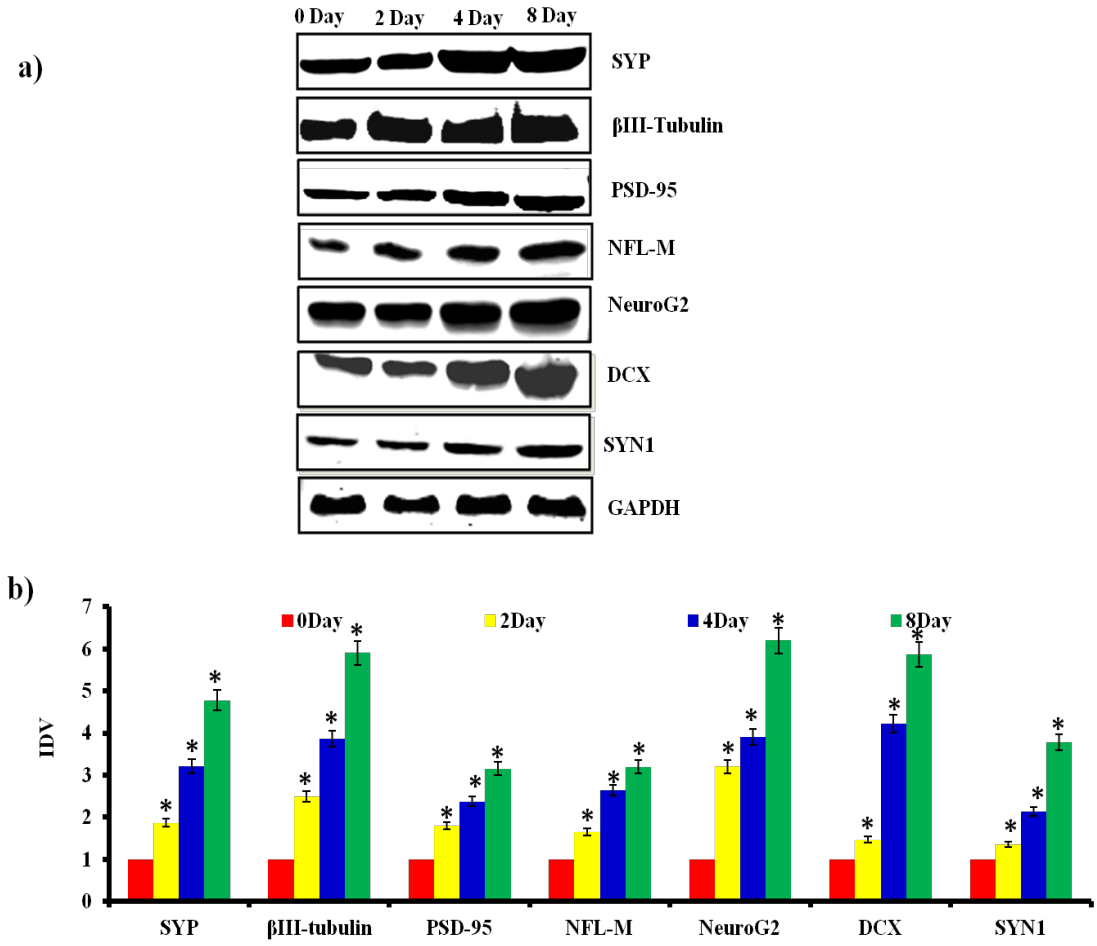


Fig 4.31: Expression pattern of different neuronal marker proteins during NGF induced differentiation in PC12 cells: (a) Western blots of SYP, βIII-Tubulin, PSD-95, NFL-M/NEFM, NeuroG2, DCX, SYN1 and GAPDH at 0 Day, 2 Day, 4 Day or 8 Day in NGF induced differentiated PC12 cells. (b) Densitometry of western blots of SYP, βIII-Tubulin, PSD-95, NFL-M/NEFM, NeuroG2, DCX, SYN1 and β-actin in NGF induced 0 Day, 2 Day, 4 Day or 8 Day differentiated PC12 cells. (IDV: Integrated Density Value pValue* <0.05, densitometry of western blots was performed using Image J software).

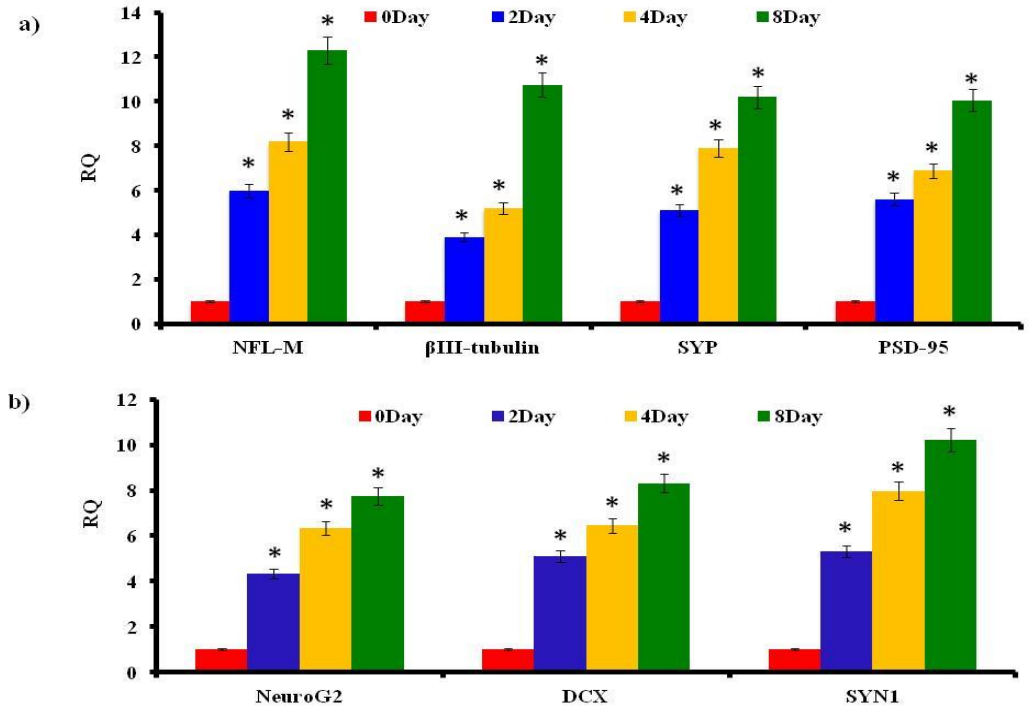


Fig 4.32: Expression pattern of different neuronal markers at mRNA level during NGF induced differentiation in PC12 cells: (a) Real time PCR of NFL-M/NEFM, βIII-Tubulin, SYP and PSD-95 at 0 Day, 2 Day, 4 Day or 8 Day in NGF induced differentiated PC12 cells. (b) Real time PCR of NeuroG2, DCX and SYN1 at 0 Day, 2 Day, 4 Day or 8 Day in NGF induced differentiated PC12 cells. (RQ: Relative quantification).

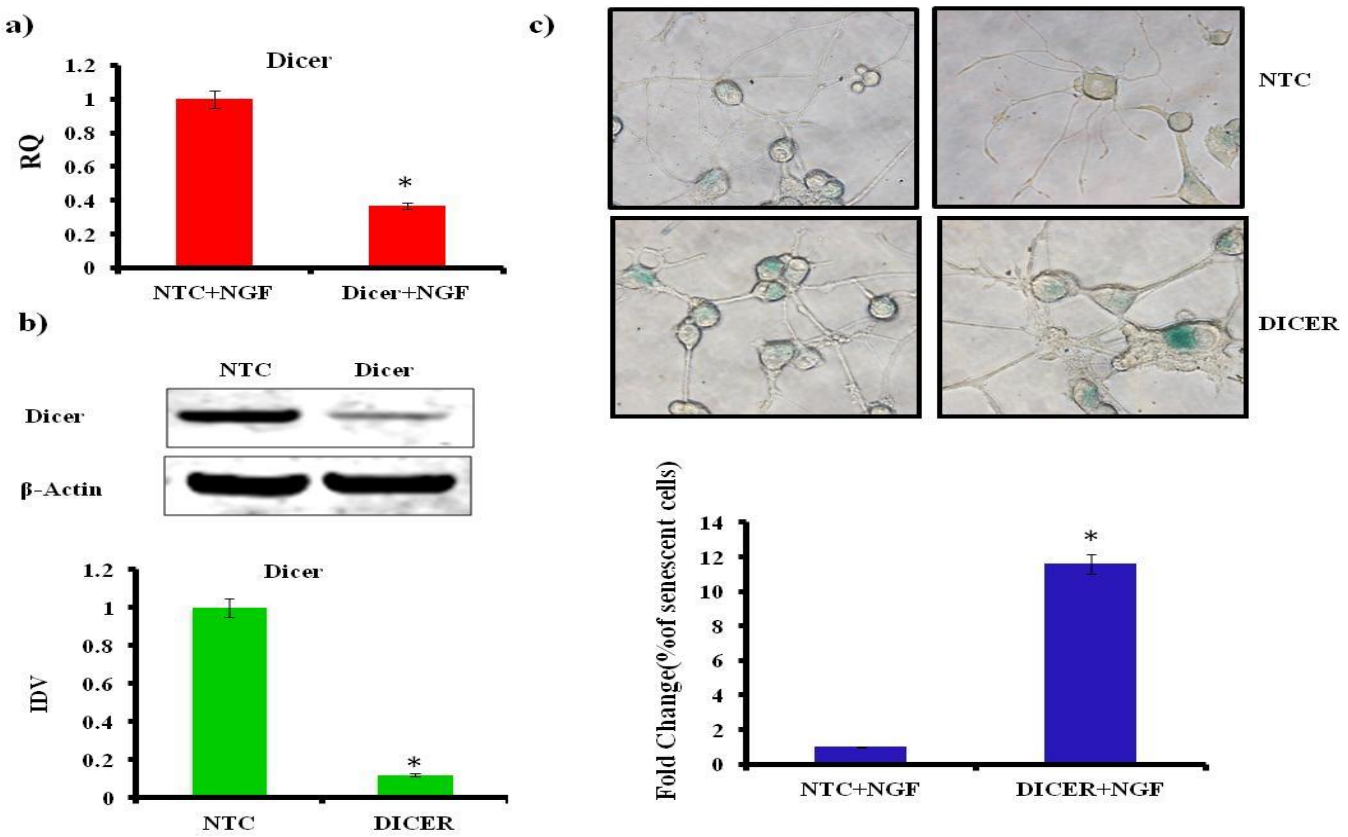


Fig 4.33: Dicer knockdown induced senescence in NGF induced differentiating PC12 cells. (a) Relative expression of Dicer in mRNA isolated from PC12 cells transfected with NTC or Dicer siRNA,(b) Western blot of Dicer in total cell lysates prepared from PC12 cells transfected with NTC or Dicer siRNA, and densitometry.(c) cell senescence assay by β-galactosidase staining in PC12 cells transfected with NTC or Dicer siRNA and exposed to NGF for 5 days and percentage of senescent cells. (IDV: integrated density value, RQ relative quantification; *$p \leq 0.05$, NGF (50ng/ml) for 5 days)

4.21 Regulation of "brain specific miRNAs" in differentiated PC12 cells.

On the basis of global miRNAs profiling in differentiated neurons, our earlier published studies reported that expression of miR-200, miR-221/222 and miR-34 family increased maximally during NGF induced differentiation of PC12 cells. In addition, to study the regulation of brain specific miRNAs in differentiated PC12 cells, customized "brain specific miRNA" array was used to identify the alteration in brain specific miRNAs profile during neuronal differentiation process in compare to naïve cells. Brain specific miRNA array profiling shown that expression of 14 miRNAs significantly altered including, miR-222 (44 folds), miR-221 (23 folds), miR-34a (18 folds) and miR-200a/c (6.5 & 5.2folds) are maximally up-regulated, while miR-503 was found to be significantly maximally down-regulated in NGF induced differentiated PC12 cells (Fig 4.34). Our earlier published studies have identified the crucial role of miR-200 family in neuronal differentiation and critical process of neuronal development. Our previous published and present results suggested miR-34 family as one of the highly up-regulated miRNAs family identified in differentiated PC12 cells. Our earlier published studies have identified the crucial role of miR-200 family in neuronal differentiation and critical process of neuronal development.MiR-34 family was one of the highly up-regulated miRNAs identified in differentiated PC12 cells.

Members of miR-34 family (miR-34a/b/c) were found to be significantly up-regulated in NGF induced differentiated PC12 cells (Fig 4.35). Similarly, miR-34 family (miR-34a/b/c) was also found to be significantly up-regulated during rat brain development.

(Fig 4.35). Among the miR-34 family members, miR-34a has shown highest increase in differentiated neurons and developing rat brain (Fig. 4.35).

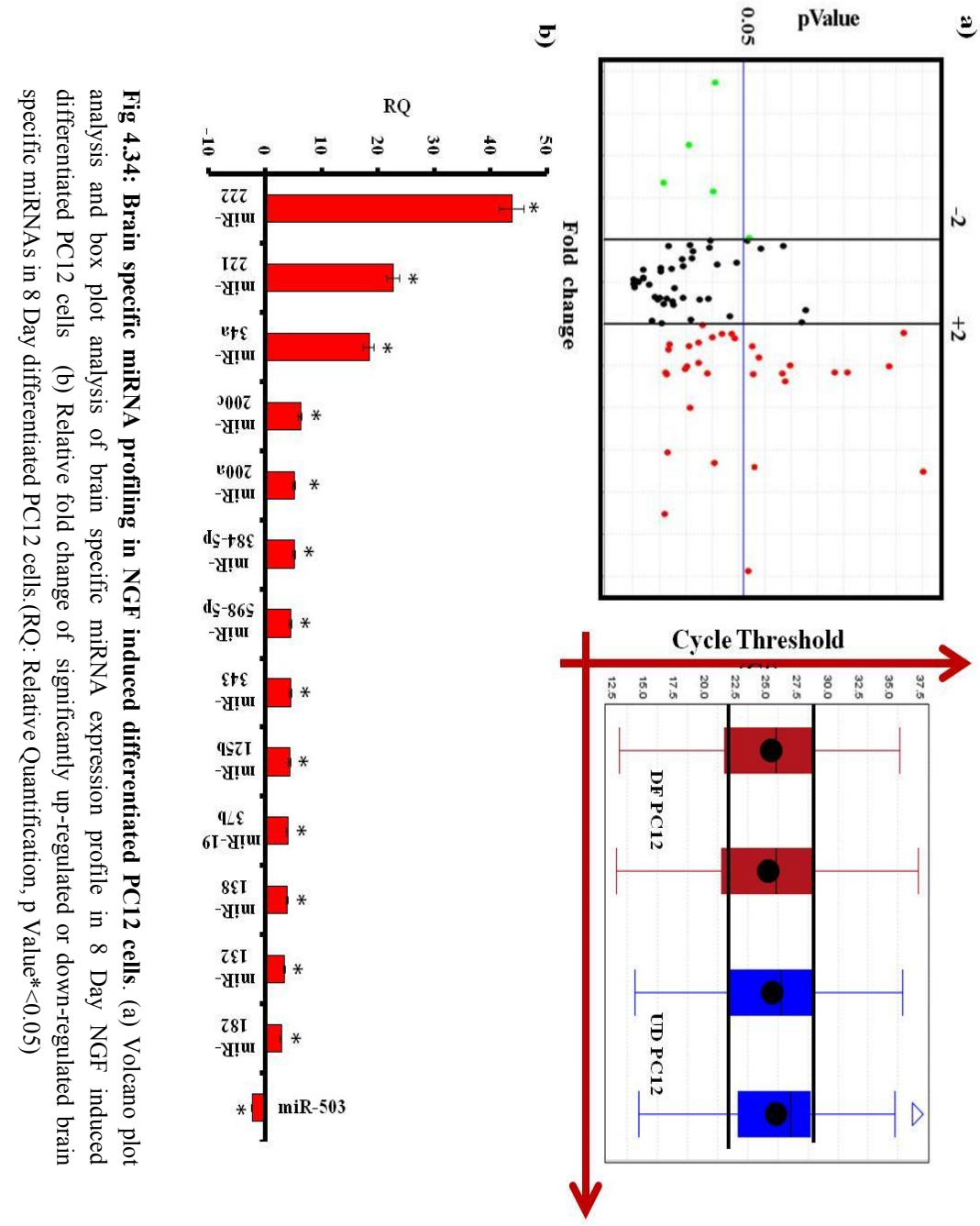


Fig 4.34: Brain specific miRNA profiling in NGF induced differentiated PC12 cells. (a) Volcano plot analysis and box plot analysis of brain specific miRNA expression profile in 8 Day NGF induced differentiated PC12 cells (b) Relative fold change of significantly up-regulated or down-regulated brain specific miRNAs in 8 Day differentiated PC12 cells.(RQ: Relative Quantification, p Value*<0.05)

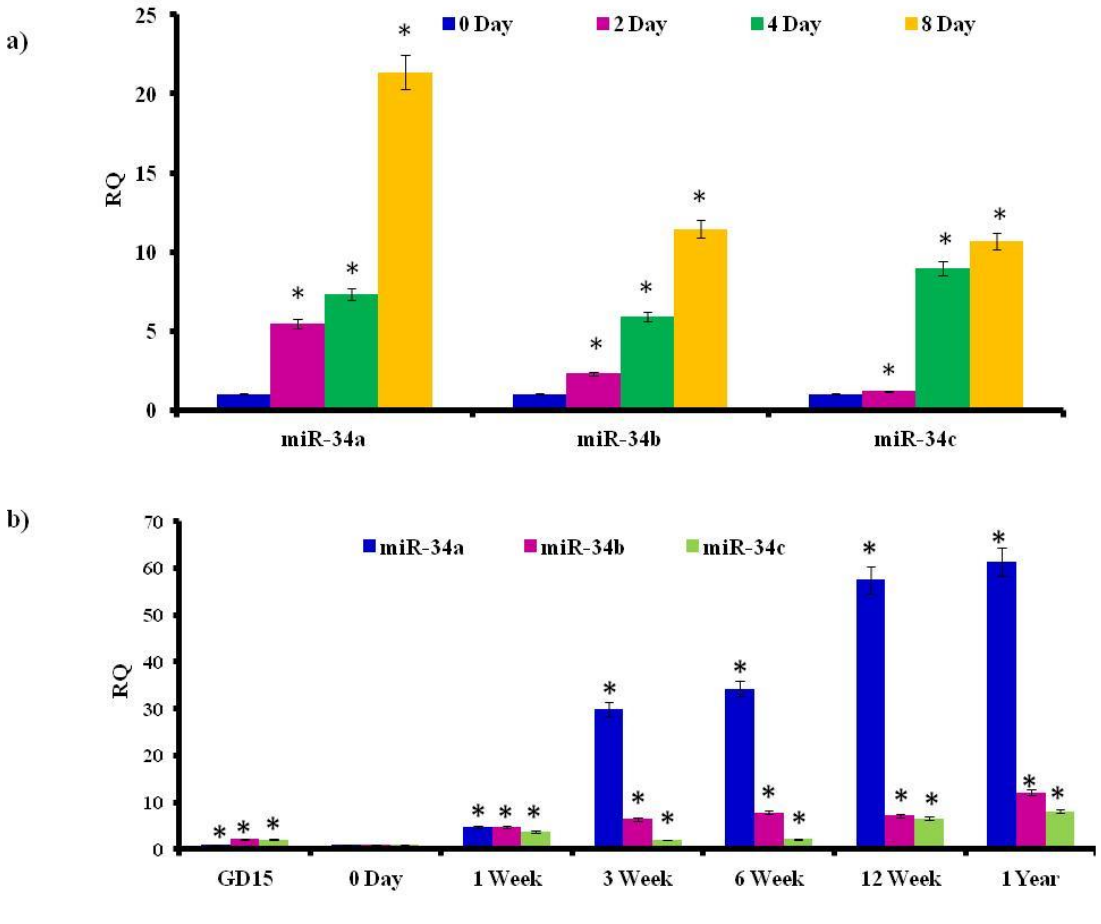


Fig 4.35: Expression of miR-34 family in NGF induced differentiated PC12 cells. (a) Relative quantification of miR-34 family (miR-34a, miR-34b and miR-34c) in NGF induced 0 Day, 2 Day, 4 Day and 8 Day differentiated PC12 cells. (b) Relative quantification of miR-34 family (miR-34a, miR-34b and miR-34c) in developing rat brain from GD15 to 1 year aged rat. (GD: Gestational day, RQ: Relative quantification)

4.22 Expression of miR-34 family in tissues of different origin:

Real time PCR of miR-34a, miR-34b and miR-34c was carried out in equal amount of total RNA isolated from brain, liver and lung tissues have identified brain as richest source of miR-34a and miR-34c (Fig 4.36). Interestingly, miR-34b is expressed maximally in lung (5.3 Folds of whole brain). Analysis of brain parts results have shown that miR-34a and miR-34c have almost same Ct values throughout the brain, but miR-34b have 71.31 folds more (in comparison to whole brain) expression in cerebellum (Fig 4.36).

Tissue	U6 Ct Value	miR-34a		miR-34b		miR-34c	
		Ct Value	RQ	Ct Value	RQ	Ct Value	RQ
Hypothalamus (Adult)	11.781±0.02	20.337±0.14	0.687	26.820±0.17	4.666	20.268±0.24	2.836
Hippocampus (Adult)	11.705±0.12	21.208±0.23	4.405	29.306±0.19	.625	22.578±0.28	.526
Cerebellum (Adult)	11.790±0.45	17.917±0.15	1.426	20.535±0.21	71.318	26.470±0.23	0.048
Frontal Cortex (Adult)	11.938±0.15	17.782±0.17	1.766	28.493±0.22	1.126	22.131±0.21	0.895
Pons medulla (Adult)	11.651±0.36	20.495±0.19	0.522	28.402±0.16	1.129	21.318±0.27	0.403
Mid brain (Adult)	11.789±0.45	20.275±0.21	0.732	28.039±0.18	1.130	21.357±0.25	0.354
Whole brain (Adult)	11.766±0.25	19.111±0.23	1	28.980±0.24	1	21.952±0.25	1
Liver (Adult)	11.653±0.36	31.915±0.29	0.006	30.868±0.29	0.118	34.231±0.31	0.006
Lung (Adult)	11.652±.24	29.559±0.28	0.008	26.665±0.21	5.359	29.903±0.24	0.015

Fig 4.36: Expression of miR-34 family in adult rat whole brain or brain regions. Relative quantification of miR-34 family (miR-34a, miR-34b and miR-34c) in whole brain along with its regions including hypothalamus, hippocampus, cerebellum, frontal cortex, pons medulla, mid brain, Along with brain, adult liver tissue and lung tissue was also quantified. (U6: snRNA for normalization, Ct: cycle threshold)

4.23 Expression of miR-34a supports NGF induced differentiation of PC12 cells:

Expression of miR-34a was significantly up-regulated during neuronal differentiation of differentiated PC12 cells. However, immunostaining with βIII-Tubulin (marker of mature neurons) in PC12 cells transfected with mimics of miR-34a have not shown any significant change in immunoreactivity of βIII-Tubulin or size and number of neurites (Fig 4.37).

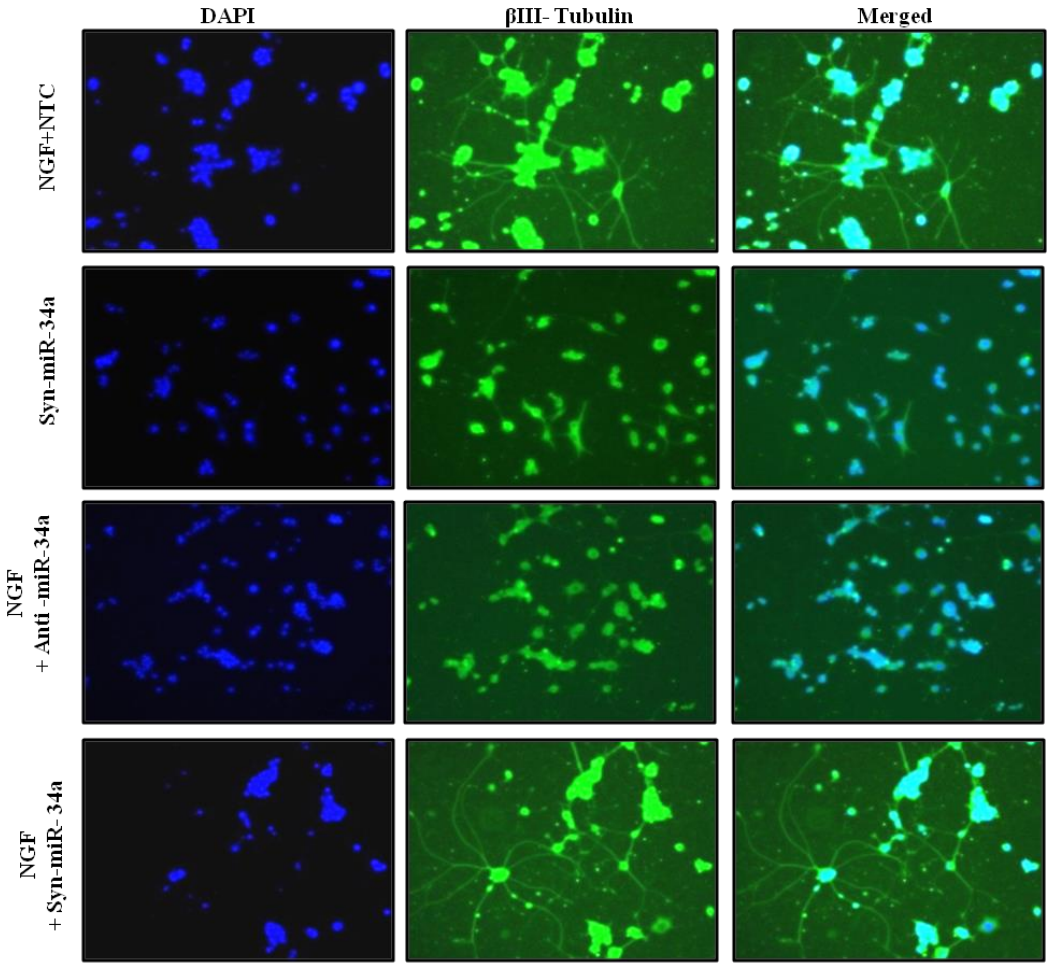


Fig 4.37: Effect of miR-34a on neuronal differentiation of PC12 cells. Immunocytochemical analysis of neuritogenesis in PC12 cells transfected with miR-34a mimics or exposed with NGF and transfected with either NTC or Syn-miR-34a or Anti-miR-34a expression using βIII-Tubulin as neuronal marker.

So, we have studied the additive effect of miR-34a over-expression in differentiation of PC12 cells pre-exposed with NGF. Measurements of neurite length and number have shown a significant increase in neurite number and length in PC12 cells transfected with miR-34a mimics, which are pre-exposed with NGF and transfected with miR-34a in comparison to only NGF exposed PC12 cells and NGF with anti-miR-34a transfected PC12 cells (Fig.4.38). Induction of βIII-Tubulin and NFL-M/NEFM in NGF exposed and miR-34a transfected PC12 cells have also observed in immunoblotting and real time PCR studies (Fig.4.39).

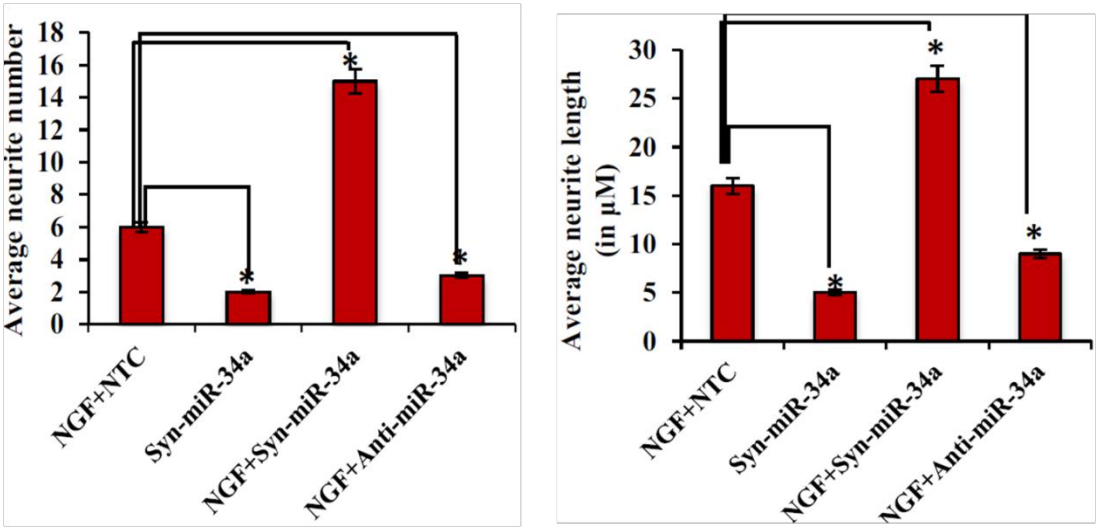


Fig 4.38: Analysis of average neurite number and length in immunocytochemical images carried out in miR-34a transfected differentiated PC12 cells. Measurement of neurite length and number measured using Nikon-BR software of Nikon-NIS microscope.

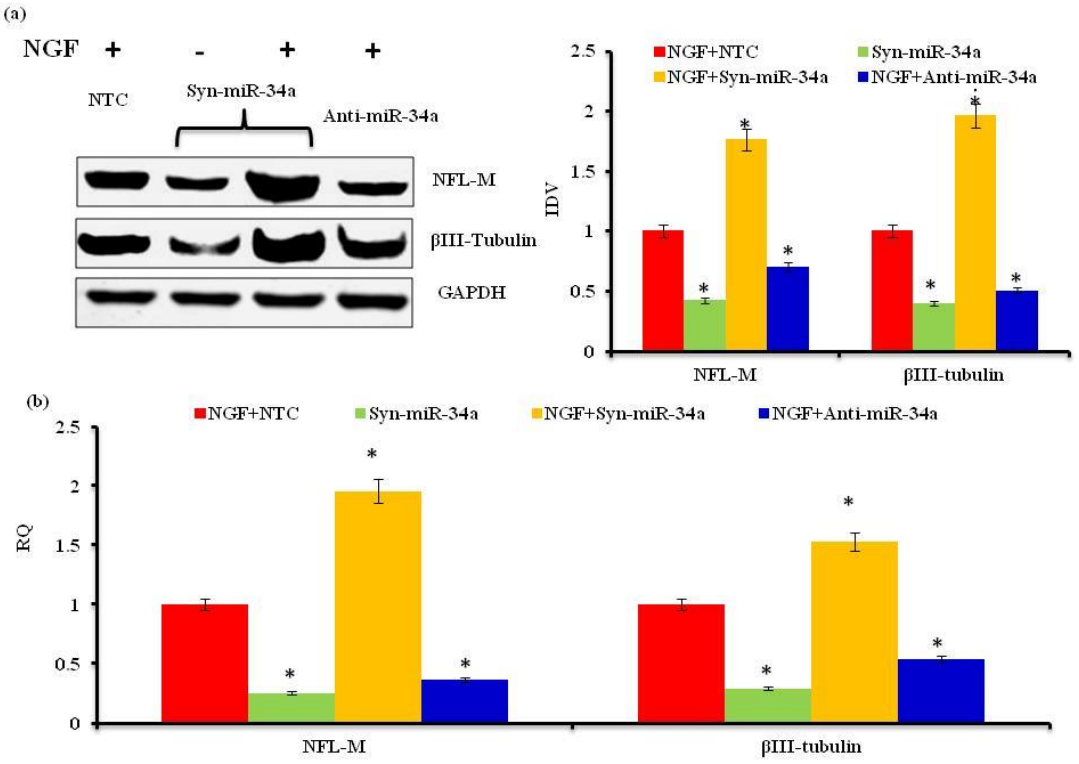


Fig 4.39: Effect of miR-34a on expression of neuronal markers: (a) western blotting of, βIII-Tubulin and NFL-M/NEFM in total cell lysates prepared from PC12 cells transfected with either NTC or mimic or inhibitor of miR-34a or exposed with NGF and, (b) Real time PCR of, βIII-Tubulin and NFL-M/NEFM in total cell lysates of NGF exposed PC12 cells transfected with Syn-miR-34a or Anti-miR-34a or NTC. (RQ: Relative Quantification; pValue* < 0.05, IDV: Integrated density value, Densitometry of western blot carried out by Image J software)

4.24 Ectopic overexpression of miR-34a induced G1 Phase arrest in PC12 cells.

Flow-cytometric analysis of cell cycle phase in PC12 cells transfected with miR-34a mimics have shown significant increase in number of cells falling in G1 phase of cell cycle similar to NTC transfected or NGF exposed PC12 cells (Fig 4.40). In NTC transfected PC12 cells , approx 40.62% cells falls in G1 phase, while in NGF exposed PC12 cells approx 73.12% cells were in G1 phase. Transfection of miR-34a mimics increased % of G1 phase cells from 40 to 61.47%. (Fig 4.40).

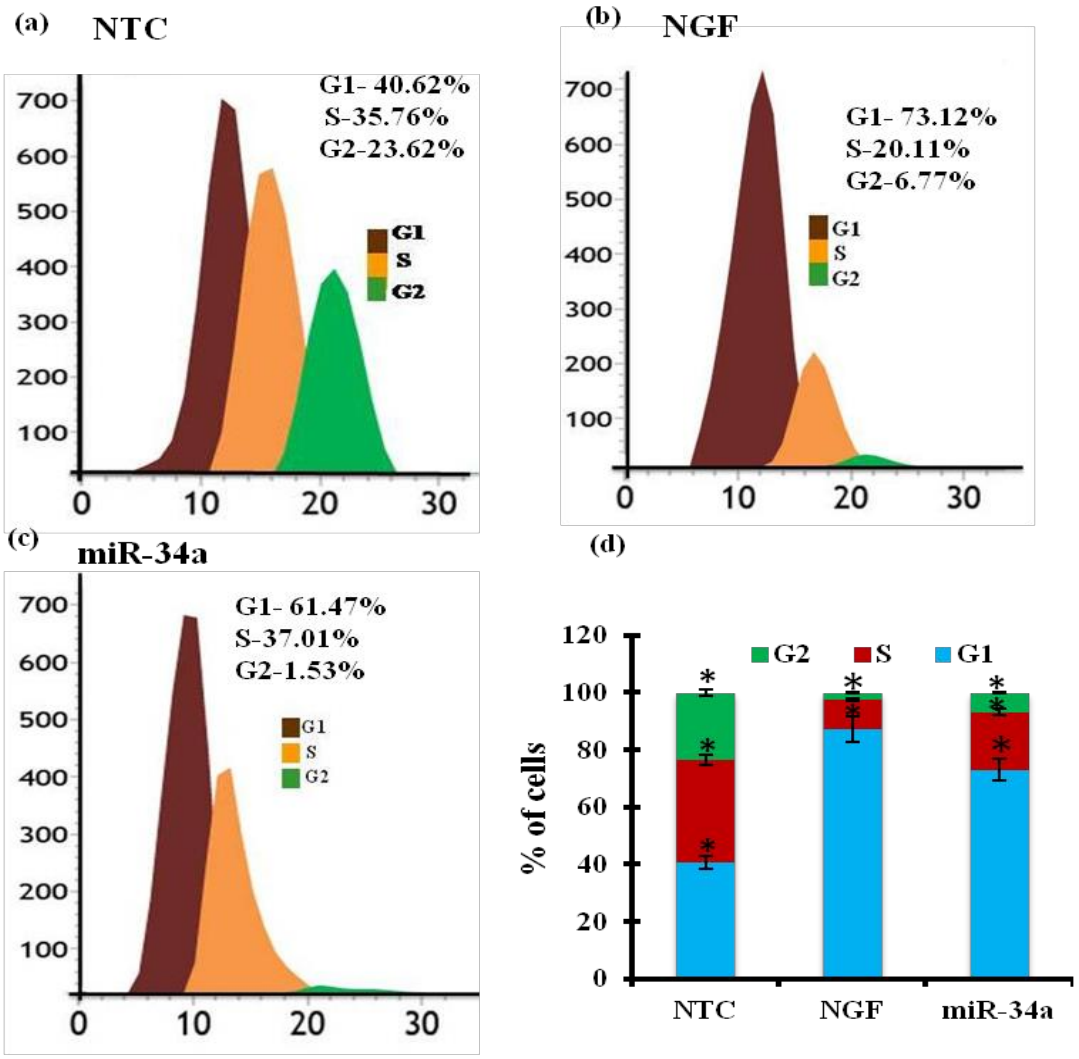


Fig 4.40 Transfection of miR-34a induces G1 Phase arrest in PC12 cells: Flow cytometry based PI uptake assay for cell cycle analysis of dividing PC12 cells NTC, (a) cells were transfected with NTC (b) cells exposed with NGF (c) cells transfected with mimics of miR-34a (25 nM) (d) Bar diagram of cell cycle analysis in NTC or NGF exposed or Syn-miR-34a transfected PC12 cells. (All values are the mean of three individual experiments. Significant changes are calculated by Student's t test. pValue* < 0.05)

4.25 Feedback loop operates between P53 and miR-34a in PC12 cells

Our earlier studies have shown a time dependent increase in level of P53, when PC12 cells were differentiated with NGF. Induction of P53 by exposure of CP-31398 (a P53

stabilizer) up-regulated P53 levels in PC12 cells (Fig 4.41a). Along with P53, exposure of CP-31398 to PC12 cells has also shown dose dependent increase in expression of miR-34a, while expression of miR-34b and miR-34c were unchanged (Fig 4.41b). Scanning of 3'UTR of P53 in rat, mice and human have shown presence of miR-34a targeting site in rats, however human and mice P53 do not harbor miR-34a target site (Fig 4.41c).

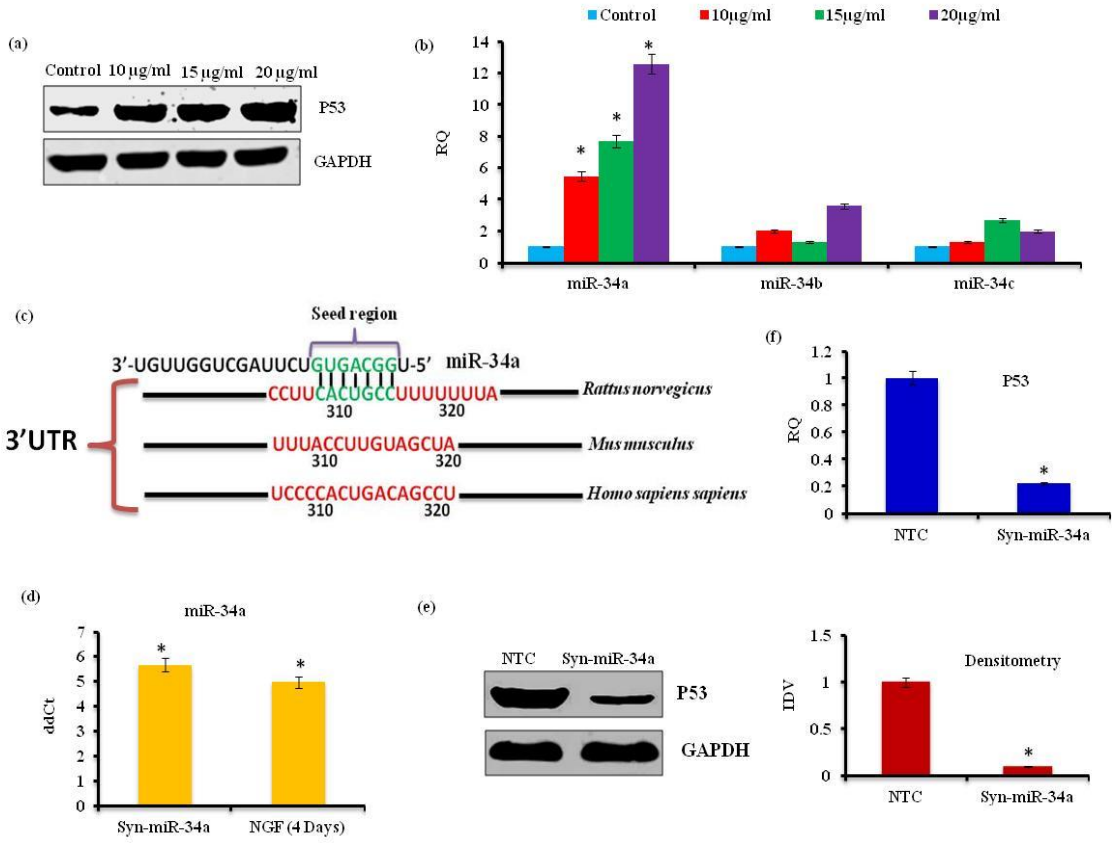


Fig 4.41. Feedback loop operates between P53 and miR-34a in PC12 cells: (a) Western blotting of P53 in total cell lysates of PC12 cells exposed with either 10, 15 or 20 µg/ml of CP-31398 for 8 h, (b) Real time PCR of miR-34 family in PC12 cells exposed with CP- 31398 (10 or 15 or 20 µg/ml for 8 h), (c) In silico analysis of 3'UTR of P53 gene for binding site of miR-34a in Rat, Mouse and Human using TargetScan web portal, (d) Real time PCR of miR- 34a in PC12 cells transfected with mimics of miR-34a or exposed with NGF, (e) Western blotting and densitometry of P53 in total cell lysates prepared from PC12 cells transfected with mimics of miR-34a, (f) Real time PCR of P53 in PC12 cells transfected with mimics of miR-34a. (RQ: Relative Quantification; pValue* >0.05)

Transfection of PC12 cells with mimics of miR-34a (25nM) induced the expression of miR-34a similar to 4 days NGF differentiated PC12 cells (Fig 4.41d). Interestingly, in miR-34a mimic transfected cells, levels of P53 down-regulated at both mRNA and protein levels (Fig 4.41e&f).

4.26 *In silico* pathway analysis of miR-34a of miR-34 family.

Online In silico gene ontology pathway analysis for the all possible predicted targets of miR-34a has predicted, nervous system development, head development, brain development, regulation of cell communication and central nervous system development as most five significant pathways.

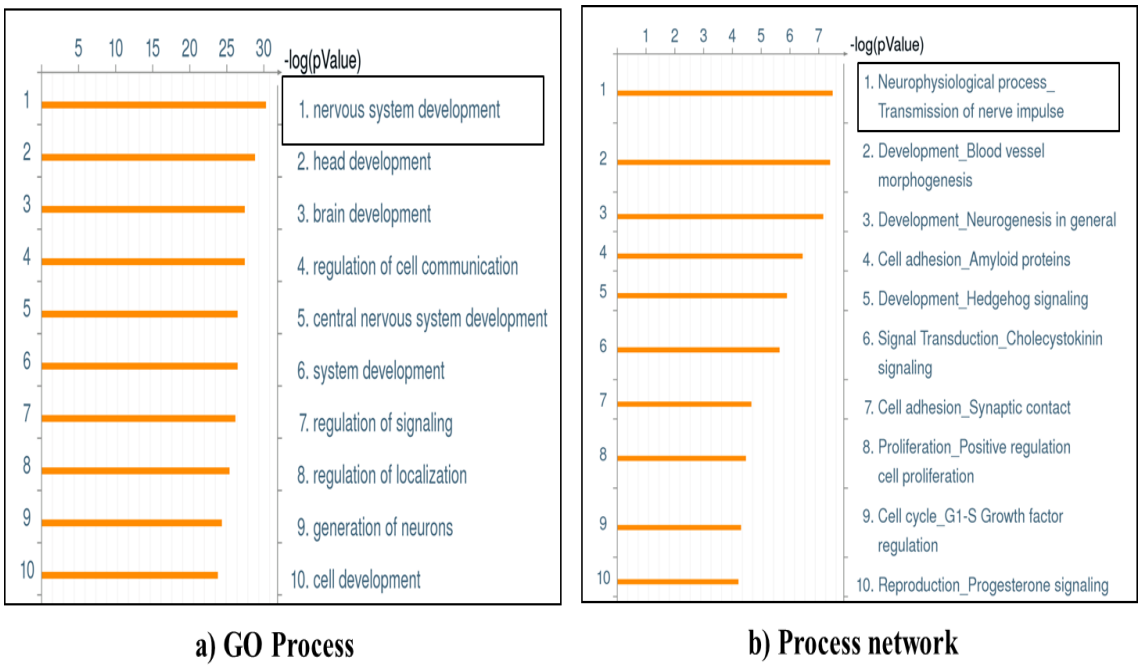


Fig.4.42: *In silico* pathway analysis of miR-34a of miR-34 family. Most significant top 10 biological processes targeted by miR-34a identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.

In addition neurophysiological process transmission of nerve impulse, development of blood vessel morphogenesis, development of neurogenesis in general, cell adhesion, amyloid proteins, and development of hedgehog signaling as the top five most significantly targeted process networks (Fig 4.42).

4.27 Identification of non-cytotoxic dose of sodium arsenite or paraquat in PC12 cells.

For the identification of non-cytotoxic dose of sodium arsenite or paraquat, MTT assay and Alamar Blue Assay were performed. PC12 cells were incubated with sodium arsenite (2.5μM-40μM) or Paraquat (25μM- 400μM) for 24, 48 and 72 hours. Exposure of PC12 cells with sodium arsenite or paraquat did not produced any

significant alterations at 2.5μM in case of sodium arsenite or 25μM in case of paraquat in both MTT and AlamarBlue assay. Concentration 5μM (sodium arsenite) and 50μM (paraquat) were identified for further experiments based on their non-cytotoxicity nature (Fig 4.43).

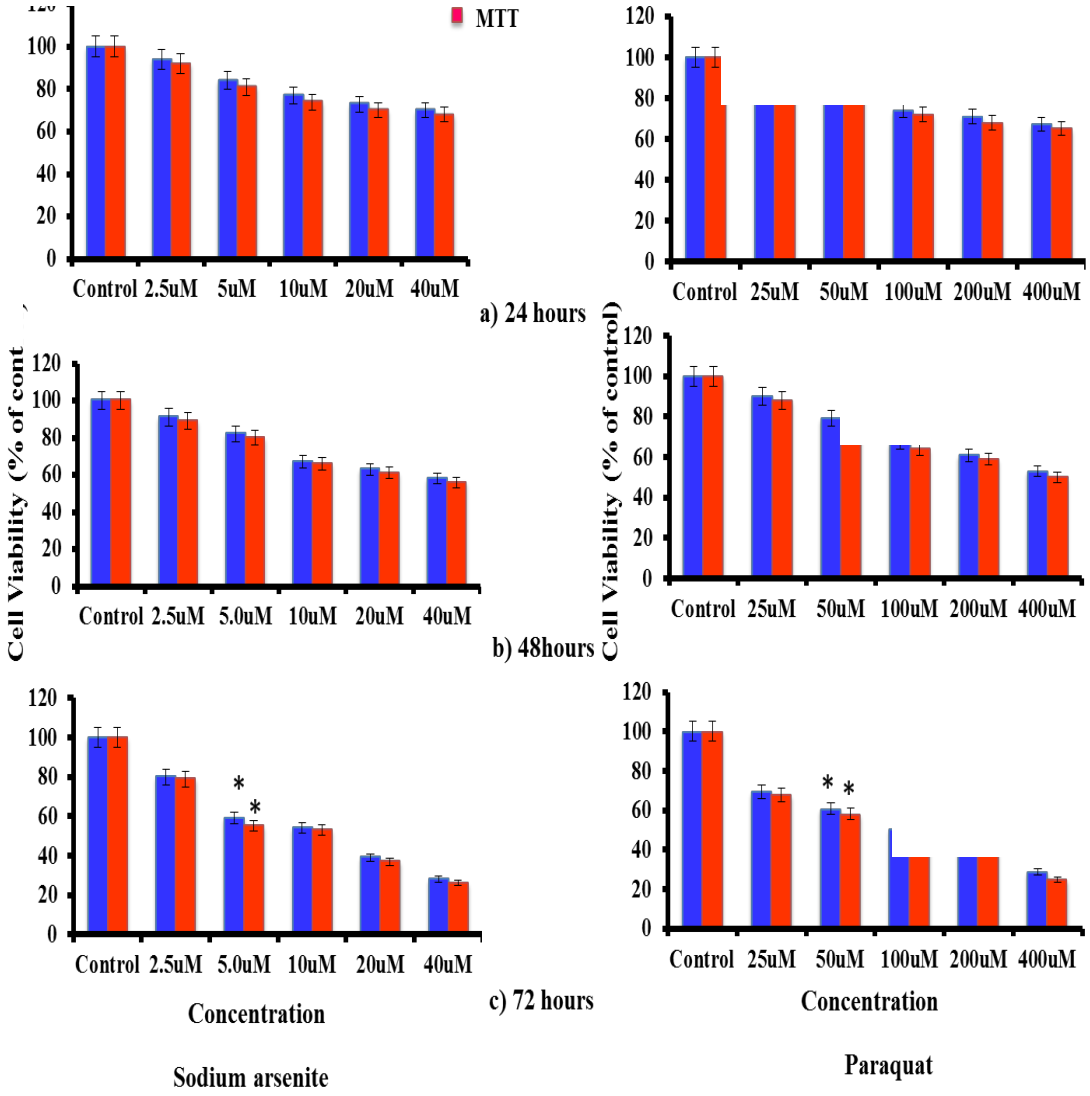


Fig 4.43. Identification of non-cytotoxic dose of sodium arsenite or paraquat in PC12 cells: Alamar blue and MTT assay were done to identify the non-cytotoxic dose of sodium arsenite or paraquat in PC12 cells at a) 24hours b) 48hours and c) 72 hours.

4.28 Flow cytometric studies for apoptosis, Reactive oxygen species (ROS) and mitochondrial membrane potential (MMP) measurements.

PC12 cells were exposed with sodium arsenite (5µM) or Paraquat (50µM) and analyzed the cellular apoptosis using flow-cytometry. Apoptotic studies revealed that exposure of sodium arsenite (5µM) induced early apoptosis (24.02%), late apoptosis (8.12%) and necrosis (5.41%) in PC12 cells while in case of Paraquat, cells undergone early apoptosis (14.12%), late apoptosis (0.60%) and necrosis (1.01%) in compare to unexposed or control cells (Fig 4.44).

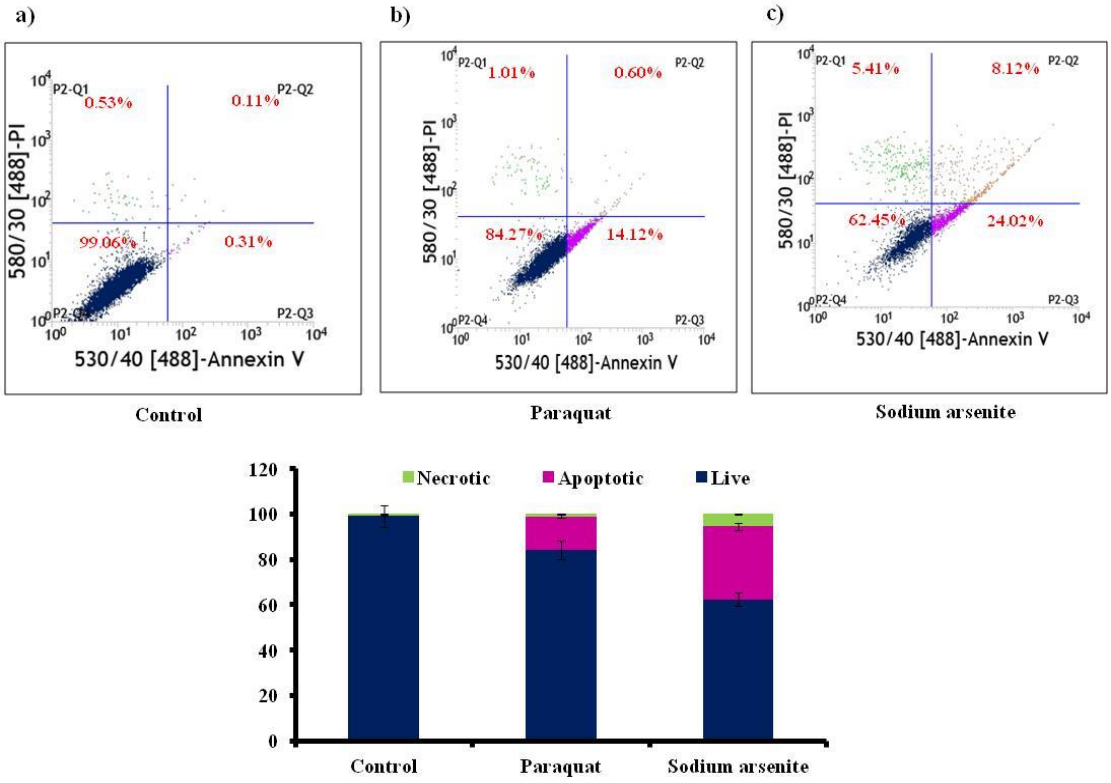


Fig 4.44. Exposure of sodium arsenite or paraquat induced apotosis inPC12 cells. Dot plot of control (a), Paraquat (b) and sodium arsenite (c) exposed PC12 cells for 72 hours stained with Annexin V and Propidium iodide. Bar diagram represents the total percentage of cells exposed to sodium arsenite or paraquat undergone apoptosis. Flowcytometry was performed on BD Influx after staining of experimental cells with Annexin V-FITC and Propidium Iodide for 15 minute. All experiments were performed in triplicates only representative images are shown here.

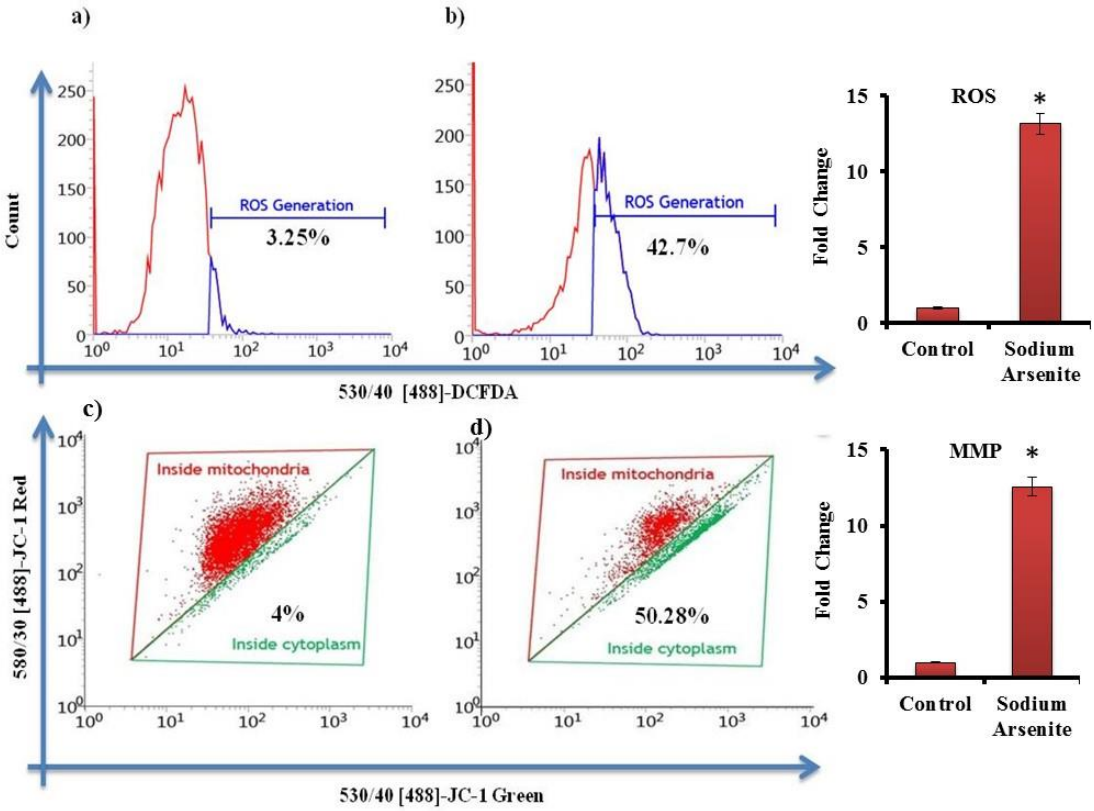


Fig 4.45 Flow-cytometric studies carried out for ROS and MMP in PC12 cells: ROS was measured using DCFDA dye in a) Control and b) Sodium arsenite exposed PC12 cells at 72 hours while MMP was measured using JC1 dye in c) Control and d) Sodium arsenite exposed P12 cells at 72 hours. Bar diagram represents the total percentage of cells exposed to sodium arsenite producing ROS and loss of MMP.

Reactive oxygen species (ROS) formation was measured by flow cytometry in PC12 cells exposed with sodium arsenite (5µM) or paraquat (50µM) using DCFDA dye. Sodium arsenite generated a higher amount of ROS (42.7%) while paraquat produced (23.2%) of ROS in compare to control cells (Fig 4.45-4.46).

Loss of mitochondrial membrane potential (MMP) studies suggested that sodium arsenite or paraquat hampers the normal mitochondrial function by altering the MMP of PC12 cells. Sodium arsenite (5µM) inverts MMP by 50.28% while paraquat (50µM) leads to loss of 25.54% in PC12 cells using JC1 dye (Fig 4.45-4.46).

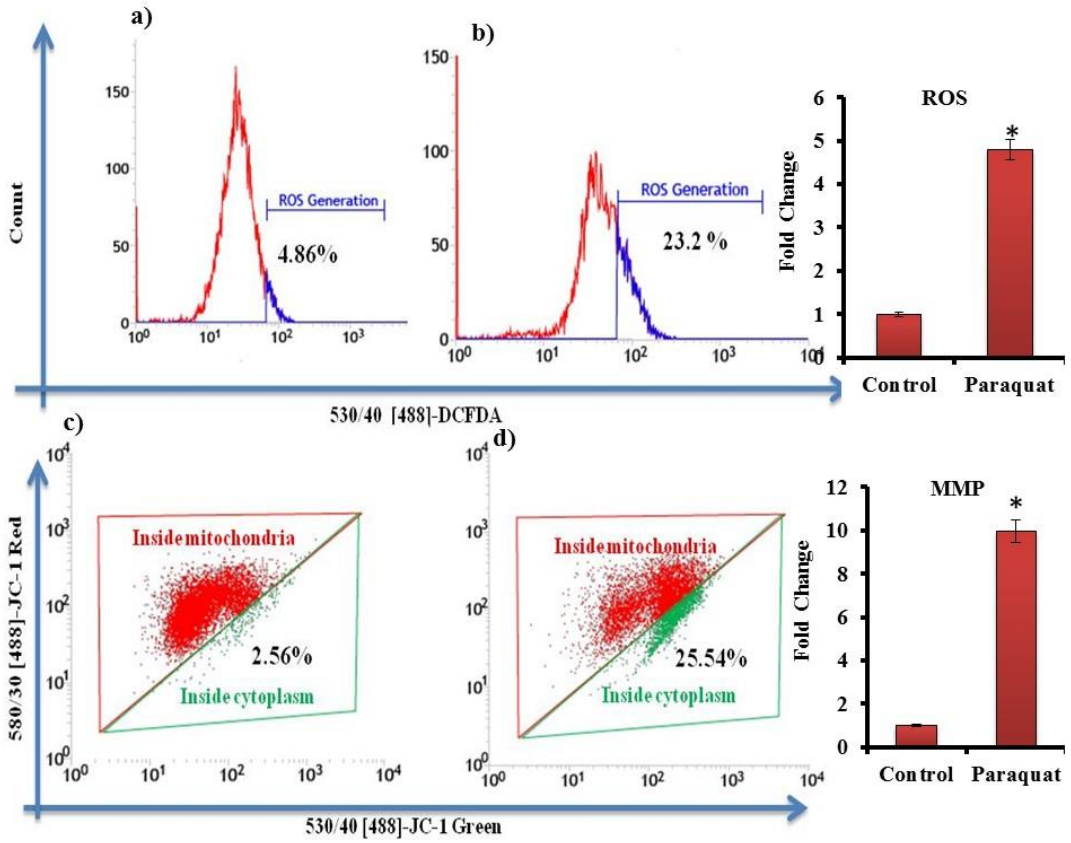


Fig 4.46 Flow-cytometric studies carried out for ROS and MMP in PC12 cells: ROS was measured using DCFDA dye in a) Control and b) Paraquat exposed PC12 cells at 72 hours while MMP was measured using JC1 dye in c) Control and d) Paraquat exposed PC12 cells at 72 hours. Bar diagram represents the total percentage of cells exposed to paraquat producing ROS and loss of MMP.

4.29 Morphological studies of neurites integrity in sodium arsenite or paraquat exposed differentiated PC12 cells.

8 Day NGF exposure to PC12 cells produced fully differentiated PC12 cells, which behaves like mature neurons. To study the effect of sodium arsenite or paraquat on neuronal integrity, differentiated PC12 cells were exposed to sodium arsenite (5µM) or Paraquat (50µM). Calcein-Am dye based staining has demonstrated that exposure of sodium arsenite (5µM) or paraquat (50µM) significantly decreased the number and

length of neurites in differentiated PC12 cells in compare to their matched controls (Fig 4.47). Moreover, effect of sodium arsenite has shown more disruption in neurites in compare to control and paraquat exposed differentiated PC12 cells (Fig 4.47).

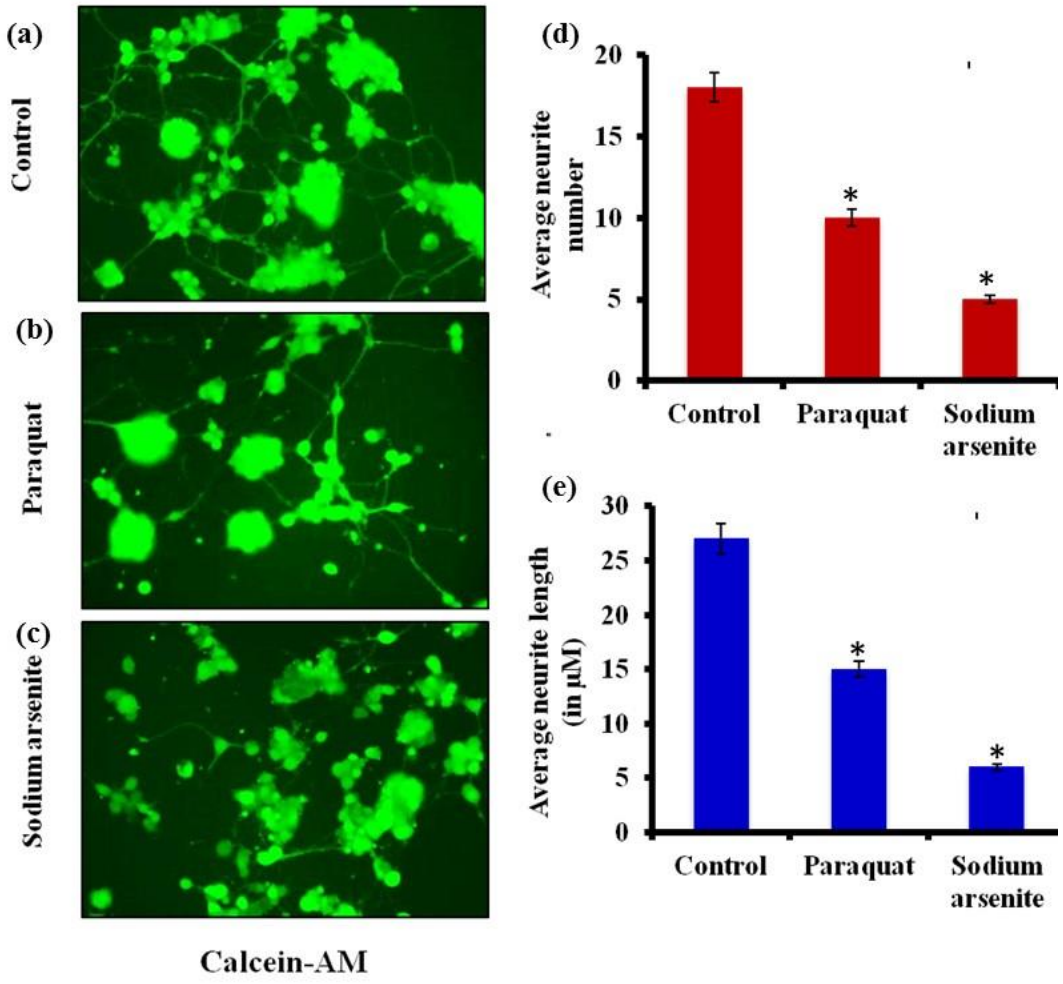


Fig 4.47. Calcein AM dye studies carried out for neurite outgrowths in differentiated PC12 cells: Neurite outgrowth was measured using calcein dye in a) Control ,b) Paraquat and c) Sodium arsenite exposed differentiated PC12 cells at 72 hours. d) Average number and and e) average neurite length of NGF induced differentiated cells exposed to paraquat or sodium arsenite was calculated using Nikon NIS-Br software.

4.30 Immunocytochemical studies carried out in sodium arsenite or paraquat exposed NGF differentiated PC12 cells.

Exposure of NGF for 8days results in the formation of mature and functional neurons. Moreover, when mature neurons were exposed to sodium arsenite or paraquat, disruption of neurites takes place which decreases the number and length of neurons. Immunocytochemical studies have shown an induction of neurofilament (NFL-M/NEFM), a neuronal marker in NGF induced differentiated PC12 cells. Further, exposure of sodium arsenite in NGF differentiated PC12 cells; expression of NFL-M/NEFM was significantly down-regulated in compare to control cells. Furthermore, paraquat exposure to differentiated PC12 cells has also shown down-regulation in NFL-M/NEFM expression in compare to control differentiated PC12 cells (Fig 4.48).

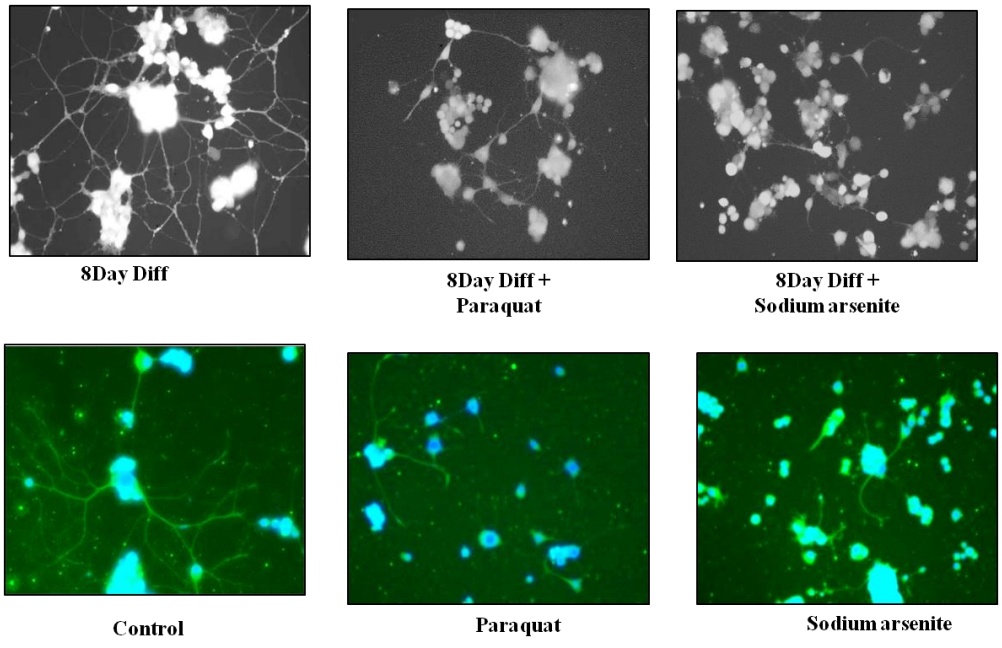


Fig 4.48: Phase contrast and immunocytochemical images of NGF induced differentiated PC12 cells exposed to paraquat or sodium arsenite. Brightfield images were captured after the exposure of sodium arsenite or paraquat for 72 hours while immunocytochemistry was carried out in sodium arsenite or paraquat exposed differentiated PC12 cells using NFL-M/NEFM as neuronal marker.

4.31 Expression of neuronal and synaptic markers in NGF induced differentiated PC12 cells exposed to sodium arsenite or paraquat.

Expression of neuronal and synaptic markers was significantly down-regulated after the exposure of sodium arsenite (5µM) or paraquat (50µM) in differentiated PC12 cells at transcriptional as well as at translational level. Real time PCR studies suggested that sodium arsenite altered the expression of PSD95 (-4.5folds), SYP (-2.7folds), βIII-Tubulin (-5folds), NFL-M/NEFM (-6.25folds), DCX (-1.73 folds), NeuroG2 (-2.17 folds) and SYN1 (-4.34 folds)

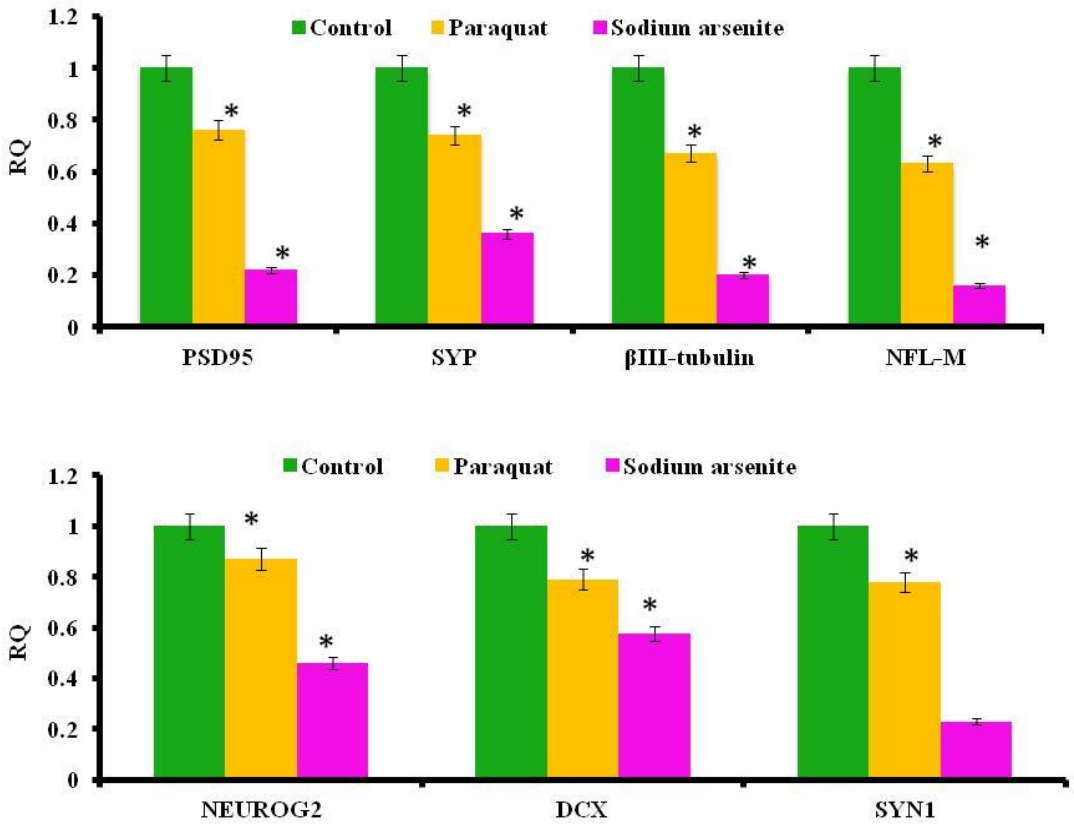


Fig 4.49: Effect of sodium arsenite or paraquat on expression of neuronal and synaptic markers at mRNA level in NGF induced differentiated PC12 cells. Real time PCR of SYP, PSD95, βIII-Tubulin, DCX, NeuroG2, Syn1 and NFL-M/NEFM in cellular RNA exposed with sodium arsenite or paraquat (RQ: Relative quantification).

while paraquat deregulates PSD95 (-1.31), SYP (-1.35), βIII-Tubulin (-1.49), NFL-M/NEFM (-1.58), DCX (-1.26), NeuroG2 (-1.14) and SYN1 (-1.28) in compare to NGF induced differentiated cells (Fig 4.49)

Immunoblotting studies have further shown the downregulation of neuronal and synaptic markers in sodium arsenite or paraquat exposed NGF differentiated PC12 cells in compare to their control. (Fig 4.50).

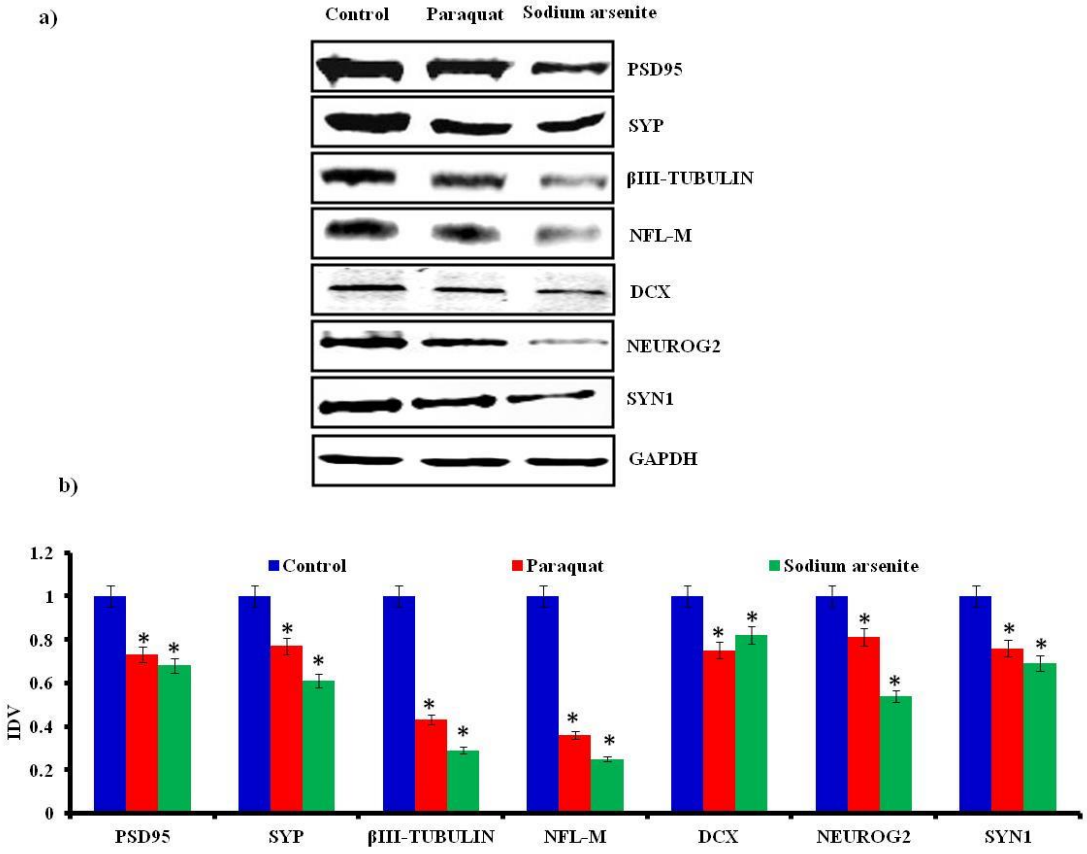


Fig 4.50: Effect of sodium arsenite or paraquat on expression of neuronal and synaptic markers at Protein level NGF induced differentiated PC12 cells. Western blots and densitometry of SYP, PSD95, βIII-Tubulin, DCX, NeuroG2, SYN1 and NFL-M/NEFM in cellular lysates exposed with sodium arsenite or paraquat (IDV: Integrated Density Value pValue* <0.05, densitometry of western blots was performed using Image J software).

4.32 Regulation of miR-34 family in neurotoxicants induced degeneration.

Our studies have identified miR-34 family in NGF induced differentiation of PC12 cells as well as in developing brain which justifies its critical role in brain development. Moreover, when we exposed our differentiated PC12 cells with sodium arsenite or paraquat, miR-34 family members get altered transcriptionally (Fig 4.51). Out of miR-34 family members, maximum alteration was found to be in miR-34c in compare to miR-34a and miR-34b. Sodium arsenite deregulated the expression of miR-34c by -6.17 folds while paraquat altered miR-34c by -4.21 folds. (Fig 4.51).

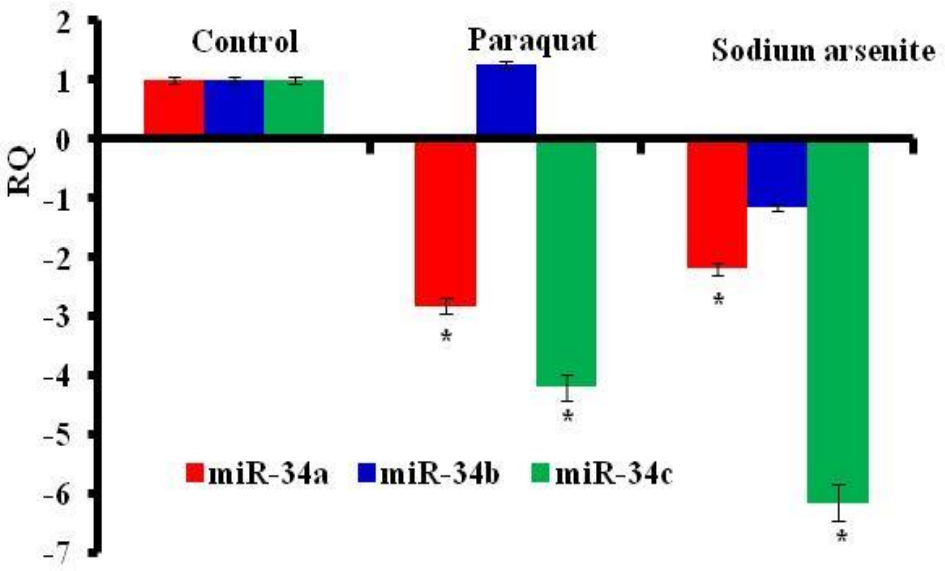


Fig 4.51: Regulation of miR-34 family in NGF induced differentiated PC12 cells exposed to sodium arsenite or paraquat. Real time PCR of miR-34 family members was carried out in sodium arsenite or paraquat exposed NGF induced differentiated PC12 cells. (RQ: Relative quantification)

Moreover, we identified the biological pathways targeted by miR-34 family using MetaCore web portal, nervous system development, head development and brain development were among the top 3 pathways regulated by miR-34 family. In addition, when disease pathways using MetaCore web portal identified, cell differentiation, neurofibromatoses and Huntington disease rated the top 3 maximally regulated by miR-34 family members (Fig 4.52).

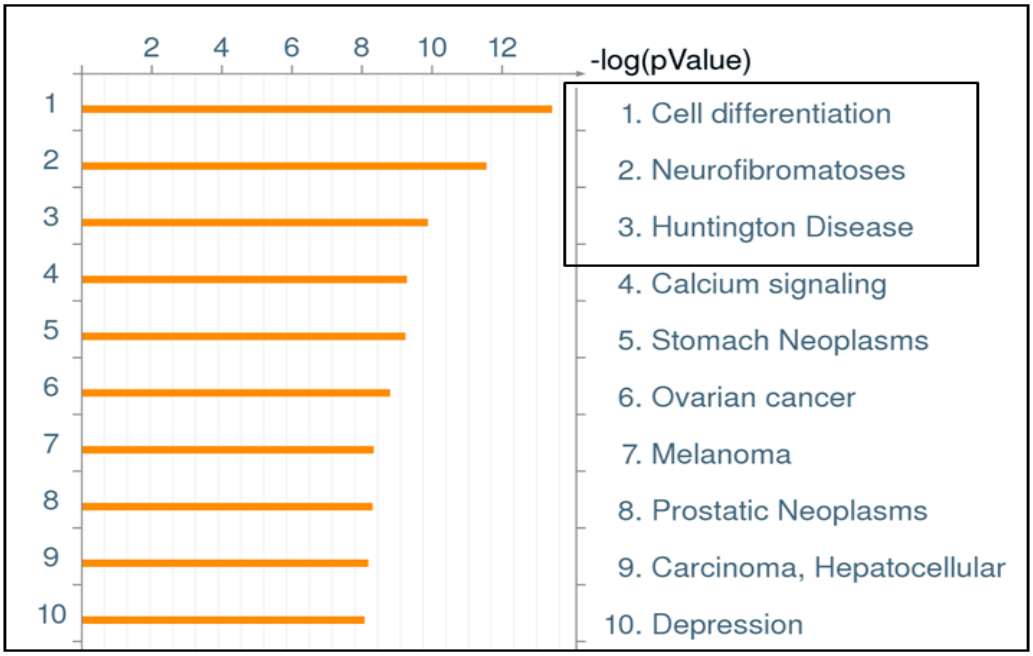


Fig.4.52: *In silico* pathway analysis of miR-34 family members up or down regulated by sodium arsenite exposure or paraquat exposure in differentiated PC12 cells. Most significant top 10 disease pathways targeted by miRNA by sodium arsenite or paraquat identified using the platform of MetaCore web portal.

4.33 Regulation of "brain specific miRNAs" by sodium arsenite or paraquat in NGF induced differentiated PC12 cells

Customized "brain specific miRNA array" was used to study the effect of sodium arsenite or paraquat on regulation of "brain specific miRNAs" in NGF induced differentiated PC12 cells. Box plot and volcano plot analysis of "brain specific miRNAs" carried out in which Ct mean in sodium arsenite or paraquat exposed differentiated PC12 cells have been done to study the overall trend in regulation of "brain specific miRNAs" (Fig4.53and Fig4.55). Sodium arsenite exposure significantly altered 17 miRNAs out of which it up-regulated the expression of miR-150 and miR-136* while expression of miR-126, miR-182, miR-29a, miR-17, miR-29c, miR-194, miR-200c, miR-200a, miR-7b, miR-664, miR-29b, miR-26a, miR-598-5p, miR-7e and miR-33a* were significantly down-regulated by sodium arsenite in NGF induced differentiated PC12 cells (≥2folds and pValue ≤0.05) (Fig. 4.54). Out of them, miR-150 (8.87folds) was maximally up-regulated while miR-33a* (9.09folds) was maximally down-regulated (Fig. 4.54).

Paraquat exposure up-regulated the expression of various miRNAs including Let-7i*, miR-136*, miR-150, miR-487b, miR-483*, miR-384-5p, miR-34a, miR-29c, miR-29b, miR-9, miR-138, miR-338-3p and miR-1937c (≥2folds and pValue ≤0.05). Out of which Let-7i* is maximally up-regulated with 13.20 folds change (Fig 4.56).

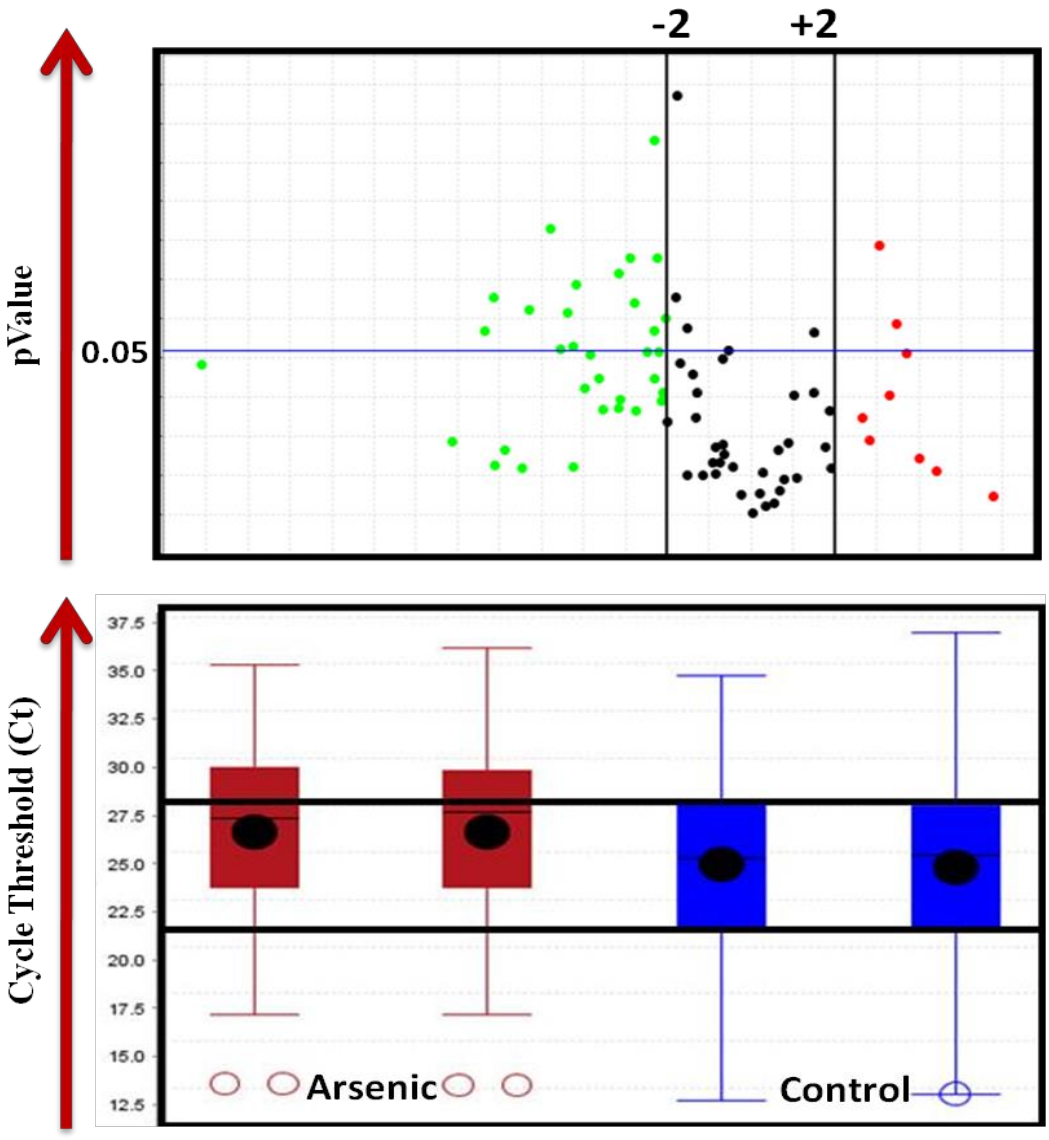


Fig 4.53: Effect of sodium arsenite on the expression of brain specific miRNAs in NGF induced differentiated PC12 cells. Volcano plot and Box plot analysis of "brain specific miRNAs" profile of differentiated PC12 cells exposed with sodium arsenite (Endogenous control; U6 snRNA, pValue*<0.05, Fold Change boundary 2 folds).

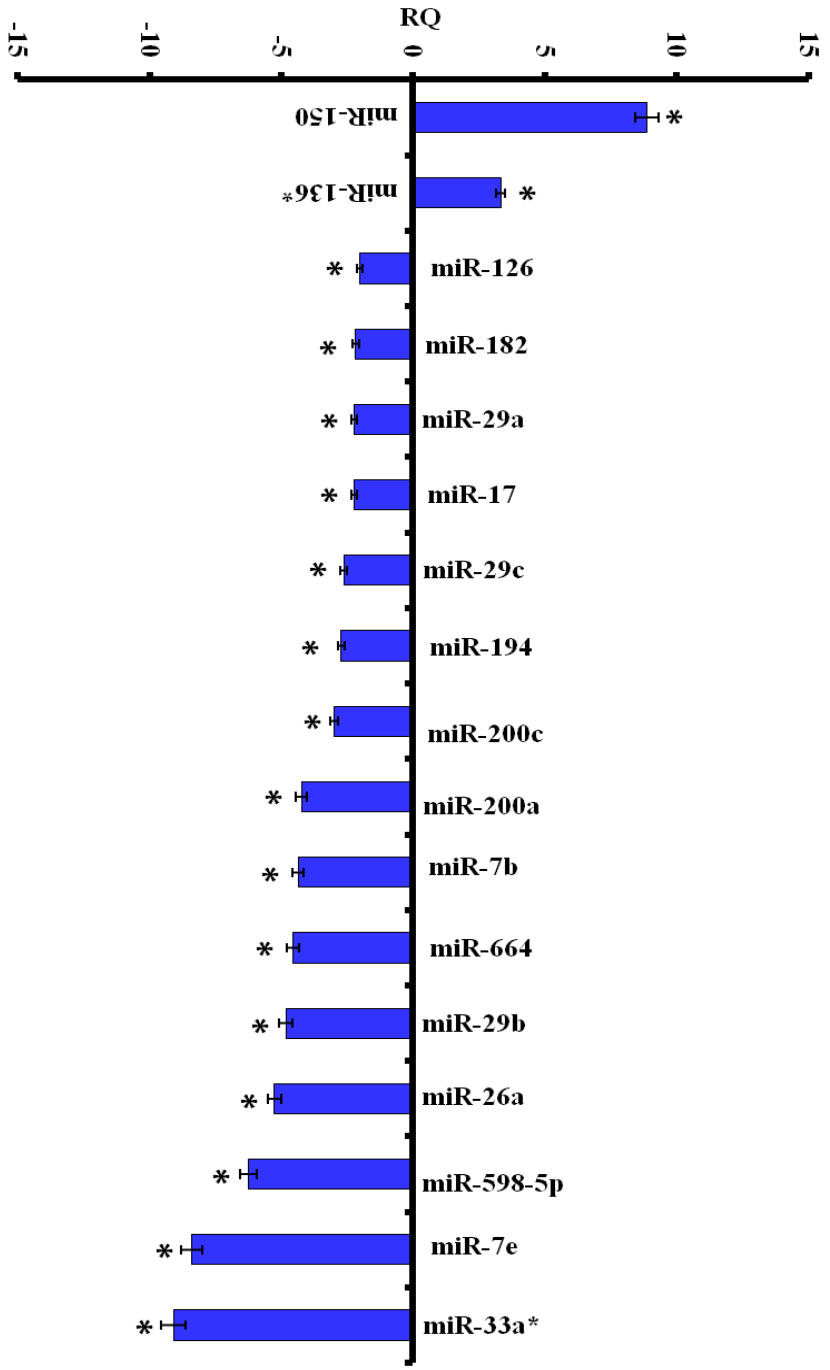


Fig.4.54: Effect of sodium arsenite on expression of brain specific miRNAs in NGF induced differentiated PC12 cells. (RQ: Relative quantification, pValue* <0.05).

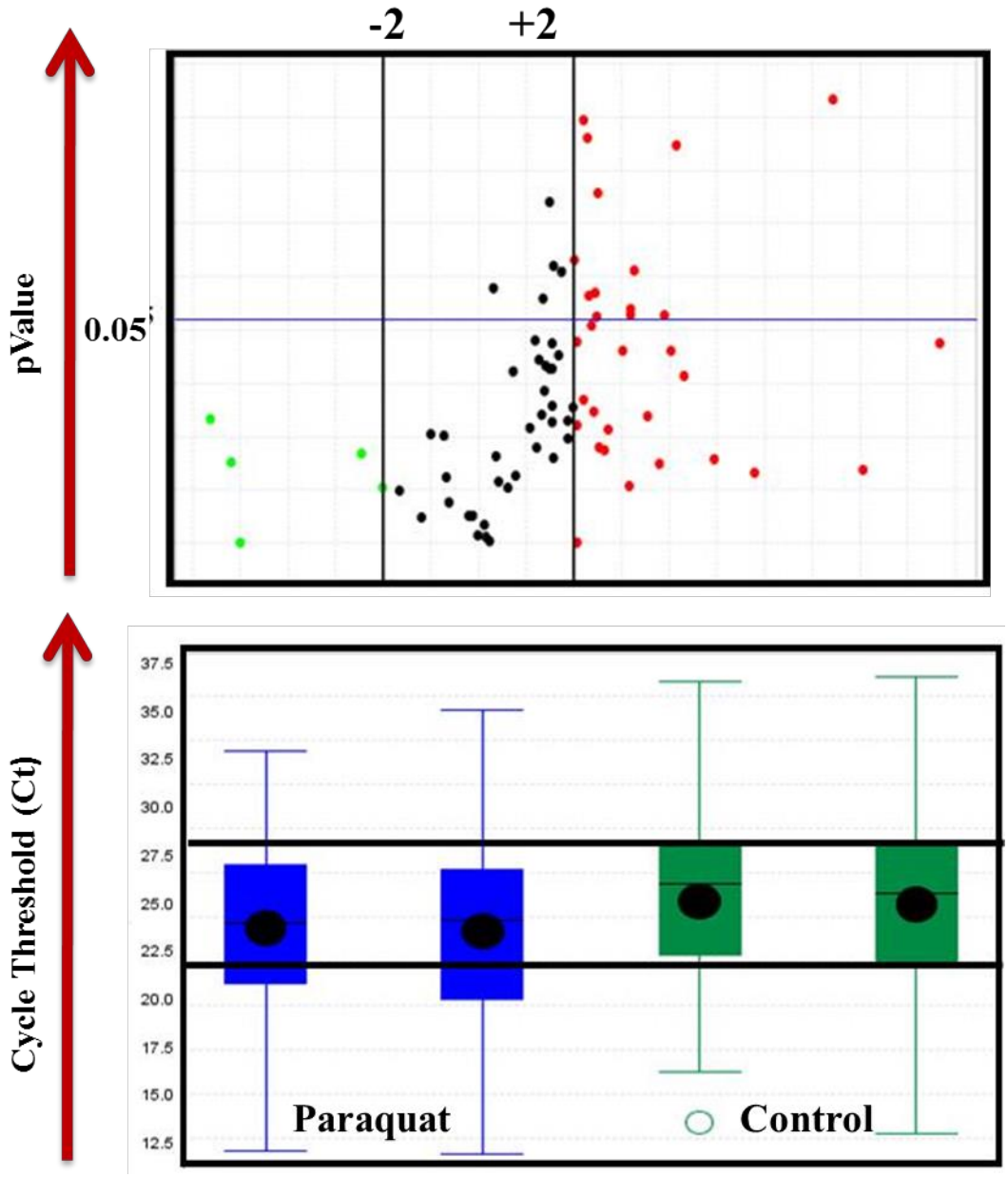


Fig 4.55: Effect of paraquat on the expression of "brain specific miRNAs" in NGF induced differentiated PC12 cells. Volcano plot and Box plot analysis of brain specific miRNA profile of differentiated PC12 cells exposed with paraquat (Endogenous control; U6 snRNA, pValue<0.05, Fold Change boundary 2 folds).

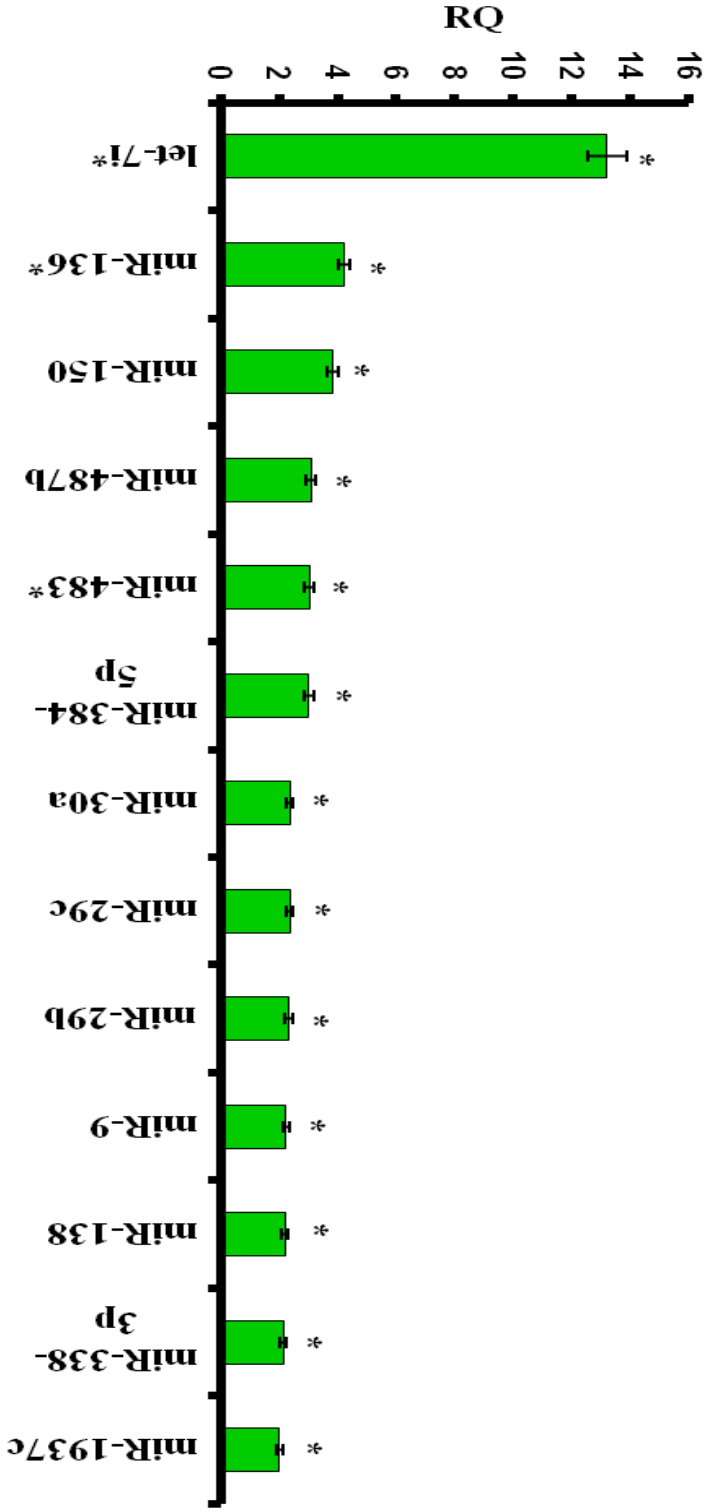


Fig.4.56: Effect of paraquat on expression of brain specific miRNAs in NGF induced differentiated PC12 cells.
(RQ: Relative quantification, pValue* <0.05).

4.34 *In silico* pathway analysis of targets of up or down-regulated miRNAs by sodium arsenite or paraquat.

To identify the possible biological pathways targeted by sodium arsenite or paraquat in NGF induced differentiated PC12 cells, *In silico* target based pathway analysis was performed using MetaCore web portal. All the potential targets of up-regulated and down-regulated miRNAs by sodium arsenite or paraquat were identified using TargetScan web portal and fetched on GO process analysis option of MetaCore platform.

GO process Process network

Fig.4.57: *In silico* pathway analysis of miRNAs up or down regulated by sodium arsenite exposure in differentiated PC12 cells. Most significant top 10 biological processes targeted by up or down regulated miRNA by sodium arsenite in differentiated PC12 cells identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.

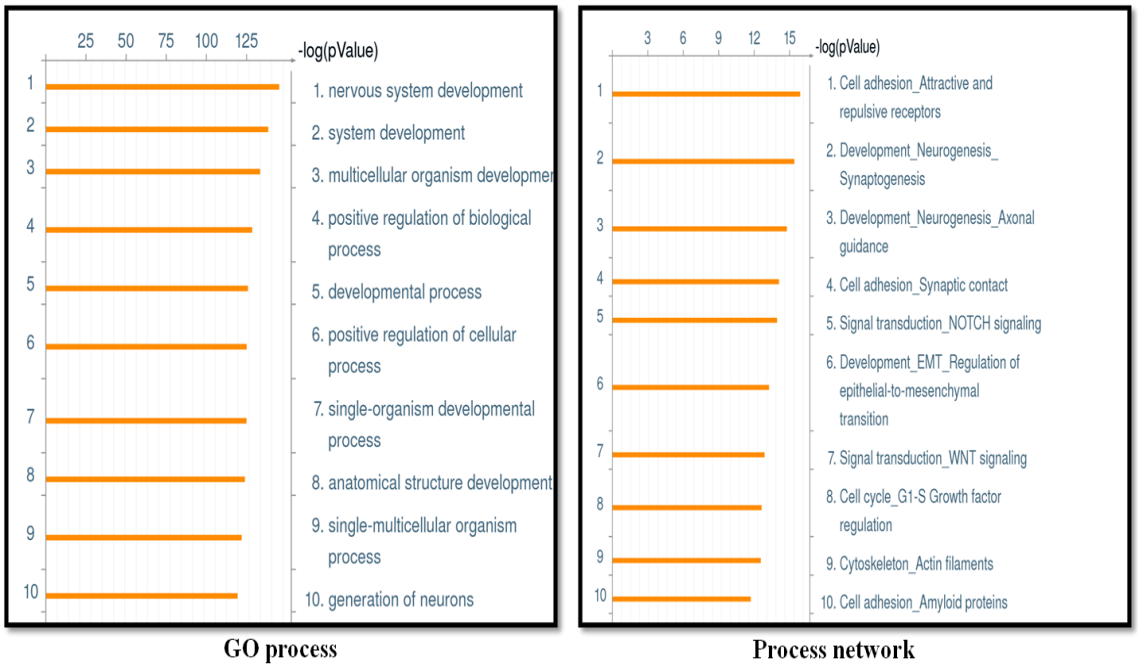


Fig.4.58: *In silico* pathway analysis of miRNAs up or down regulated by paraquat exposure in differentiated PC12 cells. Most significant top 10 biological processes targeted by up regulated miRNA by paraquat in differentiated PC12 cells identified by using the GO process (a) and Process Network (b) platform of MetaCore web portal.

Exposure of sodium arsenite or paraquat identified nervous system development, system development, development-neurogenesis-axonal guidance, cell adhesion-attractive & repulsive receptors, positive regulation of biological process, development-neurogenesis & synaptogenesis and multicellular organism development are the most targeted pathways (Fig 4.57-4.58).

4.35 Comparative regulation of miRNAs between 12week aged rat brain and NGF induced differentiated PC12 cells exposed to sodium arsenite or paraquat

Exposure of neurotoxicants (sodium arsenite or PQ+MB) altered the expression of various miRNAs with more than 2 folds in 12 week aged rat brain and NGF induced differentiated PC12 cells exposed to sodium arsenite or paraquat (PQ+MB). 8day

NGF exposed PC12 cells behave like a mature neuron and express various neurotransmitters similar to young adult rat brain. Among the identified miRNAs, some miRNAs has shown similarity in fold change in both the cases (Fig 4.60).

After exposure of sodium arsenite in PC12 cells, miR-150 (8.87 folds), miR-126 (-2.02 folds), miR-182 (-2.17folds), miR-17 (-2.23 folds) and miR-194 (-2.73 folds) were found which are almost similar in case of sodium arsenite exposed 12 week aged rats having fold change identified miR-150 (7.06 folds), miR-126 (-3.02 folds), miR-182 (-14.08 folds), miR-17 (-2.23 folds) and miR-194 (-2.73 folds).

Exposure of paraquat in differentiated PC12 cells up-regulated the expression of miR-136* (4.21 folds), miR-29c (2.36 folds), miR-29b (2.32 folds), miR-138 (2.20 folds), miR-338-3p (2.14 folds) and miR-1937c (2.05 folds) which is also found to be altered in PQ+MB exposed 12 week aged rats having fold change approx miR-136* (4.49 folds), miR-29c (2.12 folds), miR-29b (2.33 folds), miR-138 (2.05 folds), miR-338-3p (2.36 folds) and miR-1937c (2.19 folds) (Fig.4.60)

4.36 *In silico* pathway analysis of targets of up or down regulated miRNAs by sodium arsenite or paraquat in both the models.

The possible biological pathways targeted by common miRNAs altered by sodium arsenite or paraquat exposure in NGF induced differentiated PC12 cells as well as in 12week aged rat brain have shown neurofibromatosis, cell differentiation, development_neurogenesis, axonal guidance, cell adhesion_attractive and repulsive receptors using *in silico* pathway analysis on MetaCore web portal (Fig 4.61&4.62).

S No	MicroRNAs altered in Sodium arsenite exposed 12w aged rats	Fold Change (pValue * ≤0.05)	MicroRNAs altered in Sodium arsenite exposed differentiated PC12 cells	Fold Change (pValue * ≤0.05)
1	miR-150	7.06	miR-150	8.87
2	miR-126	-2.02	miR-126	-2.02
3	miR-182	-14.08	miR-182	-2.17
4	miR-17	-2.08	miR-17	-2.23
5	miR-194	-2.19	miR-194	-2.73

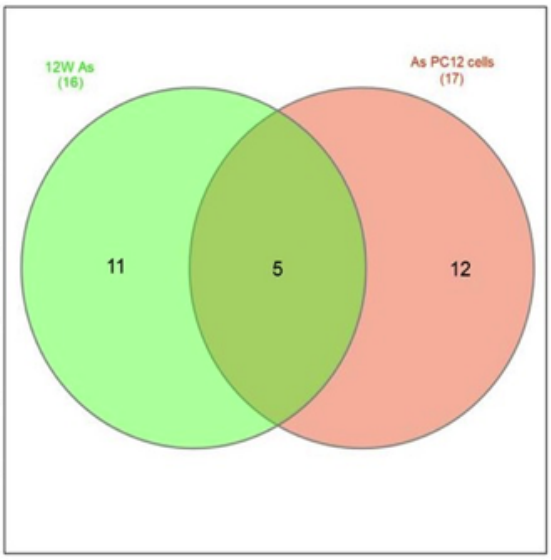


Fig 4.59: Comparative regulation of miRNAs altered by sodium arsenite exposure both in cellular and animal model. (a) Table containing common identified miRNAs altered in animal and cellular model with fold change, (b) Venn diagram represents similar number of miRNAs altered in animal and cellular model.with fold change

S No	MicroRNAs altered in PQ+MB exposed 12w aged rats	Fold Change (pValue *≤0.05)	MicroRNAs altered in Paraquat exposed differentiated PC12 cells	Fold Change (pValue *≤0.05)
1	miR-136*	4.21	miR-136*	4.49
2	miR-29c	2.36	miR-29c	2.12
3	miR-29b	2.32	miR-29b	2.33
4	miR-138	2.20	miR-138	2.05
5	miR-338-3p	2.14	miR-338-3p	2.36
6	miR-1937c	2.05	miR-1937c	2.19

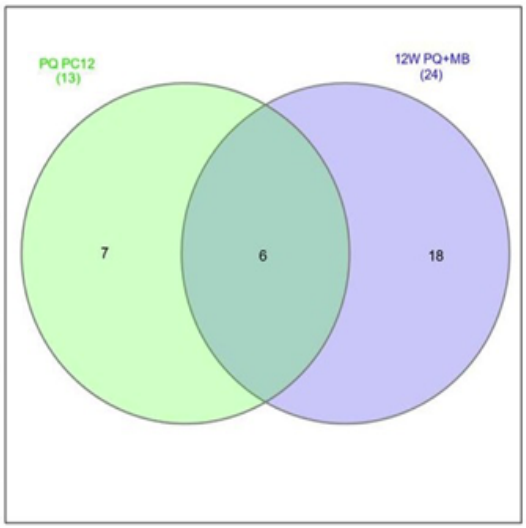
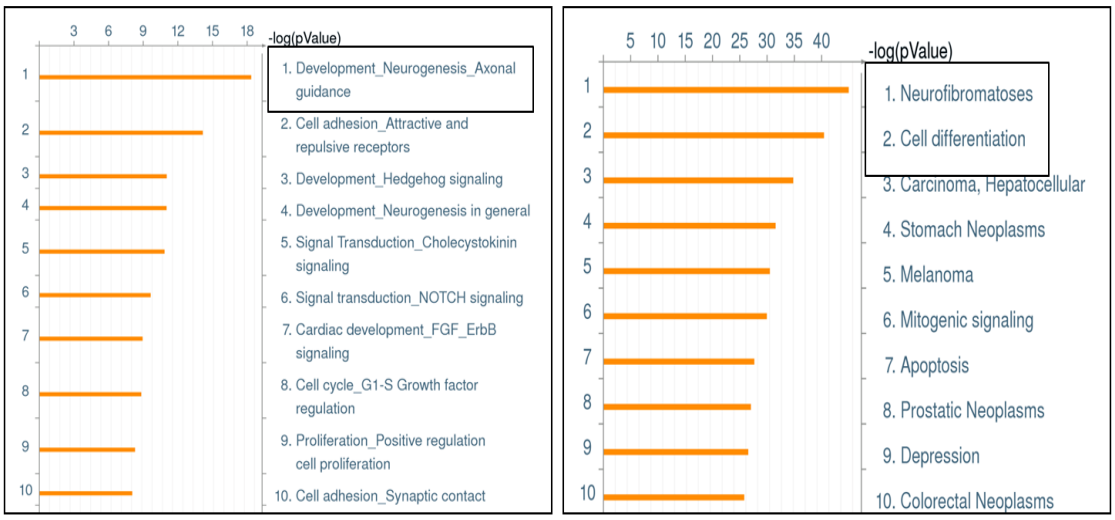
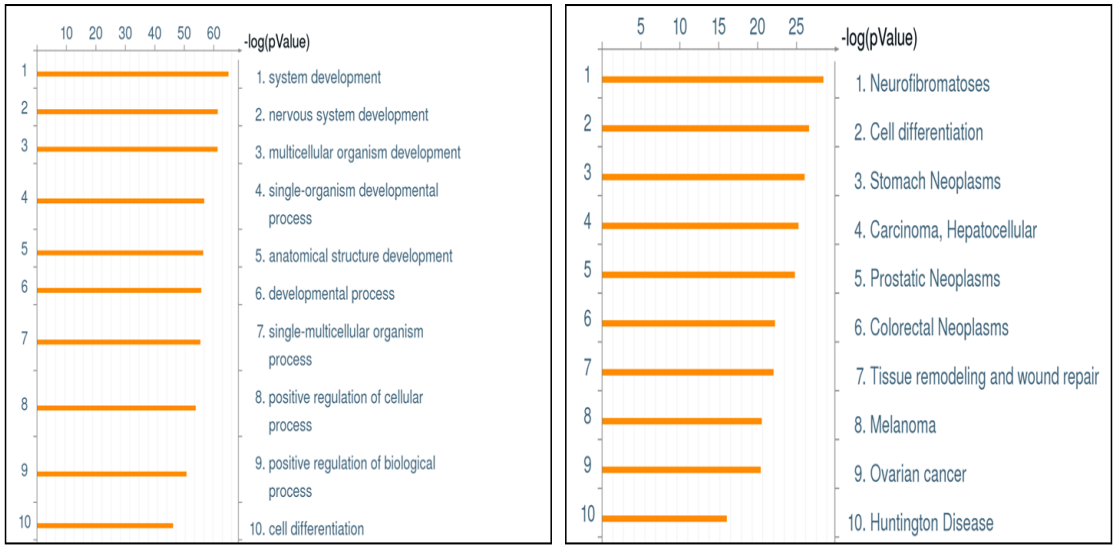


Fig 4.60 Comparative regulation of miRNAs altered by paraquat or PQ+MB exposure both in cellular and animal model (a) Table containing common identified miRNAs altered in animal and cellular model with fold change, b) Venn diagram represents similar miRNAs altered in animal and cellular model with fold change.



a)GO Process b)Disease Pathway Map

Fig.4.61: *In silico* pathway analysis of miRNAs up or down regulated by sodium arsenite exposure in differentiated PC12 cells and 12 week aged rat brain. Most significant top 10 biological processes targeted by up or down-regulated miRNA by sodium arsenite in both the models identified by using the GO process (a) and Disease pathway map (b) platform of MetaCore web portal.



a)GO Process b)Disease Pathway Map

Fig.4.62: *In silico* pathway analysis of miRNAs up or down regulated by paraquat exposure in differentiated PC12 cells and 12 week aged rat brain. Most significant top 10 biological processes targeted by up regulated miRNA by paraquat in both the models identified by using the GO process (a) and Disease pathway map (b) platform of MetaCore web portal.

4.37 *In silico* analysis of 3'UTR of NFL-M/NEFM using TargetScan web portal.

Studies has been identified that expression of neuronal and synaptic markers including NFL-M/NEFM, PSD95, SYP, TUBB3, NeuroG2, SYN1 and DCX were found to be significantly decreased during rat brain development and differentiated neurons. Therefore, we wish to analyze the 3'UTR of these genes to find out possible binding sites of miRNAs. *In silico* target prediction was performed using online available platform, TargetScan. Interestingly, 3'UTR of NFL-M/NEFM gene has shown binding sites for different miRNAs including miR-150 which was highly and commonly altered in both the models exposed by sodium arsenite (Fig 4.63).

4.38 TargetScan of NFL-M/NEFM gene and its 3'UTR analysis

TargetScan web portal was used for the identification of miRNAs found at 3ÚTR of NEFM gene. The identification of miR-150 at conserved site of NEFM suggested its role in neuronal development as well as in neurodegeneration (Fig 4.63). TargetScan web portal reported that NFL-M/NEFM gene of rat consists miR-150 in broadly conserved region (Fig 4.64). Interestingly, binding site of miR-150 in NFL-M is conserved throughout the mammalian system.

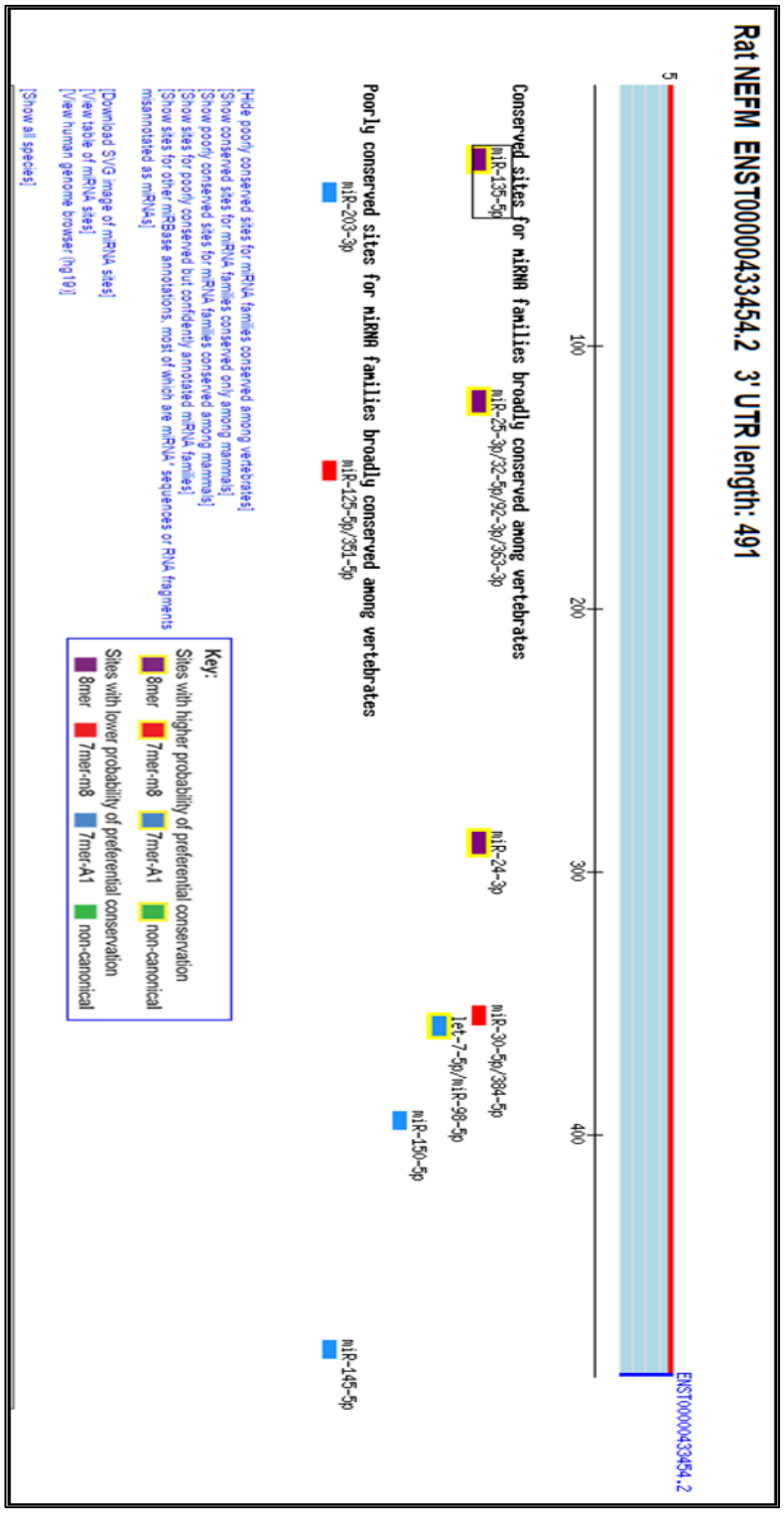


Fig. 4.63: *In silico* analysis of 3'UTR of NEFM gene: *In silico* scanning of 3'UTR of NEFM gene was performed by TargetScan web portal.

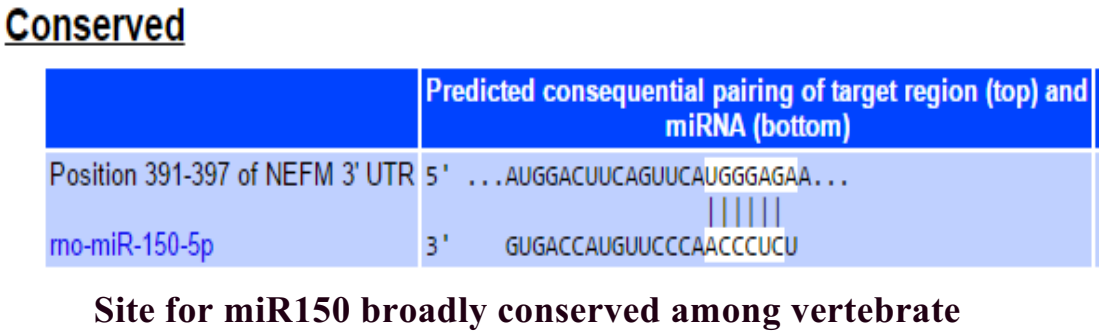


Fig 4.64: TargetScan web portal reported that NFL-M/NEFM gene of rat consists miR-150 in broadly conserved region

4.39 Regulation of expression of NFL-M/NEFM by miR-150 in sodium arsenite exposed differentiated PC12 cells.

To identify the role of miR-150 in the regulation of NFL-M/NEFM level, gain of function and loss of function studies were performed in PC12 cells. Gain of functions for miR-150 family was achieved by transfection of mimics of miR-150 (miR-150m) and loss of function was achieved by knocking-down of miR-150 by transfection of inhibitors of miR-150 (miR-150i) in PC12 cells.(Fig 4.65 -4.66). Brightfield images were taken after transfection of mimics and inhibitors of miR-150 in NGF exposed differentiated PC12 cells which has shown dramatic decrease in neurite length and number when cells were exposed to sodium arsenite ($NaAsO_2$) in comparison to NTC. Moreover, transfection of miR-150(m)/Syn-miR-150 also impaired the process of neuritogenesis when compared to NTC. Furthermore, when these miR-150(i)/ Anti-miR-150 transfected cells were exposed to $NaAsO_2$, protection has shown from

neuritodegeneration induced by sodium arsenite. In addition, disruption of neurites has shown in miR-150(m)/Syn-miR-150 transfected cells exposed to $NaAsO_2$ (Fig 4.65).

In confirming the regulation of miR-150, expression of NFL-M/NEFM was studied at transcriptional as well as at translational level which has shown that expression of NFL-M/NEFM was found to be down-regulated in sodium arsenite exposed differentiated PC12 cells at mRNAs well as at protein level. Moreover, further down-regulation in NFL-M/NEFM expression has been observed in miR-150(m)/Syn-miR-150 transfected cells exposed to $NaAsO_2$. Interestingly, NFL-M/NEFM expression was found to be up-regulated in miR-150(i)/ Anti-miR-150 transfected cells in compare to only sodium arsenite exposed, miR-150(m)/Syn-miR-150 transfected or miR-150(m)/Syn-miR-150 transfected exposed to $NaAsO_2$ differentiated PC12cells (Fig 4.66).

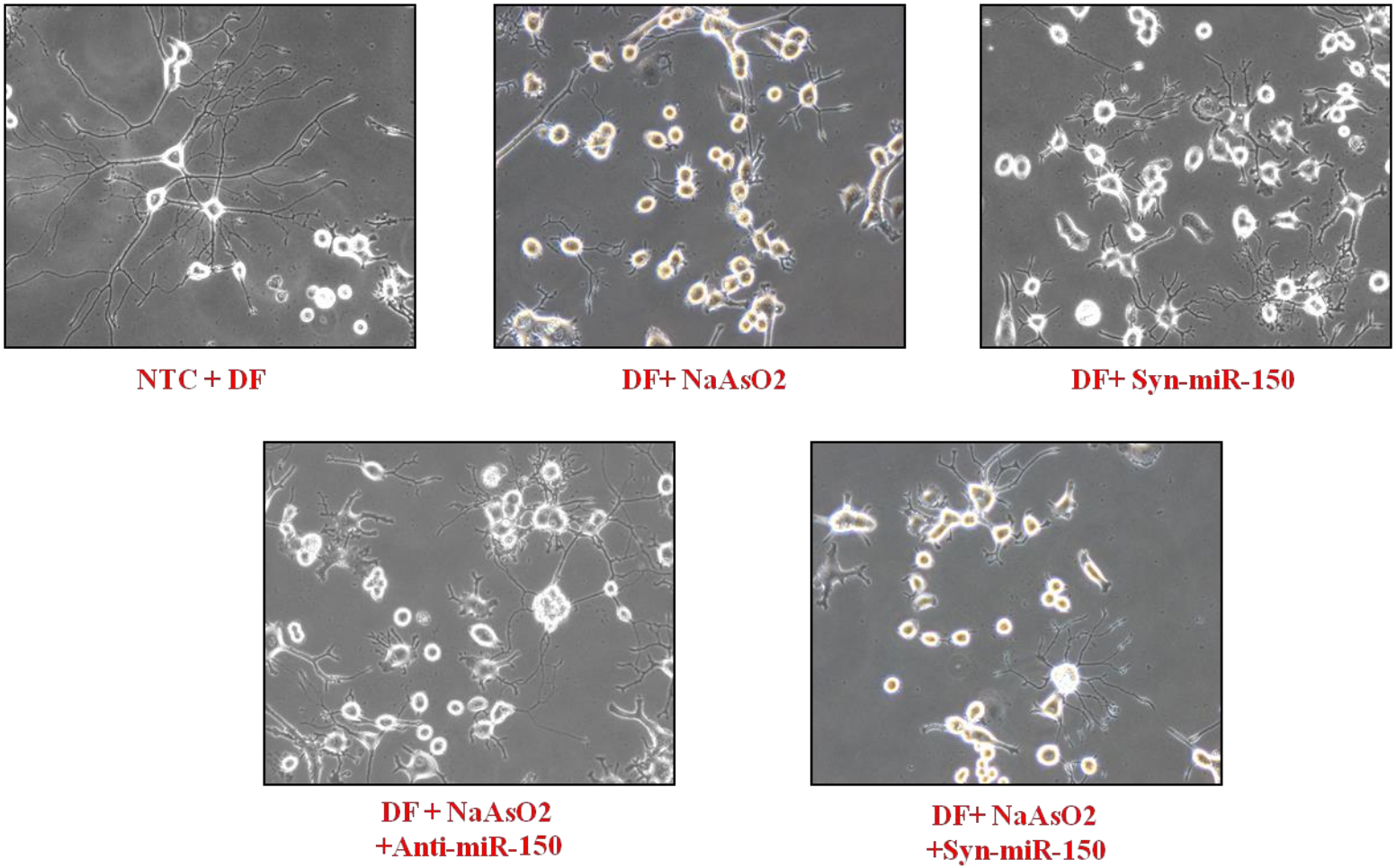


Fig 4.65. Phase contrast images captured after gain of function(miR-150(m)/Syn-miR-150) and loss of function(miR-150(i)/ Anti-miR-150) studies in sodium arsenite exposed differentiated PC12 cells .Images were captured using Nikon NIS microscope. (DF: Differentiated Fully)

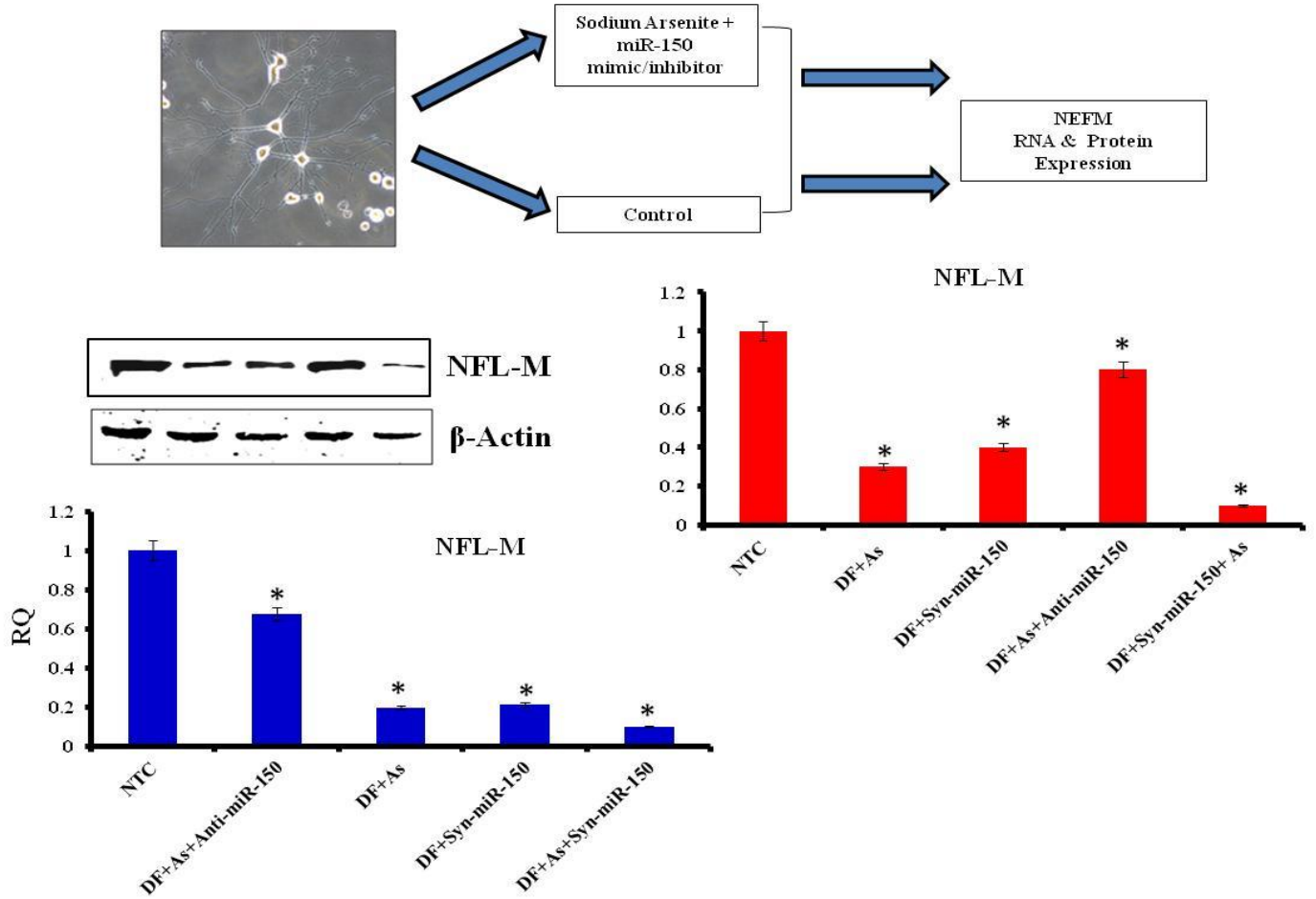


Fig 4.66 MiR-150 regulate expression ofNFL-M/NEFM: Expression of NFL-M/NEFM at mRNA(a) or at protein level with densitometry(b) after Gain of function(miR-150(m)/Syn-miR-150) and loss of function(miR-150(i)/ Anti-miR-150) studies in sodium arsenite exposed differentiated PC12 cells. (RQ: Relative quantification)

4.40 *In silico* pathway analysis of miR-150 in sodium arsenite exposed differentiated PC12 cells.

Interestingly, *in silico* pathway analysis using MetaCore web portal identified regulation of development process, regulation of cell differentiation, system development, nervous system development and its regulation are the maximum targeted biological processes. The major identified disease pathways regulated by miR-150 includes cell differentiation, neurotransmission and Huntington disease fetched on disease pathway map analysis option of MetaCore platform (Fig 4.67).

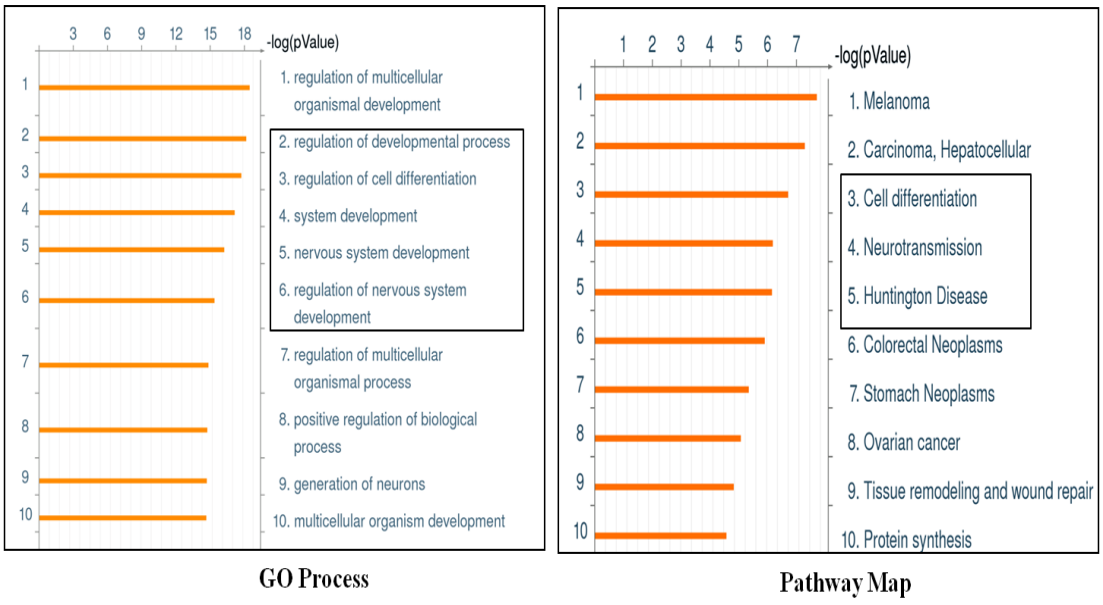
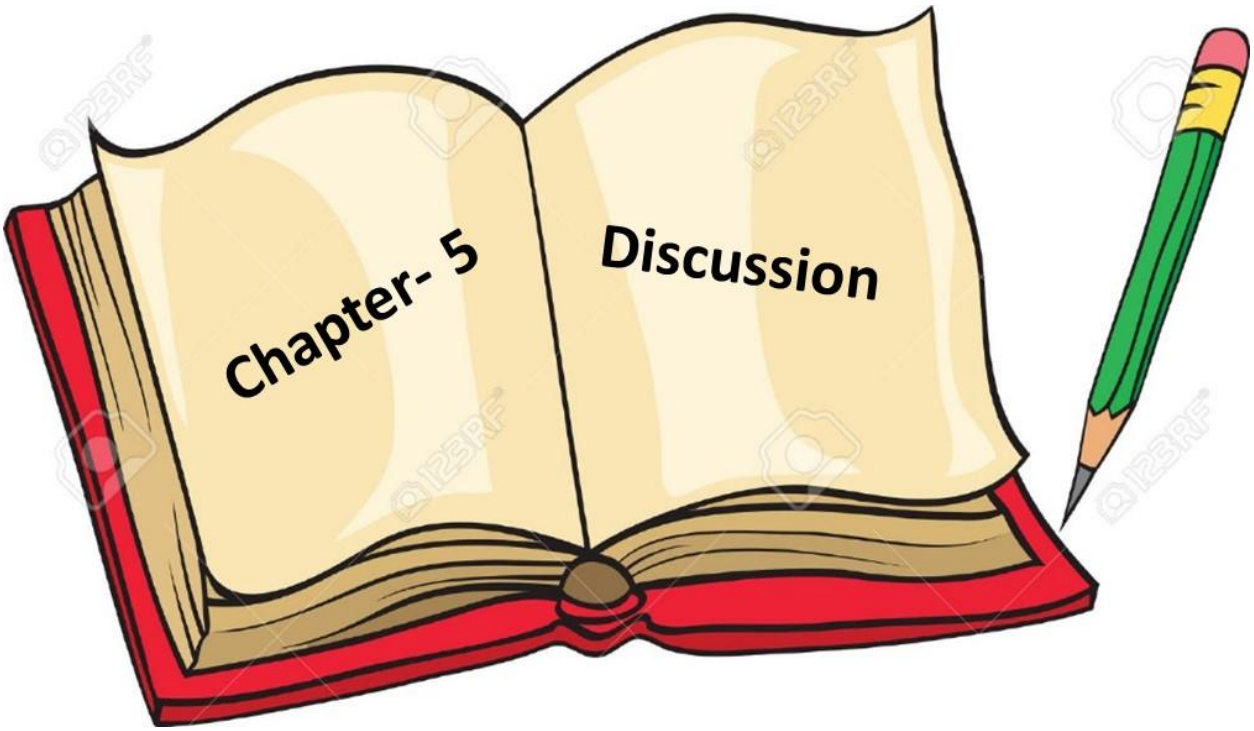


Fig.4.67: *In silico* pathway analysis of miR-150 by sodium arsenite exposure in differentiated PC12 cells. Most significant top 10 biological processes targeted by up regulated miRNA by sodium arsenite identified by using the GO process (a) and disease pathway map (b) platform of MetaCore web portal.


Chapter- 5
Discussion

CHAPTER 5: DISCUSSION

The current study was commenced to understand the role of miRNAs in neurotoxicity and develop a cellular model for screening potential neurotoxicants. Profiling of miRNAs using "brain specific miRNAs" array in developing rat brain and NGF differentiated PC12 cells have identified set of miRNAs significantly deregulated by sodium arsenite or paraquat+maneb (PQ+MB) in developing rat brain and differentiating neurons. Interestingly, sodium arsenite maximally up-regulated the expression of miR-150 in both cellular and animal models, which targets NFL-M/NEFM (neurofilament-medium chain), a protein required for maintaining axonal structure and neuronal calibre. So, expression of miR-150 in peripheral blood can be probed as an indicator against sodium arsenite exposure.

***In Vivo* Studies:**

Present study provides substantial evidences for role of miRNAs in neurotoxicants induced degeneration of developing and adult neurons. Earlier studies from our lab have shown that miRNAs are regulated by neurotoxicants and target the expression of proteins like BCL2, CCND2 and which regulates neuronal cell death (10). For *in vivo*studies, rats of 3week (pre-adolescence), 6week (adolescence) and 12week (young adults) age were selected for identification of neurotoxicants regulated miRNAs. Apart from these, present study also identified neurotoxicants regulated miRNAs in 9week (post-adolescence) aged rats. However, in cellular studies, 8day NGF differentiated PC12 cells are used as cellular model for neuron maturation, which corresponds to 12 week brain neurons.

Sodium arsenite or PQ+MB were selected due to their known neurotoxicity potential and different mechanism of action. Sodium arsenite is widely used in pesticides, hide preservative, antiseptics, dyeing, and soaps (222). Sodium arsenite, a heavy metal induces neurotoxicity by generating reactive oxygen species (ROS)and creating redox imbalance which disrupts neurocytoskeletal structure, while paraquat induces mitochondrial dysfunction and leads to cellular death. Sodium arsenite induces neurotoxicity both in adult as well as in neonatal brain cells(177). Studies have shown that arsenic reaches to developing brain by passing blood-brain barrier and cause neurotoxicity (223). Animal studies using prenatal and early postnatal exposure of arsenic have identified reductions in brain weight, numbers of glia and neuronal cells, and alterations in neurotransmitter system (224). Studies have also shown the potential of sodium arsenite in producing deformities by increasing mortality rate, decreasing prenatal growth and enhance the neural tube defects (184,225-227). Several studies have shown that repeated exposure of sodium arsenite reduces the body and brain weight in young animals (181-183,228,229). Role of sodium arsenite has also been explored in impairing neuronal development and leads towards neurodegenerative disorders like Alzheimer's (AD) and Amyotrophic lateral sclerosis (ALS) (230).

Paraquat, a known neurotoxicantinduces Parkinson's like symptoms (231). Excessive use of paraquat causes loss of dopaminergic neurons in the nigrostriatal dopamine systems which are the pathological features of Parkinson's disease (PD) (232). In present study, postnatal rats of 3week (pre-adolescence), 6week (adolescence), 9week (post-adolescent) and 12week (post-adolescence) were exposed with sodium arsenite

or PQ+MB for 7 consecutive days and expression of brain specific miRNAs was studied using TaqMan Low Density Array(TLDA). Before miRNA profiling, neurobehavioral studies were performed in sodium arsenite or PQ+MB exposed rats have shown significant impairment in motor functions in adolescent rats, which suggests that adolescent period is more vulnerable than pre- and post-adolescent rats. However, mild changes in motor functions were also observed in sodium arsenite or PQ+MB exposed young adult rats. In response to other toxicants, i.e. ethanol, nicotine and other drugs, adolescence period has already reported as more sensitivity period (233,234).Similar to our study, significant studies have been suggested that exposure of PQ+MB (maneb used to increase the uptake of PQ) alters the locomotory functions(183,235). Decreased locomotor activity observed in sodium arsenite or PQ+MB exposed adolescent rats indicates towards regulation of neuronal and synaptic proteins at transcriptional, post-transcriptional or translational level. Exposure of neurotoxicants (sodium arsenite or PQ+MB) deregulates the expression of SYP,PSD95, βIII-Tubulin, NFL-M/NEFM, DCX, NeuroG2 andSYN1 (markers of neural development) in adolescence rat brains. However, no significant changes were observed at mRNA or protein levels in pre or post adolescent rat brains, which again indicate that adolescence brain, are more vulnerable to neurotoxicants exposure.

Exposure of sodium arsenite or PQ+MB, down-regulated the expression of neuronal and synaptic markers and suggested that 6 week aged or adolescent brain are more sensitive towards neurotoxicants exposure.In humans, it is widely accepted that developing brain is much more susceptible than adult brain. Moreover, human brain has a prolonged period of postnatal maturation, continuing through adolescence,

which cooperates neurotoxicants for disturbing the nervous system (236). Studies have shown that ethanol consumption during adolescence affects large number of brain cells in the medial prefrontal cortex and basolateral amygdala of adult male and female rats (237). In addition,Evrard*et al*., (2005) have shown that ethanol exposure induces neurotoxicity in adolescent brain by reducing the expression of neurofilament proteins (238).

Analysis of miRNA profiling done with "brain specific miRNAs array" has shown that sodium arsenitederegulated maximum miRNAs in adolescent(23 miRNAs), followed by pre-adolescent(19 miRNAs), post-adolescent (10 miRNAs), and young adult (16 miRNAs) rats respectively. Similarly, PQ+MB also deregulated maximum miRNAs at adolescent(41 miRNAs) followed by pre-adolescent(27 miRNAs), post-adolescent (8 miRNAs), and young adult (25 miRNAs) rats respectively. Studies carried out with sodium arsenite or PQ+MB on different postnatal rat brain development phases (pre-adolescent, adolescent, post-adolescent and young adults) have identified adolescence period as most sensitive period of post-transcriptional regulation.

Interestingly, sodium arsenite or PQ+MB produced maximum up-regulation in expression of miR-29 family in adolescent rat brain. Earlier studies reported the importance of miR-29 family in ALS and demonstrated as a marker for its progression (239). Moreover, exposure of neurotoxicantsat adolescence brain deregulated the expression of miR-29 and promotes ageing like molecular changes(240). Maximum vulnerability of adolescent period in miRNA expression

supports the earlier findings that during adolescent age brain keeps developing and is very active transcriptionally and translationally. At cellular level, adolescence period involves maximum synaptogenesis, which comprises both generation and establishment of new synapses and removal of extra synapses known as synaptic pruning.

In post adolescent rat brains, both sodium arsenite or PQ+MB significantly induced the expression of miR-200 family, miRNA family which directly regulates neuronal differentiation (8). Interestingly, studies from our lab have demonstrated the crucial role of miR-200 family in neuronal differentiation as well as in cypermethrin induced neurotoxicity in differentiated neurons (8,9,29). Moreover, exposure ofcypermethrin, known pyrethroid induced expression of miR-200 in P53 dependent manner, which inhibited the expression of BCL2 and induced apoptosis in differentiated neurons (29).

Increased expression of miR-674* was observed in adult rats exposed with PQ+MB or sodium arsenite correlates to earlier findings that it is deregulated in Huntington disease patients(241). Moreover, in adult rats, exposure of sodium arsenite produced maximum up-regulation in miR-150 which is recently reported to be up-regulated in Alzheimer's disease (AD) and Prion disease(242).

In silico pathway analysis of target proteins of miRNAs de-regulated by sodium arsenite or PQ+MB in adolescent brain has identified developmental neurogenesis, synaptogenesis, nervous system development are the most significantly targeted biological pathways, which further confirms that sodium arsenite or PQ+MB

regulated miRNAs which regulates functions in nervous system development or degeneration.

***In Vitro* Studies:**

To further validate the *in vivo* studies, we have studied the effect of sodium arsenite or paraquat exposure on cellular model of neuronal development using NGF induced differentiated PC12 cellsand explored the developmental neurotoxicity of sodium arsenite or paraquat by various mechanisms. Various studies have identified NGF induced differentiated PC12 as a best model for studying neurodevelopment as well as neurotoxicity. Exposure of NGF in PC12 cells ceases proliferation and extends multiple neurites, which acquire the properties of sympathetic neurons(26). Moreover, the expression of βIII-Tubulin, the neuronal marker during differentiation has shown that NGF induced differentiation of PC12 cells leads to formation of fully mature neurons by day 8. This is consistent with the previous reports indicating the role of NGF in the differentiation of PC12 cells to mature neurons (243). Exposure of NGF to naive PC12 results in the formation of mature and functional neurons, which was confirmed by the expression of neuronal and synaptic markers(23,244). Up-regulation was observed at mRNA and protein levels of synaptic and neuronal markers(SYP, NFL-M/NEFM, PSD95, SYN1, NeuroG2, DCX, and βIII-Tubulin) in NGF induced differentiated PC12 cells have further confirmed the formation of mature and functional neurons.

Our earlier studies have shown that, knocking down Dicer gene significantly impairs the NGF induced differentiation of PC12 cells, which suggests that expression of

mature miRNAs is crucial for generation of functional neurons from proliferating neurons(245,246). Our earlier studies also shown that knocking down Dicer gene, in PC12 cells affects its NGF induced differentiation both qualitatively and quantitatively (8). Moreover, exposure of NGF in Dicer knocked down PC12 cells induced the formation of multiple nuclei, which is commonly seen in senescent cells (8). Our recent studies using differentiating SH-SY5Y cells and SA-β-gal assay(marker of senescence) have shown that absence of mature miRNAs promotes cells to enter in senescence, when they are forced to differentiate(14). Similar to SH-SY5Y cells, knocking down Dicer gene in PC12 cells also induced senescence when they are exposed with NGF. Increased amount of senescenceobserved in cells after Dicer knock down, confirms that miRNAs are essential for survival of mature neurons. Similarly, earlier studies of Kawase-Koga *et al*., (2009) have also shown that deletion of Dicer gene affects neurogenesis and gliogenesis of developing mouse brain (246). Our findings support earlier studies that expression of mature miRNAs and Dicer is crucial for developing neurons and brain.

MiRNAs profiling carried out using "brain specific miRNA array" have identified up-regulation as dominant phenomenon than down-regulation in miRNA expression. Volcano plot analysis of "brain specific miRNAs" using undifferentiated and differentiated PC12 cells have shown similar changes as reported in our earlier studies done with global miRNA profiling of PC12 cells (8). Box plot analysis of global miRNA profile have shown that differentiation decreased mean and median of Ct values, suggestive of overall increase in miRNA transcripts in differentiated PC12 cells. Our earlier studies have identified that out of the 19 up-regulated miRNAs,

seven miRNAs belongs to three miRNA families i.e. miR-200 (miR-200a and miR-200b), miR-221/222 (miR-221 and miR-222) and miR-34 (miR-34a, miR-34b-3p, miR-34c) (PMID: 25753155). Consistently increased expression of miR-200 and miR-34 families in differentiating PC12 and NSCs, indicates their involvement in either initiation of differentiation or maintenance of differentiated neurons (8).

***In Vitro* Studies: Role of miR-34 family in neuronal development and degeneration**

In present study, attempts were made to further identify the role and regulation of miR-34 family in developing neurons. Expression profiling of miR-34 family carried out in different tissues, cells, parts of brain and at different developing stages of rat brain provided a holistic view of miR-34 expression in mammals. Comparison of the members of miR-34 family expression, between different tissues and cell types have identified brain and mature brain cells as richest source of miR-34 family, which supports earlier studies that miR-34 family is essential for normal brain development (247). Comparative analysis of miR-34 family members has identified miR-34a as maximally expressed miR-34 miRNA in brain tissue and cells. Interestingly, in C6 (Rat Glioblastoma), liver and lung tissues, miR-34b is expressed maximally. Very high expression of miR-34a in brain tissues and cells indicates role of endogenous regulator or transcription factor in its higher transcription rate. Among three miR-34 members, miR-34a is transcribed from chromosome 1, while miR-34b and miR-34c are co-transcribed from chromosome 11, which explains differences observed between expression pattern of miR-34a with miR-34b and miR-34c. Only striking observation was expression of miR-34b and miR-34c in cerebellum, part of brain

which controls motor functions and also known as little brain. Cerebellum expresses highest amount of miR-34b and lowest amount of miR-34c among different brain regions, which suggest that these co-transcribed miRNAs are regulating different function in cerebellum. Difference observed in amount of miR-34b and miR-34c in cerebellum could also be due to short half life of miR-34c or its involvement in sponging some circular RNA. Recent study have shown that circular RNA circCDR1 acts as a sponge for miR-7, as it has 17 binding site for miR-7 (248).

Consistently higher expression of miR-34a observed in aged brain and mature neurons indicates its role in functioning and survival of mature neurons. Recent studies have shown that miR-34a also regulates proliferation, morphology and function of newborn neurons, which enhances behavioural outputs and abilities of brain (249). Comparative analysis of miR-34 expression suggests that brain is one of the major target tissues of miR-34 mediated regulations. Higher cerebral expression of miR-34a and dramatic increase in its expression during differentiation and ageing, suggests that miR-34a is most important member of miR-34 family for brain and its cells.

Members of miR-34 family are known to be up-regulated by P53 levels in different types of cancer cells (250). Several studies have shown that miR-34 is tumour suppressor and over-expression of miR-34 produces anti-proliferative effects (251). In our earlier studies, we have observed up-regulation in expression of P53 protein, when PC12 and SH-SY5Y cells are differentiated into mature neurons (14,29).

In order to understand the role of P53 in regulation of miR-34, we have measured the expression of miR-34 family members in PC12 cells. Exposure with CP-31398 to

PC12 cells selectively induced the expression of miR-34a, which again suggests that in brain, miR-34a is most important miR-34 family member.

Scanning of 3'UTR of P53 of different mammals (*Homo sapiens, Rattusnorvegicus, Musmusculus*) has shown presence of miR-34a site only in 3'UTR of *Rattusnorvegicus*. Interestingly, ectopic expression of miR-34a, significantly down regulated P53 in PC12 cells at both mRNA and protein levels, which suggests that expression of miR-34a can regulate P53 levels. Earlier studies have also suggested that miR-34a is induced by P53 binding on promoter region of miR-34a (252). Earlier studies have demonstrated multiple functions and targets of miR-34family and identified tumour suppression and development as major functions regulated by miR-34 family (9,131,253,254). Present study shows that increased expression of miR-34a in differentiated neurons controls further increase of P53, probably to protect the cells from P53 induced apoptosis.

In our earlier studies, we have observed that ectopic expression of miR-200 family induces differentiation in PC12 cells similar to NGF exposure but at lesser extent (8). However, over-expression of miR-34a mimics up to levels it reaches by NGF did not significantly induce the differentiation in PC12 cells. However inhibition of miR-34a substantially inhibited the differentiation of PC12 cells, which suggests towards its indirect role in neuronal differentiation. Interestingly, over-expression of miR-34a in PC12 cells pre-exposed with NGF(50ng/ml) promoted differentiation, which indicated that miR-34a regulate other cellular processes related or required for neuronal differentiation. Increased protein level of NFL-M/NEFM and βIII-Tubulin by miR-34a transfection in NGF exposed PC12 cells, further confirmed the role of

miR-34a in neuronal differentiation. Earlier studies of Aranha*et al*., (2011) have identified role of miR-34a in differentiation of neural stem cells and proposed SIRT1 protein as its probable target (255). G1 arrest observed in PC12 cells transfected with miR-34a mimics have suggested that miR-34a induces cell cycle arrest and helps priming of cells for differentiation. Probably, increased expression of miR-34a helps in maintaining the cells in unproliferative stage. Higher expression of miR-34a observed in aged brain, which contains most of post-mitotic cells also supports its role in maintaining the neurons in post-mitotic stage.

Further, present study also suggests that brain and brain cells expresses maximum amount of miR-34, which increases with maturation of neurons and ageing of brain. Expression of miR-34 cannot initiate neuritogenesisindependently; however it promotes neuritogenesis and is required for differentiation of PC12 cells into mature neurons. In differentiating PC12 cells, an inhibitory feedback loop operates between P53 and miR-34a, which probably prevents P53 induced apoptosis of differentiated neurons. Conclusively, expression of miR-34a is very crucial for neural development and survival of mature neurons.

Exposure of neurotoxicants can impair the process of neuronal development and results in neuronal cell death. The exposure of sodium arsenite or paraquat to PC12 cells was found to induce aberrations in the structure as well as function of neurons. Earlier studies delineated the role of sodium arsenite or paraquat as developmental neurotoxicants(152,256). Various reports have identified that sodium arsenite or paraquat induce oxidative stress in experimental animals as well as in *in vitro* conditions(257,258).Induction of apoptosis, excessive ROS generation and abnormal

mitochondrial function was observed in present study using PC12 cells exposed with sodium arsenite or paraquat. Similar to our studies, significant studies were carried out using *in vitro* models, reported that paraquat has a potential to induce ROS and dopaminergic cell death (152). Moreover, sodium arsenite is able to significantly decrease cell proliferation and increase the level of ROS in PC12 cells(157,259). Moreover, present study also identified excessive ROS generation along with mitochondrial dysfunction as evident by MMP loss, following the exposure of sodium arsenite or paraquat in PC12 cells, confirming their involvement in inducing neuronal damage.

Previous studies identified that sodium arsenite or paraquatdisrupts the neurites structure and suppresses the neuronal differentiation (257,260). Similarly, our study also confirmed that sodium arsenite (5µM) and paraquat (50µM) disrupts the neuronal structure in differentiated cells compare to control differentiated PC12 cells using Calcein AM dye. Exposure of sodium arsenite and paraquat hampers the neuronal number and length of NGF induced differentiated PC12 cells. Deregulation of neuronal markers in response to neurotoxicants is well reported. Moreover, immunocytochemical analysis also indicated the down-regulation of neurofilament medium chain (NFL-M/NEFM) in differentiated PC12 cells exposed to sodium arsenite or paraquat. The significant down-regulation in the mRNA and protein expression of the neuronal markers (SYP, NEFM, PSD95, SYN1, NeuroG2, DCX, and βIII-Tubulin) in differentiated PC12 cells exposed to sodium arsenite (5µM) and paraquat (50µM) were shown in present study.

The present study revealed the decisive role of miR-34 family in neuronal development. To further illustrate the role of miR-34 in neurodegeneration, we studied expression of miR-34 family in sodium arsenite or paraquat exposed cells and observed a decrease in miR-34a and miR-34c. Studies of Miñones-Moyano E *et al* (2011) identified that down-regulation of miR-34b/c in SH-SY5Y cells results in decreasing viability, increasing oxidative stress and mitochondrial dysfunction. This down-regulation of miR-4b/c further decreases the DJ1 and Parkin protein expression which are related to PD (75). Additionally, studies were identified in relation to miR-34a targeting Tau expression, major protein linked to AD (261). *In silico* studies were performed to predict the disease pathways regulated by miR-34 family, where cell differentiation, neurofibromatosis and Huntington disease were found on top of the list.

Expression profiling of sodium arsenite exposed differentiated PC12 cells has identified up-regulation in expression of miR-150 and miR-136* while down-regulation have been observed in miR-126, miR-182, miR-29a, miR-17, miR-29c, miR-194, miR-200c, miR-200a, miR-7b, miR-664, miR-29b, miR-26a, miR-598-5p, miR-7e and miR-33a*. Recent study has identified up-regulation in expression of miR-150 in cerebrospinal fluid (CSF) of multiple sclerosis patients and suggested miR-150 as a novel candidate biomarker for multiple sclerosis(242).*In silico*pathway analysis of potential targets of miRNAs up or down regulated by sodium arsenite exposure in differentiated PC12 cells has shown nervous system development and development-neurogenesis, axonal guidance are the top two significantly targeted

pathways indicates that sodium arsenite exposure deregulates nervous system development and neuronal maturation process.

Paraquat induced neurodegeneration is well documented, but the role and regulation of miRNAs in paraquat induced neurodegeneration is not well studied. Exposure of paraquat to NGF differentiated PC12 cells induced the expression of 13 miRNAs. Volcano plot and box plot analysis have shown that up-regulation in expression of miRNAs is more prominent than down-regulation after paraquat exposure in differentiated PC12 cells. Significant up-regulation observed in expression of Let-7i*, miR-136*, miR-150, miR-487b, miR-483*, miR-384-5p, miR-34a, miR-29c, miR-29b, miR-9, miR-138, miR-338-3p and miR-1937c in paraquat exposed differentiated PC12 cells. Let-7i* was maximally up-regulated in paraquat exposed cells. Similar to our study, earlier studies has shown that up-regulation in Let-7i* induces CNS damage by activating TLR7 (RNA sensing toll like receptor)(262). *In silico*pathway mapping of potential targets of paraquat regulated miRNAs have identified nervous system development and cell adhesion-attractive and repulsive receptors as the top two targeted pathways.

Similarity among the expression of miRNAs altered in young adult rat brain and mature neurons.

Comparing the sodium arsenite induced regulation of miRNAs in adult brain with differentiated neurons, similarity has been observed in miR-150, miR-126, miR-182, miR-17 and miR-194 in both the models. Exposure of sodium arsenite has induced the expression of above mentioned miRNAs in both differentiated PC12 cells and adult

rat brain. Furthermore, among all the similar miRNAs, miR-150 was the maximally up-regulated in both animal and cellular model. Earlier studies have shown the role of miR-126, miR-182, miR-17 and miR-194 in development of neurodegenerative disorders and identified their potential as biomarkers of neurodegeneration (263,264). Role of miR-126 has been explored recently in metabolic dysfunction and neurotoxicity especially during aging and in the pathogenesis of specific neurological disorders(265). Another miRNA, miR-182 has reported to play a role in axon outgrowth and dendrite maturation involving activation of the PTEN/AKT pathway(266). Moreover, miR-182 has also identified in prion disease (267). Expression profiling identified a significant down-regulation of miR-194 in blood and cerebrospinal fluid of Alzheimer patients (268). *In silico*pathway analysis of miRNAs deregulated by sodium arsenitein adult rat brain and differentiated PC12 cells have identified development-neurogenesis and axonal guidance are the most significantly targeted pathways. In addition, *in silico*disease pathway analysis of sodium arsenite deregulated miRNAs have identifiedneurofibromatosis and cell differentiation as the most significantly targeted pathways.

Comparing the paraquat induced regulation of miRNAs between adult brain and differentiated neurons, similarity has been observed in regulation of miR-136*, miR-29b, miR-29c, miR-138, miR-338-3p, and miR-1937c.Similar to our study, other studies have also reported the up-regulation of miR-136*, miR-29b, miR-29c, miR-138, miR-338-3p, and miR-1937c during neurodegeneration. Expression of miR-136 has identified as significantly up-regulated in prion disease (267). Studies have shown up-regulation of miR-138 in Alzheimer's disease (AD) and miR-338-3p in blood,

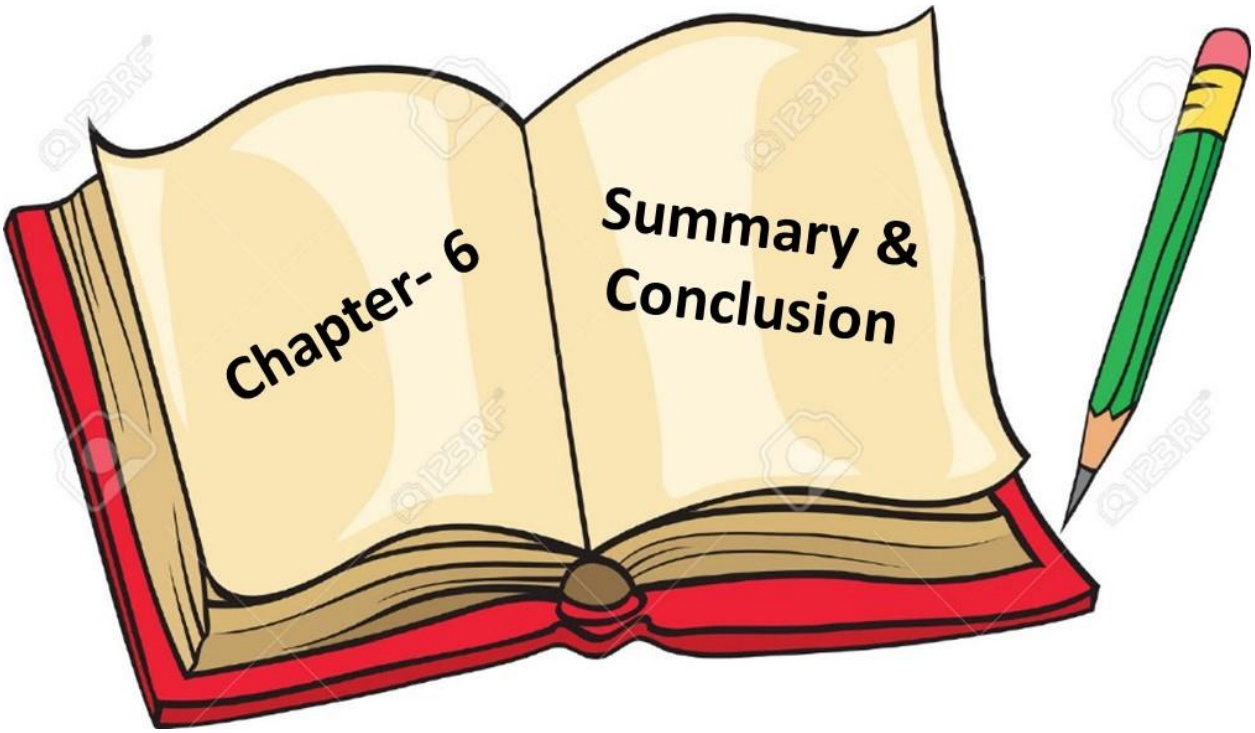
CSF, serum and spinal cord of sporadic amyotrophic lateral sclerosis (ALS) patients(269,270).In addition, *insilico* pathway analysis of altered miRNAs carried out in paraquat exposed 12week rat brain and PC12 cells identified nervous system and system development are the most regulated pathways. Likewise to sodium arsenite, *in silico*disease pathway analysis of altered miRNAs carried out in paraquat exposed 12week rat brain and differentiated PC12 cells have identified neurofibromatosis and cell differentiation as the most significantly regulated pathways.

To further confirm the role of miRNAs deregulated byneurotoxicants, 3'UTR of neuronal and synaptic proteins were analyzed for identification of binding sites for specific miRNAs. Binding sites of miR-150 were identified in 3'UTR of NFL-M/NEFM after screening, the 3'UTR of SYP, SYN1, βIII-Tubulin, PSD95, DCX and NeuroG2 genes.

TargetScan web portal reported that NFL-M/NEFM gene consists miR-150 in broadly conserved region. Interestingly, binding site of miR-150 in NFL-M is conserved throughout the mammalian system. Present study identified down-regulation of NEFM in cells exposed to sodium arsenite. Furthermore, loss of function studies of miR-150 in PC12 cells exposed with sodium arsenite has shown that knockdown of miR-150 (Anti-miR-150) protects PC12 cells from sodium arsenite induced neurite degeneration. Moreover, when differentiated PC12 cells are exposed to sodium arsenite and transfected with Syn-miR-150, levels of NEFM were further down-regulated observed at transcriptional and translation levels. Furthermore, earlier study have identified up-regulation of miR-150 and suggested NFL-L as a probable target

protein(271). Similar to our results, studies of Vahidnia*et al* (2006) reported that sodium arsenite downregulates the neurofilament levels, which results in lowering the nerve conduction velocities in nerves (272). Their studies using animal model suggested that neurites disruption is induced by sodium arsenite, which results in neuronal damage (272). Moreover, studies of Erika García-Chávez *et al* (2007) also revealed the impairment in peripheral nervous system due to arsenic exposure. They suggested that arsenic exposure induces oxidative damage, severedemyelination, and other morphological alterations in axons of peripheral nerves(193).

Additionally, maximum possible biological pathways targeted by miR-150 regulated by NFL-M/NEFM in sodium arsenite exposure were identified using *in silico* studies are regulation of development process, regulation of cell differentiation, system development, nervous system development and its regulation. The major identified disease pathways regulated by miR-150 and NFL-M/NEFM includes cell differentiation, neurotransmission and Huntington disease were also identified using MetaCore platform which further confirmed the crucial role of miR-150 in neurodegeneration.


Chapter- 6
Summary &
Conclusion

CHAPTER 6: SUMMARY AND CONCLUSION

Present study has identified the indispensable role of miRNAs in neuronal development and degeneration using both animal and cellular model. Increased senescence observed in PC12 cells exposed with NGF confirmed earlier findings of our lab that expression of mature miRNAs is essential for generation of mature and functional neurons. In continuation to earlier studies of our group, present study demonstrated role of miR-34 family in brain development and identified brain cells as richest source of miR-34 family in rats. Neurobehavioral assays carried out in rats exposed to PQ+MB or sodium arsenite during different stages of post-natal rat brain development has identified maximum alterations in locomotor activity during adolescence age. Expression profiling of "brain specific miRNAs" in brain of rats of postnatal age exposed to sodium arsenite have shown maximum alterations in miRNAs expression during adolescence age (17 up-regulated & 6 down-regulated).Similarly, exposure of PQ+MB have also produced maximum alterations in miRNA expression during adolescence brain (9 up-regulated & 32 down-regulated). Interestingly, in adolescent rat brain expression of both sodium arsenite or PQ+MB maximally induced miR-29 family which is earlier shown to deregulated in neurodegenerative disorders like Alzheimer's disease.

Expression profiling of "brain specific miRNAs" carried out in NGF differentiated PC12 cells, exposed to sodium arsenite have shown alterations in total expression of 17 miRNAs (2 up-regulated and 15 down-regulated) while exposure of PQ induced alterations in total 13 miRNAs (all up-regulated). Comparison of cellular data with animal studies has identified miR-150 as common miRNA up-regulated in differentiated PC12 cells and adult

brain by exposure of sodium arsenite. Sodium arsenite significantly induced the expression of miR-150 in young adult rat brain (7.06 folds) which found to be similar in NGF differentiated PC12 cells (8.87 folds). Moreover, binding site of miR-150 was identified at 3'UTR of neurofilament (NFL-M/NEFM) which is broadly conserved among mammals. Transfection studies in PC12 cells have shown that inhibition of miR-150 protects neuronal structure and function from sodium arsenite induced neuronal degeneration. *In silico* studies also confirmed the importance of miR-150 in regulating cellular differentiation, neurotransmission and Huntington disease.

In conclusion, present study demonstrated essential role of miRNAs in brain development and neurodegeneration and identified adolescent period as most vulnerable post-natal time of brain development. During adolescent period, miR-29 family is maximally up-regulated by sodium arsenite or PQ+MB exposure. Comparison of cellular model of neural development with animal model has identified miR-150 as common miRNA up-regulated by neurotoxicants. MiR-150 is known to regulate neurofilament proteins whose abnormalities are common features of a number of neurodegenerative diseases. These results indicate that the absence of neurofilament because of sodium arsenite exposure can lead to abnormalities in neurodegenerative disease, which protects by regulating the expression of miR-150. Moreover, miR-150 can be a probable biomarker against sodium arsenite induced neuronal death.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Synthesis in Enterohaemorrhagic Escherichia coli Serotype O157: Characterization of a Novel Glucosyltransferase Structural Characterisation of Sulfolobus Glycoproteins by Mass Spectrometry Human Milk Oligosaccharides Affect Growth of Infant Fecal Microbiota in vitro Glycosylation and Its Function in Aspergillus fumigatus Moving Malaria Glycobiology Forward: Recent Approaches to Addressing the Existing Gaps in Knowledge viz. O-GlcNAc Modifications in Parasite Development The Synthesis and Role of Modified Glycoconjugates Comprising the Surface Glycocalyx in the Infectivity of the Protozoan Parasite Leishmania major Modulation of Immunity by Helminth Parasites: From Molecules to Effector Mechanisms Effect of Inhibitors of Sphingolipids Synthesis on Leishmania Growth and (glyco)(sphingo) Lipid Expression Identification of Glycotransferases Involved in the Synthesis of Functional O-Man Glycans A Missense Mutation in ST3GAL5 Results in a Severe Intellectual Disability Syndrome Associated with Altered Glycosphingolipid and O-linked Glycan Expression Stem Cell Glycomics Identifies Differentiation Stage-specific Markers and Novel Glycan Functions Multifaceted Role of Heparan Sulfate in Mouse Embryonic Stem Cell Biology Midas Touch: Heparan Sulfate Proteoglycans Regulate the Stem Cell Niche via Novel Contact-Dependent Signaling The Cell Surface Glycan LacdiNAc (GalNAcβ1-4GlcNAc) Contributes to Self-Renewal of Mouse Embryonic Stem Cells by Regulating LIF/STAT3 Signaling Hematopoietic Stem/Progenitor Cell Surface α2, 6-sialyl Glycans are Constructed by Non-Self ST6Gal-1 via a Non-Canonical 'Extrinsic' Glycosylation Pathway Natural Antibodies and Tumor-Associated Carbohydrate Antigens as Target Structures Chemical Probes of Glycan Assembly in Mycobacteria Roles of N-glycan in Integrin-Mediated Cell Adhesion and Cellular Signaling The Human Zona Pellucida: An Extracellular Matrix that Mediates Sperm Binding and Immune Recognition? Apes, S gar and Sperm–Glycans as Agents of Evolution Increased Polysialylation in Lung Tissue of Patients with Idiopathic Pulmonary Fibrosis Targeted Drug Delivery to Tumor Vasculature by a Carbohydrate Mimetic Peptide Enhancement of Epitelial-Mesenchymal Transition-Like Phenotype in N-acetylglucosaminyltransferase V Transgenic Mouse Skin Promotes Wound Healing A Systems Approach to Dissecting Immunity Downregulation of Fer Tyrosine Kinase Signaling Increases a-dystroglycan-mediated Cell Adhesion Thereby Suppresses Tumor Formation Chemical Methods for Biomarker Discovery Glycobiology Successes and Opportunities in Biotechnology and

PharmaAnalytical Services and Training at the Complex Carbohydrate Research CenterRapid Glycoprotein Sialic Acid Determination by High Performance Anion Exchange Chromatography with Pulsed Amperometric DetectionN-linked Glycosylation on Non-Consensus Protein MotifsNeutron Structure of Retaining Glycosyltransferase GTAGlycan Analysis by Solid-Phase Capture and Release using Reversible Reaction to HydrazideDatabases for N-Glycosylated Proteins Identified by Solid Phase Extraction of Glycosylated Peptides (SPEG) Coupled with LC/MSMonosaccharide Notation in Glycoinformatics: Problems, Pitfalls, and SolutionsGlycoPattern: A Platform for Motif Discovery and Glycan Array ExplorationImproving Standard N-Glycan Sample Preparation with Manual Automation using Microchromatography to Improve Efficiency, Accuracy, and Reproducibility "Efficient Synthesis of Aminopropyl-Blood Group A Triaose in Engineered Escherichia coli" New Features and Improvements in Carbohydrate 3D Structure ValidationHigh-Resolution Crystal Structure of N-Acetylmannosamine Kinase: Insights about Substrate Specificity, Activity and Inhibitor ModellingGlycan Map® Analysis for High-throughput Glycomic Profiling of Clinical and Other SpecimensRole of Quorum Sensing ΔaviR gene in Lipooligosaccharide Structure Modulation in Agrobacterium vitis F2/5. Examining the Role of the ppGalNAc-T Lectin Domain in Modulating ppGalNAc-T Glycopeptide SpecificityThe Initiation of Mucin Type O-glycosylation: Development of a Web-Based Tool for the Prediction of ppGalNAc T Isoform Specific O-GlycosylationDouble Mutants as Tools for Elucidating N-glycosylation Pathways in Caenorhabditis elegansThio-galactoside Glycolipid Analogs; Enzymatic Syntheses, Their Galactosidase Resistance and Application for Metabolism Studies in Single CellsMicroscale Analytical Platforms for Screening Carbohydrate-Active EnzymesCyberinfrastructure for Glycome Research: neoGRID for Motif AnalysisSynthesis of α-D-Glc1, 2α-D-Glc1, 3α-D-Glc-OCH3 and Overexpression of Processing Alpha Glucosidase IComplement L-ficolin Binds to Surface Glycans of HCV and Reduces the Viral Infectivity, and Functions as an Antiviral OpsoninStudies on Site-Specific N-Glycosylation of Hemagglutinin from Influenza A Viruse Phil82 and Surfactant Protein DAnti-HIV Effect in vitro of Human Milk GlycoconjugatesYeast-Binding Lectin with Antibacterial Activity from Scapharca broughtoniiA GlcNAc/GalNAc-specific Lectin from the Ascidian Didemnum ternatanum with Effects on the Scacharomyces cerevisiae in Ethanol

Fermentation ProcessThree Recognition Mechanisms for Agaricus bisporus Agglutinin–Glycan InteractionsA Role for the ST3Gal-VI Sialyltransferases in Selectin Ligand Formation in vivoDevelopment of Peptide Mimics of Ligand for anti-GM1 Antibody and Cholera Toxin B SubunitNew Fucooligosaccharides from Human Milk: Occurrence of LeY AntigenBinding Free Energy Calculations of Dimannose to Cyanovirin-N High and Low Affinity Sites using the Jarzynski's Equality and Umbrella SamplingLectins as Pattern Recognition Molecules: The Effects of Epitope Density in Innate ImmunityMonoclonal Antibody WN1 222-5 Binds a Conserved Core Region of Enterobacterial LPSThe Role of lman2la, an L-type Lectin Gene, in the Escape Behavior in ZebrafishInvariants Vα14 Natural Killer T Cell Activation by Edible Mushroom Acidic GlycosphingolipidsMannose-Binding Lectin Activity of Lactobacilli and Its Potential in Pathogen ExclusionRegulatory unctions of Activation-Dependent Reduction of N-glycolylneuraminic Acid in Mouse T CellsNMR Analysis of an Anti-Carbohydrate Antibody MLS128 Single-Chain Fv Fragment toward Elucidation of the Multivalent Recognition MechanismImidazolium Compounds: A New Generation of Galactosyltransferase InhibitorsMutational Analyses of Human acetyl-CoA: Glucosamine-6-phosphate N-acetyltransferase, hGNA1. Slit3 C-terminal Binds Heparin and Neutralizes Heparin's Anticoagulant ActivityStructural Investigation of Chlamydiaceae-Specific AntibodiesFunctional Characterization of Histoplasma capsulatum Lipid RaftsExploiting Multivalency: A Designed Dimer of CV-N Shows Improved anti-HIV ActivityLectenz®: Carbohydrate-Binding Biomolecules Engineered via Computational Modeling and Directed EvolutionPro-Inflammatory Phenotype Associated with ST6Gal-1 Deficiency is Reversed by Hepatic Expression of a Secretory ST6Gal-1 TransgeneReengineering the Glycan Binding Pocket of Cyanovirin by Directed EvolutionCarbohydrate-Dependent Uptake of Glycopolymers by Macrophages and the Implications for Targeted Drug DeliveryInsights into the Mechanism of Oncofetal Thomsen-Friedenreich (TF) Antigen Recognition by Galectin-3Golgi Phosphopotein 3 Regulates Cell Adhesion to Selectins and ICAM-1 by Controlling Golgi Retention of C2GnT1Use of Siglec-F-Fc and a Novel IgY Antibody Recognizing 6'-sulfated-sialyl Lewis X to Identify Endogenous Lung Ligands for Siglec-FDistinct Flow Cytometric Lectin-Binding Profiles of Urinary Exosomes and Purified Tamm-Horsfall ProteinModular Labeling of Monoclonal Antibodies using Click

ChemistryStudy of Fluorinated SLeX–Selectin Binding by ITC and SPRImmunological Functions of Cholesteryl α-glucosides in Helicobacter pylori-associated InflammationSmall Molecule Recognition of Tumor-Associated Carbohydrate Antigens sLex/aHow the Autophagy Receptor NDP52 Defends the Cellular Cytosol against Bacterial InvasionThe Fine-Specificity of Mannose-Binding and Galactose-Binding Lectins Revealed using Outlier-Motif Analysis of Glycan Array DataWhen Two Biomarkers of Thyroid Cancer Meet: Interaction between galectin-3 and ThyroglobulinMechanistic Basis of Polysaccharide Recognition by LectinsEvidence for Direct Cross-Talk between Two Essential Nutrient-Sensing Enzymes: O-GlcNAc Transferase (OGT) and AMP-activated Kinase (AMPK) Human Cosmc and T-synthase are Transcriptionally Regulated by SP1 Transcription FactorMolecular Mechanism of Cosmc Function in the Biosynthesis of Active T-synthaseCD68's Glycosylation is Modified during RANKL-Induced OsteoclastogenesisTargeted Knockouts of Enzymes of O-GlcNAc Cycling and Epigenetic RegulationEctopic Expression of Core 3 Synthase in Human Pancreatic Cancer Cells Suppresses Tumor Formation and MetastasisGAG-specific Endoglycosidase Assay using 35S-Labeled ProteoglycansPhosphoproteomic Analysis of Drosophila Embryos Deficient in Neural-Specific GlycosylationThe Skp1 Prolyl 4-hydroxylase of Dictyostelium Contributes Glycosylation-Independent and-Dependent Effects on O2-dependent Development without Affecting Skp1 TurnoverDependence of G-Protein Coupled Receptor Agonists Transactivation of Trk Tyrosine Kinase and TOLL-Like Receptors on Neuraminidase-1 and Matrix Metalloproteinase-9 Cross-TalkPathway to Diet–and Obesity–Associated Diabetes through Attenuation of Pancreatic Beta Cell Glycosylation and Glucose TransportIdentification of Specific Sequences in the Polysialyltransferase, ST8Sia IV (PST), that are Required for Substrate RecognitionExcessive Aggregation of Ganglioside GM2 Can Transmit Proliferation Signals via c-Src Kinase DirectlyMucin-Type O-glycosylation is Required for Digestive System Formation and Function in DrosophilaThe Effect of O-GlcNAcylation on hnRNP A1 from Colorectal CancerO-GlcNAc Modification of Proteins and Cancer MetastasisA Sialidase Specificity Assay Reveals Restricted Glycan Tolerance by NEU2Evaluation of the Metabolic Responses Related to Fructose as a Primary Carbon SourceDisruption of Protein O-glycosylation Alters FGF Signaling by Modulating

Basement Membrane CompositionFr nge Effects on Delta1-induced Notch Signaling are Differentially Regulated by GalactoseBrain Specific Expression of GnT-IX (GnT-Vb) is Controlled by Epigenetic Chromatin Regulation and Transcriptional Factors CTCF and NeuroD1Total Internal Reflection Fluorescence (TIRF) Microscopy for the Study of Carbohydrate-Carbohydrate InteractionsN-glycosylation Enzymes in B Cells are Regulated by Innate and Adaptive SignalsAn O-GlcNAc Cycling Complex Associates with the Ribosome in Response to Proteasome InhibitionO-GlcNAcylation of HIPK1 Regulates Its Function towards p53 during Fas-mediated ApoptosisCOG Complex Specifically Regulates the Maintenance of Golgi Glycosylation MachineryA Role for O-fucosylation in Quality Control of Thrombospondin Type-I Repeat FoldingGlycosaminoglycans, New Regulators of α-synuclein Aggregation and Apoptosis in Parkinson DiseaseThe Decrease of O-GlcNAcylated Protein Level is Crucial for Human Hepatoma Cell Death Induced by Protozoan Parasite Entamoeba histolyticaCombining Essential Dynamics and Binding Statistics to Rationalize Glycosaminoglycan Sulfation by N-Sulfotransferase and MutantsO-GlcNAc and the Progression of Prostate CancerStructural and Functional Studies of the Dehydrogenases Required for the Biosynthesis of 2, 3-Diacetamido-2, 3-dideoxy-D-mannuronic AcidGlycobiology of the Probiotic Lactobacillus rhamnosus GG (LGG): A Network-Based ApproachStructural Characterisation of Burkholderia pseudomallei 576 O-Antigen by Mass SpectrometryMutagensis of Conserved Cysteines in Hyaluronan Synthase Can Uncouple Polymerizing Activity and Product Size Control, Two Discrete Enzyme FunctionsA Novel UDP-GlcNAc/GalNAc 4-epimerase, MMP1090, from the Archaeon Methanococcus MaripaludisSubcomponent Analysis of Multivalent Polysaccharide-Based Meningococcal Vaccines from ChinaSynthesis of 2-FL and LDFT by metabolically engineered E. coli through the fkp gene from Bacteroides fragilisMethanosarcina mazei Sweet Uniqueness at its Cell Surface Protein GlycansGlycoinositolphospholipids from Leishmania braziliensis and Leishmania infantum: Modulation of Innate Immune System and Variations in Carbohydrate StructureLeishmania amazonensis Glycosphingolipids, Glycosylinositolphospholipids and Inositol Phosphorylceramide Expression during Amastigote-Promastigote DifferentiationN-glycan Specific Lectin (RVL) from Remusatia vivipara with Potent Insecticidal ActivityToxoplasma Skp1 is Modified by

a Hydroxyproline-Dependent Cytoplasmic Glycosylation Pathway Similar to that Which Modulates Oxygen-Sensing in DictyosteliumSelective Mild Acid Hydrolysis and Structure of a Novel Fucan from Sea Cucumber, as well as Antithrombotic ActivityRedesigning the Carbohydrate Recognition Site of Hen LysozymeThe Anti-angiogenic Activity of the Extracts and Polysaccharides from Karenia mikimotoiComparison of Structure and Bioactivity of Two Sea Cucumber Fucosylated Chondroitin Sulfates with Slight Difference in Sulfation of Fucose BranchesOptimized Linkage Analysis of Oligo-and Polysialic Acids by Fluorescence Labeling and PermethylationProduction of an Antibody against Long-Form N-acetylglucosamine-6-O-sulfotransferase 1 that is Expressed in Human High Endothelial VenulesGlycoengineering Insect Cells for α 2, 3-sialylation of Recombinant GlycoproteinsExamining the Sialic Acid Salvaging Pathway in Lepidopteran Insect CellsPhosphatase-coupled Sulfotransferase AssayA Role for Endothelial Mannose Residues in Inflammation Dependent Monocyte Adhesion Under FlowGlycoengineered Insect Cells Can Use a Bacterial N-acetylglucosamine-6-phosphate 2′-epimerase to Produce Sialic Acid without Exogenous PrecursorsA Key Role for Mgat1 and Complex N-glycans during SpermatogenesisDetection of LacNAc and GlcNAc Moieties on the Cell's Surface with GlycosyltransferasesO-GalNAc Glycosylation of alpha DystroglycanDoes POMGnT1 Activity Influence O-Man-6-PO4 Glycans of alpha-DystroglycanDifferentiation of Oligosaccharide Linkage Isomers by Electron Activated DissociationStudy of Corneal Keratan Sulfate Biosynthesis in vivo using Sulfotransferase Gene Knockout MiceL -MS and LC-MS/MS Study of Heparan Sulfate Oligosaccharides Processed by Human Sulf-2Structural Investigation of 13C Labeled N-linked Glycans from Trichomonas vaginalis by Electron Transfer DissociationPhotocrosslinking of O-GlcNAc-modified Proteins to Neighboring MoleculesScreening of Complex-Fucoidans from Different Species of Brown Algae as Procoagulant Drug Candidates Based on Their Activity, Structural Properties, ImpuritiesSulfotransferase and Fucosyltransferase that Regulate Expression of the 5D4 Keratan Sulfate Epitope in Early Postnatal Mouse BrainImprovement in Production of Secreted Proteins by Use of the Glycosylation TagDevelopmental Roles of Putative Polypeptide GalNAc-Transferases in ZebrafishExploration of Disease-Specific Epitopes by using MUC1 Glycopeptide MicroarrayDevelopment and Application of Versatile Glycan MicroarrayRecognition

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Analysis by Capillary ElectrophoresisGlycosubstrates for Discovery of Selective Inhibitors of Cell Surface Proteolysis by Adam ProteasesP1 Antigen is Present on the Serous Ovarian Cancer Cell Line, IGROV1, Correlating with A4GALT Overexpression and Altered Cell BehaviourMechanism for Discrimination between Neu5Ac and Neu5Gc IncorporationEngineered GFP as a Sensitive Sensor of Glycosylation Site OccupancyQuantitative Proteomics Identifies ICAM-1 as an N-glycosylation Deficiency MarkerTumor Gangliosides Promote Tumor Infiltration by Myeloid Suppressor CellsEngaging the Ashwell-Morell Receptor Elicits Protection against Organ Damage and Disseminated Intravascular Coagulopthy in Fatal SepsisG neral Patterns of Fibronectin Glycosylation Change in Pathological StatesEffective Enrichment of Cancer Stem Cells by anti-CD133 Antibody and SSA LectinMetabolic Studies of a Pathogenic Pathway to DiabetesMultiorgan Toxicity Reviewed in Blood Toxicoprotome via Glycocapture Assisted Global Quantitative Proteomics (gagQP) Identification of Glycosylation on Specific Glycosites using Lectin and Chemical Immobilizations and Mass SpectrometryA Physiological Approach to Assess the Affinity of Lectin Carbohydrate Interactions using Cancer Cells Immobilised on a Biosensor SurfaceLectin Microarray Glycoprofiling of Intact Urine Exosomes and Tamm-Horsfall ProteinHigh-Throughput HPLC-based N-Glycan Analysis of Human Plasma Proteins Identifies Potential Biomarkers for Maturity Onset Diabetes of the Young (MODY) A Toolbox of Human Cell Lines for the Screening and Production of Fully Human and Glycooptimized BiotherapeuticsCommensal Bacteria Expressing the Carbohydrate Human Tumour-Specific Antigen Galß1-3GalNAcα-(Thomsen-Friedenreich) as Potential Tumour VaccineBacterial-Derived Thomsen-Friedenreich Antigen Activates Specific T Cells via Presentation on Dendritic CellsBiochemical Characterization Of PMM2-depleted Zebrafish Suggests An Unexpected Mechanism For Glycosylation Deficiency In CDG-IaColorectal Cancer Desmoplastic Reaction Up-Regulates Collagen Synthesis and Restricts Cancer Cell InvasionVisualizing Alterations in the Trafficking of Glycoconjugates within Niemann-Pick Type C Cells using Cu-Free Click TechnologyStrategies for Glycomics and Glycoproteomics and Glycosaminoglycan Analysis by Mass Spectrometry and HPLCCharacterization of O-glycosylation of the β-amyloid Precursor Protein from Wild Type and CRND8 Transgenic Mice Models of Alzheimer's DiseaseExogenous Mannose Supplementation Rescues Mpi-deficient Zebrafish in a Model of Congenital Disorder

of GlycosylationTSG-6 Irreversibly Transfers Heavy Chains from the Pathological Hyaluronan-Heavy Chain Complex onto Hyaluronan OligosaccharidesTSG-6: Novel Regulator of Viral-Induced Hyaluronan Synthesis in Inflamed AirwaysChemical Inhibition of O-GlcNAc Processing: Role of O-GlcNAc in Tau-Driven NeurodegenerationER Stress Reduces Angiogenesis and the Breast Tumor ProgressionInhibition of U937-Cell Adhesion to Human Endothelial Cells by Glycosylated Lysozyme MutantsPolysialyltransferases STX and PST are Involved in Generating Neuraminic-Acid-Containing Polysialic Acid (NeuPSA), a Modification Found Abundantly in Human Cancer CellsHPLC MS/MS Analyses of Neu5Ac, Neu5Gc and KDN in Head and Neck (H&N) Tumors of the ThroatExpression of TriSia & PolySia in Human Cancers: Potential Role for Diagnostic & Prognostic Biomarkers for Cancer ScreeningHNK-1 Glycan Functions as a Tumor Suppressor for Astrocytic TumorA Simple and Fast Protocol to Obtain Urine N-glycansThe O-MannomeMechanisms for Cancer Metastasis in the Formation of Trimeric Tn Antigen Modified by pp-GalNAc-T13 GeneGlycomics Analysis of Dried Blood Spots. *Glycobiology***21**, 1454-1531

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List of Publications

Published

- **Tanisha Singh,** Abhishek Jauhari, Ankita Pandey, Parul Singh, Aditya B Pant, Devendra Parmar, Sanjay Yadav. Regulatory triangle of neurodegeneration, adult neurogenesis and microRNAs.(***CNS & Neurological Disorders - Drug Targets, 2014, 13, 96-103***)

- Abhishek Jauhari#, **Tanisha Singh#**, Parul Singh, Devendra Parmar and Sanjay Yadav* Regulation of miR-34 Family in Neuronal Development (First author contributed equally) (***Mol Neurobiology (2017). doi:10.1007/s12035-016-0359-4***).

- Abhishek Jauhari#, **Tanisha Singh#**, Ankita Pandey, Parul Singh, Nishant Singh, Ankur Kumar Srivastava, Farah Khan, Aditya Bhushan Pant, Devendra Parmar* and Sanjay Yadav* Differentiation induces dramatic changes in miRNA profile, where loss of Dicer diverts differentiating SH-SY5Y cells towards senescence (First author contributed equally) (***Mol Neurobiology (2016). doi:10.1007/s12035-016-0042-9***).

- Ankita Pandey#, Abhishek Jauhari#, **Tanisha Singh**, Parul Singh, Nishant Singh, Ankur Kumar Srivastava, Farah Khan, Aditya Bhushan Pant, Devendra Parmar* and Sanjay Yadav* Transactivation of P53 by cypermethrin induced miR-200 and apoptosis in neuronal cells (***Toxicology Research, (2015) 4,6, 1578-1586***).

- Ankita Pandey, Parul Singh, Abhishek Jauhari, **Tanisha Singh**, AB Pant, D. Parmar, Sanjay Yadav. Critical role of the miR-200 family in regulating differentiation and proliferation of neurons (***Journal of Neurochemistry, 2015, 133, 640–652***).

- Sanjay Yadav, Abhishek Jauhari, Nishant Singh, **Tanisha Singh**, Ankur Kumar Srivastav, Parul Singh, AB Pant and Devendra Parmar MicroRNAs are Emerging as Most Potential Molecular Biomarkers (***Biochem Anal Biochem 2015, 4:3***)

Communicated

- Abhishek Jauhari, **Tanisha Singh** and Sanjay Yadav*. MiR-29b controls expression of miR-145 and its target proteins during neuronal differentiation (Communicated in ***Experimental Neurology*** IITR Com. No.3475)

Manuscript under Preparation

- **Tanisha Singh**, Devendra Parmar and Sanjay Yadav* MiR-150 regulate cytoskeleton rearrangement and induced axonal degeneration in mature neurons.

- **Tanisha Singh** and Sanjay Yadav. MicroRNAs: Linkers of Aging and Age Associated Neurological Disorders.

- **Tanisha Singh**, Devendra Parmar and Sanjay Yadav. Development of an *In-Vitro* Model for Studying Role of MicroRNAs in Neurodegeneration

Papers presented at Conferences

- **T. Singh,** A. Pandey, P. Singh, A. Jauhari, A. B. Pant, D. Parmar and S. Yadav. Regulation and role of MIR-34 family in neuronal differentiation and apoptosis. ***J. Neurochem. (Suppl. 1) (2015), 102—242***

- **T Singh**, AB Pant, D Parmar, S Yadav. Development of an *In-Vitro* Model for Studying Role of MicroRNAs in Neurodegeneration. ***Indian Journal of Pharmacology, 2013.***

- S. Yadav, A. Jauhari, **T Singh**, P Singh. Understanding Neural Development and Neural Degeneration by targeting microRNAs. LUCSON, 2017.

- S. Yadav, A. Jauhari, **T Singh**, P Singh, A Pandey, AB Pant and D Parmar. microRNAs as macromolecule in regulatory circuit of Neuronal differentiation. ***Journal of Neurochemistry (2013), 125 (Suppl. 1) 106-193. Doi: 10.1111/jnc.12185.***

- A Pandey, P Singh, A Jauhari, **T Singh**, A B Pant, D Parmar and S Yadav. Development of in vitro model for studying role of microRNAs in development of neurotoxicity. ***Journal of Neurochemistry (2013), 125 (Suppl. 1) 106-193. Doi: 10.1111/jnc.12185.***

- A Jauhari, A Pandey, P Singh, **T Singh**, A B Pant, D Parmar, S Yadav. Studies on role of microRNAs in apoptosis of Neuronal cells. Advances in Free Radicals, Redox Signaling and Translational Antioxidant Research & XII Annual Meeting of the Society for Free Radical Research- India (SFRR STAR-2013).

- P Singh, A Pandey, A Jauhari, **T Singh**, A B Pant, D Parmar and S Yadav. Effect of ethanol on Glial cell Apoptosis: Role and regulation of miRNAs. ***Journal of Neurochemistry (2013), 125 (Suppl. 1) 106-193. Doi: 10.1111/jnc.12185.***

ACADEMIC HONORS AND AWARDS

- Awarded ***Women Scientist A*** by **Department of Science and Technology, New Delhi**, India in February 2016.
- Awarded ***Senior Research Fellowship*** by ***Indian Council of Medical Research, New Delhi,*** India.
- **Travel Award** from ***International Society for Neurochemistry*** to attend "The 25th ISN-APSN Neurochemistry Conference held at Cairns, Australia in 2015.
- **CET 2012:** Qualified CET 2012 conducted by Ram Manohar Lohia Faizabad University, Uttar Pradesh, India.