STUDIES ON MOLECULAR MECHANISMS OF IRON MOBILIZATION IN ASTROGLIAL CELLS IN RESPONSE TO CATECHOLAMINE NEUROTRANSMITTER DOPAMINE

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PRATIBHA

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CERTIFICATE

The research work embodied in this thesis entitled "**Studies on molecular mechanisms of iron mobilization in astroglial cells in response to catecholamine neurotransmitter dopamine**" has been carried out at the Special Centre for Molecular Medicine, Jawaharlal Nehru University, New-Delhi, 110067, India. The work presented here is original and has not been submitted in part or full for any degree or diploma of any University/Institution elsewhere.

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I dedicate this thesis to lady science and the guru.

The last one.....

Dear Dopamine.....You are one hell of a molecule

You may drive world crazy.....to me....you were just a tool.

You neglected, son of the gun, Corpus ASTROGLIAL

You were innocent but I had to put you under trial

I was on a quest, to seek the little trouble maker

Known as mitochondria

The heartthrob, the geek, the energy generator

He was keeping secrets. He was producing heme.

“In a non-erythroidcell”..Outrageous at first, then you got me keen.

This conspiracy had Frataxin involved

Who has his own issues...unresolved.

I know your secrets, HemeOxygenase.

Away from crime scene. Sitting...in cytosolic space.

Took me long, to collect the clue

All of you played with me but I can`t sue

Coz, game was good. Kudos to you.

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PRATIBHA

CONTENTS

ABBREVIATIONS

µg	Microgram
µl	Microliter
µM	Micromolar
ACO2	Mitochondrial aconitase
bFGF	Basic Fibroblast growth factor
βME	β-mercaptoethanol
bp	Base Pair
BPS	Bathophenanthroline-disulphonic acid
BSA	Bovine serum albumin
cDNA	Complementary DNA
δ-ALAS	Delta Aminolevulinatesynthase
DA	Dopamine
DCT1	Divalent cation transporter 1
DFO	Desferrioxamine
DMT1	Divalent metal transporter
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
DTNB	Dithiobisnitrobenzoic acid
ECL	Enhanced chemiluminescence
EDTA	Ethylene diaminetetra acetic acid
EGF	Epidermal growth factor
FAC	Ferric Ammnonium citrate
FBS	Fetal bovine serum
FBXL5	F-box and leucine-rich repeat protein 5
Fe	Iron
Fpn1	Ferroportin 1
h	Hour

hfNSC	Human Fetal Neural Stem Cells
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HO-1	Heme oxygenase-1
ISC	Iron Sulfur Cluster
ISCU	Iron-sulphur cluster unit scaffold protein
IRE	Iron response element
IRES	Internal ribosomal entry site
IRP	Iron regulatory proteins
LIP	Labile iron pool
kb	kilobase
kDa	kiloDalton
M	Molar
Mfrn	Mitoferrin
mg	Milligram
ml	Mililiter
mM	Millimolar
MPP	Mitochondrial processing peptidase
mRNA	Messenger RNA
mtDNA	Mitochondrial DNA
Mt-Ft	MitochondrialFerritin
Na-Azide	Sodium Azide
NaCl	Sodium Chloride
nmol	Nano mole
NCCS	National centre for Cell Science
NCOA4	Nuclear Receptor Coactivator 4
OAA	Oxaloacetic acid
OXPHOS	Oxidative phosphorylation
nM	Nanomolar

NTBI	Non-transferrin bound iron
PAGE	Poly acrylamide gel electrophoresis
PBS	Phosphate buffer saline
PCBP	Poly-r(c)-binding protein
PCR	Polymerase chain reaction
PIH	Pyridoxalisonicotinoylhydrazone
PMSF	Phenylmethylsulfonyl fluoride
PVDF	Polyvinylidenedifluoride
qRT-PCR	Qualitative Polymerase chain reaction
RBC	Red blood cells
RISP	Reiske Iron sulphur cluster unit protein
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SD	Standard deviation
SDS	Sodium dodecyl sulfate
STEAP3	Six-Transmemberane Epithelial Antigen of the prostate 3
TBE	Tris borate EDTA buffer
Tf	Transferrin
TfR	Transferrin receptor
TRIS	Tris (hydroxylmethyl)-aminomethane
UTR	Untranslated region
UV	Ultraviolet

Synopsis:

Iron as micronutrient holds paramount importance in cellular survival. The indispensable nature of iron lies in its ability to actively participate in wide variety of cellular processes.

Iron incorporated in proteins as co-factor often acts as redox active centre in biological system. Iron containing prosthetic groups i.e. heme and Iron sulphur clusters (ISC) play versatile roles in all cellular systems. They are known to play crucial role in energy generation, DNA repair, nucleotide biosynthesis and hence all cells require iron for survival. Certain cells carry out processes to utilise significantly high amount of iron such as hemoglobinization in reticulocytes, hepatocytes as they are major stores for systemic iron pool, myelinogenesis in oligodendrocytes. In addition to these major consumers of iron, metabolically active organs consume high level of iron that includes brain, heart, testes and kidney.

Iron exhibits two redox states and exists as ferrous (Fe^{2+}) and ferric (Fe^{3+}) in biological systems. The ferrous form of iron is toxic for cellular system because of their ability to take part in Fenton reaction and to generate reactive oxygen species (ROS). Hence excess iron levels make cells prone for oxidative damages which might be detrimental to cells. But the Janus face of iron will only appear when the level of iron exceeds than the cell`s threshold to maintain the iron homeostasis.

All cellular systems have evolved elegant regulatory proteins that maintain the iron levels in accordance of need. These regulatory proteins are responsible for iron import, export and storage.

Mitochondria are the primary sub-cellular compartment that houses the iron consumption processes. Hence, mitochondria handle significant amount of iron in cells. Both heme and ISC biosynthesis take place in mitochondrion and they apparently share the same iron pool. In erythroid system, cellular iron regulatory proteins govern the mitochondrial iron consumption. The major product of mitochondrial iron consumption machinery is heme. In fact, erythroid mitochondria have developed iron regulatory proteins specific for them. Such system allows high heme biosynthesis required for efficient systemic functioning and fulfils higher iron demands. Heme synthesis is vital for all cells. Heme levels in non-erythroid cells are not directly regulated by iron levels. And not much is known about regulation of heme levels in non-erythroid cells.

Iron sulphur clusters are also required by all cells and cellular iron levels regulate ISC content. But how do mitochondria maintain the balance for flux of iron in the generation of these two iron containing co-factors for efficient functioning of non-erythroid cell is not known in detail.

Brain`s requirement for iron is very high and brain consumes 20% of total body O_2. Thus, if iron level exceeds in brain, they are more prone to oxidative damage. Also, neurons have limited regenerative capacity. Thus oxidative damage in brain might be lethal for body. Hence, brain is provided with the structural barrier that regulates the iron flux and maintains iron pool surrounding brain. This structural barrier is known as Blood brain barrier (BBB) and it is composed of vascular endothelial cells surrounded by projections of astrocytes. Astrocytes are the most populous cells in brain and due to their close contact with neurons as well as BMVECs (Brain microvasculature endothelial cells) they are known to regulate iron transport across BBB and act as gate keeper for iron.

Iron accumulation in brain has been considered as centre of pathology of several neurodegenerative diseases that include Parkinson`s disease (PD), Alzheimer`s disease (AD), Freidrich ataxia (FRDA), neuroferritinopathy, porphyria etc. The brain iron accumulation is not necessarily related to higher systemic iron levels. Rather, most of these neurodegenerative disorders are associated with dysfunctional iron regulatory pathway. The contribution of iron accumulation as cause or consequence of these diseases is still not clear. Also, since mitochondria handle significant amount of iron for synthesising iron dependant co-factors. Along with iron accumulation, mitochondrial dysfunction is also observed to be the common pathological feature that several neurodegenerative diseases share. The brain iron accumulation and mitochondrial dysfunction associated with several neurodegenerative disorders might imply imbalance in mitochondrial iron homeostasis as well.

Iron distribution in brain is not uniform. There are regions which are characterised for higher iron levels such as globuspallidus, caudate nucleus, putamen and substantianigra. Incidentally, these regions are also accounted for dopaminergic neurotransmission. Dopamine is a catecholamine neurotransmitter and controls several functions that include locomotor activity, cognition, learning, reward seeking and endocrine regulation. Imbalance in DA metabolism has been implicated in several neurodegenerative disorders and psychotic conditions such as Parkinson`s disease (PD), Schizophrenia, Attention

deficit hyperactivity disorder, drug addiction and depression. Parkinson`s disease is well studied neurodegenerative disorder and has provided insight on DA metabolism. DA biosynthesis is an iron dependant process and mainly takes place in neurons. Also, iron is abundantly present in DA-ergic neurons to fulfil the high iron demand in carrying out both iron dependant process i.e. energy generation and DA synthesis. This might be the cause of DA-ergic neurons to be more susceptible for oxidative insults. There are reports stating the presence of DA receptors and transporters in astrocytes along with metabolising enzymes. They might be involved in clearing up DA from synaptic regions and thus regulating neurotransmission.

Astrocytes are known to provide physical and nutritional support to neurons. They also exert neuroprotective role against wide variety of physiological stresses including redox stress, glutamate toxicity, metabolic toxicity, and iron toxicity as well as psychological stress such as depression. Hence the well being of astrocytes is very important for efficient brain functioning. Like all other cells, astrocytes also need to maintain its iron homeostasis for cellular survival. But existing literature in context of iron regulation in astrocytes is very limited.

The physiological regulation of iron homeostasis in astrocytes has not been much explored. It is now established that astrocytes might play important role in neurodegenerative diseases. Thus, imbalance in astrocytic iron regulation may lead to drastic consequences. Astrocytes expresse GPI-anchored Ceruloplasmin (Cp), which stabilises the iron exporter Ferroportin on membrane. The neurodegenerative disorder Aceruloplasminemia is caused due to dysfunctional Ceruloplasmin and is characterised by accumulation of redox active iron more prominently in astrocytes than neurons. Also, in cerebral ischemia, levels of Cp increases and higher levels of Cp were correlated to lesser damaged area of brain. This implies that astrocytes have ability to regulate its iron regulation machinery in response to stress stimuli not just for their own survival but for also to confer neuroprotection.

Studies have shown that 6-Hydroxydopamine (6-OHDA), a DA metabolite, modulates iron regulatory protein in astrocytes implying role of astrocytes in PD models. Another study reported that DA elevates levels of iron in mitochondria of astrocytes. Unpublished data from current laboratory showed that DA suppresses the levels of Cp that might suggest decrease in iron export machinery. DA also seems to mobilise iron from iron

stores of ferritin. Furthermore, previous report suggested an increased HO-1 level with simultaneous increase in mitochondrial iron. All these data suggest altered iron metabolism in astrocytes in response to DA.

Mitochondrial iron homeostasis is maintained by regulation of mitchondrial iron transporter, mitochondrial iron storage protein as well as components of iron utilising machinery. But these proteins do not seem to be regulated by cellular IRE-IRP system. The exact mechanism of how these components get regulated is still not clear.

All cells express mitochondrial iron importer Mitoferrin 2 (Mfrn2), which is an isoform of Mfrn 1. Mfrn1 is expressed in erythroid cells and forms stable complex with proteins involved as donor of iron (Ferrochelatase) and exporter of heme (ABCB10) on mitochondrial membrane. This complex formation allows efficient heme synthesis. Also, half life Mfrn1 is very high. Thus, Mfrn 1 contributes in iron influx of mitochondria to sustain heme synthesis in erythroid cells. But Mfrn2 does not exhibit any such property as per the existing literature.

The metabolically active organs like testes, brain, heart, kidney and spinal cord express mitochondrial ferritin (Mt-Ft) potentially to store excess iron. The role of Mt-Ft has been suggested in over-expression studies and it might induce mitochondrial iron accumulation.

Mitochondrial iron is used for synthesis of heme and ISC cofactors. Hence, increase in mitochondrial iron level might suggest increase in both heme and ISC content. Defects involved in assembly of ISC or heme synthesis are reported to be associated with mitochondrial iron accumulation, dysfunction, oxidative stress and neurodegeneration. Although both heme and ISC share the same iron pool, how mitochondria manage to maintain the delicate balance between the two essential biosynthesis processes, is not known.

The present study has provided the insight on fate of elevated iron in mitochondria in astrocytes in response to DA. It was found that DA could induce the levels of heme in astroglial cells with simultaneous reduction of ISC content. There has been no change in Mt-Ft expression; however, DA has been found to increase Mfrn2 expression both at protein and mRNA level.

There are speculations that a regulator of ISC assembly, Frataxin, may affect heme synthesis pathway. It is observed that in neurodegenerative disease Friedreich's ataxia, where Frataxin levels are drastically reduced are characterised by hampered ISC assembly pathway and mitochondrial iron accumulation. The defect in heme synthesis was also reported in FRDA. Also, in Frataxin over-expressed transgenic mice, erythroid differentiation process was observed to be altered. Frataxin is speculated as both enigmatic protein with potential to act as iron storage, metabolic switch, and iron chaperone, as well as mysterious protein as it holds hidden potential to regulate cellular processes in tissue specific manner as well. The half life of Frataxin in neuron is very high than that of astrocytes. Relative abundance of Frataxin varies in normal cells and cancer cells. As cancer cells are known to have lower levels of Frataxin. Also, Frataxin expression is very high in metabolically active cells only. The erythroid cells express an isoform of Frataxin, which does not have mitochondrial targeting sequence. The cytosolic expression of Frataxin in erythroid cells might suggest assembly of ISC in cytosol. This may rule out the possibility of sharing iron pool for heme and ISC synthesis in erythroid cell type.

The current study reveals that DA decreases Frataxin expression in astrocytes. Data suggest that DA mediated down-regulation in Frataxin is due to transcriptional repression. DA also induces Ferrochelatase expression. By using Frataxin over-expression system established by transient transfection of Frataxin flag construct in C6 astroglial cells, DA mediated repression in ISC levels was restored. In fact the ISC levels were found to be induced in Frataxin over-expression scenario. Similarly, DA mediated increase in heme level was decreased in case of Frataxin over-expression. However, there was no alteration of mitochondrial iron as well as Ferrochelatase in response to DA. This suggested that DA might regulate Frataxin level in astrocytes to induce heme synthesis. The complete understanding of DA-induced heme levels inastroglial cells and other consequences of decreased Frataxin in terms of ISC homeostasis needs further study.

In summary, the present study reveals an insight on mitochondrial iron homeostasis taking place in astrocytes in response to DA. While dopamine biosynthesis takes place in neurons, astrocytes are majorly responsible for DA metabolism in synaptic regions. While DA induces the expression of HO-1 and increases the levels of mitochondrial iron, the present study gives an insight on fate of elevated iron level in mitochondria in response to

dopamine and reveals a molecular mechanism that induces heme levels and suppresses ISC content mediated by regulation of Frataxin.

The current study opens up the possibility of understanding the equilibrium between heme and ISC biosynthesis in a non-erythroid cell that has so far been eluded in the literature.

Iron in biological system

Iron is ubiquitously present in environment and it`s abundance is second highest amongst metals in earth`s crust (Crichton, R.R et al, 2001). This micronutrient plays key role in fundamental biological processes. Almost all organisms require iron. It is involved in critical cellular processes like energy generation, nucleotide biosynthesis, DNA repair etc. Hence, iron deficiency is a major concern for efficient cellular functioning. However, excess of iron can cause toxicity due to its redox active nature and ability to participate in Fenton reactions and generate reactive oxygen species (ROS) to cause cellular damage. Hence, iron levels in cells have to be maintained for cellular survival and efficient functioning.

Cellular iron homeostasis:

All cells have evolved elegant regulatory pathways to monitor cellular iron levels and balance the iron pool in accordance of their needs. In general, iron regulatory components include iron importers, iron exporters, iron storage proteins, iron consumers, and chaperone proteins mobilising intracellular iron.

- **IRON IMPORT**: There are several mechanisms known for iron import that takes place in different cells to provide with iron demand. The pathwaythat most cellsutiliseis Tf-TfR1 pathway(Cheng, Y et al., 2004). Transferrin (Tf) binds to iron with extraordinary affinity and forms diferric iron complex. This iron bound transferrin gets internalized in cell after docked on transferrin receptor 1 (TfR1) by endocytosis. Once internalized, in endosomes, the acidified surrounding leads to conformational change in Tf-TfR1 to release iron. The ferrireductase subsequently reduces iron and divalent metal transporter (DMT1) transfers reduced iron from endosomes to cytoplasm (Fleming, M.D et al., 1998). Although there are several other mechanisms exist specific for different cells and specific conditions by which import of iron takes place e.g. in hepatocytes and duodenal crypt cells import occurs through TfR2 (Kawabata, H.Eet al, 1999). Erythroid cells also express TfR2 (Levy, J.E eta l, 1999). The affinity of transferrin receptor 2 (TfR2) is 30 fold lower than that of TfR1 (Trinder, H., et al, 2003). Many cells utilise DMT1 for intake of non-transferrin bound iron such as apical surface of enterocytes and astrocytes (Gunshin, H et al., 1997; Ferguson, C.J et al., 2001).

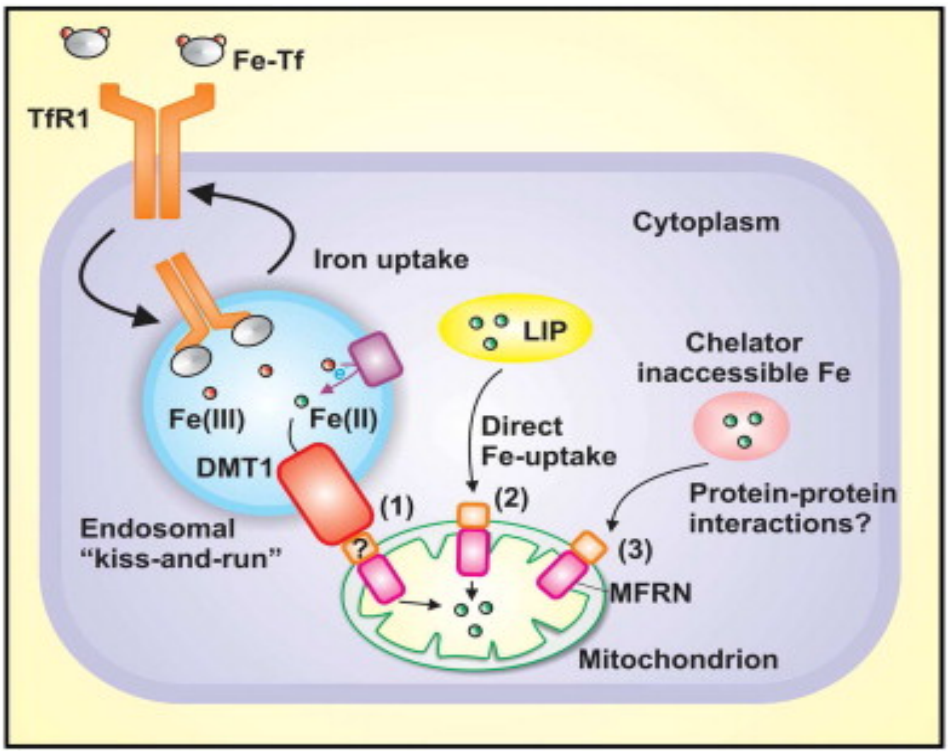
- **IRON STORAGE**: Once inside cell, excess iron gets stored in iron storage protein ferritin which has the ability to oligomerize and form nanocage structure (Torti, F.M et al, 2002).

- **IRON EXPORT**:Till date, the only identified iron exporter in mammalian cells is ferroportin (Torti, F.M et al, 2002). It requires ferroxidase activity for exporting iron from cells. The ferroxidase activity is provided by hephaestin in intestinal cells (Vulpe, C.D et al, 1999), amyloid precursor protein (APP) in neuronal cells (Duce, J.A et al, 2010) and ceruloplasmin in astroglial cells (Harris, et al, 1999).

- **CELLULAR LABILE IRON POOL**: Iron from endosome fluxed into pool of labile iron which is characterised as low molecular weight molecule bound iron accessible for membrane permeable chelators (Hider, R.C et al, 2013). It acts as transit pool for iron, from where iron may get exported out from cell, or stored in ferritin or processed in mitochondria.

- **IRON CONSUMPTION**: Most of the iron gets fluxed into mitochondria where it is used for heme synthesis and Iron sulphur cluster (ISC). Although the import of iron in mitochondria is not well understood in non-erythroid cells. In erythroid cells, the fusion of endosomal membrane and mitochondria has been reported (Ponka, P et al, 1997). The isoforms of mitoferrin, 1 and 2, expressed in erythroid cells and non-erythroid cells respectively regulate iron import in mitochondria (Richardson, D.R et al, 2010).

- **IRON SENSORS**: Iron regulatory pathways are well regulated at transcriptional, post transcriptional, translational and post translational level but post-transcription mediated regulation is the only well characterised mechanism known. Iron import, export and storage is synchronised according to cellular iron levels.



Adapted from Lane, D.J.R et al, 2015

Figure 2.1: The major cell iron import pathways, transferrin and non-transferrin bound iron mediated uptake of iron in cell. Some cells takes in transferrin bound iron under physiological conditions and under excess iron condition, takes up non-transferrin bound iron as well. While some cells imports iron primarily by non-transferrin bound iron only. Transferrin bound iron once internalized in endosomes, gets liberated from TfR by acidification induced conformational modification. Liberated iron fluxed out of endosomes by divalaent metal transporter,DMT after undergoing reduction by STEAP3, six transmembrane epithelial antigen of the prostate 3 or by ascorbate. The fluxed iron is divalent ferrous which is potentially toxic and has to be sorted out in order to avoid toxicity. The intake of non-transferrin iron also occurs through DMT1 present on membrane. Iron from cytosol may get mobilised into mitochondria where iron gets incorporated into heme or iron-sulfur clusters (ISCs). Iron may get stored into ferritin as well or can be exported outside.

The *Trans*-acting iron regulatory protein (IRP) interacts with Iron-responsive elements (IRE). The IREs are conserved elements with ability to form hair pin structures in untranslated regions (UTR) of mRNA of iron regulatory protein. The presence of IRE in 5`-UTR allows translational inhibition due to close proximity of transcription start site (Paraskeva et al., 1999) with IRE occupied with IRP. The transcript coding for Ferritin Heavy and light chain (Hentze and Kühn 1996), the first

enzyme of heme biosynthesis (erythroid 5-aminolevulinic acid) (Donovan et al. 2000), citric acid cycle enzyme (mitochondrial aconitase) (McKie et al. 2000) and ferroportin(Eisenstein and Ross 2003) contain single IRE in 5`-UTR and hence, IRP bound to IRE for such genes allows suppression of their expressions. However, the presence of IRE in 3`-UTR allows mRNA to stabilize upon IRP binding (Hentze and Kühn, 1996). Multiple IREs exists on TfR while single IRE is present in one of the isoform of DMT1 (Hubert and Hentze, 2002).

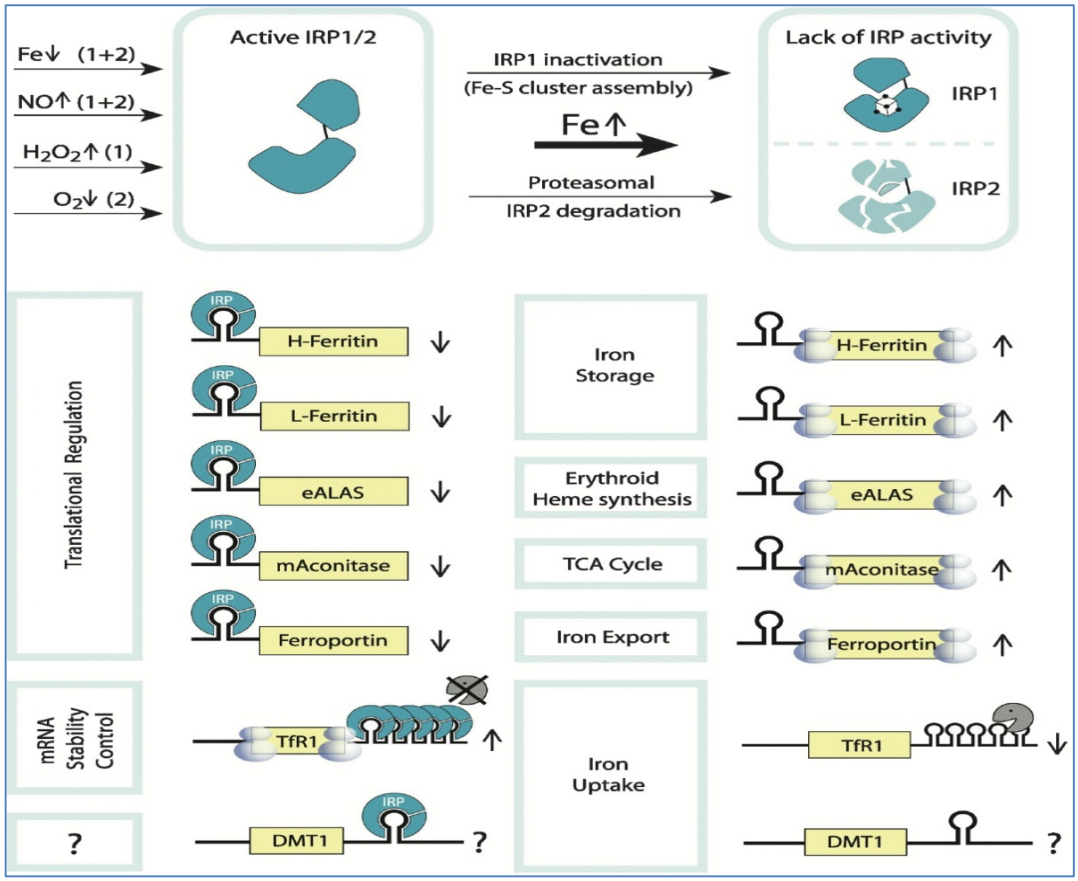
Iron sensor activity of IRPs lie in their ability to modulate their binding ability on IRE and to undergo degradation depending upon cellular iron levels. There are two IRPs exist, IRP1 and IRP2.

The ability of IRP1to interacts with IRE lies in its iron sulphur cluster level. An intact 4Fe-4S cluster of IRP1 enables it to exert enzymatic activity of cytosolic aconitase. The higher cellular iron levels maintain ISC of IRP1 and hence do not allow them to bind on IRE.

While protein stability of IRP2 is maintained by cellular iron level independent of ISC. The iron repleted condition induces protein degradation of IRP2 (Hentze and Kühn, 1996).

In addition to iron, hypoxia and nitric oxide also regulates IRP levels (Hanson, E.S et al, 1999).

- **IRON CHAPERONES**: The role of iron chaperones was acknowledged when the questions regarding how cytosolic iron dependant enzymes utilises iron from ferritin stores. In this context, the chaperonic activity of Poly C binding proteins, PCBPs were unravelled recently; these were originally identified as RNA binding proteins (Chaudhay, A et al, 2010). There are four PCBP`s exists, PCBP1-4. The chaperonic activity of PCBP1 and 2 lies in their ability to bind ferrous iron with lower micromolar affinity (Philpott, C et al, 2017) and to interactwith DMT1 (Lane, DJR, 2014), Ferroportin (Yanatori, I et al, 2016) and ferritin (Leidgens S et al, 2013). And facilitate iron mobilization in cytosol.



Adapted from Hentze, M et al., 2004

Figure 2.2: **Transcriptional regulation mediated by IRE-IRP system on cellular iron homeostasis.** The components of iron regulatory pathways have conserved iron responsive element (IRE) in their untranslated region (UTR) of transcripts. The IRE sequences form specific hairpin loop structure on which iron regulatory proteins (IRP) binds. The presence of IRE in 5`-UTR and proximity of IRE to translational initiation site blocks the translation of transcripts. Such IRE exists in ferritin (H and L chain), erythroid specific amino-levulinate synthase (e-ALAS), mitochondrial aconitase (m-aconitase) and ferroportin. While the presence of 5`-IRE in the presence of IRP leads to translational blockage, the presence of IRE in 3`-IRE leads to additional mRNA stability in transcripts such as Transferrin receptor 1 and DMT1. The ability of iron regulatory proteins to undergo degradation or maintain its stability by cellular iron levels allows them to act as iron sensors. Under iron deprived condition, Iron sulphur clusters of IRP1 loses integrity and hence become active to bind on IREs. Also, IRP2 proteins gain stability by inhibiting FBXL5 mediated degradation. Hence, in iron deprived conditions, transferrin receptor 1 and divalent metal transporter protein level increases while the levels of exporter, storage or mitochondrial consumption proteins decrease.

Iron in brain:

Brain is one of the major consumers of iron. It offers home to metabolically hyperactive neurons and iron dependant processes such as myelinogenesis, and neurotransmitter biosynthesis. 20% of total O_2 consumption takes place by brain only. As a result, brain cells are very sensitive for oxidative stress. Hence, to protect itself from potential damage that iron may cause, brain has evolved a homeostatic pathway for iron that differs significantly from systemic iron pathway. Blood brain barrier contributes in separating iron regulatory proteins in cerebrospinal fluid from serum iron regulatory proteins. In this way, iron levels in brain doesn`t get significantly affected by peripheral iron pool.

Iron in brain is not uniformly distributed. There are regions in which iron abundance reach as high as that of liver such as globuspallidus, substantianigra pars compacta, interpeduncular nucleus, dentate nucleus, and interpositus nucleus (Hill and Switzer, 1984). The abundance of iron is highest in oligodendrocytes in comparison to other brain cells. The high content of brain makes it prone for oxidative damage too. Several neurodegenerative disorders are reported with excess iron accumulation but whether it's a part of cause or consequence is not clear so far.

In brain, consumption of iron varies in different cells e.g. consumption of iron by oligodendrocytes is very high due to myelinogenesis. While neuronal iron need is high due to hyper mitochondrial activity.

Iron mobilisation from blood to brain:

The brain is protected from direct systemic blood supply by blood brain barrier. Such biological barrier allows the surrounding of brain to not experience fluctuations and maintains its own homeostasis. The blood brain barrier is composed of micro vascular endothelial cells (BMVECs) held together by tight junctions.

- The barrier is surrounded by basement membrane as well as end-foot processes of astrocytes (Abbott, N.J et al, 2006). Iron uptake across BBB occurs primarily by Tf-TfR1 pathway (Roberts, R.L et al, 1993).
- The non-transferrin mediated uptake of iron through DMT1 is also reported in BBB and known to play important role in brain physiology (Burdo, J.R et al, 2003).

- There are transferrin homologues reported in BMVECs that are known to mediate iron uptake such as lactoferrin, melanotransferrin but how much they contribute in iron flux is not known (Demule, M et al 2002; Fillebeen, C et al, 1999).

Neurons:

Neurons are the cell with intrinsic electrophysiological ability. They can excite and send neurotransmission across other cells which is the basis of our body`s ability to process emotions, locomotion and multiple external stimuli. Iron is essential for neuronal functioning as neurons are considered as one of the major metabolically active cells.

- Iron uptake in neurons primarily occurs through Tf-TfR1 pathway (Oh, T.H et al, 1986).Neurons also express high levels of DMT1in their membranes (Garrick, M.D et al, 2003).
- Several Ferrireductases are known to be expressed on neurons namely Steap 1,Steap 2 and stromal cell derived receptor 2, SDR2; but their precise membrane localisations are still not clear (Ohgami, R.S et al, 2006).

Astrocytes:

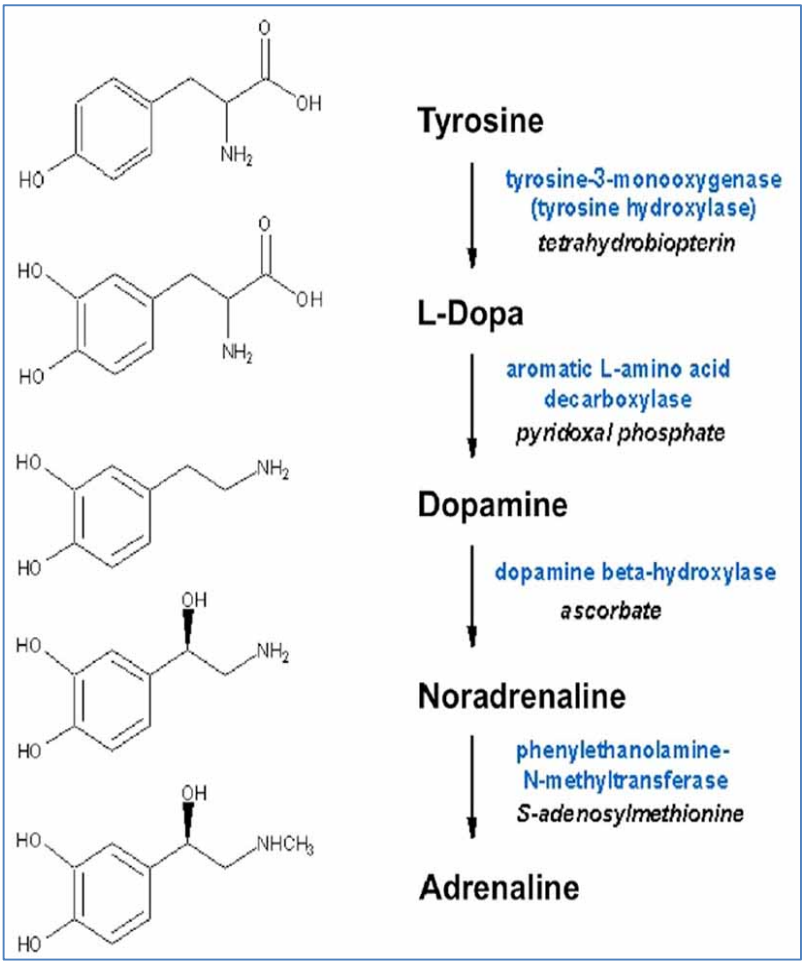
Astrocytes, the major cell type among non-neuronal brain cells, are vital regulators of neuronal function, synapse formation and brain developmental process.

- Astrocytes at tripartite junction are active contributor of neurotransmission, as they also express receptors for neurotransmitters, initiate signalling pathways and modulate synaptic behaviour (Papouin, T et al, 2017).
- Besides regulating neurotransmission at synapse they are connected with neurons for providing them with metabolic support, neurotransmitter precursors and buffering ions. Astrocytes are also connected well among each other by gap junctions and specialized channels to allow exchange of nutrition and ions for further expanding astrocytic network to confer stronger regulation of neuronal neurotransmission and synaptic behaviour (Pannasch, U et al, 2013).
- During embryonic development of brain, neurogenesis precedes astrogenesis in cortex but synaptic formation takes place after astrocytic generation at the site (Farhy-Tselnicker et al, 2018).

Dopamine:

Dopamine (DA), a catecholamine neurotransmitter, is responsible for reward seeking behaviour and motor controls. It is also involved in pleasure embracing behaviour and often associated with drug addiction.

- **Biosynthesis**: DA biosynthesis takes place in catecholaminergic neurons located in ventral tegmental area of the substantianigra, midbrain and the arcuate nucleus of the hypothalamus. Once synthesised, it can be sequestered in vesicles in site of synthesis only, before it gets released during neurotransmission.
- It is synthesised from amino acid tyrosine. Tyrosine undergoes hydroxylation catalysed by tyrosine hydroxylase and converted into DOPA. This reaction is an iron dependant reaction along with tetrahydrobioptren and O_2 as co-factor as well. This is a rate limiting reaction. DOPA gets converted into dopamine by enzyme aromatic acid decarboxylase (Meiser, J et al 2013). D-DOPA is also known to get converted into D- amino acid oxidase which acts as alternative pathway for biosynthesis (Kawazoe, T et al, 2007)
- Dopamine is one the catecholamine neurotransmitter. The classical biosynthesis process for catecholamine neurotransmitter follows similar steps and depending upon the presence of biosynthetic enzymes, end-product varies (Schulz, C et al, 2004).
- Once synthesised, DA gets internalised into vesicles by vesicular monoaminergic synaptic transporter (VMAT-2) which is present on vesicular membranes. In the vesicles, the concentration of DA may reach up to 0.1M (Gonzalez-Alvear, G.M.et al, 1994).



Adapted from Cosentino, M et al, 2015

Figure 2.3:The classical pathway for dopamine biosynthesis. Tyrosine is the precursor molecule for DA biosynthesis which undergoes sequential processing catalysed by first Tyrosine hydroxylase and then aromatic L-amino acid Decarboxylase. Tyrosine hydroxylase is the rate limiting enzyme.

Dopamine and Astrocytes

- Once released in synaptic cleft, it exerts neuro-modulatory activity mediated by post-synaptic neuronal uptake as well as pre-synaptic regulatory uptake. To stop the signalling, DA undergoes removal by surrounding glial cells. In general, Neuronal catecholamine turnover is mostly dependant on vesicular leakage rather than neuronal activity and there metabolism occur mostly intra-neuronally after leakage from stores (Eisenhofer, G et al, 2004).

- The surrounding glial cells readily degrade DA by MAO and also by catechol-O methyl transferase (COMT). COMT transfers methyl groups from S-adenosylmethionine (SAM) to hydroxyl groups of various catecholic compounds. 3-O-methylation of DOPAC by COMT leads to homovanilic acid (HVA), one of the main degradation products of DA. COMT operates in glial cells but there is no COMT activity in DAergicnigro-striatal neurons (Meiser, J et al, 2013).
- There is a subset of cortical astrocytes identified that are found to be competent for regulating Dopamine (DA) homeostasis during postnatal development of the prefrontal cortex (PFC), allowing for optimal DA mediated maturation of excitatory circuits. Such control of DA homeostasis occurs through the coordinated activity of astroglial vesicular monoamine transporter 2 (VMAT2) together with organic cation transporter 3 and monoamine oxidase type B, two key proteins for DA uptake and metabolism.
- Astrocytes throughout the brain express dopamine receptors. Extracellular dopamine triggers rapid concentration-dependent stellation of astrocytic processes that is not a result of dopamine oxidation but instead relied on both cAMP-dependent and cAMP-independent dopamine receptor signalling (Galloway,A et al, 2004).

Mitochondrial iron homeostasis:

Mitochondrion is the major consumer of iron in all cells. It houses the biosynthesis machinery of two major iron containing co-factors, heme and Iron-sulphur clusters (ISC). Liver is the major consumer of iron (Wang, Z et al, 2010). In rat hepatocytes, metabolically available iron was measured in mitochondria and cytosol. It was found that mitochondrial iron content as twice of that in cytosol as measured by fluorescent chelators (Rauen, U et al, 2008). Apparently both heme and ISC biosynthesis machinery shares same mitochondrial iron pool. They might compete for iron if balance between the two processes is hampered. Both heme and ISC are indispensable components of proteins which are involved in vital processes such as energy generation, TCA cycle, DNA repair, and nucleotide biosynthesis. Hence, mitochondrial iron homeostasis is the necessity for efficient mitochondrial functioning as well as cellular survival.

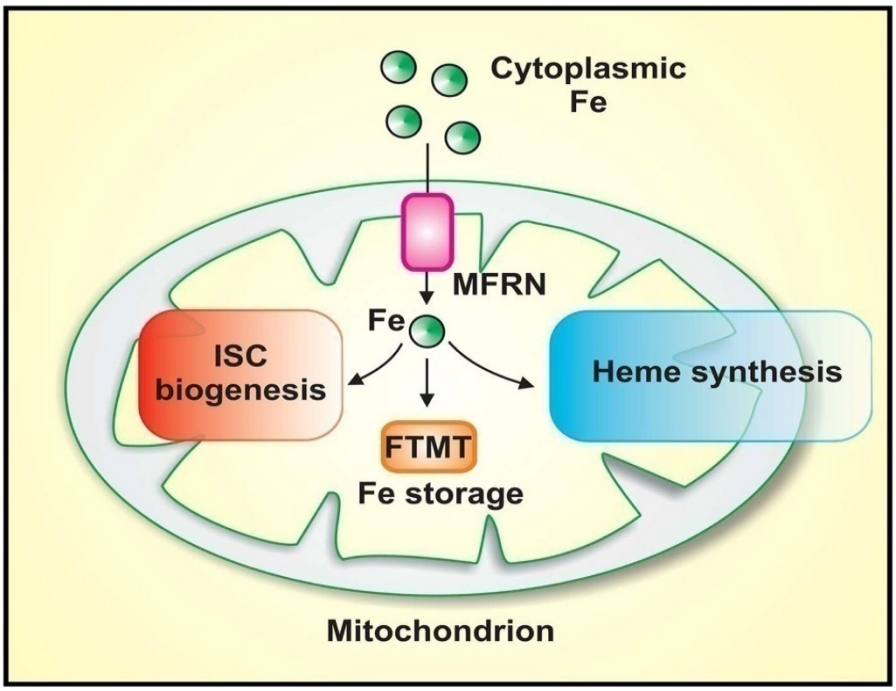


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Figure 2.4:Components of mitochondrial iron homeostasis: Cytosolic iron gets fluxed into mitochondria by mitoferrin (MFRN). Iron in mitochondria may get stored in mitochondrial iron storage protein, mitochondrial ferritin (FTMT). Or it can be used in consumption in biogenesis of iron sulphur clusters (ISC) or heme.

Brain is the second most metabolically active organ and consumes 20% of body`s oxygen (Wang, Z et al, 2010). This suggests that mitochondrial activity of brain will be very high along with high mitochondrial iron content.

Iron-sulphur cluster biosynthesis:

Iron sulphur clusters are elegant complex of iron and sulphur which gets incorporated in wide variety of proteins. These redox active centres may act as centre of catalysis, may regulate centre of enzymatic activity, or regulator of stability.

The ISC may exist as simple (2Fe-2S) and (4Fe-4S) complex while degree of complexity may increase using the simple ISC as basic unit providing wide range of redox potential (-700 to 400 mV) (Beinert, H et al 2000).

ISCs are involved in biological systems which are critical for cellular survival e.g. electron transport chain, TCA cycle, and iron homeostasis.

- Briefly, ISC are assembled by sulphur transfer and iron acquisition on a scaffold protein, ISCU (Iron-sulphur cluster unit protein). While sulphur transfer is catalysed by protein complex Cysteine desulfurase (Schwartz, C.J et al, 2000), Nfs1-Isd11, iron donor in ISC assembly is still a matter of debate. Initially Frataxin was thought to donate iron on ISCU but then reports proposed its role as regulator of the assembly (Yoon, T et al, 2003).
- Recent literature suggested that Frataxin interacts with ISCU and along with $Fe^{2+,}$ and modulates the activity of Cysteine desulfurase (Tsai, T.L et al, 2010).
- Both Frataxin and ISCU are essential for ISC biosynthesis and deficiency of either of proteins affect ISC assembly in cells. Since ISC based proteins are active participants of crucial processes, misregulated ISC assembly has pathological implications.

FRATAXIN

- Frataxin is an enigmatic protein which has the ability to participate in ISC biogenesis as well as to undergo oligomerization.
- Frataxin is translated to be 210 amino acid precursor proteins containing mitochondrial localisation signal. Once precursor peptide reaches mitochondria, it undergoes step wise proteolytic cleavage by mitochondrial processing peptidase (MPP) to process it first to intermediate form, $hFXN_{56\text{-}210}$ and finally to mature functional form, $hFXN_{81\text{-}210}$ (Condo, I et al, 2007).
- Frataxin is an evolutionarily conserved protein but its precise role has been long closeted and even till now clarity has not been achieved. Frataxin was initially reported to be co-immunoprecipitated with components of ISC assembly machinery along with MPP-β (Schmucker, S et al, 2011). Because of its ability to bind to

multiple Fe (II) ions on exposed regions, it was suggested to act as iron chaperone in ISC biosynthesis (Lane, D.J et al, 2015).

- The Frataxin protein also has ability to interact with multiple proteins including ferrochelatase (He, Y et al, 2004), mitochondrial aconitase (Bulteau, A.L et al, 2004), succinate dehydrogenase and chaperone proteins like HSC20 (Shan,Y et al, 2007).

Table 1: Disorders related to ISCU biosynthesis

Disease	Protein	Characteristic feature
Freidrich`s ataxia	Frataxin, FXN	Neurodegeneration at basal ganglion region and hypertrophic cardiomyopathy (De Biase, I et al, 2007)
SideroblasticAnemia	Glutaredoxin 5, GLRX5	Characterised by mitochondrial iron overload in erythroid precursor cells (Camaschella, C et al, 2007).
Hereditary myopathy with severe intolerance for exercise.	ISCU	Characterised by lactic acidosis, episodes of rhabdomyolysis and myoglobinuria (Mochel, F et al, 2008)
X-Linked sideroblasticanaemia and ataxia syndrome.	ATP-binding cassette protein, ABCB7	Characterised by cerebellar ataxia and hypochromic microcytic anaemia (Allikmets, R et al, 1999)

Heme biosynthesis

Existing literature of heme-iron biology in brain is very slim as compared to erythroid systems. As in erythroid cell, mitochondrial iron homeostasis is dedicated to efficiently synthesise heme to support total systemic functioning.Because the erythroid mitochondrial iron homeostatic pathways are well understood, hence the knowledge of erythroid mitochondrial iron regulatory pathways is generally used for exploring heme biosynthesis pathways in non-erythroid cells.

Cellular iron import:

Table 2: comparison of cellular iron import in erythroid cells and Brain cells

Erythroid system	Brain
1. In erythroid cells, iron import takes place through endocytosis of diferricTf mediated by TfR1 (Bruno, M et al., 2015). Serum transferrin saturation is ~30% (Bradbury, M.W et al, 1997). 2. Patients of atransferrinemia shows signs of severe anemia (Beutler, E et al, 2000) 3. Pinocytosis of extracellular ferritin has been reported in erythrocytes (Leimberg, M.J et al, 2008). 4. Erythroblasts also express heme importer Heme-Responsive Gene 1(HRG1), but whether it contributes in iron acquisition or not, is not known (An, X et al, 2014).	1. Astrocytes import Non-transferrin bound iron mediated by Divalent metal transporter, DMT1.Interestingly, astroglioma are reported to express TfR1 as well (Lis, A et al, 2004). 2. Neurons take up Transferrin bound iron mediated by TfR1. In CSF, transferring saturation is ~100 % (Moos, T et al, 2000; Moos, T et al, 2007).

Mitochondrial iron storage:

Mitochondrion contains a protein having structural and functional similarity with ferritin called mitochondrial ferritin (Mt-Ft) (Levi, S et al, 2001). It has tissue specific expression and is known to be expressed only in highly metabolically active tissue such as brain, heart, testes, pancreas (Santambrogio, P et al, 2007). Mitochondria are the primary organ of iron consumption and hence the presence of iron stores in mitochondria is surprising. The precise role of mitochondrial ferritin is not known.

- Mt-Ft is coded by an intronless gene present on Chromosome 5q23.1. The encoded product is a 242-amino acid precursor whose sequence is 79% similar to cytosolic ferritin heavy chain (Bou-Abdallah, F et al, 2005). This 30-kDa protein is targeted to mitochondria and processed to be a 22-kDa protein (Levi, S et al, 2001).
- Unlike cytosolic ferritin, Mt-Ft lacks IRE and does not follows regulation by cellular IRE-IRP system (Levi, S et al, 2001)

Table 3: Comparison of mitochondrial ferritin in erythroid and brain cell

Erythroid system	Brain
1. Erythroid cells expresses lower levels of Mt-Ft but in sideroblasticanemia, Mt-Ft levels are reported to be increased and this is thought to avoid iron toxicity (Cazzola, M et al, 2003)	1. Mt-Ft expressions are significant in neurons as compared to glia (Santambrogio, P et al, 2007). 2. The level of Mt-Ft increases in neurons of restless legs syndrome victims, the neurodegenerative disease characterised by lower iron levels in substantianigra (Snyder, A.M et al, 2009).

- A study reported that over-expression of Mt-Ft on H_2O_2 treated HeLa cells limits oxidative damage to cells. A study conducted in over-expressed recombinant Mt-Ft in Hela cells showed the presence of ferroxidase activity in Mt-Ft, similar to cytosolic Ft-H chain. They also showed the incorporation of radiolabelled iron, Fe^{55}

in immunoprecipitated Mt-Ft which suggested its role akin to that of ferritin i.e. as storage protein for iron in mitochondria (Corsi, B et al, 2002).

- Since both role and regulation arenot clear for Mt-Ft, the over-expression of Mt-Ft in cell line has been used as tool to unravel the potential of this protein.
- The over-expression of Mt-Ft induces shunting of iron in by altering iron regulatory system which includes IRE-IRP system. There`s an increase in TfR1,and decrease in ferritin. Hence over-expression of Mt-Ft induces iron influx in mitochondria by both increasing cellular iron uptake and depleting cytosolic iron stores (Nie, G et al, 2005).
- Due to potential role of Mt-Ft as iron storage protein, over-expression of Mt-Ft was expected to be cytoprotective against oxidative damage.
- A study reported that over-expression of Mt-Ft increases the cell`s susceptibility towards oxidative stress as depicted by reduced glutathione levels and increased ROS in cells pre-treated with *tert*-butyl-hydroperoxide (t-BHP) (Lu, Z et al, 2009).
- Hence, existing literature in context of mitochondrial ferritin have scarce knowledge about its role as well as regulation.Contradictory reports suggest its role as cytoprotective.
- Mutation in Mt-Ft is known to be associated with age related macular degeneration (AMD). It is also noted that ageing increases Mt-Ft levels in retinal pigment epithelial cells while suppressing it's conversion into mature form. Over-expression of Mt-Ft in retinal pigment epithelial cells induces mitophagy in HIF-1α dependant manner (Wang, X et al, 2016)

Cellular iron storage:

Ferritin stores iron by forming nanocage structure. The ratio of composition of ferritin in heavy and light chain unit's oligomerization is cells specific. The mobilization of iron from ferritin is not well understood so far.

Table 4: Comparison of cellular iron store in ferritin in erythroid and brain cell

Erythroid system	Brain
1. Erythroblasts express Ferritin but erythrocytes have higher expression of NCOA4 (An, X et al, 2014). Ferritin acts as	1. Ferritin abundance is least in neurons and maximum in microglia (Mackenzie, E.L et al, 2008). While astrocytes comes in

cargo for NCOA4 by forming complex in iron deficient condition and targets to autolysosome for degradation. This may acts as mechanism by which iron stores of ferritin supports erythropoiesis.	between. The moderate level of ferritin in astrocytes suggests its role in maintaining homeostasis instead of having either higher energy need providing with the function of storage.

Cellular iron export:

Ferroportin is the only iron exporter but it required the activity of ferroxidase for fluxing iron out of cell. The ferroxidase activity is provided by different proteins and they are cells specific.

Table 5: Comparison of exporter, Ferroportin in erythroid and brain cell

Erythroid system	Brain
1. Erythroid cells are reported to expressFerroportin (Fpn) (Cianetti, L et al, 2005). But the biological significance of Fpn expression in erythroid cells is not known, whether it is to contribute in systemic iron pool or to protect from iron toxicity.	1. Ferroportin is reported to be actively involved in maintaining iron homeostasis in both neurons and astrocytes but ferroxidase activity is provided by different proteins. In neurons, Amyloid precursor protein (APP) is known to provide for ferroxidase activity while GPI-Cp(GPI anchored Ceruloplasmin) is reported to be expressed by astrocytes (Duce, J.A et al, 2010; Dringen, R et al, 2007)

Mitochondrial iron import:

A mitochondrial iron importer Mitoferrin (Mfrn) belongs to the family of mitochondrial carrier superfamily. Mfrn 1(SLC25A37) and Mfrn 2(SLC25A28) are homologous proteins with 75% homology to each other (Paradkar, P et al, 2009).

Table 6: Comparison of mitochondrial iron import in erythroid and brain cell

Erythroid system	Brain
1. A kiss and run mechanism has been demonstrated in erythroid cells where iron directly gets transferred into mitochondria form lysosome bypassing cytosol (Hamdi, A et al, 2016) 2. Erythroblasts express iron importer Mitoferrin 1 (Mfrn1) on inner mitochondrial membrane (Shaw, G.C et al, 2006). Mfrn1 gets stabilised on membrane by ABCB10 (ATP-Binding Cassette Sub-Family B Member 10). The deficiency of either of these proteins causes severe anaemia (Chung, J et al, 2014.).	1. Mitochondrial iron import in brain is known to occur by Mitoferrin 2 (Mfrn2). But the abundance, regulation and cell specific localization of Mitoferrin 2 is still not clear. 2. In gliomas, it is reported that Mfrn 2 levels undergo up regulation and contribute in cells death (Wang, C et al, 2014).

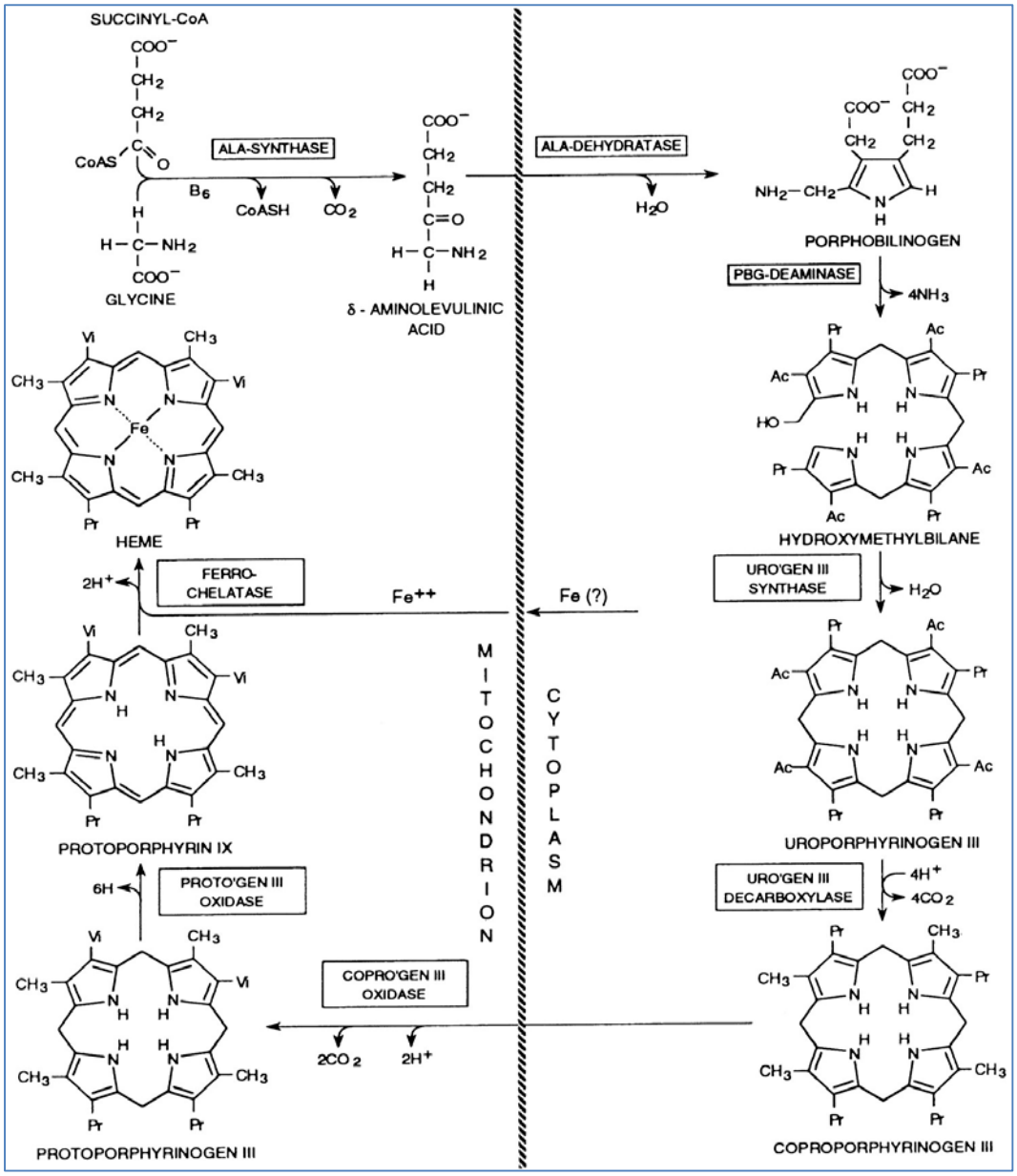
Components of Heme synthesis machinery:

- Heme biosynthesis is a multistep process catalysed by eight different enzymes and takes place in two different subcellular compartments. The pathway for heme synthesis is conserved and takes place in all cells but for many steps, isoforms exist that differentiate erythroidheme synthesis to that of non-erythroidheme synthesis.
- The first step involves condensation reaction of glycine and succinylcoA by decarboxylation. This step is catalysed by δ-aminolevulinate synthase (ALAS). The two isoforms for ALAS exist. Erythroid specific ALAS, e-ALAS/ALAS-1 have IRE in its 5`-UTR, which allows it to undergo regulation by cellular IRE-IRP system (Kikuchi G et, al, 1958).
- The condensation product of glycine and succinylcoA is 5-aminolevulinic acid. This molecule gets transported into cytosol and undergoes processing to form co-proporphyriogen III catalysed by ALA- dehydratase.
- The final three steps are catalysed in mitochondria for which co-proporphyrinogenIII gets transported into mitochondria. It undergoes transformation by undergoing oxidation catalysed first by co-proporphyriongen oxidase and then proporphyrinogen oxidase.
- The final insertion of iron in Protoporphyrin ring is catalysed by ferrochelatase.

- Free porphyrin does not have any biological activity and thus heme biosynthesis efficiency is generally measured by ability of cell to not leave any porphyrin intermediate unconsumed.
- Heme synthesis in erythroid cell is well studied but in non-erythroid cell pathways are poorly explored.

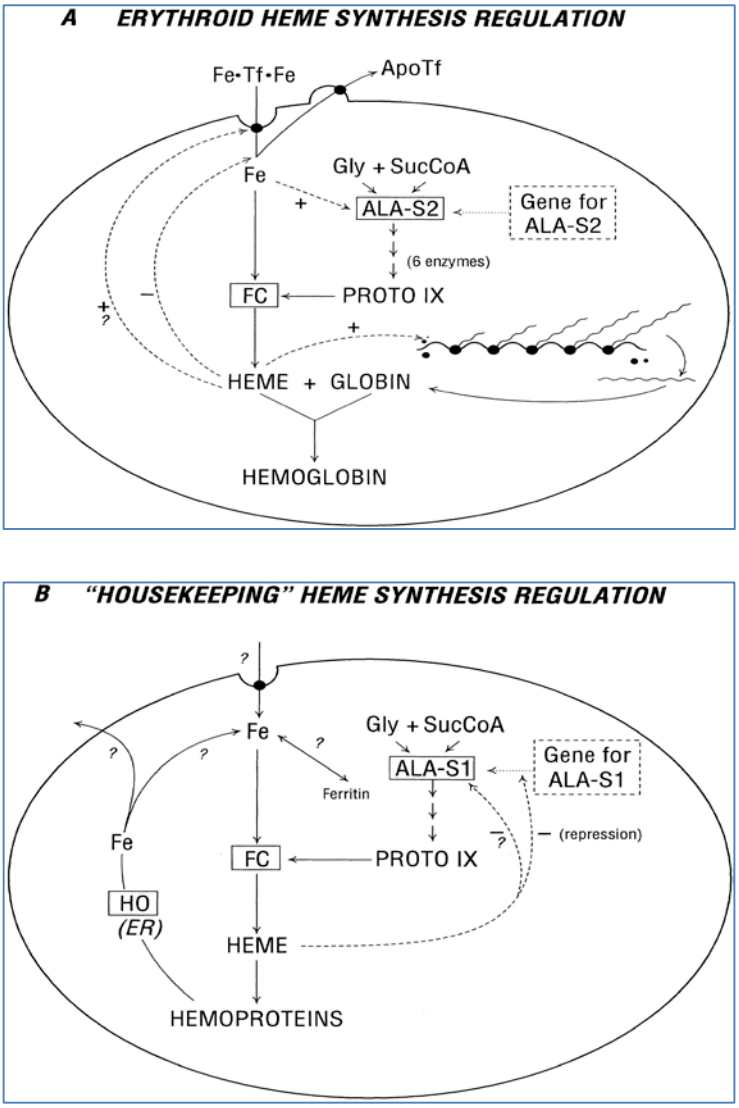
Heme in brain:

- Although the heme toxicity has been reported in cerebral haemorrhages (Aronowski, J et al, 2011). It is observed that free heme may cause more damage to the brain than free iron due to hydrophobic nature, which allows free diffusion and more accessibility of iron bound to it (Gaasch, J.A et al, 2007).
- While excess heme has been associated with damage, any defect in heme synthesis pathway is also known to be associated with disorders e.g. porphyrias (Takeda, A et al,2004)
- Recently, hemoglobin RNA and protein are shown to be expressed in DA-ergic neurons in SNPC. And post mortem reports of PD patients have shown reduced expressionof both Hb- α and β chains. Similar phenomenon is reported during aging with respect to haemoglobin expression in neuronal cell population. Rotenone treated nigrostriatal DA-ergic neurons have also shown suppressed levels for Hb mRNA and protein expression (Freed, J et al, 2016).



Adapted from Ponka, P et al, 1999

Figure 2.5:The heme biosynthesis pathway. Pyridoxal-5′-phosphate is cofactor for Aminolevulinate synthase, ALA-S; ALA represents 5-aminolevulinic acid while PBG represents porphobilinogen; URO'GEN, uroporphyrinogen; COPRO'GEN, coproporphyrinogen; PROTO'GEN, protoporphyrinogen; Ac stands for acetate; Pr for propionate; Vi for vinyl.



Adapted from Ponka, P et al, 1999

Figure 2.6.Distinct and different aspects of heme synthesis in erythroid cells and non-erythroid cells. The first and foremost enzyme of heme synthesis, aminolevulinate synthase accounts for differential responses of heme synthesis in accordance of cytosolic iron pool. ALA-S is the rate-limiting step of heme anabolism. The ability to undergo negative feedback regulation by heme exists in both A) erythroid cell and B) non-erythroid cells but presence of IRE in ALAS makes heme synthesis in erythroid cells to synchronise according to iron pool. Such mechanism does not occur in non-erythroid cells. Also, heme synthesis in erythroid cells are regulated according to globin synthesis as well but in non-erythroid cells, the major hemoprotein is cytochrome. Tf, transferrin; Gly, glycine; PROTO IX, protoporphyrin IX; HO, hemeoxygenase; FC, ferrochelatase; *ER*, endoplasmic reticulum.)

Neurodegenerative disorders involing imbalance in DA metabolism, iron overload and mitochondrial dysfunction:

Disorders involving deficient DA signalling:

- The disorders like attention deficit hyperactivity disorder (ADHD) are associated with deficient DA signallingand simultaneous loss of mitochondrial efficiency. The administration of DA agonists is known to alleviate the symptoms (Lou, H.C et al, 2004).
- GTP Cyclohydrolase 1 deficiency (GTPCH) is an enzyme deficiency disorder that causes hyperphenylalaninenemia. The deficiency of enzyme is associated with deficient DA production and disease is manifested as childhood dystonia (Mencacci, E.N et al, 2014).

Parkinsons disease

- Parkinson`s disease is characterised as degeneration of dopaminergic neurons in substantianigra pars compacta (SNPC) (Lang, A.E et al, 1998). It is manifested as rigidity and postural instability, bradykinesia and tremor. Iron and pathology of PD is intimately associated but precise mechanism is still not known. Iron overload has been observed in the region of neurodegeneration.
- Since, PD is associated with DA-ergic neuronal cell death. Administration of DA analogue is one of the therapies to alleviate the symptoms, which suggests deficiency of DA in brain (Chaudhary, K.R et al, 2009).
- Parkinson`s disease is characterised by the presence of lewy bodies, which appears to be composed of structurally altered neurofilament wherever there is neuronal cell loss.
- There are reports that have shown increased bilirubin levels in PD patients (D Marcia, Garcia, et al, 2017) which may suggest involvement of heme catabolic pathways. But heme pathways in brain are poorly understood. Hence exploring the potential of heme metabolism in pathology of PD is far from reach as of now.
- It has been observed that PD is also characterised with depleted levels of reduced glutathione and decreased complex I activity in dopaminergic neurons of SNPC (Horowitz and Greenamyre, 2010). In a study, it was reported that inhibition of activity of Grx2 might be due to substrate restriction. Grx2 is a mitochondrial

monothioloxidoreductase and known to be involved in ISC assembly. It might be involved in decreased activity of complex I and aconitase (cytosolic and mitochondrial) in DA-ergic neurons from PD cell line model implicating mitochondrial dysfunction associated with PD (Lee, W.D et al, 2009).

Schizophrenia:

- In schizophrenia, increased DA release is observed and treatment of schizophrenia is associated with PD like side effect.
- Many clinical reports have shown the correlation of hyperbilirubinemia in schizophrenics.
- Schizophrenia has also been correlated with mitochondrial dysfunction and oxidative stress (Muller, N et al, 1991; Macias Garcia, D et al, 2017).

Iron overload, mitochondrial dysfunction and neurodegeneration:

There are several disorders characterised by mitochondrial iron overload, oxidative stress and neurodegeneration. Mitochondrial dysfunction results in imbalance in ISC and heme synthesis pathways. As a result, disorders with defect in either of iron consumption pathway affect the synthesis of the other pathway as well. Till date, pathology of neurodegenerative disorders is not clear enough to address the role of mitochondrial dysfunction.

Freidrich ataxia (FRDA):

It is characterised by diminished expression of Frataxin (Fxn) (Rotig, A et al, 1997). FRDA is the most common ataxia characterised as autosomal recessive disorder due to hyper expansion of homozygous GAA triplet repeats in first intron of *frataxin* gene located at chromosome 9q13 causing its transcriptional silencing. FRDA is manifested as progressive cardiac and neurodegeneration along with gait abnormality and diabetes mellitus.

The regions of neurodegeneration are localised to dorsal root ganglia where the expression of Frataxin is diminished. Frataxin is a kinetic regulator of ISC synthesis machinery. The effect of hampered ISC biogenesis is seems to be affecting transcripts of heme pathway. Heme levels are also reported to be declined. (Schonfeld, R.A et al, 2005).

Fowler syndrome:

It is associated with loss of function mutation in heme importer, Feline leukemia virus subgroup C receptor superfamily, FLVCR2. FLVCR2 is ubiquitously expressed in central nervous system and spinal cord; although the subcellular localisation is not precisely clear (Duffy, S.P et al, 2010). The disease is characterised by proliferative vasculopathy. The disease has been recently reported to be associated with mitochondrial dysfunction (Usta, I.M 2005).

Neuropathic Porphyrias:

The disorders in heme synthesis pathway resulting in neurodegeneration, heme deficiency and excess deposition of iron are in combination termed as neuropathic porphyrias. They are characterised as tachycardia, abdominal pain, psychosis, anxiety and depression. In such disorders, the stimulus that induces endogenous heme synthesis aggravates the pathology. Such disorders include deficiency in aminolevulinatedehydratase, mutations in hydroxymethylbilane synthase (*HMBS*) gene etc (Chiabrando, F et al, 2018).

Imbalance in mitochondrial iron homeostasis has been reported in most of the neurodegenerative disorders that potentially could alter ISC and heme synthesis in brain cell types. The clear link for cross roads between ISC and heme synthesis has not been established yet but evidences derived from neurodegenerative diseases such asFreidrich ataxia (FRDA) strongly advocated that link (Schonfeld, R.A et al, 2005). The imbalance in DA metabolism has been reported with mitochondrial dysfunction and iron overload particularly in Parkinson's disease (Lin, M.T et al, 2006). DA metabolism is also known to take place in astrocytes (Lin, M.T et al, 1993; Schipper, H.M et al, 1996) in which iron overload is detected in PD patients and animal models. DA is reported to increase HO-1 activity and increased mitochondrial iron level in astroglial cells. However, the precise role of DA in astroglial iron homeostasis in general and mitochondrial iron homeostasis in particular remains unexplored so far. The research from current laboratory revealed DA could reduce iron release and induce ferritin degradation with elevated mitochondrial iron level in astroglial cells. This provides an opportunity to explore the fate of iron transported within the mitochondria in DA-exposed astroglial cells. Therefore, the following study was conducted entitled "**Studies on molecular mechanisms of iron mobilisation in astroglial cells in response to catecholamine neurotransmitter dopamine"** with the following aims.

1. *To analyse Dopamine induced mitochondrial influx of iron and the fate of elevated iron levels in Astroglial cells.*

2. To understand the molecular mechanism of induced heme levels in Astroglial cells in response to Dopamine.

Materials and Methods:

Reagents and chemicals

Most of the chemicals were obtained from Sigma-Aldrich unless otherwise stated. Tissue culture plastic wares were obtained from Corning and Greiner. The TriPure reagent, T4 DNA ligase and protease inhibitor cocktail were from Roche Applied Science. The restriction enzymes were purchased from New England Biolabs (NEB). Phusion polymerase and Taq DNA polymerase were purchased from Thermo Scientific. MMLV reverse transcriptase was purchased from Epicentre Biotechnologies. Oligo$(dT)_{18}$ primer and pre-stained page ruler were obtained from Thermo Scientific. The gene ruler for 1Kb ladder and 100bp ladder were from Genedirex; SnPP from Calbiochem; CMX-ROS mitotracker red from Thermo Scientific. Lipofectamine 2000 was obtained from Invitrogen.

Kits used

Following kits were used; Mitochondrial isolation kit was purchased from Thermo Scientific, QuantiChromeheme assay kit was obtained from BioAssay Systems, High capacity reverse transcriptase kit and Power SYBR Green PCR master mix from Applied Biosystems. Mini prep kit from QIAGEN and PCR clean up and Gel elution kit from GeNei.

Antibodies

Mitochondrial ferritin, actin, GAPDH, Frataxin, Ferrochelatase were purchased from Santa Cruz. Aconitase 2 from Cell Signalling. UQCRFS1/Reiske iron sulphur cluster protein and Cytochrome C from Invitrogen. ALAS-1, Porin, from Abcam.ISCU from Proteintech.Flag antibody from Sigma-Aldrich. Peroxidise-conjugated anti-IgG from Bio-Rad.

Table 1: List of antibodies used in experimental procedures.

Santa cruz	Mitochondrial Ferritin	sc-366719
	Actin	sc-1615
	GAPDH	sc-20357
	Porin	sc-32063
	Frataxin	sc-25820
	Ferrochelatase	sc-377377
Abcam	ALAS-1	ab101681
	SOD2	ab11888
Cell Signalling	Aconitase 2	6922S
Proteintech	ISCU	14812-AP
Sigma-Aldrich	Flag	F1804
Invitrogen	UQCRFS1/RISP	PA5-21420
Novus Biologicals	Mitoferrin 2	NBP1-59562
Novexlifetechnology	Cytochrome C	456100

Cell lines and culture conditions

Rat C6 astroglioma and human U87MG astrocytoma cell line were obtained from National centre for Cell Science (NCCS), Pune. The cells were cultured in Dulbecco`s Modified Eagle`s medium (Sigma-Aldrich) with 10% heat inactivated fetal bovine serum (Cell Clone), 100 units/ml penicillin, 100µg/ml streptomycin in humidified atmosphere containing 5% CO_2 at 37^o C in incubator. Cells were plated to achieve 50-60% confluency and then kept in serum free media for 4hr for all experiment involving DA treatments.

Human Primary Progenitor-derived Astrocyte culture

Human Fetal Neural Stem Cells (hfNSC) derived from the telencephelon region of 10-15 week-old aborted human foetus was obtained from National Brain Research Centre. Briefly, hfNSC were passaged on poly-D-lysine coated flasks in neurobasal media (Invitrogen) containing N2 supplement (Invitrogen, USA), Neural Survival Factor-1 (Lonza, Charles City, IA), EGF: 20 ng/ml (Peprotech, USA), bFGF:25 ng/ml (Peprotech, USA). The hfNSCs were then assessed for theexpression of markers such as Nestin and

SOX2, and 99% of the cellsexpressed these markers. Astrocytic differentiation was induced byreplacing the hfNSC media with Minimum Essential Medium (Sigma-Aldrich, USA) supplemented with heat inactivated 10% fetal bovine serum (Invitrogen, USA). Cells were maintained by replacing half of themedium by fresh media every alternate day. Then cells were assessedfor the expression of astrocytic markers after 3 weeks and >95% of thecells were immune-positive for GFAP, S100b and Glutamine Synthase (Santa Cruz Biotechnology, SantaCruz, CA, USA). The mature astrocyteswere then used for experiments within 3–4 passages post differentiation.

Western blot analysi Cell lysates were prepared in buffer containing 50mM HEPES, pH 7.5, 150 mMNaCl, 1 mM EDTA, 2 mM sodium vanadate, 1 mM PMSF, 0.5% NP-40 and 1X protease inhibitor cocktail. Harvested cell pellet were suspended in lysis buffer for 30 min to 1 hr. Protein supernatant was collected after spin and concentration was estimated using BioRad Bradford reagent. 65µg of cell extracts were subjected to SDS-PAGE and transferred to PVDF membrane (Millipore). The PVDF membrane was blocked with 1% skimmed milk as blocking agent. The membrane was incubated with primary antibody in 1:1000 dilution ratios for all antibodies except for actin (1:2000). This is followed by incubation with peroxidise conjugated secondary antibody (1:5000). The specific immune-reactive bands were detected by chemiluminescence using ECL reagent.

RNA isolation and mRNA quantification

RNA was isolated using TriPure reagent following manufacturer`s protocol. RNA was dissolved in DEPC water and quantitated. cDNA was prepared from RNA using Applied Biosystem high capacity cDNA synthesis kit. Real time PCR was performed on Applied Biosystem; 7500 Real time PCR system.

Table 2: Thermal profile for real time PCR

	Stage 1	Stage 2	Stage 3		Stage 4
	50º	95º	95º	60º	95º-60º-95º
Cycles	1	1	40		1
Duration	2 min	10 min	15 sec	1 min	

Table 3: Sequences of Primers used

Rat Frataxin FP	5`-CCCAAGCGCTATGACTGGAC-3`	NM_001191952.1
Rat Frataxin RP	5`-CAGTCAAGTGCCCTTTCCAGA-3`	
Human Frataxin FP	5`-TAGCAGAGGAAACGCTGGAC-3`	NM_000144.5
Human Frataxin FP	5`-ACGCTTAGGTCCACTGGATG-3`	
Rat Heme-oxygenase-1FP	5`-AGTCTTCGCCCCTGTCTACT-3`	NM_012580.2
Rat Heme-oxygenase-1RP	5`-CTGGTGTGTAGGGATGACC-3`	
Rat Ferrochelatase FP	5`-GGCGCTCTGTTCCGCGC-3`	NM_001108434.1
Rat Ferrochelatase RP	5`-AAGGGTCATGAGGTCTCGGT-3`	
Rat GAPDH FP	5`-CATTGGGGGTAGGAACACGG-3`	NM_017008.4
Rat GAPDH RP	5`-TCATGACCACAGTCCATGCC-3`	
Rat Actin FP	5`-CTGGCTTTGCCGGGATGATG-3`	NM_019212
Rat Actin RP	5`-GTGGTACGGCCGGAGGCATAG-3`	
Rat Mitoferrin 2 FP	5`-TATCAGACCCCTGTACGGCT-3`	NM_001013996.1
Rat Mitoferrin 2 RP	5`-TCTCCAGAGAGCCTCCAACA-3`	

Mitochondrial iron estimation

Mitochondria was isolated from C6 astroglial cells treated with DA (0-30µM) using mitochondrial isolation kit (Thermo Scientific) following manufacturer`s protocol. Mitochondrial pellet was re-suspended in mitochondrial suspension buffer (10 mMTris-HCl. pH 6.7, 0.15 mM $MgCl_2$, 0.25 M sucrose, 1 mM PMSF, 1 mM DTT).Isolated fractions were estimated using Bradford reagent (Bio-Rad). 30µg (20 µl) mitochondrial fractions was mixed with 180 µl of 10% (TCA+ HCl) and incubated at 45^o C overnight at gentle rotation (Tapryal, N et al, 2015; Galy, B et al, 2010). Then sample was centrifuged for 10 min with five volumes of 3M Na-acetate, 0.01% Bathophenanthroline-disulphonic acid and 0.1% thioglycolic acid. The OD was measured at 535 nm after 15 min. For each sample, the blank absorbance was determined by omitting the bathophenanthrolinechromogen. The amount of iron was inferred from the serial dilution of standard iron solution, (Iron atomic spectroscopy standard, Fluka)

Determination of Enzyme activity

C6 cells were treated with DA (0 or 30 µM) for 16hrs and harvested in 1ml suspension buffer containing 10mM Tris 7.6, 1.5M sucrose solution. Suspended solution was mixed gently and centrifuged at 600 g for 10 min. Collected supernatant was again subjected to centrifugation at 14,000 g for 15 min. The mitochondrial pellets were re-suspended in 500µl of 10mM Tris 7.6, 1X EDTA free Protease inhibitor. Aliquotes of 10 µl were made and kept in -80^{o}C for estimating enzyme activity (Spinazzi, M et al, 2012).

Succinate dehydrogenase activity assay: 10 µl aliquot was mixed with assay mixture containing 50 µl of 0.5M Potassium Phosphate buffer pH7.5, 20 µl of BSA (50mg/ml), 500 µM of Na-Azide, 100µl of 200mM succinate and 145µl of 0.015% DCPIP w/v. The 1 ml mixture was incubated at 37^o C for 10 min and then baseline activity was taken for 2 min. Reaction was started by adding 4µl of 12.5mM Decylubiquinone. Reaction was mixed and absorbance was taken at 600nm. The decrease in absorbance at every min was noted for 5 min. Specificity of reaction was verified by adding 10 µl of 1M malonate.

Mitochondrial aconitase activity assay: 10 µl of aliquot was mixed with 0.2mM cis-aconitate in 100mM Tris-Cl (pH 7.4), 100mM NaCl, 0.02% BSA and disappearance

of cis-aconitate was followed at 25 °C at 240 nm. Blank was taken as assay mixture without cis-aconitate.

Cytochrome c oxidase activity assay: Theassay mixture was prepared by adding 250 µl of 100mM Potassium Phosphate buffer pH7.0 and 50 µl of 1mM reduced cytochrome C. Volume was made up to 1 ml. Absorbance for Blank measured at 550 nm. Reaction was started by adding 10µl aliquot and decrease in absorbance was monitored at every minute for 3 min. Specificity of assay was verified by running parallel reaction with addition of 500µM Na-Azide.

Citrate synthase activity assay: 10 µl of aliquot was mixed with assay mixture of 500 µl 20mM pH 8.0, 0.2% Triton-X100, 10 µl of 10 mMDithiobisnitrobenzoicacid (DTNB), 30 µl of 10 mM acetyl coA. Baseline activity was recorded at 412 nm for 2min. Reaction was started by adding 50 µl of 10 mMOxaloacetic acid and decrease in absorbance was recorded at every min for 3 min at 412 nm.

Enzyme activity was calculated using following equation and then normalized with citrate synthase activity. The normalized activity of enzyme/mg of mitochondrial fraction in DA treated C6 cells were compared with controls.

Enzyme activity (nmol $min^{-1}mg^{-1}$) = (Δ Absorbance/min × 1,000)/[(extinction coefficient × volume of sample used in ml) × (sample protein concentration in mg ml^{-1})].

The Extinction coefficient for DCPIP, reduced Cytochrome C and Citrate synthase is 19.1 $mM^{-1}cm^{-1}$, 18.5 $mM^{-1}cm^{-1}$, 13.6 $mM^{-1}cm^{-1}$.

Estimation of Heme:

C6 cell treated with DA (0-30µM) were harvested for heme estimation in lysis buffer 50mM Tris pH 7.5, 150 mMNaCl, and 1X protease inhibitor cocktail. 100 µl samples were mixed with 900 µl of 2M oxalic acid. Then sample was heated at 95^{o} C for 30 min. Heme was measured as fluorescence of Protoporphyrin measured at excitation 400 nm and emission at 620 nm. Sample subjected to no heat treatment was kept as blank for every sample. The standard curve of heme was prepared by serial dilution of hemin solution prepared and fluorescence was measured in the same way (Beena, K.R et al, 1984).

Heme was also measured by Quantichromheme assay kit (Bioassay system) as per manufacturer`s protocol (Ye, H et al, 2010).

Actinomycin D chase assay:

C6 astroglial cells were treated with none or DA (30 µM) for 8hrs and then chased with actinomycin D (7.5 µg/ml). Cells were harvested at various time points after addition of actinomycin D (0, 3, 6, 9 and 12hrs) and transcript of Frataxin was quantified by real time PCR. Actin was used as normalization control.

Frataxin CDS-flag cloning:

Rat Frataxin clone was prepared by PCR amplification of the coding region (NM_001191952.1). cDNA for Coding region was inserted in Bam HI and Kpn I restriction site of p3x-FLAG-CMV-14 expression vector (Sigma-Aldrich, E7908).

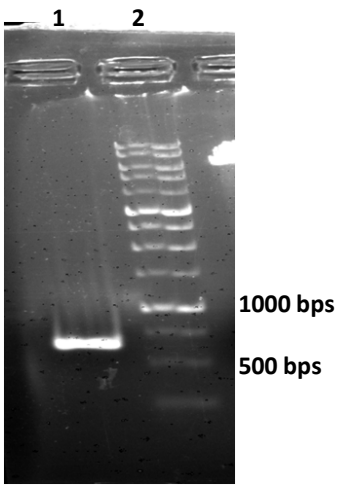
Primers used for cloning:

FXN-KpnI-FP:

5`-TTGG**GGTACC**GCCATGTGGACGTTCGGACG-3`

FXN-BamHI-RP:

5`CGC**GGATCC**GGAAGTGCCCTTTCCAGAATAGGCCA-3`



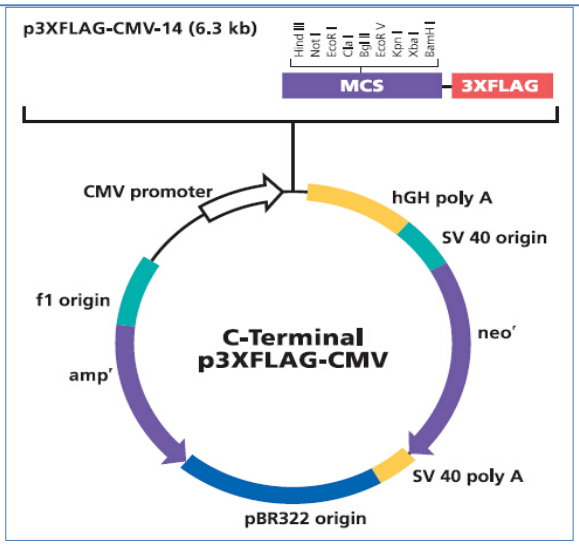
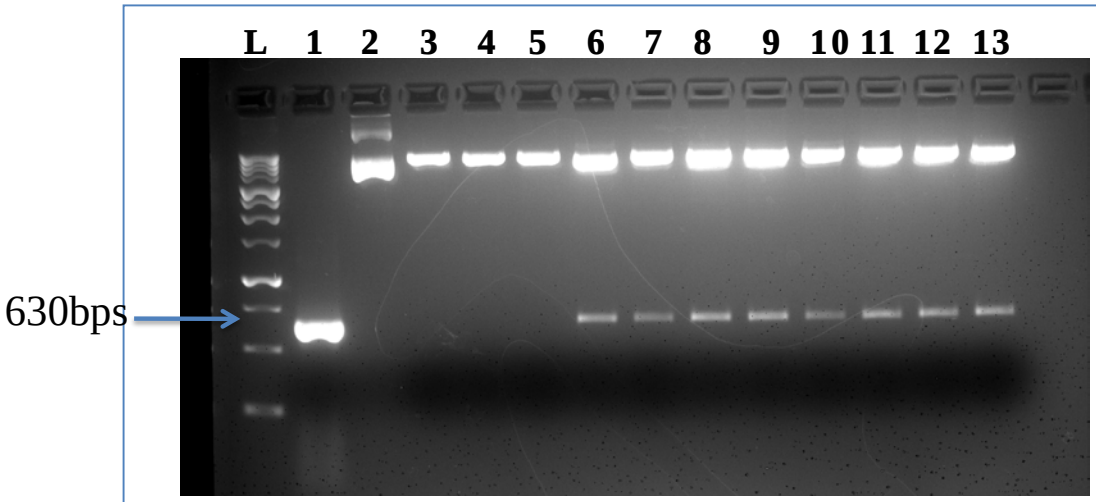


Figure 3.1: **ratFXN-CDS cloning in P3xflag-CMV vector**

A. cDNA from total RNA was PCR amplified with primers specific for rat frataxin incorporating flanking restriction site. Amplified product was subjected to run on 1% agarose gel. Lane 1 represents rat-Fxn amplicon. Lane 2 represents 1kb ladder.

B. Vector map for p3XFLAG-CMV representing multiple cloning sites. Amplicon containing Fxn CDS flanked by KpnI and BamHI was inserted in vector.



C: Colony PCR confirming Fxn-CDS cloning in vector p3XFLAG-CMV by double digesting potential colonies with BamHI and KpnI. Digestion mixture was subjected to 1% agarose gel.

1:1Kb ladder; 1:PCR amplicon; 2:uncut vetor; 3: BamHI digested vetor; 4: KpnI digested vector 5-13: BamHI and KpnI digested colinies

Statistical analysis:

All experiments were performed at least three times with similar results and representative experiments are shown. Densitometric analysis was performed by Image J software and represented with respect to normalization by internal controls. Data are expressed as mean ± SE. The statistical analysis was performed by student`s t-test for comparison between control and DA treatments.

Introduction:

Iron is a redox active metal. In biological system, it is involved in diverse array of cellular processes essential for survival e.g. mitochondrial respiratory chain, O_2 transport, DNA repair, ribosome biogenesis etc. (Ward, R.J et al, 2014). Brain is the second most metabolically active organ and consumes 20% of body`s oxygen (Wang, Z et al, 2010). It`s iron requirement is very high. Besides playing key role in many vital cellular processes, iron also holds potential risk as damaging agent for cells. Iron has ability to participate in Fenton chemistry, which generates reactive oxygen species (Singh, N et al, 2014). Hence in excess i.e. beyond cell`s capacity to store or consume, it may pose as threat. During course of evolution, every cell type has worked up in a way to design well defined regulatory machinery that not just regulates iron levels to avoid toxicity but also fulfils specific need of iron in accordance to the functionality. The iron requirement of both neurons and oligodendrocytes is very high; while iron in neuron is mainly needed for the generation of higher energy but oligodendrocytes harbour iron mainly for mylinogenesis. Hence, both cells exert regulation in iron homeostasis process with specific adaptations to fulfil significant iron import in accordance to the consumption.

Astrocytes on the other hand, are neither considered as metabolically very active cell types, nor do harbour specific process with higher iron need. The astrocytes are the most abundant glial cell type (Tower and Young, 1973). They are of utmost important for neurotransmission signalling as well as survival of neuronal cells. Most of the iron in brain is concentrated at *Substantianigra pars compacta* (SNPC) and basal ganglia. The former region is the centre of neurodegeneration in Parkinson`s disease (PD). PD is characterised by dopaminergic neurodegeneration observed at SNPC with manifestations of bradykinesia, rigidity and postural instability. While the cause or consequence dilemma is still not clear about PD, dopamine`s (DA) requirement as a vital neurotransmitter has been made clear as administration of DA analogue, DA mimetic or MAO inhibitors are generally considered as line of treatment (Cools, R , 2006; Schapira, A.H, 2007). Abnormal DA metabolism is also associated with schizophrenia and attention deficit hyperactivity disorder (ADHD).

Several neurodegenerative disorders are associated with increased expression of heme oxygenase-1 (HO-1) in affected areas that include Parkinson`s disease and schizophrenia. Increased expression of heme-oxygenase-1 is also observed in astroglial compartments of

affected regions Schipper, H.M et al, 1998; Schipper, H.M et al, 2015). HO-1 is a heme catabolism enzyme with exquisite sensitivity to undergo induction for wide variety of oxidative and inflammatory insults. The by-products of heme catabolised by HO-1 under these stimuli are known to contribute towards cytoprotection.

It`s been earlier reported that DA could induce HO-1 expression of astroglial cells with simultaneous elevation of mitochondrial iron (Schipper, H.M et al, 1999). Unpublished data from the current laboratory found that DA also could suppress the iron export machinery by translational inhibition of Ceruloplasmin (Cp) causing down-regulation of iron exporter Ferroportin (Fpn) (Mukherjee, R et al, unpublished data). Simultaneously, DA also reduces ferritin (Ft) protein abundance by increasing degradation (Dev, S et al, unpublished data). All these earlier observations support the elevation in mitochondrial iron levels in DA exposed astroglial cells. However, the elevated iron is utilized within mitochondria is not known so far.

Mitochondria are the major consumer of iron. It houses the biosynthesis machinery for two major iron dependent cofactors namely iron-sulphur cluster (ISCs) and heme. Iron-Sulphur clusters are ancient cofactors that play essential role in mitochondrial respiratory chain, enzyme catalysis and homeostatic regulation. Their composition varies from simple 2Fe-2S to complex 4Fe-4S and 3Fe-4S and further more complex clusters. While ISCs are abundant in all cell types. Their role for cellular survival became more clear with understanding of disease associated with disrupted ISC biogenesis i.e. Freidrich ataxia (FRDA). FRDA is associated with impaired ISC assembly and characterized by autosomal recessive neurodegeneration and cardiomyopathy (Delatycki, M.B et al, 2000).

Other way, heme biosynthesis also takes place in mitochondria apparently sharing same iron pool. There`s possibility that DA may modulate these iron consumption pathways in astroglial cells.

Hence, the current study was undertaken to understand the balance between ISC synthesis and heme synthesis in mitochondria of astroglial cells in response to DA. The results showed an increased heme content and heme containing protein or enzyme activity while decreased activities were detected for ISC containing enzymes suggesting DA could promote heme synthesis and suppress ISC synthesis.

Results:

Dopamine elevates mitochondrial iron content but not affects Mitochondrial Ferritin (Mt-Ft) in astroglial cells

Schipper et al (1999) reported that Dopamine (DA) induces heme catabolising enzyme, heme oxygenase-1 (HO-1) with simultaneous elevation of mitochondrial iron level in astroglial cell. A similar result was also obtained earlier in our laboratory (Thesis SomDev; Dev et al, unpublished data). To reconfirm the observation, C6 rat astroglial cell was treated with dopamine (0-30µM) for 16hrs, mitochondrial fractions were isolated and iron content was estimated. Mitochondrial iron levels were found to be elevated by 1.5-fold in 10µM DA treated cells and further increased by more than two fold in 30µM DA treated cells after 16h (Fig 1A). The time dependant increase in mitochondrial iron content was estimated by harvesting the mitochondrial fractions at different time intervals (at 4h, 8h and 16h) after DA (30µM) treatment. Mitochondrial iron levels showed mild but gradual elevation of about 1.2-fold at 4h, about 1.4-fold at 8h and about 2-fold at 16h in response to DA (30µM) treatment (Fig 1B).

Mitochondria import iron through Mitoferrin protein. Mitoferrin 2 (Mfrn2) has ubiquitous expression in all cells and contributes for mitochondrial iron influx in non-erythroid cells. To examine that whether DA has any role on Mfrn2 protein expression, C6 astroglioma cells were treated with DA and subjected to immunoblotting. It was found that DA (0-30µM) could induce Mfrn2 protein level (Fig. 1C). Further transcript level of Mfrn2 in response to DA in C6 astroglioma was also examined and a similar up-regulation was also observed like protein level (Fig. 1D).

Mitochondria contain mitochondrial Ferritin (Mt-Ft), which is predicted to have 79% homology with Ferritin heavy chain (Ft-H) and postulated to store iron in mitochondria in similar manner as cytosolic ferritin. In brain, neurons show significant Mt-Ft expression while glia shows lesser abundance (Wang et al, 2011). One of the fates of increased mitochondrial iron could be stored in Mt-Ft during DA exposure. In that case Mt-Ft expression might be elevated in response to DA. To verify that immunoblot was performed using cell lysate

Fig. 1A

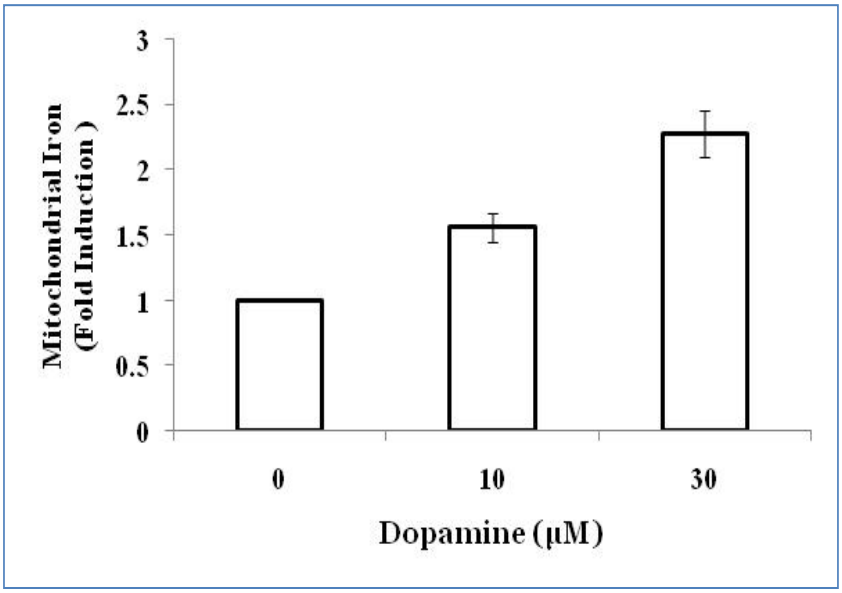
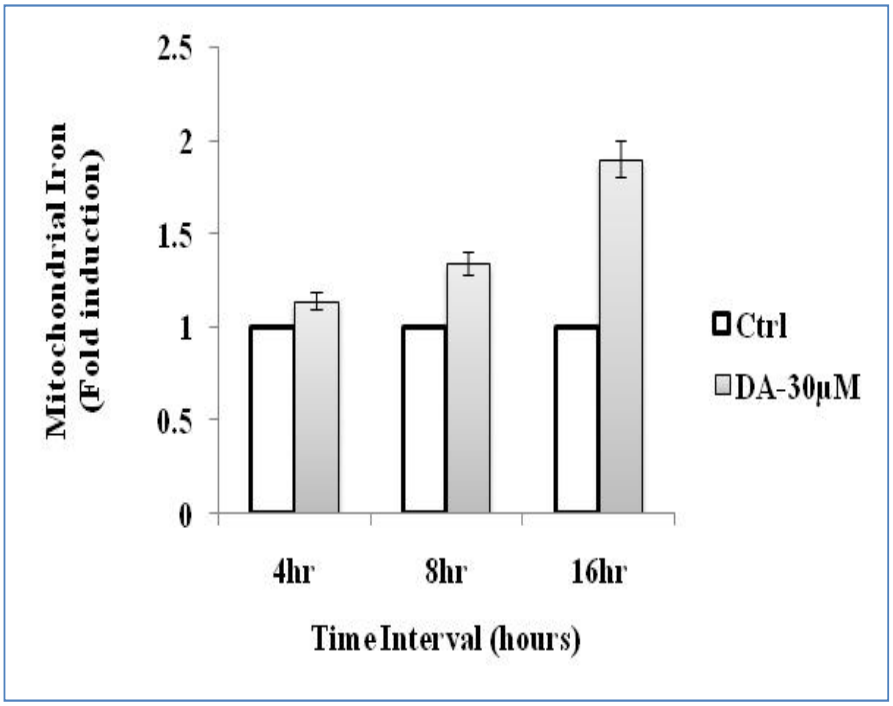


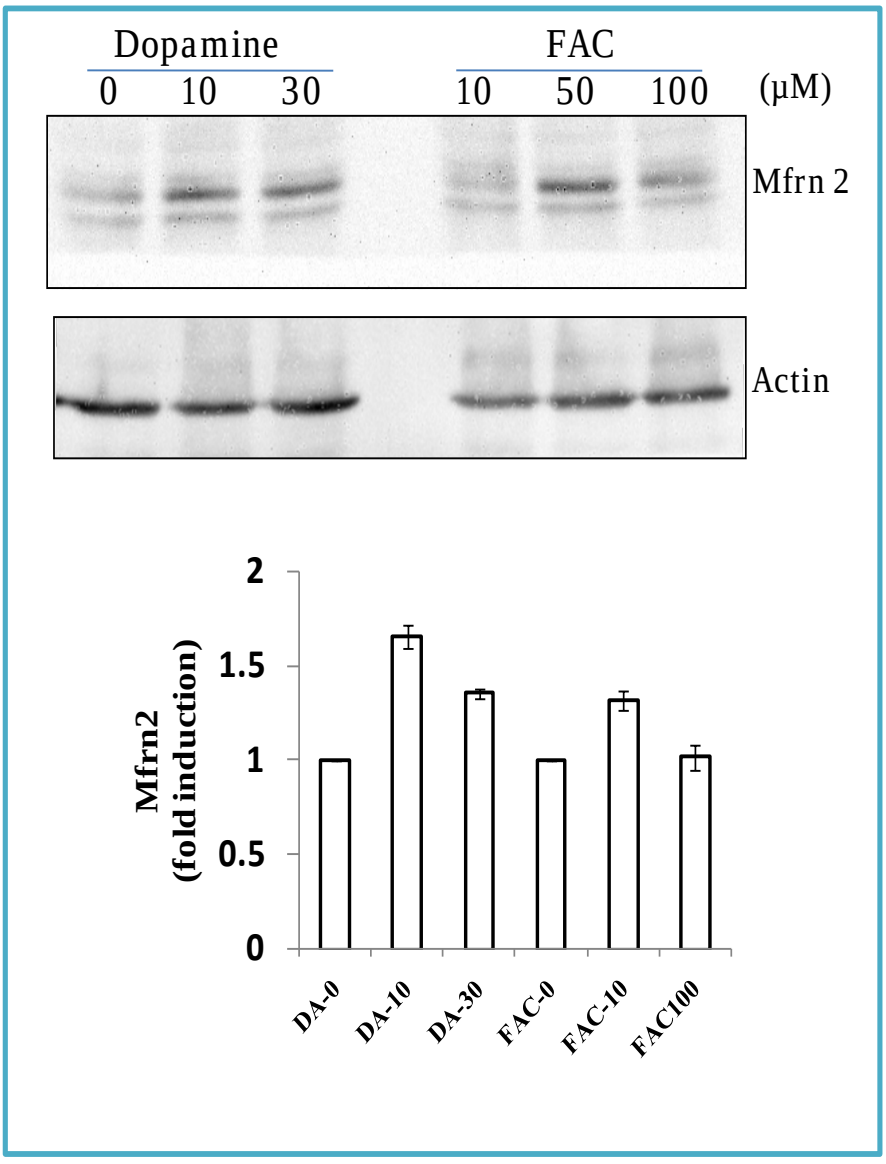
Fig. 1: **Effect Of Dopamine On Mitochondrial Iron Content, mitochondrial iron importer (Mfrn 2) and storage protein, (Mt-Ft) in Astroglioma.** C6 rat astroglioma cells were treated with Dopamine after which cells were harvested for mitochondrial fractionation. Harvested fractions were assayed for iron estimation by colorimetry. (A) Quantification of iron content from mitochondrial fractions isolated from C6 glioma cells subjected to increasing concentration of dopamine (0-30µM) for 16h.

Fig. 1B



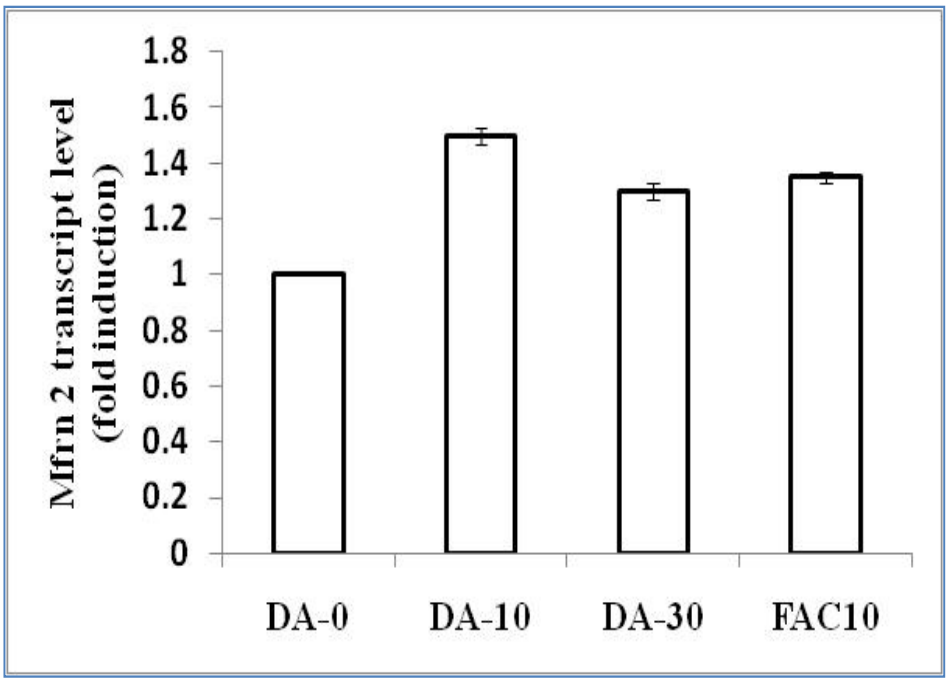
(B) Quantification of Iron content from mitochondrial fractions isolated from C6 cells treated with o µM and 30µM Dopamine for different time points (4, 8 and 16 hrs)`. Results are represented as mean of three independent experiments ± SE. Continue...

Fig. 1C



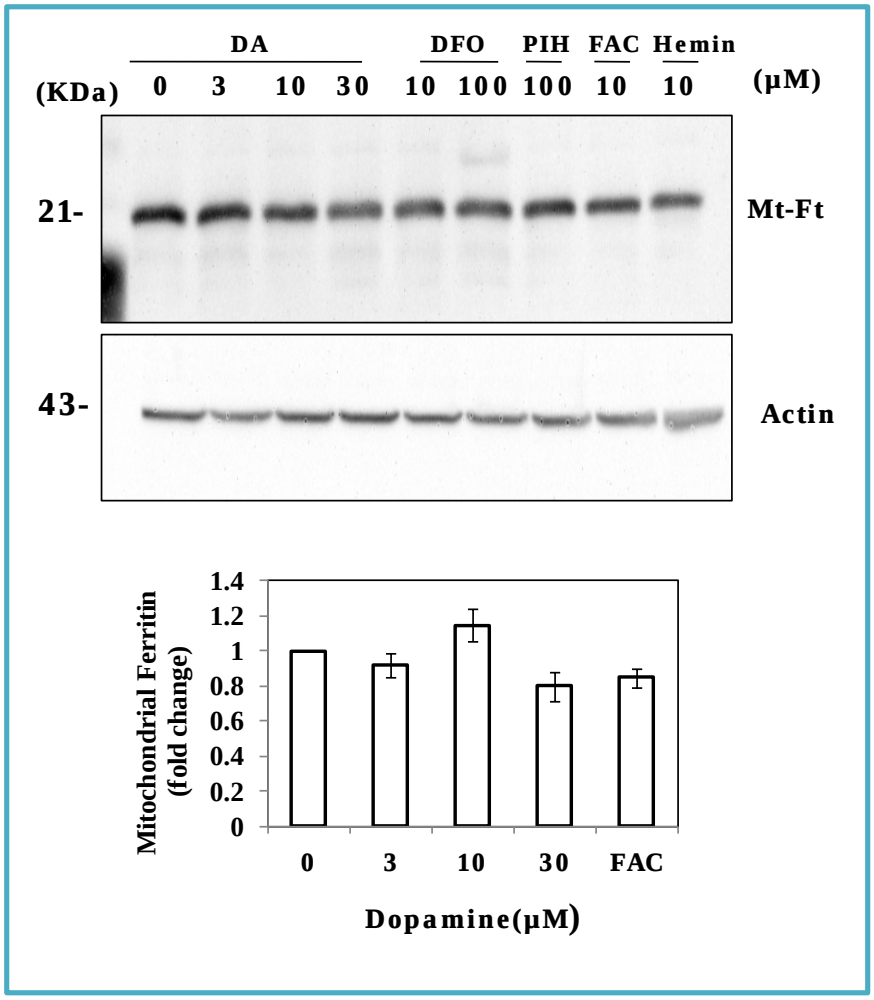
(C). C6 cells were treated with DA (0-30 µM), iron salt FAC 10 and 100µM for 16 h. Mfrn2 (upper lane) and actin (lower lane) expressions were detected in cell lysates by immunoblot analysis (top panel). Bottom panel represents Densitometric analysis of immunoblots as mean from three independent experiments ± SE. Continue..

Fig. 1D



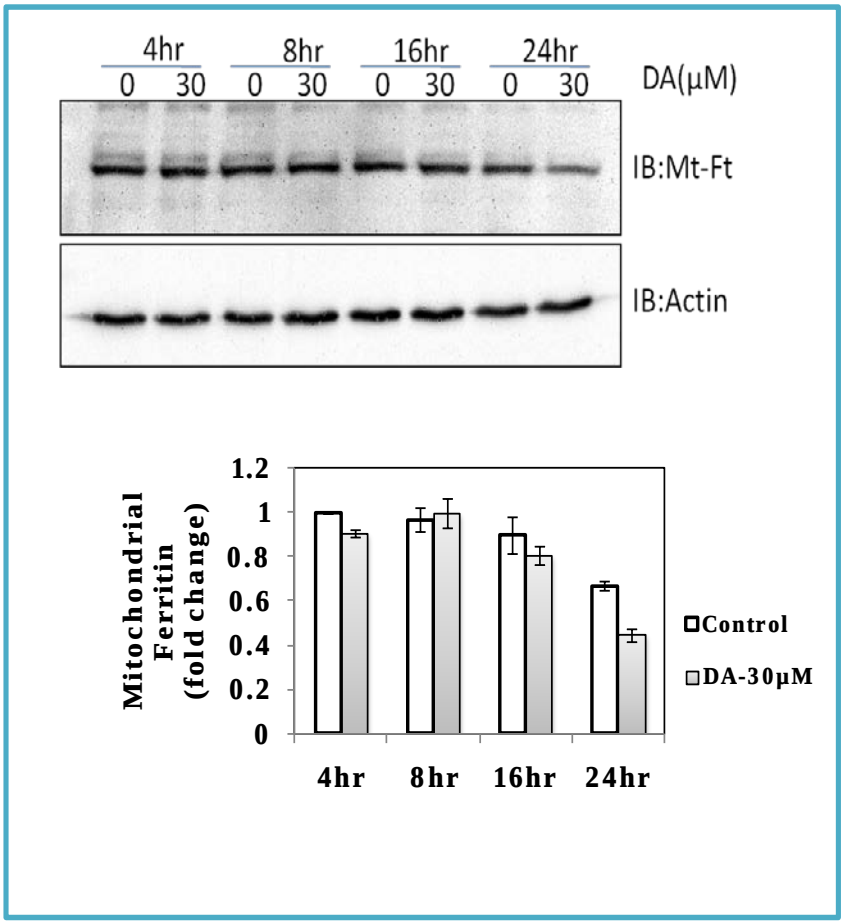
(**D).** C6 cells were treated with DA (0-30μM) and FAC were harvested for transcript level analysis of Mfrn2 by qRT-PCR. Results are represented as means from three independent experiments ±SE.

Fig. 1E



(**E**) C6 cells were treated with DA (0-30 µM), iron salt FAC 10µM, Hemin 10µM, iron chelators DFO, 100µM and PIH 100µM for 16 h. Mt-Ft (upper lane) and actin (lower lane) expressions were detected in cell lysates by immunoblot analysis (top panel). Bottom panel represents densitometric analysis of immunoblots as mean from three independent experiments ± SE.

Fig. 1F



(**F**) C6 cell lysates obtained after dopamine treatment (0 and 30µM) for different time points were subjected to immunoblot analysis for Mt-Ft (upper lane) and actin (lower lane), top panel. Bottom panel representing densitometric analysis of immunoblots as mean from three independent experiments ± SE .

harvested from C6 rat astroglial cell line. Results showed no alteration of Mit-Ft in response to increasing concentration of DA (0-30 µM) treatment (Fig. 1E). Cytosolic iron chelatorDesferroxamine, (DFO) (10 and 100µM) and membrane permeable iron chelatorPyridoxalisonicotinoylhydrazone (PIH) (100µM) and hemin (10 µM) were also showed no change in Mit-Ft expression. Cytoslic ferritins are known to be regulated by cellular iron levels but no such regulation is known for mitochondrial ferritin. Since, immunoblot showed no change in expression of Mt-Ft in astrocytes in these treatments that ruled out the possibility of antibody for cross reactivity for cytosolic ferritin. Time dependant expressions of Mt-Ft were also examined by immunoblotting of cell lysates harvested at different time intervals after DA (30µM) treatment. There was no significant alteration observed (Fig 1F).

Dopamine suppresses Iron sulphur cluster`s content (ISC):

Iron sulphur clusters are versatile cofactors of proteins involved in electron transport chain, DNA repair, and ribosome biogenesis. They may regulate the enzymatic activity of proteins by acting as redox active centres at catalytic site as in the case of mitochondrial aconitase (ACO2) (Bulteau, A.L et al, 2011) and succinate dehydrogenase (SDH) (Mochel, F. et al, 2008). Also, they may regulate the stability of proteins as in the case of Reiske iron sulphur cluster protein (RISP) (Hall, R.E et al, 1993). Hence, analysing the enzymatic activity or expression levels of such ISC dependant proteins may represent the ISC levels of cell. An increase in mitochondrial Fe level by DA treatment might increase ISC content of astroglial cells that needed to be examined to understand the destination of iron within the mitochondria. Thus, ACO2 and SDH enzymatic activities were determined in DA treated C6 astroglial cells. Interestingly, results showed that DA actually inhibited the enzymatic activities of ACO2 and SDH by ~40% and ~55% respectively (Fig. 2A). The protein level of ACO2 by immunoblot was determined and found no change in its expression. Further, the protein abundance of RISP in response to DA treatment in C6 astroglioma was decreased gradually with increasing concentration of DA. About 35% down-regulation of RISP was observed by 30µM DA treatment (Fig 2C). These results strongly suggest that DA treatment does not promote ISC biogenesis despite increase in mitochondrial iron content.

Fig. 2A

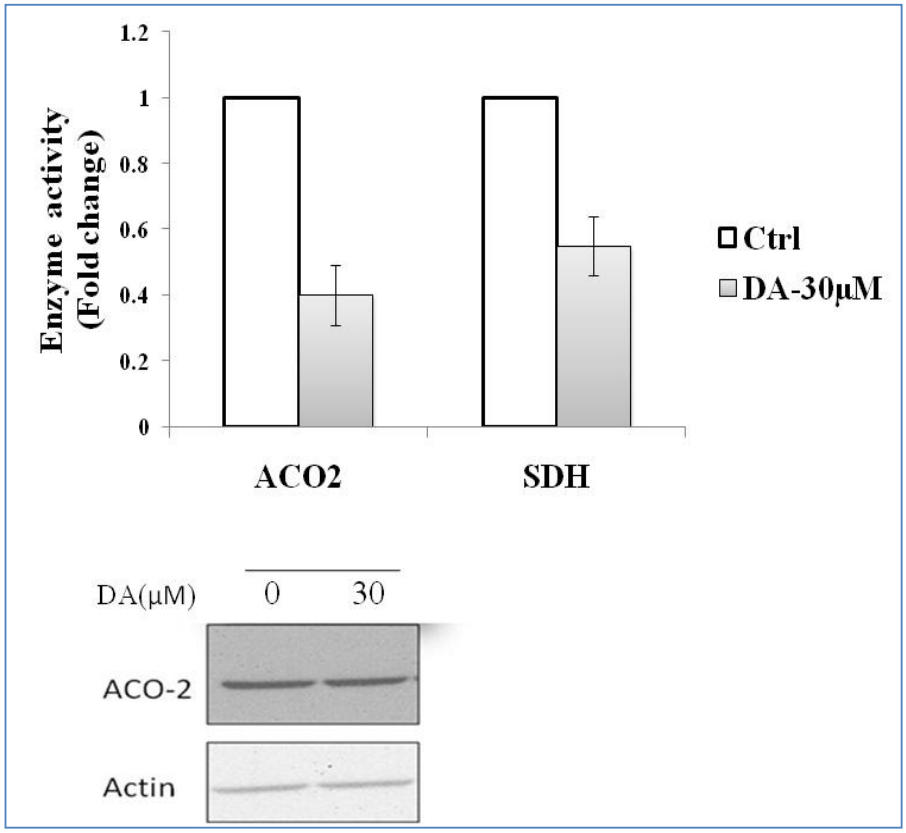
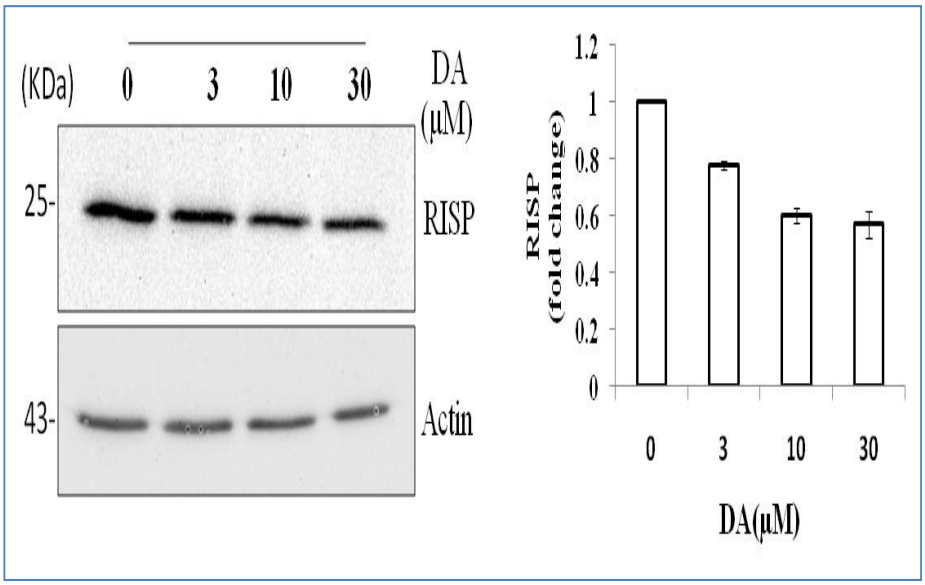


Figure 2: Effect of dopamine on Iron sulfur cluster(ISC) content in C6 astroglia cells. (A) Cells were treated with dopamine (0 and 30µM, 16 hours). Harvested cells were fractionated for mitochondrial isolation. Fractionated mitochondria were analysed for enzymatic activity of ISC dependant mitochondrial enzyme mitochondrial aconitase (ACO2) and Succinate dehydrogenase (SDH). Results are represented as mean ± SE from three independent experiments. (top panel). Bottom panel represents the immunoblot analysis of ACO2 (upper lane) and actin (lower lane). Result represented as one of the three independant experiment with similar results. Continue..

Fig. 2B



(B). C6 cells were treated with increasing concentration of DA (0-30µM) for 16 hours. Cells were harvested for immunoblotting analysis for expression levels of ISC dependant Reiske iron sulfur cluster protein (RISP) (upper lane) and actin (lower lane), left panel. Right panel represented densitometric analysis of immunoblot from three independant experiments ± SE**.**

Dopamine elevates heme levels in astrocytes

Heme is an iron bound prosthetic group whose synthesis takes place in mitochondria. Since, DA treatment decreased ISC level raising possibility of increased heme content in astroglial cells. To determine that C6 rat astroglial cells were treated with increasing concentration of DA (0-30 μM) and heme levels were estimated in whole cell lysate. Heme levels were found to be gradually elevated by ~10% and ~30% after 10μM and 30μM of DA treatment respectively (Fig 3A). When primary rat astrocytes were exposed to increasing concentration of DA, heme levels were also found to be induced (Fig. 3B). Interestingly, 3μM DA induced heme levels by ~50% (Fig 3B).

Heme content in whole cell lysate may represent steady state levels as both biosynthesis as well as catabolism might be taking place simultaneously. Since mitochondria house the heme biosynthesis pathway, while heme catabolism takes place in cytosol, thus it is important to estimate heme levels both at mitochondria and cytosol. Thus, compartmental elevation in heme levels i.e. both at the site of synthesis as well as the site of catabolism was determined. Mitochondrial and cytosolic fractions were isolated from DA treated astrocytes and heme contents were estimated in both these fractions. The heme level in mitochondrial fraction was elevated by ~50% in response to DA treatment. But heme in both cytosolic fraction as well as whole cell lysate showed elevation to similar levels (about 30 %) (Fig 3C).

DA is known to induce heme catabolism protein HO-1 (Schipper et al, 1999) suggesting degradation of heme in cytosol. In order to analyse if DA mediated elevation in heme levels is due to induction in heme synthesis, we inhibited the enzyme activity of HO-1by pre-treating cells with antagonist, Tin Protoporphyrin IX, SnPP (Jozkowicz, A et al, 2003). Astrocytes treated with only SnPP in increasing concentration (0, 10, 15 and 20μM) for 16 hrs showed increase in heme levels when calculated in whole cell lysate (Fig 3D). This act as control for estimating heme in astrocytes pre-treated with SnPP for 6hrs in increased concentration and then with DA for 12 hrs.

Fig. 3A

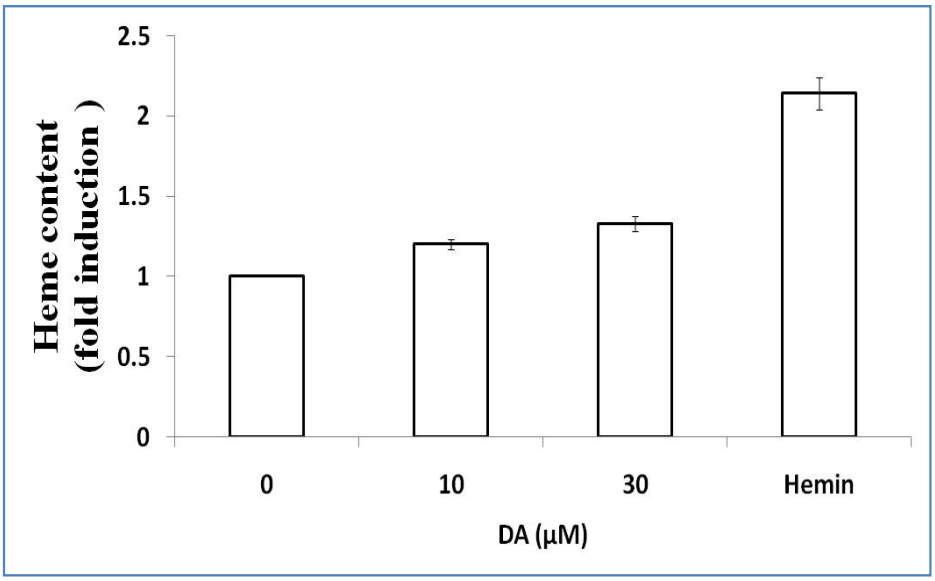
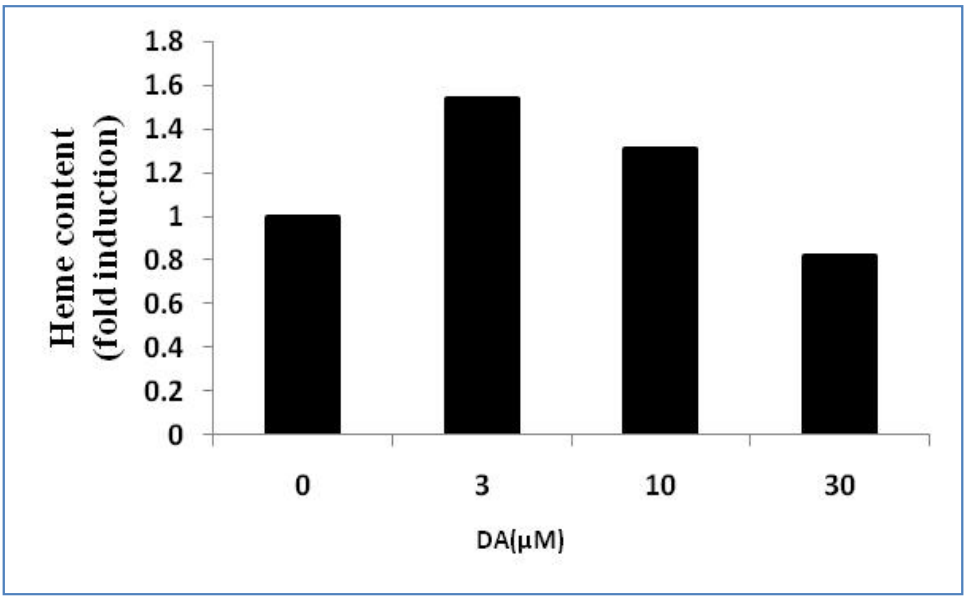


Fig 3: **Effect Of Dopamine On Heme Content and heme markers in astrocytic cells**. (**A**) C6 cells were treated with increasing concentration of DA(0-30µM) for 16 h. Cell lysate was harvested for heme estimation. Hemin 10µM was used as positive control. Results represent means ± SE of three independent sets of experiments. Continue..

Fig. 3B



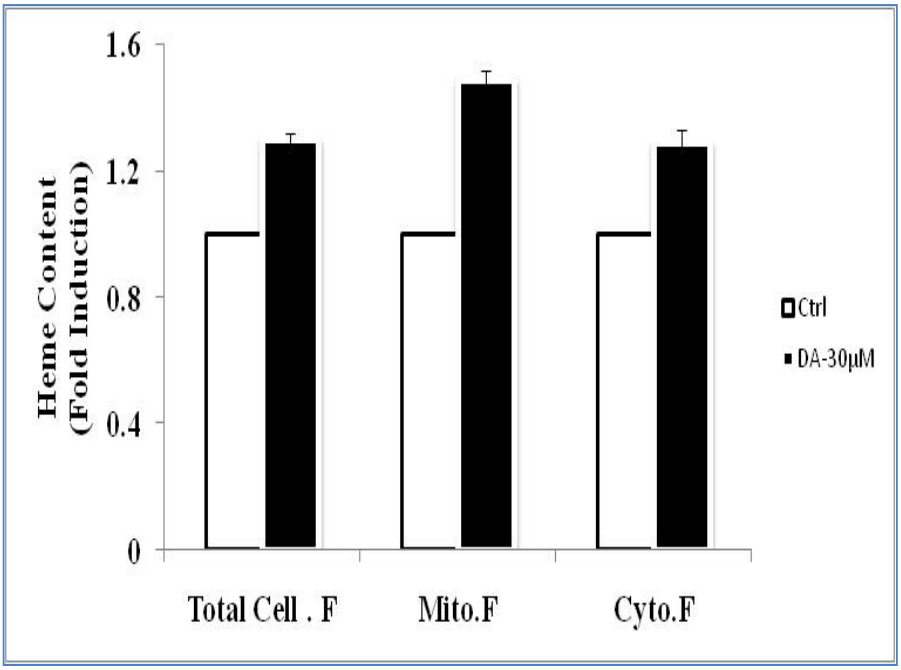
(**B**) Rat primary astrocytes were treated with DA (0-30µM) for 16h and cells were harvested for heme estimation. Results represents heme levels in total cell lysate with error bars representing ± SE from three independent experiments. Continue...

The pre-treatment of SnPP in increased concentration was made in order to inhibit the elevation in degradation pathway caused by HO-1 as it seemed that DA is inducing both synthesis (in mitochondria) as well as catabolism (by HO-1) of heme. We found that as compared to control, 10µM SnPP induced heme levels by 50% and higher concentrations of SnPP i.e. 15 and 20µM did not elevate heme levels any further. This might suggested that 10µM SnPP was enough to establish the inhibitor`s excess concentration. The DA-30µM treatments were given in all of these SnPP pre-treated samples. We found that in the presence of 10µM of inhibitor SnPP, DA induced the heme levels by more than two fold. Further increase in inhibitor`s concentration did not affected DA mediated induction in heme levels by inducing anymore elevation. It seemed that DA induces heme synthesis significantly (analysed by student`s t test $p<0.01$) but maintains the steady state levels to ~30% DA elevation by simultaneous induction of heme degradation pathway.

δ-Aminolevulinate synthase-N (ALAS-N) is the first enzyme of heme biosynthesis pathway. It undergoes negative feedback regulation by cellular heme levels (Ponka, P et al, 1999; Munakata, H et al, 2004). When heme level is increased, ALAS-N becomes low and stays in cytosol. While decreased cellular heme level up-regulates ALAS-N protein abundance, it also gets translocated to mitochondria. Thus, ALAS-N protein was used as marker of cellular heme levels and it was immunoblotted in both mitochondrial and cytosolic fraction obtained from DA (30µM, 16h) treated C6 astroglial cells (Fig 3E). A very faint expression of ALAS-N was found in mitochondrial fraction as compared to cytosolic fraction. In both mitochondrial as well as cytosolic fraction, DA treatment resulted into significant decrease in protein level. This result also suggests an increase in heme level in DA treated for C6 astroglial.

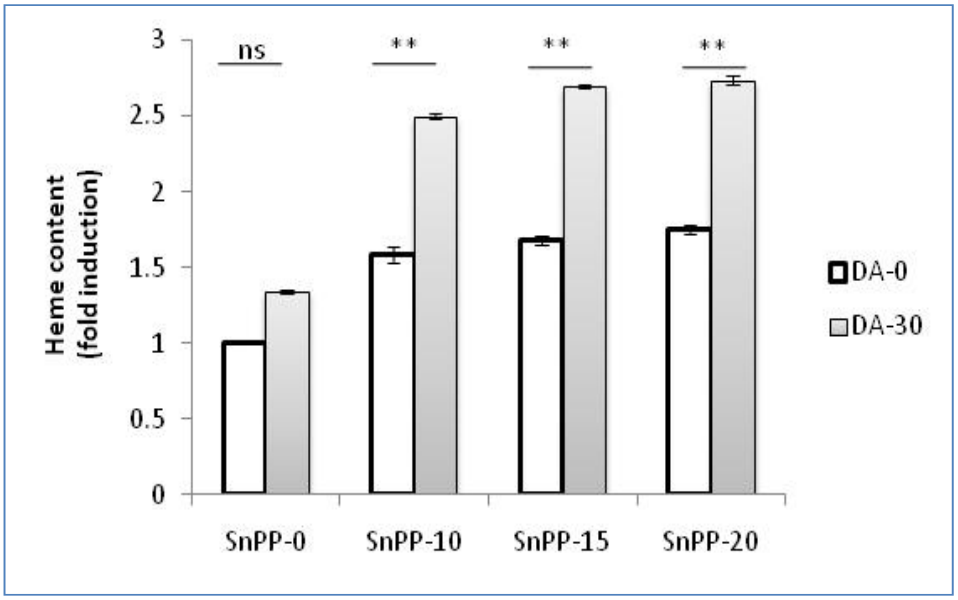
Cytochrome C oxidase is the terminal complex of mitochondrial respiratory chain. It is a complex of 13 subunits of which 3 are catalytic. The enzymatic centre of this complex requires 2 heme molecules and 2 Cu atoms for its activity. Heme levels are known to regulate Cytochrome C oxidase activity as well as transcription of mitochondrial subunits of the complex (Soto, I.C et al, 2012; Srinivasan, S et al, 2012).

Fig. 3C



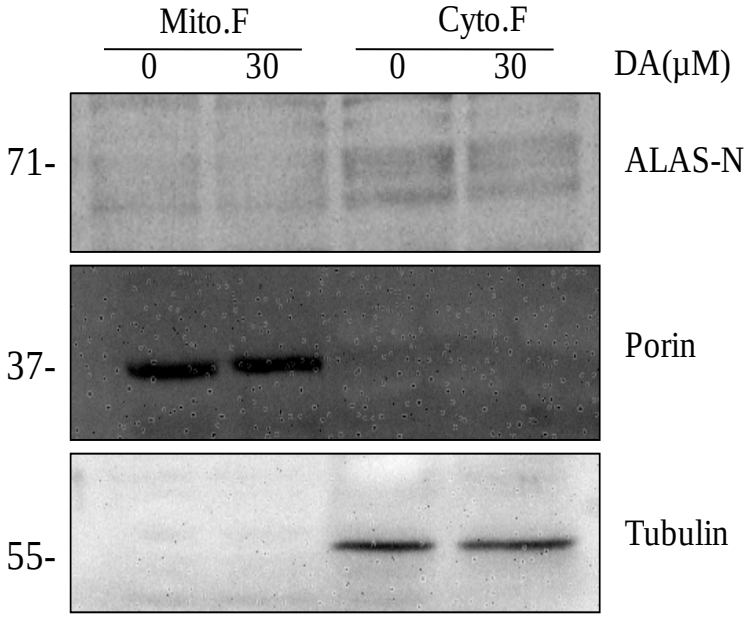
(**C**) C6 cells were treated with none or DA (30µM) for 16 hours. Cells were harvested and heme content was estimated in total cellular fraction, mitochondrial fraction (Mt. F) and cytosolic fraction (Cyt. F). Results represent means ± SE of three independent sets of experiments.

Fig. 3D



(**D**) C6 cells were treated with heme oxygenase-1 inhibitor (HO-1) SnPP in increasing concentration (0 to 20µM along with DA-30µM. Only SnPP treatment were kept as control for 16 hours and heme content was estimated. Data represent mean ± SE of three independent experiments. Statistical analysis was performed by student t test $p<0.01$

Fig. 3E



(E) Mitochondrial and cytosolic fractions were harvested from C6 cells treated with DA (0 & 30μM) and analysed by immunoblotting of ALAS-1 (top panel). Purity of fractions were analysed by immunoblotting with porin (middle panel) and tubulin (bottom panel).

Fig. 3F

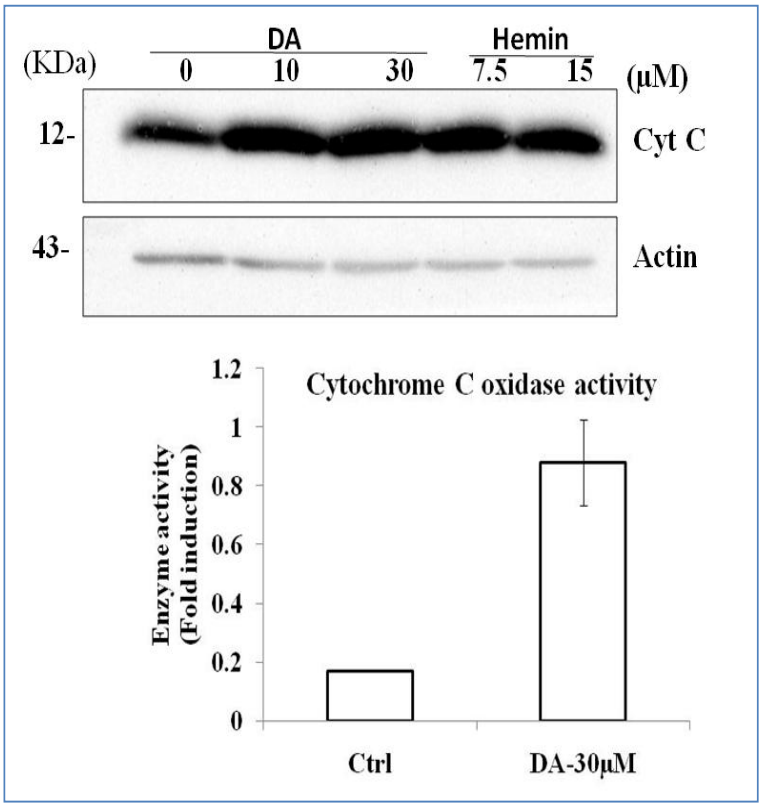


Fig. 3G

(**F**) Similarly, DA (0-30µM) treated cell lysate was immunoblotted for cytochrome C (Upper) and actin (lower). Hemin (7.5 and 15µM) was used as positive control. (**G**) Cytochrome C oxidase activity was estimated in C6 cells treated with DA (0 &30µM for 16 hours. Results are represented as mean of three independent experiments with similar results ± SE.

Heme is also known to regulate the transcription of cytochrome c protein (Guarente, L et al, 1983). When the complex IV activity and cytochrome c protein level were analysed in response to DA in C6 astroglial cells, both the complex IV activity and cytochrome c protein level showed significant increase (Fig 3F and 3G) further suggesting increase in content of cellular heme.

Discussion:

Mitochondria are the major consumer of iron in cells. They harbour biosynthesis machinery of two iron dependant co-factors i.e. iron sulphur clusters and heme. Since both these co-factors are essential for cellular survival and maintaining homeostasis, mitochondria have to maintain balance for biosynthesis of both these processes from the available mitochondrial iron pool. Mitochondrial iron homeostasis is well studied in erythroid cell lineages where cellular IRE-IRP system is involved in mitochondrial iron regulatory proteins. In erythroid cell system, iron importer Mitoferrin 1 (Mfrn1) and δ-Aminolevulinate synthase-E have IRE in their transcripts. Hence, at higher cellular iron levels, Mfrn1 and ALAS-E expression levels also go up (Paradkar, P.N et al, 2009). Thus cellular iron levels are directly related to heme biosynthesis in erythroid cells. While heme biosynthetic process takes place at maximal rate in erythroid cells, hepatocytes are second most heme consuming cells owing to their abilities to process xenobiotics transformation that needs high synthesis and activity of cytochrome P450. But unlike erythroid cells, hepatocytic mitochondrial iron homeostasis is not directly related with heme synthesis. Rather labile heme pool regulates heme biosynthesis in hepatocytic system (Ponka, P et al. 1997). The hemo-proteins like cytochromes, catalase, and peroxidase are involved in biological processes essential for all cells. Hence, heme biosynthesis is crucial for every cell type. Interestingly, astroglialheme biosynthesis and metabolism are far less studied and understood.

Whereas, Mfrn1 is responsible for mitochondrial iron influx to satisfy higher iron need of erythrocytic cells, mitoferrin2 (Mfrn2), the isoform of Mfrn1, regulates mitochondrial iron transport in non-erythroid cells. Mfrn2 is known to undergo up-regulation in head and squamous cancer cell lines in response to photodynamic therapy and contributes to mitochondrial dysfunction (Hung, H.I et al, 2013). The physiological regulation of mitoferrin2 has not been reported so far in the literature.

Iron sulphur clusters are indispensable for cellular survival. Studies on disease with affected ISC biosynthesis process reported in the case of Freidrich ataxia (Rotig, A et al, 1997) and mitochondrial myopathy (Mochel, F et al, 2008) showed severe manifestation as progressive neuropathy and cardiomyopathy. ISC biosynthesis is complex process involving series of proteins in sequential action taking place in mitochondria. ISCs levels are regulated by cellular iron levels (Rault, T et al, 1996). The two crucial proteins for

ISC assembly (Frataxin and Iron sulphur cluster scaffold protein) are reported to have whose endogenous levels regulated by iron deprivation (studied in chapter 2). Briefly, lower cellular iron level suppresses ISC assembly.

In brain, astrocytes are the most abundant glial cell type. They regulate neuronal iron transport across BBB as well as neurotransmission signalling at tripartite junction. The DA biosynthesis is iron dependant process and takes place in DA-ergic neurons of SNPC. The SNPC is the region of brain with abundant iron concentration. Hence, astrocytes around DA-ergic neurons have to maintain DA metabolism by regulating iron transport to neurons as well as catabolising DA from synaptic regions. The presence of DA transporters, receptors and enzymes catabolising neurotransmitters (MAO-A and B) are reported in astrocytes (Porter, T et al, 1997; Zhang, Y et al, 2010) so astrocytes might play crucial role in DA metabolism. The DA metabolism mainly takes place in neurons where it is an iron dependant process. DA is known to contribute in neuronal iron homeostasis by regulating synthesis of neuromelanin (Zucca, A.F et al, 2018).

But the role of DA on regulating iron homeostasis in astrocytes is not much clear so far except the reported finding of elevated mitochondrial iron level along with induction in heme-oxygenase-1 level (Schipper et al, 1999). Since, heme oxygenase-1 is catabolising heme; this may suggest a state of heme deprivation in cytosol in response to DA in astrocytes. Earlier unpublished work from the current laboratory found that DA could down-regulateiron efflux machinery. DA blocks ceruloplasmin (Cp) to affect stability of iron release component ferroportin in primary astrocytes and different glioma cells. Further, DA also affects protein stability of storage protein ferritin (Ft). These reflect the ability of DA in releasing intracellular iron pool from storage and also blocking the release. This is probably to meet the iron demand in mitochondria. Since, mitochondria have machinery to utilise iron to synthesize heme and ISCs, iron may be utilized to form both or any one of them. The work described in this chapter thus explored both these possibilities.

- In this chapter, the mitochondrial iron levels in astrocytes were estimated in response to dopamine in both dose dependant and time dependant manner. A gradual increase in mitochondrial iron content

in both concentration and dose dependant manner was found (Fig 1A and B). This confirmed the earlier observation of increased mitochondrial iron content by DA treatment. Elevated mitochondrial iron levels are reported in case of neurodegenerative diseases like Parkinson`s disease, Alzheimer`s disease, Schizophrenia etc (Horowitz, M.P et al, 2010; Rouault, T et al, 2013). Such scenario relates iron levels to mitochondrial dysfunction and defects in both ISC synthesis and heme synthesis.

- Iron import in to mitochondria is a well described process in erythroid cells (Ponka, P et al, 1997); where, normally mitochondrial iron importer protein mitoferrin 1 (Mfrn1) forms a stable complex with ferrochelatase and mitochondrial membrane ATP binding cassette ABCB10, the protein involved in last step of heme synthesis and heme export respectively (Chen, W et al, 2010). This oligomeric complex ensures the efficient iron transport across mitochondria to support efficient iron supply to mitochondria for sustained heme synthesis in erythroid cells. But in non-erythroid cells, iron import in to mitochondria is not well described. The Mitoferrin2, the homologous of Mfrn1, exists in all non-erythroid cell types. The expression of Mfrn2 in astrocytic cell was determined in response of dopamine in this study. An induction in both protein and transcript levels of Mfrn2 was detected raising the possibility of its involvement in DA-induced iron import in to mitochondria in astrocytic cells.
- The expression of Mt-Ft is tissue specific and is not known to have direct regulation by iron levels. Mt-Ft is known to have abundant expression in testes, neurons, spinal cord, cardiomyocytes, kidney cells and islets of langerhans (Santambrogio, P et al, 2007). The expression of Mt-Ft in glial cells is found to be very low while no expression was found in hepatocytes and splenocytes (Paul, B.T et al, 2017). The over-expression of Mt-Ft induces depletion in cytosolic iron levels leading to increase in IRE activity, increase in transferrin receptor levels and down-regulation of ferritin level. This is also accompanied with increase in mitochondrial iron levels (Nie, G et al, 2005). Hence, induction in Mt-Ft levels seems to enhancing the sequestration of iron in to mitochondria. The expression of

Mt-Ft in response to DA in astrocytic cells was not altered but it did not rule out whether Mt-Ft was able to store part of elevated level of DA-induced Mit-Fe.

- The DA induced HO-1 expression implicates degradation of heme in cytosol. Heme synthesis in non-erythroid cells is regulated by labile heme pool of cytosol (Ponka, P et al, 1997). When the heme content in whole cell lysate of C6 astroglial cells was estimated in response to DA and an induction of ~20% was detected. However, mitochondrial heme levels were induced by ~50% while ~30% increase in cytosolic heme levels was found. This suggests a simultaneous degradation of heme in cytosol by HO-1. HO-1 activity was inhibited using irreversible competitive inhibitor, tin Protoporphyrin IX (SnPP) then the total heme content in whole cell lysate of both DA-treated and untreated astrocytic cells were increased (Fig. 3D). Interestingly, DA-mediated increase in heme content was more in comparison to SnPP untreated cells (Fig. 3D) suggesting DA-induced HO-1 was accountable for modest induction of heme level by DA-treatment. All these strongly suggest an increase in mitochondrial heme content in DA-induced astroglial cells.
- Mitochondria also harbours ISC biogenesis pathway. However, the decrease in activities of ISC containing enzymes like aconitase 2 and succinate dehydrogenase in DA-treated astroglial cell or decreased abundance of ISC-containing RISP suggest lowering of ISC content. These results suggest a preference of heme synthesis in DA-exposed astroglial cells. The further work described in the next chapter shall elaborate on the mechanism on the preferred heme synthesis and decreased ISC.

Introduction:

The biosynthesis of iron bound cofactors i.e. Iron-sulphur clusters (ISCs) and heme, both takes place in mitochondrion. Hence, mitochondrion has to share the iron pool for generation of these two essential co-factors. Heme is the major product of mitochondrial iron consumption pathway in erythroid cell and hepatic cell lineages. Still, all cells require heme as well as iron-sulphur clusters for their survival. Existing literature is still not clear of how mitochondria maintain the delicate balance between the two iron consumption pathways.

The indispensable nature of Iron-sulphur clusters was made clear when disorders relating defective ISC biogenesis pathways were reported. Freidrich ataxia, FRDA is one such disorder which is characterised by diminished expression of protein, Frataxin, Fxn (Rotig, A et al, 1997). FRDA is an autosomal recessive disorder which is characterised by diminished expression of Frataxin. This is the due to hyper expansion of homozygous GAA triplet repeats in first intron of *frataxin* gene located at chromosome 9q13 causing transcriptional silencing of Frataxin. FRDA is manifested as progressive cardiac and neuro-degeneration along with gait abnormality and diabetes mellitus.

The studies on Frataxin showed that this enigmatic protein has ability to participate in ISC biogenesis, undergo oligomerization and to regulate heme biosynthesis. Frataxin is translated to be 210 amino acid precursor proteins containing mitochondrial localisation signal. Once precursor peptide reaches mitochondria, it undergoes step wise proteolytic cleavage by mitochondrial processing peptidase (MPP) to process it in stepwise manner to generate first an intermediate form, $hFXN_{56\text{-}210}$ and finally an mature functional form, $hFXN_{81\text{-}210}$ (Condo,I et al, 2007). Frataxin is an evolutionarily conserved protein but its precise role has been long closeted and even till now clarity has not been achieved. Frataxin was initially reported to be co-immunoprecipitated with components of ISC assembly machinery along with MPP-β (Schmucker,S et al, 2011). Thus, because of ability of Frataxin to bind to multiple Fe (II) ions on exposed regions, it was suggested to act as iron chaperone in ISC biosynthesis (Lane, D.J et al, 2015). Later reports have shown that interaction of Frataxin with ISC assembly components is independent of iron concentrations. And studies have shown that Frataxin has ability to act as kinetic regulator of ISC biogenesis. Frataxin interact with preformed complex of ISCU and NFS1/ISD11. ISCU is the iron sulphur cluster unit scaffold protein; NFS1 is cysteine

desulfurase which donates sulphur in ISC biogenesis while ISD11 acts as chaperone protein for NFS1. Frataxin accelerates the NFS1/ISD11 activity on ISCU. This interaction of Frataxin on ternary complex is independent of iron. But, Fe^{2+} induces the cysteine desulfurase`s activity and Fe^{2+} acts as allosteric modulator of the ISC assembly (Tsai, C.L et al, 2010). The in vivo data suggests that Frataxin mutants lacking ability to interact with ternary complex of ISCU and NFS1/ISD11 showed the levels of ISC to be strongly suppressed rather than completely abolished (Schmucker, S et al, 2011). That might suggest that Frataxin is required for efficient ISC biogenesis to sustain cellular survival as Frataxin mutants lacking ability to interact with components of ISC assembly machinery triggers cell death (W155R or W155A) or FRDA phenotype (N146A). The Frataxin protein also has ability to interact with multiple proteins including ferrochelatase (He, Y et al, 2004), mitochondrial aconitase (Bulteau, A.L et al, 2004), succinate dehydrogenase and chaperone proteins like HSC20 (Shan,Y et al, 2007).

The diminished expression of Frataxin in Freidrich ataxia is known to cause mitochondrial iron overloading resulting in mitochondrial dysfunction and oxidative stress. The insight on molecular dysfunction associated with suppressed levels of Frataxin includes quelling in the generation of iron–sulphur cluster-containing proteins (including complex I, II, and III of the mitochondrial electron transport chain and aconitase), an mitochondrial iron overload, decrease of mitochondrial ATP generation, increase in generation of reactive oxygen species, decrease of mitochondrial biogenesis, and induction of autophagy (Gonzalez-Cabo and Palau 2013).

The role of Frataxin has been well implicated in FRDA. The therapeutic strategies for alleviating pathology include drug treatments to mitigate oxidative stress, mitochondrial dysfunction (Idebenone) and chelation of mitochondrial iron (Deferiprone). Frataxin is known to be regulated under metabolic condition of iron starvation at transcriptional level but transcription factors involved in triggering such repression are not known (Li, K et al, 2008). It is reported that transcription factors, SRF (Serum response factor) and TFAP2 (Transcription factor AP2) regulates Frataxin in different cells in iron starved condition. In neuronal cell line, SHSY-5Y TFAP2 is known to be down-regulated in iron depleted condition and does not affect Frataxin expression. On the contrary, in HEK293 cells, iron depletion induced TFAP2 expression and increases Frataxin levels. Hence, this depicts tissue specific regulation of Frataxin expression in response to iron depletion (Li, K et al,

2010). Frataxin is also known to act under regulation of HIF-1α in response to hypoxia as reported in cardiocytes (Nanayakkara,G et al, 2015).

Another component of Iron Sulphur Cluster assembly machinery whose defect is known to be responsible for hereditary myopathy is ISCU, the major scaffold protein. The ISCU depletion in myopathy is characterised by muscle pathology, exercise intolerance and causes suppression in ISC levels and mitochondrial iron loading.

Heme as prosthetic group has been well explored in case of haemoglobin and Cytochrome P450. But heme in brain is not a well-studied area of research. In brain, hemo-proteins are involved in promoting neuronal growth, Neurferricin (Kimura, I et al, 2010), energy production through incorporation of mitochondrial cytochromes (Kim, H.J et al, 2015), regulating metabolism of neurotransmitters and neurosteroids, to detoxify neurotoxicants and oxidative insults, Neuroglobin (Trent, J.T et al, 2001). Since, heme is the prosthetic group which allows iron to be encompassed in a hydrophobic moiety Protoporphyrin-IX; it can passively diffuse across membranes. But heme mobilization is brain is mediated by transporters expressed on plasma membrane and intracellular organelles on both neurons and glial cells (Latunde-Dada Go et al, 2006). Heme cytotoxicity has been reported in neurodegenerative diseases like Parkinson`s disease (Ayuso,P et al, 2011). Also, Heme levels decrease with age (Atamna, H et al, 2002). Hence, Altered Heme levels might be involved in contributing mitochondrial iron accumulation and dysfunction associated with neurodegeneration.

Heme biosynthesis is an enzymatic process involving 8 steps, half of which takes place in mitochondrion and other half in cytosol. The first step and last step, catalysed by δ-Aminolevulinate synthase-1, ALAS-1 and ferrochelatase takes place in mitochondrion. While the first step is the rate limiting step and enzyme ALAS-1 is reported to be decreased during aging. The last step, Ferrochelatase, Fchase which catalyses last step of inserting iron in Protoporphyrin ring is reported to be increased during aging (Atamna, H et al, 2002).

The dysregulation in iron homeostasis is well reported in neurodegenerative disorders involving imbalance in DA metabolism. Dopamine is known to elevate mitochondrial iron levels in astrocytes along with increase in expression of heme-oxygenase-1 (HO-1). Heme-oxygenase-1 is the enzyme responsible for breaking down heme and acting as anti-oxidant component of cellular defence machinery against oxidative insults. In the

previous chapter, Dopamine induced heme levels in mitochondria were reported. It seems like elevated iron levels of mitochondrion are fluxed into heme synthesis specifically. As iron sulphur cluster levels were observed to be rather down-regulated.

Hence in the present chapter, the components of ISC and heme synthesis machinery are to be analysed in order to understand the molecular mechanism that DA might be exerting in mitochondria of astrocytes. The dependence of heme for DA mediated HO-1 induction will also be addressed.

Results:

Dopamine suppresses Frataxin levels but does not affect ISCU expression in astrocytes

In previous chapter, it was found that DA is suppressing Iron sulphur cluster levels in astrocytes. The ISC biosynthesis involves assembly of an iron donor protein and sulphur donor protein on a scaffold protein catalysed. Although the role of iron-donor protein in ISC assembly was initially thought to be played by Frataxin, Recent studies showed the role of Frataxin as regulator of this essential biosynthesis process. And ISCU is the scaffold protein on which assembly takes place. Both Frataxin and ISCU have been known to be involved in pathology associated with defect in ISC biosynthesis process. The expression levels of both the proteins were analysed in C6 astroglioma cells in response to DA treatment for 16hrs. The immunoblotting analysis of Frataxin showed a gradual decrease in protein expression with ~40 % down-regulation observed in DA 30μM (Fig 4A), although no such regulation was observed for ISCU protein (Fig 4B). Both the proteins showed decrease in protein levels when treated with iron chelator, DFO 100μM and was taken as negative control.

It was further checked that dopamine was affecting the Frataxin expression in different astroglial cell line from different origin. This might suggest that DA mediated Frataxin down-regulation is conserved in all species. The levels of Frataxin were also assayed in concentration and time dependent manner in response to dopamine in these cell lines. It was observed that Frataxin expression in U373MG human astroglial cell line (fig 4C); underwent DA mediated down-regulation in samples harvested for all time intervals (4, 8 and 16hrs). DA induced the down-regulation in Frataxin in human astroglioma U87MG as well, but suppressed levels were preceded by up-regulation first (fig 4D). In samples harvested at 4hr after DA treatment in U87MG cell line, DA induced the up-regulation in Frataxin levels with increasing concentration of DA. While in samples harvested at 16hr of DA treatment, down-regulation was observed. Also, there were two bands for intermediate peptide of Frataxin observed in human U373MG cell line. While in both C6 rat astroglioma and U87MG human astroglioma, single band for intermediate were observed.

Fig. 4A

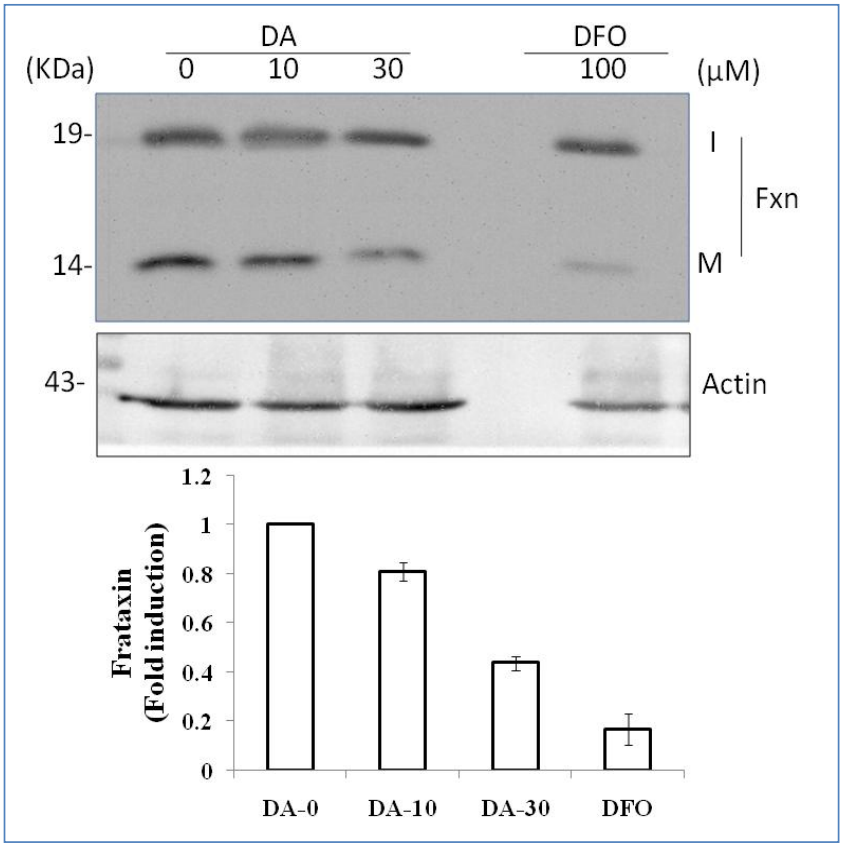
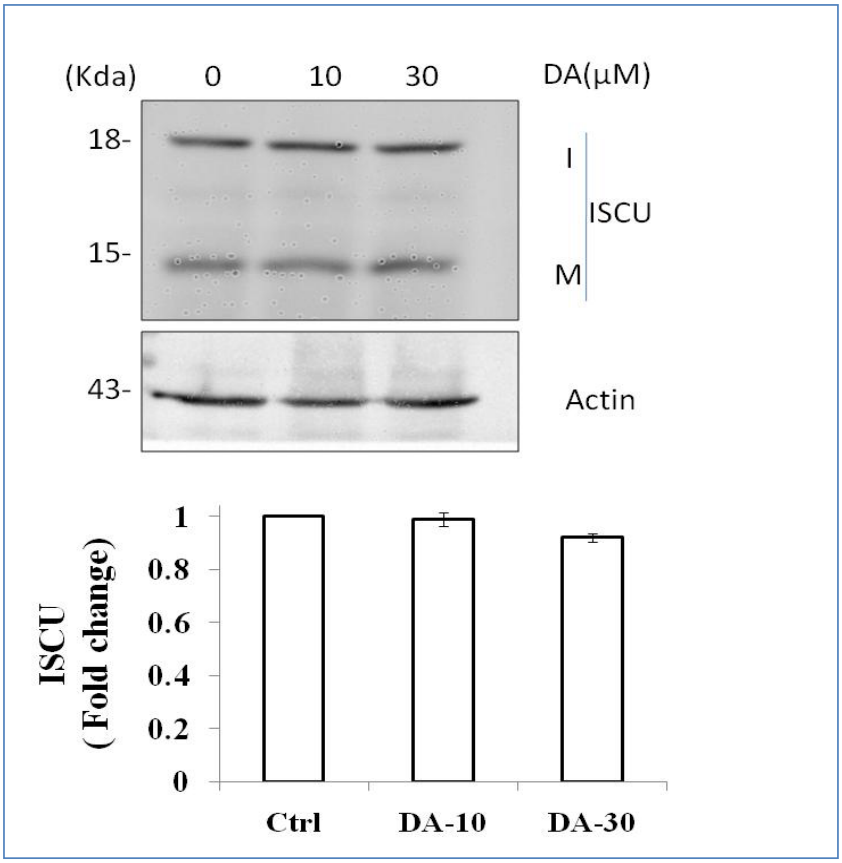


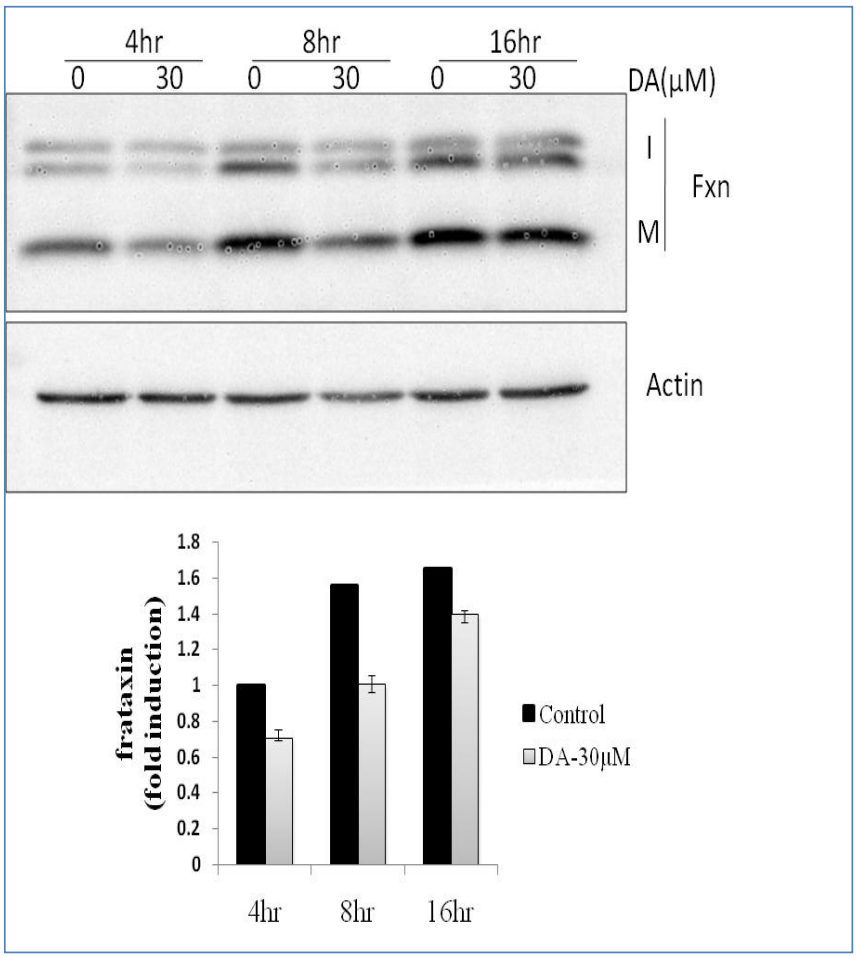
Fig 4: **Effect Of Dopamine on Components of Iron-sulphur Cluster(ISC) Biosynthesis Machinery:** C6 cells were treated with increasing concentration of DA(0-30µM). Iron chelator DFO 100µM was used as negative regulator of ISC biosynthesis machinery. A) **Effect on frataxin:** Harvested cell lysate were analysed by immunoblotting for frataxin, (upper lane) and actin (lower lane.) Bottom panel represents densitometric analysis of immunoblots as mean from three independent experiments ± SE. I and M denote as Intermediate and matured forms respectively.

Fig. 4B



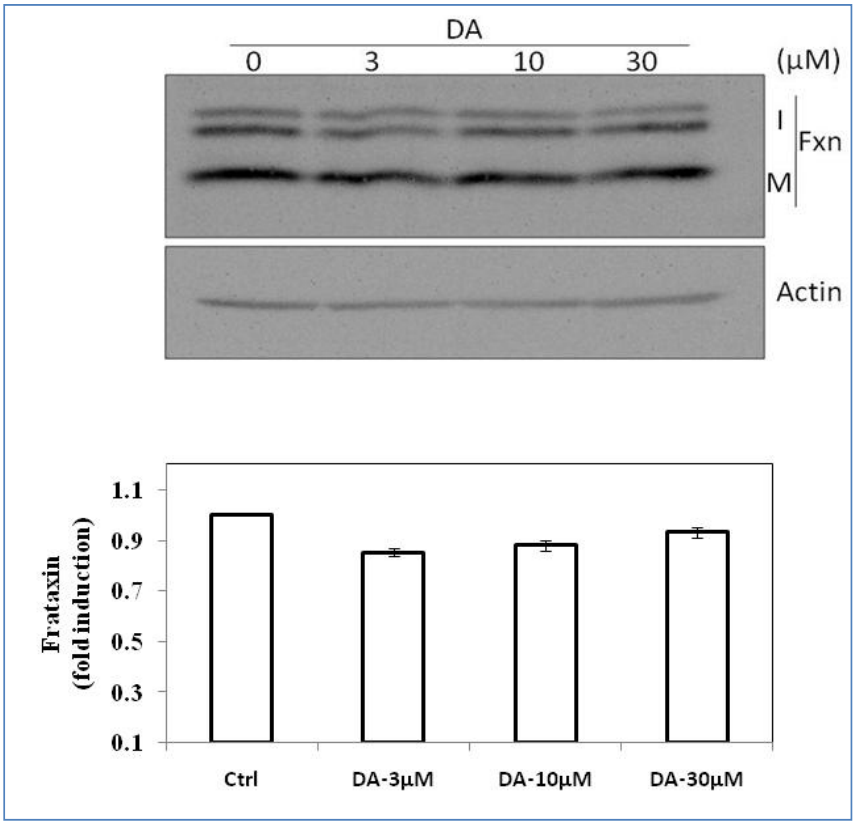
B) Effect on ISCU: Harvested cell lysate were analysed for immunoblotting of Iron sulfur cluster assembly scaffold protein, ISCU (upper lane) and actin (lower lane). Bottom panel represents densitometric analysis of immunoblots as mean from three independent experiments ± SE.

Fig. 4C



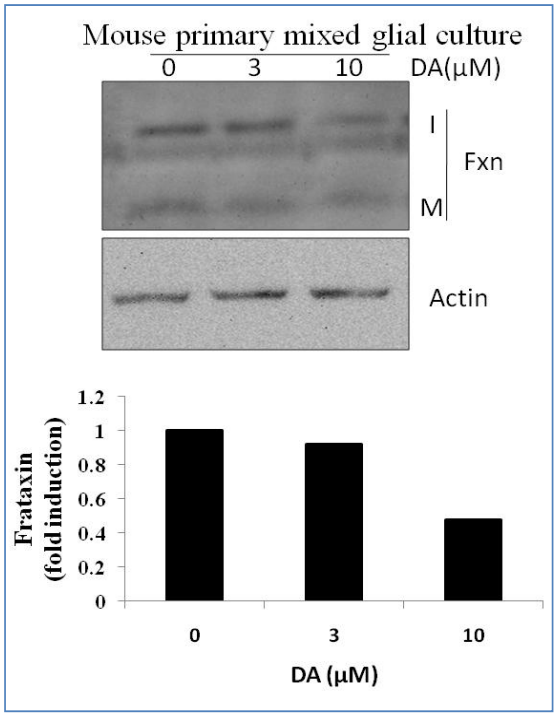
C) Top panel representing Immunoblot for frataxin (upper lane) and actin (lower lane) showing time dependant expression of frataxin in response to DA (0 & 30μM in U373MG human astroglioma. Bottom panel represents densitometric analysis of immunoblots as mean from three independent experiments ± SE.

Fig. 4D



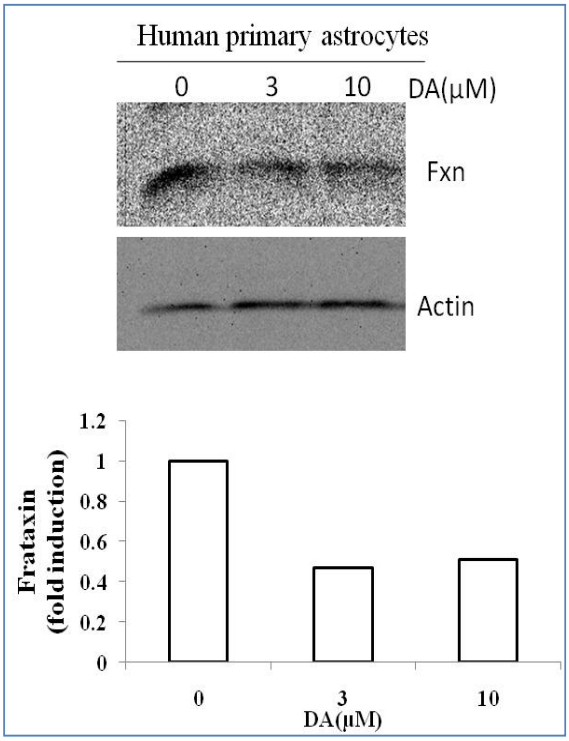
D) Human neuroblastoma cell line SHSY-5Y were treated with increasing concentration of DA(0-30μM) for 16hrs. Top panel, Upper lane represent frataxin while lower lane represent actin. Bottom panel represents densitometric analysis of immunoblots as mean from three independent experiments ± SE.

Fig. 4E



E) Immunoblots were analysed for frataxin in cell lysates harvested from mouse primary mixed glial culture treated with DA (0-10µM). Upper panel represents Fxn and lower represents actin. Bottom panel represents Densitometric analysis of blot.

Fig. 4F



F) Human primary astrocytes were treated with increasing concentration of DA(0-10µM) for 16hrs. Top panel represents immunoblot for Fxn(upper) and actin(lower). Bottom panel represents densitometric analysis of immunoblot.

Since, in literature, there are two sites for cleavage of precursor peptide has been reported 41/42 and 55/56 amino acid of 210 amino acid long precursor peptide of hFXN (Cavadini, P et al, 2000). It seemed like in human U373MG cell line; both kinds of cleavage pattern are predominant while in other cell type, Frataxin might follow only one maturation process.

The Frataxin expression in neuroblastoma cell line SHSY-5Y in response to DA was analysed by immunoblotting (fig 4E). The increasing concentration of DA did not affect the expression levels of Frataxin. This might suggest that DA mediated regulation on Frataxin is specific for astroglia.

After observing DA mediated regulation of Frataxin in astroglioma cell lines, DA`s response on Frataxin was examined on human primary astrocytes as well as mouse primary mixed glial culture (fig 4F and G). It was found that DA induced the down-regulation in Frataxin levels in primary astrocytes as well. Interestingly, it was also observed that even lower concentration of DA (3µM) was sufficient to induce the down-regulation in Frataxin.

All these data might suggests that DA induces down-regulation in Frataxin specifically in astrocytes, not in neurons. And this is not a cell line or species specific phenomenon. DA does not exert any regulation on ISCU levels. Hence, the suppression in ISC levels of astrocytes in response to DA might to due to down-regulation of Frataxin protein.

Dopamine induces Ferrochelatase, Fchase expression levels in astrocytes

Since in previous chapter, it is reported that DA induced the heme levels in astrocytes and which was further confirmed by checking the expression of rate limiting enzyme, ALAS-1. ALAS-1 expression was found to be down-regulated. The expression level of iron donor for heme, Ferrochelatase was examined. Ferrochelatase is an ISC (2Fe-2S) containing protein but its enzymatic activity is not dependent on it. Rather, Ferrochelatase`s stability is regulated by cellular iron levels at protein level. Also, Ferrochelatase`s expression is known to suppressed in ISCU myopathy as well that might suggest role of intact ISC on Fchase`s expression (Crooks, D.R et al, 2010).

Fig. 5A

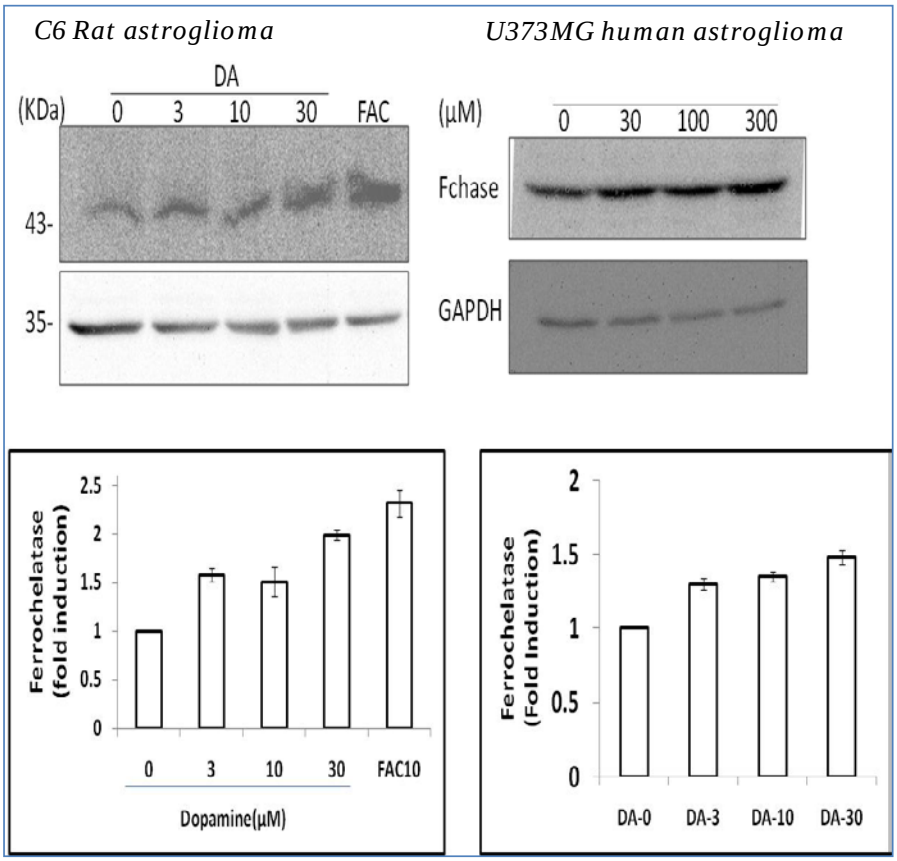
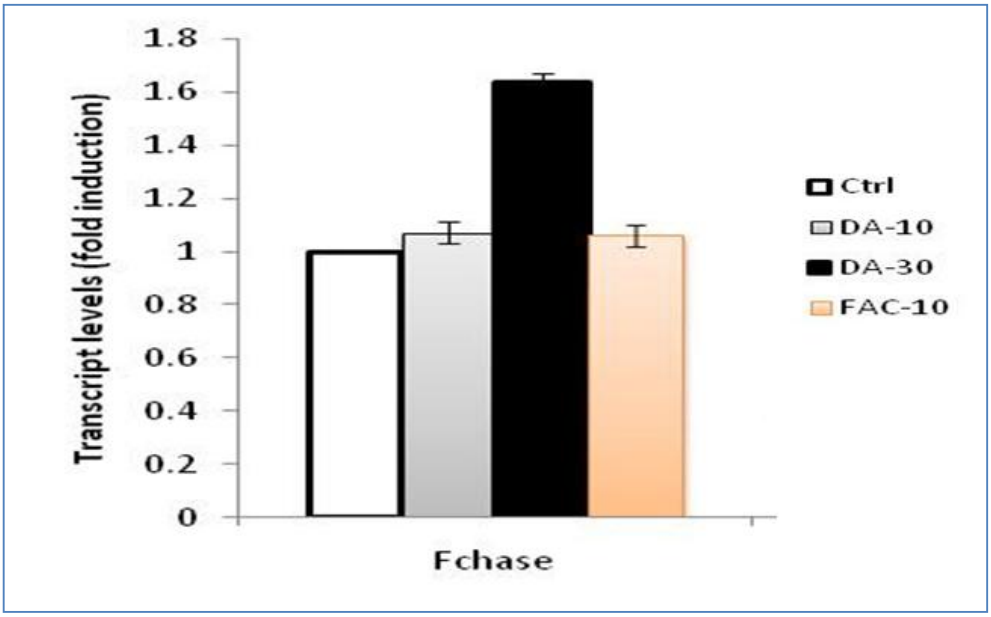


Fig 5: **Effect Of Dopamine On Heme Synthesis Machinery component, Ferrochelatase** A) C6 cells were treated with increasing concentration of dopamine (0-30µM) for 16hrs . FAC(10-100µM) treatment was given as positive control for Ferrochelatase, Fchase (Left panel). Right panel represents immunoblot for Fchase in U373MG cells in response to DA(0-30µM) for 16hrs. Top panel represent Immunoblotting analysis of Fchase (upper lane) and actin(lower lane). Bottom panel represents densitometric analysis of immunoblots as mean from three independent experiments ± SE.

Fig. 5B



B) C6 cells treated with Dopamine(0-30µM) and FAC 10µM were harvested for quantifying the transcript levels for Ferrochelatase, Fchase. Data represent mean ± SE of three independent experiments.

Hence, knowing the suppressed levels of ISC along with induced levels of heme in DA treated astrocytes, expression of Fchase was assayed. And it was found that DA induces Ferrochelatase`s protein levels as well as mRNA levels in C6 rat astroglioma. DA increased the Fchase expression in concentration dependent manner and levels were induced by two fold in response to DA-30µM (fig 5A). Iron salt, FAC was also treated to astrocytes as positive control and Fchase protein levels were found to be up-regulated. The Transcript level of Fchase were also measured and found to be up-regulated in similar manner as protein level. FAC did not induce mRNA level of Fchase in astrocytes (fig 5B).

This suggests that induction in heme synthesis machinery component, Ferrochelatase is mediated by Dopamine directly (transcriptionally).

Dopamine exerts transcriptional regulation on Frataxin to regulate its expression in astrocytes

Frataxin acts as kinetic regulator of ISC biogenesis machinery. The suppressed levels of Frataxin are known to affect ISC assembly and hence regulate ISC content of cells. The disrupted ISC levels as reported in ISCU myopathy as well as in FRDA and known to affect heme levels. It was hypothesised that if DA mediated lower levels of Frataxin were restored, that might restore ISC levels as well, and Will that affect heme levels or not? To restore the levels of Frataxin in DA treated astrocytes, the mechanism by which DA mediates down-regulation was explored.

The transcript level for Frataxin was quantified in C6 rat astroglioma in response to increasing concentration of Dopamine (0-30µM). It seemed that Frataxin transcript was down-regulated to ~40% in DA-30µM in similar manner as Frataxin protein levels (fig 6A). The transcripts for Frataxin were checked in samples harvested at increasing time interval in after dopamine treatment in C6 rat astroglioma (fig 6B). The Frataxin transcript was slightly down regulated in time intervals of as early as 4hr but significant down-regulation was observed in time interval of 16hr.

Fig. 6A

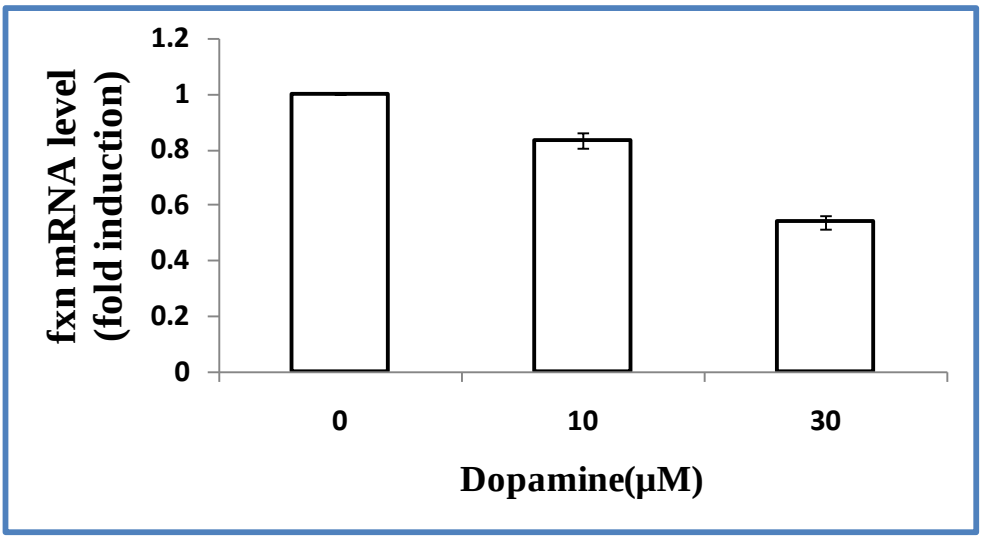
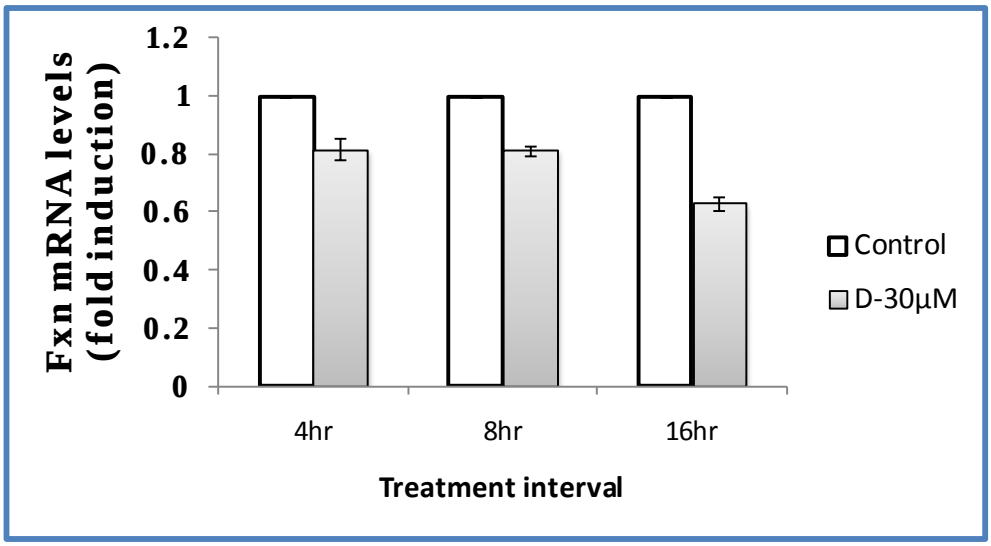


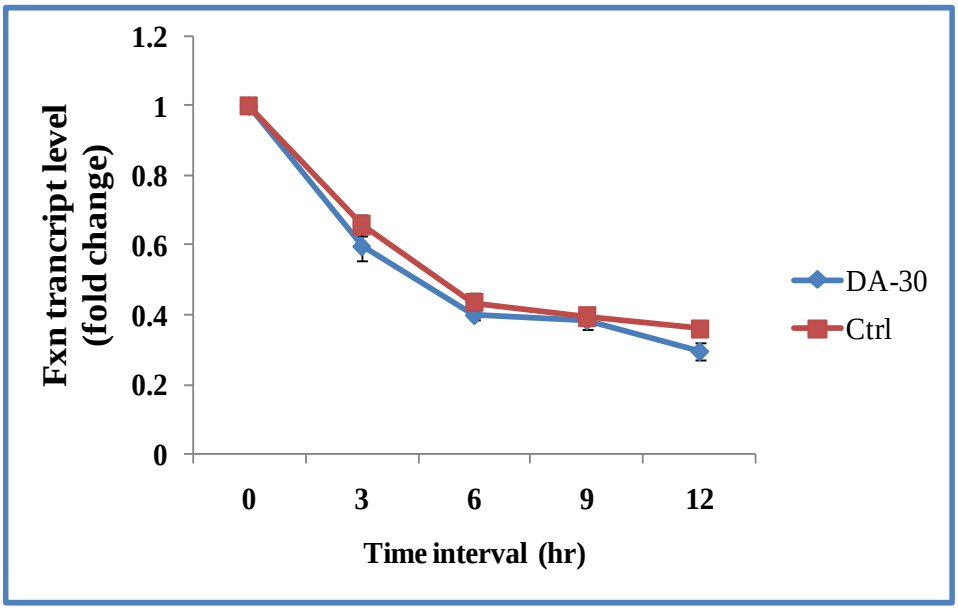
Fig 6: **Molecular Mechanism Of DA Mediated Frataxin Down-regulation A)** upper panel representing mRNA levels for frataxin, Fxn in C6 cells in response to DA (0-30µM) for 16 hrs performed by real time PCR. Continue..

Fig. 6B



B) lower panel representing mRNA expression of Fxn in time dependent manner(4,8 16 hrs) in response to DA-30µM. Results are represented as mean of three independent experiments with similar results ±SE.

Fig. 6C



C) Data representing mRNA stability of Fxn in response to DA in C6 cells. C6 cells were treated with DA-30µM for 8hrs then chased for different time intervals after treating actinomycin D (10 µg/ml). Cells harvested at different time points were analysed for mRNA levels by real time PCR. Results represented as mean from three independent experiment±SE.

DA might be regulating the Frataxin expression in a way to suppress mRNA levels which might be causing decrease in protein levels. Hence, the mRNA stability of Frataxin was assayed in response to DA. C6 astroglioma were treated with DA-30µM for 8hrs and then chased with actinomycin D (10µg/ml). Samples were harvested for transcript analysis of Frataxin at time interval of 0, 3, 6, 9 and 12hrs after actinomycin D treatment. There was no change in stability of mRNA observed for Frataxin in response to dopamine in astrocytes (fig 6C).

Interestingly, in the actinomycin D chase experiment, it was also observed that DA induced down-regulation of Frataxin was not observed in actinomycin D treated samples. This might suggest that transcriptional inhibition mediated by actinomycin D abolished the DA induced down-regulation of Frataxin in astrocytes.

Altogether, DA suppresses mRNA levels of Frataxin without affect its stability. This suggests that Dopamine mediates transcriptional suppression on Frataxin in astrocytes.

Frataxin over-expression restores the elevated levels of heme in astrocytes mediated by Dopamine and does not affect mitochondrial iron influx.

To restore the Frataxin levels in DA mediated Frataxin depleted astrocytes, rat Frataxin CDS (devoid of both 5` and 3` Untranslated regulatory regions) was cloned in flag-14 vector. This construct allowed the translation of precursor peptide of Frataxin to fuse with carboxy terminus flag peptide and bypass any regulation that Dopamine might exerts on Frataxin through UTR`s. And it has already been established that DA does not affect mRNA stability of Frataxin, so transfection of this construct might induce the over-expression of Frataxin, which was needed for extending the understanding of role of Frataxin in DA mediated change in mitochondrial iron homeostasis.

The transfection of 1.5µg Fxn-flag DNA/10^5 cells were made on C6 cells while only flag vector was taken as mock control. Both groups were treated with DA-30µM for 16hrs and cells were harvested for immunoblotting,

Fig. 7A

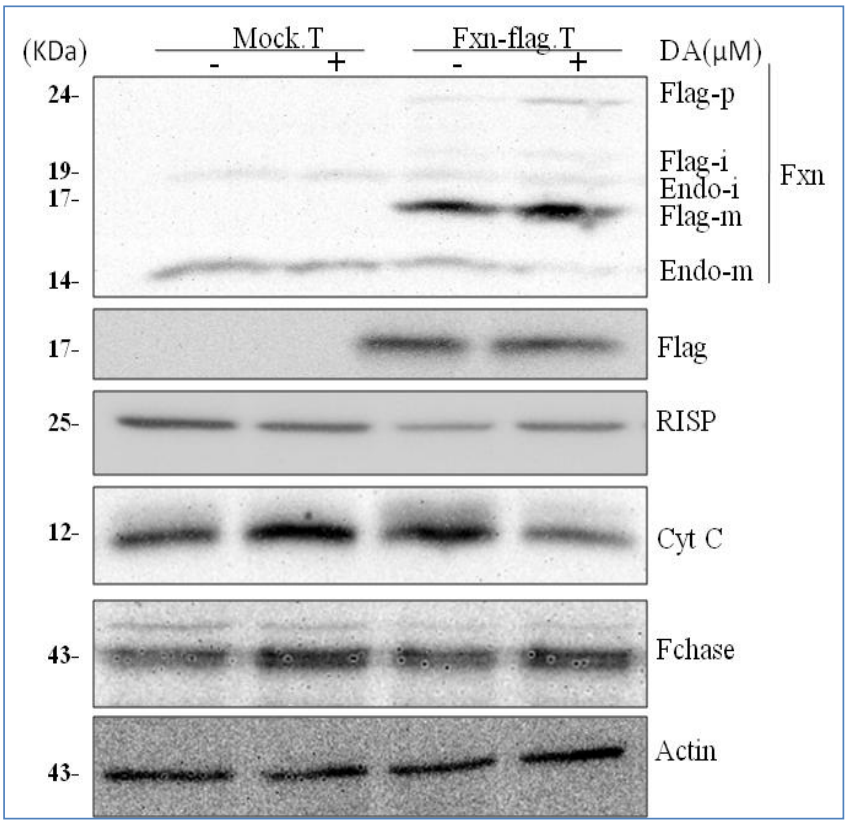
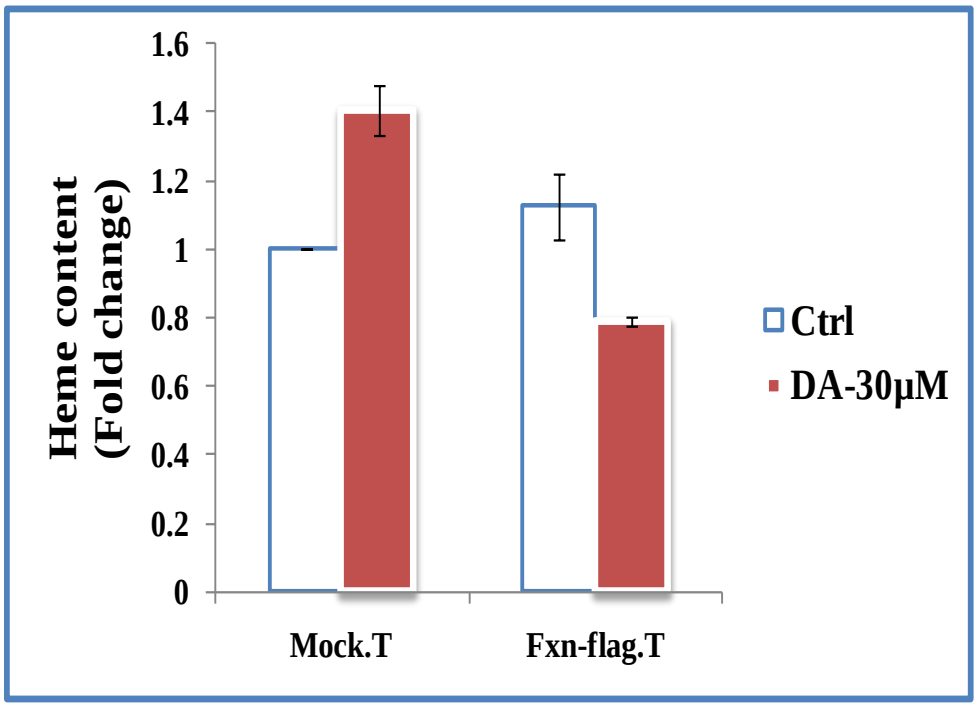


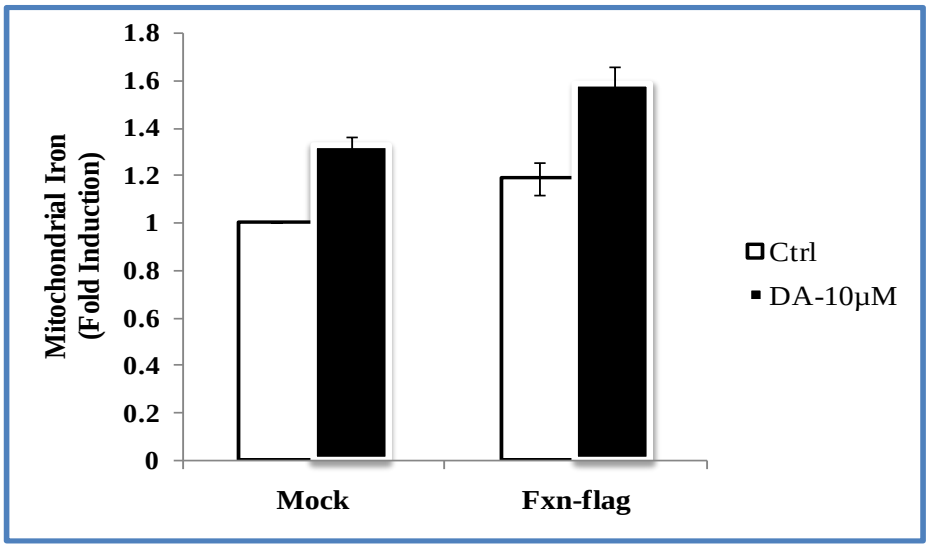
Fig 7: **Effect of restoring frataxin expression on DA mediated induction in heme content** C6 cells were transfected with construct containing rat Fxn-CDS fused with N`- flag peptide. Only flag vector was used as mock transfection control. Both fxn-CDS and mock transfected cells were subjected with DA-30μM treatment for 16 hrs. A) Cells were harvested for immunoblotting analysis of Fxn, flag, RISP, Cytochrome C, Fchase and actin as normalization control. Continue..

Fig. 7C



B) C6 cells were transfected with fxn-CDS flag. Post-transfecton, cells were treated with DA-30µM for 16hrs. Lysate was harvested for heme estimation.

Fig. 7D



C) mitochondrial fractions were harvested from transfected samples post DA treatment and analysed were iron estimation. Results are represented as mean from three independent experiments ± SE.

mitochondrial iron estimation and heme estimation. Both Fxn-flag and mock transfected samples were immunoblotted with Frataxin antibody as well flag antibody (fig 7A). The Fxn-flag treated lane in top panel showed appearance of extra bands of intermediate and mature peptide that corresponds with flag tagged Fxn band. Also, appearance of precursor peptide in Fxn-flag lane confirms the exogenous expression of Frataxin. The down-regulation in lower-most band that correspond to endogenous mature Frataxin showed that DA is mediating regulation on endogenous protein. While equal intensity of flag tagged mature peptide band is bypassing any such regulation as showed in blot. This is further confirmed by flag probed blots that is not showing any regulation and was only observed in Fxn-flag transfected lane.

To check the levels of ISC and heme, we used Reiske iron sulphur cluster protein, RISP as marker for Iron sulphur cluster levels and Cytochrome C, Cyt C as marker for heme. The Immunoblotting of RISP and Cyt C in mock lane showed suppressed levels of ISC and induced levels of heme. While Fxn-flag transfected lane showed reversal in expression of protein. While RISP levels were induced representing increase in ISC levels. Cyt C levels were reduced that might suggest decrease in heme levels.

This observation was surprising. Hence it was further confirmed by estimating heme in whole cell lysate. It was observed that induced heme levels mediated by dopamine were not just restored in Fxn over-expressed samples; they rather underwent down-regulation (fig 9B). The possible explanation for this observation was that exogenously added Frataxin induced ISC levels to the extent that it`s restricted the supply of iron to heme biosynthesis now.

To check that this reversal in flux of iron in iron consumption pathway is due intra-mitochondrial switch or altogether altered mitochondrial iron inflow mediated by Frataxin over-expression in response to DA. Mitochondrial iron levels were quantified in Frataxin over-expression and found to have same response as mediated by DA. Also, it was noted that Frataxin over-expressed cells showed increase in basal levels of mitochondrial iron. The possible explanation for this observation is due to increase in mitochondrial iron need as higher levels of Frataxin are consuming more iron.

Altogether, the present study showed that Frataxin does not affect DA mediated iron sequestration in mitochondrion. But Frataxin is indeed involved in DA mediated

alteration in mitochondrial iron consumption pathway and hence affecting mitochondrial iron homeostasis in astrocytes exposed with dopamine.

Dopamine induces heme levels to sustain the elevated HO-1 levels in astrocytes

Schipper et al reported that DA induces mitochondrial iron levels in conjunction with induction in heme-oxygenase-1 protein. Heme-oxyegnase-1(HO-1) is an inducible protein and is known to play role as anti-oxidant component of cells. The substrate for HO-1is heme and its breakdown products are known to contribute for its anti-oxidant property. Hence it was examined that whether DA is inducing heme levels in mitochondrion to synthesise the substrate for HO-1. This was studied by using Fxn-flag over-expression system. Cell lysates harvested after Fxn-flag overexpression and DA treatments were analysed for transcript estimation of HO-1(fig 8). It was found that mock transfection controls had induced HO-1 transcript levels in DA treatments, while no such induction was observed in DA treatments of Fxn-flag transfected lanes.

This suggests that DA exerted its regulation for inducing heme content in mitochondrion might be to induce the levels of HO-1 in astrocytes.

Discussion:

Mitochondria are the energy house of cells. Besides supplying energy currency to the cells, mitochondria also houses the process of fatty acid oxidation, steroid synthesis and synthesis of iron based cofactors ISC and heme. Since mitochondrion is the major consumer of iron, it has to handle significant amount of iron. Also, both iron consumption process of mitochondria shares the same mitochondrial iron pool but how does it maintain the balance between the two processes to continue the steady synthesis of both co-factors is not known. Iron sulphur clusters as well as heme acts as versatile cofactor and plays vital role in essential cellular processes. They are involved in diverse physiological processes like oxidative phosphorylation (complex I, II and III), nucleotide biosynthesis, TCA cycle, DNA repair and heme synthesis, O_2 transport (haemoglobin, neuroglobin and myoglobin), H_2O_2 detoxification, nitric oxide generation, cytochrome c and cytochrome c oxidase activity, cellular iron homeostasis (IRP1).

Since mitochondrion lies at the heart of cellular survival, any impairment related to mitochondrial functioning might affect the whole cellular system. Mitochondrial dysfunction have been well documented in case of neurodegenerative disorders like Parkinson`s disease, schizophrenia and attention deficit hyperactivity disorders. All these diseases are related to aberrant dopamine metabolism.

Existing literature has ample documentation that relates impaired DA metabolism to neurons especially in context of neurodegenerative disorders. However, earlier research did not consider the role of other brain cell like astroglia that might play role in regulating DA homeostasis. Astroglia being the most abundant glial cell type, also reported to express DA transporters along with DA catabolising enzymes.

New findings are bringing the role of astroglia in maintaining DA balance. It been reported that a subset of cortical astrocytes in prefrontal cortex plays vital role in regulating dopamine homeostasis during post-natal development. The regulation by astrocytes is exerted by proteins involved in DA transport (Vesicular monoamine transporter, VMAT2) and metabolism (Monoamine oxidase type B). The deletion of these proteins in astrocytes leads to defective synaptic transmission, plasticity and executive functions (Petrelli, F et al, 2018). A recent report compared the transcriptome of several genes involved in PD pathology and found that eight genes are that lies at the heart of pathology have more expression in astrocytes as compared to neurons. These genes include PARKJ and PINK 1 and 2 etc. (Booth, H.D et al, 2017).

Now researchers are considering the role of astrocytes that might play importance in understanding pathology associated with disorders involving abrupt DA metabolism. AthoughSchipper et al reported that DA induces mitochondrial iron levels in astrocytes in conjunction with increase in heme-oxygenase-1 levels. No follow up study came after that showed the fate of elevated iron levels in mitochondria. DA induces heme-oxygenase-1 levels in transcriptional manner but what might be the signalling molecule which is regulating the protein expression in astrocytes is not known.

In earlier chapter, some light was shed in direction of possible fate of induced iron levels in mitochondria mediated by dopamine. It was found that DA induces heme levels and this increase in heme is mediated by DA specific pathway, not by feedback regulation exerted by regulatory labile heme pool. The induction in heme pool was associated with simultaneous suppression in ISC levels.

In the present chapter, the molecular mechanism behind this shift in iron consumption pathway was explored. It was expected that the levels of both iron consumption pathways will be up-regulated as both shares the same mitochondrial iron pool. But specific induction of only one pathway and suppression of other suggest the possibility of DA specific sensor with the potential act as metabolic switch.

Hence, the components of ISC assembly machinery were analysed. Frataxin and ISCU are two proteins which are already reported to be involved in pathology associated with deficiency in ISC levels. The depleted levels of Frataxin are reported in FRDA. Frataxin is the protein with potential to be involved in multiple process and it`s precise role is still under closet. Initially it was thought to act as iron chaperone in ISC assembly but iron concentration did not affect its interaction on scaffold protein (Gonzalez Cabo P et al, 2013). Now Frataxin role is rather more acceptable as kinetic regulator of assembly of ISC on scaffold protein. The Lower levels of Frataxin suppress ISC levels. It was found that DA down regulates Frataxin without affecting ISCU levels in C6 rat astroglioma. The expression of Frataxin in astroglial cell lines from human origin as well showed similar down-regulation in response to dopamine treatment for 16hrs. Although in human U87MG cell line, cells treated with DA for 4hrs, levels of Frataxin were initially found to be up regulated and then underwent down regulation.

The Expression of Frataxin in neurons is significantly higher than astrocytes. Also the half-life of Frataxin protein in astrocytes is significantly higher than neurons. Infact,

Insulin like growth factor 1 (IGF-1) acts as positive regulator of Frataxin and induces its expression through canonical Akt/mTOR pathway in normal astrocytes but not in normal neurons. Although neurons pre-depleted with endogenous Frataxin does respond to IGF-1 in similar manner as astrocytes (Franco, C et al, 2012). This suggests the differential regulation of Frataxin in neuron and astrocytes. It was noted that dopamine only regulates Frataxin in astrocytes, not in neurons.

The Frataxin`s expression were also analysed in mouse and rat primary astrocytes and found decreased levels in response to DA. It was noted that lower concentration of DA was inducing were sufficient to alter the levels. This suggested higher sensitivity of Frataxin in primary astrocytes. Interestingly, Frataxin levels are found to be higher in cancerous tissues that include glioblastomas and colon cancers (Guccini, I et al, 2011). This might explain the poor detection of Frataxin bands in lysates from primary astrocytes.

Ferrochelatase is a 2Fe-2S cluster containing enzyme, which catalyzes the last step of iron insertion in Protoporphyrin ring. The ISC in ferrochelatase is not required for catalysis but in ISCU myopathy, lower levels of ferrochelatase were observed. It's been reported that cellular iron levels regulates stability of apo-protein. The Frataxin is known to interact with Ferrochelatase as well and might regulate heme synthesis. It's been reported that Frataxin have different affinity for ISCU and ferrochelatase protein. The affinity of Frataxin for ISCU is very low as compared to its affinity for ferrochelatase protein which might suggest that lowering of Frataxin levels immediately affects ISC levels as compared to heme levels.

The expression of ferrochelatase in astrocytes in response to DA was found to be induced at levels of both protein and mRNA. The induction in mRNA levels might suggest DA specific regulation on ferrochelatase. The protein stability of Ferrochelatase is known to be affected by iron status as depicted by treating k562 cells with iron chelator, DFO (Taketani, S et al, 2000). The iron starvation induces down-regulation in protein stability. Although depleting iron levels with DFO decreases the level of mitochondrial iron as well so it`s not clear whether mitochondrial iron levels or cytosolic iron level regulates ferrochelatse protein stability.

DA down-regulates Frataxin level and induces ferrochelatase`s level. Since Frataxin has potential to regulate ISC as well as heme synthesis, experiments were performed to

understand the molecular mechanism by which DA is regulating Frataxin. The aim of these experiments was to find the way to restore the levels of DA mediated depleted levels of Frataxin. It was found that DA does not affect mRNA stability of Frataxin in astrocytes. Interestingly, it was also noted that treatment of transcriptional blocker was abolishing the DA mediated suppression on Frataxin. This raises the possibility of a complex regulation that might involve expression of a repressor molecule by dopamine in astrocytes. And actinomycin D induced inhibited the expression of that repressor which was suppressing DA mediated Frataxin levels. So far so, literature for regulation over Frataxin is still limited. Since, studies on Frataxin are mainly conducted in context of FRDA; only inducers for Frataxin expression are main concern in present research society.

Frataxin CDS clone lacking regulatory region were expected to bypass the DA mediated repression. The transfection of this construct was used to establish over-expression of Frataxin in DA induced depleted levels of Frataxin. The Over-expression of Frataxin has been a strategy well exploited in number of reports. The transgenic mice over-expressing Frataxin showed no change in systemic iron parameters but altered the erythroid differentiation pathway which suggests direct involvement of Frataxin in regulating heme synthesis (Miranda, C.J et al, 2004). Existing literature regarding Frataxin over-expression in drosophila have reported contradictory results (Navarro, J.A et al, 2011). But all these studies were performed in transgenic mice with altogether higher levels of Frataxin. On the other hand, DA induced down-regulation in Frataxin and depleted its levels in astrocytes. One study reported the protective role of tat-Frataxin transduced in neuronal cell SHSY-5Y in MPTP induced toxicity (Kim, M.J et al, 2012). This might suggest significance of maintaining optimal level of Frataxin.

In present study, it was found that Frataxin over-expression restored the levels of ISC in astrocytes. Supplying exogenous Frataxin now reduced the levels of heme in astrocytes. It might be possible that higher levels of Frataxin consumed iron in synthesising ISC that it has depleted iron supply for heme synthesis. In erythroid cell types, where heme is the major product of mitochondrial iron consumption machinery, an extra-mitochondrial form of Frataxin exists to support the ISC assembly (Guo, L et al, 2018). This might suggest the necessity of both co-factors in all cell types. The restored levels of ISC and heme in Frataxin over-expressed cells were not accompanied with altered mitochondrial influx mediated DA. The excess Frataxin levels in astrocytes might lead to increase in

iron consumption as well and as a result there was increase in basal levels of mitochondrial iron.

By now, it has been established that DA is mediating induction in heme levels by regulating Frataxin levels. But DA is also catabolising heme in cytosol. The induction in both degradation and synthesis process might raise the question of futility of whole cycle. Heme degradation is catalysed by ER anchored enzyme Heme-oxygenase-1. The by-products of heme catabolism have known to exert anti-oxidant property but it generates the by-products, there has to maintain a steady supply of substrate as well. The substrate for heme-oxygenase-1 is heme hence DA is inducing the heme levels to sustained the expression of HO-1. As DA metabolism is known to generate ROS and astrocytes are the major cell type responsible for metabolising excess DA from synaptic cleft. The DA mediated iron flux and increase in heme levels might act as defence mechanism against ROS generated.

Summary and Discussion:

Iron overload, oxidative stress and mitochondrial dysfunction are the triad of evil and are often associated as cause or consequence dilemma behind neurodegeneration disease pathology. Excess iron levels in brain are often associated with neurodegenerative disorders such as Parkinson`s diseases (PD), Alzheimer`s disease (AD), Huntington's disease (HD) and others (Garlach, M et al, 1994; Zecca, L et al, 1994). These neurodegenerative disorders are mostly characterised by focal accumulation of iron at specific site in brain such as globus pallidus, substantia nigra, basal ganglia, putamen (Dusek,P et al, 2012). The mitochondrial dysfunction and iron accumulation has been a common characteristic feature of most of this neurodegenerative diseases but the exact mechanism behind pathology is still not clear.

Mitochondrion is the major consumer of iron in all cellular systems. It houses the two major iron utilising biosynthesis process. It is the site of heme synthesis as well as iron sulphur clusters. Heme and iron sulphur clusters are versatile prosthetic groups. They are involved in vital physiological processes essential for all cellular systems like energy generation, DNA repair and nucleotide biosynthesis. Hence, tissues and organs with higher metabolic activity have higher iron demand. The heart and brain are considered as major consumers of iron, (besides liver and reticulo-endothelial system) based on their need of high energy (Hill, J.M et al, 1984).

The mitochondrial share on total cellular iron content is significantly higher than the rest of cellular compartments, hence mitochondria are more prone to iron overload mediated oxidative insults. The regulations on mitochondrial iron pool in erythroid cell lineages have been well studied. In erythroid system, the major product of iron consumption machinery is heme, which is incorporated in hemoglobin, to enable RBC to have O_2 carrier function. The mitochondrial iron pool in erythroid cell is directly regulated by cellular iron levels. However, such is not the case of non-erythroid cell types (Ponka, P et al, 1999).

All cells are required to maintain balance on mitochondrial iron pool. As both heme and ISC shares same cellular compartment for synthesis i.e. mitochondria, there has to be a balance between the two process as they share the same iron pool. So, mitochondrion must have a delicate regulatory mechanism for maintaining iron levels as well as balancing them within the mitochondrion. Mitochondrial iron level increases with ageing

and the regions where iron accumulation is seen are the sites affected in Parkinson`s disease and Alzheimer`s disease (Hirose,W et al,2003; Schipper, H.M et al, 2004). This raises the need to understand the regulation on mitochondrial iron homeostasis and how its function gets affected in disease scenario.

While iron deficiency affects the efficiency of mitochondria to sustain the energy demands of cells, overloading in mitochondria may generate oxidative stress that can affect the integrity of membrane lipid, protein and mitochondrial DNA (Kowaltowski, A.J et al, 1997). A study compared the effect of iron deficiency and iron overload on mitochondrial parameters in rats by altering dietary iron supplementation. It was found that both conditions, iron deficiency as well as iron excess, induced lowering of liver mitochondrial respiratory control ratios. Although oxidant induced damage was only observed in iron excess supplemented group (Walter, P.B et al, 2001).

Astrocytes are the most populous cells in the central nervous system (Tower and Young, 1973) and regulate iron distribution in brain (Zhang et al, 2013). Astrocytes are uniquely positioned in brain, which allows them to contact both neurons and blood vessels in a way to provide neuroprotection as well as nutritional support (Abbott, N.J et al, 2006). Recent literatures have addressed the possibility that astrocytes are contributing significant role in neurodegenerative diseases as not only they are required for neuronal survival but they can also release neurotrophic factors that can cause toxicity to neuronal cells (Lee, M et al, 2013). Since these hidden potentials of astrocytes have emerged, they are also focus of therapeutic target (Rappold, P.M et al, 2010)

Dopamine (DA) is a catecholamine neurotransmitter, contributes for learning, motivation and reward seeking nature of human (Wise, R.A et al, 2004). Imbalance in DA metabolism has been implicated in several neurodegenerative disorders such as PD, schizophrenia and attention-deficit hyperactivity disorder (ADHD). PD is characterised by mitochondrial dysfunction, iron accumulation, neurodegeneration at *Substantia nigra pars compacta* (SNPC) (Abou-Sleiman, P.M et al, 2006). SNPC is the site of dopaminergic neuronal signalling. Dopamine biosynthesis which takes place in neurons is an iron-dependant process and SNPC is region of high iron levels in brain. Hence, this raised the possibility of DA metabolism and iron homeostasis to have some link. A study showed that DA metabolite aminochrome induced mitochondrial dysfunction and iron accumulation in neurons by inhibiting Complex I, increasing expression of iron importer,

DMT1 and decreasing expression of exporter, Ferroportin (Aguirre, P et al, 2012). Another study showed that in primary astroglial cell, hydroxydopamine (6-OHDA) could induce the expression of DMT1, Fpn and Ferritin L expression in a way to induce the rate of iron transport in astrocytes. While in neurons 6-HODA are known to contribute for iron accumulation and regulates iron importer and exporter accordingly (Jiang, H et al, 2010). This is one of a few studies which has shown that astroglial cell`s response is different than that of neurons and serving its role as neuroprotector.

It has been reported that astroglial cells challenged with DA have elevated mitochondrial iron levels along with induced expression of heme oxygenase-1 (HO-1) (Schipper, H.M et al,1999) while in a different study DA mediated induction in transcript level of HO-1 was also reported (Schmidt, J et al, 1999).

Heme oxygenase-1 is an ER anchored heme catabolising enzyme that breaks heme in cytosol and releases biliverdin, carbon monoxide and iron. While carbon monoxide and biliverdin have anti-oxidant effect, free iron might have deleterious effect on cell.

The earlier unpublished data from current laboratory have found that DA could degrade iron storage protein ferritin (Unpublished data, Som Dev thesis). DA also suppresses iron efflux machinery in astroglial cells by down-regulating ceruloplasmin (Cp), the stabilizer of iron efflux protein ferroportin (Fpn) (Unpublished data, Reshmi Mukherjee Thesis). Hence, DA induces the mobilisation of iron from ferritin and heme and sequesters iron in mitochondria. But in mitochondria, there are iron regulatory proteins and iron consumption pathways, both of which are least explored in astroglia cells.

Therefore, this study aimed to understand the DA induced influx of iron and fate of elevated iron levels in mitochondria.

The major findings of this study are as follows:

- Dopamine elevates mitochondrial iron levels in both concentration and time dependant manner in C6 rat astroglial cells. This confirms the earlier study that reported the increase in iron content in rat Primary cultured astrocytes (Schipper,H.M et al, 1999). Mitochondrial dysfunction has been implicated in neurodegenerative disorders involving imbalance in DA metabolism such as PD, Schizophrenia and ADHD. Most of the existing literature that explores the link of DA signalling with mitochondrial iron accumulation and dysfunction is based on

studies of PD. PD is characterised by degeneration of DA-ergic neurons and actual status of DA levels in PD is still unclear. Mitochondrial dysfunction in PD is well reported along with loss of complex I activity and mtDNA damage (Schapira, A.H.V et al, 1990; Henchcliffe, C et al, 2008). Schizophrenia is characterised to have hyper-dopaminergic signalling (Seeman, P et al, 2000) and recent reports have showed dysfunction in mitochondria along with mitochondrial hypoplasia and drastically affected oxidative phosphorylation in patient brain samples (Ben-Shachar, D et al, 2004). In contrast, ADHD involves suppressed DA-ergic signalling (Bymaster, F.P et al, 2002) and ADHD`s pathology is suggested to involve energy deficiency in brain (low ATP production in neurons and low supply of lactate to oligodendrocytes). This may suggest inefficient astrocytic function (Russell, V.A et al, 2006). Thus DA mediated elevation in iron level in astroglial cells may be linked to mitochondrial dysfunction to neurodegenerative disorders and psychosis in regards to altered DA homeostasis.

- All cells expresses mitochondrial iron importer Mitoferrin (Mfrn). There are two isoform exists for Mfrn, 1 and 2. Mfrn1 is responsible for efficient iron supply in erythroid cells to sustain hemoglobinization but Mfrn2 cannot sustain erythroid heme synthesis (Paradkar, P et al, 2009). Mfrn 2 is not well studied so far. There are no cellular regulators known for Mfrn2. It has been reported to undergo up-regulation in response to arsenic trioxide and photodynamic therapy in human glioma (Wang, C et al, 2014) and squamous carcinoma (Hung, H.I et al, 2013) respectively. Hence, the role of Mfrn2 in inducing iron-dependant mitochondrial dysfunction in cancer has been studied. In the present study, when DA`s response on Mfrn2 was studied, an induction in both protein and transcript level was found. This may implicate the involvement of mitoferrin2 in inducing DA mediated elevated iron levels.

- Mitochondrion expresses structurally and functionally similar form of cytosolic Ft. Although expression of Mt-Ft is tissue specific and is known to have abundant expression in testes, neurons, spinal cord, cardiomyocytes, kidney cells and islets of langerhans (Santambrogio, P et al, 2007). The expression of Mt-Ft in glial cells is found to be very low while no expression was found in hepatocytes and

splenocytes (Paul, B.T et al, 2017). The over-expression of Mt-Ft induces depletion in cytosolic iron levels leading to increase in IRE activity, increase in transferrin receptor levels and down-regulation of ferritin level. This is also accompanied with increase in mitochondrial iron levels (Nie, G et al, 2005). Hence, induction in Mt-Ft levels seems to enhancing the sequestration of iron in to mitochondria. When DA mediated Mt-Ft expression levels were analysed, no change was observed.

- Since, Mitochondria house the pathway for synthesis of two major iron based co-factors i.e. Heme and Iron sulphur clusters (ISCs). Both the pathways were separately analysed to understand the effect of elevated mitochondrial iron levels on heme and ISC content.

- Disruption in Iron sulphur cluster or biosynthesis machinery have been implicated in brain in several neurodegenerative disorders such as Freidreich`s ataxia and ISCU myopathy. In both these diseases, inability of iron to incorporate in ISC lead to mitochondrial iron overload and dysfunction. The ISC levels were analysed in DA treated C6 cells using quantification of ISC dependant enzyme activity (mitochondrial aconitase and succinate dehydrogenase) or protein levels (Reiske iron sulphur cluster protein). Thus, ISC levels were suppressed by DA. Many reports have shown that PD models having low aconitase and complex I activities along with depleted glutathione levels suggesting suppressed ISC pathway in Parkinson`s disease (Lee, W.D et al, 2009).
- Heme is an indispensable molecule for brain functioning. Heme supports neuronal growth and survival by incorporation in hemoproteins like cytochromes, catalase, neuroglobin. The toxicity associated with excess heme in brain has been extensively studied in haemorrhages. The hypometabolism of heme has been associated in neurodegenerative diseases such as FRDA, AD, Porphyrias, Fowler syndrome (due to defect in heme transporter Feline leukemia virus subgroup C receptor 2, FLVCR2), Retinitis pigmentosa (defect in heme exporter, FLVCR1) (Mormolino, D et al, 2011;Besur, S et al, 2014;Lalonde, E et al, 2010; Yusuf, I.H et al, 2018). Heme level also declines with ageing contributing decreased activity of complex IV and increased iron accumulation (Atamna, H et al, 2004). In the

present study, heme levels were quantified in response to DA in C6 cells and primary astrocytes. Heme content was found to be induced by DA treatment. Heme levels were also analysed by markers for heme pool such as cytochrome C oxidase activity, cytochrome c expression, and compartmental expression of ALAS-1. All suggested increase in heme levels.

- Since heme and iron sulphur cluster biosynthesis takes place in mitochondria, both might share same iron pool. The decreased levels of ISC and increase in heme content in astroglia in response to DA might indicate a metabolic shift. Hence the components for synthesis of both pathways were analysed.

- The ISC synthesis involves assembly of iron donor and sulphur donor proteins on a scaffold protein. ISCU is the scaffold protein on which assembly takes place. Frataxin was initially thought to be the iron donor, is now accepted as kinetic regulator of ISC assembly pathway. Cysteine desulfurase is the sulphur donor for ISC. Iron and Frataxin acts as allosteric modulator for assembly of ISC on ISCU (Tsai, C.L et al, 2010). Both Frataxin and ISCU expression alters in iron deprived condition (Li, K et al, 2008; Tong, W.H et al, 2006). It was found that DA down-regulated Frataxin through transcriptional repression mechanism but did not affect ISCU. Frataxin deficiency has been implicated in FRDA and contributes in mitochondrial iron accumulation and dysfunction (Campuzano, V et al, 1997). Also, DA down-regulates Frataxin in astrocytes only, not in neuronal cells which may suggest cell specific regulation of DA on Frataxin. Such regulation has already been reported for Frataxin in response to Insulin like growth factor, IGF-1 (Franco, C et al, 2012).

- Ferrochelatase, Fchase is the terminal enzyme of heme synthesis pathway and incorporates iron in protoporphrin ring. Fchase protein is known to be destabilised in iron deprived condition (Taketani, S et al, 2000). A study reported transcriptomic analysis of PD patients and found levels of heme metabolising enzyme significantly down-regulated including ferrochelatase (Santiago,J.A et al, 2017). DA mediated expression levels for ferrochelatase was analysed for both protein and mRNA expression and was found to be increased. This finding supports the increase in heme in astrocytes in response to DA.

- DA mediated down-regulation of Frataxin was found to be mediated at transcriptional level. To understand the significance of it, Frataxin was cloned and transiently transfected in C6 cells. It was found that Frataxin over-expression restored the levels of ISC, in response to DA. Also, the level of heme was reversed without affecting ferrochelatase expression or mitochondrial iron content. The over-expression of Frataxin has been studied in different model organisms (Miranda, C.J et al, 2004). In mouse model, Frataxin over-expression affected erythroid differentiation. In drosophila, contradictory findings were reported (Navarro, J.A et al, 2011). The exogenous addition of tat-frataxin in PD model was reported to have neuroprotective role (Kim, M.J et al, 2012). Altogether, the present study suggests that DA may be regulating Frataxin expression in astrocytes to influence heme levels.

- It has been reported that DA mediates transcriptional induction of heme oxygenase-1 in astroglial cell (Schipper,H.M et al, 1999; Schmidt, J et al, 1999). Heme oxygenase- 1 is a heme catabolising enzyme but because of anti-oxidant property of heme breakdown products, induction of HO-1 is considered as neuroprotective. Studies have shown increased expression of heme oxygenase-1 in ageing. (Hirose, W et al, 2003). HO-1 levels are also shown to be upregulated in PD (Schipper, H.M et al, 2000), Schizophrenia (Song,W et al, 2012) and ADHD (Di Tommaso, M.C et al, 2012). One of theimplications of DA-induced heme level may be to provide heme for HO-1 catabolising activity for continuous supply of antioxidants.

In conclusion, the present study reveals an insight on unexplored mechanisms that take place in astrocytes in response to DA. While dopamine biosynthesis takes place in neurons, astrocytes are majorly responsible for DA metabolism in synaptic regions. While DA induces the expression of HO-1 and increases the levels of mitochondrial iron, the present study gives an insight on fate of elevated iron level in mitochondria in response to dopamine and reveals a molecular mechanism to induce heme level and to suppress ISC content by regulating Frataxin.

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- **Singh. P**, Mukhopadhyay, C.K Dopamine favours heme synthesis by regulating Frataxin in Astrocytes (Manuscript under preparation)

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- Dev, S. **Singh, P***, Mukhopadhyay, C.K. Dopamine promotes ferritin degradation in astroglial cells in International conference on neuroscience and 36th annual meeting of Indian academy of neuroscience, 2018 at Banaras Hindu university, Banaras, India

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Phosphoinositide-3-kinase inhibition elevates ferritin level resulting depletion of labile iron pool and blocking of glioma cell proliferation

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ABSTRACT

Background: Elevated endogenous phosphoinositide-3-kinase (PI3K) activity is critical for cell proliferation in gliomas. Iron availability is one of the essential factors for cell growth and proliferation. However, any relation between PI3K and cellular iron homeostasis has not been understood so far.

Methods: Glioma cells and human primary astrocytes were treated with class I PI3K inhibitors to examine regulation of iron homeostasis components. Regulation of ferritin was detected at mRNA and translational level. Labile iron pool (LIP) and cell proliferation were examined in glioma cells and human primary astrocytes.

Results: Blocking of PI3K activity elevated ferritin level by 6–10 folds in glioma cells by augmenting mRNA expression of ferritin subunits and also by influencing ferritin translation. IRE-IRP interaction was affected due to conversion of IRP1 to cytosolic aconitase that was influenced by increased iron-sulfur scaffold protein iron-sulfur cluster assembly enzyme (ISCU) level. Elevated ferritin sequestered LIP to affect cell proliferation that was reversed in silencing ferritin by siRNAs of ferritin-H and ISCU. Human primary astrocyte with little PI3K activity did not show any change in ferritin level, LIP and cell proliferation by PI3K inhibitors.

Conclusions: PI3K inhibition promotes ferritin synthesis by dual mechanism resulting sequestration of iron to limit its availability for cell proliferation in glioma cells but not in primary astrocytes.

General Significance: This observation establishes a relation between PI3K signalling and iron homeostasis in glioma cells. It also implies that activated PI3K controls ferritin expression to ensure availability of adequate iron required for cell proliferation.

1. Introduction

The phosphoinositide-3-kinase family of genes encodes lipid and protein kinases to regulate multiple cellular processes including cell survival, cell proliferation, cell cycle progression, angiogenesis, invasion and metastasis [1–3]. PI3Ks are divided into three classes based on their structure and substrate specificity [4]. The class I PI3Ks phosphorylate and activate Akt (also known as PKB) to participate in cell signalling pathways involved in cell proliferation and several other important cellular mechanisms [5,6]. Class IA PI3Ks are diverse in mammals as they have three catalytic p110 isoforms (p110α, p110β, and p110δ; each encoded by a separate gene) and seven regulatory adaptor proteins. The p110γ is the sole class IB PI3K and differs from class IA enzymes in the N-terminus end (lacking p85 binding site) with a p101 or p84 regulatory subunit [7]. Class I PI3Ks are major focus of research as they are coupled to extracellular stimuli and involved in a wide range of cellular processes [8–10]. PI3K phosphorylates the 3'-OH position of the inositol ring of inositol phospholipids to generate phosphatidylinositol-3-phosphate (PI-3-P), phosphatidylinositol-3,4-bisphosphate (PI-3,4-P2), and phosphatidylinositol-3,4,5-trisphosphate (PI-3,4,5-P3) [11]. Phosphorylation and activation of Akt (PKB), a serine/threonine kinase, the key mediator of signalling downstream of PI3 kinase, is principally dependent on the production of PIP3 [12].

Glioblastoma multiforme (GBM) is the most common primary tumour of the central nervous system in adults [13] with limited patient survival and is considered to be among the most lethal cancers [14].

Abbreviations: BPS, Bathophenanthrolinedisulfonic acid disodium salt hydrate; DFO, Deferrioxamine; DMT1, Divalent Metal Ion Transporter; EMSA, Electrophoretic mobility shift assay; FAC, Ferric ammonium citrate; Ft, Ferritin; FPN, Ferroportin; ISCU, Iron-sulfur cluster assembly enzyme; IRE, Iron Response Element; IRPs, Iron Regulatory Proteins; LIP, Labile iron pool; PCBP, Poly-r (C) binding protein; PI3K, Phosphoinositide 3-kinase; PI, Propidium Iodide; qRT-PCR, Quantitative Real time PCR; SDS-PAGE, Sodium dodecyl sulfate-polyacrylamide gel electrophoresis; UTR, Untranslated region; XTT, 2,3-bis [2-Methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxanilide inner salt

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