Role of Protein S-Nitrosylation in Unfolded Protein Response during Endoplasmic Reticulum Stress in Brain

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Azka Khan

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List of Abbreviations

%	Percentage
ºC	Degree Celsius
Aβ	Amyloid beta
AD	Alzheimer's Disease
PD	Parkinson's Disease
ER	Endoplasmic Reticulum
APP	Amyloid precursor protein
CNS	Central nervous system
HCl	Hydrochloric acid
H_2O_2	Hydrogen peroxide
IRB	Internal Review Board
Kg	Kilogram
DTT	Dithiothreitol
IRE1	Inositol Requiring Endonuclease 1
BiP	Binding immunoglobulin Protein
PERK	PKR-like ER Kinase
ATF6	Activating Transcription Factor 6
PDI	Protein Disulfide Isomerase
TM	Tunicamycin
UPR	Unfolded Protein Response
ERAD	Endoplasmic Reticulum Associated Degradation
mg	Milligram
I.P.	Intraperitoneal
MS/MS	Tandem Mass spectrometry
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
NO	Nitric Oxide

ROS/RNS	Reactive oxygen species/ Reactive nitrogen species
SNO	S-nitrosothiol
PPI	Protein-Protein Interactions
GO	Gene Ontology
GPS-SNO	Group-Based Prediction System for SNO
STRING	Search Tool for the Retrieval of Interacting Genes/Proteins
KEGG	Kyoto Encyclopedia of Genes and Genomes
rpm	Rotations per minute
µg	Microgram
ml	Microliter
µg	Microgram

Abstract

Abnormalities in endoplasmic reticulum (ER) homeostasis cause ER stress which initiates unfolded protein response (UPR). Aberrant protein S-nitrosylation and failure in UPR activation has shown be underlying causes of neurodegeneration, however exact molecular and cellular mechanism still remain elusive. Therefore, the present study elucidates the relationship between the unfolded protein response (UPR) during ER stress and aberrant S-nitrosylation levels that ultimately results in neuronal toxicity leading to neurodegeneration.

The experimental animals, Balb/c mice were divided into 2 groups (n=8, each). Dithiothreitol (DTT) was used to induce UPR following ER stress. An optimum dose of DTT (75 mg/kg) was administered after every 24hr for three consecutive days. Histological and immunohistochemical examination showed clear signs of neurodegeneration and increased S-nitrosocystine antibody reactivity in cortex and hippocampal sections of mice brain respectively, along with atrophied neuronal morphology, following DDT exposure. Data of differentially expressed proteins during ER stress from ESI-QTOFMS/MS was used to perform *in silico* analysis to predict the plausible S-nitrosylation sites in proteomic data set. Many plausible S-nitrocystine sites were predicted via Group-based Prediction System-S-nitrosothiols (GPS-SNO 1.0.) while protein-protein interactions of proteomic data set showed interactions with some important proteins involved in signaling cascades associated with neurodegeneration.

In conclusion, the findings of the present work may contribute to the existing pool of knowledge and provide a better understanding of complex molecular association of aberrant S-nitrosylation of proteins in neurodegeneration. This may help in further elucidation of plausible aberrant molecular/signaling pathways during ER stress which may lead to neurodegeneration.

Introduction

Chapter 1

Introduction

Neurodegenerative disorders, such as Alzheimer's disease (AD), Parkinson's disease (PD), Huntington's disease (HD) and amyotrophic lateral sclerosis (ALS), have been posing a substantial burden on public health worldwide. These disorders are commonly associated with a progressive declining of neuronal and synaptic functions and subsequent neuronal cell death. Characteristic pathological features of neurodegenerative diseases are aggregation of misfolded proteins (e.g., α-synuclein in PD and amyloid-β in AD), excessive oxidative and nitrosatve stress followed by mitochondria dysfunction and synaptic damage with reduced span of neuronal survival (Taylor et al., 2002). However, exact cellular and molecular events underlying the phenomena of neurodegeneration still remain enigmatic and needs to be investigated. During past decade, growing evidences have shown that in addition to oxidative stress nitrosative stress remarkably contributes in manifestation of pathological mechanisms involved in neurodegeneration. Signaling cascades mediating nitrosative stress may appear to be a common determinant mediating pathogenesis and prognosis of neurodegenerative disorders (Nakamura and Lipton, 2007).

Accumulation of misfolded proteins is one of the hallmarks for neurodegenerative diseases which adversely influence neuronal connectivity and synaptic plasticity, consequently activating the apoptotic signaling cascades (Gu, Z, 2010). Degenerating brain of AD shows intracellular neurofibrillary tangles containing accumulation of hyperphosphorylated tau and extracellular plaques containing aberrantly misfolded β-amyloid proteins (Pereira et al., 2005). Moreover, inclusion known as Lewy bodies mostly found in cytoplasm of neurons in the brain of PD are composed of misfolded and aggregated synuclein and synphilin. These aggregates usually entail oligomeric complexes with non-native secondary protein structures and exhibit low solubility in aqueous as well as detergent solvent (Alvarez-Castelao and Castaño, 2011). Huntington's disease, amyotrophic lateral sclerosis, and prion disease belong to the category of neurodegenerative diseases manifesting protein aggregation. Group of aforementioned diseases are also known as

conformational diseases due to the relevance of protein aggregation and neurodegeneration in these disorders (Carrell and Lomas 2002).

Anomalies in accurate protein folding in pathology of neurodegenerative disorders usually occur due to some rare mutations in genes encoding protein associated with disorder. Furthermore, aberration in protein folding also occurs due to conformational changes in protein structure after posttranslational modification induced by oxidative and nitrosative stress (Gu et al., 2010). During normal physiological conditions, very low levels of reactive oxygen/nitrogen species (ROS/RNS) are normally generated in most of the mammalian cells. ROS/RNS function as important physiological messengers of various cellular signaling pathways. Nevertheless exposure of certain environmental toxic agents or even during the normal process of brain aging can lead to an overabundance of ROS/RNS (Yao et al., 2004). Usually increased levels of ROS/RNS occur due to imbalance of their production and counteracting mechanisms which normalize their level such as antioxidant enzymes, glutathione and molecular chaperones. Typically, neurons are extremely susceptible to oxidative as well as nitrosative stress because they possess fewer levels of many antioxidants as compared to the other cells and high energy demand from ROS/RNS producing mitochondrial metabolism (Mattson et al., 2002).

During neurodegenerative disorders the accumulation of immature or misfolded proteins leads to excessive ER stress, failure of unfolded protein response (UPR) and ultimately neuronal death. Protein misfolding/unfolding induces ER stress which stimulates a critical intracellular response UPR which reduces the ER stress and restores its function (Lindholm et al., 2006). UPR activates the expression of specific chaperones proteins which prevent the abnormal aggregation of other misfolded/unfolded proteins implicated in general protein folding, post translational protein complexes, and proteomic degradation. UPR initiates downstream signaling cascades which are believed to be involved in maintenance of the cellular homeostasis to withstand the fluctuations in environmental conditions. Moreover, UPR activation results in attenuation of global translation by triggering the signaling cascades featuring eukaryotic initiation factor (eIF2) kinase (Ron and Walter, 2007).

The lumen of ER is the major site where nascent and secretory proteins are folded and processed and transferred to Golgi bodies and then exported to the target sites. Excessive protein folding burdens ER function which leads to activation of UPR, however under severe ER stress UPR fails to function properly and induces apoptosis via an ER-specific caspase (Holcik and Sonenberg, 2005). ER has ability to withstand relatively mild stress by manipulating the expression of stress transducers which behave as molecular chaperones such as glucose regulated protein (GRP) and protein disulphide isomerase (PDI) (Wilkinson and Gilbert 2004).

Likewise, PDI serves as catalyst in a thiol/disulfide exchange including reactions formation and rearrangement of disulfide bond on target protein during protein folding and processing in ER. PDI constitutes two predominant domains with great homology to small redox-active protein thioredoxin (TRX) which functions as independent active sites (Wang et al., 2013). Notably ER and UPR dysfunction has resulted due to accumulation of misfolded/unfoled proteins during neurodegenerative diseases. Expression of GRP, PDI, and other ER chaperones has shown to contribute significantly in cellular performance and survival under sever ER stress condition (Uehara et al., 2006). Therefore, PDI may be the key player which functions in activation of adaptive responses during cellular stress, which ultimately lead to increased neuronal survival.

In consistent with the nitrosative stress, aberrant S-Nitrosylation (a post translation modification) have been appeared to be one for factor involved during pathology of various neurodegenerative diseases. S-nitrosylation is a reversible covalent chemical reaction in which an NO moiety specifically combines with a cysteine thiol (-SH) group (or more accurately thiolate anion, $-S^-$) on the target protein in order to regulate its function. This is a nitrosation reaction which attaches S-nitrosothiol (-SNO) group to the proteins, and thus S-nitrosylated proteins are referred to as SNO-proteins. S-nitrosylation of cysteine thiol is an entirely different redox reaction from the nitration of tyrosine residues which represent another important NO-dependent post-translational modification reaction where reaction of tyrosine with peroxynitrite ($ONOO^-$) occurs (Halloran, M et al., 2013).

During normal physiological conditions, S-nitrosylation plays a dynamic role in a number of biological processes by modulating the functions of substrate proteins. Like other posttranslational

modifications, S-nitrosylation triggers conformational changes in protein structures, regulate protein activity, change protein-protein interactions, and affect localization and aggregation of proteins as well. Such mechanisms subsequently influence overall cellular signaling transduction cascades involved in neuronal function (Shi et al., 2013).

On the other hand, under some pathological conditions, aberrantion in S-nitrosylation of specific proteins activates such mechanisms which induce severe cellular stress followed by cell death, hence amplifying the process of neurodegeneration. Aberrant S-nitrosylation disrupts many intracellular phenomena including protein misfolding, ER stress, mitochondrial dysfunction, synaptic loss, and apoptosis (Nakamura and Lipton, 2007). Nitrosative stress-linked excitotoxicity seems to be implicated in a variety of neurological disorders, which range from acute cerebral ischemia to chronic neurodegenerative disorders. Alteration in rate of S-nitrosylation has shown to affect neural pathology because neuroprotection has been observed by pharmacological nNOS inhibition or by using nNOS gene knockouts in neurological disorders (Huang et al., 1994).

For in vitro studies, UPR is commonly activated upon the treatment of model with various ER stress agents such as tunicamycin (TM) or Dithithreitol (DTT). Tunicamycin induces ER stress by hampering the exchange of oligosaccharides with nascent ER resident proteins (Hoyer-Hansen and Jaattela, 2007). Dithithreitol, C4H10O2S2 (DTT) is the commonly known as Cleland's reagent, a strong reducing agent which undergoes oxidation reaction, conforming into a stable six-membered ring, with a core disulphide bond (S=S) (Cleland, 1964). DTT acts by disrupting the redox reactions necessary for the development of disulfide bridges of newly synthesized proteins in the cytosol.

Likewise, DTT initiates ER stress by disturbing the redox conditions which are prerequisite for the formation of disulfide bridges in proteins quacking leading to ER stress (Ryoo et al., 2007). Both TM and DTT bring about misfolded protein conformations accumulating in ER lumen, they are essential intermediaries for the characteristic natural conditions which initiate UPR in cells as well. The current study proposes to observe the role of S-nitrosylation in brain of mouse model exhibiting ER stress induced by DTT by using advance proteomics approaches.

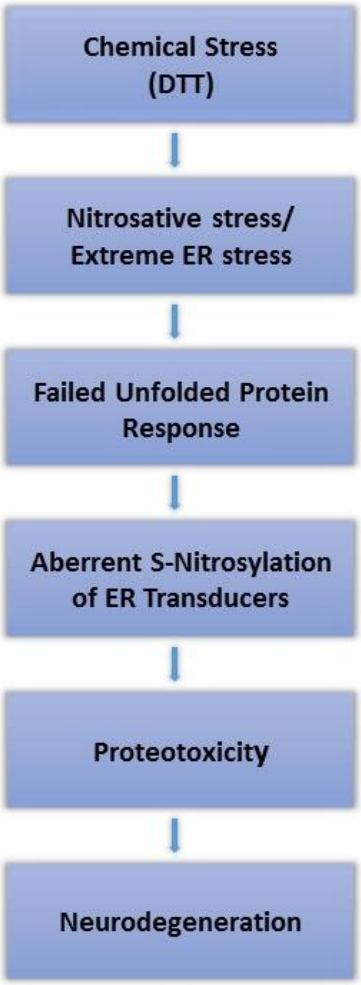


Figure 1.1: Mechanistic representation of chemically induced ER stress and aberrant S-nitrosylation leading to neurodegeneration. Extracellular environmental and chemical as well as aberrant S-nitrosylation causes neuronal proteotoxicity in the mammalian brain, which consequently leads to neurodegeneration**.**

1.1 Research Objectives

Following are the objectives of the study:

- To investigate the role of S-nitrosylation during ER stress induced by DTT in mice model in comparison with controls.
- To evaluate the extent of neuronal cell death in mouse model exhibiting ER stress induced by DTT.
- To observe the S-nitrosylation levels of total brain proteome of mouse model during ER stress in comparison to controls.
- To conduct *in silico* analysis in order to predict the S- nitrosylation sites in differentially expressed protein during ER stress induced by DTT.
- To investigates the protein-protein interactions of differentially expressed proteins with S-nitrosylation sites in human and mouse signaling pathways involved in neurodegeneration

Literature Review

Chapter 2

Literature Review

Neurodegenerative disorders like Alzheimer's disease (AD), Parkinson's disease (PD), prion disease, Huntington's disease (HD), frontotemporal dementia (FTD), and amyotrophic lateral sclerosis (ALS) shared a common pathological feature of intracellular as well as extracellular accumulation of misfolded proteins. The aggregates of misfolded proteins are however, found in different regions of the brain exclusive to each neurodegenerative disease. The principal site for protein synthesis is the endoplasmic reticulum (ER), where secretory, transmembrane and organelle-targeted proteins are produced and processed, covering nearly 30% of the overall proteome. The fundamental component of protein quality control in ER is the unfolded protein response (UPR) which ensures the ER homeostasis, the most important factor in play, if the proteostasis in ER is compromised (Scheper and Hoozemans, 2015).

2.1 Endoplasmic Reticulum Stress and Unfolded Protein Response UPR

Endoplasmic reticulum (ER) is a specialized organelle responsible for synthesis, folding and processing, post translation modifications and transport of nascent proteins to their target sites. Along with protein folding, ER plays a crucial role in cell survival by maintaining calcium and redox hemostasis. The lumen of the ER serves as the central site for its functions as it harbors many important molecular chaperones and protein folding enzymes including Grp78 (BiP), Grp94 and protein disulfide isomerase (PDI) (Roussel et al., 2013).

ER exerts a quality control mechanism which ensures the export of only properly folded proteins to Golgi organelle. While incompletely folded or misfolded proteins reside in ER where they are either properly folded or they are transported to cytosol for degradation by the process of endoplasmic reticulum–associated degradation (ERAD). Protein load and folding capacity of ER remain in equilibrium in normal physiologic conditions however high protein

load, buildup of misfolded proteins, or disturbance in calcium or redox hemostasis result in perturbed ER function, a condition termed as ER stress (Maly and Papa, 2014).

Mammalian cells have developed an adaptive signaling pathway called unfolded protein response which ameliorates ER stress and restores the ER hemostasis back to its equilibrium state. Initially UPR reinstates ER hemostasis by increasing the expression of protein-folding chaperones which enhances the protein folding capacity and by decreasing the protein load in ER by inhibition of general protein translation and degradation of misfolded/unfolded proteins (Zhang and Kaufman, 2006).

During many pathological conditions, UPR fails to alleviate ER stress and activates apoptotic signaling cascade resulting in programmed cell death. Nevertheless, perturbations in ER function and chronic ER stress have been linked with a number of pathologies ranging from diabetes and neurodegenerative disorders to cancer and inflammatory diseases. UPR-mediated cell death has shown to contribute to pathogenesis of both AD and PD (Hoozemans et al., 2005).

2.2 The Unfolded Protein Response (UPR) Effector Proteins

Inositol-requiring protein-1 (IRE1), protein kinase RNA-like ER kinase (PERK), and activating transcription factor 6 (ATF6) are key players of which sense ER stress. These are ER-localized sensor proteins which upon sensing ER stress perform function after dissociation of ER-resident chaperone glucose-regulated protein (GRP78), also known as immunoglobulin heavy chain–binding protein (BiP) from their ER luminal domains and activate ER-to-nucleus signaling cascades to balance ER homeostasis (Okada et al., 2002).

2.2.1 Inositol-requiring protein-1 (IRE1)

IRE1 is the most evolutionarily conserved sensor of ER stress, which detects the stress signal, dissociates from BiP and finally performs its function by transautophosphorylation. Directly binding of IRE1 to unfolded/misfolded proteins has shown to be one of the mechanisms responsible for UPR activation (Chen and Brandizzi 2013). IRE1 constitutes a cytoplasmic

endoribonuclease domain which activates X-box binding protein–1 (XBP1) by selectively splicing 26-base intron from mRNA encoding XBP1 and thus inducing a more efficient translation. XBP1 is a transcription factor responsible for expression of many genes associated with UPR and its downstream pathways such as ER-associated degradation pathway (ERAD) which reduce client load on the ER (Yoshida et al., 2001).

Other mechanisms lower ER stress by increasing the expression of several ER resident chaperones, such as GRP78, expanding ER membrane surface area, hence ER folding capacity. Other than activation of XBP1s, IRE1 has been shown to be crucial for splicing and post-transcriptional degradation of mRNAs encoding other necessary proteins which may be accountable for decreasing the unfolded/misfolded protein load ER (Sriburi et al., 2004).
IRE1 also possesses an intrinsic kinase activity which appears to be involved in activation of signaling cascades leading to apoptosis. Activated IRE1 binds the TNF receptor–associated factor–2 (TRAF2) an adaptor protein, which through activation of apoptosis signal–regulating kinase–1 (ASK1) initiates c-Jun N-terminal kinase (JNK) pathway (Urano et al., 2000). The intrinsic kinase activity has been reported to be critical for regulation its nuclease function, however the exact mechanisms still remain elusive.

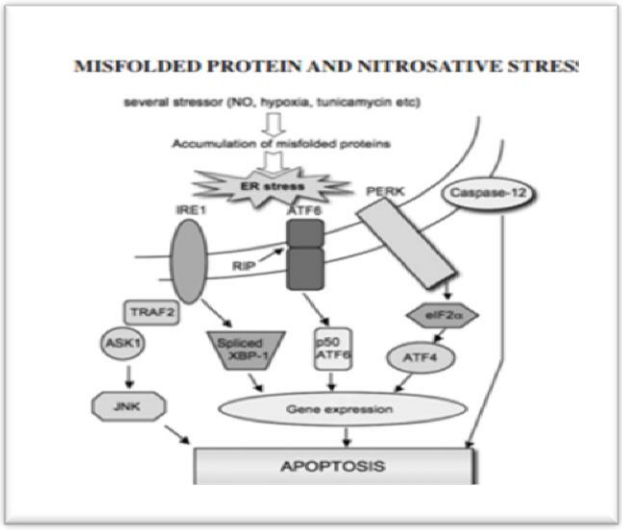


Fig 2.1: Misfolded protein and nitrosative stress: Stresses such as NO, hypoxia or chemical agents induce ER stress by causing accumulation of misfolded proteins. Persistent ER stress stimulates activation of various apoptotic signaling cascades which ultimately lead to cell death (Urano et al., 2000).

2.2.2 Protein kinase RNA-like ER kinase (PERK)

PERK is a serine threonine which constitutes luminal ER-stress-sensing domain and similar to IRE1, it is also activated through transautophosphorylation and dimerization. Upon ER stress, activated PERK inactivates the α subunit of eukaryotic translation initiation factor 2 alpha (eIF2α) by phosphorylating it at Ser51 (prerequisite for 80S ribosome assembly), which results in attenuation of overall protein translation rate through a decrease in GTP-bound form of eIF2α, thus reducing ER protein load (Harding et al., 2000).

Phosphorylation of eIF2α has also shown to trigger the translation of activating transcription factor–4 (ATF4), which promptly enhances the transcription of many UPR effector proteins such as CAAT/enhancer binding protein homologous protein (CHOP) and DNA damage-inducible protein-34 (GADD34). Through negative feedback GADD34 dephosphorylates eIF2α which efficiently perform its role UPR and cellular growth (Kouroku et al., 2007).

2.2.3 Activating transcription factor 6 (ATF6)

ATF6, a basic leucine zipper transcription factor commences another important UPR pathway upon sensing ER stress signals. Upon activation, ATF6 translocates to the Golgi complex where site 1 and site 2 proteases cleave it to generate a transcription factor which is responsible for upregulation of genes encoding various ER chaperones involved in amplification of ER folding capacity such as Grp78, protein disulfide isomerases (PDI), CHOP and XBP-1 (Shen et al., (2002).

2.2.4 Dysfunction of Unfolded Protein Response (UPR) effectors protein

During prolonged and severe ER stress, UPR fails to restore ER hemostasis which triggers apoptotic cascades resulting in cell death. In some cell types, activated IRE1 has also been shown to trigger the recruitment of a pro-apoptotic ER-resident cysteine protease, caspase 12 (in murine which is homologue caspase-4 in humans) (Szegezdi et al., 2003)

During persistent ER stress, caspase 12 along with prolonged increased CHOP expression induce apoptotic cell death by JNK pathway. Under normal physiological condition, CHOP

performs its function by blocking G1 to S phase transition from during cell cycle progression. Prolonged and upregulated transcription of CHOP in pathological conditions found to be triggering apoptosis through various signaling cascades (Schröder, and Kaufman, 2005).
Noticeable cascades include activation of ER oxidase–1α, a CHOP transcriptional target and GADD34 which increases oxidation reactions at the ER and translocation of Bcl associated X protein (Bax) to mitochondria due to downregulation of B cell lymphoma-2 (Bcl-2), along with subsequent release of cytochrome c, hence activation of apoptosis-effector caspase-3, resulting in cell death (Sano and Reed, 2013). As ER and mitochondria lie in close proximity and in normal conditions Ca2+ released from ER is taken up by mitochondria to increase ATP production. However, during ER stress excessive Ca2+ release from ER and overload in Ca2+ uptake by mitochondria accompanied by apoptotic stimuli could induce release of cytochrome c and other caspase cofactors resulting in programmed cell death (Pinton et al., 2008).

2.3 S-nitrosylation and UPR Effector Proteins

2.3.1 Protein Disulphide Isomerase

S-nitrosylation of PDI is observed in a NOS dependent manner when NMDA is used to induce Ca+ influx and NO production in primary cultures of rat cerebrocortical neurons. This S-nitrosylation of PDI is also induced when such neuronal cultures are exposed to calcium ionophore and rotenone, a complex I inhibitor molecule in mitochondria. PDI is present in the lumen of endoplasmic reticulum which increases in its proportion in many stress conditions. (Tanaka et al., 2000).

In many cases it is found that this increased in the expression of PDI, particularly the wild-type PDI protects against many stress conditions involving ischemia and hypoxia especially when treated with NMDA or any other ER stressors such as tunicamycin, thapsigargin, which are basically the inhibitors of glycosylation and Ca+ ATPase in ER, respectively. Moreover, similar protective effect is also observed by overexpression Pael receptors or by reducing neurons which are positive for polyunbiquitin (Lipton and Nicotera, 1998). It has been found that in contrast to patients with peripheral disorders, significant S-nitrosylation of PDI is found

in the tissue samples of brain taken from the patients suffering from either Alzheimer or sporadic Parkinson disease (Lipton et al., 2006).

S-nitrosylation of the PDI is observed particularly with in the cysteine residues of its thioredoxin like domains. S-nitrosylation of endogenously present PDI is also known to inhibit its isomerase and chaperone like activity which may result in the formation of impaired pathological structures called Lewy bodies which are considered one of the hallmarks in Parkinson disease due to their ability to accumulate aberrantly ubiquinated synphilin-1 and other related protein structures with in the cells. NO might play a detrimental role by reversing the preventive role of over expressed PDI against the intracellular formation of unbiquitinated aggregates of synphilin-1 and this leads to accumulation of aberrant proteins and neuronal cell death as seen in the case of Parkinson (Uehara et al., 2006).

PDI is therefore a potential and novel therapeutic target for several neurodegenerative disorders involving protein aggregates. In this regard, explicitly understanding NO-mediated pathways and their role in apoptosis of neurons is as important to discover novel therapeutic approaches towards neurodegenerative disorders dominantly caused by nitrosative and oxidative stresses. Perhaps, a therapeutic approach to abrogate S-nitrosylation of PDI, induce over expression of PDI and increase its enzymatic activity and suppress NO production would be desirable (Foster et al., 2009).

Moreover, as NO is known to play a crucial role in several neurodegenerative disorders, that is, Alzheimer's, Huntington's, ALS, and several forms of strokes, it is also important to determine the role of NO induced PDI dysfunction and its implications in protein aggregates accumulation and neurons death (Dawson et al.,1991).

It has been found that in contrast to patients with peripheral disorders, significant S-nitrosylation of PDI is found in the tissue samples of brain taken from the patients suffering from either Alzheimer or sporadic Parkinson disease. S-nitrosylation of PDI is also observed in a NOS dependent manner when NMDA is used to induce Ca+ influx and NO production in primary cultures of rat cerebrocortical neurons. This S-nitrosylation of PDI is also induced

when such neuronal cultures are exposed to calcium ionophore and rotenone, a complex I inhibitor molecule in mitochondria (Choi et al., 2000). This S-nitrosylation of the PDI is observed particularly with in the cysteine residues of its thioredoxin like domains.

S-nitrosylation of endogenously present PDI thus inhibits its isomerase and chaperone like activity which may result in the formation of impaired pathological structures called Lewy bodies which are considered one of the hallmark in Parkinson and Alzheimer due to their ability to accumulate aberrant ubiquinated synphilin-1 and other related protein structures with in the cells (Chung et al., 2001). These S-nitrosylation induced loss of PDI functions results in both accumulations of aberrant (unfolded) proteins and induction of UPR related events such as pathway involving IRE1-XBI and chop mRNA which can induce apoptosis in the presence of sever ER stressors as UPR is ER specific signal transduction pathway involves in cellular homeostasis during imbalance in environmental ques (Ko et al., 2002). Although, the exact role of PDI in pathophysiology of neurodegenerative disorders such as its role in neuronal death and protein accumulation is unknown, there are increasing evidence to hint its possible role in accumulation of misfolded aggregates of protein in ER and its subsequent link to Parkinson disease (Yao et al., 2002).

There is also a possibility that PDI might be a potential target of NO mediated upregulation of glutamate release in the case of brain ischemia or mitochondrial injury as seen in Parkinson Disease. NO might play a detrimental role by reversing the preventive role of over expressed PDI against the intracellular formation of unbiquitinated aggregates of synphilin-1 and thus leading to accumulation of aberrant protiens and neuronal cell death as seen in the case of Parkinson (Nakamura and Lipton 2009).

Interestingly, PDI is present in the lumen of endoplasmic reticulum which increases in its proportion in many stress conditions (Mori, 2000). For instance, in many cases it is found that this increase in the expression of PDI, particularly the wild-type PDI protects against many stress conditions involving ischemia and hypoxia especially when treated with NMDA or any other ER Stressors such as tunicamycin, thapsigargin, which are basically the inhibitors of

glycosylation and Ca+ ATPase in ER, respectively. Moreover, similar protective effect is also observed by overexpression Pael receptors (Pael-R) or by reducing neurons which are positive for polyunbiquitin (Larsen et al., 2001).

S-nitrosylation primarily disturbs the ER hemostasis by inhibiting the activity protein disulfide isomerase (PDI), belongs to family of ER chaperones and through thiol–disulfide exchange it carries out the process of protein folding. Due to important role of in protein synthesis and maturation, PDI contributes a major role in cell defense system and increased expression of PDI helps in attenuating burden of misfolded protein, maintaining normal proteostasis hence, decreasing ER stress. S-Nitrosylation of PDI (SNO-PDI) impairs ER stress which leads to the level of ubiquitinated proteins and subsequent neuronal cell death in many neurodegenerative disorders (Obukuro et al., 2013).

Initially formation of SNO-PDI has found to be significantly increased in the brains of patient with AD and PD (Uehara et al., 2006). Environmental toxin such as rotenone, believed to induce Parkinson's-like symptoms, promotes SNO-PDI formation in cell-based models which suggests that accumulation of misfolded proteins and severe ER stress may occur duo to aberrantly S-nitrosylated PDI. Compromised functioning of PDI thus leads to accumulation of misfolded α-synuclein and synphilin proteins which ultimately activate the apoptotic signaling cascades via activation of UPR, resulting in neuronal death (Walker et al., 2010). If the level of misfolded/unfolded proteins remains high UPR fail to maintain ER hemostasis leading to apoptosis, however PDI but not SNO-PDI, can efficiently reduce proteotoxicity and repair proteasome dysfunction and persistent UPR (Uehara et al., 2006).

S-nitrosylated PDI fails to protect neurons from accumulation of mutant or misfolded superoxide dismutase 1 (SOD1) in experimental models of familial ALS. Normally wild type SOD1 protein structure is homodimer and a highly conserved intra-molecular disulfide bonds stabilized its structure. Contrary to wildtype structure, mutant SOD1 in familial ALS forms either monomers with less number of disulfide bonds or multimers of poor solubility with excessive disulfide bonds. Therefore, partial mislocalization of mutant SOD1 at ER results in its

aggregation in intracellular inclusions thus contributing to neuronal death (Wang et al., 2006). Upregulation of PDI causes decline in mutant SOD1 aggregation, less inclusion formation and cell death whereas PDI knockdowns escalates mutant SOD1 level and inclusion formation, strengthening the concept of PDI as a neuroprotective chaperone (Walker et al., 2010).

PDI is therefore a potential and novel therapeutic target in several neurodegenerative disorders involving protein aggregates. In this regard, explicitly understanding NO-mediated pathways and their role in apoptosis of neurons is also as important to discover novel therapeutic approaches towards neurodegenerative disorders dominantly caused by nitrosative and oxidative stresses (Uehara et al., 2006). Therapeutic agents which inhibit S-nitrosylation of PDI could potentially be used to reduce severe ER stress linked protein misfolding and neuronal death. Moreover, aberrant S-nitrosylation of PDI occurs only in neurodegeneration related events, SNO-PDI presents to be a biomarker specific to several neurodegenerative diseases (Nakamura and Lipton 2008).

S-Nitrosylation of PDI disturbs the neuroprotective effects of PDI due to accumulation of mutant SOD1 aggregates. In neuronal cell culture models, mutant SOD1 enhances the aggregation by upregulating the expression of iNOS and stimulates SNO-PDI formation. However, treating such neuronal cell lines with Nω-nitro-L-arginine (a broad spectrum NOS inhibitor) reduces the SNO-PDI-linked cell damage (Chen et al., 2013). SNO-PDI has been observed in the spinal cords of experimental animals modeling ALS and stroke and in lumbar segments of spinal cord in the human patients of sporadic ALS (Walker et al., 2010).

2.3.2 Parkin

Parkin is a ligase protein belonging to the class of E3 ubiquitin which is involved in ubiquitin-protease system. This system degrades specific tagged proteins (Shimura et al., 2000). Parkin also participates in ER-associated degradation (ERAD) by degrading protein during ER stress through its interaction with several (Hsp)70 heat shock proteins, chaperones, and carboxyl-terminus of Hsp70 interacting protein (CHIP) (Wang and Takahashi, 2007) as perturbing the normal structure of protein disrupt its protein degradation property which results in the

aggregate formation of neurotoxic protein and its accumulation which inevitably causes ER stress (Dawson and Dawson, 2003; Lindholm et al., 2006). Moreover, it has also been demonstrated in several studies that neurotoxins such as MPTP and pesticides such as rotenone causes s-nitrosylation of parkin (Chung et al., 2004). S-nitrosylation subsequently leads to diminish expression of E3 ligase activity of Parkin, possibly due to the phenomenon known as autoubiquitination. As a result of the loss of E3 ligase activity protein degradation system is impaired. It thus plays a vital role in Lewy body formations and subsequent neuronal cell death as seen in many neurodegenerative disorders (Yao et al., 2004).

Several neurotoxic environmental factors such as pesticides and herbicides can also disrupt normal functioning of parkin in protein degradation possibly by producing oxidative stress via over production of NO and ROS. High level of these NO and ROS are reported in several studies which can destroy parkin function by its excessive s-nitrosylation or oxidation (Meng et al., 2011). Multiple cysteine residues of parkin react with NO to produce a complex called SNO-parkin. Such SNO-parkin are found to be significantly over expressed in the brains of patients suffering from sporadic PD. More-over these s-nitrosylated parkin are further confirmed by animal model of PD in several studies (Chung et al., 2004).

2.4 Chemical Induction of Unfolded Protein Response (UPR)

(DTT) has been established as chemical agent for induction of UPR. DTT is a commonly used laboratory reagent known as Cleland's Reagent where it is generally used to denature proteins in techniques like SDS-PAGE. However, recently it has been used to induce the UPR in various cell lines, C. elegans and zebrafish. Dithiothreitol is a reducing agent and reduces the disulphide bridges by causing thiol formation between cysteine residues, which causes protein build up in the endoplasmic reticulum, which leads to the initiation of UPR. DTT has been utilized as an ER stress inducer in numerous cell lines; Mouse Embryonic Fibroblasts, multiple myeloma cell line, β cell line, MIN6, rat β cell lines and human pancreatic β cells and plasma cells (Miyazaki et al., 1990; Asfari et al., 1992; Hohmeier et al., 2000; Schindler and Schekman, 2009; Chen et al., 2011; Schuiki et al., 2012; Gao et al., 2014). Furthermore, recently DTT has also been used to induce stress in a transgenic zebrafish model (Li et al., 2015).

The ER and activation of UPR can be induced in all cell types by various other chemicals as well. Tunicamycin and thapsigargin have been known as UPR activating agents corresponding to the mode of action on living cells (Oslowskia and Urano, 2011). Tunicamycin functions by inhibiting N-linked glycosylation of nascent proteins in ER and developing cellular stress including ER stress and finally causes to cell cycle arrest (Chan and Egan, 2005). Whereas thapsigargin chemically interrupts the calcium homeostasis of the cell, by blocking the activity of an important enzyme, Sarco/Endoplasmic Reticulum Ca2+-ATPase (SERCA) localized in ER (Dixon and Stockwell, (2014). Besides aforementioned chemicals, diothiothreitol, brefeldin A has known to be implicated in initiation of the UPR, as it acts as an inhibitor of calcium homoestasis (Kitamura, 2011).

Materials and Methods

Chapter 3

Materials and Methods

3.1 Chemical Reagents

Reverse transcriptase (RT), Deoxynucleotide triphosphate (dNTPs) and Taq polymerase were acquired from Fermentas (Thermo Scientific, USA). Trizol was obtained from Invitrogen (USA). Dithiothreitol (DTT) and all the other chemicals were procured from Sigma-Aldrich, USA, unless indicated otherwise.

3.2 Animals

BALB/c mice were obtained from National Institute of Health (NIH) Islamabad, Pakistan and housed in the Laboratory Animal House of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). After acclimatization time of two weeks, the mice were bred and kept in cages at a steady temperature (25±2 ºC) and regular light-dark cycles (12-12h). The mice were provided with distilled water and a standard regimen comprising of 30% crude protein, 9% crude fat, 4% crude fiber and 10% moisture. 20 male mice (35-45 g and 10-12 weeks of age) were utilized in the experiments.

3.3 Ethics Statement

The mice were housed in the Laboratory Animal House of Atta-ur-Rahman School of Applied Biosciences (ASAB), National University of Science and Technology (NUST), under a controlled environment. All the experiments performed were in compliance with the rulings of the Institute of Laboratory Animal Research, Division on Earth and Life Sciences, National Institute of Health, USA (Guide for the Care and Use of Laboratory Animals: Eighth Edition, 2011). The protocol was approved from the Internal Review Board (IRB) of Atta-ur-Rahman School of Applied Biosciences, NUST.

3.4 Study Design

The experimental animals were divided into 2 groups (n=5, each) and were provided with unadulterated food and water regimen. DTT was administered intraperitoneally as a cellular stress-inducing agent with the time intervals of 24 hours.

Serial No.	Group Treatment	Duration (days)
1. Control	Normal feed and water placebo i.p. administration	4
2. DTT treated (24h)	Normal feed and water i.p. injection of 75mg/kg dose of DTT	4

Table 3.1: Experimental Design. Untreated Balb/c mice were used as the control. Other group was given DTT treatment for 72h. (n=5)

3.5 Brain Dissection and Isolation of Cortex and Hippocampus

Mice were anesthetized and sacrificed consecutively by neck dislocation and the cortex and hippocampus, were dissected out and immediately frozen in the liquid nitrogen. These samples were stored at -80 ºC until further processing.

3.6 Histological Examination of Brain Regional Tissues

Tissue Perfusion/Fixation for Histological Assessment Heart perfusion was performed in accordance with the protocol of (Gage et al., 2012). The excised brain tissue was then placed in 4% paraformaldehyde for 24h at 4˚C before being processed further for paraffin processing and embedding. After 24h, the brain tissue was dehydrated through a series of alcohols (isopropanol), 70% (1h), 95% (1h), and 100% (1h) before paraffin infiltration. The brain tissues were then placed in xylene (4h) and paraffin embedding was performed by keeping the tissue in molten paraffin (4h at 60 °C and left to solidify (4°C) in mould (block formation) prior to cutting**.**

3.7 Haemotoxylin and Eosin Staining (H&E)

Standard haematoxylin-eosin staining was performed on 5 µ tissue sections. Tissue was de-paraffinized and incubated for 8 minutes in Mayer's haematoxylin solution and washed in water for 10 minutes. Sections were dipped in 95% ethanol and counterstained with eosin for 30 seconds. These sections were visualized using inverted microscope (Labomed, USA) at 10X,

40X and 100X resolutions. The images were captured by Pixel Pro™ image analysis software (Labomed, USA).

3.8 Immunohistochemical Staining

5 µ sagital sections were mounted on Poly Lysine coated adhesive slides. Graded concentrations of ethanol were used to rehydrate the sections followed by heat mediated antigen retrieval that was performed by incubating sections for 35 min in sodium citrate (pH: 6) in a pressure cooker. The sections were washed subsequently and incubated in 35% H_2O_2 to quench endogenous H_2O_2. The slides were incubated in 5% bovine serum albumin in PBS for 10 minutes to avoid non-specific binding of antibody followed by overnight incubation in 4°C in 0.1% bovine serum albumin in PBS containing rabbit polyclonal antibody for S-Nitrocysteine (1:100; ab50185). The sections were washed and incubated in HRP conjugated anti-rabbit IgG (1:100; ab97051) for 1h at room tempertaure. The sections were placed in a solution containing 0.025% of 3,3′ diaminobenzidine (DAB, ab50185) for 10 minutes to visualize the peroxidase reaction product. The sections were counter stained with haematoxylin and cover slips were mounted. The slides were visualized using inverted microscope (Labomed, USA) at 4X, 10X and 40X resolutions. The images were captured by Pixel Pro™ image analysis software (Labomed, USA).

3.9 In Silico Analysis of modification sites and protein association network analysis

The plausible S-nitrosylation sites on the identified differentially expressed proteins were predicted using Group-based Prediction System for SNO 1.0 (GPS SNO 1.0) with default medium threshold (Xue et al., 2010) at http://sno.biocuckoo.org/. GPS-SNO 1.0, a software which uses computational methods for prediction of putative *S*-nitrosylation sites in potentially *S*-nitrosylated proteins for which the exact *S*-nitrosylation sites had not been experimentally determined so far. In all analyses, amino acid sequences of differentially expressed protein were submitted in FASTA format for use in predicting S-nitrosylation sites under the medium threshold condition using the batch prediction tool of the GPS-SNO 1.0 software

3.10 Pathway enrichment analysis in the protein data set

To classify the molecular function, biological process and signaling pathways connected with targeted proteins, KEGG pathway analysis using 'WEB-based Gene SeT AnaLysis Toolkit" (WEBGESTALT) at http://bioinfo.vanderbilt.edu/webgestalt was carried out where "KEGG Analysis" category was selected. KEGG stands for Kyoto Encyclopedia of Genes and Genomes, a bioinformatics database that contains information on genes, proteins, reaction and pathways. The following parameters were used to make KEGG tables, reference set, 'mus_musculus', significance level, $p<0.01$; minimum numbers of genes,2 and statistical method applied was "hypergeometric test".

3.11 Protein-Protein interactions of protein data set

3.11.1 Functional association of interacting partners via STRING 8.3

UniProtKB accession numbers of predicted S-nitrosylated proteins were submitted to STRING 8.3 database (http://string-db.org/; Jensen et al., 2009) to generate and to specifically study biological association network among neighboring partner.

3.11.2 Gene Ontology (GO) analysis of protein data set

Gene Ontology (GO) analysis was done to functionally annotate the proteins with predicted S-nitrosylation sites, which categorized these proteins on the basis of biological process, molecular and cellular cataloging. GO analysis of our proteins data set was carried out using BioGRID 3.4 (http://thebiogrid.org/). BioGRID, "Biological General Repository for Interaction Dataset" is a biological database that houses genetic interaction, chemical interaction and protein-protein interactions curated from the primary biomedical literature for all major model organism species and humans and it attempts to remove redundancy to create a single mapping of data (Chatr-Aryamontri, et al., 2015).

3.11.3 Protein-protein interactions via BioGRID 3.4

To elucidate the protein-protein interaction of differentially expressed proteins with predicted S-nitrosylation sites among all major model organism species, BioGRID 3.4 (http://thebiogrid.org/) was used.

Results

Chapter 4

Results

4.1 Comparative Histological Assessment of Neurodegeneration induced by DTT

The morphological changes occurred in the cortex and hippocampus after the administration of DTT, were assessed by Haemotoxylin and Eosin (H&E) staining (Figure 4.1). The results showed that Dithiothreitol, a potent ER stress inducer has caused neurodegeneration in the cortical and hippocampal regions. Previous studies showed that at the dose of 75 mg/kg, the extent of neurodegeneration was moderate with significant alteration in cellular architecture. Therefore, a dose of 75 mg/kg was used as an optimum dose to induce ER stress. Furthermore, marked cellular degeneration was observed after 72 hours of DTT administration, at a dose of 75 mg/kg in H&E stained sections.

4.2 Immunoreactivity of S-Nitrocysteine in mouse brain tissues

Immunoreactivity of hippocampal tissue with S-Nitrocysteine antibody was assessed through comparative immunohistochemistry analysis between DTT treated and control mice group. Brain tissue with DTT induced ER stress shown to have more binding of S- Nitrocysteine in comparison with control tissues.

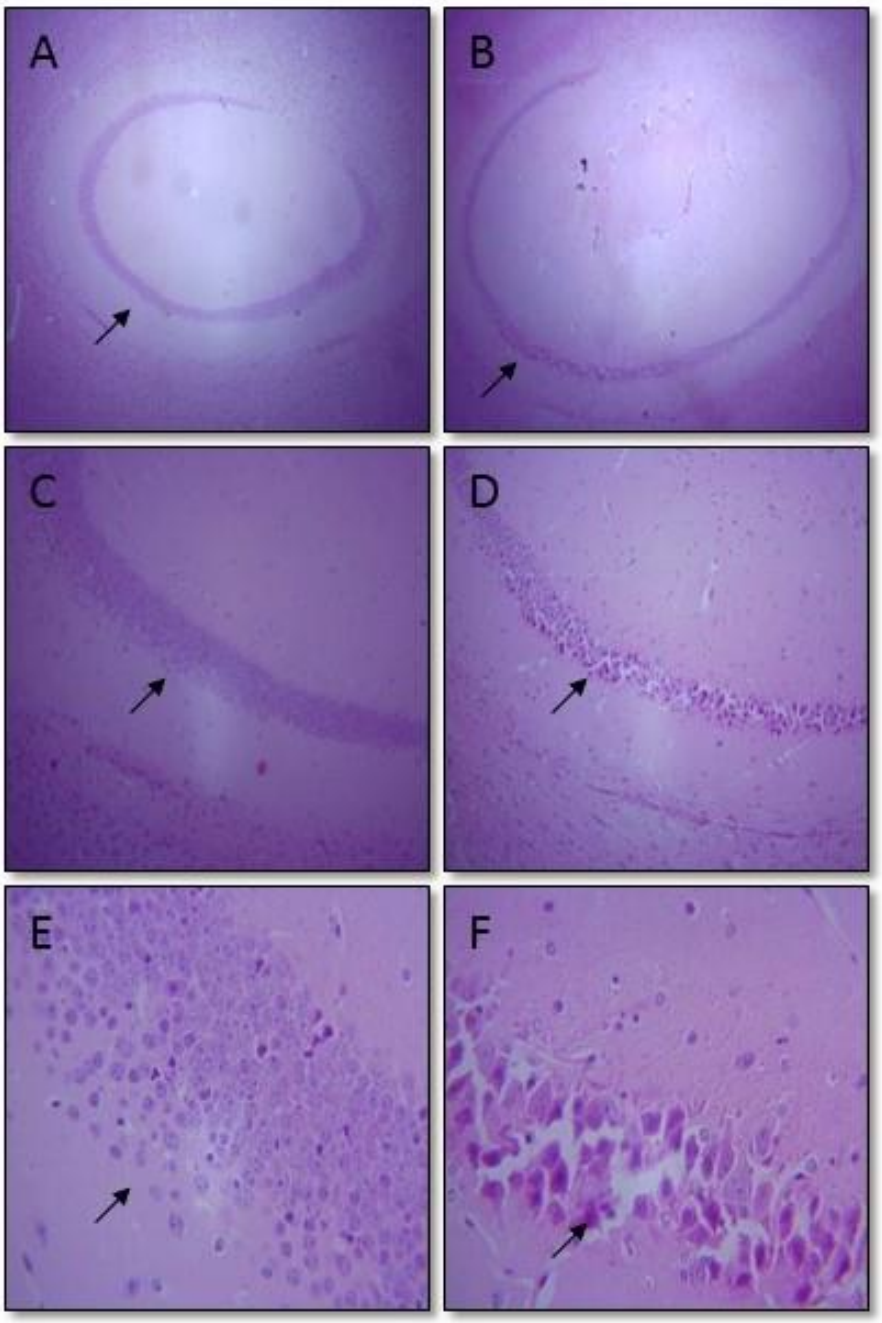


Figure 4.1: Histological assessment of DTT treated hippocampus in comparison with control by Haemotoxylin and Eosin (H&E) staining. Evaluation of extent of neurodegeneration in hippocampus at a dose of 75mg/kg of DTT observed at different resolution. A: Control (4X), B: DTT treated group (4X), C: Control (10X) D: DTT treated group (10X) E: Control (40X) F: DTT treated group (40X)

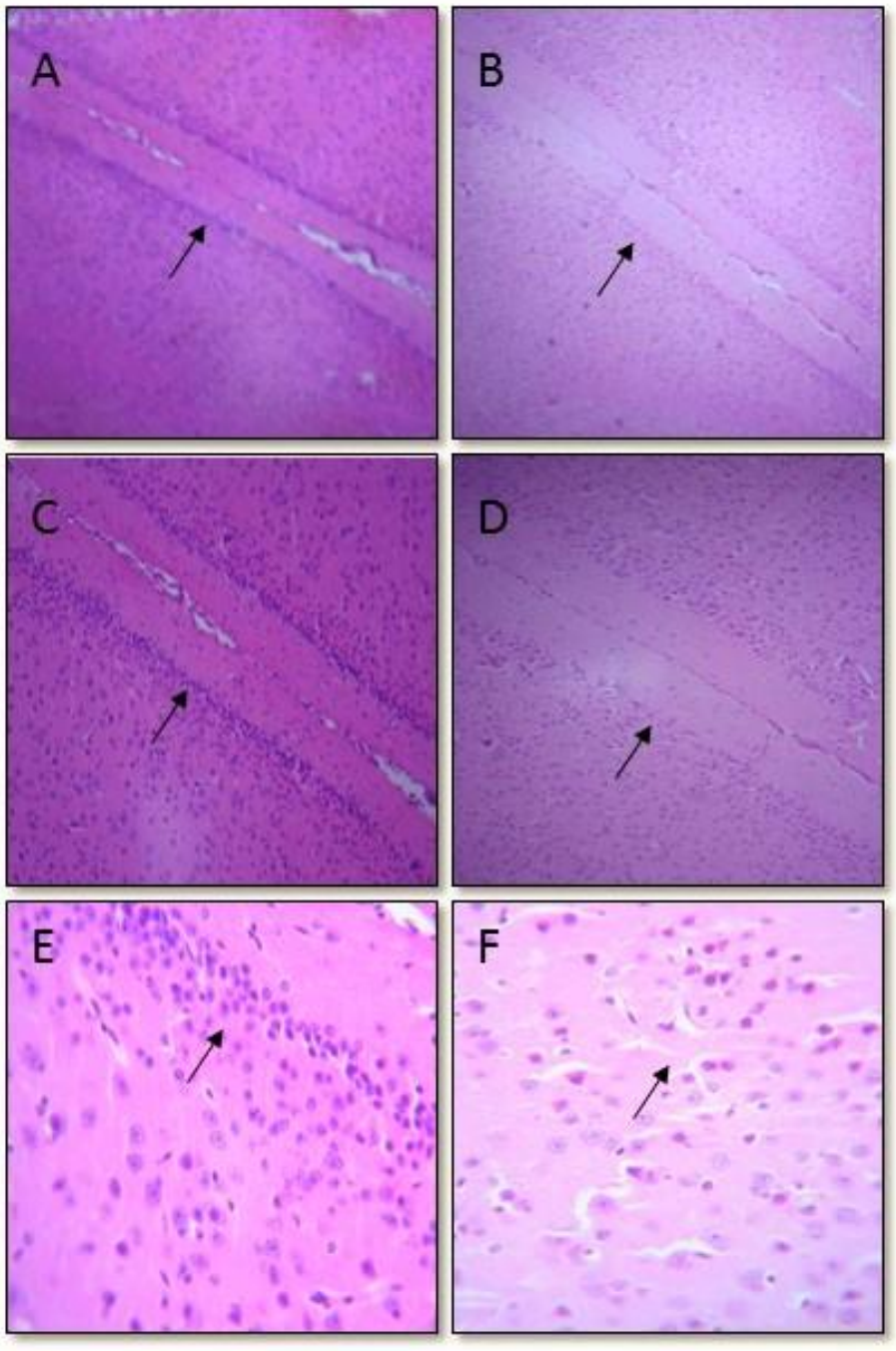


Figure 4.2: Histological assessment of DTT treated cortex in comparison with control by Haemotoxylin and Eosin (H&E) staining. Evaluation of the extent of neurodegeneration in hippocampus at a dose of 75mg/kg of DTT observed at different resolution. A: Control (4X), B: DTT treated group (4X), C: Control (10X) D: DTT treated group (10X) E: Control (40X) F: DTT treated group (40X).

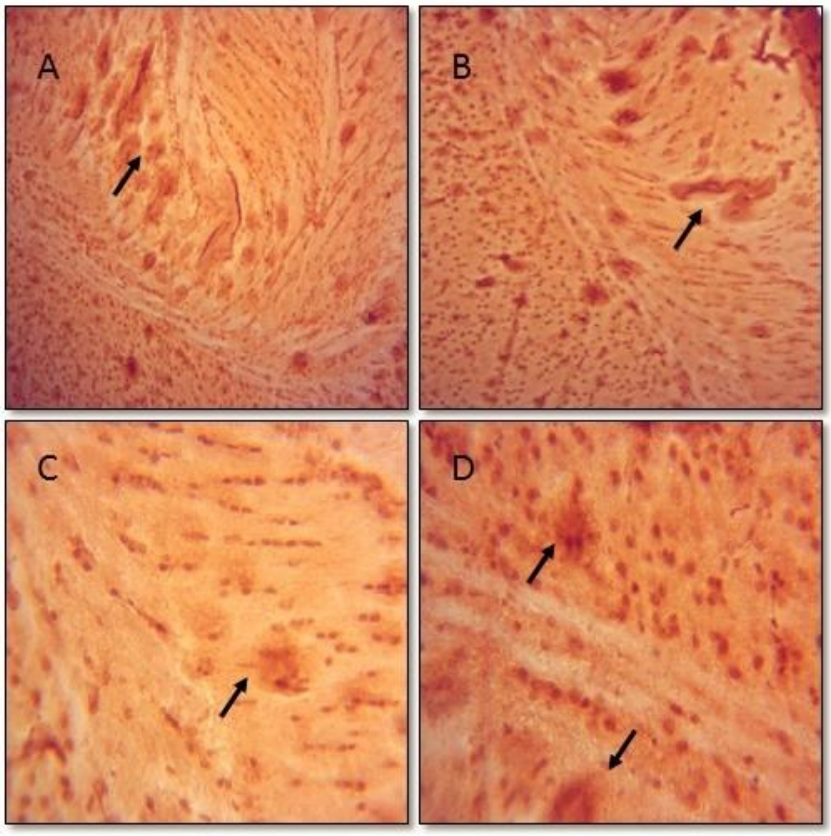


Figure 4.3: Immunohistochemical Observation of S-nitrocysteine Antibody. The brain sections were fixed and stained with S-Nitrocysteine antibody and DAB, respectively. S-nitrocysteine immunoreactivity in the brain sections of Balb/c mice in control and DTT treated group at different resolution. A: Control (10X) B: DTT treated group (10X), C: Control (40 X) D: DTT treated group (40 X).

4.3 Proteomic data set of differentially expressed Proteins in DTT induced ER stress

Acc #	Protein	Mol. Wt. (kDa)	Peptide Matches	Percent Coverage (%)	Peptides
Q91VD9	NADH Ubiquinone Oxidoreductase	79	18	29.70	ALSEIAGITLPYDTLDQVR
Q03265	ATP Synthase, subunit α	59.7	33	70.70	AVDSLVPIGR
			43	59.70	AVDSLVPIGR
Q64521	Glycrol-3-Phosphate Dehydrogenase	80.8	27	36.90	AIMNLDVEQYR
P05064	Fructose Bisphosphate Aldolase A	39.3	24	53.60	AAQEEYIK
Q9Z1G3	V-type Proton ATPase, Subunit C	43	11	25.70	ASAYNNLK
P62984	Ubiquitin-60S Ribosomal Protein L40	14.7	4	24.20	EGIPPDQQR
P06837	Neuromodulin	23.6	3	15.40	IEQDGVKPEDK
P07724	Serum Albumin	68	24	46.20	AADKDTCFSTEGPNLVTR
Q8BWF0	Succinate Semi Aldehyde Dehydrogenase	55.2	15	34.60	AAYDAFNSWK
P11798	Calcium/ Calmodulin dependent kinase type II subunit α.	54.1	15	38.10	AGAYDFPSPEWDTVTPEAK

Table 4.1: Differentially expressed proteins during ER stress treated with DTT in mice cortex and hippocampus as identified by ESI-QTOF MS/MS. Accession number and functional categories have been obtained by UniProt and the percent coverage refers to the percentage of protein sequence coverage, determined by the number of matched peptides.

4.4 *In silico* analysis of modifications sites and protein association network analysis

The plausible S-nitrosylation sites on differentially expressed proteins were predicted using Group-based Prediction System for SNO 1.0 (GPS SNO 1.0) with default medium threshold (Xue et al., 2010) at http://sno.biocuckoo.org/.

Acc. #	Position	Peptides with predicted S-nitrosylation sites	Predicted Cysteine modification sites	Score	Cutoff
Q91VD9	75	RLSVAGN**C**RMCLVEI	5	20.854	20.743
	92	APKVVAA**C**AMPVMKG		21.226	20.743
	564	RQDLPKD**C**FIVYQGH		21.27	20.743
	710	ASQTMAK**C**VKAVTEG		3.576	2.454
	727	AVEEPSI**C**		3.168	2.454
Q03265		No site was predicted	0		
Q64521	285	EFDVRAK**C**VINASGP	1	21.628	20.743
P05064	339	ALANSLA**C**QGKYTPS	1	3.674	2.454
Q9Z1G3	225	EDQDSYL**C**NVTLFRK	1	23.036	20.743
P62984		No site was predicted	0		
P06837	3	ML**C**CMRRTK	2	25.226	20.743
	4	MLC**C**MRRTKQV		3.495	2.454
P07724	58	FSQYLQK**C**SYDEHAK	8	1.694	1.67
	125	QEPERNE**C**FLQHKDD		21.664	20.743
	277	CHGDLLE**C**ADDRAEL		2.788	2.454
	289	AELAKYM**C**ENQATIS		2.016	1.6
	393	AEANPPA**C**YGTVLAE		4.163	2.454
	416	KNLVKTN**C**DLYEKLG		21.847	20.743
	501	SEHVTK**CC**SGSLVER		3.027	2.454
	603	GPNLVTR**C**KDALA**		3.587	2.454
Q8BWF0	4	****MAT**C**FLLRSFW	4	26.518	20.743
	81	KLGTVAD**C**GVPEARA		20.971	20.743
	247	GVYNVIP**C**SRNKAKE		2.755	2.454
	260	KEVGEVL**C**TDPLVSK		2.603	2.454
P11798	6	**MATIT**C**TRFTEEY	4	2.957	2.454
	115	SEADASH**C**IQQILEA		3.082	2.454
	280	HRSTVAS**C**MHRQETV		1.891	1.67
	289	HRQETVD**C**LKKFNAR		3.076	2.454

Table 4.2: Identified S-nitrosylated proteins: Predicted S-nitrosyation sites of differentially expressed proteins in DTT induced ER stress in mice cortex and hippocampus by GPS-SNO 1.0 with medium threshold.

4.5 Interaction network of differentially expressed proteins with predicted S-nitrosylation sites

Differentially expressed proteins with predicted S-nitrosylation sites were submitted to STRING 8.3 database (http://string-db.org/; Jensen et al., 2009) to generate and to specifically figure out functional association among proteins of interest.

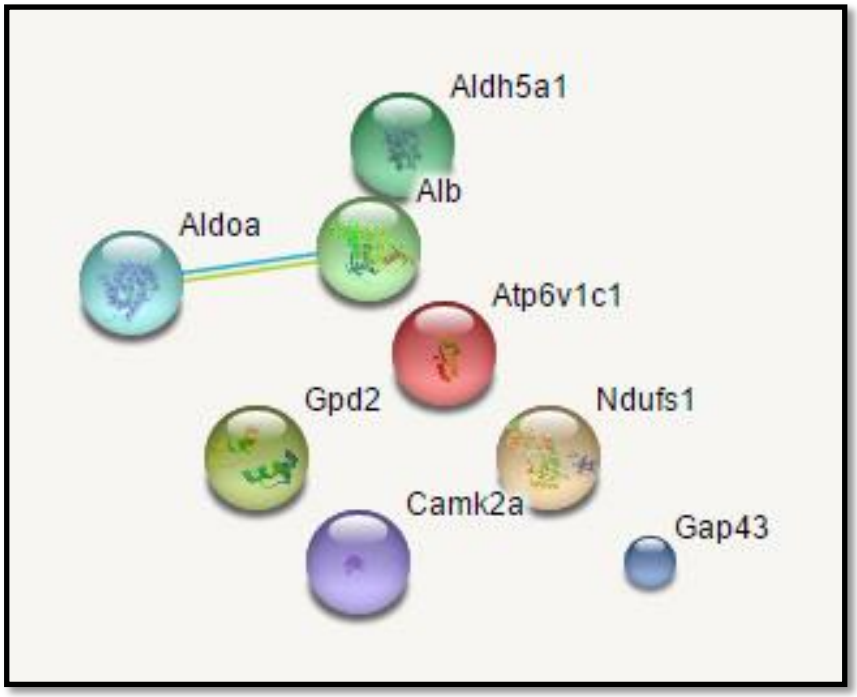


Figure 4.4: Functional association network of identified proteins with predicted S-nitrosylation sites in the mouse brain. High confidence protein-protein interaction network of identified proteins derived from the STRING database (http://stringdb.org). Each protein is represented as a node with edged interactions.

4.6 Pathway enrichment analysis of proteins with predicted S-nitrosylation sites

To investigate the molecular function, biological process and signaling pathway associated with each identified protein, KEGG, pathway analysis using WEB-based Gene SeT AnaLysis Toolkit (WEBGESTALT) was performed (http://bioinfo.vanderbilt.edu/webgestalt; Zhang et al., 2005a and Zhang et al., 2005b).

Pathways	Proteins	Number of Proteins
Metabolic pathways	Ndufs1, Atp5a1, Aldh5a1, Aldoa, Atp6v1c1	5
Oxidative Phosphorylation	Ndufs1, Atp5a1, Atp6v1c1	3
Huntington's disease	Ndufs1, Atp5a1	2
Parkinson's disease	Ndufs1, Atp5a1	2
Alzheimer's disease	Ndufs1, Atp5a1	2

Table 4.3: KEGG pathway analysis for differentially expressed proteins of DDT induced ER stress in mice brain (hippocampus and cortex). KEGG pathway analysis was carried out using WEBGESTALT (http://bioinfo.vanerbilt.edu/webgestalt/) and only pathways with 2 or more proteins and $p<0.01$were included.

4.7 Functional characteristics of identified proteins

To investigate the molecular function, biological process and cellular components associated with each identified protein, Gene Ontology (GO) analysis was done using BioGRID 3.4 (http://thebiogrid.org/).

Protein	Biological process	Molecular function	Cellular component
NADH Ubiquinone Oxidoreductase	ATP Metabolic Process Apoptotic Mitochondrial Changes Cellular Respiration Reactive Oxygen Species Metabolic Process Regulation of Mitochondrial Membrane Potential	NADH Dehydrogenase (Ubiquinone) Activity	Mitochondrial Intermembrane Space Mitochondrial Respiratory Chain Complex I Mitochondrion Myelin Sheath
Glycerol-3-Phosphate Dehydrogenase	NADH Metabolic Process Camera-Type Eye Development Gluconeogenesis Glycerol-3-Phosphate Metabolic Process Multicellular Organism Growth Oxidation-Reduction Process	Glycerol-3-Phosphate Dehydrogenase [NAD+] Activity	Glycerol-3-Phosphate Dehydrogenase Complex Mitochondrial Inner Membrane Mitochondrion
Fructose Bisphosphate Aldolase A	ATP Biosynthetic Process Fructose 1,6-Bisphosphate Metabolic Process Fructose Metabolic Process Glycolytic Process Muscle Cell Cellular Homeostasis Regulation of Cell Shape Striated Muscle Contraction	Cytoskeletal protein binding Fructose binding Fructose-bisphosphate aldolase activity Poly(a) RNA binding	Actin Cytoskeleton, Cytoplasm Extracellular Space Extracellular Vesicular Exosome Membrane Mitochondrion Nucleus Sperm Fibrous Sheath
V-type Proton ATPase, Subunit C	ATP Catabolic Process Hydrogen Ion Transmembrane Transport	Hydrogen-Exporting ATPase Activity, Phosphorylative Mechanism	Apical Part of Cell, Cytoplasm Cytoplasmic Vesicle Extracellular Vesicular Exosome Lysosomal Membrane ,Plasma Membrane

Table 4.4: Gene Ontology (GO) analysis of proteins with predicted S-nitrosylation sites using BioGRID 3.4. Biological processes, Molecular Processes and Cellular process of each protein with predicted S-Nitrosylation sites are categorized.

Protein	Biological process	Molecular function	Cellular component
Neuromodulin	Axon Choice Point Recognition Axon Guidance Cell Fate Commitment Glial Cell Differentiation Regulation of Filopodium Assembly	Calmodulin Binding	Axon, Cell Periphery Cytoplasm Filopodium Membrane Neuronal Postsynaptic Density Plasma Membrane, Postsynaptic Density
Serum Albumin	Cellular Response to Starvation Hemolysis by Symbiont of Host Erythrocytes Maintenance of Mitochondrion Location Negative Regulation of Apoptotic Process Positive Regulation of Circadian Sleep/Wake Cycle, Non-REM Sleep	DNA binding, Chaperone binding Drug binding, Enzyme binding Fatty acid binding, Oxygen binding Pyridoxal phosphate binding Toxic substance binding Zinc ion binding	Basement Membrane , Blood Microparticle, Cytoplasm Extracellular Region, Extracellular Space, Extracellular Vesicular Exosome, Myelin Sheath, Nucleus, Protein Complex
Succinate Semi Aldehyde Dehydrogenase	Central Nervous System Development Galactosylceramide Metabolic Process Gamma-Aminobutyric Acid Catabolic/ Metabolic Process Glucose/ Glucosylceramide Metabolic Process Glutamate/Glutamine/ Glutathione Metabolic Process Glycerophospholipid Metabolic Process Neurotransmitter Catabolic Process Oxidation-Reduction Process Post-Embryonic Development Protein Homotetramerization Respiratory Electron Transport Chain Short-Chain Fatty Acid Metabolic Process Acetate/Succinate Metabolic Process	NAD binding Aldehyde dehydrogenase (NAD) activity Carboxylic acid binding Succinate-semialdehyde dehydrogenase (NAD+) activity Succinate-semialdehyde dehydrogenase	Mitochondrion

Table 4.4: Gene Ontology (GO) analysis of proteins with predicted S-nitrosylation sites using BioGRID 3.4. Biological processes, Molecular Processes and Cellular process of each protein with predicted S-Nitrosylation sites are categorized.

4.8. Protein-protein interactions of differentially expressed proteins with predicted S-Nitrosylation sites

To elucidate the protein-protein interaction of differentially expressed proteins with predicted S-Nitrosylation sites among different species, BioGRID 3.4 (http://thebiogrid.org/) was used. Physical interactions with various proteins in muns musculus and homo sapiens were found out.

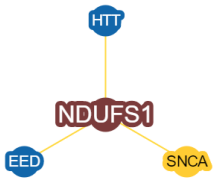
Proteins	Interactors	Organism	Throughput	Interactions
NDUFS1	EED Embryonic ectoderm development HTT Hungtingtin SNCA Synuclein,alpha (non A4 component of amyloid precursor)	M. Musculus M. Musculus H. Sapians	High High low	
GD2	No physical interaction	---	---	---
ATP6V1C1	EED Embryonic ectoderm development	M. musculus	High	

Table 4.5: Protein-Protein Interaction analysis: Identification of interacting partners and their interactions of proteins with predicted S-nitrosylation sites in Mus musculus and Homo sapiens using BioGRID 3.4. A network of interactions of protein in *Mus musculus and Homo sapians.* Nodes represent proteins, and edges represent physical protein interactions.

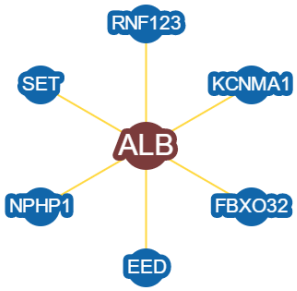
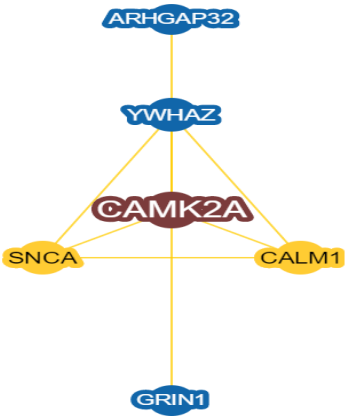
Proteins	Interactors	Organism	Throughput	Interactions
Alb1	KCNMA1	M. musculus	High	
	EED	M.musculus	Low	
	FBXO32	M. musculus	Low	
	NPHP1	M. musculus	High	
	RNF123	M. musculus	Low	
	SET	M. musculus	Low	
GAP43	Calmodulin 1	M. musculus	Low	
ALDH5 A1	**No physical interactions**	---	---	---
CAMK 2A	ARHGAP32	M. musculus	Low	
	CALM1	H. sapiens	High	
	YWHAZ	M. musculus	Low	
	GRIN1	H. sapiens	Low	
	SNCA	M. musculus	High	

Table 4.5: Protein-Protein Interaction analysis: Identification of interacting partners and their interactions of proteins with predicted S-nitrosylation sites in Mus musculus and Homo sapiens using BioGRID 3.4. A network of interactions of protein in *Mus musculus and Homo sapians.* Nodes represent proteins, and edges represent physical protein interactions.

Discussion

Chapter 5

Discussion

The study was conducted to highlight the role of S-nitrosylation in DTT induced ER stress in brain tissue of mouse model. A dose of 75mg/kg was selected to induce ER stress in mouse model and its effects exhibited moderate neurodegeneration with coincident alteration in cellular architecture. Histological analysis of brain section was carried out by using H&E staining, which confirmed the deleterious effects of DTT, showing deteriorated neuronal architecture in hippocampus and cortical region after 72 hours of its treatment.

Immunohistochemical analysis was carried to determine the level of immunoreactivity of S-nitrocysteine antibody in hippocampus and cortical region of mouse brain. S-nitrocysteine reactivity was found to be increased in DTT treated mouse brain as compare to control mouse brain which indicates presence of excessively S-nitrosylated proteins during ER stress. In an attempt to better understand the molecular mechanisms of chemically induced ER stress, initiation of UPR and decline in neuronal survival, a proteomic approach was employed to examine the changes in the brain proteome profile of mice treated with DTT. Significant differences in the expression of 10 proteins were observed after the 72 h treatment group and the control group. From this, 10 proteins were successfully identified via the ESI-QTOF MS/MS experiments. The main functions of these proteins were energy metabolism and transport.

The S-nitrosylation status of the proteins identified using proteomic technique was further validated using *in silico* method for the prediction of cysteine modification sites on the primary sequences of these proteins. The computational prediction of S-nitrosylation sites performed with GPS-SNO 1.0 reveals the number of plausible cysteine modification sites for the identified proteins.

The interacting patterns of the identified S-nitrosylated proteins predict a mutual mechanism of action to mediate various cellular and molecular processes. Recently, a number of methods have been developed for SNO protein characterization, identification and quantification that focus on direct detection of NO-modified thiol or chemical reduction/photolytic breakdown of SNO or labeling S-nitrosylated Cys Thiol (Foster, 2012). Detail description of S-nitrosylation sites on identified proteins, their functional significances, as well as the potential roles are discussed below.

A number of proteins affected by the DTT treatment were the energy metabolism-related proteins. *In silico* analysis through GPS-SNO 1.0 predicted plausible cysteine sites on primary sequence of protein data set. NADH ubiquinone oxidoreductase and succinate semi aldehyde dehydrogenase have 4 plausible cysteine sites, only one site on glycerol-3-phosphate dehydrogenase, fructose bisphosphate aldolase A and V-type proton ATPase subunit C whereas no cysteine sites were predicted on ATP synthase subunit α.
KEGG pathway analysis of these differentially expressed protein indicated enrichment of these aforementioned proteins in various signaling pathways related to metabolism, oxidative phosphorylation, Huntington disease, Alzheimer's disease and Parkinson's disease. Out of all five proteins, NADH ubiquinone oxidoreductase and ATP synthase subunit α were common in all of these important pathways.

GO analysis revealed the biological, molecular and cellular functions of these proteins in which NADH ubiquinone oxidoreductase has shown to possess vital role during metabolic processes such as ATP metabolic process, apoptotic mitochondrial changes, reactive oxygen species and regulation of mitochondrial membrane potential (Hroudová et al. 2014). Reduced levels of NADH ubiquinone oxidoreductase (complex I) resulting in mitochondrial impairment have been observed in patients with AD (Kin et al, 2001). The perturbation in NADH ubiquinone oxidoreductase levels may possibly due to the plausible cysteine sites in peptide sequence with possibility of aberrant S-nitrosylation that may lead to mitochondrial damage. Protein-protein interactions via BioGRID 3.4 showed that interacting partners of NADH ubiquinone oxidoreductase are Hungtingtin protein in mouse and synuclein alpha in human, which are the

main pathological factors of HD and PD respectively. Moreover, as an important component of various biological processes, aberrant S-nitrosylation may affect the normal functions of NADH ubiquinone oxidoreductase and may exacerbate the underlying causes of neurodegeneration by interacting with these proteins such as Huntingtin protein or synuclein alpha in disease state.
Biological function through GO analysis showed that succinate semi aldehyde dehydrogenase play crucial role in central nervous system development, galactosylceramide, glycerophospholipid, glucose/ glucosylceramide, and GABA, metabolic process, neurotransmitter catabolic process and oxidation-reduction process. Anomalies in S-nitrosylation of this protein may significantly influence the normal cascades of aforementioned metabolic process of undergoing during neuronal survival. Succinate semi aldehyde dehydrogenase is a negative regulator of the inhibitory neurotransmitter, GABA (Wang et al., 2013). The GABA neurotransmitter reduces the function of hyper activated neurons. Hyperactive neurons lead to emotions like fear or anxiety, which makes succinate semi aldehyde dehydrogenase one of the key enzymes for synaptic neurotransmission (Pearl et al., 2014). Under ER stress and aberrant S-nitrosylation levels may affect the inhibition of GABA which possibly could halt neurotransmission between neurons. On the other side, if succinate semi aldehyde dehydrogenase levels up regulate possibly due to aberrant S-Nitrosylation , inhibition of GABA may be at a great extent that can lead to sustained hyperactivity of neurons (Del Pino 2015).

Neuromodulin has 2 predicted cysteine sites and Calmodulin/Calcium dependent kinase type have 4 plausible cysteines residues which can undergo S-nitrosylation. GO analysis revealed that neuromodulin plays an important role in the nerve growth, development, repair and neurogenesis. Interacting partners of neuromodulin is calmodulin 1 because in the absence of Ca2+ ions neuromodulin binds to calmodulin (Benowitz and Routtenburg, 1997). In ER stress conditions, neuromodulin incorporates in the ER and contributes to the ER protein load (Kim et al., 2006).

Calmodulin/Calcium dependent kinase are important component of calcium ion transport, ionotropic glutamate receptor signaling pathway, regulation of neuronal synaptic plasticity and

neurotransmitter secretion and response to ischemia indicated by GO analysis. Interacting partners of calmodulin/calcium dependent kinase are synuclein alpha and calmodulin 1 in human indicating its significant role in the process of neurodegeneration. As it possesses more cysteine residues which presents increased chances of aberrant S-nitrosylation under nitrosative stress or ER stress, which may disturb its critical role and augment the process of neurodegeneration during prognosis of neurodegenerative diseases. Calcium/calmodulin dependent kinase type-II alpha is a prominent kinase found in the central nervous system (CNS) and its function is long-term potentiation and neurotransmitter release. Moreover, ER is the reservoir of Ca2+ ions, which are released during neurotransmitter release (Liu and Murray, 2012). Under normal conditions, calmodulin binds to 4 Ca2+ ions for activation and furthermore, phosphorylates other kinases and proteases that help in autophagy. Autophagy is normally a cell survival tool, however under cellular stress; it leads to cell death (Ryan et al., 2014). Recent evidence points to the fact that ER stress triggers an efflux of Ca2+ ions into the cytoplasm, which bind to calmodulin, which becomes hyper activated that ultimately leads to sustained autophagy in the neuron that may lead to cellular apoptosis (Roe and Ren, 2013). However, the mechanisms behind neurotransmission dysfunction in ER stress remains to be further clarified.

Ubiquitin- 60S ribosomal protein and ATP synthase, subunit alpha play key role in the degradation of cellular proteins via the proteasome and oxidative phosphorylation respectively (Caldeira et al., 2014), however no plausible cysteine residues were found in peptide sequences of both proteins suggesting no effect on signaling cascades involved in neurodegeneration.

5.4 Conclusion

There is strong evidence for activation of ER-stress-responsive pathways in a range of neurological disorders. What remains to be discovered is how successful will the strategies be that target these responses, in the treatment of neurological disorders. This study has revealed an insight on the basic molecular mechanism underlying the relationship between UPR and neurodegeneration. From this preliminary data, we can conclude that environmental toxicity may lead to a buildup of misfolded proteins in the ER, which causes a temporary halt in all cellular processes. Moreover, continuous stress leads the ER stress sensors to activate apoptotic pathways. Research into unfolded-protein-response signaling has matured to a point at which small-molecule inhibitors of its components are under development, which can be used as bio-markers for early onset neurological disorders like AD, PD or ALS.

References

Chapter 6

References

Alvarez-Castelao, B., & Castaño, J. G. (2011). Synphilin-1 inhibits alpha-synuclein degradation by the proteasome. Cellular and Molecular Life Sciences, 68(15), 2643-2654.

Carrell, R. W., & Lomas, D. A. (2002). Alpha1-antitrypsin deficiency—a model for conformational diseases. New England Journal of Medicine,346(1), 45-53.

Castrillo, J. I., Oliver, S. G. (2015). Alzheimer's as a Systems-Level Disease Involving the Interplay of Multiple Cellular Networks. Systems Biology of Alzheimer's Disease, 3-48.

Chatr-Aryamontri, A., Breitkreutz, B. J., Oughtred, R., Boucher, L., Heinicke, S., Chen, D., & Reguly, T. (2015). The BioGRID interaction database: 2015 update. *Nucleic acids research*, *43*(D1), D470-D478.

Chen X, Li C, Guan T, Shang H, Cui L, Li XM, Kong J. S-Nitrosylated protein disulphide isomerase contributes to mutant SOD1 aggregates in amyotrophic lateral sclerosis. J Neurochem.2013;124:45–58.

Chen, Y., & Brandizzi, F. (2013). IRE1: ER stress sensor and cell fate executor. Trends in cell biology, 23(11), 547-555.

Chung KK, Thomas B, Li X, Pletnikova O, Troncoso JC, Marsh L, Dawson VL, Dawson TM. S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. Science.2004;304:1328–1331.

Chung KK, Zhang Y, Lim KL, Tanaka Y, Huang H, Gao J, Ross CA, Dawson VL, and Dawson TM. Parkin ubiquitinates the alpha-synuclein-interacting protein, synphilin-1: implications for Lewy-body formation in Parkinson disease. Nat Med 7: 1144–1150, 2001.

Cleland, W. W. (1964). Dithiothreitol, a new protective reagent for SH groups. Biochemistry, 3(4), 480-482

Dawson TM, Dawson VL. Molecular pathways of neurodegeneration in Parkinson's disease. Science. 2003;302:819–822.

Dawson VL, Dawson TM, London ED, Bredt DS, and Snyder SH. Nitric oxide mediates glutamate neurotoxicity in primary cortical cultures. Proc Natl Acad Sci USA 88: 6368–6371, 1991

Del Pino, J., Frejo, M. T., Baselga, M. J. A., Moyano, P., & Díaz, M. J. (2015). Impaired glutamatergic and GABAergic transmission by amitraz in primary hippocampal cells. Neurotoxicology And Teratology, 50, 82-87. (Benowitz and Routtenburg, 1997).

Dixon, S. J., & Stockwell, B. R. (2014). The role of iron and reactive oxygen species in cell death. Nature Chemical Biology, 10(1), 9-17. (Chan and Egan, 2005)

Foster, M. W. (2012). Methodologies for the characterization, identification and quantification of S-nitrosylated proteins. Biochimica et Biophysica Acta (BBA)-General Subjects, 1820(6), 675-683.

Foster, M. W., Hess, D. T., & Stamler, J. S. (2009). Protein S-nitrosylation in health and disease: a current perspective. Trends in molecular medicine, 15(9), 391-404.

Gage, G. J., Kipke, D. R., & Shain, W. (2012). Whole animal perfusion fixation for rodents. JoVE (Journal of Visualized Experiments), (65), e3564-e3564.

Gu, Z., Nakamura, T., & Lipton, S. A. (2010). Redox reactions induced by nitrosative stress mediate protein misfolding and mitochondrial dysfunction in neurodegenerative diseases. Molecular neurobiology, 41(2-3), 55-72.

Halloran, M., Parakh, S., & Atkin, J. D. (2013). The role of s-nitrosylation and s-glutathionylation of protein disulphide isomerase in protein misfolding and neurodegeneration. International journal of cell biology.

Harding, H. P., Zhang, Y., Bertolotti, A., Zeng, H., & Ron, D. (2000). Perk is essential for translational regulation and cell survival during the unfolded protein response. Molecular cell, 5(5), 897-904.

Holcik, M., & Sonenberg, N. (2005). Translational control in stress and apoptosis. Nature reviews Molecular cell biology, 6(4), 318-327.

Hoozemans, J. J. M., Veerhuis, R., Van Haastert, E. S., Rozemuller, J. M., Baas, F., Eikelenboom, P., & Scheper, W. (2005). The unfolded protein response is activated in Alzheimer's disease. Acta Neuropathologica, 110(2), 165-172.Huang Z, Huang PL, Panahian N,

Dalkara T, Fishman MC, Moskowitz MA. Effects of cerebral ischemia in mice deficient in neuronal nitric oxide synthase. Science. 1994;265:1883–1885.

Hroudová, J., Singh, N., & Fišar, Z. (2014). Mitochondrial dysfunctions in neurodegenerative diseases: relevance to Alzheimer's disease. BioMed research international, 2014.

Høyer-Hansen, M., Jäättelä, M. (2007). Connecting endoplasmic reticulum stress to autophagy by unfolded protein response and calcium. Cell Death & Differentiation, 14(9), 1576-1582.

Jung, E. S., Hong, H., Kim, C., & Mook-Jung, I. (2015). Acute ER stress regulates amyloid precursor protein processing through ubiquitin-dependent degradation. Scientific Reports, 5;5:8805.

Kim, S. J., Zhang, Z., Hitomi, E., Lee, Y. C., & Mukherjee, A. B. (2006). Endoplasmic reticulum stress-induced caspase-4 activation mediates apoptosis and neurodegeneration in INCL. Human Molecular Genetics, 15(11), 1826-1834.

Kim, S. H., Vlkolinsky, R., Cairns, N., Fountoulakis, M., & Lubec, G. (2001). The reduction of NADH: Ubiquinone oxidoreductase 24-and 75-kDa subunits in brains of patients with Down syndrome and Alzheimer's disease. *Life sciences*, *68*(24), 2741-2750.

Kitamura, K., Taki, M., Tanaka, N., & Yamashita, I. (2011). Fission yeast Ubr1 ubiquitin ligase influences the oxidative stress response via degradation of active Pap1 bZIP transcription factor in the nucleus. Molecular Microbiology, 80(3), 739-755.

Ko HS, Uehara T, and Nomura Y. Role of ubiquilin associated with protein-disulfide isomerase in the endoplasmic reticulum in stress-induced apoptotic cell death. J Biol Chem 277: 35386–35392, 2002.

Kouroku, Y., Fujita, E., Tanida, I., Ueno, T., Isoai, A., Kumagai, H., & Momoi, T. (2007). ER stress (PERK/eIF2α phosphorylation) mediates the polyglutamine-induced LC3 conversion, an essential step for autophagy formation. Cell Death & Differentiation, 14(2), 230-239.

Larsen, A. H., Frandsen, A., & Treiman, M. (2001). Upregulation of the SERCA-type Ca 2+ pump activity in response to endoplasmic reticulum stress in PC12 cells. BMC biochemistry, 2(1), 1.

Lindholm D, Wootz H, Korhonen L. ER stress and neurodegenerative diseases. Cell Death Differ. 2006;13:385–392.

Lindholm, D., Wootz, H., & Korhonen, L. (2006). ER stress and neurodegenerative diseases. Cell Death & Differentiation, 13(3), 385-392.

Lipton SA and Nicotera P. Calcium, free radicals and excitotoxins in neuronal apoptosis. Cell Calcium 23: 165–171, 1998.

Lipton SA, Nakamura T, Uehara T, Shi ZQ, Gu Z. Comment on S-nitrosylation of parkin regulates ubiquitination and compromises parkin's protective function. Science. 2005;308:1870.

Lipton SA, Nakamura T, Yao D, Shi Z-Q, Uehara T, and Gu Z. Comment on "S-Nitrosylation of Parkin regulates ubiquitination and compromises Parkin's protective function". Science 308: 1870, 2005

Liu, X. B., Murray, K. D. (2012). Neuronal excitability and calcium/calmodulindependent protein kinase type II: Location, location, location. Epilepsia, 53(1), 45-52.

Maly, D. J., Papa, F. R. (2014). Druggable sensors of the unfolded protein response. Nature Chemical Biology, 10(11), 892-901Choi YB, Tenneti L, Le DA, Ortiz J, Bai G, Chen HS, and Lipton SA. Molecular basis of NMDA receptor-coupled ion channel modulation by S-nitrosylation. Nat Neurosci 3: 15–21, 2000.

Mattson, M. P., Chan, S. L., & Duan, W. (2002). Modification of brain aging and neurodegenerative disorders by genes, diet, and behavior. Physiological reviews, 82(3), 637-672.

Meng F, Yao D, Shi Y, Kabakoff J, Wu W, Reicher J, Ma Y, Moosmann B, Masliah E, Lipton SA, Gu Z. Oxidation of the cysteine-rich regions of parkin perturbs its E3 ligase activity and contributes to protein aggregation. Mol Neurodegener. 2011;6:34.

Mori K. Tripartite management of unfolded proteins in the endoplasmic reticulum. Cell 101: 451–454, 2000

Nakamura T., Lipton S. (2007). Molecular mechanisms of nitrosative stress-mediated protein misfolding in neurodegenerative diseases. Cell Mol. Life Sci. 64, 1609–1620. 10.1007/s00018-007-6525-0.

Nakamura T., Lipton S. (2008). Emerging roles of S-nitrosylation in protein misfolding and neurodegenerative diseases. Antioxid. Redox Signal. 10, 87–101. 10.1089/ars.2007.1858.

Nakamura T., Lipton S. (2009). Cell death:protein misfolding and neurodegenerative diseases.Apoptosis 14, 455–468. 10.1007/s10495-008-0301-y.

Nakamura T., Lipton S. (2011). Redox modulation by S-nitrosylation contributes to protein misfolding, mitochondrial dynamics, and neuronal synaptic damage in neurodegenerative diseases. Cell Death Differ. 18, 1478–1486. 10.1038/cdd.2011.65.

Nakamura T., Tu S., Akhtar M. W., Sunico C., Okamoto S., Lipton S. (2013). Aberrant protein S-nitrosylation in neurodegenerative disease. Neuron 78, 596–614. 10.1016/j.neuron.2013.05.005

Obukuro K, Nobunaga M, Takigawa M, Morioka H, Hisatsune A, Isohama Y, Shimokawa H, Tsutsui M, Katsuki H. Nitric oxide mediates selective degeneration of hypothalamic orexin neurons through dysfunction of protein disulfide isomerase. J Neurosci. 2013;33:12557–12568.

Okada, T., Yoshida, H., Akazawa, R., Negishi, M., & Kazutoshi, M. O. R. I. (2002). Distinct roles of activating transcription factor 6 (ATF6) and double-stranded RNA-activated protein kinase-like endoplasmic reticulum kinase (PERK) in transcription during the mammalian unfolded protein response. Biochemical Journal, 366(2), 585-594.

Oslowski, C. M., & Urano, F. (2011). Measuring ER stress and the unfolded protein response using mammalian tissue culture system. Methods in enzymology, 490, 71. Li, J., Chen, Z., Gao, L. Y., Colorni, A., Ucko, M., Fang, S., & Du, S. J. (2015). A transgenic zebrafish model for monitoring xbp1 splicing and endoplasmic reticulum stress in vivo. Mechanisms Of Development. 137:33-44. (Miyazaki et al., 1990; Asfari et al., 1992; Hohmeier et al., 2000; Schindler and Schekman, 2009; Chen et al., 2011; Schuiki et al., 2012; Gao et al., 2014)

Pearl, P. L., Schreiber, J., Theodore, W. H., McCarter, R., Barrios, E. S., Yu, J., & Gibson, K. M. (2014). Taurine trial in succinic semialdehyde dehydrogenase deficiency and elevated CNS GABA. Neurology, 82(11), 940-944.

Pereira C, Agostinho P, Moreira PI, Cardoso SM, Oliveira CR (2005) Alzheimer's disease-associated neurotoxic mechanisms and neuroprotective strategies. Curr Drug Targets CNS Neurol Disord 4: 383-403.

Pinton, P., Giorgi, C., Siviero, R., Zecchini, E., & Rizzuto, R. (2008). Calcium and apoptosis: ER-mitochondria Ca2+ transfer in the control of apoptosis. Oncogene, 27(50), 6407-6418.

Roe, N. D., Ren, J. (2013). Oxidative activation of Ca2+/calmodulin-activated kinase II mediates ER stress-induced cardiac dysfunction and apoptosis. American Journal of Physiology-Heart and Circulatory Physiology, 304(6), 828839. Caldeira, M. V., Salazar, I. L., Curcio, M., Canzoniero, L. M., & Duarte, C. B. (2014). Role of the ubiquitin–proteasome system in brain ischemia: Friend or foe? Progress In Neurobiology, 112, 50-69.

Ron, D., & Walter, P. (2007). Signal integration in the endoplasmic reticulum unfolded protein response. Nature reviews Molecular cell biology, 8(7), 519-529.

Roussel, B. D., Kruppa, A. J., Miranda, E., Crowther, D. C., Lomas, D. A., & Marciniak, S. J. (2013). Endoplasmic reticulum dysfunction in neurological disease. The Lancet Neurology, 12(1), 105-118.

Ryan, A. J., Larson-Casey, J. L., He, C., Murthy, S., & Carter, A. B. (2014). Asbestos-induced Disruption of Calcium Homeostasis Induces Endoplasmic Reticulum Stress in Macrophages. Journal Of Biological Chemistry, 289(48), 33391-33403.

Ryoo, H. D., Domingos, P. M., Kang, M. J., & Steller, H. (2007). Unfolded protein response in a Drosophila model for retinal degeneration. The EMBO Journal, 26(1), 242-252

Sano, R., & Reed, J. C. (2013). ER stress-induced cell death mechanisms. Biochimica et Biophysica Acta (BBA)-Molecular Cell Research, 1833(12), 3460-3470.

Scheper, W., Hoozemans, J. J. (2015). The unfolded protein response in neurodegenerative diseases: a neuropathological perspective. Acta Neuropathologica, 130(3):315-31

Schröder, M., & Kaufman, R. J. (2005). ER stress and the unfolded protein response. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 569(1), 29-63.

Shen, J., Chen, X., Hendershot, L., & Prywes, R. (2002). ER stress regulation of ATF6 localization by dissociation of BiP/GRP78 binding and unmasking of Golgi localization signals. Developmental cell, 3(1), 99-111.

Shi ZQ, Sunico CR, McKercher SR, Cui J, Feng GS, Nakamura T, Lipton SA. S-Nitrosylated SHP-2 contributes to NMDA receptor-mediated excitotoxicity in acute ischemic stroke. Proc Natl Acad Sci USA. 2013;110:3137–3142.

Shimura H, Hattori N, Kubo S, Mizuno Y, Asakawa S, Minoshima S, Shimizu N, Iwai K, Chiba T, Tanaka K, Suzuki T. Familial Parkinson disease gene product, parkin, is a ubiquitin-protein igase. Nat Genet. 2000;25:302–305.

Singh, S., Brocker, C., Koppaka, V., Chen, Y., Jackson, B. C., Matsumoto, A., ... & Vasiliou, V. (2013). Aldehyde dehydrogenases in cellular responses to oxidative/electrophilicstress. *Free Radical Biology and Medicine*, *56*, 89-101.

Sriburi, R., Jackowski, S., Mori, K., & Brewer, J. W. (2004). XBP1 a link between the unfolded protein response, lipid biosynthesis, and biogenesis of the endoplasmic reticulum. The Journal of cell biology, 167(1), 35-41.

SZEGEZDI, E., FITZGERALD, U., & SAMALI, A. (2003). Caspase-12 and ER-Stress-Mediated Apoptosis. Annals of the New York Academy of Sciences, 1010(1), 186-194.

Tanaka S, Uehara T, and Nomura Y. Up-regulation of proteindisulfide isomerase in response to hypoxia/brain ischemia and its protective effect against apoptotic cell death. J Biol Chem 275: 10388–10393, 2000.

Taylor, J. P., Hardy, J., & Fischbeck, K. H. (2002). Toxic proteins in neurodegenerative disease. Science, 296(5575), 1991-1995.

Uehara, T., Nakamura, T., Yao, D., Shi, Z. Q., Gu, Z., Ma, Y., & Lipton, S. A. (2006). S-nitrosylated protein-disulphide isomerase links protein misfolding to neurodegeneration. Nature, 441(7092), 513-517.

Urano, F., Wang, X., Bertolotti, A., Zhang, Y., Chung, P., Harding, H. P., & Ron, D. (2000). Coupling of stress in the ER to activation of JNK protein kinases by transmembrane protein kinase IRE1. Science, 287(5453), 664-666.

Walker K, Farg MA, Bye CR, McLean CA, Horne MK, Atkin JD. Protein disulphide isomerase protects against protein aggregation and is S-nitrosylated in amyotrophic lateral sclerosis. Brain.2010;133:105–116.

Wang C, Li W, Ren J, Fang J, Ke H, Gong W, Feng W, Wang CC Antioxid RedoxSignal. 2013 Jul 1; 19(1):36-45.

Wang G, Moniri NH, Ozawa K, Stamler JS, Daaka Y. Nitric oxide regulatesendocytosis by S-nitrosylation of dynamin. Proc Natl Acad Sci USA. 2006;103:1295–1300.

Wang HQ, Takahashi R. Expanding insights on the involvement of endoplasmic reticulum stress in Parkinson's disease. Antioxid Redox Signal. 2007;9:553–561.

Wang, X. L., Liang, J. Y., Liu, R. Z., Dong, Z., & Yu, S. Y. (2013). The expression of succinate semialdehyde dehydrogenase in the caudal part of the spinal trigeminal nucleus is down-

regulated after electrical stimulation of the duramater surrounding the superior sagittal sinus in conscious rats. Neuroscience, 248:145-153

Wilkinson B., Gilbert H. F. (2004). Protein disulfide isomerase. Biochim. Biophys. Acta 1699, 35–44. 10.1016/j.bbapap.2004.02.017

Yao D, Gu Z, Nakamura T, Shi ZQ, Ma Y, Gaston B, Palmer LA, Rockenstein EM, Zhang Z,Masliah E, Uehara T, Lipton SA. Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. Proc Natl Acad Sci USA. 2004;101:10810–10814.

Yao, D., Gu, Z., Nakamura, T., Shi, Z. Q., Ma, Y., Gaston, B., & Uehara, T. (2004). Nitrosative stress linked to sporadic Parkinson's disease: S-nitrosylation of parkin regulates its E3 ubiquitin ligase activity. Proceedings of the National Academy of Sciences of the United States of America,101(29), 10810-10814

Zhang, K., & Kaufman, R. J. (2006). The unfolded protein response A stress signaling pathway critical for health and disease. Neurology, 66(1 suppl 1), S102-S109.

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