“ROLE OF FOXO3 IN REGULATION OF NEURODEGENERATIVE DISORDERS.”

A DISSERTATION

SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE AWARD OF THE DEGREE OF

MASTER OF SCIENCE

IN

BIOTECHNOLOGY

Submitted by:

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Title of paper: FOXO and related transcription factors binding elements in the regulation of neurodegenerative disorders.

Authors name: Vaibhav Oli and Pravir Kumar

Name of Journal: Journal of Chemical Neuroanatomy

Conference date with Venue (if applicable): NA
Have you registered for the conference (yes/no)?: NA
Status of paper: Communicated

Date of paper communicated: 16th May, 2021

Date of paper acceptance: --
Date of paper Publication: --

(Name: Vaibhav Oli and Roll No. 2K19/MSCBIO/34)

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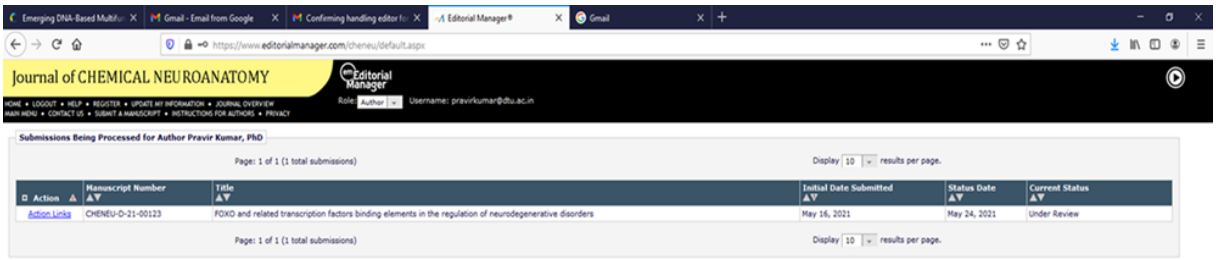

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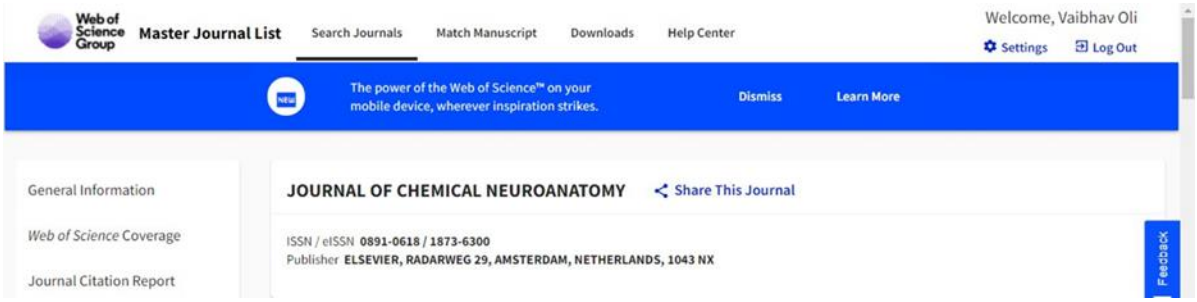

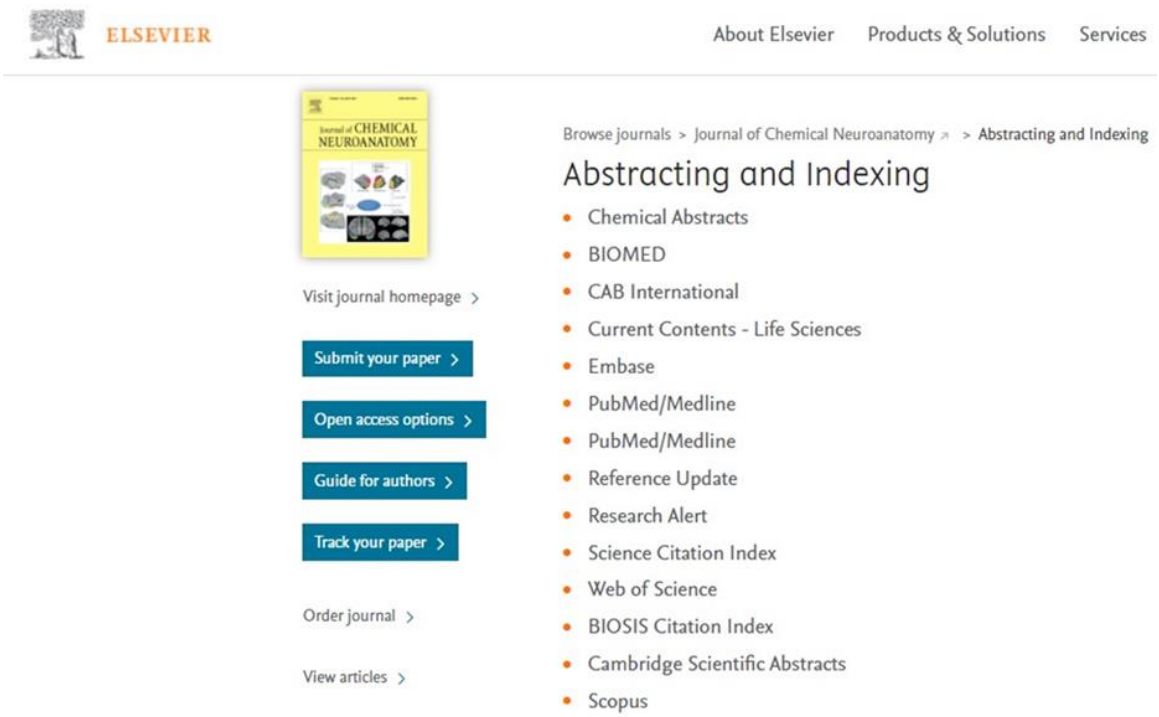

ACKNOWLEDGEMENT

It is my privilege to express my profound sense of gratitude and indebtedness to my mentor **Prof. Pravir Kumar**, Professor and Head in the Department of Biotechnology, Delhi Technological University for his valuable guidance and consistent encouragement during the progress of the project work. The dissertation wouldn't be completed within a short period without his insightful suggestions and support. I am equally grateful and wish to express my wholehearted thanks to respected research scholars of our lab, Mr. Rohan Gupta, Mr. Rahul Tripathi, Mr. Sudhanshu Sharma, Ms. Smita Modi and Ms. Dia advani for their kind support and help in the course of my research work. I also express my deepest gratitude to all the faculty members of Department of Biotechnology for constant motivation. I am highly thankful to Mr. Chhail Bihari and Mr. Jitendra Singh for their support. On a personal note, I wish to express my gratitude and affection to my family for their constant love and support. I would like to thank all my friends for their boundless love and trust which motivated me to complete the project work in the given time.

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ABSTRACT

Neurodegenerative disorders are conditions arising from the loss of structure and function of neurons. An alarming number of people are affected by them around the world. These disorders cause serious health conditions and ultimately death. Some of these include Parkinson's disease, Alzheimer's disease, and Huntington's disease among others. Currently, there are no treatments that can cure these diseases but their progression can be slowed down. Our approach is to study the use of FOXO proteins as therapeutic targets for the effective treatment of such neurodegenerative disorders. FOX family proteins are a group of forkhead transcription factors consisting of several subclasses from 'A' to 'S' but will keep our focus on the FOXO subclass which are present in all cells of the body and help in the growth, development, and maintenance of cells. FOXO proteins are basically transcription factors that bind to DNA through their DNA binding domain and bring about expression regulation in the cells. FOXO proteins subclass is further divided into 6 more members i.e. FOXO1 to FOXO6. But in neuronal cells, their specific expression was observed that makes a scope for them to facilitate neuronal cell protection by regulating and interacting with different signal transduction pathways. In the FOX family, the most relevant and effective targets will be FOXO proteins for neurodegenerative disorders because the pathways they regulate can actually help in blocking neurodegeneration and provide scope for treatment.

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ABBREVIATIONS

AD: Alzheimer’s disease

PD: Parkinson’s disease

HD: Huntington’s disease

IGF: Insulin like growth factors

EPC: Endothelial progenitor cells

ROS: Reactive oxygen species

NADPH: Nicotinamide adenine dinucleotide phosphate

XO: Xanthine oxidase

ER: Endoplasmic reticulum

SOD: Super oxide dismutase

GSH: Glutathione

Aβ: Amyloid plaques

NFT: Neurofibrillary tangles

DA: Dopaminergic neuronal

SNc: Substantia nigra pars compacta

PINK: Tensin homolog-induced putative kinase

TFBEs: Transcription factor binding events

DG: Denate gyrus

CHAPTER 1

INTRODUCTION

The life expectancy has increased drastically owing to the advances in medical sciences but with this, the diseases that are old age-dependent also have started to become predominant [1] Alzheimer's disease, Parkinson's disease, Huntington's disease, frontotemporal dementia, spinocerebellar ataxias, and amyotrophic lateral sclerosis are some examples of neurodegenerative disorders. These disorders have different etiology and cause either loss of memory or loss of motor functions. Researchers around the globe are working on model organisms of these disorders to understand the etiology and mechanism so that therapeutic approaches can be designed [2]. Model organisms have provided deep insights into the development and progression of neurodegenerative disorders [2]. Using this data therapeutic approaches are designed to treat such neurodegenerative disorders.

In neurodegenerative disorders, where neurons are affected as the prone population of neurons are gradually destroyed and is different from the invariable loss of neurons in diseases that are due to toxicity or metabolic imbalances. Neuronal dysfunction in neurodegenerative disorders is generally due to oxidative stress, neuroinflammation, protein accumulation, proteotoxic stress, and apoptosis [3]. Apart from the above-mentioned neurodegenerative disorders, there are many more which are not discussed and even are ignored in neuroscience as a huge focus is on only a few popular ones but those not discussed are equally serious conditions [4]. At the beginning of these disorders, different parts of the brain are affected and may show overlapping mechanisms and symptoms which makes them difficult to accurately classify. So the predominant symptom

or lesion in the brain part can be used to classify these disorders. Like when we talk about dementia then the main problem lies with some damage in the cerebral cortex which can have clinical signs that can be either dementia-like conditions or no dementia-like symptoms at all. If in this case dementia is a symptom then in most similar cases the Alzheimer's disease (AD) can be a predominant reason for this but there are at least 50 more diseases where dementia can be a clinical sign [5]. Also suffering from dementia cannot always be related to neurodegenerative disorders because damage to the brain due to other factors like toxicity, trauma, infections, etc., are also possible causes. The causes of neurodegenerative disorders are a topic of research and scientific debates but there is an understanding that heredity and environmental conditions are important areas to search for answers for this problem. If we study its genetic basis then it was found to be an autosomal dominant trait to be passed on to the next generation. But in contrast to this in few cases X-linked, autosomal recessive, maternal inheritance traits have been observed in patients when their family tree is studied. These studies support molecular bases for neurodegenerative disorders but when AD and PD patients are studied for this then irregularities have been observed. Only a slight percentage of patients are found to be following this basis strictly others have gaps that are needed to be filled with more focused research [4].

Various physiological and pathological mechanisms of the body are regulated by FOXO transcription factors like cancer development, aging, and the development of neurological disorders [6][7]. In the study of FOXO, it has been found that there is a correlation between its activation and the increased life of a cell and so the organism [8]. So they can help increase the lifespan of the organism and can also be studied as therapeutic molecules for age-related disorders. Forkhead transcription factors (FOX family) are a set of transcription factors that have a winged helix-like structure for its DNA binding domain which is named forkhead box and the FOXO are a subclass of the complete FOX family [9]. FOXO family in mammals include FOXO1, FOXO3, FOXO4, FOXO6 but other organisms like drosophila contain only one member and they are different from each other only in their expression within different cellular and tissue-specific environments. So basically they are transcriptional activators and they are regulated by signaling cascades of insulin and growth factors. The PI3K-AKT signaling pathway is upregulated by AKT and other similar

pathways like SGK which phosphorylates three conserved residues of FOXO when insulin or insulin-like growth factors (IGF) are present due to which FOXO cannot be retained in the nucleus and loses its transcriptional function [10][11][12][13]. The targets of FOXO which were discovered first include genes for metabolism and stress resistance as whenever the cells are not generating enough energy or when insulin or insulin-like growth factors (IGF) are not present then FOXO accumulates in the nucleus and stimulates activation of such genes [12][14][15]. FOXO responds to different kinds of cellular stimuli like oxidative stress, so it is proposed that they undergo post-translational modification that can be recognized by the molecules they bind and can regulate specific gene expression [16]. Protein kinase that responds to stress also phosphorylates FOXO at different sites to regulate it. FOXO can be observed as link between many pathways that respond to different kind of stimuli in the cell, so understanding the complete mechanism can help in use of these factors to increase the life of cells. Thus FOXO regulates the expression of genes that do quality control for the cell and its role in maintaining proteostasis maybe because it functions as a pro-longevity factor in the cell. FOXO interacts with many other regulatory pathways in the cell that control aging, apoptosis, autophagy and also its interaction with mTOR pathway give it a main role in neurodegenerative disorder regulation as this pathway regulates aging in cells [17]. Various mouse models have been developed to understand the etiology of neurodegenerative disorders and to find new effective therapeutic approaches for them. **Table 1.1** summarizes few important mouse models of Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD). **Figure 1.1** gives a schematic representation of etiopathology, risk factors and development of neurodegenerative disorders with key therapeutic approaches for their treatment.

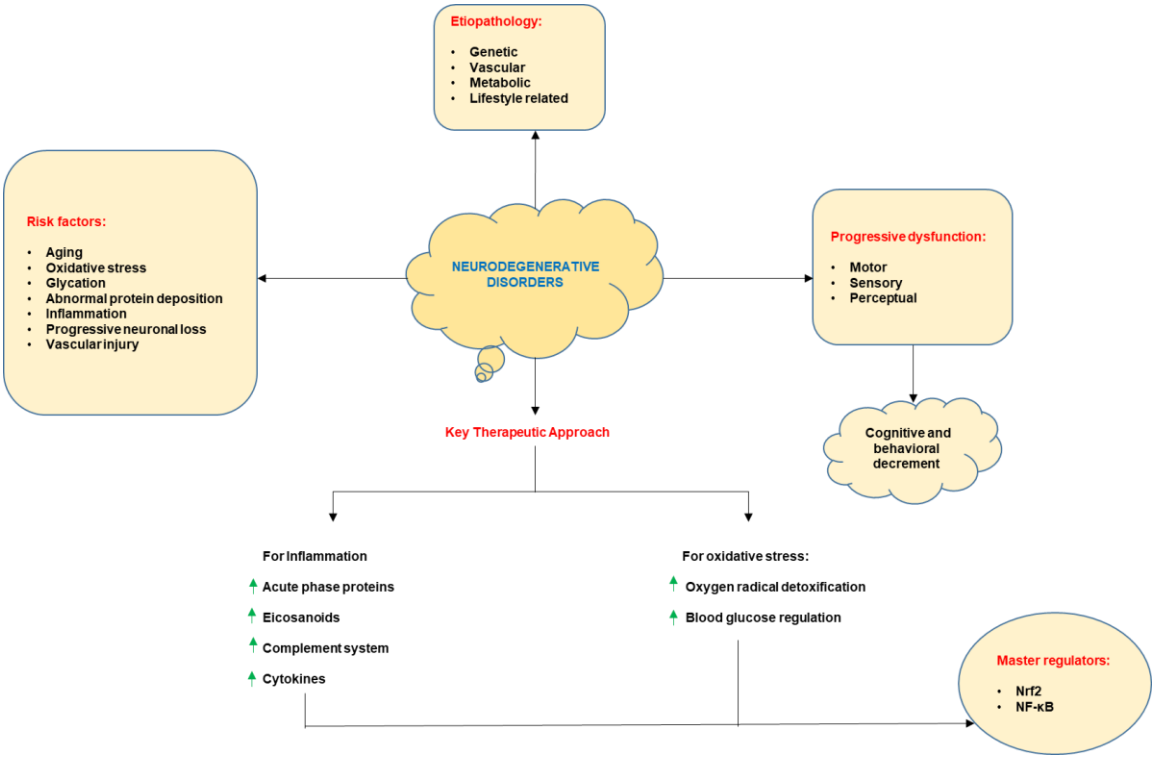

Figure 1.1: Schematic diagram of the risk factors, etiopathology, causes and key therapeutic approaches for neurodegenerative disorders. Aging, oxidative stress and inflammation among others are the factors which make an individual prone to neurodegenerative disorders. Also certain lifestyle adopted, genetic factors, vascular and metabolic factors can induce neurodegenerative diseases. Motor, sensory and perceptual function of the brain is affected in such disorders which leads to cognitive and behavioral decrement which is a major symptom of these disorders. Therapeutic approaches can be designed based on reducing inflammation and oxidative stress in the neuronal cells. Key approaches can be cytokine therapy for inflammation and oxygen radical detoxification for oxidative stress. Nrf2 and NF-κB can regulate both inflammation and oxidative stress.

Table 1.1: Some important mouse models for Alzheimer's disease (AD), Parkinson's disease (PD) and Huntington's disease (HD).

Mouse models	Disease	Mechanism	Type	References
rTg4510 mice	AD	Reversible binary transactivator system produces tau overexpression.	Genetic (Random transgene integration)	[18]
KO based mice	AD	Open reading frames of genes like APP, MAPT, BACE1 are disrupted.	Genetic (HDR-based)	[19][20][21]
3 × Tg strain of mice	AD	Two constructs are injected simultaneously, one is APP expressing and the other is P301L mutant tau type.	Combinatorial genetic	[22]
hTau mice (trauma based)	AD	Injury or trauma to the brain for tau pathology development.	Non- genetic	[23]
LRRK2 mutations based mice	PD	Inclusion formation is affected and slight dopaminergic neurodegeneration.	Genetic (Viral vector mediated)	[24][25]
SNCA transgenic mice	PD	Large amount of α-synuclein aggregation.	Genetic (point mutation)	[26]
UCH-L1 mutations based mice	PD	Dopaminergic neurodegeneration induced.	Genetic (I93M mutation)	[27]

Nurr1 deficient mice	PD	Dopaminergic neurodegeneration is induced.	Genetic (gene knock-out)	[28]
R6/2 mice	HD	N-terminal fragment of htt gene (exon-1) with 144-150 CAG repeats at exon-1 is expressed.	Genetic (transgenic)	[29]
N171-82Q mice	HD	N-terminal fragment with exon-1 and exon-2 of htt gene withy 82 polyglutamines are expressed.	Genetic (transgenic)	[30]
HdhQIII	HD	111 CAG repeats are inserted into murine HD gene.	Genetic (gene knock-in)	[31]
BACHD mice	HD	170 kb of human huntingtin locus is expressed.	Genetic (transgenic)	[32]

CHAPTER 2

FOXO FACTORS IN CELL PROLIFERATION, CELL CYCLE, AND REGULATORY PROTEINS.

In coronary heart disease, it is known that the destruction of vascular endothelial cells is an important vascular physiopathological aspect. Endothelial progenitor cells (EPCs) that circulate in the blood can move towards the injured site of ischemic tissue or blood vessels and keep the integrity of endothelial cells thereby differentiating into mature ones. This can be seen as a regenerative medicine approach [33]. But patients of this disease usually have impaired EPCs and their number is also low in circulation [34] due to aging and other cardiovascular risk factors [35]. So by therapeutic intervention, we need to increase the number of EPCs and make them functional. Many studies have demonstrated in glioma cells, endothelial cells, and vascular smooth muscle cells that FOXO factors have the ability to negatively regulate proliferation [36]. Also, it was found that FOXO3a expression is increased during oxidative stress in the EPCs but there is no effect on FOXO1 and FOXO4 [37]. FOXO3a is activated upon dephosphorylation by Akt and inhibits cell proliferation as it enters into the nucleus from the cytoplasm. Cyclin-dependent kinase inhibitor p27kip1 at the protein level is accumulated due to which cyclin/CDK complexes are prevented which are required to progress into S phase by the cell, as a result, the cell cycle is arrested at Go/G1 phase [37]. In a study by Tiantian Sang, Qing Cao et al, they have explored the mechanism of the proliferation of EPCs and regulation of cell cycle proteins by overexpression and inhibition of FOXO3a respectively. In this study, EPCs were first isolated from the umbilical cord of a human donor and were then cultured in vitro. 3 recombinant adenovirus vectors were made, namely Ad-TM (triple mutant)-FOXO3a, Ad-shRNA-FOXO3a, and Ad-GFP. In the Ad-TM (triple mutant)-FOXO3a, Thr-32, Ser-253, and Ser-315 are the sites of Akt phosphorylation are replaced by alanine residues so that Akt cannot phosphorylate this type of construct. After that transfection was done, then the transfected cells were stored for further investigation. Flow cytometry was performed using propidium iodide stain for cell cycle assay and fluorescence-activated cell

sorting (FACS) was used to measure DNA content. Finally the western blot analysis was performed to obtain results [38].

Brief transfection of CCK-8 assay was done to check the effect of FOXO3a on EPC proliferation and for that cell, viability readings were taken after 24, 48, and 72 hours. At the three-time points, transfection with Ad-TM-FOXO3a resulted in a strong reduction in EPC proliferation whereas compared to Ad-GFP transfection. Also, the results with transfection with Ad-shRNA-FOXO3a show strong enhancement in the proliferation of EPCs. So it was concluded from the CCK-8 assay that EPC proliferation is reduced by the presence of FOXO3a [23].

Flow cytometry observations to check the effect of FOXO3a on the cell cycle show that Ad-TM-FOXO3a transfected EPCs clearly show arrest at the G1 phase. When FOXO3a is silenced then there is a significant increase in proliferation of EPCs and also the percentage of cells in S and G2 phases i.e. the proliferation index is very high compared to Ad-GFP transfected EPCs. This means the silencing of FOXO3a, triggers the cell cycle progression. So this can be explained in this way that, PI3K/Akt signaling pathway which is related to FOXO3a, prevents cell cycle progression from G1 to S phase, and thus the proliferation of EPCs is blocked [23].

After transfection, a western blot was performed to check the effects of FOXO3a on regulatory proteins of the cell cycle. The expression of PCNA protein, which is related to the synthesis of DNA in the EPCs was significantly decreased in the Ad-TM group and significantly increased in the Ad-shRNA group when compared to GFP, the control group. Thus it was concluded that FOXO3a can reduce cell proliferation in EPCs and can be related to PCNA protein levels. In addition to this, it was found that p27kip1 which regulates cell cycle transition from G1 to S phase (Toyoshima & Hunter, 1994) and is a cyclin-dependent kinase inhibitor was upregulated by overexpression of FOXO3a and was downregulated when FOXO3a was inhibited. Thus it can be concluded that in EPCs expression of p27kip1 protein is seen when there is overexpression of FOXO3a. CDs like p27kip1 suppress complexes like cyclin D1/CDK6 and cyclin E/CDK2 in G1 to S phase progression of the cell cycle (Chu et al., 2008). Western blots for cyclin D1 and CDK2 expression show that EPCs transfected with Ad-TM-FOXO3a and Ad-shRNA-FOXO3a

downregulate and upregulate levels of their expression respectively. So based on the presence or absence of FOXO3a it may be possible that cell cycle regulatory proteins can be regulated by it and thus it can control the transition of the G1 to S phase, blocking the proliferation of EPCs (Sang et al., 2014). This may be applied to other cells of the body and the neuronal cells too as a regenerative medicinal approach. Various protein kinases and signaling molecules that activate and inhibit FOXO which leads to regulation of genes that control major cellular processes are shown in **Figure 2.1**

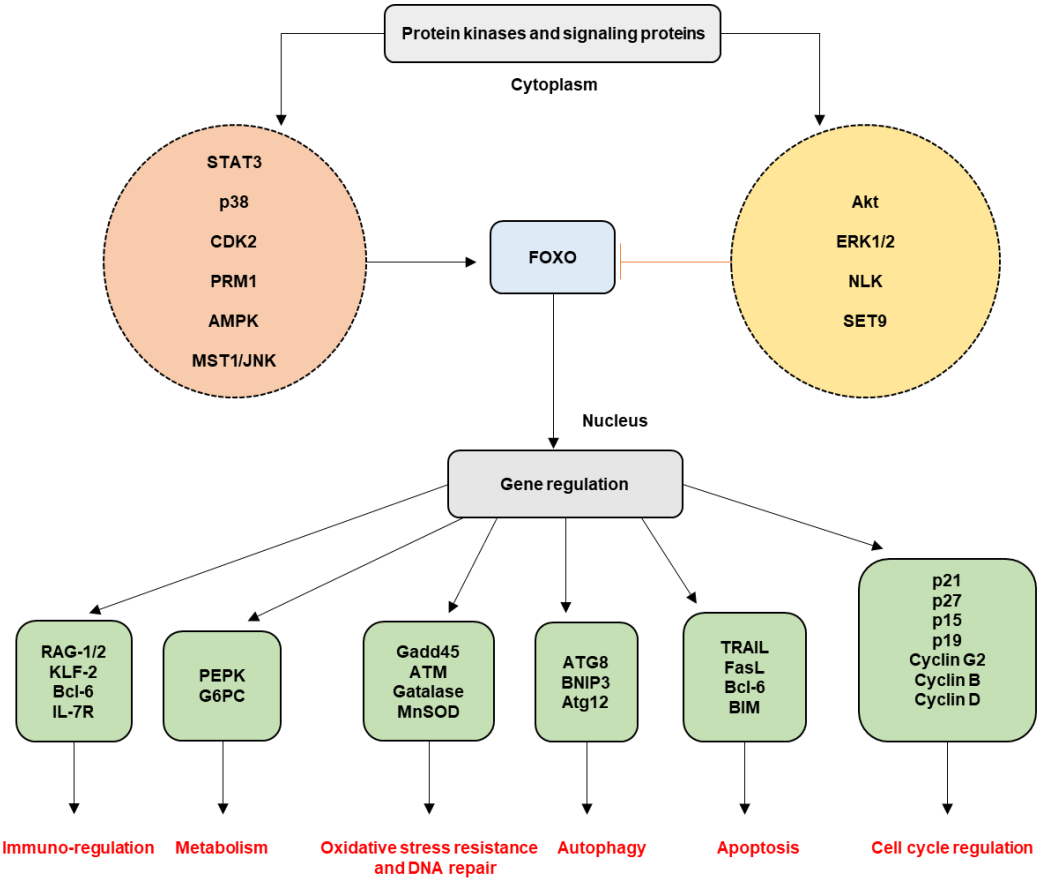

Figure 2.1: FOXO regulates major cellular processes by regulating expression of downstream genes. Various protein kinases and signaling proteins interact with FOXO in the cytoplasm of the cell to activate or inhibit it for nuclear translocation. STAT3, p38 among others can activate FOXO in the cytoplasm whereas Akt, SET9, NLK, ERK1/2 can inhibit activation of FOXO. After the translocation of FOXO in the nucleus, it interacts with downstream genes and regulates them to control major cellular processes like metabolism, apoptosis and various others for maintaining the necessary functions and life of the cell.

CHAPTER 3

FOXO PROTEIN EXPRESSION AND FUNCTION IN NEURONAL CELLS

Recent studies on *C.elegans* have helped us to understand the expression and function of FOXO in neuronal cells. In these organisms, FOXO ortholog, called DAF-16 is present, which is responsible for regeneration, stress resistance, memory, and learning in neurons. To investigate similar features of FOXO in neurons of mammals should be the next step for its better understanding. A therapeutic approach for neurodegenerative diseases can be designed if know the function of FOXO in neurons which are healthy, aged, or damaged by any one of such disorders [39]. In neuroscience, *C.elegans* has emerged as a suitable model for FOXO studies because of its simplicity, neuronal wiring that is stereotyped, cell lineage invariance, and behavioral plasticity. Each of these worms has exactly 302 neurons and consistent connectivity of cells between these animals can be observed [40]. So based on its specific position, morphology, and connectivity each neuron can be identified. Studies on the behavior of this worm and new approaches in genomics have identified the neuronal function of DAF-16/FOXO in memory and learning [41].

A study by Murakami et al. found that associative learning is regulated by the insulin/IGF1 pathway. They used an isothermal tracking assay in which the worm learns to associate food with temperature and this activity declines with age. The authors found that age-1 (PI3K) and daf-2 mutants have delayed age-related decay in this assay by increasing daf-16 activity. Suppression in the delay can be observed with any mutation in DAF-16. So it was concluded that daf-2 activity reduces associative learning and DAF-16 activity increases it in the insulin/IGF pathway [42]. Another method i.e. is salt chemotaxis learning in which salt starvation conditioning is used, where organisms learn to associate salt with the absence of food. The salt aversion that is learned in this assay requires insulin/IGF-1 signaling because age-1, daf-2, and akt-1 can chemotaxis towards salt in normal conditions but salt aversion cannot be developed by them. Worms that have PI3K/Akt signaling enhanced by daf-18/PTEN mutation have better associative learning than wild types in this

assay. DAF-16 in isothermal tracking experiments enhance the effect of associative learning while in salt chemotaxis experiments it has a slight effect to overcome the defect of daf-2. This suggests that other factors work downstream in insulin/IGF-1 signaling in the salt chemotaxis experiments. So it can be concluded that different types of the neuron are used for the two different learning skill set [43]. Tomioka et al. showed that AFD neuronal subtype is responsible for isothermal tracking and ASER neuronal subtype for salt chemotaxis learning [43].

Aging in *C.elegans* like in humans affect memory performance, but it was observed that daf-16 can regulate that too. In a study by Kauffman et al., a positive olfactory assay was developed to assess the age-related effect on learning and memory in these animals. This chemosensation is developed with AWS neurons which associate food with a particular odorant like butanone in the assay [44]. The first neuronal function that is lost due to age is long olfactory memory which is regulated by CREB and is similar in mammals. Daf-2 mutants show 3 times more short-term memory and enhanced long-term memory when compared to wild types. Although in such mutants longevity can be observed they do not show long-term memory performance. Also, it is observed that insulin/IGF-1 signaling regulates longevity in intestine cells but not in neurons. This suggests that daf-16 regulates a particular set of genes that are specific to the neurons [45]. More studies are ongoing to ascertain specific neurons that are directly regulated by DAF-16. And also it will be important to map the downstream mechanism of DAF-16 in different neuronal subtypes to understand why its expression is different for some neurons in which they maintain longevity and young age-dependent expression [39]. Also, studies on *Drosophila* found that FOXO ortholog in them i.e. dFOXO regulates life span by its activity on the fat body and not the neurons in the brain [46].

So the interesting question here is that can we compare this DAF-16 expression and function with FOXO in mammals and humans. Although studies have yet not given any strong evidence experiments with knockout mice do tell about its function in humans. Also, studies on cognitive processes have established a strong link between them and insulin/IGF signaling in humans. A specific allele of FOXO3 can be directly related to the increased cognitive ability of humans with age [47]. In the human brain, the four isoforms of FOXO

i.e. FOXO1, FOXO3, FOXO4, and FOXO6 are expressed [48]. Knockout mouse models for all the FOXOs have been made and it is observed that they can survive with a complete knockout of FOXO3, FOXO4, and FOXO6 but knockout of FOXO1 results in rare survival of the embryo [49][50]. The mouse models to study the function of FOXOs in the brain have several results. FOXO1 and FOXO3 have been found to regulate the anxiety-based behavior of the mouse [50]. Nucleus accumbens (ventral striatum) this also called the reward center of the brain is also regulated by FOXO3 and this was tested with behavioral response to cocaine [51]. And finally, FOXO6 is limited to regulate learning and memory in mammals and humans [52]. FOXO4 is the least expressed of all in the neurons of the brain [53]. Isoforms of the FOXOs have similar DNA binding domains but very different in other regions. The FOXOs can interact with different cofactors to regulate the expression of the genes in the hippocampus in the brain and also a large number of cellular processes [54].

Our main aim is to investigate whether like *C.elegans*, can FOXOs in mammals also can maintain neuronal function and protect them from the effects of aging. The cognitive ability of mammals is reduced with age as the activity of the neural system is disrupted with age [55]. The role of FOXOs in aging and its regulation will be complex but the expression profiling of the brain of humans has shown that there is dysregulation of synaptic genes due to increasing age [56]. These genes are homologous to FOXO6 in humans is not confirmed but FOXO6 does help in maintaining age-related effects of synaptic stability. Also, insulin/IGF signaling is known to be protective for an aging brain [57]. Survival of neurons, neurotransmission, synaptic stability, memory, and formation of neurons is assisted by insulin/IGF signaling but in Alzheimer's model mouse the knockout of IGF-1 receptor or Irs2 signaling has therapeutic effects [58][59]. So for now it can be deduced from these studies that insulin/IGF signaling and FOXO activation in the neurons of the brain can maintain cognitive function with aging. We can use their intentional stimulation as a therapeutic method to treat neurodegenerative disorders but for that more understanding of the underlying mechanisms is needed with extensive research. This also will enable us to more precisely define different function of all the isoforms of FOXO in neurons in different regions of brain and spinal cord [39]. **Table 3.1** has tried to summarize

expression of FOXO proteins in neuronal system and its corresponding effects that can help to design therapeutic approach for neurodegenerative disorders.

Table 3.1: Regulation of FOXO protein expression in neuronal system and its corresponding therapeutic effect.

FOXO protein	Neuronal system	Expression	Effect	References
FOXO1	Neural stem/progenitor cells (NSPCs)	Downregulated	Promotes NSPCs differentiation into neurons	[60]
FOXO3	Neuronal reprogramming	Downregulated	Production of induced neuronal cells (iN) from aged mice fibroblasts	[61]
FOXO3a	Mitochondrial activity regulation (for all cells including neurons)	Upregulated	Limits ROS production from mitochondria during hypoxic condition	[62]
FOXO4	Oxidative stress response	Upregulated	FOXO4 and ATXN3 interaction with SOD2 increases antioxidative response	[63]
FOXO6	Memory consolidation	Upregulated	Regulation of gene expression for	[52]

			synaptic function and number control and also neuronal coordination in hippocampus after memorizing	
DAF-16/ FOXO	Neurotransmission of pheromone signals	Upregulated	Maintains glutamate homeostasis in hippocampus of mouse and head of *C.elegans*	[64]
dFOXO	Oxidative stress sensitivity	Downregulated	DJ-1 inhibits dFOXO through PI3K/AKT which leads to inhibition of DLP production and apoptosis in drosophila	[65]

CHAPTER 4

OXIDATIVE STRESS AND ROS IN NEURONAL CELLS IN NEURODEGENERATIVE DISORDERS

Neurodegenerative disorders are those in which there is a progressive loss of neurons. The correct mechanism for these disorders is yet not confirmed but oxidative stress is a major cause [66][67]. Oxidative stress can be defined as an imbalanced redox state due to disruption in antioxidant processes or the production of large numbers of ROS (reactive oxygen species). It can cause cell damage and can malfunction the DNA repair mechanism of the cell [68]. Also, it can cause a malfunction in mitochondria and all these factors lead to the process of aging and progression of neurodegenerative disorders [69][70]. The brain has plenty of peroxidation susceptible lipid cells and has excessive oxygen demand so is at risk for damage by ROS. In both Alzheimer's disease and Parkinson's disease, oxidative stress plays a major role and it is anticipated that antioxidants can be used to treat neurodegenerative disorders but the clinical outcomes have been very inconsistent [71]. To treat neurodegenerative disorders efforts are made to overcome oxidative stress.

4.1 Reactive Oxygen Species (ROS)

The outer shell of the oxygen contains two unpaired electrons due to which it can take part in radical formation. So very reactive molecules that are formed from oxygen are called reactive oxygen species (ROS) [72], they have 2 unpaired valence electrons so they are very reactive and have a short life [70]. Some examples of ROS are superoxide (O_2^-), hydroxyl radical (·OH), and hydrogen peroxide (H_2O_2). –OH is the main ROS produced and has cytotoxic effects [72]. It can be produced from H2O2 and O2- where iron ions act as catalysts formed from Fe2+ based decomposition of H2O2 [73].

4.2 ROS in Brain

Endogenous and exogenous sources both are responsible for ROS production in cells [74]. Drugs, ionizing radiations, UV rays are exogenous sources of ROS production. Toxic substances in chemicals and the environment also cause the production of ROS in the form of byproducts upon metabolism [74]. Mitochondria and enzymes like Nicotinamide adenine dinucleotide phosphate (NADPH), Oxidase (Nox), Xanthine oxidase (XO), Flavin oxidases from peroxisomes, and Cytochrome P450 present in the endoplasmic reticulum (ER) are the endogenous sources of ROS formation. The main source of ROS formation remains the mitochondrial respiratory pathway and the Nox system [75]. Antioxidants can be used as a treatment method to protect from the damage caused by excessive ROs accumulation. For this treatment antioxidants in the form of enzymes or small molecules can be used [66]. Superoxide dismutase (SOD) can be used to break down reactive oxygen into more stable H2O2 and O2 [76]. SOD inactivates oxygen radicals and produces H2O2. Then by the activity of glutathione peroxidases, peroxiredoxins, and catalase, further degradation of H2O2 takes place [77].

4.3 Physiology of ROS

Certain levels of ROS are necessary for maintaining metabolic pathways and cellular signaling [78][79]. It helps in regulating different cellular activities like mitosis, proliferation, survival, migration, gene expression, and apoptosis [70][79][80]. Transcription factors that regulate cellular responses to ROS stimulations are also activated by ROS [70]. Also in some cases, the increased level of ROS can support an antioxidant-based defense mechanism, like Nrf2 is a redox-based transcription factor. It is activated by the presence of high levels of ROS and in turn regulates the expression of enzymes like SOD, PRX, GPX [81][82]. Under normal conditions, Nrf2 is inactive due to a protein Keap1 which suppresses its action by blocking its translocation to the nucleus [82]. When levels of ROS are high the interaction between Keap1 and Nrf2 is hindered so it can translocate to the nucleus and gets activated [83]. Nuclear factor-kappa B is another important transcription factor that is regulated by ROS, NF-κB is present in an inactivated form in the cytoplasm due to interaction with its inhibitor present there. ROS presence in moderate levels stimulates, phosphorylation and degradation of the inhibitor and in turn

activating NF-κB [84]. And this in return helps to block the caspase-based cell death pathway and production of anti-apoptotic proteins [85]. Also, excessive ROS can again inactivate NF-κB as it can block its interaction with DNA and support apoptosis [86]. So it can be concluded that the amount of ROS formation can regulate pro-survival transcription factors [85].

4.4 Oxidative stress

Oxidative stress is a condition in which the levels of ROS are excessively formed due to imbalances in between its formation and antioxidant mechanism [87]. Oxidative stress can cause damage to the protein structure and function of the cell as a result of protein oxidation, the membrane due to lipid peroxidation, and damage to the structure of DNA [66]. In the brain, many metabolic activities take place due to which it is at high risk from damage with oxidative stress. Firstly it has high oxygen demand which is almost 20% of the oxygen needed by the human body. Second, iron and copper are present in the brain in enough quantity to readily catalyze ROS production. Third, the polyunsaturated fatty acids present in cell membranes of the brain, act as lipid peroxidation substrates [88]. And finally the antioxidant enzyme GSH, in present in low quantity in the brain, can degrade ROS if present in adequate levels and act as an endogenous antioxidant [89].

4.5 Oxidative stress and Neurodegenerative disorders

The most common neurodegenerative disorder is Alzheimer's disease (AD), and it can be identified by gradual loss of neurons and accumulation of extracellular amyloid plaques (Aβ) and intracellular tau tangles (neurofibrillary tangles, NFT) can be observed that are proteins that can be considered as hallmarks of AD [90]. So from studies, it is suggested that imbalance in the oxidative stress in neurons leads to neuronal damage which may be the main cause of the initiation and progression of AD [91]. Although the source of increased ROS formation is yet to be identified and the mechanism of redox imbalance remains unknown but it can be confirmed that mitochondrial dysfunction is caused in the patients suffering from AD due to the accumulation of high levels of ROS [92]. In mouse models of AD, that express mutant amyloid precursor protein (APP) and presenilin-1 (PS-1) it is observed that excessive accumulation of H_2O_2 and lipid and protein oxidation, suggesting that Aβ may increase oxidative stress in AD [93]. The formation and

aggregation of extracellular amyloid plaques (Aβ) and enhanced phosphorylation of tau proteins due to oxidative stress and causes dangerous events of pathogenesis in AD [92]. Also in the AD mouse model with APP overexpression with deletion of cytoplasmic/zinc SOD in the Tg2576 is because of increased oligomerization Aβ and serious memory disruption [94]. Enhanced formation of Aβ in neurons can be related to downregulation of activity of α-secretase and upregulation of the expression and activity of β and γ-secretase by oxidative stress [95]. There is evidence that cells with excessive tau proteins are prone to oxidative stress due to a decrease in the number of peroxisomes [96]. Also, the mouse model that expresses mutant (P301S and P301L) tau protein has less activity of NADH-ubiquinone oxidoreductase and mitochondrial dysfunction, which are due to excessive ROS accumulation in neurons [97][98][99]. Mainly Aβ can be found in extracellular regions but it also accumulates in the endoplasmic reticulum, mitochondria, and Golgi apparatus [100]. Mitochondrial dysfunction that is caused by Aβ, blocks sufficient production of ATP and enhances ROS production in AD [101]. This is supported by observations that show reduced metabolism of energy in the brain during AD [91].

A neurodegenerative disorder in which there is a specific loss of neurons (dopaminergic neurons) in the substantia nigra pars compacta (SNc) and in the nigrostriatal DA (dopaminergic neuronal) pathway in the brain, levels of DA are declined is Parkinson's disease (PD) [102]. Oxidative stress, like in AD, here also plays a major role in initiation and progression [103][104]. Studies have found that in the respiratory chain in substantia nigra pars compacta (SNc) of patients with PD, there is a decrease in the activity of Complex I due to which high levels of ROS are produced and this leads to apoptosis [103][104][105]. It was observed that mutations in proteins like α-synuclein, tensin homolog-induced putative kinase (PINK), parkin, and phosphatase are related to familial forms of PD. Disruption of mitochondrial function and a high level of oxidative stress are observed due to these mutations [105]. In PD, the iron presence is high in DA neurons causes the formation of very toxic hydroxyl radicals (OH) which are formed by the interaction of ferrous ions with H2O2. The chance of survival of DA neurons is low in presence of increased levels of iron in substantia nigra pars compacta (SNc) [106]. Deletions and point mutations have been observed in the subunit of mitochondrial DNA encoding Complex I in patients of PD [107][108]. **Figure 4.1** summarizes various ROS

sources for brain that can lead to high levels of ROS in brain which may induce neurodegeneration.

Figure 4.1: Major sources of ROS production and their effects in the brain. NADPH oxidase, Xanthine oxisae, Monoamine oxidase and mitochondria are major ROS producers for the brain and lead to accumulation of superoxide (O2-) and hydrogen peroxide (H2O2) in the brain. ROS accumulation in the brain results into oxidative stress in the neuronal cells. This results into increase of 8-hydroxydeoxyguanosine, malondyaldehyde, 4-hydroxynonenal and protein carbonyls in the brain. ROS mediated injury and protein oxidative damage in the brain leads to neurodegeneration.

CHAPTER 5

REGULATION OF OXIDATIVE STRESS AND ROS BY FOXO AND RELATED TFBES

FOX transcription factors regulate multiple cellular processes including stress resistance, metabolism, proliferation, immune response, and apoptosis [109]. The activation or deactivation of the FOX family can be regulated by growth factors like IGF (insulin-like growth factor), which can support FOXO phosphorylation at the C-terminal side with the help of protein kinase B (AKT/PKB) due to which it cannot be retained in the nucleus and cannot perform its function as its nuclear localization signal is masked(NLS) [110]. Also, posttranslational modifications like phosphorylation through the JNK pathway can help the inactivation of FOXO with ROS presence [111]. Jun N-terminus kinase (JNK) and STE20-like protein kinase 1 (MST1), phosphorylates FOXO at sites other than the 14-3-3 site, so that the FOXO can be retained in the nucleus and this effect is dominant over the inhibitory effect of Akt on FOXO in the nucleus [112]. Also, methylation and ubiquitination [113] induce regulation of FOXO, that's why they have a major function in redox signaling [114]. FOXO has a very conserved DNA binding domain and has a definite function in a particular tissue. Thus the pathway that regulates FOXO in that cell will determine its role for the cell [115]. The expression of glucose-6-phosphatase and phosphoenolpyruvate carboxykinase is controlled by FOXO1a, thereby regulating gluconeogenesis and glycogenolysis and controls the checkpoints in the cell cycle by decreasing the cyclin D levels [116][117]. Enzymes like catalase and Mn-SOD which act as antioxidants are upregulated by FOXO1a and FOXO3a and protects against oxidative stress [118][119]. Apoptosis is also affected by FOXO3a as it can regulate the expression of proapoptotic factors like Bim, PUMA, and antiapoptotic factors like FLIP [110]. In a study by Nancy P. Gómez-Crisóstomo, Erika Rodríguez Martínez, and Selva Rivas-Arancibia [120], a mouse model for neurodegeneration which was exposed to low doses of ozone. This model concluded that with ozone's chronic exposure, the brain can get damaged due to oxidative stress induced [115]. Further, it was used to study the role of FOXO and related TFBEs in

the regulation of oxidative stress. pFOXO3a is the phosphorylated form of FOXO3a and is used to detect its activation. In the rat hippocampus, an increase in immunoreactivity of the dentate gyrus (DG) was observed and it was visible from 15 to 60th day of exposure to ozone. FOXO3a protein is present in the nucleus of the DG cells. In the western blot, it was evident that activation of FOXO3a takes place very significantly after 30 days and 60 days of ozone exposure which causes neurodegeneration [108]. After all the ozone exposure was done in the rat hippocampus, immunoreactive cells to FOXO1a were detected. As we increase the exposure time for ozone, a slight increase in such immunoreactive cells can be observed. The western blot for FOXO1a shows its increase in 15, 30 and 60 days of ozone exposure when compared with the control [108]. **Figure 5.1** shows AKT, mTORC1 and mTORC2 pathways that can regulate cell survival, proliferation and growth.

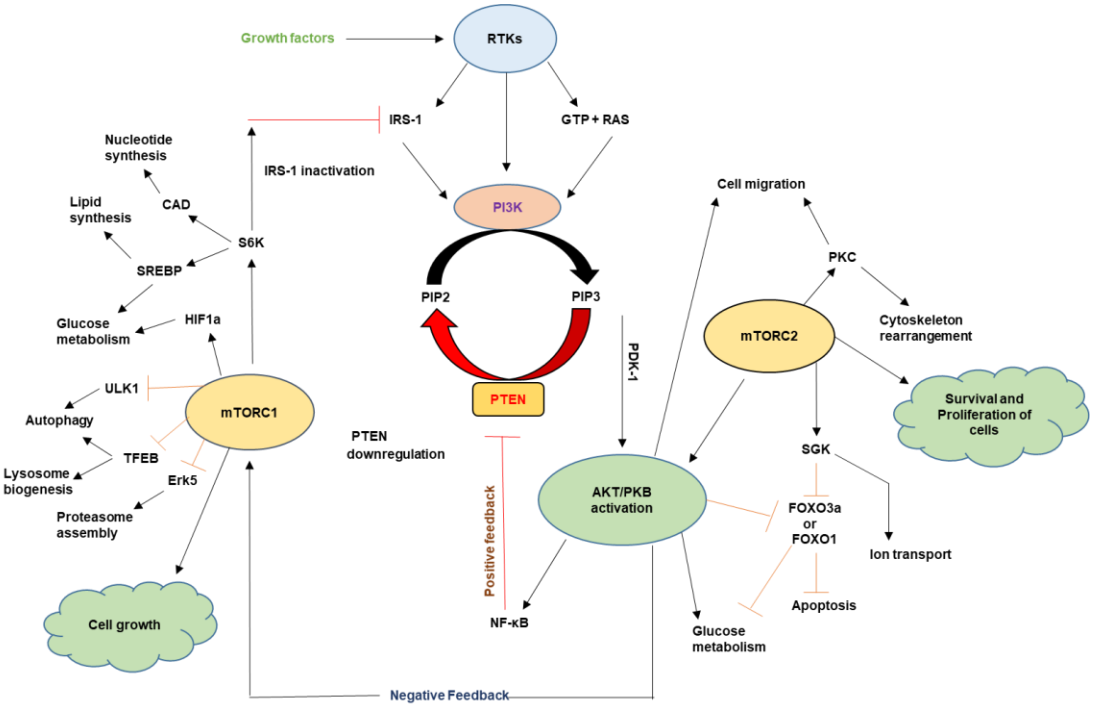

Figure 5.1: AKT and mTORC mediated signaling in cell survival, proliferation and growth. Growth factors activates the signaling cascade through RTKs which in turn through P13K forms PIP3 that activates AKT/PKB pathway. Further this pathway supports its own regulation by positive and negative feedback. In positive feedback, after AKT/PKB activation, NF-κB is produced which inhibits PTEN, that can convert PIP3 to PIP2 as it can block AKT. In negative feedback, mTORC1 after AKT/PKB activation can induce

S6K to inactivate IRS-1 so that no P13K is available and AKT/PKB remain in inactivated form. AKT and mTORC2 through intercellular signaling and interaction with FOXO1 and FOXO3a regulate glucose metabolism and apoptosis in the cell. mTORC2 through different signaling proteins maintain survival and proliferation of cells. mTORC1 through its interaction with signaling proteins and AKT/PKB aid in cellular growth.

A study by Kops et al. also found that [121] the increase in the activated form of FOXO3a can be related to an increase in the amount of Mn-SOD. This suggests that in response to the oxidative stress generated due to ozone exposure, FOXO3a has a regulatory role. Also, this study found that it may be possible that the activity of this enzyme is restricted due to damage to the structure of the enzyme caused by oxidative stress [122]. In many studies, it has been investigated that damage caused to neurons like in Alzheimer's disease, upregulates the expression and activity of proteins that regulate the cell cycle [123]. This mechanism can be related to neuron formation in the DG of the hippocampus from neuroblasts [124]. But due to the presence of such proteins in fully formed neurons, apoptotic pathways are activated [125]. Expression of cyclin D2 in the neurons can be observed after 7 days of exposure to ozone but this protein translocates to the nucleus after 30 days of exposure. The translocation of cyclin D2 can be linked with increased levels of active caspase 3 at 30 and 60 days of exposure which suggests that apoptotic pathways have been activated. Also due to neurogenesis, increase in cyclin D2 after short intervals of ozone exposure can be observed [120]. The number of neuroblast expand from 7 to 30 days of exposure but after 30 days, decrease in their numbers can be seen, concluding that neuronal repair mechanism is disrupted [126]. When there is no exposure, the neurons that are matured block signals that facilitate cell cycle reentry by phosphorylation of FOXO1a as it activates p27 which stops the production of cyclin D2 [117]. The significant formation of pFOXO1a cannot be directly linked to the downregulation of cyclin D2 by it [127]. The translocation of p53 to the nucleus, suggests the initiation of apoptosis in matured neurons [126]. Many studies have concluded that p53 and FOXO1a are linked with similar cell signaling pathways and can regulate the survival and apoptosis of cells [128]. As studied earlier, redox balance is very important for the regulation of these pathways, otherwise, the disruption in balance induces disruption in the pathways. So if DNA damage takes place due to it, then p53 can also block the function of FOXO1a [128]. So mature neurons face cell damage due to redox imbalance and the resulting activation of apoptosis by it

[120][126]. The same process can take place in neurodegenerative disorders that are due to chronic redox imbalance. **Table 5.1** represents clinical significance of FOXO transcription factors in some neurodegenerative disorders.

Table 5.1: Clinical significance of FOXO transcription factors in some neurodegenerative disorders.

Disease	Targeted pathology	Clinical significance of FOXO	References
Alzheimer's disease (AD)	Neurotoxic Aβ processing	Blocking of FOXO3 phosphorylation has a neuroprotective role	[129]
Parkinson's disease (PD)	Impaired autophagy	FOXO6 promotes expression of genes and proteins needed for autophagy	[130]
Huntington's disease (HD)	Cellular senescence	FOXO3 may stop cellular senescence by suppressing ETS2 and reducing p16INK4a levels	[131]
Spinocerebellar ataxia (SA)	Decreased antioxidant capacity	FOXO4 dependent SOD2 expression helps in reducing ROS levels	[132]
Frontotemporal dementia (FD)	Stress induced protein misfolding	TDP-43 promotes activation of FOXO that helps in expression of stress resistant genes and protein homeostasis	[133]
Amyotrophic lateral sclerosis (ALS)	Stress induced protein misfolding	TDP-43 promotes activation of FOXO that helps in expression of stress resistant	[133]

		genes and protein homeostasis	
Lewy body dementia (LBD)	Lewy body formation	FOXO3a, α-synuclein and 14-3-3 protein may interact to form a complex that can promote neuronal survival	[134]

CHAPTER 6

METHODOLOGY

1. FOXO3 and SOD2 genes were chosen as data sets.
2. Functional enrichment analysis was done using g:Profiler by entering the data sets and analyzing for results.
3. After the query run g:Profiler gives us a graph between significance and data obtained from query run.
4. Then this data was saved in gem and gmt files respectively for visualization analysis in cytoscape software.
5. Enrichment map is a app available in cytoscape which can be downloaded and helps in visualization of pathway from available data from g:Profiler.
6. This pathway can be analyzed, edited and results can be conferred for the selected data sets.
7. Autoannotate is another app present in cytoscape which was used to draw a summary network of the previously drawn pathway.
8. Finally all the output files were saved as pdf for publication purpose.
9. These outputs were then analyzed for interactions and mechanism of FOXO3 and SOD2 with neurodegenerative disorders.

CHAPTER 7

RESULTS

1. g:Profiler data analysis graph

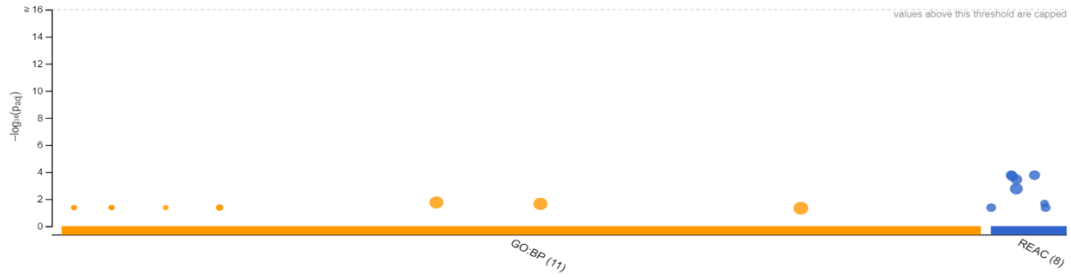

Figure 7.1: Graph between (significance) -log10 Padj Vs. GO:BP and pathways

- g:Profiler used to perform functional enrichment analysis (Figure 7.1).
- FOXO3 and SOD2 genes taken as data set.
- Gene ontology based on biological process was selected.
- Reactome pathways used in analysis of data.
- No electronic GO annotations allowed to avoid any false result.

2. Detailed P value analysis of biological processes and related pathways

- Both the genes are significantly involved in different biological processes and pathways related to neurodegenerative diseases (Figure 7.2).
- Overlaps were observed in gene ontology between them.
- Results were based on P values of significance.
- Bonferroni correction used to avoid any insignificant result during the search.
- Threshhold value taken as 0.05 for calculating significance.

GO:BP		stats						
Term name	Term ID	p_{adj}	$-\log_{10}(p_{adj})$ 0 ≤16	T	Q	T∩Q	U	FOXO3 SOD2
regulation of neuron apoptotic process	GO:0043523	1.761×10^{-2}		118	2	2	16566	
neuron apoptotic process	GO:0051402	2.239×10^{-2}		133	2	2	16566	
regulation of muscle atrophy	GO:0014735	4.226×10^{-2}		2	1	1	16566	
positive regulation of muscle atrophy	GO:0014737	4.226×10^{-2}		2	1	1	16566	
age-dependent response to oxidative stress	GO:0001306	4.226×10^{-2}		1	2	1	16566	
age-dependent general metabolic decline	GO:0007571	4.226×10^{-2}		1	2	1	16566	
regulation of systemic arterial blood pressure by neurotrans...	GO:0003070	4.226×10^{-2}		1	2	1	16566	
acetylcholine-mediated vasodilation involved in regulation ...	GO:0003069	4.226×10^{-2}		1	2	1	16566	
regulation of systemic arterial blood pressure by acetylcholi...	GO:0003068	4.226×10^{-2}		1	2	1	16566	
age-dependent response to reactive oxygen species	GO:0001315	4.226×10^{-2}		1	2	1	16566	
regulation of neuron death	GO:1901214	4.726×10^{-2}		193	2	2	16566	

1 to 11 of 11 Page 1 of 1

REAC		stats						
Term name	Term ID	p_{adj}	$-\log_{10}(p_{adj})$ 0 ≤16	T	Q	T∩Q	U	FOXO3 SOD2
Neurodegenerative Diseases	REAC:R-HSA-88...	1.700×10^{-4}		21	2	2	10543	
Deregulated CDK5 triggers multiple neurodegenerative pat...	REAC:R-HSA-88...	1.700×10^{-4}		21	2	2	10543	
Diseases of programmed cell death	REAC:R-HSA-96...	2.049×10^{-4}		23	2	2	10543	
FOXO-mediated transcription of oxidative stress, metabolic ...	REAC:R-HSA-96...	3.522×10^{-4}		30	2	2	10543	
FOXO-mediated transcription	REAC:R-HSA-96...	1.737×10^{-3}		66	2	2	10543	
RUNX3 regulates BCL2L11 (BIM) transcription	REAC:R-HSA-89...	2.134×10^{-2}		5	1	1	10543	
AKT phosphorylates targets in the nucleus	REAC:R-HSA-19...	4.268×10^{-2}		10	1	1	10543	
Regulation of FOXO transcriptional activity by acetylation	REAC:R-HSA-96...	4.268×10^{-2}		10	1	1	10543	

Figure 7.2: Biological processes and pathways with ID and P values which represents significance and overlap.

3. Enriched pathway of data analyzed

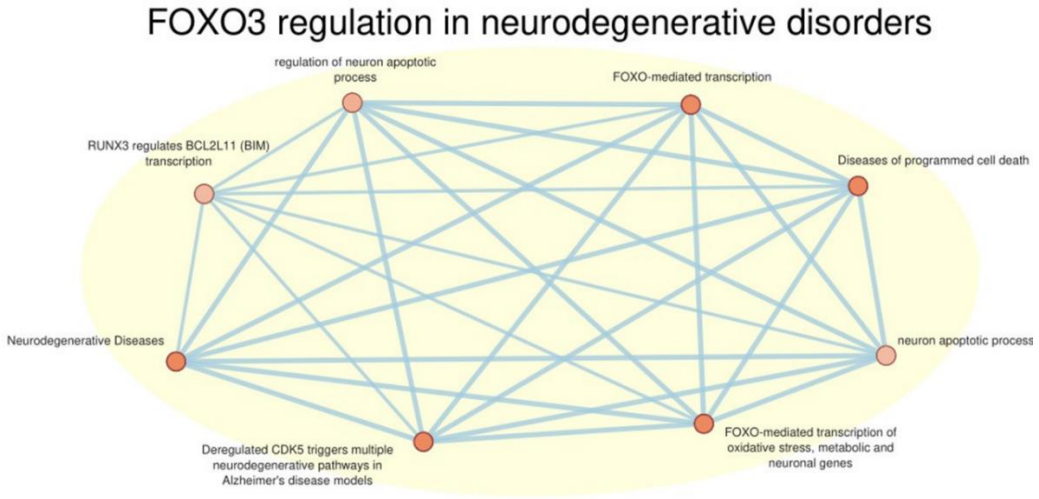

Figure 7.3: Enriched pathway Of FOXO3 regulation in neurodegenerative disorders.

- Enrichment map was constructed by using cytoscape (Figure 7.3).
- It is basically visualization of results and pathway enrichment from g:Profiler data.
- Darker nodes signify overrepresented data of gene ontology.
- Pathway shows FOXO3 is involved in regulation of major processes and pathways related to neurodegeneration.

4. Summary network of the drawn pathway

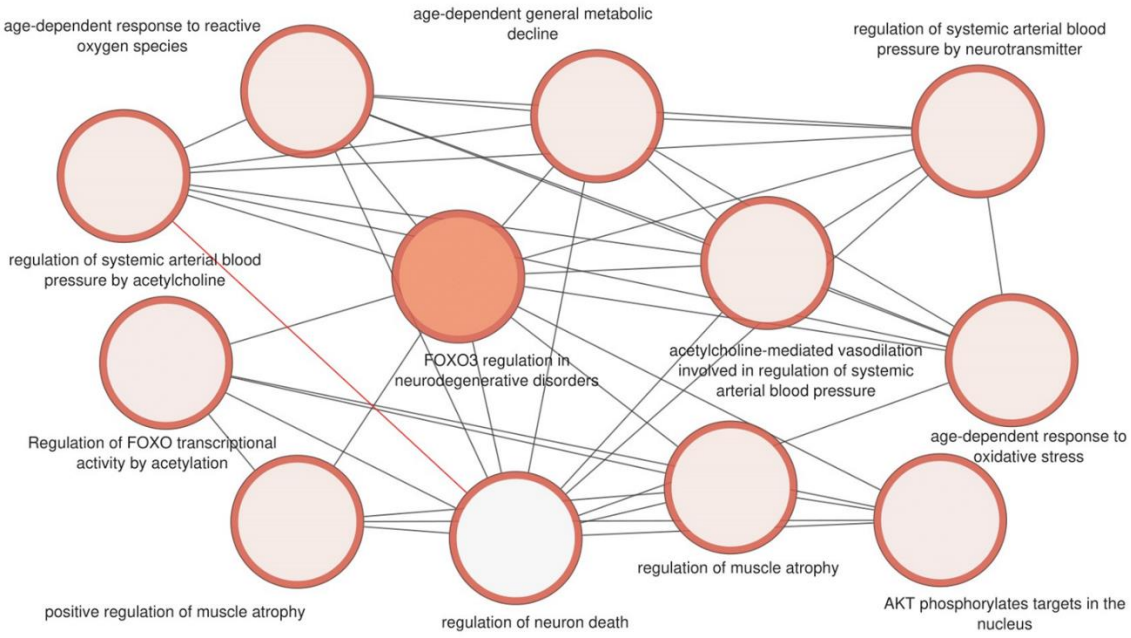

Figure 7.4: Summary network of the previously enriched pathway.

- Summary network of the enriched pathway was constructed in cytoscape (Figure7.4).
- For this Construction us AutoAnnotate app is present in cytoscape software.
- Nodes in the summary network represent major regulatory processes.
- Edges represent interrelation between the different processes an pathways.
- 12 nodes and 42 edges present in this summary network.

CHAPTER 8

DISCUSSION

- **FOXO3 and SOD2 genes are involved in theses pathways:**

1. Neurodegenerative diseases
2. Deregulated CDK5 triggers multiple neurodegenerative pathways in Alzheimer's disease models
3. Diseases of programmed cell death
4. FOXO-mediated transcription of oxidative stress, metabolic and neuronal genes
5. FOXO-mediated transcription

- **These genes were together involved in the regulation of following biological processes:**
    1. Neuron apoptotic process
    2. Neuronal death

- **FOXO3 was found significant in:**
    1. Positive regulation of muscle atrophy

- **SOD2 was found significant in:**
    1. Age-dependent response to oxidative stress
    2. Age-dependent general metabolic decline
    3. Age-dependent response to reactive oxygen species

CHAPTER 9

CONCLUSIONS AND FUTURE CONSIDERATION

FOXO transcription factors have specific functions in neurons that are tightly regulated by signaling pathways and environmental stimulations. In this project, our focus was on the role of FOXO3 and related transcription factor binding events on neurodegenerative disorders. FOXO family proteins play an important role in regulating cell proliferation. Also, the expression of regulatory proteins of the cell cycle is controlled by FOXO3a which in turn makes it control the cell cycle. In the future, this characteristic can be used to develop a regenerative medicinal approach. FOXOs have a major regulatory function in learning, memory, and pro-longevity effects on neurons. The cognitive ability of the brain can be maintained with aging with the help of FOXOs but this needs more evidence and research. The role of all isoforms of FOXO in different parts of the brain should be ascertained to propose a therapeutic method for neurodegenerative disorders.

Furthermore, oxidative stress and excessive ROS presence in the neurons support the initiation and progression of neurodegenerative disorders like AD and PD. Due to ROS production in high levels, the neurons get damaged, and also it induces signals that result in apoptosis. The activated FOXOs can regulate oxidative stress and metabolic pathways to support neuronal survival. Also, it is necessary to maintain redox balance in neurons to treat or avoid neurodegenerative disorders.

In conclusion, the FOXO3 can be used as therapeutic targets to treat neurodegenerative disorders in the future. With the help of experimental procedures on mouse models, it is needed to investigate the use of FOXO3 in the regulation of oxidative stress, metabolic pathways, and maintenance of redox balance in neurons.

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