A Study to Investigate the Potential Role of Anti Diabetic versus Classical Acetylcholinesterase Inhibitor in Neurogenesis

BY

Sara Ahmed

(NUST201361722MASAB92513F)

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology,

Islamabad, Pakistan.

2015.

A Study to Investigate the Potential Role of Anti Diabetic versus Classical Acetylcholinesterase Inhibitor in Neurogenesis

A thesis submitted as a final year project in partial fulfillment of the requirement for the degree of Masters of Science

By

Sara Ahmed

(NUST201361722MASAB92513F)

Supervised by: Dr. Saadia Zahid

Atta-ur-Rahman School of Applied Biosciences,

National University of Sciences and Technology,

Islamabad, Pakistan.

2015

Dedicated to

My beloved parents and Sana, Nomaira & Hassan

ACKNOWLEDGEMENTS

All praises to Almighty Allah, the authority of knowledge and creator of resources, skills and opportunities. Who guides me in congenital circumstances and blesses me to accomplish this work.

I owe my gratitude to all those people who have made this dissertation possible and because of whom my experience has been one that I will cherish forever. I feel great pleasure to take this opportunity to express my sincere regards and gratitude to my supervisor Dr. Saadia Zahid, Assistant Professor of Neurobiology, for her continuous support throughout the study, for her patience, motivation, enthusiasm, and immense knowledge and whom I got inspiration for research in proteomics associated with the clinical manifestations of Alzheimer's disease. I feel great honor to express my deep gratitude to Dr. Peter John, Principal, Atta ur Rahman School of Applied Biosciences (ASAB), for his efforts to keep the environment congenial for research and study. Special thanks to Engr. Muhammad Asghar, Rector National University of Science and Technology (NUST), for promoting his vision of science and technology by creating research opportunities in NUST. I am also indebted to my external GEC member, Dr Noreen Asim for her invaluable suggestions. I wish to thank my GEC members Dr. Aneela Javed and Dr. Touqeer Ahmed for their support guidance and invaluable suggestions. I am also thankful to Dr Saima Zafar, University of Gottingen, Germany for her help in MS/MS analysis and Brig. Sohaib Hashmi and his staff at AFIP for providing the facilities to conduct immunohistochemistry studies.

I am greatly indebted to my family and friends especially Misbah Nazir, Nayab Nawaz, Syeda Qudsia, Saleha Resham, Hafsah Akhter, Hafsa Waheed, Mariam Masood, Zahra Mahmood and Haleema Bhatti for their tireless support and cooperation.

Sara Ahmed

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

LIST OF ACRONYMS

AD	Alzheimer's disease
NFT	Neurofibrillary tangles
Aβ	Amyloid beta
SVZ	Subventricular zone
DG	Dendrate gyrus
Tg	Transgenic
IGF-1	Insulin like Growth Factor-1
CBP	CREB binding protein
aPKC	atypical protein kinase C
AMPK	AMP kinase
$AlCl_3$	Aluminium Chloride
NPCs	Neural progenitor cells
SGZ	Subgranular zone
RMS	Rostral migratory stream
DCX	Doublecortin
NeuN	Neuronal Nuclei
BDNF	Brain-derived neurotrophic factor
FGF-2	Fibroblast growth factor -2
VEGF	Vascular endothelial growth factor
siRNA	Short interfering RNA
AICD	APP intracellular domainRNA
IRS	IR substrate
ROS	Reactive oxygen species
ChAT	Choline Acetyl transferase
AcH	Acetylcholine
CREB	CAMP-response element-binding
u	Micron
%	Percentage

ABSTRACT

Alzheimer's disease (AD), the most common cause of dementia, is associated with neurodegeneration that is characterized initially by synaptic injury followed by neuronal loss. More recent studies have uncovered evidence, suggesting that another component to the neurodegenerative process in AD might include the possibility of interference with the process of adult neurogenesis in the hippocampus. Since growing evidence supports the notion that AD is fundamentally a metabolic disease with substantial and progressive derangements in brain glucose utilization and responsiveness to insulin stimulation, it can be inferred that antidiabetics may serve as an alternative therapeutic strategy for AD. The present study has investigated the pro neurogenic effects of metformin (300mg/kg) in an in house generated $AlCl_3$ induced mouse model of neurodegeneration (600mg/kg) and drawn a comparison with neurogenic potential of donepezil (15mg/kg). A morris water maze task was performed to test spatial learning, and a subsequent histopathological and immunohistochemical evaluation was conducted. Expression of neurogenesis markers (Ki67, DCX and NeuN) along with insulin expression and differential proteome analysis of hippocampus was evaluated by qRT-PCR and SDS-PAGE respectively. The aberrantly expressed proteins were subsequently identified by ESI-QTOF MS/MS and functionally associated using STRING 8.3. The results demonstrated impaired spatial memory in $AlCl_3$ group as reflected by deviant learning curves, escape latency, and impaired probe trial performance. Histopathological assessment showed that $AlCl_3$ exposure led to substantial decrease in the density of Nissl substances (indication of neurodegeneration) and classical appearance of shrunk neurons and vacuolation suggesting concurrent degeneration of neurons in hippocampus. Proteome profiling showed that a total of eight proteins, involved in several biologically important pathways, were differentially expressed in hippocampus. Interestingly, treatment with metformin normalized the structural changes, changes in protein expression pattern as well as the alterations in the expression levels of insulin, Ki67, DCX and NeuN. Metformin improved memory impairment and increased the number of post mitotic NeuN-positive neurons in the hippocampus of $AlCl_3$ exposed animals. Moreover, metformin demonstrated profound effects on hippocampal neurogenesis as compared to donepezil. The results indicate that

metformin improves cognitive function and enhances the survival of newborn neurons in the hippocampus in animal model of neurodegeneration. Furthermore, metformin associated profound pro neurogenic effects observed in this study favor metformin over donepezil with regard to neuro regenerative potential. Further elucidation of underlying molecular mechanisms of metformin mediated adult hippocampal neurogenesis is needed.

Chapter 1

INTRODUCTION

Alzheimer's disease (AD), the most common cause of dementia, is characterized by two types of lesions; intracellular neurofibrillary tangles (NFT) and amyloid-β (Aβ) plaques (Selkoe, 2001). Elevated oxidative stress, synaptic loss, regionalized neuronal death and brain atrophy have also been observed in AD pathology (Ashe and Zahs, 2010). In the past, efforts have been directed to interlink these abnormalities under a single primary pathogenic mechanism and several heavily debated hypotheses exist trying to explain the underlying factor that trigger the development of AD brain pathology (Hardy and Selkoe, 2002; Gil-Bea *et al*., 2010; Markesbery, 1997). Despite decades of intense investigation, the field lacks consensus regarding the etiology and pathogenesis of AD, and therefore the best strategies for treating and preventing it are still not known (de la Monte, 2012).

AD associated dementia has been attributed to neurodegeneration that is characterized initially by synaptic injury (Terry *et al*., 1994; Masliah *et al*., 1997; DeKosky and Scheff, 1990) followed by neuronal loss (Terry *et al*., 1981). Synaptic loss (DeKosky and Scheff, 1990, Masliah *et al*., 1997, Scheff and Price, 2001) and axonal pathology (Raff *et al*., 2002) are plausible key features that govern dementia in these neurodegenerative disorders; however, other factors may also contribute. Besides synaptic dysfunction and loss of neuronal integrity in mature neuronal circuitries, aberrant adult hippocampal neurogenesis has been implicated in AD associated neurodegenerative process (Boekhoorn *et al*., 2006; Li *et al*., 2008; Jin *et al*., 2004b; Dong *et al*., 2004; Jin *et al*., 2004a; Lee *et al*., 2004; Haughey *et al*., 2002a). Adult neurogenesis, a process that occur throughout the life (Kempermann *et al*., 1997), takes place in the olfactory bulb, the subventricular zone (SVZ) and the dendrite gyrus (DG) of the hippocampus (Zhao *et al*., 2008). Uncovering recent evidence, studies have suggested that another component that adds to AD associated neurodegeneration includes the possibility of interference with the process of adult hippocampal neurogenesis (Boekhoorn *et al*., 2006; Li *et al*., 2008). Studies have shown significant alterations in the

process of adult neurogenesis in the hippocampus in transgenic (tg) AD models (Donovan *et al*., 2006; Dong *et al*., 2004; Wen *et al*., 2004) hence suggesting that AD pathogenesis may represent a two-pronged attack on the brain, contributing to degeneration of mature neurons, and disruption of the neurogenic niches in the adult brain (Crews and Masliah, 2010).

Several controversies exist over whether neurogenesis is increased (Jin *et al*., 2004b) or decreased (Boekhoorn *et al*., 2006; Li *et al*., 2008) in the pathogenesis of AD; however, in consistence with results reported in AD patients, a number of AD animal models display significantly reduced neurogenesis compared with non- tg controls (Donovan *et al*., 2006; Dong *et al*., 2004; Haughey *et al*., 2002b; Rockenstein *et al*., 2007). The molecular mechanisms implicated in defective neurogenesis in AD remain to be fully elucidated. Mechanisms modulating fetal neurogenesis have been extensively studied, and pathways such as the wnt (Lie *et al*., 2005) and Notch (Androutsellis-Theotokis *et al*., 2006; Beatus and Lendahl, 1998) signaling have been found to play an important role in this process. Little is known about the factors regulating neurogenesis in the adult central nervous system and their role in neurodegenerative disorders.

In the past, AD therapies were directed at improving memory by activating cholinergic neurotransmission (Perry *et al*., 1978) and, more recently, anti-oxidants (Fleisher *et al*., 2007) and blockers of calcium channels (van Dyck *et al*., 2007) have been utilized. In recent years, the focus was shifted towards reducing Aβ or Tau aggregation (Crews *et al*., 2010). Reflecting a major paradigm shift, an alternative therapeutic approach is now being considered; protecting selective neuronal populations and promoting synapse formation and neurogenesis.

AD, often tagged as a heterogeneous disorder, implicates multiple aberrant signaling cascades in its pathogenesis. Insulin resistance is one such factor known to affect multiple cascades of known relevance to AD (Lester-Coll *et al*., 2006). Insulin's ability to bind to its receptors has been reportedly compromised in AD (Steen *et al*., 2005). The decline in glucose processing is thought to coincide with, or even preceded, the early stages of AD (Iwangoff *et al*., 1980). Moreover, Aβ pathology, impaired cholingeric system, tau hyperphosphorylation, pro apoptotic and pro inflammatory events have also been attributed to impaired insulin signaling (Schiöth *et al*., 2012; Craft *et al*.,

2013; Zhu *et al*., 2005). It was reported that a common finding in AD was the impairments in energy metabolism and glucose utilization and subsequently the term Type III diabetes was coined for AD. It was observed that AD brains presented perturbed insulin and Insulin like Growth Factor-1 (IGF-1) mediated neuronal development and mitochondrial dysfunction (Suzanne and Wands, 2005). Role of insulin and IGF-I was also demonstrated in neurogenesis in ex vivo and hippocampal cultured cell lines (Arsenijevic *et al*., 2001). Since growing evidence supports the concept that AD is fundamentally a metabolic disease with substantial and progressive derangements in brain glucose utilization and responsiveness to insulin and IGF stimulation (De la Monta *et al*., 2005), it can be inferred that impaired insulin signaling is responsible for in aberrant/altered neurogenesis. Moreover evidence from human studies has shown that some oral antidiabetic medications and intranasal insulin can improve memory and cognitive abilities in mild cognitive impairment (MCI) and AD patients (Alagiakrishnan *et al*., 2013).

AD pathology, recapitulated by treatment with diabetes agents led to the hypothesis that AD-pathology and cognitive deterioration could be reduced by treatment regime involving antidiabetic agents. Owing to common underlying pathological cascade governing AD and Diabetes Mellitus (DM), it was proposed that common therapeutic intervention could be effective. The significance of CREB binding protein (CBP) transcriptional coactivator in optimal differentiation of embryonic neural precursors and the fact that its ability to promote differentiation required phosphorylation by atypical protein kinase C (aPKC) was demonstrated (Wang *et al*., 2012). Intriguingly, in liver cells, the aPKC-CBP pathway is downstream of AMP kinase (AMPK) and is activated by the AMPK-activating drug metformin (He *et al*., 2009), which is widely used to treat type II diabetes and other metabolic syndromes. These findings therefore suggest that metformin might activate aPKCs in neural stem cells and, in so doing, allow their recruitment in the adult brain.

1.1 Research Objectives:

The present study intends to

1. Investigate the pro-neurogenic potential of metformin in an in house generated $AlCl_3$ induced mouse model of neurodegeneration that in addition recapitulates some other features that are basic to AD.

2. Compare pro neurogenic effects of metformin with that of donepezil, a cholinesterase inhibitor, commonly used for treating AD by

- Assessing spatial learning and memory by morris water Maize (MWM) test in experimental groups (Table 3.1).
- Hippocampal histopathological examination by Cresyl violet and Haematoxylin and Eosin (H&E) staining.
- Transcriptional analysis of genes mediating major phases of neurogenesis, that is proliferation (Ki67), migration (DCX) and maturation (NeuN).
- Mapping differential hippocampal proteome profile by performing SDS PAGE.
- Immunohistochemical labeling of post mitotic NeuN positive neurons.

Chapter 2

LITERATURE REVIEW

2.1 Alzheimer's Disease:

Alzheimer's disease, first described by the German neuropathologist Alois Alzheimer as *Dementia Praecox* in 1907, is an age-related progressive neurodegenerative disease. In the 37th Conference of German psychiatrists meeting, Alzheimer reported his findings stating that "*in the centre of an almost normal cell there stands out one or several fibres due to their characteristic thickness and peculiar impregnability. Numerous small foci are found in the superior layers. They are determined by the storage of peculiar substance in the cerebral cortex. All in all we have to face a peculiar disease process*" (Hippius and Neundörfer, 2003).

The impregnable fibres and the millary foci, later referred as the neurofibrillary tangles and amyloid based neuritic plaques respectively are now documented as characteristic hallmarks of AD (Figure 2.1). Alzheimer's discovery generated a muted response; hence senility and dementia were still attributed to aging process. It was in 1910 that Dr. Emil Kraepelin described AD as a particularly serious form of senile dementia (Graeber *et al*., 1997).

AD is characterized by dementia that typically begins with subtle and poorly recognized failure of memory and slowly becomes more severe and, eventually, incapacitating. As the brain functions continue to disintegrate, there is a decline in person's cognitive abilities, memory, mood (Sultana et al., 2007), spontaneity and socializing behavior (Pollack and Namazi, 1992). Intraneuronal accumulation of NFT produced as a result of abnormal hyperphosphorylation of cytoskeletal tau protein, extracellular aggregation of amyloid-β (Aβ) protein as senile plaques, and massive neuronal death represent important hallmarks of AD neuropathology (Selkoe, 2001).These hallmarks are evident in specific brain regions and the hippocampus is one of the earliest to be affected (Braak *et al*., 1993).

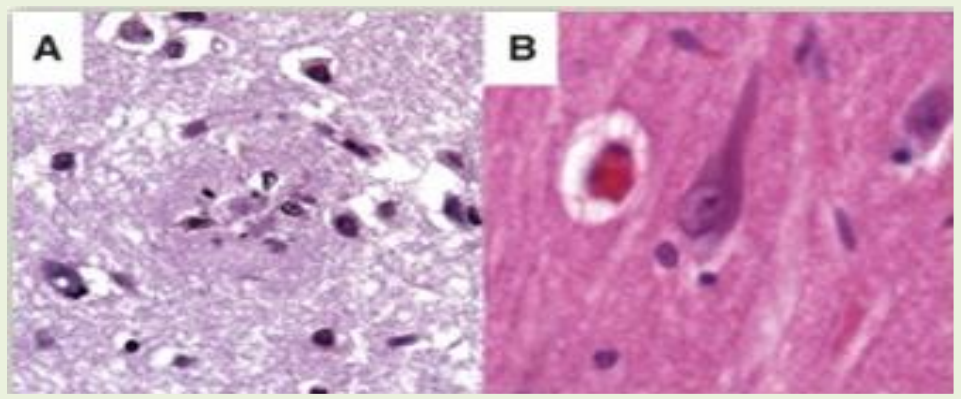


Figure 2.1: H&E stained sections of (A) Cortex, displaying neuritic plaques. (B)Hippocampus, depicting neurofibrillary tangles. (Adapted from (Serrano-Pozo *et al*., 2011))

Hippocampus, a major component of limbic system, includes subfields CA1–CA4 and the hippocampal formation (Frisoni *et al*., 2008). Hippocampal region has been implicated in the mechanisms of learning and spatial, semantic and episodic memory (Mavrogiorgou *et al*., 2011). Atrophy of the dorsal surface of the hippocampus encompassing most of the CA1 subfield was demonstrated, whereas CA2-3 showed negligible morphological changes besides a small and non-significant bulging (Frisoni *et al*., 2008). A marked reduction in CA1 hippocampus neuronal density has been reported (Padurariu *et al*., 2012). Studies have attributed this loss of neuronal density to the memory disturbances which are clinical signs in AD, even during the preclinical stages (Korbo *et al*., 2004), suggesting that cognitive decline in AD correlates with loss of neurons. Of the various neuropathological features of AD, cognitive impairment in patients with AD is also closely associated with synaptic loss in the neocortex and limbic system (Terry *et al*., 1991; DeKosky and Scheff, 1990; DeKosky *et al*., 1996). Also, during advanced stage AD, the hippocampus is the most intensely affected brain region. Researchers have used the term "hippocampal dementia" to describe the consistency of hippocampal histopathology as seen during AD (Šimić and Bogdanović, 1997). All in all, aberrant neural activity, synaptic dysfunction, and degeneration of specific neuronal populations were reported as main substrates of cognitive decline in AD.

Emerging evidence has indicated that altered neurogenesis in the adult hippocampus represents an early critical event in the course of AD. Besides synaptic dysfunction and loss of neuronal integrity in mature neuronal circuitries, alterations in

neurogenesis has been implicated in AD associated neurodegenerative process (Boekhoorn *et al*., 2006; Li *et al*., 2008; Jin *et al*., 2004b; Jin *et al*., 2004a; Dong *et al*., 2004; Lee *et al*., 2004; Haughey *et al*., 2002a). Key molecules, central in AD pathogenesis, have been shown to impact neuron generation, either positively or negatively, though causal links remain to be established. From a functional point of view, hippocampal neurogenesis has been implicated in structural plasticity and network maintenance. Therefore, early subtle disease manifestations may lead to dysfunctional neurogenesis which in turn may exacerbate neuronal vulnerability to AD hence contributing to cognitive impairment, whereas enhanced neurogenesis may represent body's compensatory response and its attempt at an endogenous brain repair mechanism. All together, these studies demonstrate that AD pathogenesis may be characterized by not only neurodegeneration but also by derangements in neural progenitor cells (NPCs) in neurogenic niches such as hippocampal DG.

2.2 Adult Hippocampal Neurogenesis:

The hippocampus, the earliest brain region known to be affected in dementia, is known for its role in learning and memory. It is exclusive in that it is one of the very few brain regions where adult neurogenesis continues to occur. Adult neurogenesis principally occurs in two brain regions: the SVZ and the subgranular zone (SGZ) of the hippocampus (Fuster-Matanzo *et al*., 2013).

Adult hippocampal progenitor cells of the DG undergo extensive proliferation in the SGZ before they migrate into the granular cell layer (Barkho *et al*., 2006). Around 9000 new cells are born per day per DG in adult rats. Many of these adult-generated cells are fated to death within the first few weeks (Biebl *et al*., 2000). Of the various factors, local neuronal activity and trophic support have been implicated in this selection. Significant proportions of the newly formed cells remain viable and differentiate into mature neurons that are later incorporated into mature hippocampal circuitry. Besides DG, neurogenesis also occurs in SVZ of the lateral ventricle. Here, via the rostral migratory stream (RMS), committed progenitor cells migrate into the olfactory bulb (OB) where they differentiate into interneurons that have a role in olfactory discrimination learning Characterization of different markers has been used to distinguish between neurogenesis stages however there is much overlap in expression of different markers and

phases themselves. Markers of cell division (Sox2, PCNA, Ki67, or BrdU in BrdU-treated cells or animals) or NPC-specific markers (nestin) are often used to identify cells in proliferative phase (Kempermann *et al*., 1997; Scholzen and Gerdes, 2000). For later stages, markers such as b-III Tubulin or doublecortin (DCX) are utilized to detect progeny in the immature neurons or early neuroblast phase (newlyborn neurons, often migratory), respectively (Rao and Shetty, 2004). Cells committed to a neuronal fate are immuno-positive with markers such as NeuN, MAP2 or synaptic markers (Figure 2.2).

Despite being prominent in young rodents, the proportions of neuronal progenitors rapidly decrease over time and are present at much lower levels in adult and particularly aged animals. Studies have observed similar levels in older primates. The very few studies on this subject indicate that the adult and elderly human brain is no exception in this respect (Thompson *et al*., 2008).

Adult neurogenesis is regulated by a wide array of environmental factors, intrinsic growth factors and hormones. Environmental factors, such as learning experiences, or physical exercise stimulate neurogenesis; however, aging, glucocorticoid hormones or stress potently inhibit neurogenesis (Kempermann *et al*., 2004). Interestingly, a variety of stimuli can affect different stages of the neurogenic process (Ming and Song, 2011), each targeting a specific developmental stage. Consistent with role of the hippocampus in cognition, several studies have reported changes in adult neurogenesis to be paralleled by changes in hippocampal functional plasticity and/or learning and memory performance (Aimone *et al*., 2006; Leuner *et al*., 2006; Saxe *et al*., 2006; Lledo *et al*., 2006). Given the functional incorporation of mature neurons in the hippocampal circuit, and observations that hippocampal learning positively modulates neurogenesis, it has been hypothesized that adult neurogenesis directly, or indirectly, contributes to adaptations in hippocampal functioning (Aimone *et al*., 2006). Conversely, any pathological alteration within the trisynaptic hippocampal circuit can induce changes in neurogenesis (Herrup and Yang, 2007, Kuhn *et al*., 2007). Of the many regulatory stimuli, neurogenesis has been shown to get spontaneously stimulated by hippocampal or cortical damage, for example, by acute excitotoxic, ischaemic or epileptic insults (Scharfman, 2002). Some of the modulating factors upregulated after ischemic damage are hypoxia-inducible peptides such as brain-derived neurotrophic factor (BDNF), IGF-1, fibroblast growth factor -2 (FGF-2) and

vascular endothelial growth factor (VEGF), also recognized as stimulators of adult neurogenesis (Larpthaveesarp *et al*., 2015). Although it is not known whether neurogenesis is also upregulated after chronic lesions, or during ‘‘slow’’ neurodegenerative processes such as expected to occur in AD, it has been speculated that either aberrant or reduced neurogenetic responses are involved in the cellular pathology in AD.

2.3 Neurogenesis Deficits in AD:

Hippocampal neurogenesis is often thought to be necessary to maintain hippocampus-dependent cognitive abilities (Deng *et al*., 2010). Recently, neurogenesis impairments were associated with the pathogenesis of AD. It is notable that quite a few molecules central to AD pathology were found to have a regulatory role in aspects of adult neurogenesis (Mu and Gage, 2011; Gadadhar *et al*., 2011). A study reported that neurogenesis impairments in the DG of hippocampus are closely associated with AD pathogenesis (Rodriguez *et al*., 2008). Indeed hippocampus is affected early in AD; impaired memory related to hippocampal damage may be associated with deregulations of neurogenesis (Abrous *et al*., 2005; Jin *et al*., 2004b; Tatebayashi *et al*., 2003; Shors *et al*., 2001).

Most investigations using tg AD animal models report a reduction in hippocampal neurogenesis (Mu and Gage, 2011) giving rise to the idea that impaired neurogenesis has an important role during the onset and progression of the disease. In many animal models of AD with familial-type mutations, this decrease in neurogenesis is associated with the deposition of toxic $A\beta_{42}$ (Haughey *et al*., 2002a). Nevertheless, some works with transgenic animals have shown that $A\beta_{42}$ aggregation increases neurogenesis (Jin *et al*., 2004a; Lopez-Toledano and Shelanski, 2007; Yu *et al*., 2009). Another study reported no conclusive results in this regard. Still, the most general view in the field is that AD related neuropathology damages hippocampal neurogenesis and in consequence impairs cognition (Ermini *et al*., 2008).

A recent study, by means of a loss-of-function animal model, has reported that lack of ApoE increased NPCs proliferation within the DG, leading to depletion of the

overall pool of Type 1 NPCs over time (Yang *et al*., 2011). Another study demonstrated that lenti viral vectors infected

NPCs, expressing short interfering RNA (siRNA) for the exclusive knockdown of presenilin -1 (PS1) showed a dramatic increase in cell differentiation in a γ- secretase-dependent manner (Oddo *et al*., 2003).

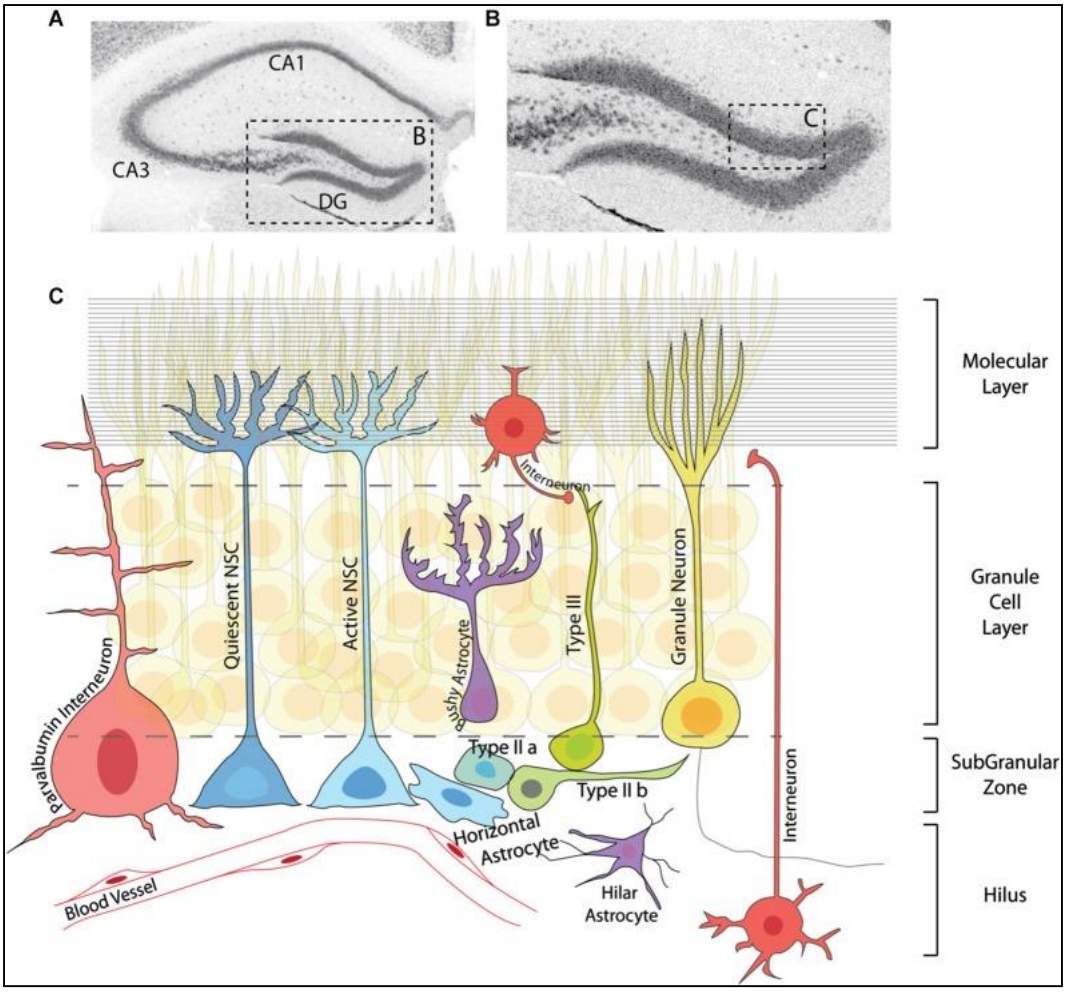


Figure 2.2: Adult neurogenesis in the dentate gyrus. (A) Immunohistochemistry for the neuronal marker NeuN showing the structure of the adult hippocampus.**(B)** Magnification of the DG region in A. **(C)** Graphic representation of the area marked in B depicting the neurogenic lineage and several elements of the DG niche. The neurogenic lineage consists of quiescent and active NSCs (including horizontal astrocytes), IPCs (type IIa, type IIb), neuroblasts (type III) and granule neurons. Neural stem cells and IPCs reside in the SGZ, while neuroblasts and neurons are found in the granule cell layer. Several types of interneurons (red) and astrocytes (purple) are located in different regions of the DG, and together with granule neurons are essential parts of the adult hippocampal niche. Blood vessels throughout the DG and axonal projections in the

molecular layer (horizontal lines) also contribute to the regulation of adult neurogenesis at different steps of the lineage. (Adapted from (Urbán and Guillemot, 2014)).

Furthermore recent findings implicated that APP could function to influence proliferation of NPCs and might regulate transcriptional activity of various genes. Studies demonstrated that influence of neurogenesis by APP is conferred differently via its two separate domains, soluble secreted APPs (sAPPs, mainly sAPPα) and APP intracellular domain (AICD) (Zhou *et al*., 2011). BrdU incorporation has shown that AICD expression results in decreased hippocampal progenitor cell proliferation and survival (Frazer *et al*., 2008). In contrast to the negative effect of AICD on neurogenesis, the sAPPα was shown to protect neurons and promote neurogenesis, possibly mediated by its ability to prevent overactivation of CDK5 and tau hyperphosphorylation (McKee *et al*., 2008). The balance between α-secretase and β-secretase determines the Aβ levels and sAPPα formation and neuronal viability. The disruption of this secretase balance leads to increased Aβ formation, decreased sAPPα and increased neuronal demise. The balance between α-secretase and γ-secretase was therefore found to influence neurogenesis in brain. Such secretase balance could affect the downstream balance between AICD induced inhibition of neurogenesis and sAPPα induced promotion of neurogenesis. Disturbance of this balance would lead to disruption of balance between neurogenesis and neuron demise and contribute to AD (Zhou *et al*., 2011). Therefore factors leading to disruption of these secretase balances might be culprits of AD onset, especially for sporadic AD.

PS1 mutations have a negative effect on the production of new neurons in general. Using PS1M146V knock-in mice, it was found that familial AD (FAD) mutation impaired contextual fear conditioning, which is correlated with reduced adult neurogenesis in the DG (Wang *et al*., 2004). Mutant PS1P117L tg mice demonstrated a decrease in survival of BrdU-labeled NPCs, resulting in fewer new β-III-tubulin-immunoreactive neurons in FAD mutant animals (Wen *et al*., 2004). Similarly, in tg mice expressing a chimeric mouse-human (mo-hu) APP-695swe (APPswe) polypeptide, the proliferation and survival of NPC were reduced, (Haughey *et al*., 2002b). An age-dependent decrease in SGZ proliferation was also observed in tg mice for human V717F mutant APP (Donovan *et al*., 2006). In 3xTg-AD mice, decreased proliferation was

found. The reduction was directly associated with an increase in the number of Aβ-containing neurons in the hippocampus (Rodriguez *et al*., 2008). Despite evidence for generalized neurogenic deficits in AD mouse models, contradictory observations have been reported. PDGF-APPswe, ind mice (J20), expressing the APP mutations driven by a PDGF promoter, showed increased BrdU immunoreactive neurons and expression of immature neuronal markers in the DG. These changes occurred before neuronal loss and amyloid deposition could be detected (Jin *et al*., 2004a). A separate study revealed an increase in not only NSC proliferation but also neuronal differentiation in J20 mice, which were induced by oligomeric Aβ (Lopez-Toledano and Shelanski, 2007). Moreover, a similar animal model exhibited longer dendrites, higher spine density and stronger functional responses in adult-born DG cells at an early developmental stage, but they were found to be impaired morphologically and functionally during later maturation. Normalized neuronal development despite the presence of high Aβ levels has been attributed to early inhibition of GABAergic signaling or late facilitation of glutamatergic signaling, suggesting that an imbalance between GABAergic and glutamatergic neurotransmission may contribute to impaired neurogenesis in AD models (Sun *et al*., 2009).

In a nut shell, over the past few years several studies using tg AD animal models have generated mounting evidence supporting alterations in neurogenesis. Cell proliferation has been reported to be enhanced during early phases of neurodegeneration, whereas the viability of newly generated neurons has been found impaired up to late stages (Chen *et al*., 2008). Owing to differences in many parameters, discrepancies exist in the current literature; with a majority of studies reporting compromised neurogenesis while others demonstrating increased neuronal generation. Nevertheless, to establish the causal relationship, the functional implications of aberrant neurogenesis in AD models need to be further characterized.

The current animal models only recapitulate partial AD pathology hence making it necessary to correlate the mouse model studies with human samples. Postmortem human brains of senile AD patients have showed increased expression of immature neuronal marker proteins in hippocampus that signal the birth of new neurons, including DCX, polysialylatednerve cell adhesion molecule, neurogenic differentiation factor and

TUC-4. It was proposed that facilitation of neurogenesis might represent a self-compensating mechanism to replace the lost neurons and that stimulating hippocampal neurogenesis might provide a new treatment strategy for AD (Jin *et al*., 2004b). However, in presenile AD cases, immunostaining for Ki-67, Glial fibrillary acidic protein (GFAP) and DCX revealed no indications of altered DG neurogenesis (Boekhoorn *et al*., 2006). Moreover, Aβ-associated increase in bone morphogenetic protein 6 (BMP6) expression was found accompanied by reduced neurogenesis markers in AD hippocampus (Crews *et al*., 2010). Needless to say, these findings are not in agreement, and the discrepancies may arise from stage of the disease, treatment provided to the patients and method for labeling proliferating cells. These findings provide compelling evidence that improved cognition, and specifically improved memory, following social housing in AD mouse model, is linked to increased hippocampal BDNF expression and associated neurogenesis. The question of whether or not the disease process induces compensatory birth of new neurons or specific changes in dentate neurogenesis in AD and related mouse models still needs to be addressed.

2.4 Therapeutic Strategies for Alzheimer's Disease:

Drugs currently approved by the food and drug administration (FDA) for AD treatment inhibit acetylcholine esterase to increase the levels of the neurotransmitter acetylcholine, which is depleted in AD brains, or antagonize N- methyl D Aspartate (NMDA)-type glutamate receptors to prevent aberrant neuronal stimulation (de la Torre *et al*., 2004). These drugs have a modest and transient impact on disease manifestations, although it is suggested that combination treatment may increase the time before patients require nursing (Lopez *et al*., 2009). There is no convincing evidence, though, that these agents can prevent, halt, or reverse the disease. Adult neurogenesis, a field still in its infancy, is likely to be affected in AD. Taking into account that adult hippocampal neurogenesis contribute to learning and memory (Van Praag *et al*., 2000, Shors *et al*., 2001, Aimone *et al*., 2006), it was speculated that an appropriate form to improve the subsequent deficits in cognitive functions associated with AD would result from modulating factors, that regulate this proneurogenic process. Finally, since adult neurogenesis declines with age, being a not so common event in the elderly (Thompson

et al., 2008), a fact that reinforces even more the idea of preserving or stimulating it as a brain repair mechanism.

2.5 Alzheimer's Disease- Emergence as Type III Diabetes:

AD has characteristic neuropathological abnormalities, including regionalized neurodegeneration; neurofibrillary tangles; Aβ deposition, activation of proapoptotic genes; and oxidative stress (de la Monte, 2012). As the brain functions continue to disintegrate, there is a decline in person's cognitive abilities, memory, mood, spontaneity and socializing behavior (Steen *et al*., 2005).A framework that sequentially interlinks all these phenomenon under one event is lacking. A common finding in AD was the impairments in energy metabolism and glucose utilization. Accumulating evidence has indicated the role of insulin deficiency and insulin resistance as mediators of AD neurodegeneration (de la Monte, 2012). Striking evidence has attributed loss of insulin receptor-bearing neurons to precede or accompany initial stage of AD (de la Monte *et al*., 2009). This state seems to progress with AD such that, in the terminal stages, it worsens and become global. Oxidative stress, tau hyperphosphorlation, APP-Aβ deposition, and impaired glucose and energy metabolism have all been linked to perturbation in insulin/IGF signaling (Steen *et al*., 2005).Insulin receptor (IR), Insulin, and IGF deficiency in AD brain further implicated insulin resistance in AD neuropathology (Steen *et al*., 2005; Craft, 2012). AD Braak stages have demonstrated an inversely proportional relationship to insulin expression. AD patients presented an 80-percent decline in insulin receptors (de la Monte *et al*., 2006). Insulin's ability to bind to its receptors was reportedly compromised. The decline in glucose processing was thought to coincide with, or even preceded, the early stages of AD (Iwangoff *et al*., 1980). Moreover, Aβ pathology, impaired cholingeric system, tau hyperphosphorylation, pro apoptotic and pro inflammatory events have all also been attributed to impaired insulin signaling (Schiöth *et al*., 2012).

Hippocampus, with its abundant IR substrate (IRS) proteins regulates the acquisition and consolidation of memories thereby suggesting the role of insulin in memory potentiation (Zhao and Alkon, 2001). In healthy adults, systemic infusion of insulin yielded a significant improvement in verbal memory and selective attention (Kern

et al., 2001). Insulin has been suggested to be neuroprotective and considered to have significant effects on memory enhancement (Nelson *et al*., 2008). In accordance with this, AD patients have shown improvement in memory and performance following insulin administration (Benedict *et al*., 2007). Evidence is growing to suggest impaired insulin signaling as the putative factor governing AD pathology hence favoring the conjecture of AD being a neuroendocrine disorder (de la Monte *et al*., 2006; Ahmed *et al*., 2015). Researchers thus concluded that perhaps Alzheimer's is a brain-specific type of diabetes which they termed "type 3 diabetes". In addition, AD pathology, recapitulated by treatment with diabetes agents led to the hypothesis that AD associated cognitive deterioration could be reduced by treatment regime involving antidiabetic agents.

2.6 Role of Insulin in Neurogenesis:

In recent years, insulin-activated signal transduction mechanisms have been implicated in stem cell differentiation induction stage in the various tissues (Wickelgren, 1998; Hoyer *et al*., 1996; McNay, 2007; Rafalski and Brunet, 2011; Stranahan *et al*., 2008). Adult neural stem cells sense and respond to changes in energy homeostasis occurring locally in the brain and systemically in the mammalian organism. The transition from an undifferentiated neural stem cell to a differentiated neuron, astrocyte, or oligodendrocyte is associated with various transcriptional changes, including genes associated with metabolism and energy sensing such as insulin signal transduction pathways and insulin receptors (Figure 2.3). Insulin also promotes induction of undifferentiated neural stem cells to differentiate into oligodendrocytes (Hsieh *et al*., 2004). Moreover, insulin promotes FGF2 mediated maintenance of neural stem cells in the undifferentiated state, besides playing a major role in the stem cell self-renewal stage (Beck *et al*., 1995; Carson *et al*., 1993; Åberg *et al*., 2000; Arsenijevic *et al*., 2001; Åberg *et al*., 2003). During reduced insulin expression, the proliferative and maintenance functions of undifferentiated stem cells (self-renewal capacity) are often consistently suppressed, even in different lineages. Diabetic rat model exhibited dendritic atrophy of hippocampal pyramidal neurons, decrease in spine density, synaptic reorganization in the DG, increased neuronal vulnerability and reductions in cell proliferation and neurogenesis (Alvarez *et al*., 2009; Beauquis *et al*., 2006; Kim *et al*., 2003). Since insulin

replacement reverses these neurological deficits in diabetes animals (Biessels *et al*., 1998; Magariños *et al*., 2001) these results support the emerging and expanding hypotheses regarding the important role of insulin in the CNS, especially for hippocampal neurons.

2.7 Metformin and Neurogenesis:

Metformin, first synthesized in the 1920s, ameliorates high blood sugar without stimulating insulin secretion or causing hypoglycemia. Its long history of efficacy and safety has made it the most commonly prescribed medication for type II diabetes worldwide (Potts and Lim, 2012). Though its therapeutic mechanisms remain to be fully elucidated, metformin has been known to activate AMPK, which subsequently phosphorylates aPKC, which in turn stimulates phosphorylation of CBP, resulting in inhibition of hepatic gluconeogenesis (He *et al*., 2009).

CBP, a ubiquitously expressed histone acetyltransferase and transcriptional coactivator, has a pivotal role in developing murine cortex. Consistently, CBP haplo-insufficiency has been shown to result in cognitive deficits (Wang *et al*., 2010). Given that phosphorylation of CBP by aPKC isoform ζ (aPKCζ) is required for CBP-mediated differentiation of cortical precursors, it was hypothesized that metformin might activate aPKCs in NSCs as well, thereby increasing neurogenesis. It was demonstrated that the aPKC-CBP pathway regulates neuronal differentiation from embryonic NPCs. Conversely, shRNA knockdown of aPKCζ or aPKCι isoforms both reduced the number of βIII-tubulin-positive neurons, co-transfection of a plasmid encoding an activated form of CBP with a phosphomimic mutation (serine to aspartic acid) at the aPKC site rescued aPKCζ knockdown, but not aPKCι knockdown (Wang *et al*., 2012). All together, the data suggested that CBP is downstream of aPKCζ. Metformin treatment appeared to modestly increase aPKC phosphorylation in NSCs. Metformin administration increased the number of βIII-tubulin-positive cells in culture by up to 50% while decreasing the proportion of Pax6- and Sox2-positive precursor cells. This pro-neurogenic effect was inhibited by aPKCζ/ι shRNAs as well as CBP siRNA. When administered to wild-type adult mice, metformin increased the number of neurons generated from NSCs in hippocampus DG.

Intriguingly, Wang *et al*. (2012) reported that metformin improved the ability to form new spatial memories, a task related to hippocampal neurogenesis (Wang *et al*.,

2012; Deng *et al*., 2010). While the study supported a mechanism that involves hippocampal neurogenesis, it is also possible that other mechanisms may contribute. Activated AMPK, for instance, increases glucose uptake by neurons (Amato and Man, 2011); metformin may affect neural metabolism more globally. CBP is a histone acetyltransferase, and as such, its activation by metformin may facilitate memory formation via synapse plasticity in a manner similar to the clinically approved histone deacetylase (HDAC) inhibitor suberoylanilidehydroxamic acid (SAHA).

2.8 Donepezil and Neurogenesis:

Functional perturbations of the cholinergic system, including a reduced expression of acetylcholine (ACh) and choline acetylltransferase (ChAT) in the cerebral cortex and hippocampus have been observed in AD patients (Hshieh *et al*., 2008). Donepezil ((±)-2-[(1-benzylpiperidin-4-yl)methyl]-5,6-dimethoxy-indan-1-one monohydrochloride) is a selective acetylcholinesterase inhibitor (AChEI) (Fuchs and Gould, 2000, Kosasa *et al*., 2000) that has been proven effective in mitigating cognitive impairment in AD patients. At present, there are no drugs available that can provide a cure for AD. However, medicines providing symptomatic relief or that can temporarily slow down AD progression have been developed. One such class of drug is AchEIs. AchEIs including donepezil and rivastigmine are currently being used for patients with mild–moderate stages of AD. Donepezil has been documented to attenuate the volume of cerebral infarction (Mohapel *et al*., 2005), protect against massive neuronal death and cognitive impairments following traumatic brain injury (TBI) (Nibuya *et al*., 1996), and enhance adult hippocampal neurogenesis by increasing cAMP-response element-binding protein (CREB) phosphorylation (Fujioka *et al*., 2004).

It is perceivable that via CREB signaling, the cholinergic system mediates hippocampal neurogenesis. Studies have demonstrated that donepezil increased p-CREB-positive cells in the DG, conversely scopolamine decreased them (Kotani *et al*., 2006). The p-CREB, expressed in newborn cells in the SGZ (Nakagawa *et al*., 2002), is involved in the survival of the cells (Nibuya *et al*., 1996; Fujioka *et al*., 2004; Thome *et al*., 2000). There are two possible ways in which phosphorylation of CREB is regulated by the cholinergic system. First, ACh, produced by AChE inhibition, directly enhances the

phosphorylation of CREB in newborn cells. The expression of mAChR in newborn cells in the DG has been reported (Mohapel *et al*., 2005) and a muscarinic agonist is known to enhance the phosphorylation of CREB (Chan *et al*., 2005, Zhao *et al*., 2003). Second, the cholinergic system via increasing hippocampal BDNF levels might activate the CREB signaling. BDNF is known to enhance the phosphorylation of CREB (Finkbeiner *et al*., 1997) and the survival of newborn cells (Lee *et al*., 2002, Sairanen *et al*., 2005). Scopolamine, on the other hand, has been shown to downregulate BDNF expression in hippocampus, which is consistent with the previous studies that have correlated deficiency of central cholinergic system with reduced levels of hippocampal BDNF mRNA (Lapchak *et al*., 1993, Berchtold *et al*., 2002). On the other hand, cholinergic activation by pilocarpine or carbachol upregulated BDNF mRNA in the hippocampus (da Penha Berzaghi *et al*., 1993; French *et al*., 1999) while donepezil, demonstrated no significant effect on the hippocampal BDNF expression levels. It is reported that the increase in BDNF mRNA is transient, and disappears 24 h after treatment with muscarinic agonists (French *et al*., 1999).

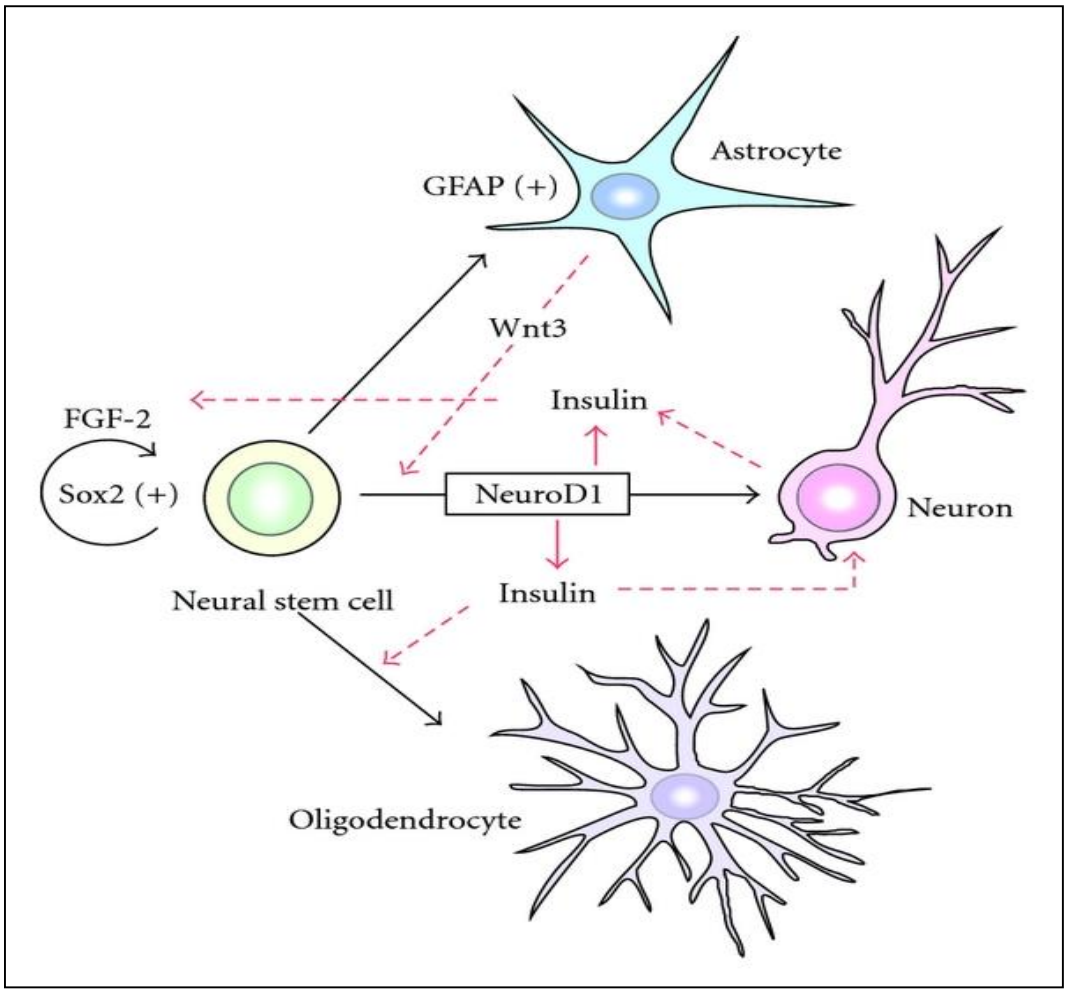


Figure 2.3: Schematic representation of the signals and transcription factors regulating adult neurogenesis. Undifferentiated adult neural stem cells express Sox2 transcription factor for the self-renewal function. FGF-2 promotes the proliferation of neural stem cells and insulin and IGF-1, and IGF-2 support the process. Astrocyte-secreted Wnt3 promotes the neuronal differentiation from neural stem cells by the activation of NeuroD1 transcription factor in the neuronal progenitor cell. The NeuroD1 transcription factor triggers the expression of insulin gene. Insulin, IGF-1, and IGF-2 promote the oligodendrocyte differentiation from neural stem cells. They also promote neuronal survival and possess the protection ability of mature neurons by preventing their natural cell death (Adapted from (Machida *et al*., 2012)).

Chapter 3

MATERIALS AND METHODS

3.1 Chemicals and Reagents

Aluminum Chloride hexahydrate ($AlCl_3.6H_2O$) was obtained from Scharlau (Product catalogue# AL0770). Metformin Hydrochloride (Glucophage) and Donepezil Hydrochloride (Donecept) were synthesized by Merck Sereno and ATCO Laboratories respectively. All other chemicals were purchased from Sigma-Aldrich (USA) unless indicated otherwise.

3.2 Animals

BALB/c mice were bred and housed in animal house of Atta urRahman School of Applied Biosciences (ASAB), National University of Sciences and Technology (NUST). Mice were kept in cages at constant temperature (25±2 ºC) and natural light-dark cycle (12-12 hours). Animals were given distilled water *ad libitum* and fed with standard diet consisting of (%): crude protein 30, crude fat 9, crude fiber 4 and moisture 10.4. Male mice (n=60) weighing 35-45 g and 2.5-3 months of age were used in experiments.

3.3 Ethics Statement

All 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), ASAB, NUST.

3.4 Study Design:

A 29 days long plan was formulated to generate an Alzheimer's disease like mouse model by injecting $AlCl_3$ and investigate the effect of Donepezil hydrochloride and Metformin hydrochloride on neurogenesis in this mouse model. Behavioral tests were performed in the last five days, following which the animals were decapitated for expression (transcriptome and proteome) and histological studies (Figure 3.1).

3.4.1 Animal Groups for Study:

Animals were randomly divided into six groups. Each group had a total of 10 animals of 2.5-3 months of age. Details of all the groups are provided in Table 3.1.

Table 3.1: Experimental design. Untreated Balb/c mice were used as control. Other groups comprised of $AlCl_3$, Metformin, Donepezil, $AlCl_3$+Metformin, and $AlCl_3$+Donepzil treated mice.

Sr No.	Groups	Treatment	Duration
1.	Control group	Distilled water and feed	29 Days
2.	$AlCl_3$ Group (Enas, 2011)	600mg/kg $AlCl_3$	15 Days
3.	Metformin Group (Hwang *et al*., 2010).	300mg/kg Metformin	14 Days
4.	$AlCl_3$+ Metformin Group	600mg/kg $AlCl_3$ 300mg/kg Metformin	15 Days 14 Days
5.	Donepezil Group (Kwon *et al*., 2014)	15mg/kg Donepezil Hydrochloride	14 Days
6.	$AlCl_3$+ Donepezil Group	600mg/kg $AlCl_3$ 15mg/kg Donepezil Hydrochloride	15 Days 14 Days

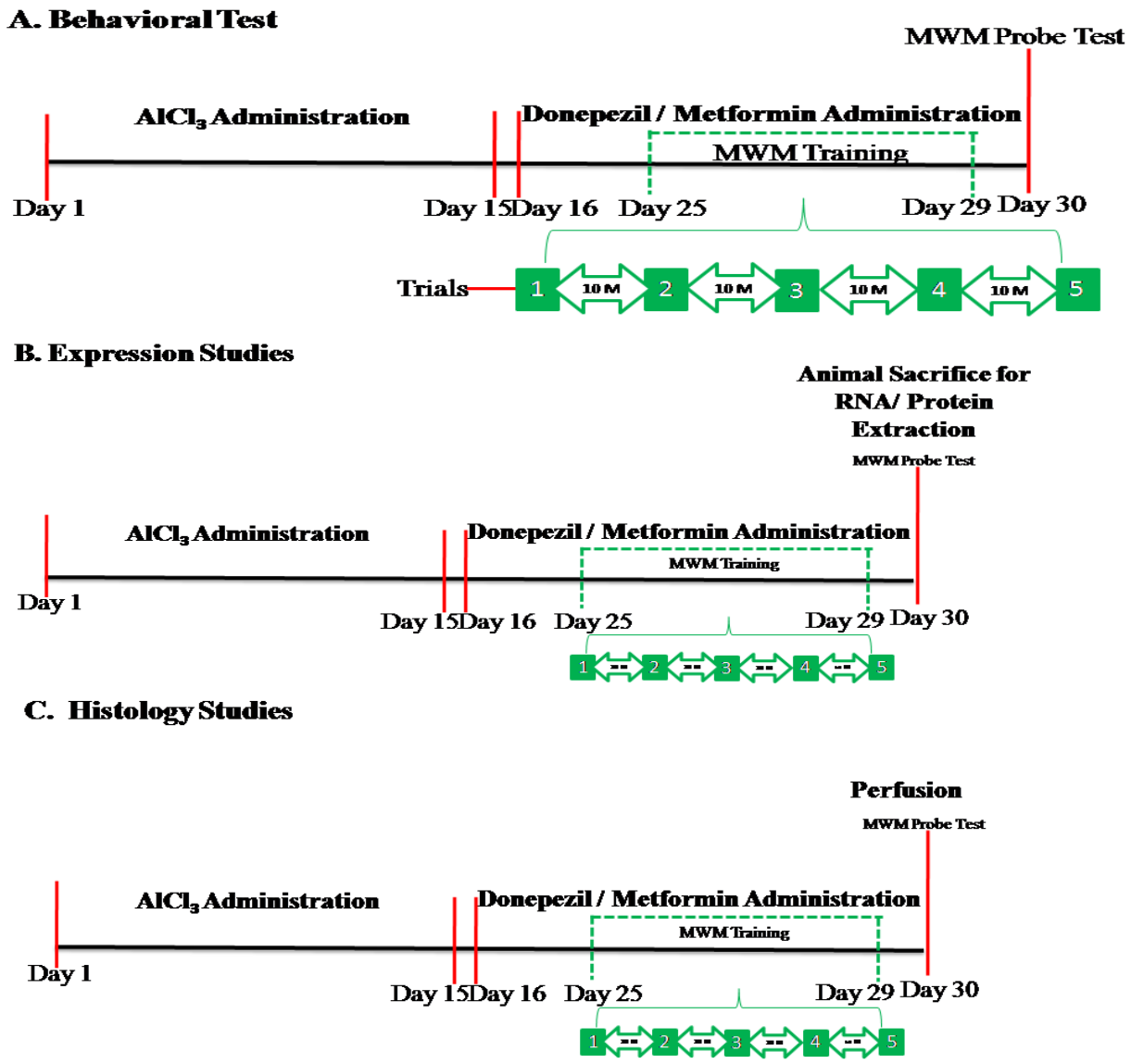


Figure 3.1: Experimental Plan: (A) Balb/c male mice of 2.5-3 months of age received $AlCl_3$ for 15 days. On day 16 mice were switched to an oral administration of Donepezil hydrochloride/Metformin hydrochloride for another 14 days. On day 25, animals were pre-trained in the MWM (five trials of 90 seconds each, inter trail interval of 10 minutes) with the platform protruding from the water surface. Between days 25 and 29 mice were trained to locate the hidden platform in the MWM. On day 30, they received a probe test in the MWM by placing them in the water without the platform for 90 seconds and assessing the amount of time they spent in the target quadrant (where the platform used to be located). Mice were sacrificed one hour later for **(B)** protein/ RNA extraction or were **(C)** transcardially perfused and their brains were processed for staining and immunohistochemistry.

3.5 Behavior Studies:

3.5.1Morris Water Maize Test:

The Morris Water Morris test was carried out in accordance with a previously described method (Bromley-Brits *et al*., 2011) with slight modifications. Briefly, a120-cm × 60-cm circular pool, which was filled with water (21 °C ± 2 °C) made opaque by the addition of blue color, was divided into four equally spaced quadrants (north, south, east, and west). A transparent platform (13-cm x 32 cm) was placed at the North-West quadrant with its surface 1 cm below the water. Distal cues were placed on the pool wall for the animals to navigate from the release position around the pool's perimeter to find the hidden platform. The mice were subjected to acquisition trial five times a day for five consecutive days with a minimum of 10-min inter-trial interval. During each trial, the mice were placed in water at one of the five starting positions; each starting position was randomly selected. The mice that failed to locate the platform within 90s were manually guided to the platform and allowed to stay for 20s. If the platform was found before 90s cut off, it was allowed to stay for 5s. The escape latency was recorded and the average for five trials for each day was calculated. The platform was removed 24 h after the last acquisition trial, and the mice were subjected to the spatial probe trial test. In this test, the mice were allowed to swim in the pool for 90 s, and the time spent in each platform quadrant was recorded.

3.6 Histological Examination of Brain Regional Tissues

3.6.1 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 24 h at 4 ˚C before being processed further for paraffin processing and embedding. After 24 h, the brain tissue was dehydrated through a series of alcohols (isopropanol), 70% (1 h), 95% (1 h), and 100% (1 h) before paraffin infiltration. The brain tissues were then placed in xylene (4 h) and paraffin embedding was performed by keeping the tissue in molten paraffin (4 h at 60 °C) and left to solidify (4 ^{o}C) in mould (block formation) prior to cutting.

3.6.2 Cresyl Violet Staining

Tissue sections (4 µ) mounted on slides were deparaffinized in xylene for 10 m before being rehydrated by 70% isopropanol (10 m), and washed with dd H_2O (5 m). Cresyl violet stain was poured over the tissues sections and left for proper staining (4 m). The sections were then washed with dd H_2O and 70 % acid alcohol (2 m) and later dried for 2 h before being mounted with cover slips. The slides were visualized by inverted microscope (Labomed, USA) at 10X and 40 X resolutions. The images were captured by Pixel Pro™ image analysis software (Labomed, USA).

3.6.3 Haematoxylin and Eosin Staining:

Standard haematoxylin–eosin staining was performed on 5 µ tissue section. Tissue was deparaffinized and incubated for 8 m in Mayer's haematoxylin solution and washed in warm water for 10 m. Sections were dipped in 95% ethanol and counterstained with eosin for 30 s.

3. 6.4 Immunohistochemistry:

5 µ sagital sections were mounted on Poly L Lysine coated adhesive slides. Following rehydration (in graded concentrations of ethanol), heat mediated antigen retrieval was performed by incubating sections for 35 m in sodium citrate (pH: 6).The sections were subsequently washed and endogenous H_2O_2 activity was quenched by incubation in 35% H_2O_2. To minimize nonspecific labeling, the sections were incubated for 10 m in 5% bovine serum albumin in PBS. The tissue sections were then incubated overnight at 4°C in 0.1% bovine serum albumin in PBS containing: rabbit monoclonal antibody for NeuN (1:100; ab177487). The following day, for NeuN labeling, sections were washed and placed in 1:100 dilution of HRP conjugated anti-rabbit IgG (ab97051) for 1 h at room temperature. The peroxidase reaction product was visualized by incubation in a solution containing 0.025% of 3,3′ diaminobenzidine (DAB Substrate Kit, ab50185) for 10 m. Following haematoxylin counter staining, cover slips were mounted and sections were visualized by inverted microscope (Labomed, USA) at 4X,20X and

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

3.7 Brain Dissection & Hippocampus Isolation:

Mouse was anesthetized using chloroform. The head was gently but assertively stretched forward and a cut, posterior to the ears, was made using surgical scissors. A small incision, starting from caudal point was made followed by making a firm cut through the anterior part of the skull. Using curved narrow pattern forceps the parietal bone of both sides was tilted and broken off. The curved narrow pattern forceps was then slid under the anterior part of the brain which was gently lifted out of the skull. The removed brain was immediately transferred to a pertri dish containing pre chilled Phosphate Buffer Saline (PBS) such that the ventral side of the brain was facing the plate.

Once the brain was transferred to pre chilled PBS the first step was to remove olfactory bulb and cerebellum using scalpel. Small curved forceps was then held in a closed position between the cerebral halves. The forceps was gently opened revealing the opening of cortical halves. Once sufficient opening along the middle line was obtained, the closed forceps, at an angle of 30-40°, was directed counterclockwise and clockwise to separate the left and right hippocampus from the cortex respectively. The dissected hippocampus was transferred in a pre-chilled eppendorf and stored at -80 °C till further use.

3.8 Gene Expression Analysis

3.8.1 RNA Extraction

RNA extraction was carried out according to the manufacturer's protocol using Tri-reagent. Briefly, tissue samples were washed with PBS (1X) and homogenized in 1 ml trizol using UP400S Ultrasonic Processor (Hielscher Ultrasound Technology) and then allowed to stand at room temperature for 5 m to ensure nucleoprotein complex dissociation. Chloroform (200 µl/ ml of Tri-reagent) was added and samples were rigorously shaken for 15 s and allowed to stand for next 10 m at room temperature. The mixture was centrifuged (HERMLE LabortechnikGmbR Germany) at 12,000 rpm for 15

m at 4˚C. After centrifugation the colorless upper aqueous phase was removed carefully and transferred to a new tube. Isopropanol (500 µl) was added to each sample and incubated for 10 m at room temperature. Samples were again centrifuged at 12,000 rpm for 10 m at 4˚C. After centrifugation, the RNA was precipitated, forming a pellet on inner side of the tube. The supernatant was removed and pellets were washed with 1 ml of 75% ethanol per 1 ml of Tri-reagent used in sample preparation. Later, it was centrifuged at 7500 rcf for 5 m at 4˚C. The final RNA sample was stored at -80˚C until further use.

3.8.2 Quality of RNA

For complementary DNA (cDNA) synthesis, the extracted RNA was centrifuged at 12000 rpm for 5 m. Pellet was allowed to dry and resuspended in 30µl of PCR water. RNA of all the samples (hippocampus) was run on 2% agarose gel to ensure the quality of RNA (mandatory for reproducibility and accuracy of the results). RNA bands were visualized on WealtechDolphen Doc (S/N470883) Gel Documentation system. The RNA quality, as judged by the ratio of 28S to 18S rRNA on the agarose gel was comparable among all samples.

3.8.3 Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) for cDNA Synthesis

Extracted RNA was quantified using BioPhotometer plus (Eppendorf, Germany) and equal quantity of RNA was used to reverse transcribe into cDNA. The protocol used for RT-PCR reaction included 4.5µl of 10mM dNTP's, 4.5µl of 5 mM oligodT (heated for 5 m at 55 °C) 12µl of 5x RT buffer, 6µl of 0.1M Dithiothreitol (DTT) and 3µl of MMLV-RT enzyme. Total volume of reaction mixture was made up to 60µl by the addition of PCR water accordingly.

3.8.4 Gene Expression Analysis by Quantitative Real Time Polymerase Chain Reaction (qPCR):

Real time PCR was performed in ABI Prism 7300 Sequence Detection System (Applied Biosystem, 7300) by using SYBR Green PCR Master Mix. The PCR reaction mixture consisted of 12.5µl of SYBR Green PCR Master Mix, 1µl of both forward and reverse primer specific for particular genes, 3µl of cDNA template and then volume was

made up to 25µl by adding DNase water. The thermocycling conditions were 50 °C for 2 m, 95 °C for 10 m followed by 40 cycles of 30 s at 95 °C, 1 m at 60 °C followed by 1 m at 72°C, and a final dissociation step. Dissociation curves, amplification plots and agarose gel electrophoresis were used to verify the quality of the PCR products. Each sample was tested in duplicate. All values were normalized to β-actin. Values obtained from three independent experiments were analyzed relative to gene expression data using the $2^{-\Delta\Delta CT}$ method (Livak and Schmittgen, 2001).The specific primer sequences of β-actin, DCX, Ki67 and NeuN and insulin are listed in Table 3.2.

Table3.2: Primer Sequences used for expression Analysis of DCX, KI67, NeuN and Insulin

Gene	Primer Sequence	Product Length
DCX	F-ATGCAGTTGTCCCTCCATTC R-ATGCCACCAAGTTGTCATCA	182bp
Ki67	F-CTGCCTGCGAAGAGAGCATC R-AGCTCCACTTCGCCTTTTGG	81bp
NeuN	F-GGCAATGGTGGGACTCAAAA R-GGGACCCGCTCCTTCAAC	65bp
Insulin	F-TGGCTTCTTCTACACACCCAT R-CTCCAGTGCCAAGGTCTGAA	112bp

3.9 Protein Expression Studies:

3.9.1 Protein Extraction:

The whole tissue lysates were prepared by suspension in 100µl of ice cold lysis buffer (7M urea, 2M thiourea, 4% CHAPS, 10mM phenyl methyl sulfonyl fluoride (PMSF), 1% d-Dithiothreitol (DTT)), followed by sonication using a UP400S Ultrasonic Processor (Hielscher Ultrasound Technology). To increase dissolubility, the homogenates were stored at room temperature for 1 h and centrifuged at 14000 rpm at 4 ºC for 10 m. The supernatant was transferred and stored at -20 ºC. In order to maximize the yield, 50µl lysis buffer was added to the pellet and the treatment was repeated. The two supernatants were then pooled and centrifuged at 14000 rpm for 90 m. The final supernatant was stored at -80 ºC until further use.

3.9.2 Protein Quantification (Bradford's Assay):

Serial dilutions of bovine serum albumin (1mg/ml) were prepared in duplicate with ddH_2O. The sample was diluted with ddH_2O (1:20) in duplicates. The final volume of each standard/sample was 20µl and 1 ml of Bradford reagent was added, followed by gentle vortexing. The samples were then incubated for 10 m at room temperature. The absorbance of each sample was measured at 595nm reagent blank using OPTIMA 300 spectrophotometer. A standard curve was derived by plotting standard absorbance against its concentration. This curve was used to estimate the protein concentration against the observed absorbance.

3.9.3 Protein Separation- Sodium Dodecyl Sulaphate-Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Sodium Dodecyl Sulaphate Polyacrylamide Gel Electrophoresis (SDS-PAGE) was used to separate the proteins based on their molecular weight using Mini Protean Tetra Cell (Bio-Rad). 10% resolving gel (distilled water; monomer solution; 1.5M Tris-HCl pH 8.8, 10% SDS, 10% ammonium persulphate (APS), TEMED) was prepared and poured immediately between the glass plates and left to polymerize for 45 m. This was followed by the preparation of the 4% stacking gel (distilled water; monomer solution; 1.5 M Tris-HCl pH 6.8, 10% SDS, 10% APS, TEMED). The gel was left for another 45 m to allow polymerization. Samples were prepared by addition of sample diluting buffer (0.125M Tris-HCl pH 6.8, 20% Glycerol, 10% 2-Mercaptoethanol) in 1:1 ratio. The samples were heated at 100 ºC for 3 m and given a short spin at 12000 rpm for 3 m. The samples were then loaded in the wells and the electrophoretic separation process was carried out at 100 volts for 90 m. After the run, the gel was placed in coomassie brilliant blue staining solution (0.025%), overnight. The gel was destained, using 10% destaining solution (75ml glacial acetic acid, distilled water, 25 ml of 100% ethanol) until a clear background was attained.

3.9.4 Image and Statistical Analysis:

Image Lab^{TM}software (Bio-RAD) was employed for gel image analysis, quantification and molecular weight calculation of the protein bands. The differential

expression of proteins was calculated on the basis of relative quantity of each protein band. The data was statistically analyzed by One Way ANOVA. A value of $p < 0.05$ was considered to be statistically significant. The histograms for differential protein expression were created with Graph Pad Prism 5.

3. 10 Protein Identification by ESI QTOF MS/MS:

Using a 22 min linear gradient (5-35% acetronitrile vs. 0.1% formic acid, 240 ml/min) on an EASY nLC-1000 system, peptide mixtures were concentrated on a Reversed Phase-C18 precolumn (0.15 mm ID x 20 mm self-packed with Reprosil-Pur 120 C18-AQ 3 µm material) and separated by Reversed Phase-C18 nano-flow chromatography (0.075 mm ID x 200 mm Picofrit column, self-packed with Reprosil-Pur 120 C18-AQ 3 µm material). Eluents were analyzed using a Top10 method in Data Dependent Acquisition mode on a Q Exactive high resolution mass spectrometry system operated under Tune 2.2 using HCD fragmentation, with a Normalized Collision Energy of 25%. Peak lists (PKL) were generated using Raw2 MSM v1.10 software (MPI for Biochemistry, Martinsried). PKL files were searched using Scaffold (4.45).

3.11 In Silico Analysis of Functional Association:

To investigate functional association network of identified differentially expressed proteins, their respective UniProtKB accession numbers were submitted in STRING 8.3 database (http://string-db.org/).

Chapter 4

RESULTS

Behavioral Analysis:

4.1 Effect of $AlCl_3.6H_2O$, Metformin and Donepezil on Cognitive Functions:

Morris water maze test, performed for cognitive assessment, was used to determine the effect of metformin and donepezil on spatial learning and reference memory. Average escape latency of mice to reach platform was a direct reflection of effect of drugs on spatial memory. During acquisition, all groups showed improvement, with control (6.1$\pm$ 0.34), metformin and $AlCl_3.6H_2O$ + Metformin (7.2 $\pm$ 1.61) treated groups consistently finding the platform at almost 6s at day 5. $AlCl_3.6H_2O$ induced neurodegeneration model (43.7 $\pm$ 1.93) demonstrated reduced retention of spatial memory than control mice with an escape latency of almost 44s at day 5. $AlCl_3.6H_2O$ + Donepezil treated group (29.75 $\pm$ 0.854) showed improved memory as compared to $AlCl_3.6H_2O$ treated group with an improved escape latency of 29s at day 5, however, the spatial memory was better restored in $AlCl_3.6H_2O$ +Metformin treated group than $AlCl_3.6H_2O$ + Donepezil treated group (figure 4.1). Escape latency for day 5 is shown separately in figure 4.2 (a)

Probe trial was performed for assessing reference memory. Time spent in the target quadrant was recorded to check the exploration time for the previously placed hidden platform (figure 4.2b). It demonstrated that $AlCl_3.6H_2O$ treated group (9.9 $\pm$ 0.1) showed significant decrease ($p< 0.001$) in reference memory as they spent less time in target quadrant as compared to Control (41 $\pm$ 1), Metformin (41.5 $\pm$ 0.5) and Donepezil treated group (37.0 $\pm$ 1). On the other hand, time spent by $AlCl_3.6H_2O$ + Metformin group (30 $\pm$ 2) was significantly increased ($p< 0.001$) relative to $AlCl_3.6H_2O$ group indicating that metformin overcame the effect of $AlCl_3.6H_2O$ on memory. When compared with $AlCl_3.6H_2O$ group, $AlCl_3.6H_2O$ + Metformin demonstrated improved reference memory than $AlCl_3.6H_2O$ + Donepezil treated group (18.5 $\pm$ 0.5; $p<0.05$).Representative mouse pathways for the probe trial are shown in figure 4.3.

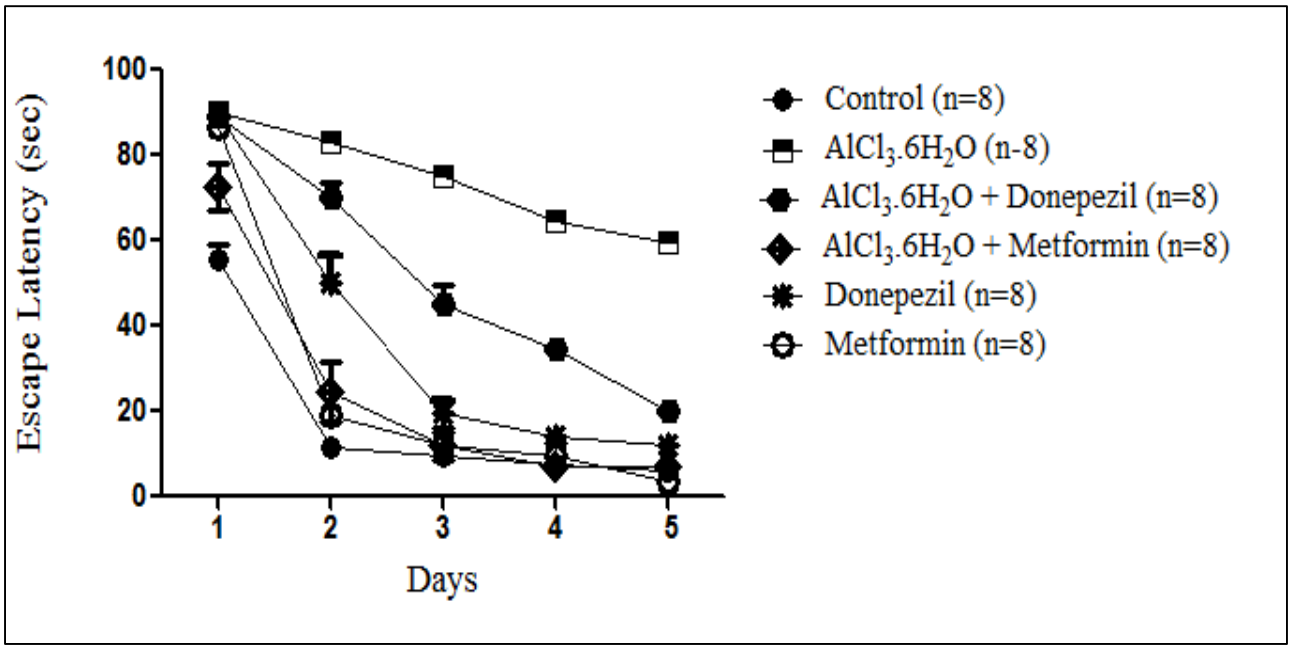
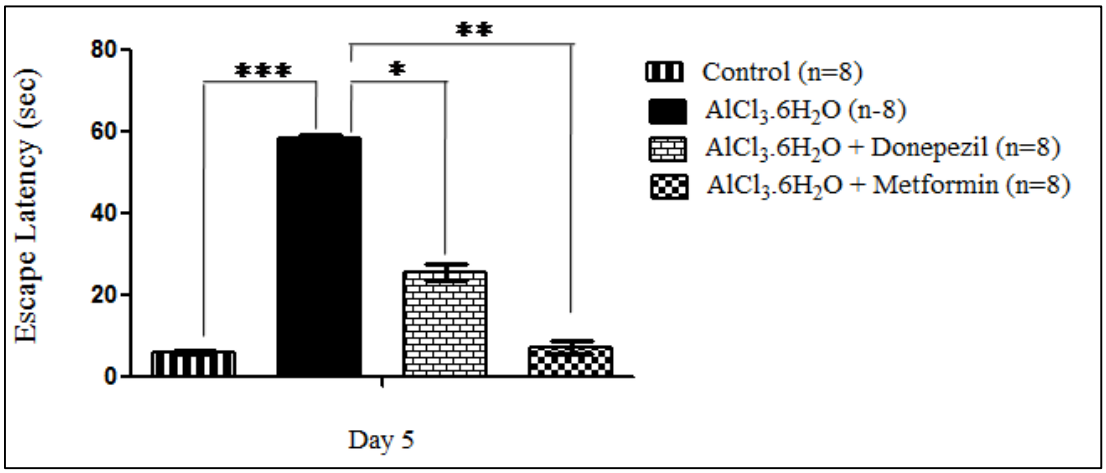


Figure 4.1: Effect of Metformin and donepezil on learning and memory using Morris water maze (MWM) test: Graph depicts escape latency (sec) to assess formation of spatial memory between Control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Metformin and Donepezil treated group. $AlCl_3.6H_2O$ treated mice demonstrate less retention of spatial memory as compared to control mice. The data indicate that $AlCl_3.6H_2O$ + Metformin treated mice find the platform much faster than $AlCl_3.6H_2O$ + Donepezil treated group. Error bars represent mean ± SEM; n=8.

(a)



(b)

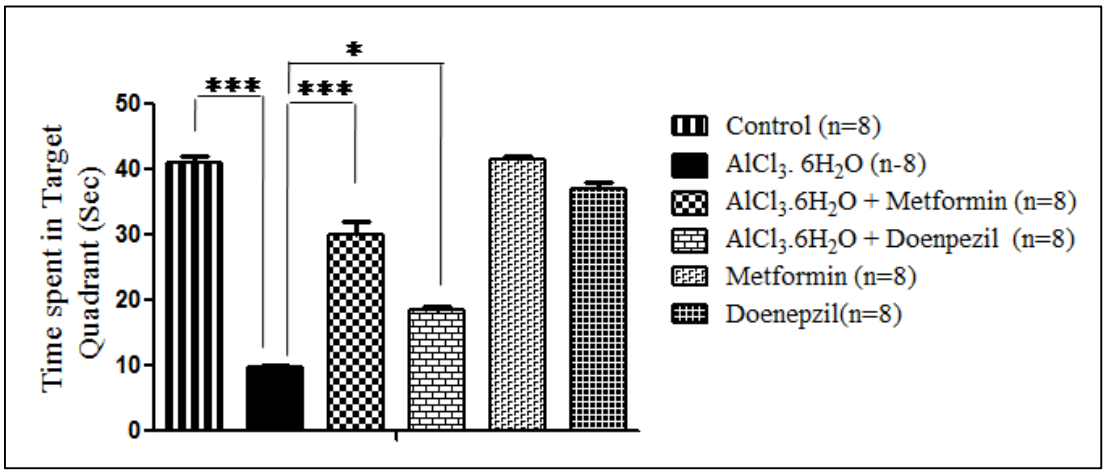


Figure 4.2 (a): Comparison of behavioral activity among different experimental groups (Day 5) Compared to $AlCl_3.6H_2O$ group more significant decrease in escape latency was observed in $AlCl_3.6H_2O$ + Metformin treated group than in $AlCl_3.6H_2O$ + Donepezil group. Error bars represent ± SEM, n=8. Data was analyzed using one ANOVA followed by Bonferroni Multiple Comparison Test. **(b)** time spent (sec) by test animals in target quadrant is shown in bar diagram.

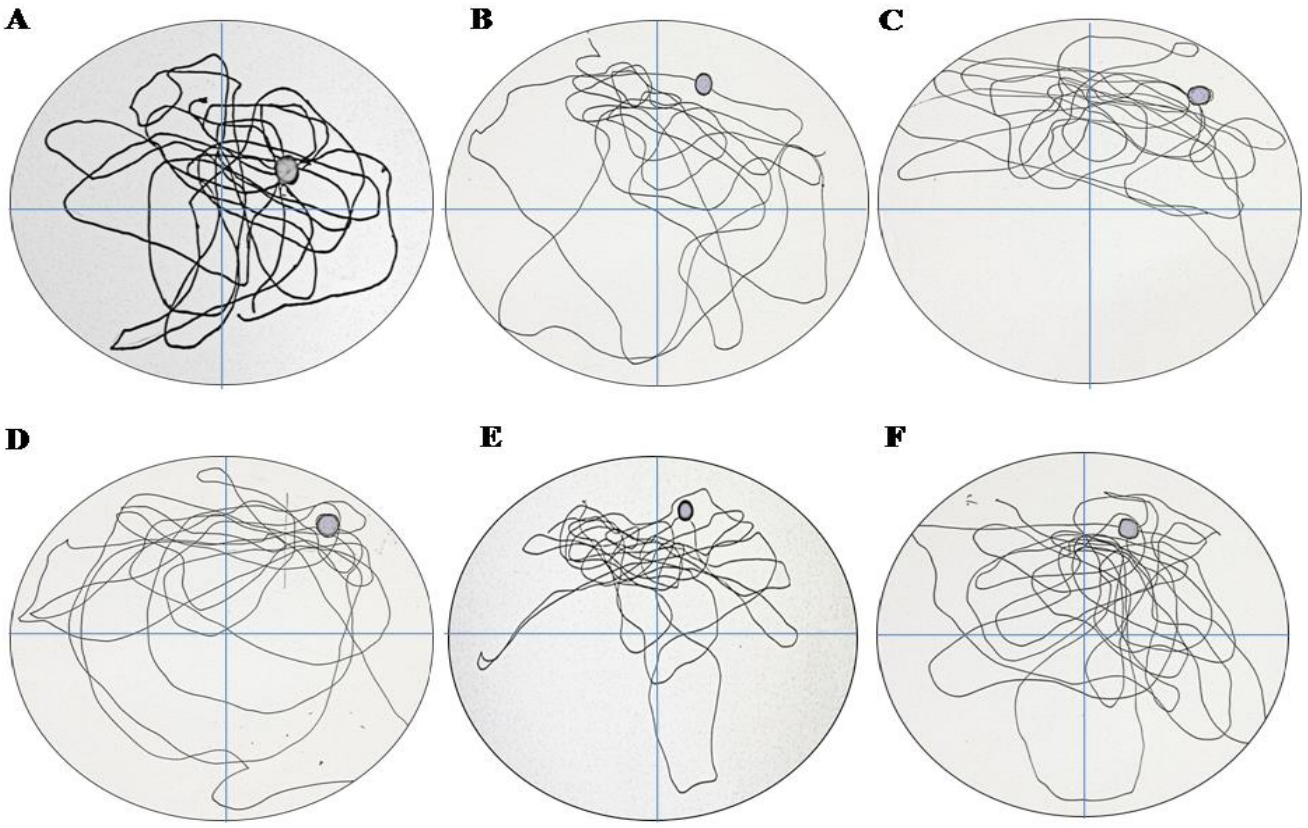


Figure 4.3: Effect of Metformin and Donepezil on reference memory (MWM test, Probe Trial): Representative pathways of mouse swimming in probe trial are shown **(A)** control mice centered their search around the previous platform location, whereas **(B)** $AlCl_3.6H_2O$ treated mice spent more time along the perimeter. Reference memory is improved by Metformin **(F)** and donepezil **(D)** administration post $AlCl_3.6H_2O$ exposure. Metformin **(C)** and donepezil **(E)** treated mice also demonstrate reference memory that is comparable to control.

4.2 Histological Studies:

4.2.1 Cresyl Violet Staining

Histopathological assessment of hippocampus of all the study groups was performed to observe morphological changes that occurred in affected region (Hippocampus). The Cresyl violet staining revealed a marked reduction in Nissl substances in $AlCl_3.6H_2O$ treated group as compared to control. Metformin treatment post aluminum exposure showed an increase in number of Nissl bodies as compared to donepezil. Number of cell bodies in metformin treated group is similar to control.

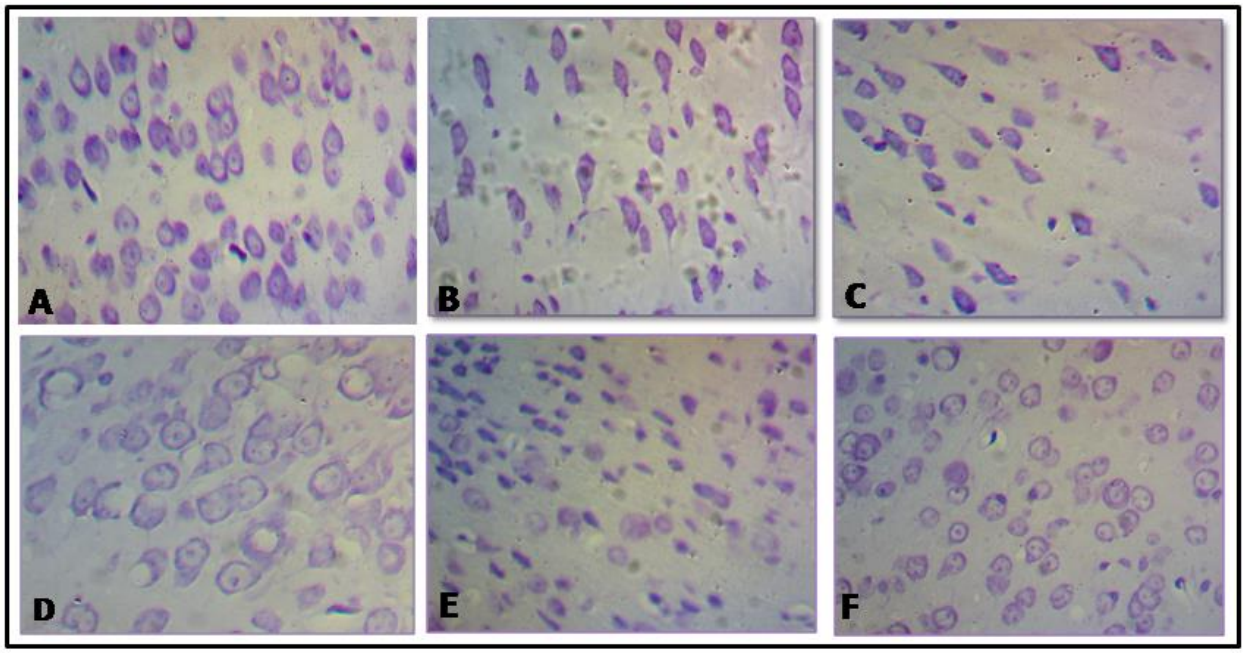


Figure 4.4: Cresyl Violet stained sections of Hippocampus: (A) Healthy neurons with intact nucleolus and predominant Nissl bodies (B) Donepezil treated group. (C) Metformin treated group (D) $AlCl_3.6H_2O$ treated group (E) $AlCl_3.6H_2O$ + Donepezil treated group (f) $AlCl_3.6H_2O$+ Metformin treated group. Original magnifications **40X.**

4.2.2 Haematoxylin and Eosin Staining:

H&E stained sections depict neuron degeneration. Control shows the pattern of dark neurons within the cortex as compared to $AlCl_3.6H_2O$ which shows classic appearance of shrunk neurons. In addition, there is vacuolation suggesting concurrent degeneration of neuron. The cellular morphology and density is better restored by metformin administration as compared to donepezil treatment post aluminium exposure.

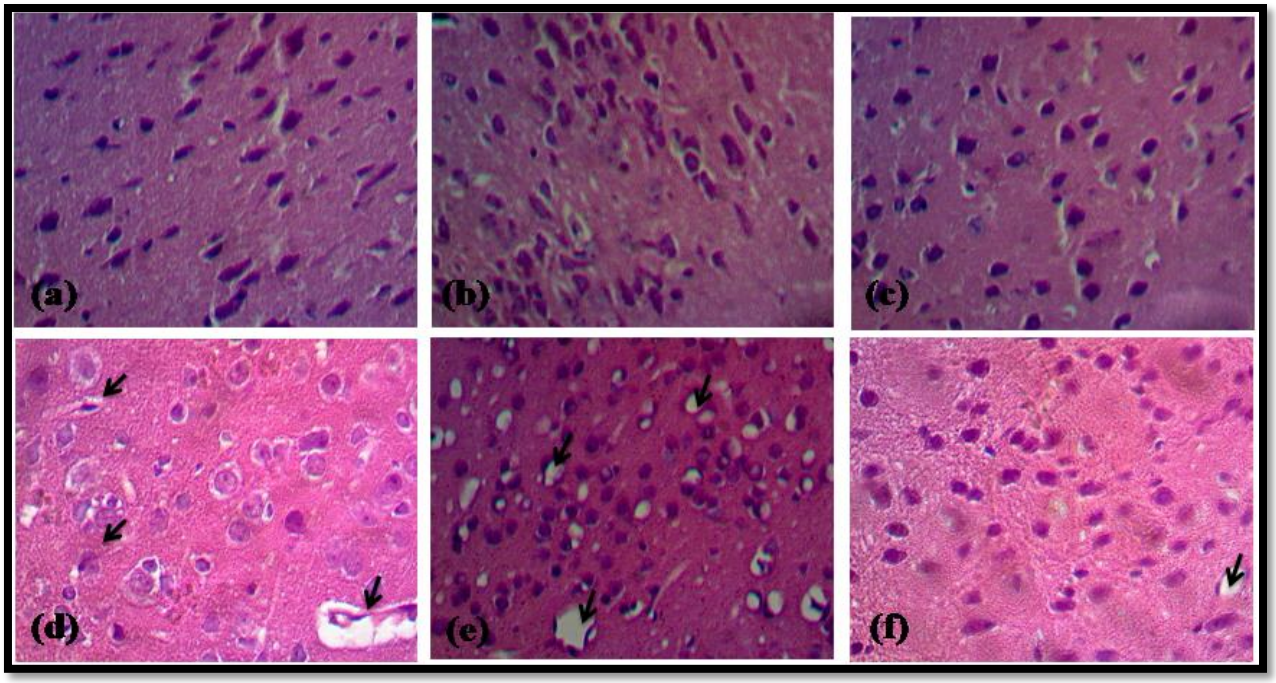


Figure 4.5: H&E stained sections of Cortex: (a) Healthy neurons of control group. **(b)** Donepezil treated group. **(c)** Metformin treated group **(d)** Arrow heads point shrunk cells in $AlCl_3.6H_2O$ treated group **(e)** $AlCl_3.6H_2O$ + Donepezil treated group. Arrow heads point vacuolization **(f)** $AlCl_3.6H_2O$ + Metformin treated group. Arrow head points vacuolization. Original Magnification: **40X**

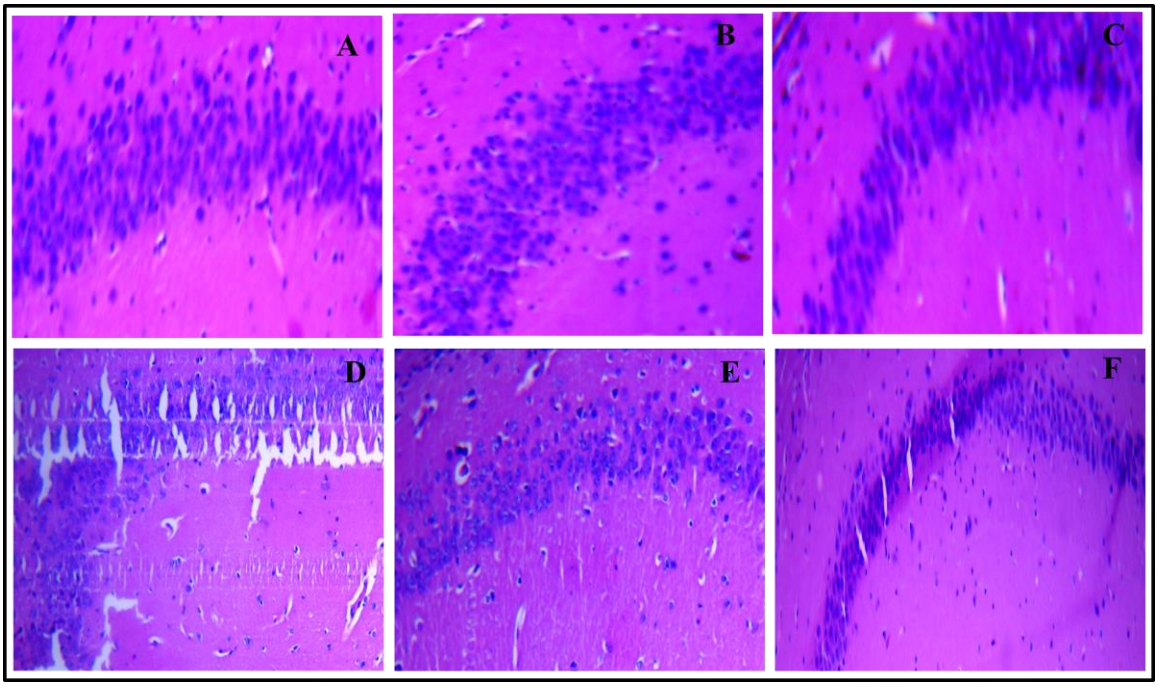


Figure 4.6: H&E stained sections of hippocampus:(a) Pattern of dark neurons within the pyramidal layer of the hippocampus of control group. **(b)** Donepezil treated group. **(c)** Metformin treated group **(d)** Classic appearance of neuron degeneration in $AlCl_3.6H_2O$ treated group. **(e)** $AlCl_3.6H_2O$ + Donepezil treated group. **(f)** Neuron density in restored in $AlCl_3.6H_2O$ + Metformin treated group. Original Magnification: **20X**

4.3Transcriptional Analysis of Insulin and Neurogenesis Markers:

4.3.1 Transcriptional Analysis of Insulin:

Quantitative real time PCR (qRT-PCR) was carried out to observe the expression of insulin in hippocampus. It was observed that the expression level of insulin was significantly decreased ($p< 0.001$) in $AlCl_3.6H_2O$ (0.05 ± 0.03) hippocampus as compared to control (1 ± 0). Upon comparison between $AlCl_3.6H_2O$+ Metformin treated group and $AlCl_3.6H_2O$ group, a significant increase ($p< 0.001$) was observed in insulin expression in hippocampus of $AlCl_3.6H_2O$+ Metformin group (0.78 ± 0.02). There was no significant change in levels of insulin in $AlCl_3.6H_2O$ + Donepezil group (0.05 ± 0.005) as compared to $AlCl_3.6H_2O$ group. No significant change in levels of insulin was

observed in metformin (0.865 ± 0.035) and donepezil treated group (1 ± 0) as compared to control.

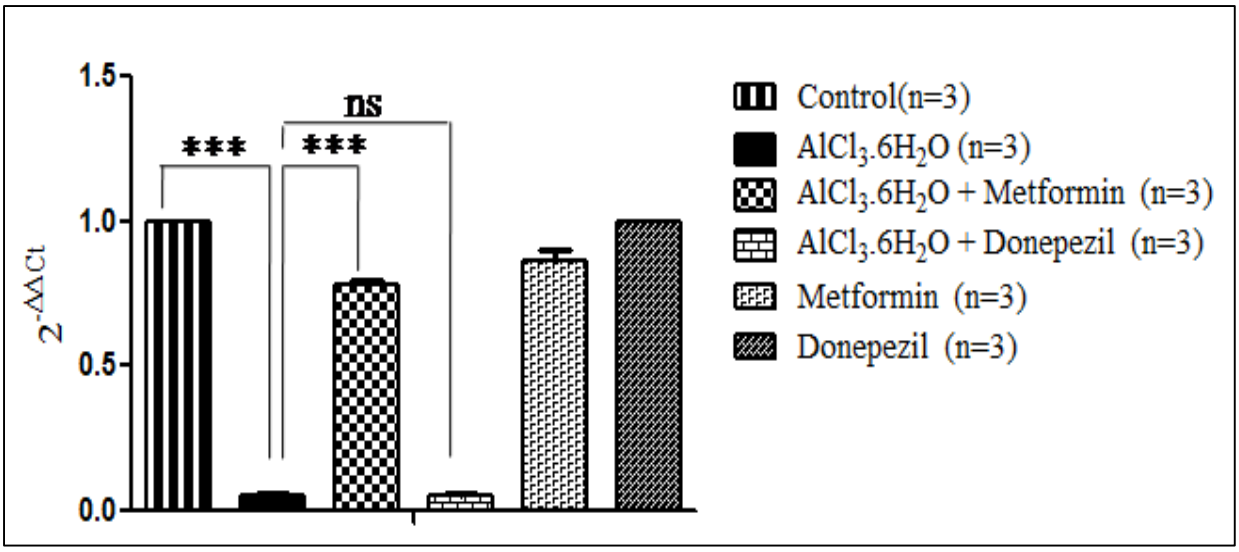


Figure 4.7: Transcriptional expression of hippocampal insulin: Histogram represent insulin levelsin control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformin and Donepezil group. The data is shown as mean ± SEM, **p< 0.01; ***p< 0.001. ns= Non significant. To check the consistency samples were run in duplicates.

4.3.2Transcriptional Analysis of Ki 67:

qRT-PCR results demonstrated significantly decreased (p< 0.001) expression level of Ki67 in $AlCl_3.6H_2O$ treated group (0..045 ± 0.015) as compared to control (1 ± 0) hippocampus while treatment with metformin (1.8 ± 0.3) post aluminum exposure caused significant increase (p< 0.01) in ki67 gene expression. As compared to $AlCl_3.6H_2O$ group, there was a more significant increase (p< 0.001) in ki67 gene expression in $AlCl_3.6H_2O$ + Donepezil group (3.6 ± 0.2) as compared to $AlCl_3.6H_2O$ + Metformin group. Both metformin (2.1 ± 0.1; p< 0.01) and donepezil treated group (3.8 ± 0.2; p< 0.001) showed significant increase in expression of ki67 as compared to $AlCl_3.6H_2O$ group.

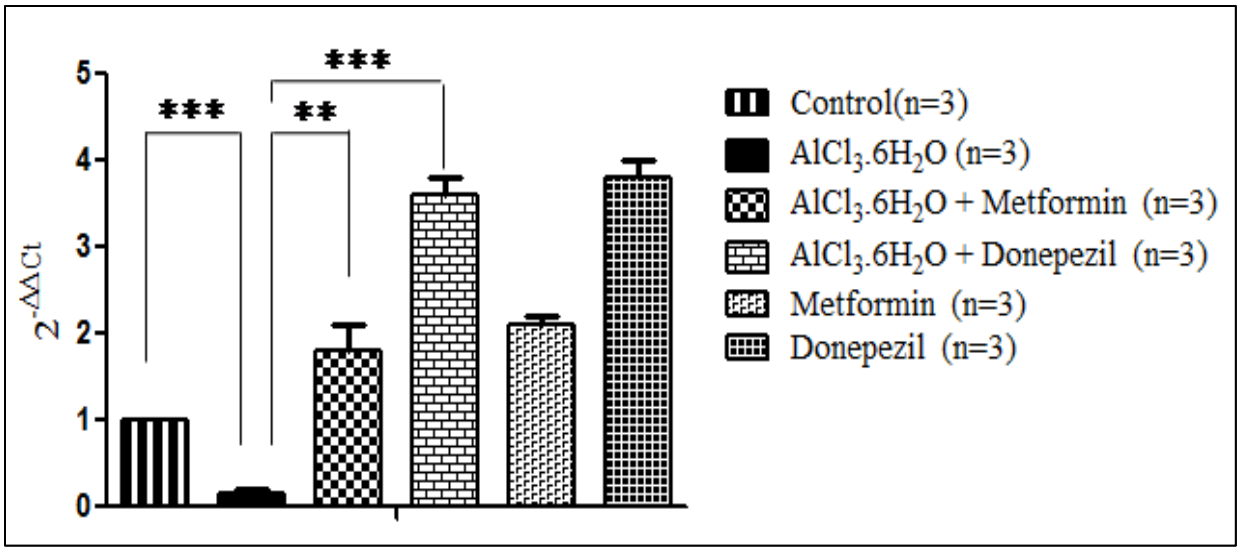


Figure 4.8: Transcriptional expression of hippocampal Ki67: Histogram representing Ki67 levels in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as mean ± SEM, ***p*< 0.01; ****p*< 0.001. To check the consistency samples were run in duplicates.

4.3.3Transcriptional Analysis of DCX:

Results of qRT-PCR showed a significant decrease (*p*< 0.001) in the expression levels of DCX in $AlCl_3.6H_2O$ group (0.022 $\pm$ 0.022) as compared to control hippocampus (1 $\pm$ 0) while metformin administration (0.57 $\pm$ 0.03) post aluminum exposure caused significant increase (*p*< 0.01) in DCX gene expression as compared to $AlCl_3.6H_2O$ group. There was a more significant increase in DCX gene expression in $AlCl_3.6H_2O$ + Metformin group as compared to $AlCl_3.6H_2O$ + Donepezil group (0.36 $\pm$ 0.02; *p*< 0.05). Both Metformin (1.225 $\pm$ 0.025; *p*< 0.001) and donepezil treated group (1.0 $\pm$ 0.1; *p*< 0.001) showed significant increase in expression of DCX as compared to $AlCl_3.6H_2O$ group.

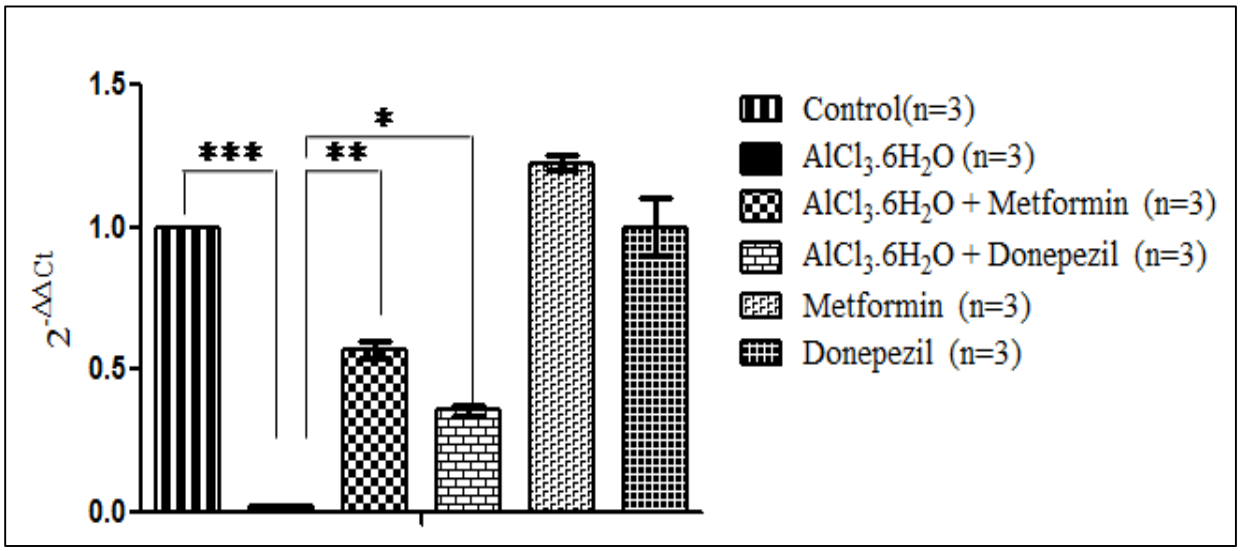
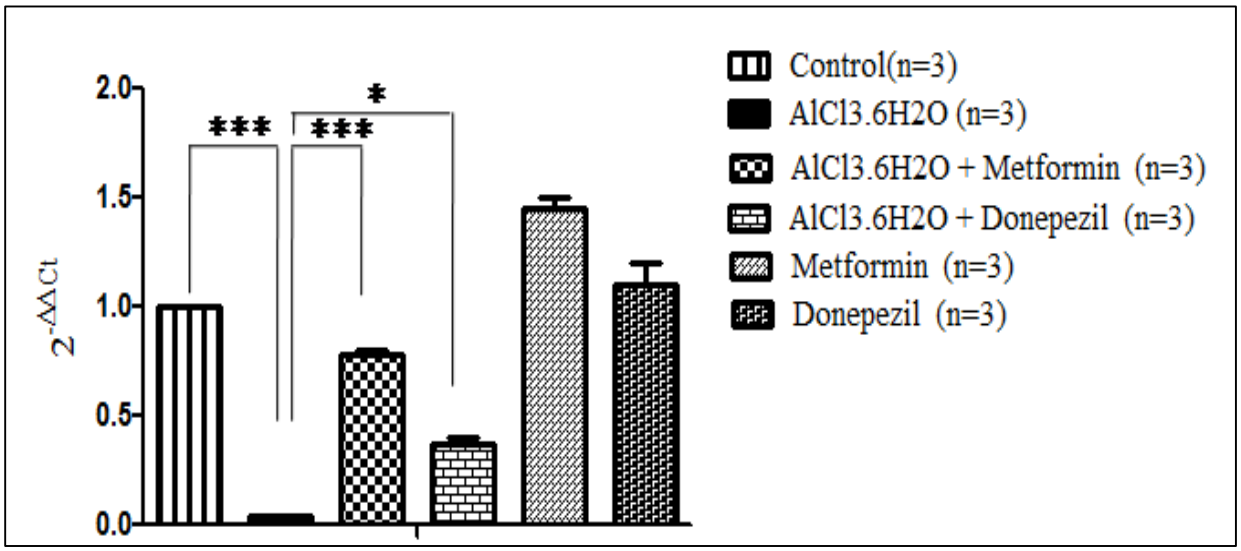


Figure 4.9: Transcriptional expression of hippocampal DCX: Histogram representing DCX levels in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as the mean ± SEM, *p< 0.05; **p<0.01; ***p<0.001. To check the consistency samples were run in duplicates.

4.3.4 Transcriptional Analysis of NeuN:

A significant decrease (p< 0.001) in the expression levels of NeuN in $AlCl_3.6H_2O$ group (0.037 $\pm$ 0.003) was observed as compared to control hippocampus (1$\pm$0) while metformin administration (0.78 $\pm$ 0.02) post aluminum exposure caused significant increase (p< 0.001) in NeuN gene expression as compared to $AlCl_3.6H_2O$ group. There was a more significant increase in NeuN gene expression in $AlCl_3.6H_2O$ + Metformin group as compared to $AlCl_3.6H_2O$ + Donepezil group (0.37 $\pm$ 0.03; p<0.05). Both metformin (1.45 $\pm$0.05; p< 0.001) and donepezil treated group (1.1 $\pm$ 0.1; p< 0.001) showed significant increase in expression of NeuN as compared to $AlCl_3.6H_2O$ group.



4.10: Transcriptional expression of Hippocampal NeuN: Histogram representing NeuN levels in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as the mean ± SEM, *p< 0.05; ***p<0.001. To check the consistency samples were run in duplicates.

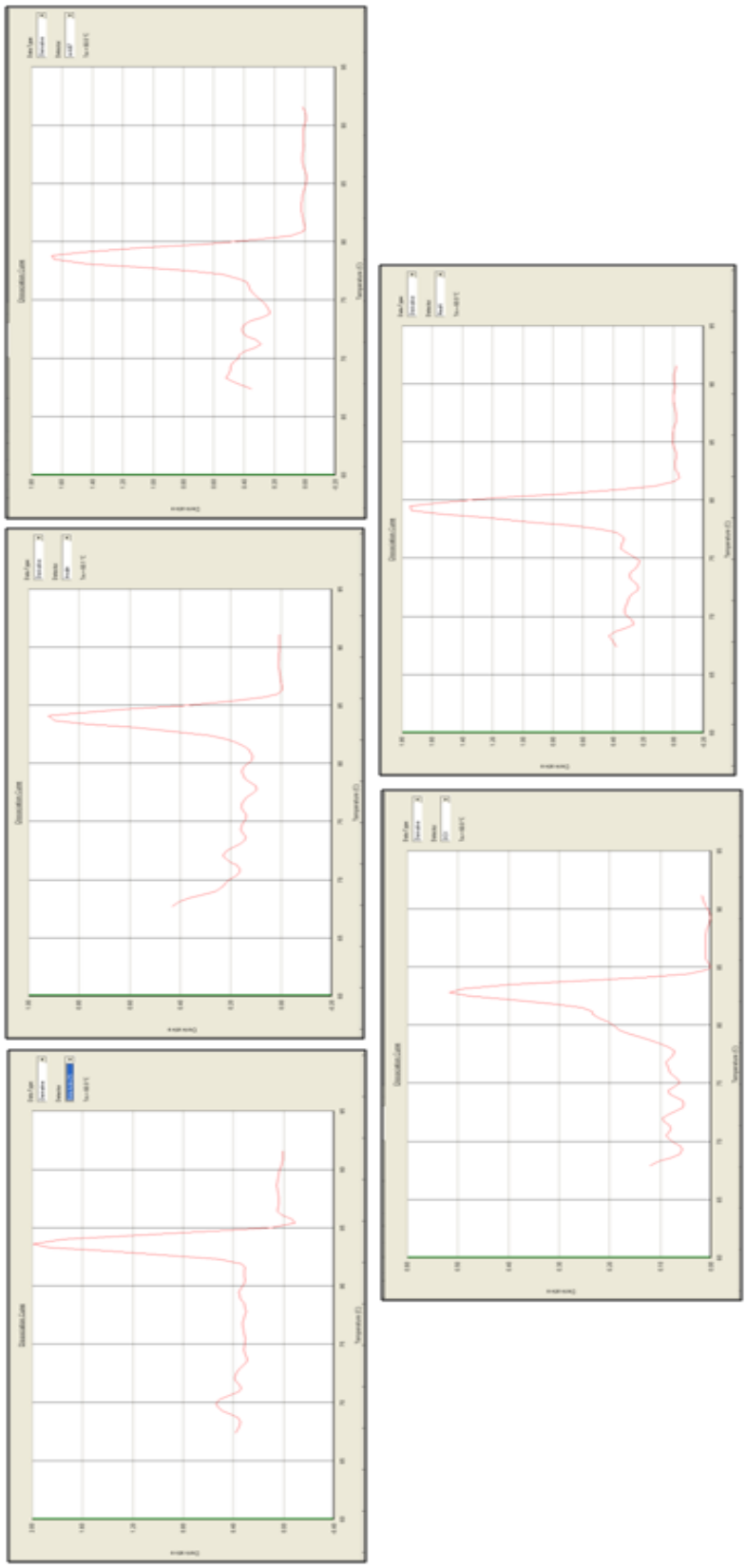


Figure 4.11: Representative dissociation curves of neurogenesis, housekeeping and insulin genes; Top left to right-Actin, Insulin, KI67, DCX and NeuN.

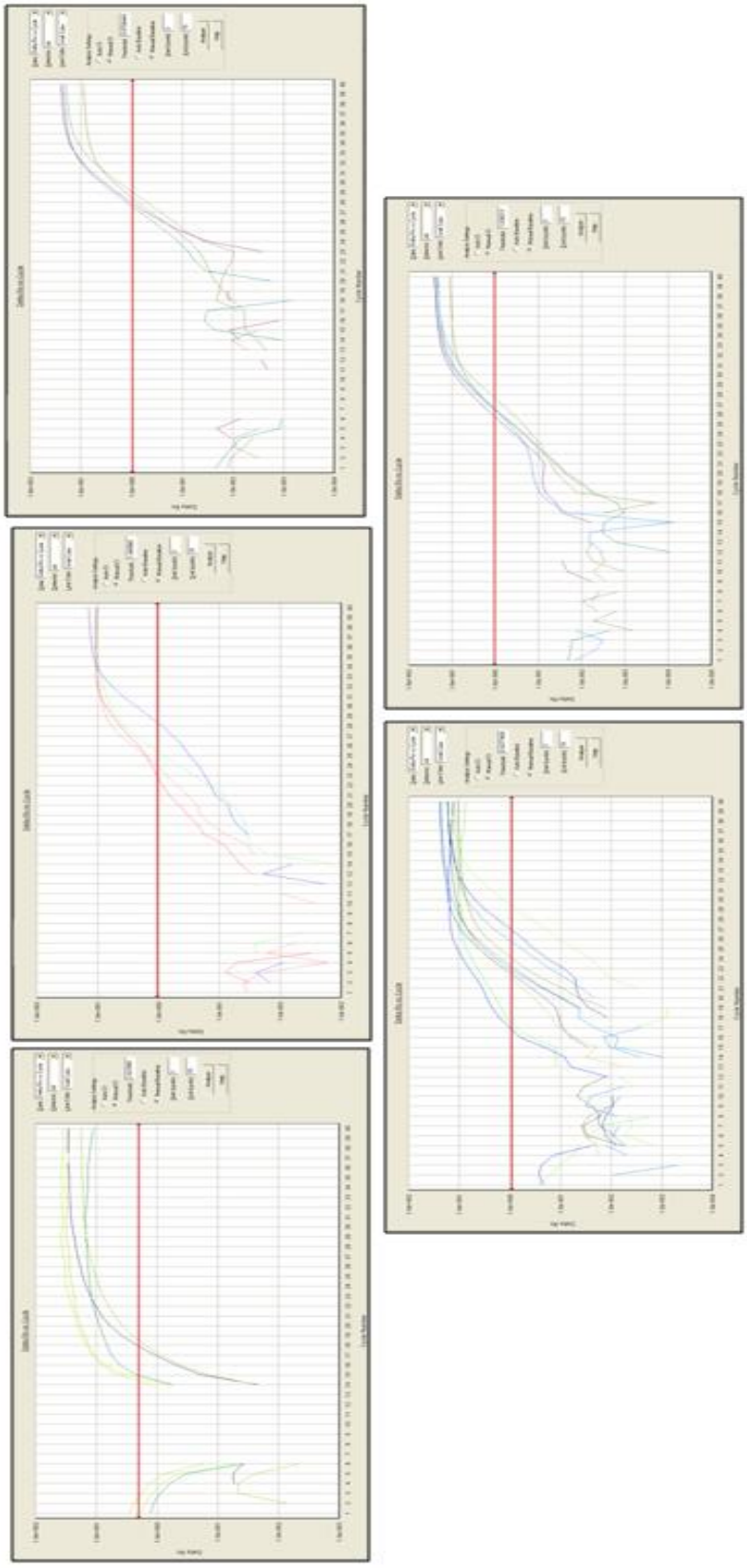


Figure 4.12: Representative amplification plots of neurogenesis, housekeeping and insulin genes; Top left to right-Actin, Insulin, KI67, DCX and NeuN.

4.4 Protein Expression Analysis:

4.4.1 Protein Quantification

Protein concentration of each sample was estimated by plotting the absorbance value of the coloured reaction product on the standard curve. The intensity of the coloured product is directly proportional to the protein content of the sample.

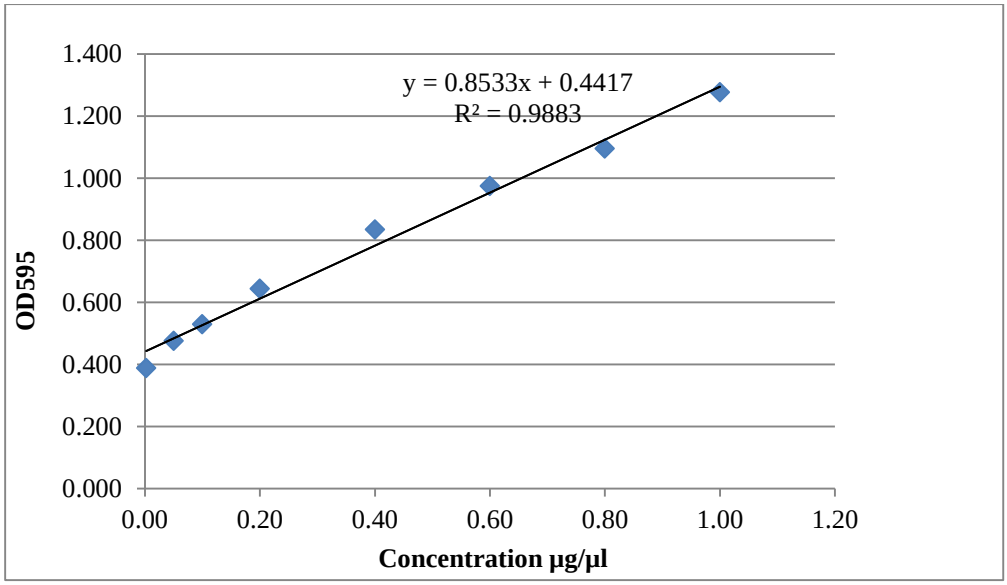


Figure 4.13: Bradford standard curve plotted for eight standard values. Concentration was plotted on the x-axis (independent variable). Absorbance measured at 595 nm was plotted on the y-axis (dependent variable). This graph represents linear regression for the eight standard points. The obtained linear regression value was 0.988 (R^2=0.988).

4.4.2 Hippocampal Proteome Profiling:

The total proteome profile of hippocampus for all six groups was attained by 1D gel separation (SDS-PAGE). The gel image analysis using Image Lab^{TM}software (Bio Rad) revealed a total of eight differnetially expressed protein bands in hippocampus (Table 4.1)

Expression levels of certain proteins were significantly altered when analyzed by 1D-Gel electrophoresis. SDS-PAGE gel images showed the proteins of hippocampus, the levels of which were altered in comparison with respective controls. (Figure 4.14). MS/MS analysis confirmed their identification, as discussed in section below. The

molecular weight, peptide matches and sequence coverage of these proteins are stated in table 4.1.

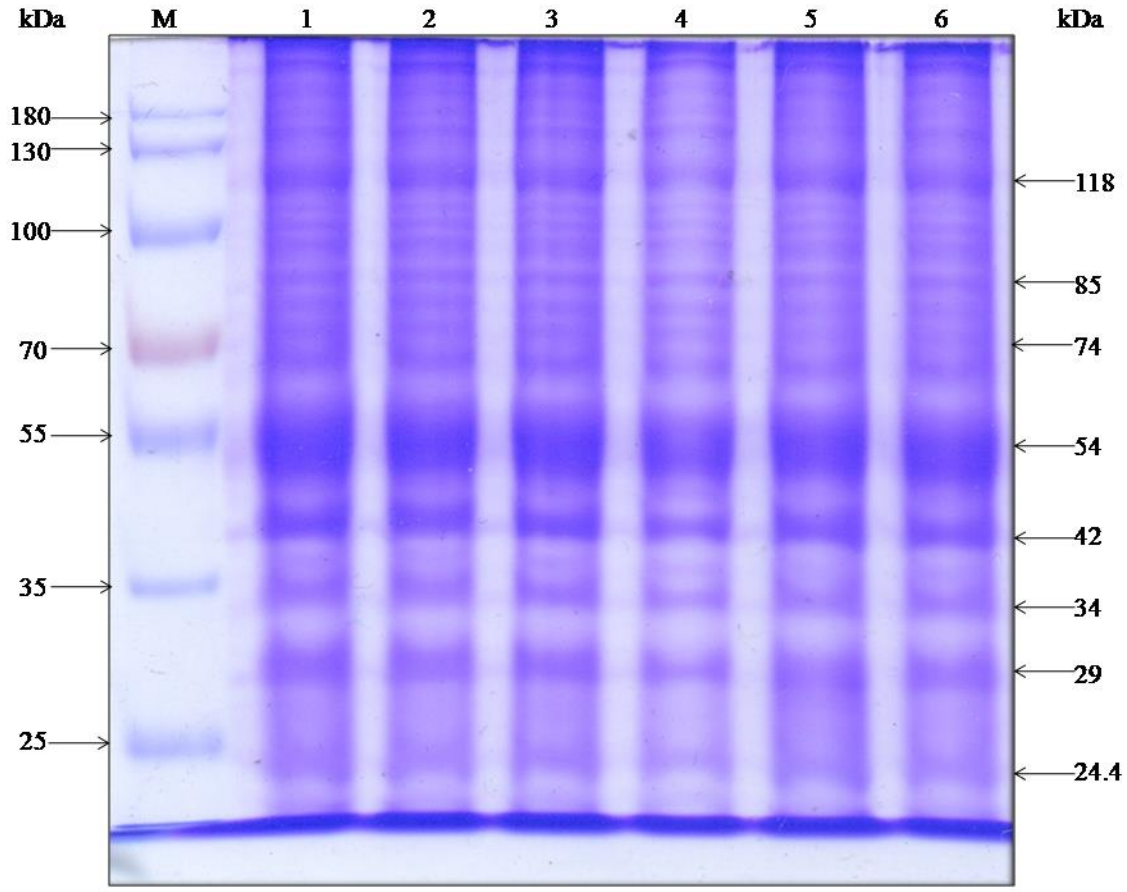


Figure 4.14:The Differential Expression pattern of hippocampal proteins. Extracted proteins were separated in 10% resolving gel and stained with Coomassie Brilliant Blue. Lane M: protein marker (Thermo Scientific Page RulerTM), Lane 1: Control group, Lane 2: $AlCl_3.6H_2O$ + Metformin treated group, Lane 3: Metformin treated group, Lane 4: $AlCl_3.6H_2O$ treated group, Lane 5: $AlCl_3.6H_2O$ + Donepezil treated group and Lane 6: Donepezil treated group. The amount of hippocampal protein loaded in each well was 50µg.

Table 4.1: Differentially expressed proteins as identified by ESI-QTOF MS/MS. Accession no. is obtained from UniProtKB and percent coverage refers to the percentage of protein sequence coverage, determined by number of matched peptides.

Accession No	Protein	Abbr.	*Mr.* (kDa)	Peptide Matches	% Coverage	Subcellular Localization	Functional Category
Q02053	Ubiquitin like Modifier Activating Enzyme -1	UBA1	118	36	42.2	Cytoplasm, Mitochondrion, Nucleus	Ubiquitination
Q99KI0	Aconitrate Hydratase	ACON	85.4	7	11.8	Mitochondrion	Metabolism
O88935	Synapsin-1	SYN-1	74	20	49.7	Cell Junction, Synapse, Golgi Apparatus	Synaptic Function
P11798	Calcium /Calmodulin Dependent Protein Kinase II subunit alpha	KCC2A	54	24	56.9	Cytoplasm, Synapse	Synaptic Transmission
Q04447	Creatine Kinase B Type	KCRB	42	29	88.7	Cytoplasm	Metabolism
Q9CWZ7	Gamma Soluble NSF Attachment Protein	SNAG	34	12	37.5	Membrane	Vesicular Transport
P97251	40S Ribosomal Protein S3a	RS3A	29	11	41.3	Cytoplasm, Nucleus	Transcription Regulation
P10649	Glutathione S Transferase Mu 1	GSTM-1	24.4	12	54.6	Cytoplasm	Metabolism

4.5 Differential Hippocampal Proteome Signature:

A total of 8 proteins showed differential expression in the understudied brain region (Figure 4.14). These included metabolic (ACON, KCRB, GSTM1), synaptosomal (SYN1, KCC2A SNAG) a ubiquitinaion linked (UBA1) and a transcriptional regualator (RS3A) protein. In $AlCl_3.6H_2O$ treated group, expression of UBA1 protein (17.5 ± 2.5)***, ACON(13.0 ± 1.0)***, SYN1 (17.5 ± 2.5)**, KCC2A (22.5 ± 2.5)***, KCRB (9 ± 1.0)**, SNAG (25 ± 2.5)**, RS3A (46 ± 3)*** and GSTM1 (15 ± 3)** were significantly decreased as comapred to control. Metfromin treatment post aluminum exposure significantly increased the levels of UBA1 (41± 2.0)**, ACON (29.0 ± 1)**, SYN1 (38.0 ± 3)*, KCC2A (52.0 ± 1)**, KCRB (36.0 ± 1)**, SNAG (50 ± 2.5)*, RS3A (74 ± 2)**, GSTM1 (39.5 ± 1.5)**. Donepezil presented a simpilar trend, demonstrating a siginificant increase in UBA1(31 ± 1.0)*,SYN1 (36.5 ± 3.0)* ,KCC2A (52± 4.0)**, KCRB (26.5 ± 1.5)*, SNAG (48 ± 5)*, RS3A (70 ± 1.0)* and GSTM1 (41 ± 2)**. No significant effect of donepezil was observed on ACON expression. In all cases, donepezil either presented an equal (SYN1$^{*/*}$, KCC2A$^{**/**}$, SNAG$^{*/*}$, GSTM1$^{**/**}$) or less signifiacnt effect (UBA1$^{**/*}$, KCRB$^{**/*}$, RS3A$^{**/*}$) on protein expression as comapred to metfomin.

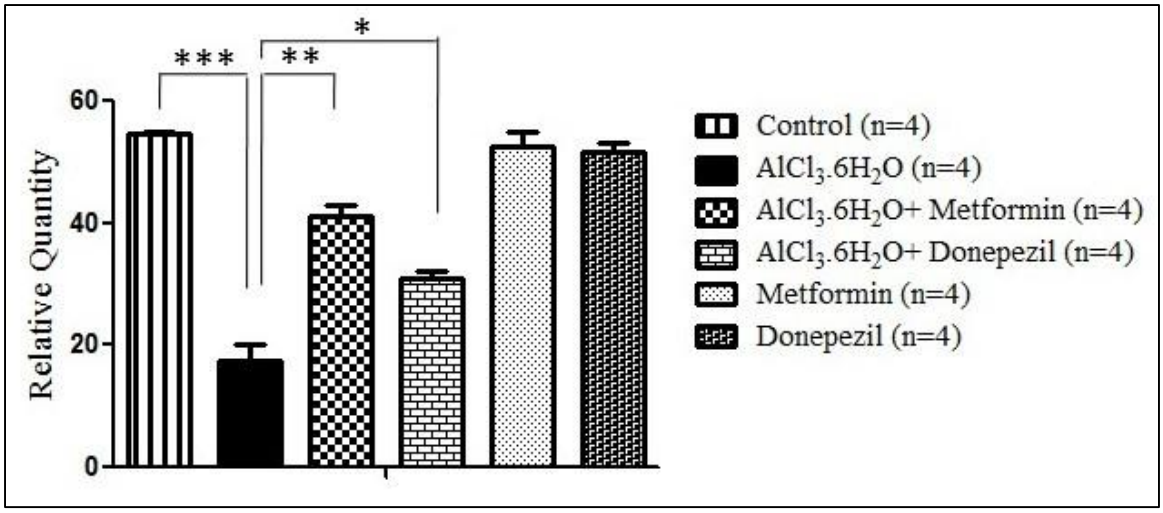


Figure 4.15: Expression graph of differentially expressedUBA1 in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformin and Donepezil group. The data is shown as mean ± SEM, *p< 0.05; **p< 0.01; ***p< 0.001. To check the consistency samples were run in duplicates.

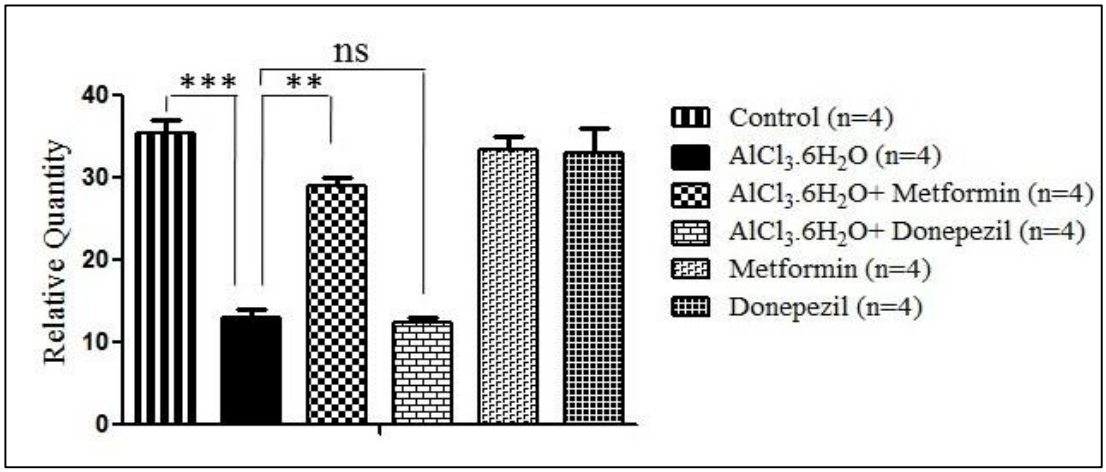


Figure 4.16: Expression graph of differentially expressed ACON in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as mean ± SEM, $^{**}p< 0.01$; $^{***}p< 0.001$. ns= Non significant. To check the consistency samples were run in duplicates

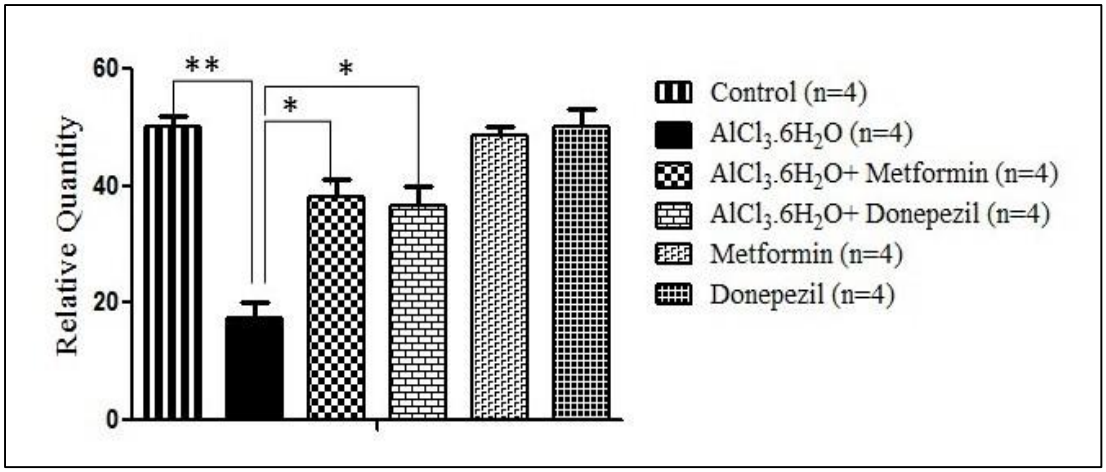


Figure 4.17: Expression graph of differentially expressed SYN1 in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as the mean ± SEM, $^{*}p< 0.05$; $^{**}p< 0.01$. To check the consistency samples were run in duplicates.

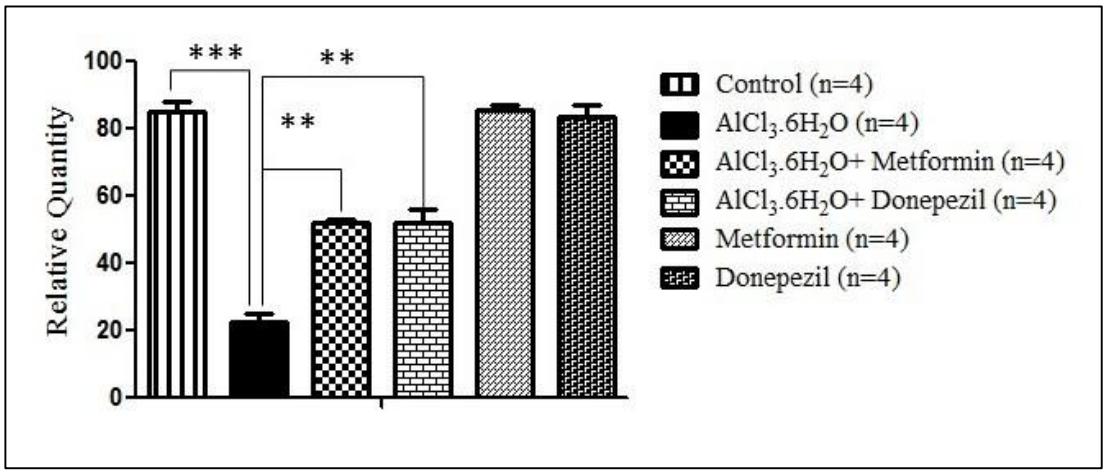


Figure 4.18: Expression graph of differentially expressed KCC2A in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as the mean ± SEM, $^{**}p< 0.01$; $^{***}p< 0.001$. To check the consistency samples were run in duplicates.

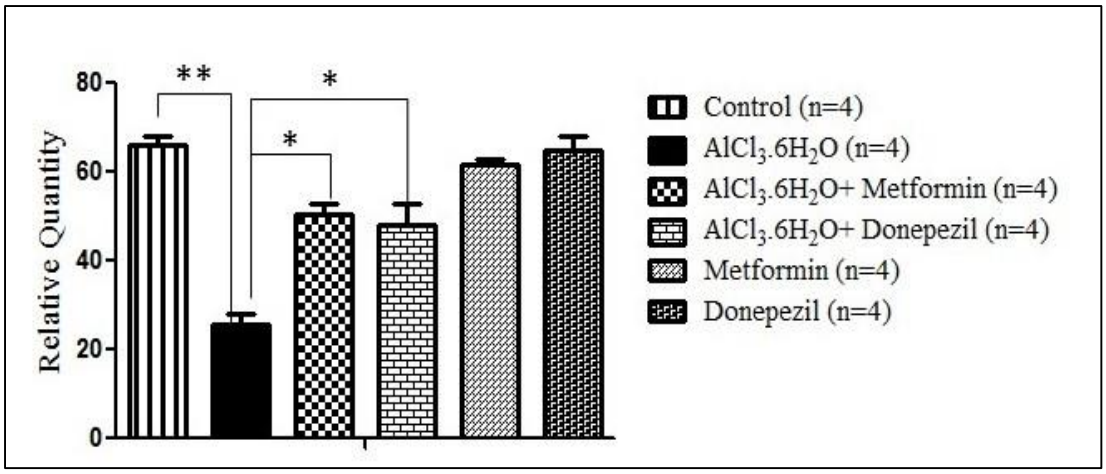


Figure 4.19: Expression graph of differentially expressed KCRB in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as the mean ± SEM, $^{*}p< 0.05$; $^{**}p< 0.01$. To check the consistency samples were run in duplicates.

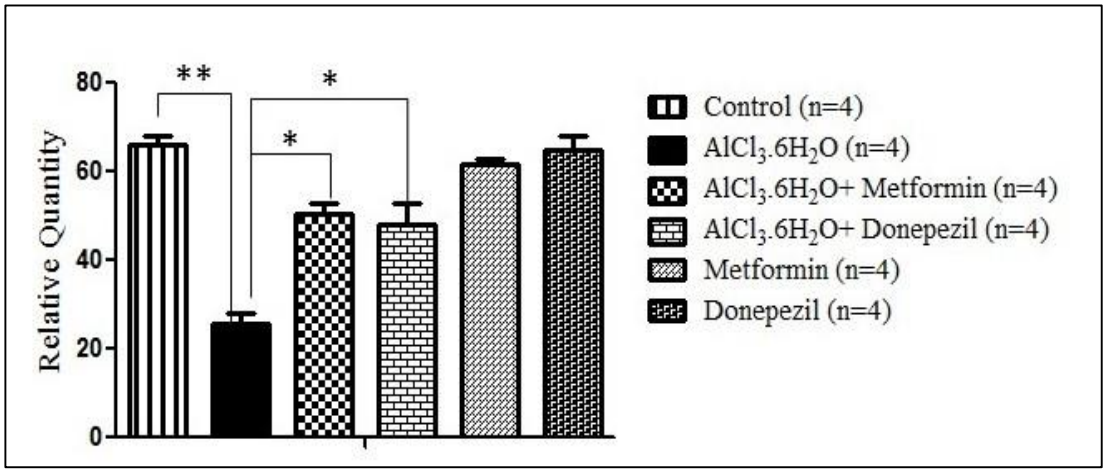


Figure 4.20: Expression graph of differentially expressed SNAG in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as the mean ± SEM, *p< 0.05; **p<0.01. To check the consistency samples were run in duplicates.

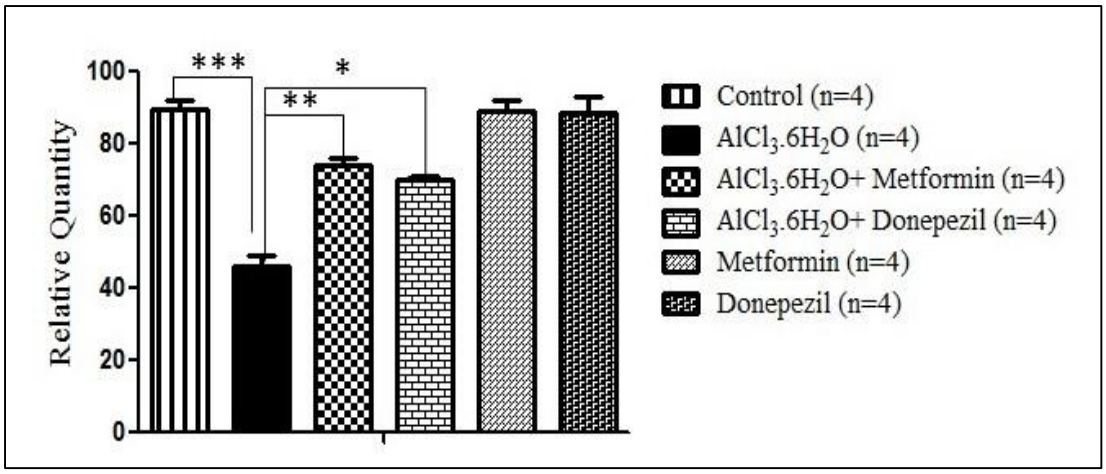


Figure 4.21: Expression graph of differentially expressed RS3A in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as the mean ± SEM, *p< 0.05; **p< 0.01; ***p< 0.001. To check the consistency samples were run in duplicates.

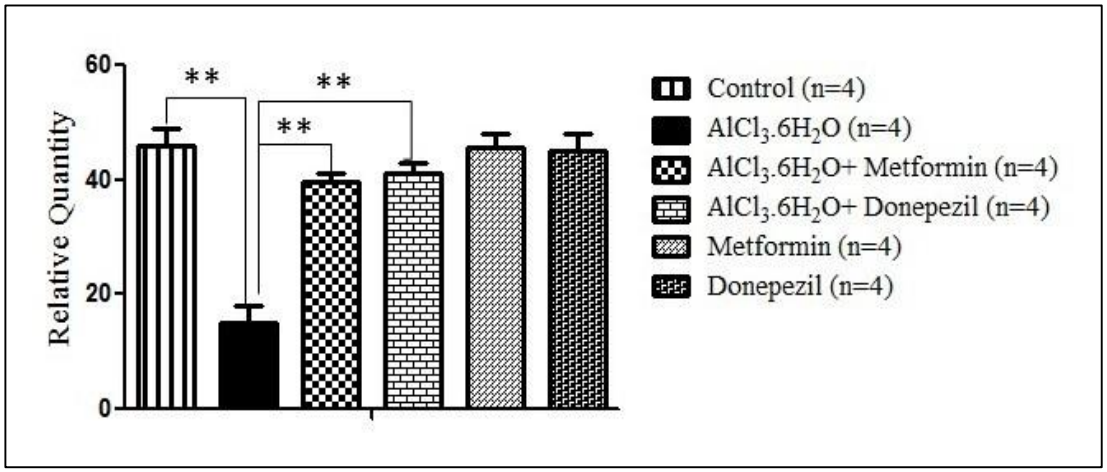


Figure 4.22: Expression graph of differentially expressed GSTM1 in control, $AlCl_3.6H_2O$, $AlCl_3.6H_2O$ + Metformin, $AlCl_3.6H_2O$ + Donepezil, Meformn and Donepezil group. The data is shown as the mean ± SEM, $^{**}p < 0.01$. To check the consistency samples were run in duplicates

4.6 Functional Association and Interaction between Differentially Expressed Proteins:

Predicting functional association networks of differentially expressed proteins could facilitate functional characterization that will further help eluciadating the participation of these proteins in various functional activities. For functional association prediction, STRING 8.3 was used (Jensen et al., 2009). The close intearction patterns are helpful to explicate disease related consequences due to functional perturbations of the differentially expressed proteins. No protein-protein interactions were predicted at high confidence score (0.7) (Figure 4.23a), however, interaction is evident (medium confidence score;0.4) between Camk2A (Calcium/ Calmodulin Dependent Protein Kinase II Subunit alpha) and SYN1 (Figure 4.23b). SYN 1 interacting partner as predicted by STRING 8.3 is shown in figure 4.24.

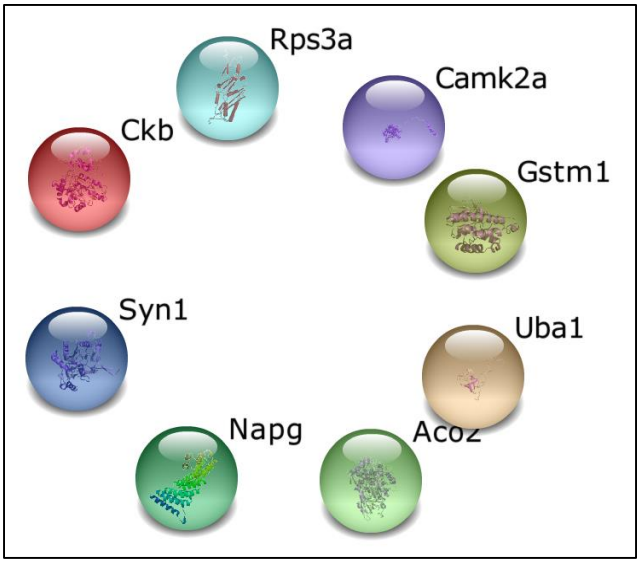


Figure 4.23 (a): Functional association network of differnetially expressed proteins: No protein-protein intearctions were predicted at high confidence score (0.7) derived from the STRING database (http://string-db.org/). Each protein, Uba1 (UBA1), Aco2 (ACON), Syn1 (SYN1), KCC2A (Camk2a), Ckb (KCRB), Napg (SNAG), Rps3a (RS3A) and Gstm1 (GSTM1) is represented as a node .

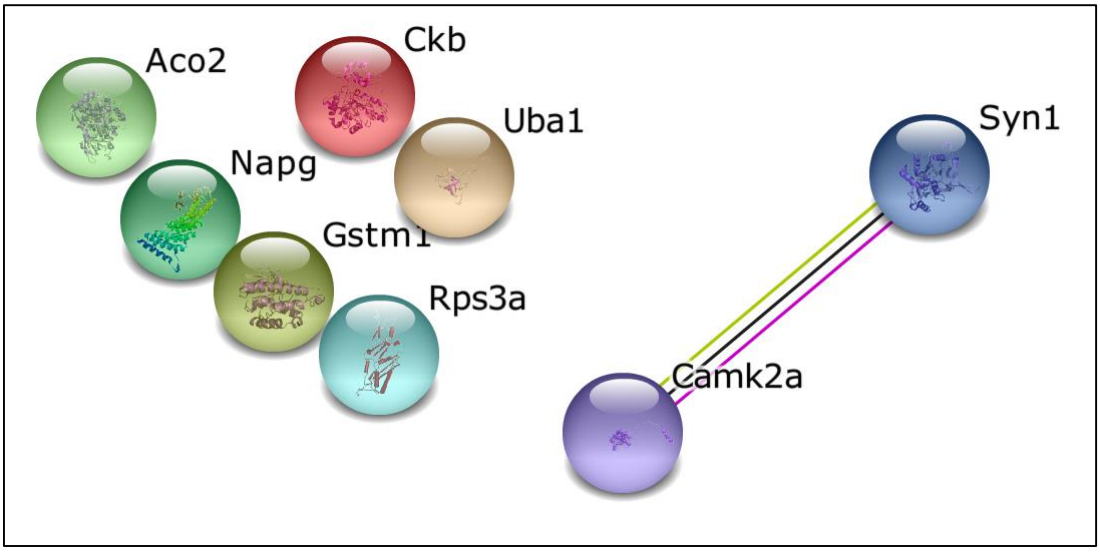


Figure 4.23 (b): Functional association network of differnetially expressed proteins: Medium confidence protein –protein interaction network of identified differentially expressed proteins derived from the STRING database (http://string-db.org/). Each protein is represented as a node with edged interactions.

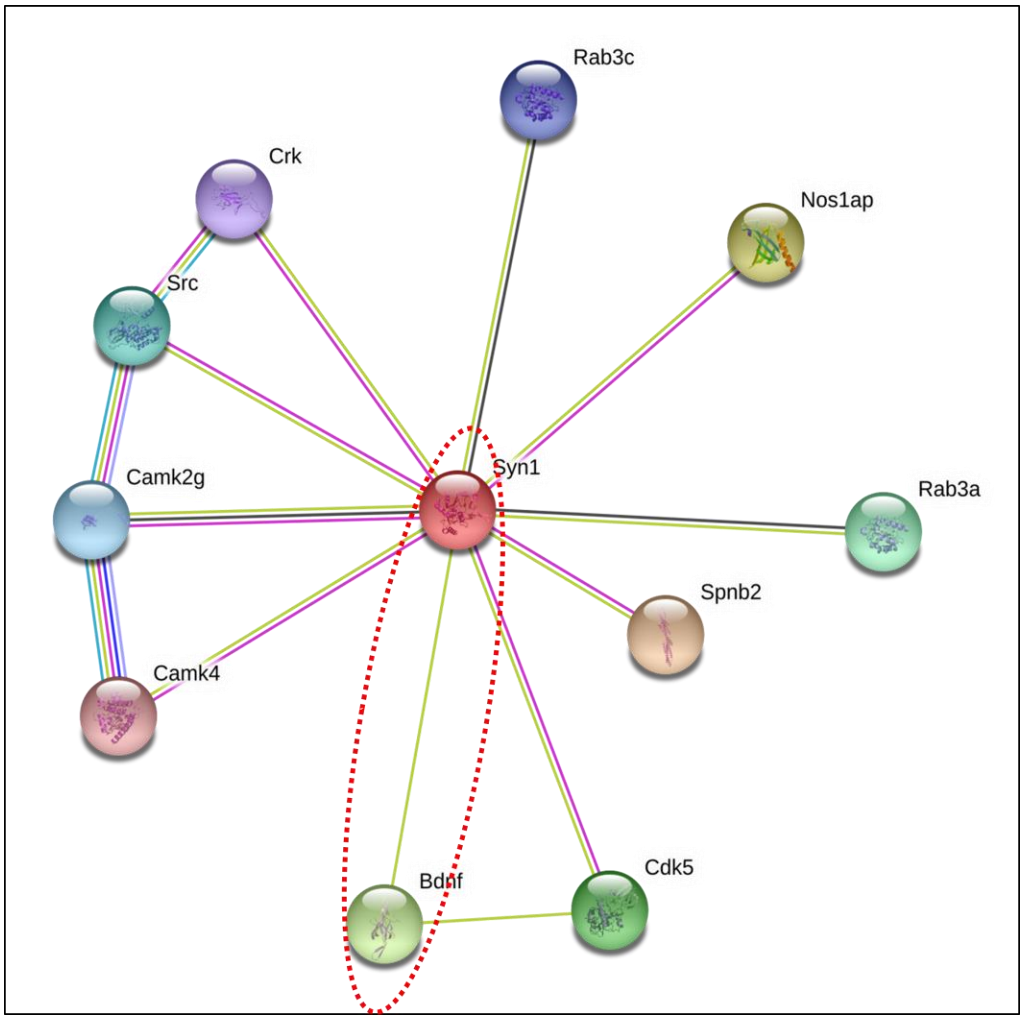


Figure: 4.24: **Functional annotation of potential interacting partner of SYN-1**derived from the STRING database (http://string-db.org/). Each protein is represented as a nodewith edged interaction.

4.7. Immunohistochemical Evidence for Increased Neurogenesis in Hippocampus:

To study the neurodegenerative consequences of $AlCl_3.6H_2O$ treatment and putative rescue by metformin and donepezil administration, tissue sections were incubated with antibody NeuN. NeuN immunoreactive neurons were detected in hippocampal pyramidal layer. There was no obvious hippocampal neuronal loss in control, metformin and donepezil treated mice. However, there was a striking degree of neuronal loss in $AlCl_3.6H_2O$ treated group. The number of NeuN immunoreactive neurons was considerably increased in both $AlCl_3.6H_2O$+ Metformin and $AlCl_3.6H_2O$ + donepezil treated group as compared to $AlCl_3.6H_2O$ treated group (Figure 4.25).

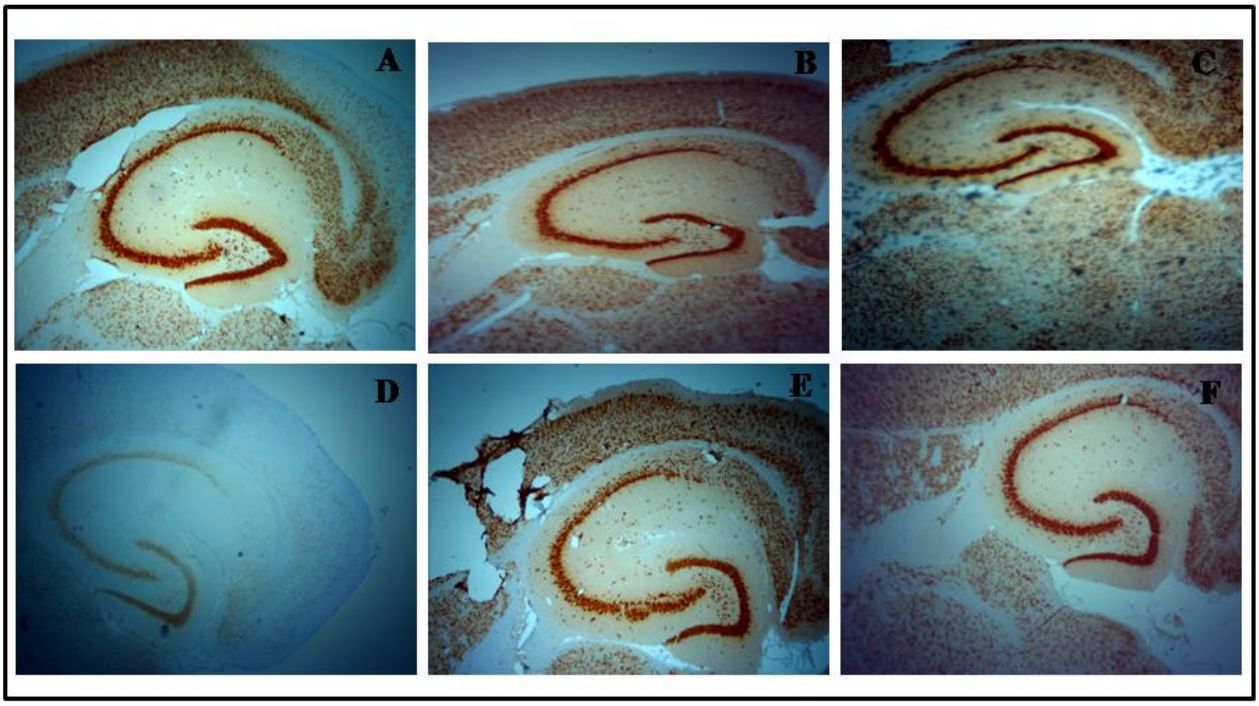


Figure 4.25- **I**

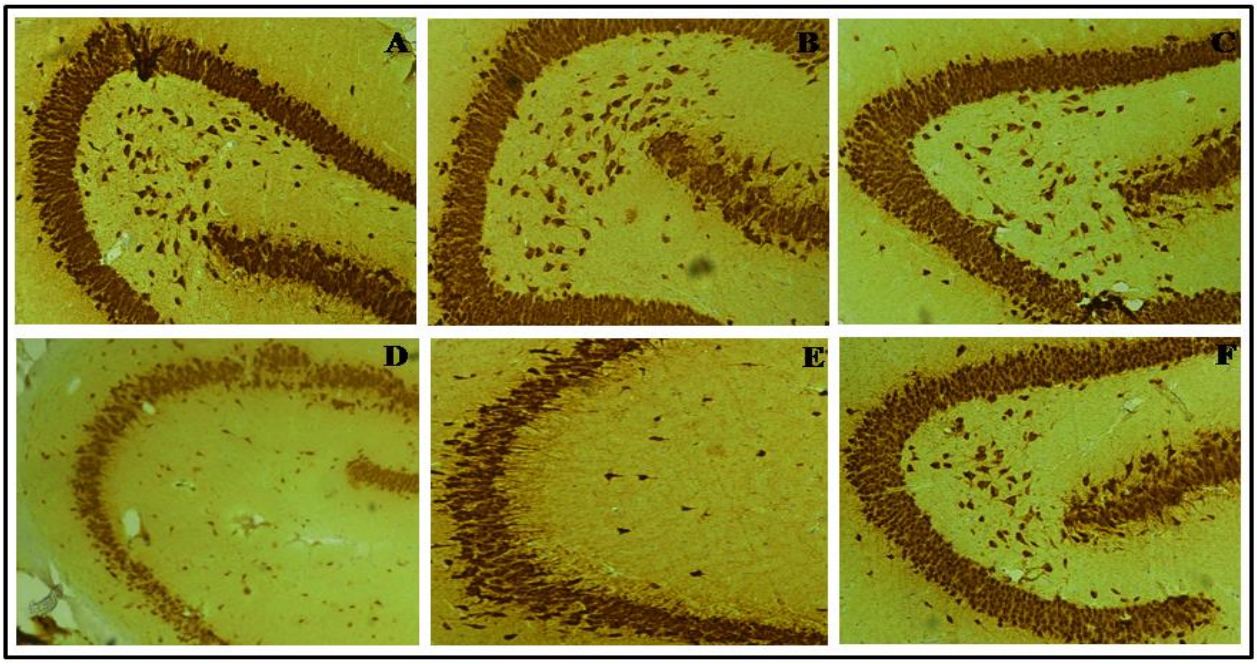


Figure 4.25-**II**

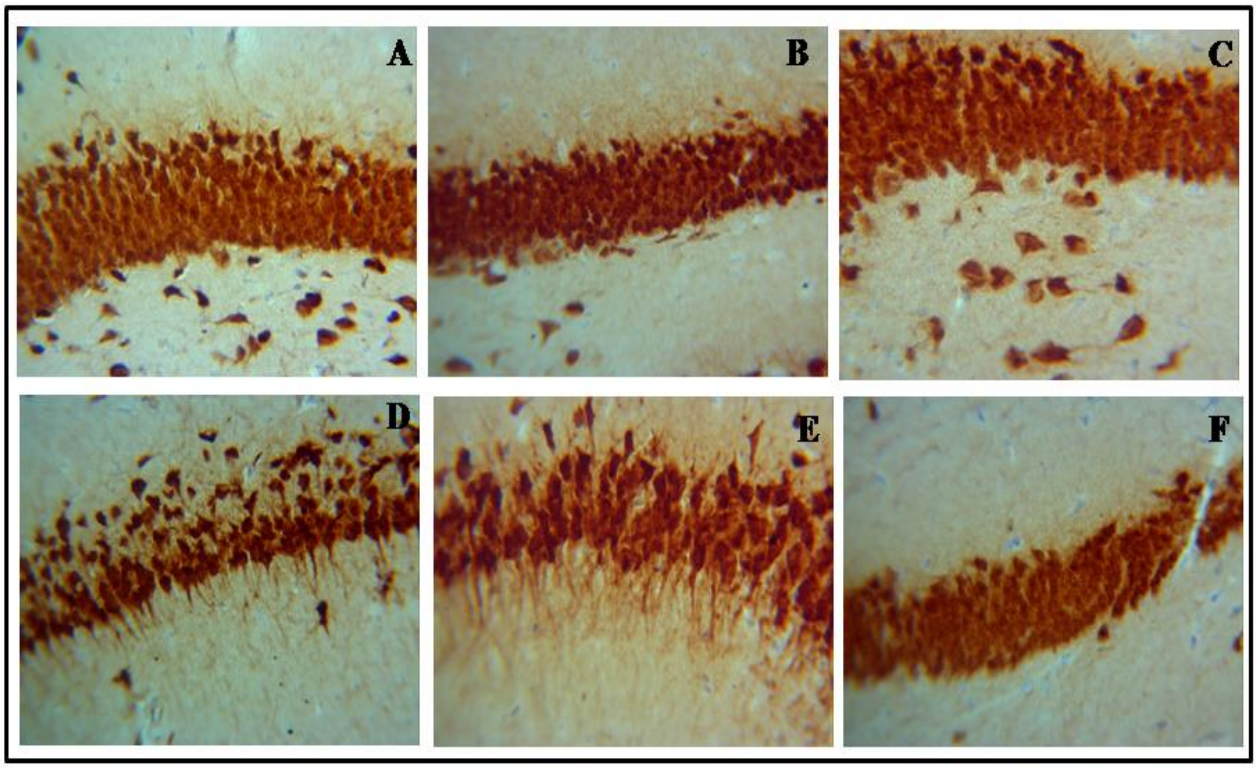


Figure 4.25- **III**

Figure 4.25: Neuron-specific NeuN labeling of Hippocampal Tissue: (A) Substantialneuronal loss in$AlCl_3.6H_2O$ treated mice hippocampus. Image indicates neuronal loss recovery in $AlCl_3.6H_2O$ + Donepezil group **(B)** and $AlCl_3.6H_2O$ + Metformin group**(C).**Intact pyramidal layer, positive for NeuN antibody, observed in control **(D)**, Donepezil **(E)** and Metformin group **(F).**Original magnifications: **I:** 4X**, II:** 20X**, III:** 40X

Chapter 5

DISCUSSION

The current study was conducted with the objective of identifying and comparing with donepezil, pro-neurogenic effects of metformin and enhancement of hippocampal-dependent memory in animal model of $AlCl_3.6H_2O$ induced neurodegeneration.

$AlCl_3.6H_2O$ treated mice have previously been reported to display cognitive deficits in a MWM paradigm (Rebai and Djebli, 2008, Rani *et al*., 2015), a widely used laboratory tool in behavioral research that assesses spatial learning and memory in rodents (D'Hooge and De Deyn, 2001). Similar acquisition and probe trial deficits as previously described were observed in $AlCl_3.6H_2O$ treated mice. The mice were unable to learn the position of the hidden platform as efficiently as $AlCl_3.6H_2O$+ Metformin and $AlCl_3.6H_2O$+ Donepezil treated group, which was reflected by deviant learning curves, escape latency, and impaired probe trial performance. This inferior performance can be attributed to cognitive deficits. The findings demonstrate that the widely used diabetes drug metformin has better chances of recuing memory deficits than acetylcholinesterase inhibitor, donepezil as shown by improved escape latency and probe trail performance. It is speculated that metformin enhances spatial memory function coincident with a long-term increase in the number of newly born adult dentate gyrus neurons.

Consistent with this, significantly increased expression of genes mediating major phases of neurogenesis, that is proliferation (Ki67), migration (DCX) and maturation (NeuN), was observed in $AlCl_3.6H_2O$ + Metformin and $AlCl_3.6H_2O$ + Donepezil group as compared to $AlCl_3.6H_2O$ group. The proliferation rate was much increased in $AlCl_3.6H_2O$ + Donepezil group as compared to $AlCl_3.6H_2O$ + Metformin group as demonstrated by increased ki67 expression. However, the pro neurogenic effects appeared to wane as demonstrated by comparatively decreased DCX and NeuN gene expression in $AlCl_3.6H_2O$ + Donepezil group as compared to $AlCl_3.6H_2O$ + Metformin. Conversely, the $AlCl_3.6H_2O$ + Metformin group sustained the increased levels of both DCX and NeuN. This could be explained by insulin mediated Akt signaling pathway and consequent anti-apoptotic-associated protein synthesis that maintains cell viabilty so long

so that they enter the late phase of neurogenesis; migration and maturation (Dudek *et al*., 1997; Duarte *et al*., 2008).

In support of recent findings (Crews and Masliah, 2010; Hwang *et al*., 2010), our immunohistochemical results demonstrated a more significant increase in post mitotic NeuN positive neurons in hippocampus of $AlCl_3.6H_2O$ + Metformin group as compared to $AlCl_3.6H_2O$ + donepezil group . Conversely, loss of immunoreactivity in $AlCl_3.6H_2O$ group demonstrated neuronal loss. The profound/intense NeuN labeling observed in $AlCl_3.6H_2O$ + Metformin group favors metformin over donepezil with regard to pro neurogenic potential.

Histopathological assessment presented more promising observations for tissues sections treated post $AlCl_3.6H_2O$ exposure with metformin as compared to donepezil. Metformin reversed $AlCl_3.6H_2O$ induced oxidative stress that mediates neuronal loss (Rao and Shetty, 2004) and demonstrated a substantial increase in density of Nissl substances in comparison to donepezil. These, and previous data (Correia *et al*., 2008; Abd-Elsameea *et al*., 2014) implicate insulin sensitizing drug metformin as a promising neuroprotective agent against oxidative stress and apoptosis associated with neurodegenerative diseases.

Loss of synaptic circuitry in the AD brain can be related to observed cognitive deficits in AD individuals (Serrano-Pozo *et al*., 2011). Metformin has been shown to mediate memory formation via synapse plasticity in a manner similar to the clinically approved histone deacetylase (HDAC) inhibitors (Crews and Masliah, 2010). This is in line with increased levels of synaptosomal proteins; KCC2A, SYN-1 and SNAG observed in $AlCl_3.6H_2O$ + Metformin group. SNAG plays an important role in vesicular transport in the constitutive secretory pathway as well as in neurotransmitter release (Zhao *et al*., 2007). Deficits in SNAG as seen in $AlCl_3.6H_2O$ group suggest its plausible role in the altered synaptic function in AD. KCC2A has been implicated as a key player in molecular mechanisms underlying learning and memory. The interaction between KCC2A and N methyl D aspartate receptor (NMDAR) is important for the induction of long term potentiation (LTP)(Barria and Malinow, 2005) which correlates with learning and memory. However disrupted interactions produced deficits in hippocampal LTP and spatial learning as evident from the results of MWM. SYN1, has a role in

neurotransmitter synthesis, homeostasis and synaptic vesicle development and functions at excitatory glutamatergic synapses in the hippocampus (Bogen *et al*., 2011). Functional association between SYN1 and KCC2A, as demonstrated by STRING, predicts disease related consequences due to synaptosomal functional perturbations assocaited with down regulation of KCC2A and SYN1. Moreover, functional association network as predicted by STRING 8.3 showed (medium confidence score, 0.4) SYN1 to interact with BDNF, a known stimulator of adult neurogenesis. It is conceivable that upregulation of SYN-1 and KCC2A in $AlCl_3.6H_2O$ + Metformin group indicates metformin facilitated memory formation via synapse plasticity and possible BDNF mediated increased neurogenesis.

Notably, decrease in UBA1 protein expression was detected in $AlCl_3.6H_2O$ group. Down-regulation of UBA1 implies a sub-optimal functioning or defective ubiquitin proteasome system (UPS).The exact mechanism that impairs the UPS in AD remains obscure, although there are several indications that the general mechanisms underlying AD might have a direct effect on the UPS(Deger *et al*., 2015). It is conceivable that proteins that accumulate in the classical hallmarks of AD are linked to UPS dysfunction. Metformin treatment significantly increased UBA1 levels. This increase in UBA1 may therefore be interpreted as a cytoprotective response to combat abnormal or aggregated proteins.

RS3A, a constituent of ribosomal assembly, is known to interact with proteins mediating cellular differentiation. Its down regulation in $AlCl_3.6H_2O$ group and positive modulation by metformin strongly suggests a decrease and increase in endogeneous neurogenesis respectively.

Several metabolism associated proteins were found to be downregulated in $AlCl_3.6H_2O$ group. ACON, an important TCA cycle enzyme, catalyzes the reversible isomerization of citrate and isocitrate via its intermediate form cis- aconitate and is one of the metabolic enzymes which are particularly sensitive to reactive oxygen species (ROS) and important in oxidative inactivation. Significant alteration of aconitase activity in several oxidative stress mediated neurodegenerative disorders mediates neurotoxicity by increasing $Fe^{2+}$and hydrogen peroxide (Cantu *et al*., 2009). Glutathione S-transferases (GSTs), a family of isoenzymes, play a critical role in providing protection against electrophiles and products of oxidative stress (Ghosh *et al*., 2012).The significant

downregulation of GSTM-1 and ACON in $AlCl_3.6H_2O$ hippocampus indicates a handicapped antioxidant defense system as a consequence of increased oxidative stress. The levels were found to be restored in $AlCl_3.6H_2O$ + Metformin group. It is highly conceivable that metformin increased ACON and GSTM1 levels are a reflection of metformin counteracting oxidative stress, a condition common to both AD and DM. Significant downregulation of KCRB in hippocampus is valuable information since creatine kinase (CK) regulates ATP levels in neuronal cells and have an integral role in energy buffering and overall cellular bioenergetics (Wallimann *et al*., 2011). Generation of ATP is critical to CNS function as neurons require a greater amount of ATP to maintain membrane polarization, Ca^{2+} influx from organelles, and processing of neurotransmitters. Creatine Kinase Protein (CKP) is associated with these important energy production processes and catalysed ATP regeneration and energy homeostasis by the reversible transfer of high energy phosphate from phosphocreatine to ADP. Alterations in metabolism associated proteins in $AlCl_3.6H_2O$ and their restoration by metformin strengthens the conjecture of AD being a metabolic disorder, imploring the need to investigate the effect of anti diabetic drugs like metformin in this heterogeneous disorder. Profound pro neurogenic effects associated with metformin as compared to donepezil further stresses the need of alternative therapeutic strategy for AD. Furthermore, SYN1 upregulation mediated BDNF activation may represent the underlying mechanism of metformin mediated neurogenesis.

Conclusion:

Although our understanding of the functional role of adult neurogenesis is limited, the notion of modulating this endogenous process with oral medications for the treatment of neurological disorders has garnered much interest. Our findings demonstrate that metformin associated profound pro neurogenic effects rule in favor of metformin over donepezil with regard to neuro regenerative potential. Thus, while metformin may have other actions in the nervous system, our findings raise the possibility that its ability to enhance neurogenesis might have a positive impact in neurodegeneration associated nervous system disorders. Further studies should take into consideration the complex interplay between metformin and neurogenesis associated proteins.

Chapter 6

REFERENCES

ABD-ELSAMEEA, A. A., MOUSTAF, A. A. & MOHAMED, A. M. 2014. Modulation of the oxidative stress by metformin in the cerebrum of rats exposed to global cerebral ischemia and ischemia/reperfusion. *European review for medical and pharmacological sciences,* 18**,** 2387-92.

ÅBERG, M. A., ÅBERG, N. D., HEDBÄCKER, H., OSCARSSON, J. & ERIKSSON, P. S. 2000. Peripheral infusion of IGF-I selectively induces neurogenesis in the adult rat hippocampus. *The Journal of Neuroscience,* 20**,** 2896-2903.

ÅBERG, M. A., ÅBERG, N. D., PALMER, T. D., ALBORN, A.-M., CARLSSON-SKWIRUT, C., BANG, P., ROSENGREN, L. E., OLSSON, T., GAGE, F. H. & ERIKSSON, P. S. 2003. IGF-I has a direct proliferative effect in adult hippocampal progenitor cells. *Molecular and Cellular Neuroscience,* 24**,** 23-40.

ABROUS, D. N., KOEHL, M. & LE MOAL, M. 2005. Adult neurogenesis: from precursors to network and physiology. *Physiological reviews,* 85**,** 523-569.

AHMED, S., MAHMOOD, Z. & ZAHID, S. 2015. Linking insulin with Alzheimer's disease: emergence as type III diabetes. *Neurological Sciences***,** 1-7.

AIMONE, J. B., WILES, J. & GAGE, F. H. 2006. Potential role for adult neurogenesis in the encoding of time in new memories. *Nature neuroscience,* 9**,** 723-727.

ALAGIAKRISHNAN, K., SANKARALINGAM, S., GHOSH, M., MEREU, L. & SENIOR, P. 2013. Antidiabetic drugs and their potential role in treating mild cognitive impairment and Alzheimer's disease. *Discovery medicine,* 16**,** 277-286.

ALVAREZ, E. O., BEAUQUIS, J., REVSIN, Y., BANZAN, A. M., ROIG, P., DE NICOLA, A. F. & SARAVIA, F. 2009. Cognitive dysfunction and hippocampal changes in experimental type 1 diabetes. *Behavioural brain research,* 198**,** 224-230.

AMATO, S. & MAN, H.-Y. 2011. Bioenergy sensing in the brain: the role of AMP-activated protein kinase in neuronal metabolism, development and neurological diseases. *Cell Cycle,* 10**,** 3452-3460.

ANDROUTSELLIS-THEOTOKIS, A., LEKER, R. R., SOLDNER, F., HOEPPNER, D. J., RAVIN, R., POSER, S. W., RUEGER, M. A., BAE, S.-K., KITTAPPA, R. &

MCKAY, R. D. 2006. Notch signalling regulates stem cell numbers in vitro and in vivo. *Nature,* 442**,** 823-826.

ARSENIJEVIC, Y., WEISS, S., SCHNEIDER, B. & AEBISCHER, P. 2001. Insulin-like growth factor-I is necessary for neural stem cell proliferation and demonstrates distinct actions of epidermal growth factor and fibroblast growth factor-2. *The Journal of Neuroscience,* 21**,** 7194-7202.

ASHE, K. H. & ZAHS, K. R. 2010. Probing the biology of Alzheimer's disease in mice. *Neuron,* 66**,** 631-45.

BARKHO, B. Z., SONG, H., AIMONE, J. B., SMRT, R. D., KUWABARA, T., NAKASHIMA, K., GAGE, F. H. & ZHAO, X. 2006. Identification of astrocyte-expressed factors that modulate neural stem/progenitor cell differentiation. *Stem cells and development,* 15**,** 407-421.

BARRIA, A. & MALINOW, R. 2005. NMDA receptor subunit composition controls synaptic plasticity by regulating binding to CaMKII. *Neuron,* 48**,** 289-301.

BEATUS, P. & LENDAHL, U. 1998. Notch and neurogenesis. *Journal of neuroscience research,* 54**,** 125-136.

BEAUQUIS, J., ROIG, P., HOMO-DELARCHE, F., DE NICOLA, A. & SARAVIA, F. 2006. Reduced hippocampal neurogenesis and number of hilar neurones in streptozotocin-induced diabetic mice: reversion by antidepressant treatment. *European Journal of Neuroscience,* 23**,** 1539-1546.

BECK, K. D., POWELL-BRAXTONT, L., WIDMER, H.-R., VALVERDE, J. & HEFTI, F. 1995. Igf1 gene disruption results in reduced brain size, CNS hypomyelination, and loss of hippocampal granule and striatal parvalbumin-containing neurons. *Neuron,* 14**,** 717-730.

BENEDICT, C., HALLSCHMID, M., SCHMITZ, K., SCHULTES, B., RATTER, F., FEHM, H. L., BORN, J. & KERN, W. 2007. Intranasal insulin improves memory in humans: superiority of insulin aspart. *Neuropsychopharmacology,* 32**,** 239-243.

BERCHTOLD, N. C., KESSLAK, J. P. & COTMAN, C. W. 2002. Hippocampal brain-derived neurotrophic factor gene regulation by exercise and the medial septum. *Journal of neuroscience research,* 68**,** 511-521.

BIEBL, M., COOPER, C. M., WINKLER, J. & KUHN, H. G. 2000. Analysis of neurogenesis and programmed cell death reveals a self-renewing capacity in the adult rat brain. *Neuroscience letters,* 291**,** 17-20.

BIESSELS, G.-J., KAMAL, A., URBAN, I. J., SPRUIJT, B. M., ERKELENS, D. W. & GISPEN, W. H. 1998. Water maze learning and hippocampal synaptic plasticity in streptozotocin-diabetic rats: effects of insulin treatment. *Brain research,* 800**,** 125-135.

BOEKHOORN, K., JOELS, M. & LUCASSEN, P. J. 2006. Increased proliferation reflects glial and vascular-associated changes, but not neurogenesis in the presenile Alzheimer hippocampus. *Neurobiology of disease,* 24**,** 1-14.

BOGEN, I. L., JENSEN, V., HVALBY, O. & WALAAS, S. I. 2011. Glutamatergic neurotransmission in the synapsin I and II double knock-out mouse. *Seminars in cell & developmental biology,* 22**,** 400-7.

BRAAK, H., BRAAK, E. & BOHL, J. 1993. Staging of Alzheimer-related cortical destruction. *European neurology,* 33**,** 403-408.

BROMLEY-BRITS, K., DENG, Y. & SONG, W. 2011. Morris water maze test for learning and memory deficits in Alzheimer's disease model mice. *Journal of visualized experiments : JoVE*.

CANTU, D., SCHAACK, J. & PATEL, M. 2009. Oxidative Inactivation of Mitochondrial Aconitase Results in Iron and H₂O₂-Mediated Neurotoxicity in Rat Primary Mesencephalic Cultures. *PLoS ONE,* 4**,** e7095.

CARSON, M. J., BEHRINGER, R. R., BRINSTER, R. L. & MCMORRIS, F. A. 1993. Insulin-like growth factor I increases brain growth and central nervous system myelination in tTransgenic mice. *Neuron,* 10**,** 729-740.

CHAN, A. S., YEUNG, W. W. & WONG, Y. H. 2005. Integration of G protein signals by extracellular signal-regulated protein kinases in SK-N-MC neuroepithelioma cells. *Journal of neurochemistry,* 94**,** 1457-1470.

CHEN, Q., NAKAJIMA, A., CHOI, S. H., XIONG, X., SISODIA, S. S. & TANG, Y.-P. 2008. Adult neurogenesis is functionally associated with AD-like neurodegeneration. *Neurobiology of disease,* 29**,** 316-326.

CORREIA, S., CARVALHO, C., SANTOS, M. S., PROENCA, T., NUNES, E., DUARTE, A. I., MONTEIRO, P., SEICA, R., OLIVEIRA, C. R. & MOREIRA, P. I. 2008. Metformin protects the brain against the oxidative imbalance promoted by type 2 diabetes. *Medicinal chemistry (Shariqah (United Arab Emirates)),* 4**,** 358-64.

CRAFT, S. 2012. Alzheimer disease: insulin resistance and AD—extending the translational path. *Nature Reviews Neurology,* 8**,** 360-362.

CRAFT, S., CHOLERTON, B. & BAKER, L. D. 2013. Insulin and Alzheimer's disease: untangling the web. *Journal of Alzheimer's disease : JAD,* 33**,** S263-S275.

CREWS, L., ADAME, A., PATRICK, C., DELANEY, A., PHAM, E., ROCKENSTEIN, E., HANSEN, L. & MASLIAH, E. 2010. Increased BMP6 levels in the brains of Alzheimer's disease patients and APP transgenic mice are accompanied by impaired neurogenesis. *The Journal of neuroscience,* 30**,** 12252-12262.

CREWS, L. & MASLIAH, E. 2010. Molecular mechanisms of neurodegeneration in Alzheimer's disease. *Human molecular genetics***,** ddq160.

D'HOOGE, R. & DE DEYN, P. P. 2001. Applications of the Morris water maze in the study of learning and memory. *Brain research reviews,* 36**,** 60-90.

DA PENHA BERZAGHI, M., COOPER, J., CASTREN, E., ZAFRA, F., SOFRONIEW, M., THOENEN, H. & LINDHOLM, D. 1993. Cholinergic regulation of brain-derived neurotrophic factor (BDNF) and nerve growth factor (NGF) but not neurotrophin-3 (NT-3) mRNA levels in the developing rat hippocampus. *The Journal of neuroscience,* 13**,** 3818-3826.

DE LA MONTE, S. M. 2012. Brain insulin resistance and deficiency as therapeutic targets in Alzheimer's disease. *Current Alzheimer Research,* 9**,** 35.

DE LA MONTE, S. M., TONG, M., LAWTON, M. & LONGATO, L. 2009. Nitrosamine exposure exacerbates high fat diet-mediated type 2 diabetes mellitus, non-alcoholic steatohepatitis, and neurodegeneration with cognitive impairment. *Molecular neurodegeneration,* 4**,** 54.

DE LA MONTE, S. M., TONG, M., LESTER-COLL, N., PLATER JR, M. & WANDS, J. R. 2006. Therapeutic rescue of neurodegeneration in experimental type 3

diabetes: relevance to Alzheimer's disease. *Journal of Alzheimer's disease: JAD,* 10**,** 89-109.

DE LA TORRE, J., ALIEV, G., PERRY, G., FINUCANE, T., MEYER, M. & CUMMINGS, J. 2004. Drug therapy in Alzheimer's disease. *The New England journal of medicine,* 351**,** 1911-1913.

DEGER, J. M., GERSON, J. E. & KAYED, R. 2015. The interrelationship of proteasome impairment and oligomeric intermediates in neurodegeneration. *Aging Cell***,** n/a-n/a.

DEKOSKY, S. T. & SCHEFF, S. W. 1990. Synapse loss in frontal cortex biopsies in Alzheimer's disease: correlation with cognitive severity. *Annals of neurology,* 27**,** 457-464.

DEKOSKY, S. T., SCHEFF, S. W. & STYREN, S. D. 1996. Structural correlates of cognition in dementia: quantification and assessment of synapse change. *Neurodegeneration,* 5**,** 417-421.

DENG, W., AIMONE, J. B. & GAGE, F. H. 2010. New neurons and new memories: how does adult hippocampal neurogenesis affect learning and memory? *Nature Reviews Neuroscience,* 11**,** 339-350.

DONG, H., GOICO, B., MARTIN, M., CSERNANSKY, C., BERTCHUME, A. & CSERNANSKY, J. 2004. Modulation of hippocampal cell proliferation, memory, and amyloid plaque deposition in APPsw (Tg2576) mutant mice by isolation stress. *Neuroscience,* 127**,** 601-609.

DONOVAN, M. H., YAZDANI, U., NORRIS, R. D., GAMES, D., GERMAN, D. C. & EISCH, A. J. 2006. Decreased adult hippocampal neurogenesis in the PDAPP mouse model of Alzheimer's disease. *Journal of Comparative Neurology,* 495**,** 70-83.

DUARTE, A. I., SANTOS, P., OLIVEIRA, C. R., SANTOS, M. S. & REGO, A. C. 2008. Insulin neuroprotection against oxidative stress is mediated by Akt and GSK-3β signaling pathways and changes in protein expression. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research,* 1783**,** 994-1002.

DUDEK, H., DATTA, S. R., FRANKE, T. F., BIRNBAUM, M. J., YAO, R., COOPER, G. M., SEGAL, R. A., KAPLAN, D. R. & GREENBERG, M. E. 1997.

Regulation of neuronal survival by the serine-threonine protein kinase Akt. *science,* 275**,** 661-665.

Enas, A.K. (2011). Evaluation of the Possible Protective and Therapeutic Influence of Coriander (Coriandum sativum L.) SeedAqueous Extract on Hippocampal Pyramidal Cells Against Alzheimer's Disease Induced by Aluminum Chloride in Adult Male Albino Rats. *Researcher,* 3(1)

ERMINI, F. V., GRATHWOHL, S., RADDE, R., YAMAGUCHI, M., STAUFENBIEL, M., PALMER, T. D. & JUCKER, M. 2008. Neurogenesis and alterations of neural stem cells in mouse models of cerebral amyloidosis. *The American journal of pathology,* 172**,** 1520-1528.

FINKBEINER, S., TAVAZOIE, S. F., MALORATSKY, A., JACOBS, K. M., HARRIS, K. M. & GREENBERG, M. E. 1997. CREB: a major mediator of neuronal neurotrophin responses. *Neuron,* 19**,** 1031-1047.

FLEISHER, A., SOWELL, B., TAYLOR, C., GAMST, A., PETERSEN, R. & THAL, L. 2007. Clinical predictors of progression to Alzheimer disease in amnestic mild cognitive impairment. *Neurology,* 68**,** 1588-1595.

FRAZER, M. E., HUGHES, J. E., MASTRANGELO, M. A., TIBBENS, J. L., FEDEROFF, H. J. & BOWERS, W. J. 2008. Reduced pathology and improved behavioral performance in Alzheimer's disease mice vaccinated with HSV amplicons expressing amyloid-β and interleukin-4. *Molecular Therapy,* 16**,** 845-853.

FRENCH, S. J., HUMBY, T., HORNER, C. H., SOFRONIEW, M. V. & RATTRAY, M. 1999. Hippocampal neurotrophin and trk receptor mRNA levels are altered by local administration of nicotine, carbachol and pilocarpine. *Molecular brain research,* 67**,** 124-136.

FRISONI, G. B., GANZOLA, R., CANU, E., RÜB, U., PIZZINI, F. B., ALESSANDRINI, F., ZOCCATELLI, G., BELTRAMELLO, A., CALTAGIRONE, C. & THOMPSON, P. M. 2008. Mapping local hippocampal changes in Alzheimer's disease and normal ageing with MRI at 3 Tesla. *Brain,* 131**,** 3266-3276.

FUCHS, E. & GOULD, E. 2000. Mini-review: in vivo neurogenesis in the adult brain: regulation and functional implications. *The European journal of neuroscience,* 12**,** 2211-4.

FUJIOKA, T., FUJIOKA, A. & DUMAN, R. S. 2004. Activation of cAMP signaling facilitates the morphological maturation of newborn neurons in adult hippocampus. *The Journal of neuroscience,* 24**,** 319-328.

FUSTER-MATANZO, A., LLORENS-MARTÍN, M., HERNÁNDEZ, F. & AVILA, J. 2013. Role of neuroinflammation in adult neurogenesis and Alzheimer disease: therapeutic approaches. *Mediators of inflammation,* 2013.

GADADHAR, A., MARR, R. & LAZAROV, O. 2011. Presenilin-1 regulates neural progenitor cell differentiation in the adult brain. *The Journal of Neuroscience,* 31**,** 2615-2623.

GAGE, G. J., KIPKE, D. R. & SHAIN, W. 2012. Whole animal perfusion fixation for rodents. *Journal of visualized experiments : JoVE*.

GHOSH, T., MUSTAFA, M., KUMAR, V., DATTA, S. K., BHATIA, M. S., SIRCAR, S. & BANERJEE, B. D. 2012. A preliminary study on the influence of glutathione S transferase T1 (GSTT1) as a risk factor for late onset Alzheimer's disease in North Indian population. *Asian journal of psychiatry,* 5**,** 160-3.

GIL-BEA, F. J., SOLAS, M., SOLOMON, A., MUGUETA, C., WINBLAD, B., KIVIPELTO, M., RAMIREZ, M. J. & CEDAZO-MINGUEZ, A. 2010. Insulin levels are decreased in the cerebrospinal fluid of women with prodomal Alzheimer's disease. *Journal of Alzheimer's disease : JAD,* 22**,** 405-13.

GOEDERT, M. & SPILLANTINI, M. G. 2006. A century of Alzheimer's disease. *science,* 314**,** 777-781.

GRAEBER, M., KÖSEL, S., EGENSPERGER, R., BANATI, R., MÜLLER, U., BISE, K., HOFF, P., MÖLLER, H., FUJISAWA, K. & MEHRAEIN, P. 1997. Rediscovery of the case described by Alois Alzheimer in 1911: historical, histological and molecular genetic analysis. *Neurogenetics,* 1**,** 73-80.

HARDY, J. & SELKOE, D. J. 2002. The Amyloid Hypothesis of Alzheimer's Disease: Progress and Problems on the Road to Therapeutics. *Science of Aging Knowledge Environment,* 2002**,** or8-.

HAUGHEY, N. J., LIU, D., NATH, A., BORCHARD, A. C. & MATTSON, M. P. 2002a. Disruption of neurogenesis in the subventricular zone of adult mice, and in human cortical neuronal precursor cells in culture, by amyloid β-peptide. *Neuromolecular medicine,* 1**,** 125-135.

HAUGHEY, N. J., NATH, A., CHAN, S. L., BORCHARD, A., RAO, M. S. & MATTSON, M. P. 2002b. Disruption of neurogenesis by amyloid β-peptide, and perturbed neural progenitor cell homeostasis, in models of Alzheimer's disease. *Journal of neurochemistry,* 83**,** 1509-1524.

HE, L., SABET, A., DJEDJOS, S., MILLER, R., SUN, X., HUSSAIN, M. A., RADOVICK, S. & WONDISFORD, F. E. 2009. Metformin and insulin suppress hepatic gluconeogenesis through phosphorylation of CREB binding protein. *Cell,* 137**,** 635-646.

HERRUP, K. & YANG, Y. 2007. Cell cycle regulation in the postmitotic neuron: oxymoron or new biology? *Nature reviews. Neuroscience,* 8**,** 368-78.

HIPPIUS, H. & NEUNDÖRFER, G. 2003. The discovery of Alzheimer's disease. *Dialogues in clinical neuroscience,* 5**,** 101.

HOYER, S., HENNEBERG, N., KNAPP, S., LANNERT, H. & MARTIN, E. 1996. Brain Glucose Metabolism Is Controlled by Amplification and Desensitization of the Neuronal Insulin Receptora. *Annals of the New York Academy of Sciences,* 777**,** 374-379.

HSHIEH, T. T., FONG, T. G., MARCANTONIO, E. R. & INOUYE, S. K. 2008. Cholinergic Deficiency Hypothesis in Delirium: A Synthesis of Current Evidence. *The journals of gerontology. Series A, Biological sciences and medical sciences,* 63**,** 764-772.

HSIEH, J., AIMONE, J. B., KASPAR, B. K., KUWABARA, T., NAKASHIMA, K. & GAGE, F. H. 2004. IGF-I instructs multipotent adult neural progenitor cells to become oligodendrocytes. *The Journal of cell biology,* 164**,** 111-122.

HWANG, I., KIM, I., JOO, E., SHIN, J., CHOI, J., WON, M.-H., YOON, Y. & SEONG, J. 2010. Metformin Normalizes Type 2 Diabetes-Induced Decrease in Cell Proliferation and Neuroblast Differentiation in the Rat Dentate Gyrus. *Neurochemical Research,* 35**,** 645-650.

IWANGOFF, P., ARMBRUSTER, R., ENZ, A. & MEIER-RUGE, W. 1980. Glycolytic enzymes from human autoptic brain cortex: normal aged and demented cases. *Mechanisms of ageing and development,* 14**,** 203-209.

JENSEN, L. J., KUHN, M., STARK, M., CHAFFRON, S., CREEVEY, C., MULLER, J., DOERKS, T., JULIEN, P., ROTH, A., SIMONOVIC, M., BORK, P. & VON MERING, C. 2009. STRING 8--a global view on proteins and their functional interactions in 630 organisms. *Nucleic acids research,* 37**,** D412-6.

JIN, K., GALVAN, V., XIE, L., MAO, X. O., GOROSTIZA, O. F., BREDESEN, D. E. & GREENBERG, D. A. 2004a. Enhanced neurogenesis in Alzheimer's disease transgenic (PDGF-APPSw, Ind) mice. *Proceedings of the National Academy of Sciences of the United States of America,* 101**,** 13363-13367.

JIN, K., PEEL, A. L., MAO, X. O., XIE, L., COTTRELL, B. A., HENSHALL, D. C. & GREENBERG, D. A. 2004b. Increased hippocampal neurogenesis in Alzheimer's disease. *Proceedings of the National Academy of Sciences,* 101**,** 343-347.

KEMPERMANN, G., KUHN, H. G. & GAGE, F. H. 1997. More hippocampal neurons in adult mice living in an enriched environment. *Nature,* 386**,** 493-495.

KEMPERMANN, G., WISKOTT, L. & GAGE, F. H. 2004. Functional significance of adult neurogenesis. *Current opinion in neurobiology,* 14**,** 186-191.

KERN, W., PETERS, A., FRUEHWALD-SCHULTES, B., DEININGER, E., BORN, J. & FEHM, H. L. 2001. Improving influence of insulin on cognitive functions in humans. *Neuroendocrinology,* 74**,** 270-280.

KIM, H.-B., JANG, M.-H., SHIN, M.-C., LIM, B.-V., KIM, Y.-P., KIM, K.-J., KIM, E.-H. & KIM, C.-J. 2003. Treadmill exercise increases cell proliferation in dentate gyrus of rats with streptozotocin-induced diabetes. *Journal of diabetes and its complications,* 17**,** 29-33.

KORBO, L., AMREIN, I., LIPP, H. P., WOLFER, D., REGEUR, L., OSTER, S. & PAKKENBERG, B. 2004. No evidence for loss of hippocampal neurons in non-Alzheimer dementia patients. *Acta neurologica scandinavica,* 109**,** 132-139.

KOSASA, T., KURIYA, Y., MATSUI, K. & YAMANISHI, Y. 2000. Inhibitory effect of orally administered donepezil hydrochloride (E2020), a novel treatment for

Alzheimer's disease, on cholinesterase activity in rats. *European journal of pharmacology,* 389**,** 173-179.

KOTANI, S., YAMAUCHI, T., TERAMOTO, T. & OGURA, H. 2006. Pharmacological evidence of cholinergic involvement in adult hippocampal neurogenesis in rats. *Neuroscience,* 142**,** 505-14.

KUHN, H. G., COOPER-KUHN, C. M., BOEKHOORN, K. & LUCASSEN, P. J. 2007. Changes in neurogenesis in dementia and Alzheimer mouse models: are they functionally relevant? *European archives of psychiatry and clinical neuroscience,* 257**,** 281-9.

KWON, K. J., KIM, M. K., LEE, E. J., KIM, J. N., CHOI, B. R., KIM, S. Y., CHO, K. S., HAN, J. S., KIM, H. Y., SHIN, C. Y. & HAN, S. H. 2014. Effects of donepezil, an acetylcholinesterase inhibitor, on neurogenesis in a rat model of vascular dementia. *Journal of the neurological sciences,* 347**,** 66-77.

LAPCHAK, P., ARAUJO, D. & HEFTI, F. 1993. Cholinergic regulation of hippocampal brain-derived neurotrophic factor mRNA expression: evidence from lesion and chronic cholinergic drug treatment studies. *Neuroscience,* 52**,** 575-585.

LARPTHAVEESARP, A., FERRIERO, D. M. & GONZALEZ, F. F. 2015. Growth Factors for the Treatment of Ischemic Brain Injury (Growth Factor Treatment). *Brain sciences,* 5**,** 165-177.

LEE, H.-G., MOREIRA, P. I., ZHU, X., SMITH, M. A. & PERRY, G. 2004. Staying connected: synapses in Alzheimer disease. *The American journal of pathology,* 165**,** 1461-1464.

LEE, J., DUAN, W. & MATTSON, M. P. 2002. Evidence that brain-derived neurotrophic factor is required for basal neurogenesis and mediates, in part, the enhancement of neurogenesis by dietary restriction in the hippocampus of adult mice. *Journal of neurochemistry,* 82**,** 1367-1375.

LESTER-COLL, N., RIVERA, E. J., SOSCIA, S. J., DOIRON, K., WANDS, J. R. & DE LA MONTE, S. M. 2006. Intracerebral streptozotocin model of type 3 diabetes: relevance to sporadic Alzheimer's disease. *Journal of Alzheimer's disease: JAD,* 9**,** 13-33.

LEUNER, B., GOULD, E. & SHORS, T. J. 2006. Is there a link between adult neurogenesis and learning? *Hippocampus,* 16**,** 216-224.

LI, B., YAMAMORI, H., TATEBAYASHI, Y., SHAFIT-ZAGARDO, B., TANIMUKAI, H., CHEN, S., IQBAL, K. & GRUNDKE-IQBAL, I. 2008. Failure of neuronal maturation in Alzheimer disease dentate gyrus. *Journal of neuropathology and experimental neurology,* 67**,** 78.

LIE, D.-C., COLAMARINO, S. A., SONG, H.-J., DÉSIRÉ, L., MIRA, H., CONSIGLIO, A., LEIN, E. S., JESSBERGER, S., LANSFORD, H. & DEARIE, A. R. 2005. Wnt signalling regulates adult hippocampal neurogenesis. *Nature,* 437**,** 1370-1375.

LIVAK, K. J. & SCHMITTGEN, T. D. 2001. Analysis of relative gene expression data using real-time quantitative PCR and the 2(-Delta Delta C(T)) Method. *Methods (San Diego, Calif.),* 25**,** 402-8.

LLEDO, P.-M., ALONSO, M. & GRUBB, M. S. 2006. Adult neurogenesis and functional plasticity in neuronal circuits. *Nature Reviews Neuroscience,* 7**,** 179-193.

LOPEZ-TOLEDANO, M. A. & SHELANSKI, M. L. 2007. Increased neurogenesis in young transgenic mice overexpressing human APP (Sw, Ind). *Journal of Alzheimer's disease: JAD,* 12**,** 229-240.

LOPEZ, O. L., BECKER, J., WAHED, A., SAXTON, J., SWEET, R., WOLK, D., KLUNK, W. & DEKOSKY, S. 2009. Long-term effects of the concomitant use of memantine with cholinesterase inhibition in Alzheimer disease. *Journal of Neurology, Neurosurgery & Psychiatry,* 80**,** 600-607.

MACHIDA, M., FUJIMAKI, S., HIDAKA, R., ASASHIMA, M. & KUWABARA, T. 2012. The insulin regulatory network in adult hippocampus and pancreatic endocrine system. *Stem cells international,* 2012.

MAGARIÑOS, A. M. A., JAIN, K., BLOUNT, E. D., REAGAN, L., SMITH, B. H. & MCEWEN, B. S. 2001. Peritoneal implantation of macroencapsulated porcine pancreatic islets in diabetic rats ameliorates severe hyperglycemia and prevents retraction and simplification of hippocampal dendrites. *Brain research,* 902**,** 282-287.

MARKESBERY, W. R. 1997. Oxidative stress hypothesis in Alzheimer's disease. *Free radical biology & medicine,* 23**,** 134-47.

MASLIAH, E., MALLORY, M., ALFORD, M., DETERESA, R., IWAI, A. & SAITOH, T. 1997. Molecular mechanisms of synaptic disconnection in Alzheimer's disease. *Connections, cognition and Alzheimer's disease.* Springer.

MAVROGIORGOU, P., GERTZ, H.-J., FERSZT, R., WOLF, R., BÄR, K.-J. & JUCKEL, G. 2011. Are routine methods good enough to stain senile plaques and neurofibrillary tangles in different brain regions of demented patients? *Psychiatria Danubina,* 23**,** 334-339.

MCKEE, A. C., CARRERAS, I., HOSSAIN, L., RYU, H., KLEIN, W. L., ODDO, S., LAFERLA, F. M., JENKINS, B. G., KOWALL, N. W. & DEDEOGLU, A. 2008. Ibuprofen reduces Aβ, hyperphosphorylated tau and memory deficits in Alzheimer mice. *Brain research,* 1207**,** 225-236.

MCNAY, E. C. 2007. Insulin and ghrelin: peripheral hormones modulating memory and hippocampal function. *Current opinion in pharmacology,* 7**,** 628-632.

MING, G.-L. & SONG, H. 2011. Adult neurogenesis in the mammalian brain: significant answers and significant questions. *Neuron,* 70**,** 687-702.

MOHAPEL, P., LEANZA, G., KOKAIA, M. & LINDVALL, O. 2005. Forebrain acetylcholine regulates adult hippocampal neurogenesis and learning. *Neurobiology of aging,* 26**,** 939-946.

MU, Y. & GAGE, F. H. 2011. Adult hippocampal neurogenesis and its role in Alzheimer's disease. *Mol Neurodegeneration,* 6**,** 85.

NAKAGAWA, S., KIM, J.-E., LEE, R., CHEN, J., FUJIOKA, T., MALBERG, J., TSUJI, S. & DUMAN, R. 2002. Localization of phosphorylated cAMP response element-binding protein in immature neurons of adult hippocampus. *The Journal of neuroscience,* 22**,** 9868-9876.

NELSON, T. J., SUN, M.-K., HONGPAISAN, J. & ALKON, D. L. 2008. Insulin, PKC signaling pathways and synaptic remodeling during memory storage and neuronal repair. *European journal of pharmacology,* 585**,** 76-87.

NIBUYA, M., NESTLER, E. J. & DUMAN, R. S. 1996. Chronic antidepressant administration increases the expression of cAMP response element binding protein (CREB) in rat hippocampus. *The Journal of Neuroscience,* 16**,** 2365-2372.

ODDO, S., CACCAMO, A., SHEPHERD, J. D., MURPHY, M. P., GOLDE, T. E., KAYED, R., METHERATE, R., MATTSON, M. P., AKBARI, Y. & LAFERLA, F. M. 2003. Triple-transgenic model of Alzheimer's disease with plaques and tangles: intracellular Aβ and synaptic dysfunction. *Neuron,* 39**,** 409-421.

PADURARIU, M., CIOBICA, A., MAVROUDIS, I., FOTIOU, D. & BALOYANNIS, S. 2012. Hippocampal neuronal loss in the CA1 and CA3 areas of Alzheimer's disease patients. *Psychiatria Danubina,* 24**,** 152-158.

PERRY, E. K., TOMLINSON, B. E., BLESSED, G., BERGMANN, K., GIBSON, P. H. & PERRY, R. H. 1978. Correlation of cholinergic abnormalities with senile plaques and mental test scores in senile dementia. *British Medical Journal,* 2**,** 1457-1459.

POLLACK, N. J. AND NAMAZI, K. H. (1992). The effect of music participation on the social behavior of Alzheimer's disease patients. *Jounal of Music Therapy,* 29: 54-67.

POTTS, M. B. & LIM, D. A. 2012. An Old Drug for New Ideas: Metformin Promotes Adult Neurogenesis and Spatial Memory Formation. *Cell stem cell,* 11**,** 5-6.

RAFALSKI, V. A. & BRUNET, A. 2011. Energy metabolism in adult neural stem cell fate. *Progress in neurobiology,* 93**,** 182-203.

RAFF, M. C., WHITMORE, A. V. & FINN, J. T. 2002. Axonal self-destruction and neurodegeneration. *Science,* 296**,** 868-71.

RANI, A., SODHI, R. K. & KAUR, A. 2015. Protective effect of a calcium channel blocker "diltiazem" on aluminum chloride-induced dementia in mice. *Naunyn-Schmiedeberg's archives of pharmacology***,** 1-11.

RAO, M. S. & SHETTY, A. K. 2004. Efficacy of doublecortin as a marker to analyse the absolute number anddendritic growth of newly generated neurons in the adult dentate gyrus. *European Journal of Neuroscience,* 19**,** 234-246.

REBAI, O. & DJEBLI, N. E. 2008. Chronic exposure to aluminum chloride in mice: exploratory behaviors and spatial learning. *Advanced Biomedical Research,* 2**,** 26-33.

ROCKENSTEIN, E., MANTE, M., ADAME, A., CREWS, L., MOESSLER, H. & MASLIAH, E. 2007. Effects of Cerebrolysin on neurogenesis in an APP transgenic model of Alzheimer's disease. *Acta Neuropathology,* 113**,** 265-75.

RODRIGUEZ, J. J., JONES, V. C., TABUCHI, M., ALLAN, S. M., KNIGHT, E. M., LAFERLA, F. M., ODDO, S. & VERKHRATSKY, A. 2008. Impaired adult neurogenesis in the dentate gyrus of a triple transgenic mouse model of Alzheimer's disease. *PloS one,* 3**,** e2935.

SAIRANEN, M., LUCAS, G., ERNFORS, P., CASTRÉN, M. & CASTRÉN, E. 2005. Brain-derived neurotrophic factor and antidepressant drugs have different but coordinated effects on neuronal turnover, proliferation, and survival in the adult dentate gyrus. *The Journal of Neuroscience,* 25**,** 1089-1094.

SAXE, M. D., BATTAGLIA, F., WANG, J. W., MALLERET, G., DAVID, D. J., MONCKTON, J. E., GARCIA, A. D., SOFRONIEW, M. V., KANDEL, E. R., SANTARELLI, L., HEN, R. & DREW, M. R. 2006. Ablation of hippocampal neurogenesis impairs contextual fear conditioning and synaptic plasticity in the dentate gyrus. *Proceedings of National Acadamey of Science U S A,* 103**,** 17501-6.

SCHARFMAN, H. E. 2002. Book review: Epilepsy as an example of neural plasticity. *The Neuroscientist,* 8**,** 154-173.

SCHEFF, S. W. & PRICE, D. A. 2001. Alzheimer's disease-related synapse loss in the cingulate cortex. *Journal of Alzheimer's disease : JAD,* 3**,** 495-505.

SCHIÖTH, H. B., CRAFT, S., BROOKS, S. J., FREY II, W. H. & BENEDICT, C. 2012. Brain insulin signaling and Alzheimer's disease: current evidence and future directions. *Molecular neurobiology,* 46**,** 4-10.

SCHOLZEN, T. & GERDES, J. 2000. The Ki-67 protein: from the known and the unknown. *Journal of cellular physiology,* 182**,** 311-322.

SELKOE, D. J. 2001. Alzheimer's disease: genes, proteins, and therapy. *Physiological reviews,* 81**,** 741-766.

SERRANO-POZO, A., FROSCH, M. P., MASLIAH, E. & HYMAN, B. T. 2011. Neuropathological alterations in Alzheimer disease. *Cold Spring Harbor perspectives in medicine,* 1**,** a006189.

SHORS, T. J., MIESEGAES, G., BEYLIN, A., ZHAO, M., RYDEL, T. & GOULD, E. 2001. Neurogenesis in the adult is involved in the formation of trace memories. *Nature,* 410**,** 372-376.

ŠIMIĆ, G. & BOGDANOVIĆ, N. 1997. Volume and number of neurons of the human hippocampal formation in normal aging and Alzheimer's disease. *Journal of Comparative Neurology,* 379**,** 482-494.

STEEN, E., TERRY, B. M., RIVERA, E. J., CANNON, J. L., NEELY, T. R., TAVARES, R., XU, X. J., WANDS, J. R. & MONTE, S. M. D. L. 2005. Impaired insulin and insulin-like growth factor expression and signaling mechanisms in Alzheimer's disease-is this type 3 diabetes? *Journal of Alzheimer's disease,* 7**,** 63-80.

STRANAHAN, A. M., ARUMUGAM, T. V., CUTLER, R. G., LEE, K., EGAN, J. M. & MATTSON, M. P. 2008. Diabetes impairs hippocampal function through glucocorticoid-mediated effects on new and mature neurons. *Nature neuroscience,* 11**,** 309-317.

SULTANA, R., BOYD-KIMBALL, D., CAI, J., PIERCE, W. M., KLEIN, J. B., MERCHANT, M. AND BUTTERFIELD, D. A. (2007). Proteomics analysis of the Alzheimer's disease hippocampal proteome. *Journal of Alzheimers Disease*., 11: 153-64.

SUN, B., HALABISKY, B., ZHOU, Y., PALOP, J. J., YU, G., MUCKE, L. & GAN, L. 2009. Imbalance between GABAergic and glutamatergic transmission impairs adult neurogenesis in an animal model of Alzheimer's disease. *Cell stem cell,* 5**,** 624-633.

SUZANNE, M. & WANDS, J. R. 2005. Review of insulin and insulin-like growth factor expression, signaling, and malfunction in the central nervous system: relevance to Alzheimer's disease. *Journal of Alzheimer's Disease,* 7**,** 45-61.

TATEBAYASHI, Y., LEE, M. H., LI, L., IQBAL, K. & GRUNDKE-IQBAL, I. 2003. The dentate gyrus neurogenesis: a therapeutic target for Alzheimer's disease. *Acta neuropathologica,* 105**,** 225-232.

TERRY, R. D., MASLIAH, E. & HANSEN, L. A. 1994. Structural basis of the cognitive alterations in Alzheimer disease. *In:* TERRY, R. D., KATZMAN, R. & BICK, K. L. (eds.) *Alzheimer disease.* New York, NY, US: Raven Press.

TERRY, R. D., MASLIAH, E., SALMON, D. P., BUTTERS, N., DETERESA, R., HILL, R., HANSEN, L. A. & KATZMAN, R. 1991. Physical basis of cognitive alterations in Alzheimer's disease: synapse loss is the major correlate of cognitive impairment. *Annals of neurology,* 30**,** 572-580.

TERRY, R. D., PECK, A., DETERESA, R., SCHECHTER, R. & HOROUPIAN, D. S. 1981. Some morphometric aspects of the brain in senile dementia of the alzheimer type. *Annals of Neurology,* 10**,** 184-192.

THOME, J., SAKAI, N., SHIN, K.-H., STEFFEN, C., ZHANG, Y.-J., IMPEY, S., STORM, D. & DUMAN, R. 2000. cAMP response element-mediated gene transcription is upregulated by chronic antidepressant treatment. *The Journal of Neuroscience,* 20**,** 4030-4036.

THOMPSON, A., BOEKHOORN, K., VAN DAM, A. M. & LUCASSEN, P. 2008. Changes in adult neurogenesis in neurodegenerative diseases: cause or consequence? *Genes, Brain and Behavior,* 7**,** 28-42.

URBÁN, N. & GUILLEMOT, F. 2014. Neurogenesis in the embryonic and adult brain: same regulators, different roles. *Frontiers in Cellular Neuroscience,* 8**,** 396.

VAN DYCK, C. H., TARIOT, P. N., MEYERS, B., RESNICK, E. M. & GROUP, M. M.-M.-S. 2007. A 24-week randomized, controlled trial of memantine in patients with moderate-to-severe Alzheimer disease. *Alzheimer Disease & Associated Disorders,* 21**,** 136-143.

VAN PRAAG, H., KEMPERMANN, G. & GAGE, F. H. 2000. Neural consequences of enviromental enrichment. *Nature Reviews Neuroscience,* 1**,** 191-198.

WALLIMANN, T., TOKARSKA-SCHLATTNER, M. & SCHLATTNER, U. 2011. The creatine kinase system and pleiotropic effects of creatine. *Amino Acids,* 40**,** 1271-1296.

WANG, J., GALLAGHER, D., DEVITO, L. M., CANCINO, G. I., TSUI, D., HE, L., KELLER, G. M., FRANKLAND, P. W., KAPLAN, D. R. & MILLER, F. D. 2012. Metformin activates an atypical PKC-CBP pathway to promote neurogenesis and enhance spatial memory formation. *Cell stem cell,* 11**,** 23-35.

WANG, J., WEAVER, I. C., GAUTHIER-FISHER, A., WANG, H., HE, L., YEOMANS, J., WONDISFORD, F., KAPLAN, D. R. & MILLER, F. D. 2010. CBP histone acetyltransferase activity regulates embryonic neural differentiation in the normal and Rubinstein-Taybi syndrome brain. *Developmental cell,* 18**,** 114-125.

WANG, R., DINELEY, K., SWEATT, J. & ZHENG, H. 2004. Presenilin 1 familial Alzheimer's disease mutation leads to defective associative learning and impaired adult neurogenesis. *Neuroscience,* 126**,** 305-312.

WEN, P. H., HOF, P. R., CHEN, X., GLUCK, K., AUSTIN, G., YOUNKIN, S. G., YOUNKIN, L. H., DEGASPERI, R., SOSA, M. A. G. & ROBAKIS, N. K. 2004. The presenilin-1 familial Alzheimer disease mutant P117L impairs neurogenesis in the hippocampus of adult mice. *Experimental neurology,* 188**,** 224-237.

WICKELGREN, I. 1998. Tracking insulin to the mind. *Science,* 280**,** 517-519.

YANG, C. P., GILLEY, J. A., ZHANG, G. & KERNIE, S. G. 2011. ApoE is required for maintenance of the dentate gyrus neural progenitor pool. *Development (Cambridge, England),* 138**,** 4351-62.

YU, Y., HE, J., ZHANG, Y., LUO, H., ZHU, S., YANG, Y., ZHAO, T., WU, J., HUANG, Y. & KONG, J. 2009. Increased hippocampal neurogenesis in the progressive stage of Alzheimer's disease phenotype in an APP/PS1 double transgenic mouse model. *Hippocampus,* 19**,** 1247-1253.

ZHAO, C., DENG, W. & GAGE, F. H. 2008. Mechanisms and functional implications of adult neurogenesis. *Cell,* 132**,** 645-660.

ZHAO, C., SLEVIN, J. T. & WHITEHEART, S. W. 2007. Cellular Functions of NSF: Not Just SNAPs and SNAREs. *Federation of European Biomedical Sciences letters,* 581**,** 2140-2149.

ZHAO, W.-Q. & ALKON, D. L. 2001. Role of insulin and insulin receptor in learning and memory. *Molecular and cellular endocrinology,* 177**,** 125-134.

ZHAO, W. Q., ALKON, D. L. & MA, W. 2003. c-Src protein tyrosine kinase activity is required for muscarinic receptor-mediated DNA synthesis and neurogenesis via ERK1/2 and c-AMP-responsive element-binding protein signaling in neural precursor cells. *Journal of neuroscience research,* 72**,** 334-342.

ZHOU, Z.-D., CHAN, C. H.-S., MA, Q.-H., XU, X.-H., XIAO, Z.-C. & TAN, E.-K. 2011. The roles of amyloid precursor protein (APP) in neurogenesis, implications to pathogenesis and therapy of Alzheimer disease (AD). *Cell Adhesion & Migration,* 5**,** 280-292.

ZHU, X., PERRY, G. & SMITH, M. A. 2005. Insulin signaling, diabetes mellitus and risk of Alzheimer disease. *Journal of Alzheimer's disease : JAD,* 7**,** 81-4.

APPENDIX

Neurol Sci
DOI 10.1007/s10072-015-2352-5
REVIEW ARTICLE

Linking insulin with Alzheimer's disease: emergence as type III diabetes

Sara Ahmed[1]• Zahra Mahmood[1]• Saadia Zahid[1]
1 Neurobiology Research Laboratory, Department of Healthcare Biotechnology, Atta-ur-Rahman School of
Applied Biosciences, National University of Sciences and Technology, Islamabad, Pakistan
Received: 9 April 2015 / Accepted: 25 July 2015

Abstract

Alzheimer's disease (AD) has characteristic neuropathological abnormalities including regionalized neurodegeneration, neurofibrillary tangles, amyloid beta (Ab) deposition, activation of pro-apoptotic genes, and oxidative stress. As the brain functions continue to disintegrate, there is a decline in person's cognitive abilities, memory, mood, spontaneity, and socializing behavior. A framework that sequentially interlinks all these phenomenons under one event is lacking. Accumulating evidence has indicated the role of insulin deficiency and insulin resistance as mediators of AD neurodegeneration. Herein, we reviewed the evidence stemming from the development of diabetes agent-induced AD animal model. Striking evidence has attributed loss of insulin receptor bearing neurons to precede or accompany initial stage of AD. This state seems to progress with AD such that, in the terminal stages, it worsens and becomes global. Oxidative stress, tau hyperphosphorylation, APP-Ab deposition, and impaired glucose and energy metabolism have all been linked to perturbation in insulin/IGF signaling. We conclude that AD could be referred to as ''type 3 diabetes''. Moreover, owing to common pathophysiology with diabetes common therapeutic regime could be effective for AD patients.

Keywords: Alzheimer's disease _ Type 3 diabetes _Insulin signaling _ Tau hyperphosphorylation _Acetylcholine _ Anti-diabetics

Introduction

Alzheimer's disease (AD), an age-related, progressive neurodegenerative disorder, is the leading cause of dementia. It is characterized by two types of lesions; intracellular neurofibrillary tangles (NFT) and amyloid-b (Ab) plaques [1]. These, together with elevated oxidative stress, synaptic loss, regionalized neuronal death, and brain atrophy have been observed in AD pathology [2]. In the past, efforts have been directed to interlink these abnormalities under a single primary pathogenic mechanism and several heavily debated hypotheses that exist trying to explain the

underlying factor that trigger the development of AD brain pathology [3–5]. AD, often tagged as a heterogeneous disorder, implicates multiple aberrant signaling cascades in its pathogenesis. Insulin resistance is one such factor known to affect multiple cascades of known relevance to AD [6, 7]. De la Monte et al. reported cerebral insulin and Insulin-like growth factor (IGF) production. De la Monte et al. later observed that a common finding in AD was the impairments in energy metabolism and glucose utilization [8]. Insulin receptors (IRs), insulin, and IGF deficiency in AD brain further implicated insulin resistance in AD neuropathology [9, 10]. AD Braak stages have demonstrated an inversely proportional relationship to insulin expression. AD patients presented an 80 % decline in insulin receptors [8]. Insulin's ability to bind to its receptors was reportedly compromised. The decline in glucose processing was thought to coincide with, or even preceded, the early & stages of AD [11]. Moreover, Ab pathology, impaired cholinergic system, tau hyperphosphorylation, pro-apoptotic and pro-inflammatory events have all also been attributed to impaired insulin signaling [12]. In a nut shell, evidence is growing to suggest impaired insulin signaling as the putative factor governing AD pathology hence favoring the conjecture of AD being a neuroendocrine disorder. Researchers thus concluded that perhaps Alzheimer's is a brain-specific type of diabetes which they termed as ''type 3 diabetes'' [8]. In the following review, we will provide a brief description about the role of insulin in brain and focus more closely on accumulating evidences implicating impaired insulin signaling in AD pathology. Finally we will discuss the potential of insulin targeting drugs in AD therapeutics.

Role of insulin in brain

The role of insulin in carbohydrate, lipid, and protein metabolism is already known [13]. Reflecting a major paradigm shift, hypothalamic actions of insulin in regulating energy homeostasis was reported [14]. This suggested insulin signaling in brain as well. IR localization in central nervous system (CNS), by ligand autoradiography was first documented by Havrankova et al. with later verification by immunohistochemistry and autoradiography [15]. Controversies still exist regarding the source of insulin in brain, where some claiming that insulin is of cerebral origin, it is however agreed upon that insulin plays a role in cerebral glucose utilization following its receptor mediated transport across the blood brain barrier [16]. IR seems to be widely distributed with most of them concentrating the synapses of astrocytes and neurons. Cerebral regions such as hippocampus, amygdala, and septum have shown higher distribution of IR [17]. Intracellular pathways such as PI3K/AKT and ERK/MAP kinase pathways are reportedly activated via IR and IGF-1 receptors stimulation [17] thereby indicating broad impact of insulin signaling in the outside of the hypothalamus. Hippocampus, with its abundant IR substrate (IRS) proteins

regulates the acquisition and consolidation of memories thereby suggesting the role of insulin in memory potentiation [18]. In healthy adults, systemic infusion of insulin yielded a significant improvement in verbal memory and selective attention [19]. Insulin has been suggested to be neuroprotective and considered to have significant effects to enhance memory [20]. In accordance with this, AD patients have shown improvement in memory and performance following insulin administration [21]. Implication of causal impaired insulin signaling in stroke, AD, and Parkinson disease [22] has made it necessary to find the putative proteins that trigger these neurological disorders [23]. IRS-1, a putative target of known relevance to proper brain function, is found to be inhibited in AD brains [24].

Insulin-mediated AD pathology

Insulin receptor deficiency and aberrant insulin signaling in AD was first reported by Frolich and colleagues [25]. Their results were further strengthened when cerebrospinal fluid (CSF) of AD and mild cognitive impairment (MCI) patients showed reduced levels of insulin [4]. Reduced levels of insulin and IGF-1 polypeptide and receptor genes have been linked to advanced stage AD. It was observed that AD brains presented perturbed insulin and IGF-1-mediated neuronal development and mitochondrial dysfunction [26]. Evidence has suggested neurodegeneration of insulin and IGF-1 receptor-bearing neurons to precede or accompany initial stage of AD [11, 27]. This insulin-mediated neurodegeneration progresses with AD such that, in the later stages, it becomes global [26]. Role of insulin has also been suggested in the development of AD pathology markers [28]. The regulation of tau phosphorylation, one of the characteristic hallmarks of AD [29], is demonstrated to be governed by b-N-acetylglucosamine (GlcNAc)-mediated O-GlcNAcylation which inversely affects tau phosphorylation. Impaired glucose metabolism, one of the features of AD, has been linked with down regulation of O-GlcNAcylation consequently leading to tau hyperphosphorylation [30]. Furthermore, due to insulin deficiency, glycogen synthase kinase (GSK-3) remains inactivated (unphosphorylated), thereby leading to tau hyperphosphorylation, as shown in Fig. 1 [31]. Duration of diabetes has been demonstrated to positively correlate with neuritic plaques [32]. Acetylcholine deficiency has long been recognized as an early irregularity in AD [33] which has now also been linked to insulin resistance [12]. Acetylcholine transferase (ChAT), involved in Ach synthesis is expressed in insulin and IGF-I receptor-positive cortical neurons [34]. Insulin deficiency and resistance have been linked to decreased ACh level owing to underlying reduced ChAT expression [35]. Reduced ChAT co-localization has been documented in insulin receptor-bearing neurons of AD patients [26]. Hippocampi of non-diabetic AD patients have shown an increase in the levels of peripheral insulin resistance biomarkers [36]. This established a link between aberrant insulin signaling and dementia

[34, 37]. In this regard antidiabetic agents-mediated prevention of hypoglycemic events can be a potential strategy to reduce cognitive decline and dementia [38].

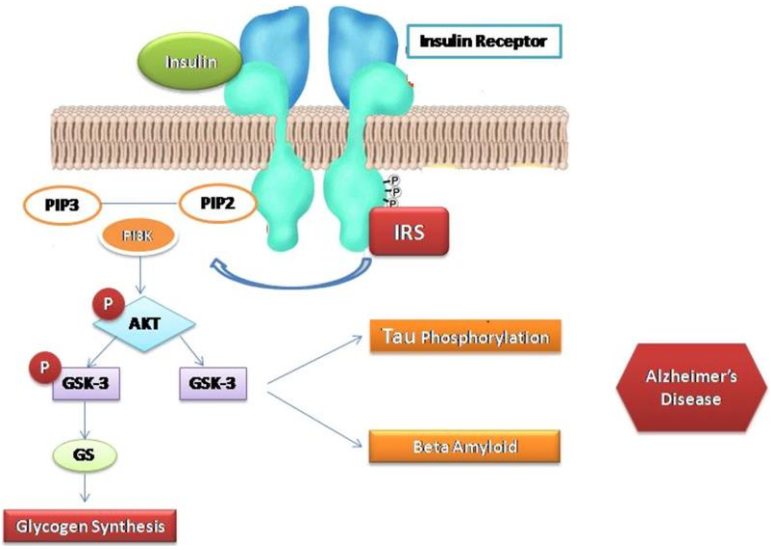


Fig. 1 Insulin receptor signaling. Insulin binds to and activates the membrane-bound insulin receptor (IR) tyrosine kinase, which consists of two asubunits and two b-subunits forming an a2b2 heterotetramer. Receptor autophosphorylation and subsequent phosphorylation of IRS activates PI3K. PI3K further activates GSK-3 hence leading to glycogen synthesis. During conditions of insufficient insulin, GSK-3 remains inactivated. Unphosphorylated GSK-3 leads to tau phosphorylation and Ab accumulation, the characteristic hallmarks of AD.

Impairment in energy metabolism with attendant increased oxidative stress and cognitive deterioration may be due to perturbation in the insulin/IGF signaling [39] where progressive brain insulin/IGF resistance tends to increase the expression of cerebral inflammatory mediators in AD. Aberrant insulin/IGF signaling-mediated increased oxidative stress and mitochondrial dysfunction enhance the APP gene expression level while APP-Ab deposition-mediated neurotoxicity further positively regulates oxidative stress-induced APP-Ab deposition [8] (Fig. 2). Evidences regarding underlying shared cascade that governs both AD and diabetes have extensively been reviewed [40, 41]. Impaired insulin signaling and inflammation appear to be shared processes in diabetes mellitus (DM) and AD. It was hence inferred that events analogous to those that result in peripheral insulin resistance in type 2 diabetes mellitus (T2DM) likely underlie aberrant insulin signaling in AD. In T2DM, c-Jun N-terminal kinase (JNK) pathway is stimulated by TNF-a cascade [42] thereby initiating peripheral insulin resistance [43]. Likewise, in cultured hippocampal neurons and AD mouse models Ab oligomers via activating the

TNF-a/JNK pathway causes IRS-1 inhibition [44–46]. Following intracerebroventricular (i.c.v.) administration of Ab oligomers, hippocampi of cynomolgus monkeys showed JNK activation and IRS-1 inhibition. Likewise, elevated IRS-1pSer and activated JNK levels have been reported in AD brains following postmortem analysis [36, 45]. Oligomer-mediated internalization of neuronal IRs [43] may facilitate IRS-1pSer following IR displacement from the cell surface. Insulin prevents both, Ab oligomers-induced IR downregulation [47] and IRS-1pSer [45]. Insoluble Ab fibrils, generated by self-association of Ab in AD brains [48] were initially thought to cause neuronal loss (hence, memory loss). Overwhelming evidence in recent past has however indicated otherwise. Of clinical relevance, MCI-negative individuals presented brain Ab deposition while, conversely, individuals displaying cognitive deterioration lacked Ab load [49]. Moreover, it was suggested that synapse loss rather than amyloid burden, is the best correlate of the extent of dementia [50]. This led to the conclusion that a factor other than fibrillar Ab mediates synapse and cognitive impairment. Lambert and coworkers demonstrated that Ab self-aggregation forms neurotoxic-soluble oligomers. These oligomers are not easily observed in pathological examination [51]. AD hippocampi analysis also revealed the presence of oligomers at the postsynapse [52]. Increased levels of Ab oligomers were reported in CSF and AD brains [53, 54]. It was hypothesized that synapse failure and neuronal dysfunction are primarily mediated by Ab oligomers [55, 56]. NMDA- and AMPA-type glutamate receptors, involved in synaptic plasticity [57, 58], upon oligomer exposure, are removed from the cell surface indicating a broad impact of oligomers on synapses. Oligomers; also implicated in AD associated neuropathology, activates the signaling pathways that lead to abnormal tau phosphorylation [59] and oxidative stress [60]. Ab oligomers, therefore, through altered neuronal IR function, mediates synaptic

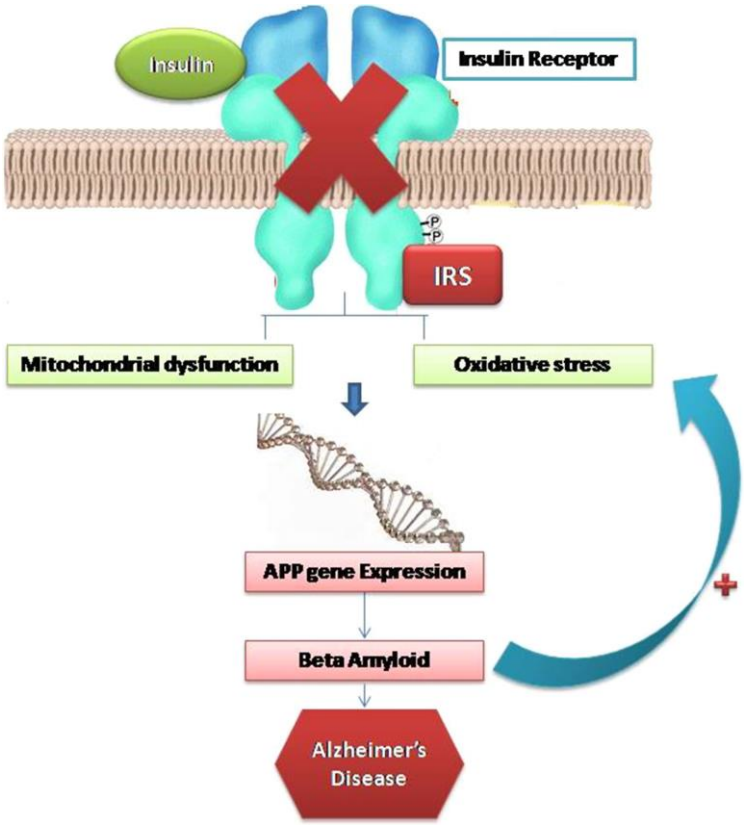


Fig. 2 Developmental profile of AD pathology. The schematic illustration depicts perturbed insulin signaling resulting in oxidative stress and mitochondrial dysfunction. Oxidative stress and attendant mitochondrial dysfunction further lead to APP gene expression and consequent APP-Aβ deposition. APP-Aβ deposition positively regulates the oxidative stress-mediated APP-Aβ deposition.

This provides a basis for brain insulin resistance in AD and is likely connected to impaired learning and memory in disease. Defective insulin signaling seems to be intimately linked to Ab oligomers. The first evidence demonstrated that Ab oligomers bind to hippocampal neurons thereby displacing IRs from the plasma membrane [43, 47]. This was subsequently verified in AD brains [9, 61]. Elevated IR levels in cell bodies of neurons with oligomers attached to their surface suggested a subcellular redistribution of IRs [62]. This redistribution and internalization of IRs mediates decreased responsiveness to insulin. The latter has been revealed by impaired insulin-induced receptor protein tyrosine kinase activity in oligomer-exposed cultured neurons [43].

Evidence of insulin resistance in AD

From the past couple of decades, evidence is being gathered by producing diabetes agent-induced experimental AD animal models. In spite of several factors known to trigger AD, overwhelming evidence suggests involvement of cerebral insulin/IGF resistance in MCI, dementia, and AD [37, 63–65]. Cerebral insulin, agreed to be of pancreatic origin, is known to modulate synaptic plasticity that regulates learning and memory. It has been shown to induce memory consolidation, retrieval and extinction of contextual memory via phosphatidylinositol 3-kinase (PI3K) pathway [31]. AD association with increasing brain insulin resistance in the absence of T2DM, indicates primary impaired insulin signaling [9, 37, 64]. Cognitive impairment in rats following i.c.v. injections of streptozotocin (STZ), with deficits in spatial memory, insulin resistance, and insulin deficiency further consolidates the hypothesis of AD being a type 3 diabetes [66]. AD hallmarks, including tau hyperphosphorylation, APPAb deposition, and decreased neuronal survival have been recapitulated by STZ. Downstream effects of STZ-induced impaired insulin and IGF signaling in the CNS could be responsible for this. Craft et al. have suggested that progressive insulin resistance, accompanied by reduced cerebral glucose metabolism and subtle cognitive impairments Fig. 2 Developmental profile of AD pathology. The schematic illustration depicts perturbed insulin signaling resulting in oxidative stress and mitochondrial dysfunction. Oxidative stress and attendant mitochondrial dysfunction further lead to APP gene expression and consequent APP-Ab deposition. APP-Ab deposition positively regulates the oxidative stress-mediated APP-Ab deposition Neurol Sci 123 Author's personal copy at initial AD stages, may serve as a marker of AD even before the onset of MCI [6]. Results from i.c.v. injection of STZ have demonstrated reduced glucose metabolism, oxidative stress, IR dysfunction, and cognitive impairment [67]. Reduced expression of insulin and IR encoding genes has been linked to STZ. Striking evidence of STZ-induced brain atrophy, increased tau phosphorylation, and APP-Ab deposition was demonstrated by De la Monta et al. [68]. Moreover, low-dose nitrosamines exposure has been shown to induce cognitive impairment, AD-type neurodegeneration, and brain insulin resistance [69], similar to the effects of STZ. This may account for progressive increase in sporadic AD (Type 3 diabetes) prevalence rates, since environmental exposures such as nitrosamines that contaminate highly processed and preserved foods and have become staples in our diets [65]. Studies on Tg2576 have linked increased APP-Ab aggregation to diet-induced insulin resistance [70]. This increased APP production coincides with increased amyloid load and poor performance in spatial water maize task [71]. These findings establish that cerebral insulin signaling is perturbed in rodent and non-human primate AD models, by mechanisms similar to those, governing insulin resistance in DM.

Anti-diabetics—a promising solution for AD patients

Alzheimer's disease pathology, recapitulated by treatment with diabetes agents led to the hypothesis that AD pathology and cognitive deterioration could be reduced by treatment regime involving anti-diabetic agents such as peroxisome proliferator-activated receptor (PPAR) agonists [68]. PPARc, a neuromodulator, has been implicated in the pathogenesis of both DM and AD. PPARc agonists, thiazolidinediones, have been shown to improve insulin resistance. Treatment with rosiglitazone yielded positive relation between insulin levels and cognition as compared with placebo. This was proved by the fact that IR expression and binding were significantly enhanced by the PPAR-agonist treatment [72]. Intranasal insulin administration exhibited improvement in memory [73] and attention on the 21st day of treatment. Likewise, Exendin-4 has been documented to activate pathways common to insulin signaling via glucagon-like peptide 1 (GLP-1) receptor simulation. This in turn has shown to block insulin signaling impairment in hippocampal cultures [45]. Exendin-4 in transgenic (Tg) mice thus reverses insulin-mediated AD pathology and cognitive improvement [74]. These results reflect paradigm shift regarding AD pathogenesis, i.e., AD is mediated by perturbed insulin signaling owing to underlying insulin resistance and insulin deficiency in the brain.

Conclusion

Initially thought to be an independent disorder, DM and AD, seemingly have shared pathophysiological mechanisms. Positive results stemming from the results of (a) development of animal model with diabetes agent-induced insulin-resistant brain state accompanied by cognitive impairment, (b) anti-diabetes agent-induced reversal of insulin signaling-associated neurodegenerative effects suggest AD to be a neuroendocrine disorder. Insulin and IGF signaling impairment, with attendant inflammatory mediators, oxidative stress, and impaired mitochondrial function contribute to AD-associated neurodegeneration. Owing to common underlying pathological cascade governing AD and DM, AD could be rightfully referred to as Type 3 diabetes thereby indicating that common therapeutic intervention could be effective. Currently a number of clinical trials based on testing effectiveness of anti-diabetic drugs against Alzheimer's are being conducted. The results, if positive, would pave ways for potential new pharmacotherapy for AD patients. Compliance with ethical standards

Conflict of interest: The authors have no conflict of interest pertaining to this review.