INVESTIGATING THE ROLE OF G1 AND S CDKS IN AMYLOID-β OLIGOMER INDUCED NEUROTOXICITY

by

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In

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APPROVAL PAGE

I certify that this thesis satisfies all the requirements as a thesis for the degree of Master of Science.

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This is to certify that I have read this thesis and that in my opinion it is fully adequate, in scope and quality, as a thesis for the degree of Master of Science.

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M. S. Thesis – Genetic and Bioengineering
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ABSTRACT

Alzheimer's disease (AD) is a neurodegenerative disorder with the hallmarks; amyloid plaques, neurofibrillary tangles, and eventual neuronal loss in brain regions. Amyloid-β proteins are believed to be at the center of Alzheimer neuropathology. Amyloid-β peptides are generated as a result of proteoliytic cleavage of amyloid precursor protein (APP) by β-secretase and γ-secretase and this peptides aggregate into amyloid oligomers,fibrils and plaques, subsequently. For a long time, plaques have been considered as a major cause of Alzheimer neuropathology. However, recent studies suggest that amyloid-β oligomers may trigger the initiation of Alzheimer disease. It has been shown that amyloid-β oligomers cause neurotoxicity and lead to apoptosis of neuron cells but it has not been very well characterized. The neurons are known to be terminally differentiated cells and lack the ability to enter cell cycle. If nerve cells in quiescent phase are forced to re-enter the cell cycle, it may cause neuronal death and/or loss of functionality. The aberrant cell cycle events are deleterious to neurons, which undergo death rather than complete the cell cycle. It has known that cell cycle proteins are increase in Alzheimer patient and it was demonstrated that amyloid-β oligomers have the ability of cell cycle re-entry in neurons. In this study I have investigated the role of cdk4 and cdk2 in amyloid-β oligomer induced cell cycle and neurotoxicity. In order to investigate the role of cdk4 and cdk2 proteins, SH-SY5Y cells were transfected with cdk4dn and cdk2dn mutant costruct. It was demonstrated that Amyloid-β_{42} oligomers induced the neuronal cell cycle in when differentiated SH-SY5Y cell but Amyloid-β_{42} oligomers did not induce cell cycle in cdk4dn or cdk2dn transfected cells. In conclusion, amyloid-β_{42} oligomer induce cell cycle re-entry via cdk4 and cdk2 as a possible mechanism of neurodegeneration.

Keywords: Alzheimer, amyloid-β, cell cycle, cdk4, cdk2

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Ayşe GÜLÜŞÜR

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ÖZ

Nörodejeneratif bir hastalık olan Alzheimer'ın belirtileri nörofibril yumaklar, amyliod plaklar ve sonrasında meydana gelen beyin bölgesindeki nöron hücresi kayıplarıdır. Amyloid-β proteinlerinin bu hastalığın patolojisinde merkezi bir rolü olduğuna inanılıyor. Amyloid-β peptidleri APP'nin β-sekretaz ve γ-sekretaz enzimleriyle proteolitik degradasyonu sonucu oluşur ve bu peptidler sırasıyla oligomer, fibril ve plak formunu oluşturur. Uzun bir süre, Alzheimer nöropatolojisinde asıl rolü plakların oynadığı düşünülüyordu. Diğer yandan araştırmaler amiloid-β oligomerlerinin Alzheimer hastalığının başlatıcısı olabileceğini gösterdi. Amyloid-β oligomerlerinin nöron hücrelerini apoptoza iterek nörotoksisiteye sebep olduğu düşünülmekteyse de bu konu tam olarak açıklığa kavuşturulamamıştır. Nöronlar farklılaşmış hücrelerdir ve hücre döngüsüne girme yeteneklerini kaybetmişlerdir. Eğer sinir hücresi sessiz fazda iken hücre döngüsüne girmeye itilirse sahip olduğu fonksiyonu kaybedebilir. Alzheimer hastalarında hücre döngüsü proteinlerinde artış olduğu bilinmektedir ve Amyloid-β oligomerleri nöron hücrelerini hücre döngüsüne girmeye ittiği gösterilmiştir. Bu tezde hücre döngüsü proteinleri olan cdk4 ve cdk2 'nun amyloid oligomer indüklü nörotoksisitede bir rolü olup olmadığı araştırıldı. Cdk4 ve cdk2 proteinlerinin rollerini araştırmak için hücre cdk4 ve cdk2 dominant negative mutantlarla transfekte edildi. Amyloid-β_{42} oligomerleri farklılaştırılmış SH-SY5Y hücrelerinde hücre döngüsünü tetikledi fakat cdk4 ve cdk2 dominant negative mutant hücrelerde hücre dögüsünü tetikleyememiştir. Sonuçta, olası bir neurodejenerasyon mekanziması olarak amyloid-β oligomer cdk4 ve cdk2 aracılığıyla hücre döngüsünü tetiklemiştir.

Anahtar Kelimeler: Alzheimer hastalığı, amiloid- β, hücre döngüsü, cdk4, cdk2

To my parents

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LIST OF SYSMBOLS AND ABBREVIATIONS

SYMBOL/ABBREVIATION

AD's	Alzheimer disesase
Aβ	Amyloid-β
$A\beta_{42}$	Amyloid-β 42 (Amyloid-β_{42})
$A\beta_{40}$	Amyloid -β 40 (Amyloid-β_{40})
APP	Amyloid precursor protein
BrdU	Bromodeoxyuridine
Cdk 4	Cyclin Dependent Kinase 4
Cdk 4 dn	Dominant negative Cyclin Dependent Kinase 4
Cdk 2	Cyclin Dependent Kinase 2
Cdk 2 dn	Dominant negative Cyclin Dependent Kinase 4
DMEM	Dulbecco's Modified Eagle Medium
dn	Dominant negative
FBS	Fetal Bovine Serum
LDH	Lactate Dehydrogenase
ml	Milliliter
mg	Milligram
ng	Nanogram
PBS	Phosphate Buffer Saline
Pen/Strep	Penniciline /streptomycine
WT	Wild type
µl	Microliter
µg	Microgram

CHAPTER I

INTRODUCTION

1.1 OVERVIEW OF ALZHEIMER DISEASE

Alzheimer disease (AD) is a neurodegenerative disorder which is first described by German psychiatrist and neuropathologist Dr. Alios Alzheimer in the first decade of 1900s. Dr. Alios Alzheimer noticed a patient, Aguste Deter who was suffering from memory loss. During six years Dr. Alzheimer observed the stages of the progressive memory loss on his patient. After six years his patient died and Dr. Alzheimer examined her brain and found some abnormal clumps, tangle formation and amyloid plaques, by silver staining method. Previously, plaque formation was observed in other neurodegenerative disorder (Blocq and Marinesco, 1892), however, tangle formation was described by Dr. Alzheimer. Initially Dr. Alios Alzheimer named the disease as a presenile dementia (Alzheimer, 1907) and his colleague, Emil Kraepelin, called the disease as Alzheimer disease. Major symptoms of the disease are loss of cognitive ability, loss of memory and behavioral changes (Katzman, 1986 and Budson 2005). Gradually the disease gets worse and a patient becomes bedridden slowly leading to the death of the patient. Researchers thought that progressive deterioration of brain cells in the cerebral cortex of the brain caused these symptoms.

Alzheimer disease is the most prevalent form of the dementia. According to the Alzheimer World Report 2011, today about 20-25 million people are thought to have Alzheimer disease in the world and according to prediction, this number will increase about the 70-150 million people in 2050. Although a lot of research paper and reviews have published on Alzheimer disease causes of the disease is not well understood.

The dominant risk factor of the disease is the increasing age (Mendez, 1992). Other factors such as are family history of dementia, education level, arthritis are related with Alzheimer disease (Lindsay, 1999 and Lindsay 2002).

1.1.1 Alzheimer Genetics

Alzheimer disease is suggested to be age dependent because most of the people who have Alzheimer are over the age of 60. In US, around 5.4 million people of all ages have Alzheimer disease and 5.2 million of them are 60 ages and older. AD is classified according to age of onset. If the signs of the disease appear after the age of 60, the disease is defined as late-onset Alzheimer disease/ sporadic AD (Hebert, 2003). 95 % of all patients are late onset Alzheimer disease. Late onset Alzheimer is related to genetic risk factors but the cause of the disease is not clear (Figure1.1). 5% of Alzheimer patients are under the age of 60 and they are classified as familial Alzheimer disease or early-onset Alzheimer. Familial Alzheimer is inherited by gene mutations: *APP, Presenilin 1 (PS1) and Preseninilin 2 (PS2) genes* (Figure 1.1).

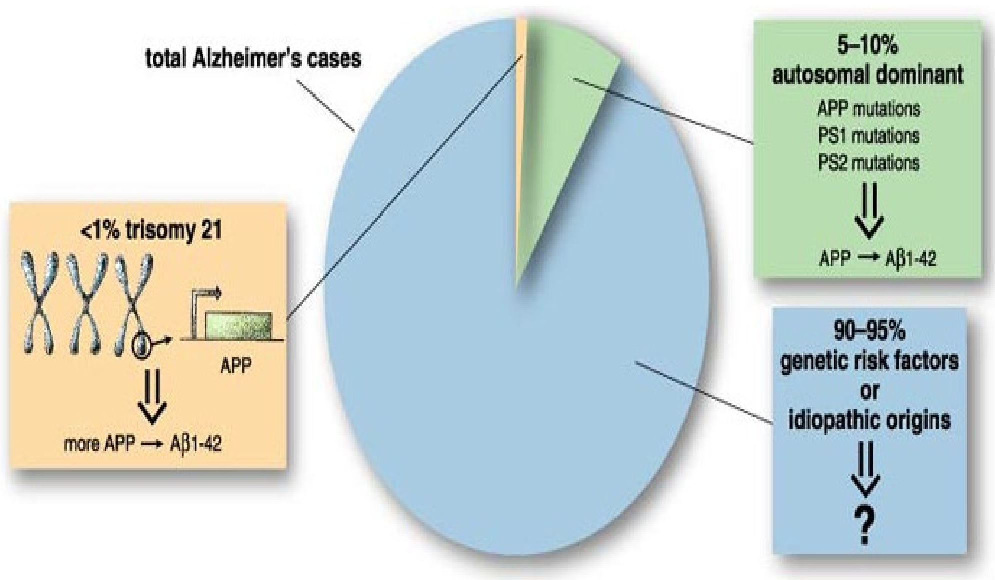


Figure 1.1 Alzheimer Genetics (Adopted from Ingram, 2003).

1.1.1.1 Early-onset/ Familial AD

Early onset Alzheimer is a rare form of dementia. Incidence of early-onset Alzheimer is about 5-10% percent of all population who have Alzheimer disease. Autosomal dominant mutations of three genes, APP (Amyloid precursor protein), presenilin 1 and presenilin 2, are associated with early-onset Alzheimer. (Goate et al., 1991; Levy-Lahad et al., 1995; Rogaev et al., 1995; Sherrington et al., 1995)

Familial Alzheimer disease shows autosomal dominant inheritance (Nee Le, 1983). Relationship between gene mutations and Alzheimer was confirmed by segregation analysis studies and the research searched out for underlying risk factor of early-onset Alzheimer (Farrer 1991; Rao, 1994). Firstly Amyloid Precursor Protein (APP) mutations which are on chromosome 21 were discovered (Goate, 1991) and next, abnormal presenlin 1 (Sherrington et al., 1995) and presenilin 2 mutations (Levy-Lahad, 1995; Rogaev, 1995) were reported, on chromosome 14 and 1, respectively. According to Alzheimer Disease and Frontotemporal Dementia Mutation Database, until now 32 different mutations in *APP*, 177 in *PSEN1*, and 14 in *PSEN2* were linked to early-onset Alzheimer in 86, 392, and 23 different families, respectively (Figure 1.2). APP mutations have been found near secretase cleavage sites of the APP and this affect directly APP metabolism and amyloid-β production. Moreover, PS2 and PS1 mutations are associated with over-production of amyloid-β_{42} peptide (reviewed by Lambert and Ammouyel, 2007) (Figure 1.2)

Autosomal dominant mutations of these genes lead to toxic amyloid-β peptides formation and subsequently formation of senile plaques which is a major Alzheimer disease characteristic.

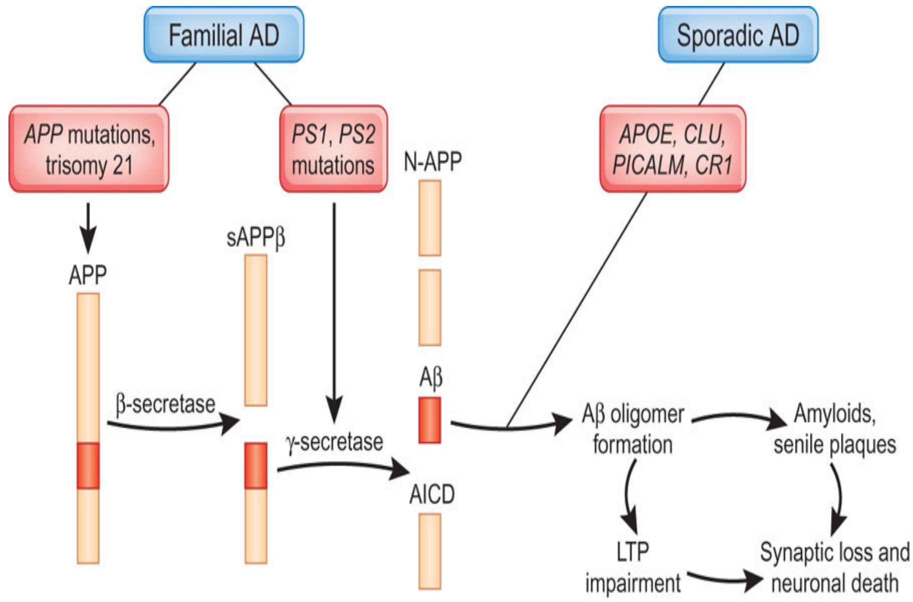


Figure 1.2 Relationship between mutated genes and Alzheimer (Van Es and Van den Berg, 2009).

1.1.1.2 Late-onset/sporadic AD

Late-onset Alzheimer is the most common form of dementia and occurs after age of 65. Up to 90% of the Alzheimer patients show late-onset Alzheimer. Genetic risk factors are related with this form of the disease. Late-onset Alzheimer is associated with *apolipoprotein E (APOE) gene* located on chromosome 19 which was firstly determined as a risk locus and subsequently related with late-onset Alzheimer. This gene product is 299 aminoacid lipoprotein binding amyloid proteins and is a main apolipoprotein in the brain that plays an important role in cholesterol homeostasis. This protein is responsible for lipid transporter in brain. (reviewed by Piaceri et.al., 2008)

Polymorphism of *APOE gene* is associated with sporadic AD (Figure 1.2) Apolipoprotein E (APOE) gene has several different forms and alleles, APOE ε2, APOE ε3, and APOE ε4. Higher frequency of the *APOE* ε4 allele was reported in Alzheimer patient and *APOE* ε4 allele is associated with late-onset Alzheimer (Corder, 1993; Deb et.al., 2000). In AD, brain apolipoprotein may have a role in amyloid-β clearance and deposition, but it is not clear (Pericak Vance, 1991; Namba et al., 1991;

Holtzman, 2000). Besides *APOE gene*, as a result of more than 1,000 association studies, more than 660 AD candidate genes were suggested since the 1990s. *SORL1* (sortilin-related receptor), *BIN1* (Bridging integrator), *CLU* (clusterin), *PICALM* (phosphatidylinositol-binding clasthrin assembly protein), and *CR1* (complement component receptor 1) gene are related to late onset Alzheimer bu they are not directly linked to amyloid-β (Abraham et. al., 2008 and rewieved by Piaceri et. al., 2008). Genetic variation in *APOE, SORL1, CLU, PICALM* and *CR1* may be involved in reducing amyloid-β clearance but their actual role in Alzheimer is not well characterized (reviewed Lambert and Ammouyel, 2011) (Figure 1.2).

1.2 PATHOLOGICAL HALLMARKS OF ALZHEIMER

When normal neuron cells are compared with neurons in Alzheimer disease, neuron cells exhibit some abnormalities that bring out the loss of neuronal function in Alzheimer disease. Alzheimer patients have two hallmarks of the disease, one of them is neurofibrillary tangles and the other is amyloid-β plaques. These lesions roles in disease are still controversial (Figure1.3).

1.2.1 Neurofibrillary tangles (NFT)

Neurofibrillary tangles (NFT) are aggregates of highly phosphorylated forms of the microtubule-associated tau protein (Wood 1986). Tau protein is part of the cells provide structural support and helps to deliver substances throughout the cell. In Alzheimer disease, tau is changed in a way that the phosphorylated tau dissociates from microtubules and agregaties into fibrillar forms which is named neurofibrillary tangles. This cause destabilized microtubules and impairs axonal transport. NFTs density in brain is linked to degree of Alzheimer disease (Brion, 1998). Despite the fact that neurofibrillay tangle formation is thought to be characteristic for AD, Tau based neurofibrillary tangles are found in other neurodegenerative disorders. According to Lambert et al., 1994 tau phosphorylation is induced by aggregated amyloid β_{42} in differentiated SH- SY5Y cells (Lambert et al.,1994), and this result has been supported with amyloid-β activated tau phosphorylation in cultured primary rat hippocampal neurons (Busciglio, 1995). These findings suggest that there is a relationship between Amyloid and tau protein.

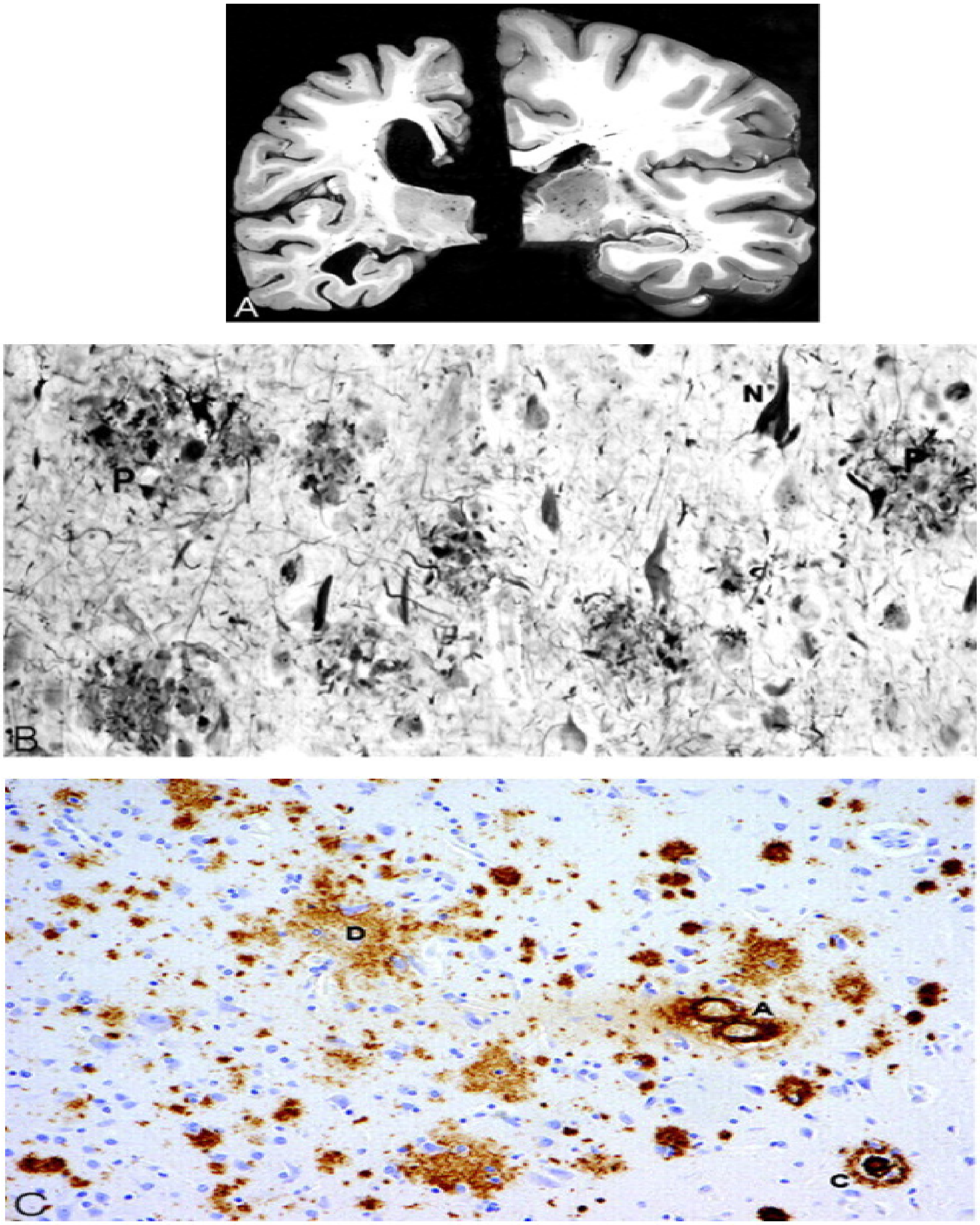


Figure 1.3 Pathological hallmarks of Alzheimer disease: neurofibrillary tangles and amyloid plaques **A)** Atrophy of the brain **B)** Neurofibrillary Tangles(N) and neuritic plaques (P) in the hippocampus **C)** Amyloid plaques : difuse plaques (D) core plaques (C) cerebral amyloid angyopathy (A) in the frontal lobe (Adopted from Wippold II, 2008).

1.2.2 Amyloid Plaques

Amyloid means starchlike which is derived from the Greek word amylon (=starch) and was coined initially in a botanical context by Schleiden (Schleiden, 1838). Then the term is transferred into medicine to describe human-pathogenic deposits that stain blue-violet with sulphuric acid and iodine solution (Virchow, 1854) but these deposits are found proteinaceous rather than polysaccharides (Friedreich, 1859) so current definitions of amyloid fibrils depend on the context of their usage.

Amyloid-β was first identified and sequenced from meningeal blood vessels of AD and Down syndrome patients in 1984 (Glenner and wong 1984; Master et al., 1985). Amyloid-β formation is also associated with major diseases, including cardiac arhythmias, atherosclerosis, rheumatoid arthritis and type II diabetes. As a result of aging, amyloid fibrils eventually accumulate in every person after a certain age. Amyloid fibril formation is found not only in brain but also other parts of the body. This formation is found in aortic vessel wall, aortic medial amyloid which of 97% of all humans aged over 50 years (Haggvist et al., 1999).

Amyloid plaques associated with AD are extracellular aggregates of β-amyloid peptide (Aβ) in the brain. Amyloid-β is a cleavage product derived from amyloid precursor protein (APP) (Korenberg et al., 1989). By different proteases, different amyloid-β peptide sizes which range in length from 39 to 43 amino acids are produced. Then amyloid peptides aggregate with each other and subsequently form oligomer, fibril structure and amyloid plaques which are characteristic of AD. Amyloid plaques are insoluble fibrillar aggregates and they are neurotoxic in vivo end in vitro(Hardy and Higgins,1992)

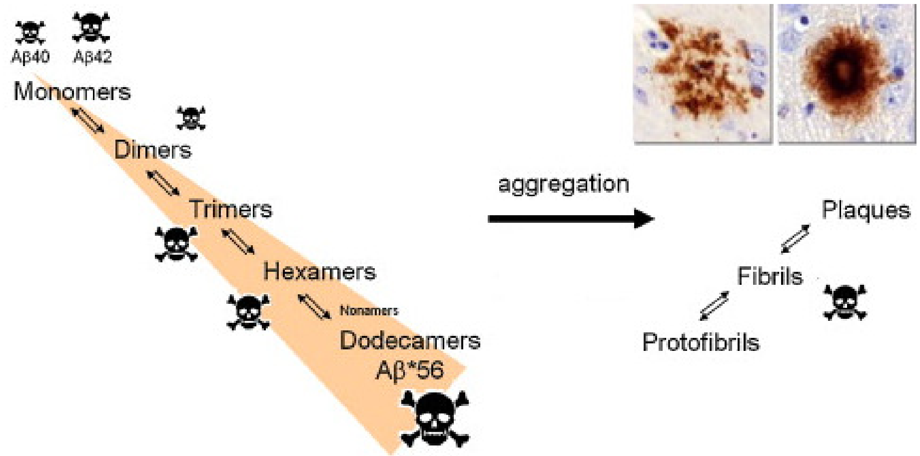


Figure 1.4 Aggregation of amyloid-β monomers (Adopted from Pahnke 2009).

1.3 APP AND PROTEOLYTIC PROCESS

Amyloid precursor protein (APP) is a ~70-kDa trans-membrane cell surface protein that is present in many cells including neuron cells and is concentrated in the synapses of neurons. APP gene is located on chromosome 21 and it contains 18 exons. 8 isoforms are generated from the APP transcripts by alternative splicing. APP is a glycoprotein of 695–770 amino acids which comprises three parts including the extracellular N-terminal region, a single hydrophobic transmembrane region and the cytoplasmic C-terminal domain. 695 amino acid, 751 amino acid and 770 amino acid forms are most common forms of APP (Bayer et. al., 1999).

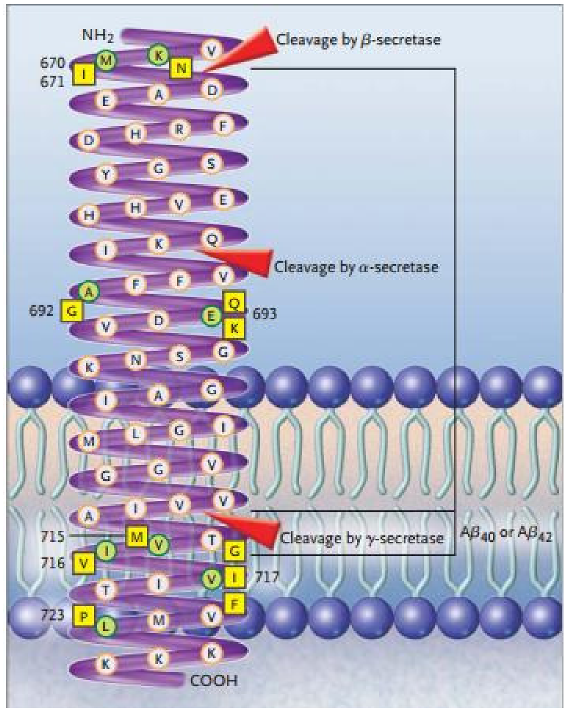


Figure 1.5 Structure of Amyloid Precursor Protein (Nussbaum, 2003).

In neurons, APP is produced in large quantities and is metabolized very rapidly but its function is unknown (Hellstrom, 2009). Gene manipulation in intact animals showed that the APP family of proteins is essential for survival (Hornsten, 2007; Herms 2004) and suggested that they may play a role in signal transduction and cell adhesion (Herms 2004; Li 2010). As a result of overexpression of APP in mice, size of cortical neurons increased (Oh et. al., 2009). APP reduction or deletion is associated with impaired neuronal viability in vitro and reduced synaptic activity in vivo (Allinquant et. al., 1995; Perez et. al., 1997; Herard et. al., 2006). Trans-synaptic interaction of APP is necessary for the proper development of motor neurons (Wang 2005; 2007; 2009; and Yang et al., 2005). Mutations in amyloid precursor protein (APP) are associated with familial Alzheimer disease (Bettens, 2010).

APP is cleaved by proteases and there are two major pathways for proteolytic cleaveage of APP: non-amyloidogenic pathway and amyloidogenic pathway (reviewed by Strooper, 2010). In non-amyloidogenic pathway, APP is cleaved by α- secretase and γ-secretase. Generation of the Aβ peptide in non-neuronal cells is prevented through non-amyloidogenic process (Sissodia, 1990). In this process α-secretase cleaves APP between Lys-16-Leu-17 in the Aβ sequence and liberates the secreted APPsα ectodomain. APP-CTF is a fragment of 83 amino acids which begins with the N-terminal aspartyl residue of amyloid. After α-secretase proteolytic cleavage, the membrane-bound carboxy-terminal fragment (APP-CTF), C83, is cleaved by γ-secretase. As a result of this process non-amyloidogenic 3 kDa fragment, p3 is formed instead of amyloid peptide (Haass, 1993). After γ-secretase cleavage, AICD (APP intracellular domain) is generated which may play a role in signal transduction (Hardy and Selkoe, 2002).

In amyloidogenic pathway, Amyloid precursor protein (APP) is cleaved following the sequential cleavage of β- and γ-secretase and leads to production of ~4 kDa amyloid-β (Aβ). γ-secretase which is responsible for the cleavage of multiple membrane proteins as well as APP, is a multisubunit enzyme composed of the proteins APH1, PEN2, nicastrin, and presenilin (PS1 and PS2). Components of the enzyme are necessary for γ-secretase maturation and function (Kimberly et al., 2003; Edbauer et al., 2003). β-secretase is known to be β-site APP cleaving enzyme I (BACEI), an aspartyl protease (Hussain 1999; Linx 2000). Amyloid-β (Aβ) production is initiated by BACE1 cleavage of APP at the Asp+1 residue of the Aβ sequence to form the N-terminus of the peptide. After this cleavage, two fragments are produced: one of them is APPsβ, a secreted APP ectodomain and the other is a membrane-bound carboxyl terminal fragment (CTF), C99. Cleavage of carboxyterminal fragment of amyloid precursor protein (APP) is achieved by γ-secretase. Membrane-bound carboxyl terminal fragment is subsequently cut by γ-secretase and the C-terminus of the Aβ peptide and an APP intracellular domain (AICD) is generated. Endoproteolysis of APP and generation of different amyloid-β peptides are achieved by γ-secretases. The majority of Aβ peptides are released by γ-secretase activity. Most of them are Amyloid-β_{40} and Amyloid-β_{42} peptides (reviewed by Pearson and Pears, 2006).

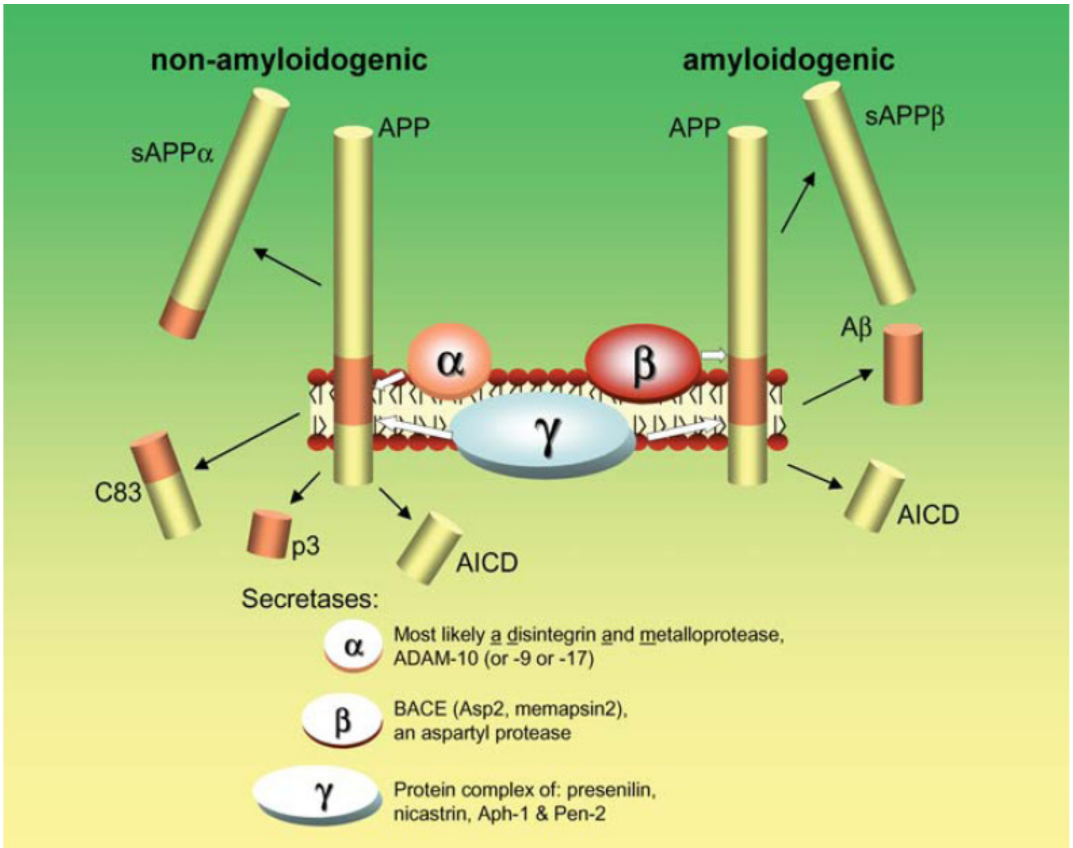


Figure 1.6 Proteolytic processing of APP via non-amyloidogenic and amyloidogenic pathway (Pearson and Pears, 2006).

1.3.1 Amyloid-β Peptides

Amyloid peptides that are group of hydrophobic peptides of 39-43 amino acid residues are main components of amyloid plaques. Amyloid-β peptides are derived from amyloid precursor protein (APP) through sequential proteolysis by β- and γ-secretases. This amyloid-β peptides can aggregate over time to produce amyloid plaques or this peptides can be cleared by enzymes such as neprilysin (Iwata et. al.,2001), insulin degrading enzyme (Kuochkin and Goto,1994) and cahthepsin B (only amyloid beta 1-42) (Mueller Steiner et. al., 2006) . Cleareancy of amyloid-β is mediated by proteolytic enzymes like neprylsin and besides mediated by chaperone molecules like apoE are involved in amyloid-β clearency (Kim, 2009), lysosomal (Bendikse, 2003) and non-lysosomal pathways (Marambaud, 2005). The imbalance between the levels of Aβ production, aggregation and clearance, amyloid-β accumulates abnormally in the brain.

Studies show that increasing level of the amyloid-β production leads to neurotoxicity, neuronal tangle formation, synaptic damage and eventually neuron loss in the pathologically affected brain regions (Selkoe 1998 ; Shankar and Walsh 2009). The peptide production is stimulated by mutations in the APP gene and/or in the gene encoding the γ-secretase complex and also increase the relative production of $A\beta_{42}$ to $A\beta_{40}$ (Suzuki et al., 1994 ; Duff et al., 1996; Scheuner et al., 1996). Mutations in presenilin affect the spectrum of the Aβ peptides generated by γ-secretase but do not essentially increase the production of Aβ (Bentahir et al., 2006). According a study which was published by Raussea and Schymkowitz in 2010, relative ratio of amyloid-β peptides including $A\beta_{42}$ and $A\beta_{40}$ is more crucial than the absolute amount of the peptides for the induction of neurotoxicity.

Amyloid–β protein fragments have an important function in the neurodegenerative process in Alzheimer disease (AD) (Hardy and Selkoe, 2002) Amyloid-β (1-42) and amyloid-β (1-40) peptides are major components of amyloid plaques. Especially 40 amino acids long ($A\beta_{40}$) peptide is major Aβ species recovered from serum, cerebrospinal fluid (CSF) and cell culture supernatants (Hass and Selkoe, 1993; Scheuner et al., 1996). $A\beta_{42}$ level is detected lower about ten fold than $A\beta_{40}$ level in AD brain (Gao et.al., 2010 ;Abromowski et.el., 2012). Amyloid-β (1-42) peptide is more hyrophobic, amyloidogenic and toxic than the others (Burdick et al., 1992). Because of amyloid- β_{42} more toxic than the other peptides, I have focused on amyloid-β_{42} in this study.

1.3.2 Relationship Between Amyloid Plaques, Amyloid-β Oligomers and Neurotoxicity

Plaques are consist of amyloid-β peptides (39-43 aminoacids in length). For long time, plaques have been considered as the major cause of Alzheimer neouropathology. Some studies have shown that Aβ fibril formation is toxic *in vitro* (Pike, 1993; Shearman, 1994; Seilheimer, 1997). In Alzheimer diseased brain, there is no correlation between fibrillar plaque density and severity of dementia (Terry, 1991 and Dickson, 1995), whereas there are strong correlations between soluble Aβ oligomer levels and the extent of synaptic loss and cognitive impairment (Lue, 1999 and Mclean, 1999). A lot of evidences support the notion that the Amyloid-β oligomers rather than the fibrils are responsible for the synapto-toxic effects (Walsh, 2004; Klein, 2001).

More recent studies have focused on the toxic effects of oligomeric forms of amyloid-β and it was showed that non-fibrillar soluble oligomers are toxic for neuronal and non-neuronal cells (Klein ,2002; Hsu, 2009). Aβ oligomers inhibit the hippocampal LTP and said to be able to impair synaptic plasticity and memory (Lambert, 1998). Solutions containing only monomeric amyloid-β demonstrated no deleterious effect on neurons in culture. Especially amyloid-β oligomers, not monomers or fibrils, disrupted synaptic plasticty *in vivo* (Walsh, 2002; Cleary, 2005). Dimeric oligomers isolated from AD brain potently impair synapse structure and function (Shanker, 2008).

1.4 CELL CYCLE

Cell division and cell differentiation are regulated and coordinated events required for normal cell growth. Extracellular and intracellular factors that act on the cell cycle machinery determine whether somatic cells become quiescent or resume active proliferation. Several factors that control cell cycle progression are responsible for this process. The cell cycle is a highly regulated process with numerous checks and balances that ensure its proper completion. Eukaryotic cell cycle process is comprised of four distinct stages which are derived by oscillating expression of cell cycle proteins and ensured by checkpoints.

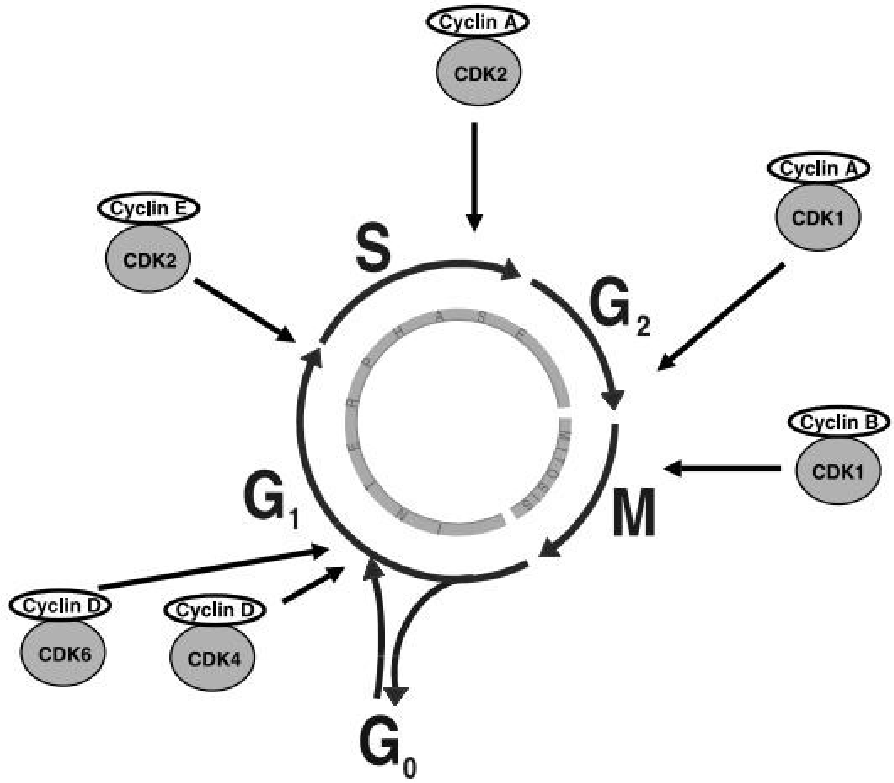


Figure 1.7 The stages of the cell cycle (Vermeulan, 2003).

The four phases of cell cycle are; S phase, where DNA replication and chromosome duplication occurs; M-phase, where mitosis and cytokinesis occurs; and the gap phases that separate the two; G1 and G2 (Murray and Hunt, 1993; Enders, 2002; Dean and Hinshelwood, 1965). Cells can exit the cell cycle to enter and stay at quiescent (G0) phase.

Cyclin dependent kinases (CDK) are Serine Threonine Kinases which are core protein complexes for cell cycle and act at distinct times. CDK activity requires binding of their regulatory subunits, cyclins, which are synthesized and destroyed at specific times during the cell cycle. This provides regulation of kinase activity in a timely manner (Tyson et al., 2003; Morgan, 2001). Progression through the mammalian cell cycle is associated with the activity of four cyclin dependent kinases including Cdc2/ CDK 1, CDK 2, CDK4, and CDK6. CDK protein levels remain stable during the cell cycle and cyclin protein levels rise and fall during the cell cycle. On the other hand cyclins are responsible as a mechanism to periodically activate CDK (Evans et al. 1983;

Pines 1991). The sequential expression and activation of cyclin and cyclin dependent kinase (CDK) complexes provide the cell cycle progression. Extracellular signals triggers the expression/activation of cyclin-CDK complexes that provide re-entry of resting cells into the G1 phase of cell cycle. Certain cyclin-CDK complexes are active at a specific phase of the cell cycle and contribute to the activation of the next cyclin/C CDK complex, this leads to transition from one phase to another. CDK4/6-cyclinD complexes control the re-entry from the resting(Go) cells to the G1 phase and CDK2 / Cyclin E complexes are responsible for transition from G1 to S phase (Sherr, 1995).

When mitogenic signals trigger the cell cycle initiation, cyclin D is expressed. Cyclin D binds to stimulate activation of CDK4 and CDK6 during G1, where cells prepare to initiate DNA synthesis (Malumbres, 2001). Activation of CDK4and CDK6 leads to E2F transcription protein activation by partial inactivation of RB protein which is known as pocket protein. E2F triggers cyclin E and cyclin A expression that allows to pass through restriction point which is defined as a point of no return in G1. E-type cyclins are expressed and cyclin E binds and stimulate CDK2 activation (Harbour, 1999). CDK2 is subsequently activated by cyclin A binding. At the end of the S phase, which is the late stage of DNA replication, is completed by CDK2-cyclin A complexes to drive the transition from S phase to mitosis which is called the G2 phase. Finally, cyclin A stimulate activation of CDK1 to promote entry into M phase and also mitosis is regulated by cyclin B in complex with CDK1 which causes breakdown of nuclear envelope (King et al., 1994; Arellano & Moreno, 1997). These complexes induce the initiation of M phase by stimulating downstream proteins involved in chromosome condensation and mitotic spindle assembly. At the end of M phase CDK1-Cyclin B complex is deactivated that causes the cell to exit mitosis.

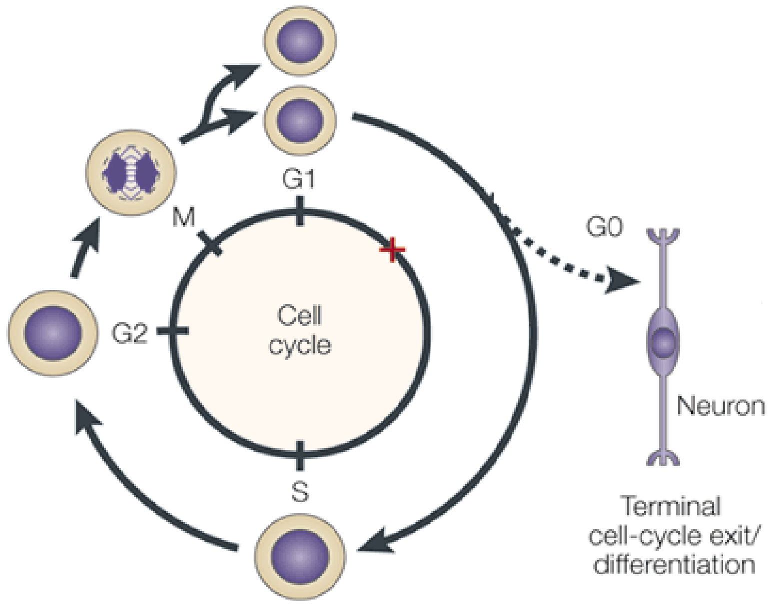


Figure 1.8 Neuronal cell cycle (Adopted from Dyer and Cepko, 2001).

1.4.1 Alzheimer and Cell Cycle

Neuron cells are terminally differentiated cells and they exit the cell cycle during embriyonic development and retained in quiscent (G0) phase at cell cycle (figure 1.7). According to the recent investigations, when neuronal cell cycle is triggered or the neuron attempts to undergo cell cycle activity or cell cycle proteins expression is induced, neuronal cell is damaged (Greene et al., 2004; Herrup and Yang, 2007; Kruman, 2004; Neve and McPhie, 2006; Raina et al., 2004) . The re-expression of various cell cycle proteins in neurons from patients with AD have been reported by a number of laboratories (Lamp, 2009). Cell cycle marker Cyclin A, cyclin B, cyclin D expression are detected in AD brain (Nagy, 1997; Yang, 2003; Hoozemans, 2002).

However, no evidence of actual mitosis has ever been found in vulnerable neurons in AD (Zhu et al., 2008). This suggests that these neurons are arrested at a point or points prior to actual event of cellular division. Cell cycle process unidirectional, once cyclin A is expressed the cells become committed to division and lack the ability to return to G0, therefore, cells must either complete the cycle or die.

Amyloid-β oligomers are mitogenic *in vitro* (McDonald et al., 1998; Pyo et al., 1998) which may have a direct role in the induction of cell cycle- mediated events in AD. Besides amyloid-β is toxic for cells *in vitro*, and this is dependent on the presence of various cell-cycle related elements (Giovanni, 1999). Amyloid-β oligomers may become neurotoxic when the neuronal cell cycle machinery is activated by amyloid- β oligomers.

Recent studies indicated that Aβ oligomers promote the expression of cell cycle proteins and the induction of neuronal cell cycle events via PI3K-AKT-mTOR pathway (Bhaskar et. al., 2009). Besides, extracellular signal-regulated kinase (ERK)1/2 signaling pathway has a role in Aβ oligomer induced neuronal death (Chong et al., 2006). These pathways are associated with cyclin D expression and increased cyclin D expression lead to activation of G1 CDKs, CDK4-6. CDK4-6 is shown to be involved in amyloid-β fibril induced neuronal death (Giovanni et al., 1999). It was also found that CDK4 mRNA levels in AD brain are higher than controls (Tsujioka, 1999).

On the other hand CDK5 which is an atypical member of cyclin dependent kinases may be involved in cell cycle re-entry. It has no cyclin partner, instead p25 and p35 are responsible for the activation of CDK 5 (Lew et al., 1994; Tsai et al., 1994; Tang et al., 1995). Some survey revealed that CDK5, Serine Threonin Kinase, is associated with Alzheimer disease. When CDK5 is silenced, tau phosphorylation is reduced and as a result of this, nerurofibrillary tangle is not formed (Piedrahita, 2010). In 1995, it was reported that CDK5 phosphorylates Rb protein (Kato G and Shuichiro M, 1995). Also, phosphorylation of Rb by CDK5 is reported *in vitro* (Lee et al., 1997; Hamdane et al., 2005). It was shown that CDK5 is associated with cell cycle induction, synaptoxicity, tau phosphorylation, apoptotic death in post-mitotis neurons in amyloid-β 1-40 peptide induced *in vivo* (Lopes J P, 2010, 2009) . In this study CDK4 protein level did not change but localization of CDK4 was changed by CDK5. It was shown that CDK5 can regulate cell cycle by Rb phosphorylation (Fututsagi et al., 2012). Lastly, another survey claims that CDK5 inhibit HDAC 1, histone deacetylase 1, by phosphorylation and cause increased expression of cell cycle proteins (Kim D et al., 2008).

In this study, I have investigated the possible role of cdk 4 in amyloid -β oligomer induced cell cycle re-entry and neurotoxicity. Further I have investigated whether cdk2 is involved in amyloid-β oligomer induced DNA replication.

CHAPTER 2

MATERIALS AND METHODS

2.1 MATERIALS

2.1.1 Constructs

Constructs are mentioned in the below chart (see table 2.1)

Table 2.1 Constructs

Constructs	Sourced by
PCMV CDK2 WT	Enders Lab
PCMV CDK2 dn	Enders Lab
PCMV CDK4WT	Enders Lab
PCMV CDK4dn	Enders Lab
EGFP	

2.1.2 Chemicals

Chemicals which used are listed with their catalog number and their company name.

Table 2.2 Chemicals

Material	Company Name	Catalog no
Lipofectamine 2000 reagent	Invitrogen	11668-027
WST1	Roche	11 644 807 00
LDH	Roche	11 644 793 001
BrDU	Roche	11 647 229 00
DMEM/high Glucose	Hyclone	SH30243.01
DMEM/Low Glucose	Invitrogen	11966025
DMEM/No Glucose	Invitrogen	11966025
F12	Invitrogen	21765029
PBS	Hyclone	
Trypsin	HyClone	
Penicillin/Streptomycin	HyClone	
FBS	HyClone	
Trypan Blue	Sigma	T 815
Dimethyl Sulphoxide	Sigma	D2650
Amyloid-β 1-42	American Peptide company	
Ham's F12	Caisson Laboratories	
Hexafluoro-2propanol	Sigma	
DMSO	Sigma	
N_2 Supplement	Invitrogen	
Retionic Acid	Sigma	
LB agar	Merck	1.10283.0500
LB broth	Merck	1.10285.0500
Gene-jet miniprep	Fermentas	
Rubidium Chloride		
Genopure Maxi Prep	Roche	
Ampicillin		
Potassium Acetate		
CaCl2		
MnCl2		
MOPS		
NaOH		

2.1.3 Equipments

Table 2.3 List of Equipments

Material	Company name
Laminar Flow	
CO2 incubator	NÜVE
Evaporator	Thermo Scientific
Ice machine	Scotsman
Freezer (-80 ºC)	Daihan
Deep Freezer (-20ºC)	ARÇELİK
Deep Freezer (-80ºC)	New Brunswick Scientific
Invert Microscopy	
Flouresence Invert microscopy	Nikon –eclipse ti-u
Fume Hood	TOLKİM
Magnetic stirrer and heater	Daihan
Centrifuge 15ml TC	NÜVE
Centrifuge 1.5 ml	Bio-Rad
ELISA reader	Shimadzu
Spectrophotometer	Grainer
Cell culture plate	Grainer
96 well plate	Grainer
6 well plate	Millipore
Distilled water machine	NÜVE
Autoclave	Biosan
Vortex	ARÇELİK
Refrigerator	Drummond
Pipette Gun	Denville
Pipette Set	Grainer
Eppendorph	Grainer
Tips	Grainer

Table 2.3 continued

Falcon tubes(10 ml-50ml)	Grainer
Serological pipette	Grainer
70% Ethanol	
Autoclave	ALP
Vortex	Biosan
Refrigerated centirfuge	Thermo Scientific
Benchtop minicentrifuge	Wisd

2.2 METHODS

2.2.1 Cell culture

2.2.1.1 Subculture of Adherent Cell

SH-SY5Y cell line was used for cell culture experiments. Cells were grown on 10 cm cell culture plate (Grainer) in growth media which included 10% FBS, 1% PS and DMEM (no glucose): F12 (1:1) until 90% confluency of population. After old media was removed and cells were washed with PBS, tyripsin was used to release the cells from cell culture plates. SHSY5y cells were dislodged from the surface by adding particular amount of trypsin and incubated for 3-4 minutes in the CO2 incubator. After detachment, media including 10% FBS was added to inhibit the activity of tyripsin. Cells were seeded at a proper ratio on new cell culture plate. SH-SY5Y cell line was subcultured in 5-6 days and their growth media was replaced once in two days.

2.2.1.2 Differentiation of the Cells

SH-SY5Y cells differentiation was triggered by treating with retinoic acid and N2 supplement. Cells were seeded in 96 well plate in a way to obtain 70% confluency in the next day. Then growth media was replaced with the differentiation media which included DMEM (low glucose), 1% PS, 1% N2 supplement, 10µM retionic acid). Retinoic acid was prepared in DMSO as a 3mg/ml of stock solution. Solution was aliquoted to the PCR tubes and then stored in light protected manner at -20^{o}C and diluted with growth media as mentioned above before use. Cells were incubated for 72h in differentiation media and during differentiation, third day old differentiation media was changed with fresh one until cells become fully differentiated (app 9 days). Retinoic acid and N2 supplement were added freshly into differentiation media.

2.2.2 Transformation

2.2.2.1 Producing Competent Cells

DH5-alpha strain of *E. coli* was used as competent cell at log (or exponential) phase and a colony grown in 3 ml LB broth at 37 ^{o}C and subsequently 1ml from this culture was subcultured into 100ml of LB broth until OD reaches 0,4-0,and 6. After this cells were spinned at 4^{o}C centrifuge at maximum (approximately 1500 rpm) for 10 minutes. TFbI buffer and TFbII were prepared according the volume (see table 2.4) and sterilized with 0.2 µm filter. TFbI buffer was added to the pellet and re-centrifuged in 4^{o}C centrifuge at maximum for 10 minutes. Pellet was resuspended with TFbII (table 2.4). Newly produced competent cells were aliquoted and could be used for transformation or quick frozen with liquid nitrogen and stored at -80 ^{o}C.

Table 2.4 Ingredients for TFbI and TFbII buffers

TFb I		TFb II	
30Mm Potassium Acetate	1.465 gr	10Mm MOPS	0.210 gr
100Mm RbCl2	6.045 gr	10Mm RbCl2	0.121 gr
10Mm CaCl2	0.735 g	75Mm CaCl2	1.100 g
50Mm MnCl2	4.954 g	15% Glycerol	75 ml
15% Glycerol	75 ml	d H2O	75ml
d H2O	500 ml	pH	6.5
pH	5.8		

2.2.2.2 Plasmid Transformation

DH5alpha competent cells were transformed with the desired construct. Appropriate volume of DNA which was determined according to corresponding DNA concentration (100 mg) was added to 50µl of competent cells. Then mixture was incubated at 4°C for 30 minutes and subsequently was heat shocked at 42°C for 45 seconds. Following the incubation, the mixture was placed on ice for 2 minutes, 950µl of LB broth was added to competent cells. Solution was incubated at 37°C with 225 rpm agitation for 45 minutes. Ampicillin containing (or other antibiotic based on the resistant gene on the vectors) plates were prepared beforehand and were used to spread the culture solution. 50 µl of culture was gently spread to the suspension around the plate. The plates were incubated at 37°C for 16 hours. After 16 hours, a colony was selected and cultured in 3 ml LB broth until 16 hours or stored at 4 °C.

2.2.2.3 Plasmid isolation with miniprep kit

After 16 hours, a single colony was selected and cultured in 3 ml LB broth until 16 hours. Cultured cells were centrifuged at 8000 rpm for 5 minutes and pellet was resuspended with 250 µl of resuspension buffer which was ready to use in miniprep kit. And subsequently 250 µl of lysis buffer was added to resuspended cells. Then 350 µl neutralization buffer was added and centrifuged at 12000 rpm for 5 minutes. After spin, supernatant was transferred to columns which were designed for plasmid DNA binding. Column was spun at 12000 rpm for 5 minutes. The column was washed twice with 500ul Wash Buffer and subsequently spun until empty. After 50 µl elution buffer is added and bound plasmid DNA was eluted from column. DNA concentration was measured by Nanodrop.

2.2.3 Transfection

Lipofectamine:DNA ratios for transfection of SH-SY5Y neuroblastoma cell line were decided according to the manufacturer's protocol. SH-SY5Y cells were seeded in 96 well plate that would be at 80-90% confluency in the next day since high cell population was necessary for optimization. Stable green flouresence protein was used for the optimization experiments. The ratio acquired high transfection efficiency for HeLa cells and was recommended in the manufacturer's protocol. Firstly lipofectamine 2000 transfection reageant and DNA were diluted in 25 µl OPTIMEM for 6 minutes. Then diluted DNA and lipofectamine combined with each other and incubated at room temperature for 20 minutes . 50 µl was added to 100 µl DMEM without pen/strep FBS. After 6 hours media was changed with fresh media.No DNA, empty vector transfected cells and neither DNA nor lipofectamine 2000 transfection reageant was added as a control.

In double transfection method, cells were cultured in 96-well micro plate that would be at 95% confluency in the next day. And the cell transfected with 0,35µg DNA and 0,8 µl Lipofectamine 2000 for 5-6 hours and subsequently media was changed with differentiation media. The transfected cell was differentiated for 72 hours and re-transfected with same ratio for 5-6 hours. And then the cell was differentiated for 48 hours. After this, cells were ready for further experiments.

2.2.4 Preparation of Amyloid-β 42 (Aβ_{42}) Oligomers

2.2.4.1 Preparation of 1,1,1,3,3,3-Hexafluoro-2-propanol (HFIP) Film of Aβ Sample

HFIP container was placed on ice because hexafluoro-2-propanol (HFIP) must be used as cold. For dissolving amyloid-β 42 peptides, enough HFIP was measured. HFIP is corrosive and very volatile so must be handled carefully since it can be evaporate very fast. For establishing monomerization and randomization of structure the peptide was incubated with the Aβ - HFIP solution at room temperature for at least 1 hour. Aβ-HFIP solution had to be clear and colorless. Then HFIP was aliquoted (0,050mg peptide/tube), left the caps of tubes open and allowed HFIP to evaporate overnight, in this step most of HFIP evaporated , followed by 15 minutes in a Savant Speed Vacuum for removing any remaining traces of HFIP or moisture. The resulting peptide should be a thin clear film at the bottom of the tubes and was stored -80 ^{o}C.

2.2.4.2. Preparation of Aβ$_{1-42}$ Oligomers

A tube of 0,05 mg Aβ-(1-42) HFIP film was dissolved in fresh DMSO because is very hygroscopic and should be fresh. For dissolving HFIP film, through a pipette, sides of the tube were washed/scraped to ensure complete resuspension of peptide film. For preventing crystallization, the tube was warmed at room temperature briefly before adding DMSO. Cold phenol-free F-12 cell culture media was added in the same tube (final concentration: 100 µM). F12 media is light sensitive so prolonged exposure to light was avoided and F12 solutions were kept at +4 °C. The tube was vortexed ~10 seconds and incubated at 4°C for 24 hours. DMSO/F12 was prepared with same ratio as a control. After 24 hours, the tube was centrifuged at 14000 g x 10 min at 4°C and followed transferring supernatant to a sterile tube. Theprotein concentration of amyloid-β oligomers was measured in cuvettes and controlled the values with BSA standart. OD was measured at 595 nm.

Table 2.5 Ratio of BSA and amyloid-β oligomer

Protein	ddH20	Coomassie Plus	Vehicle control	0.25mg/ml BSA
0 µg BSA	0,49 ml	0,5ml	10 µl	0 µl
1 µg BSA	0,49 ml	0,5ml	8 µl	2 µl
2 µg BSA	0,49 ml	0,5ml	6 µl	4 µl
3 µg BSA	0,49 ml	0,5ml	4 µl	6 µl
4 µg BSA	0,49 ml	0,5ml	2 µl	8 µl
5 µg BSA	0,49 ml	0,5ml	0 µl	10 µl
Amyloid-β$_{1-42}$ oligomer	0,49 ml	0,5 ml	5 µl	5 µl Amyloid-β$_{1-42}$ oligomer

ADDLs mass is calculated according to a standart curve which was done with BSA protein's concentration-absorbance chart.

ADDLs concentration = [µg protein (average for duplicate ADDL samples based on BSA standard)÷5 (µl tested)] ÷4514 (MW for Aβ-(1-42).

2.2.5 Measurement of Cell Proliferation and Level of Viable Cell and Cytotoxicity

WST1 cell proliferation reagent (Roche) and LDH cytotoxicity kit (Roche) was used to detect the level of cytotoxicity and percentage of viable cell. WST1 reagent could not not distinguish whether the cells were actively dividing or quiescent. Roche BrdU Labbeling reagent was used to assess measurement of replicating cells. Cultured cell in 96 well plate were examined for assessment of cell proliferation through BrdU cell proliferation kit (Roche). Firstly, 100µl of suspension media was taken as a replica to another 96 well-plate then as manufacturer's protocol, BrdU labeling reagent was diluted to a final BrdU concentration of 10 µM and added on cells in 100µl media. Cells were incubated with BrdU in incubator for 2 hours. And then WST-1 10ul/ well volume cell proliferation reagent was added on cells in same media and re-incubated for 2 hours in the incubator (37 ^{o}C, 5% CO2 and in a humidified atmosphere). And then LDH mixture which was preapared as in the manufacturer's protocol was added 100ul of suspension media and re-incubated 30 minutes at room temperature. The WST1 absorbance was measured at 480 nm and LDH absorbance was measured at 492 nm with ELISA reader. After measuring the absorbance WST1 and LDH, the media was removed by tapping and cells were added with FixDenat solution and the plate was kept at room temperature for 30 minutes. FixDenat solution was removed by tapping. Antibody was diluted with antibody dilution solution and incubated at room temperature for 90 minutes. Solution was removed and washed 3 times with PBS. Finally, substrate was added and incubated for 15-20 minutes then measured the absorbance at 370 nm.

CHAPTER 3

RESULTS

3.1 DIFFERENTIATION OF SH-SY5Y CELL LINE

SH-SY5Y cell line is ideal for neurotoxicity and neuroprotection studies and is a well characterized system for neuronal differentiation. In order to use for neuron cell model, SH-SY5Y cells were differentiated according to the method mentioned in 2.2.1.2. Briefly, cells were seeded to 96 well plate in growth media containing 1% pen/strept, %10 FBS, DMEM:F12 (1:1) and next day growth media was replaced with differentiation media including 1% pen/strep, low glucose DMEM, 1% N2 supplement, retionic acid (final concentration 10 µM) (Lambert et al., 1994). Every 72 hours differentiation media was changed with fresh differentiation media. After 72 hours the out-neurite growth was observed, as shown in Figure 3. 1 B. Cells were differentiated for nine days and the neuron network was clearly observed (Figure 3.1 C-D). Literature survey shows that neurite length is maximal in 4-5 days at RA induced protocol (reviewed by Chu CT, 2009). Accordingly in our data neuronal network at 5th day is comparable to nine days differentiation (Figure 3.1 C).

The confluency ratio of cells is one of the factors that affect the differentiation process. Initial cell confluency was optimized for differentiation process. When cells were started to differentiate in 90% cell confluency, after 24 hours cells were over-confluent and failed to differentiate. On the other hand, when the cells were differentiated at 70% confluency, they differentiated very well and neurite outgrowth extension was observed under inverted microscopy (Figure 3.1).

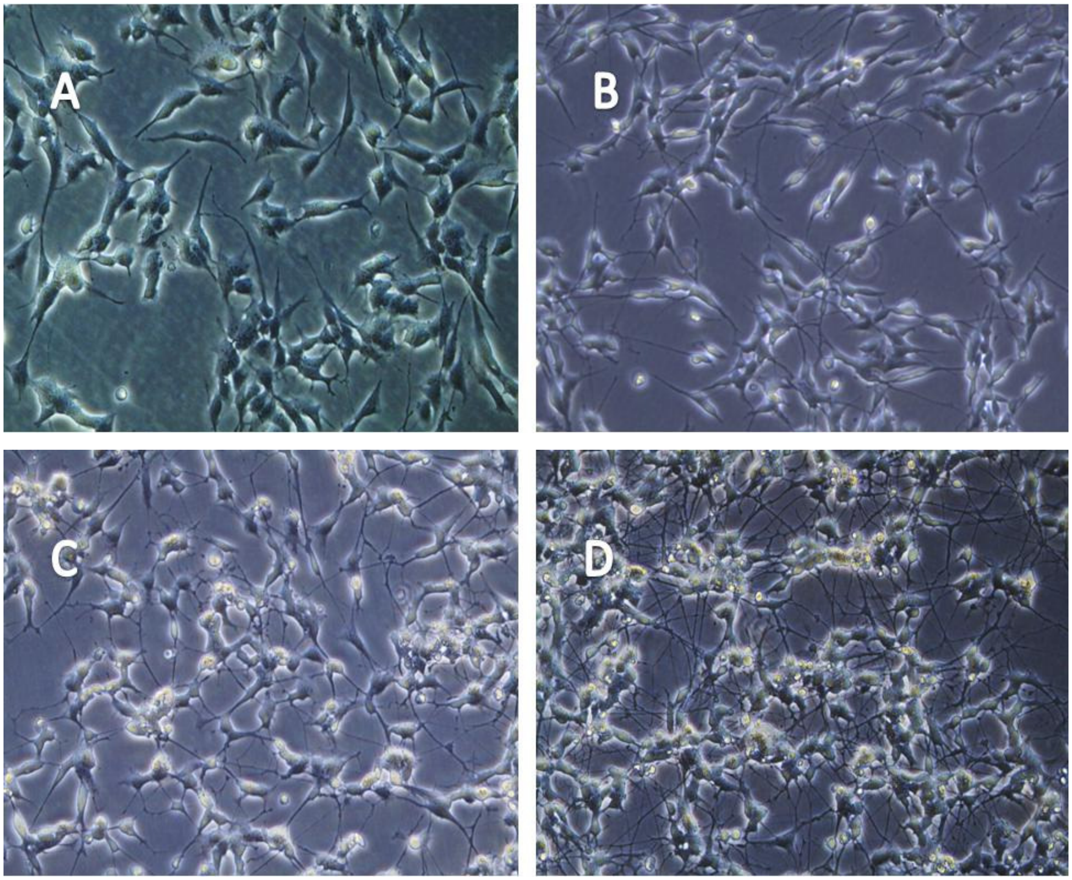


Figure 3.1 Images of differentiating SH-SY5Y cells **(A)**Undifferentiated SH-SY5Y cells **(B)** SH-SY5Y cells incubated with differentiation media for 72 hours **(C)** SH-SY5Y cell incubated with differentiation media for 5 days.**(D)** SH-SY5Y cell incubated with differentiation media for 9 days.

Neuronal cells are terminally specialized cells and they do not divide. Therefore, one can say if SH-SY5Y cells differentiate in the right way, they will exit the cell cycle. In order to test cell cycle exit of differentiated cells, I cultured cells in 96 well plate in triplicate and differentiated for 9 days. Undifferentiated cells were also seeded in 96 well plate and analyzed next day. BrdU assay and WST-1 assays were performed for the same wells. In order to compare the BrdU incorporation level of proliferating and differentiated SH-SY5Y cells. BrdU values were normalized to viable cell number based on WST1 values. I have shown that as expected, amount of replicating cells decreased five-fold in differentiated cells (Figure 3.2).

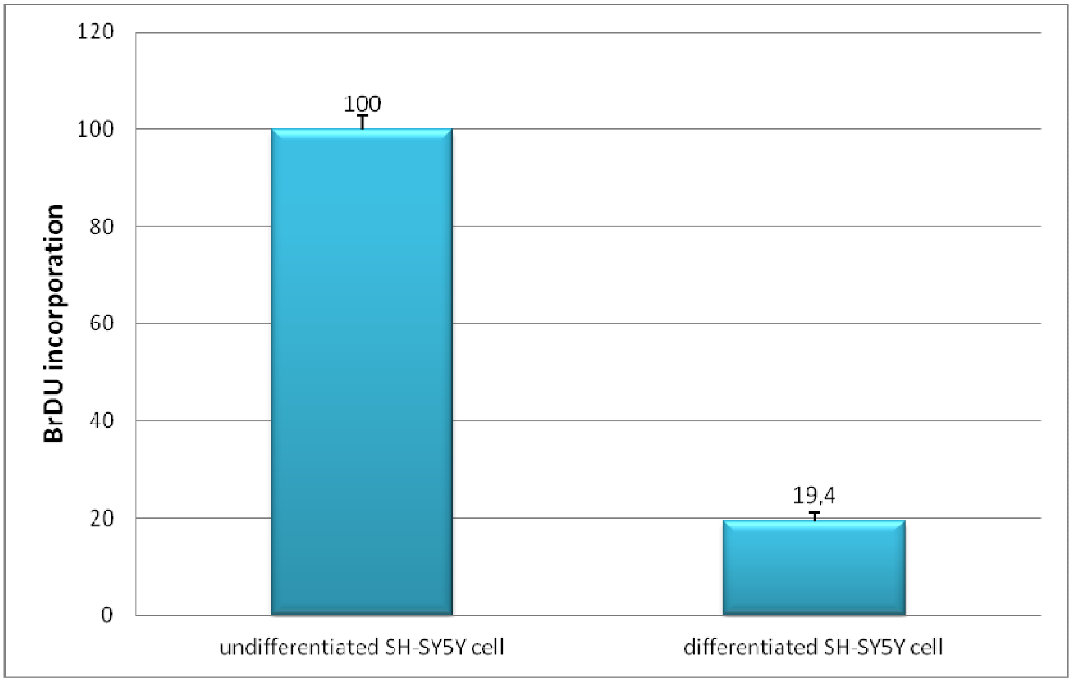


Figure 3.2 Comparison of replicating cells in undifferentiated and differentiated cell population. BrdU values were normalized to viable cell number based on WST1 values. Results of differentiated cells were presented as percentage of value obtained from undifferentiated cells.

3.2 OPTIMIZATION OF TRANSFECTION

In order to investigate the role of cyclin dependent kinases, I inhibited the protein function using dominant negative mutant version of cyclin dependent kinases. To detect whether cdks involved in oligomer induced cell cycle, SH-SY5Y cells were transfected with cdk4dn (dominant negative mutation of cyclin dependent kinase 4) and cdk2dn (dominant negative mutation of cyclin dependent kinase 2) constructs which were received from ENDER's lab. In my study, plasmid DNA was transfected *in vitro* to SH-SY5Y cells by lipofection method. As a transfection reageant, Lipofectamine™ 2000 Transfection Reagent was used which leads to form DNA-containing liposomes for fuse plasma membrane.

Transfection of SH-SY5Y neuroblastoma cell line is challenging and it was initially optimized for various transfection parameters, including DNA and lipofectamine ratio, incubation time, different transfection reagents. Green fluorescent protein (GFP) which is visualized without cell lysis using fluorescent microscopy was used for optimization.

3.2.1 Optimization of Undifferentiated SH-SY5Y Cell Line Transfection

Transfection of undifferentiated SH-SY5Y cells is a bit easier than differentiated SH-SY5Y cells. So to determine the ratio and incubation time, undifferentiated SH-SY5Y cells were used.

In our lab, HeLa cell line was tranfected succesfully with optimized ratio of 0.25 µl for lipofectamine and 0,1 µg for DNA per well of 96 well plate. Firstly, this ratio was used for the transfection of undifferentiated SH-SY5Y cells, and cells which express GFP protein are observed under flouresence microscopy. According to these observations, 2% of cells was green and this suggests that transfection efficiency was 2% (Figure 3.3).

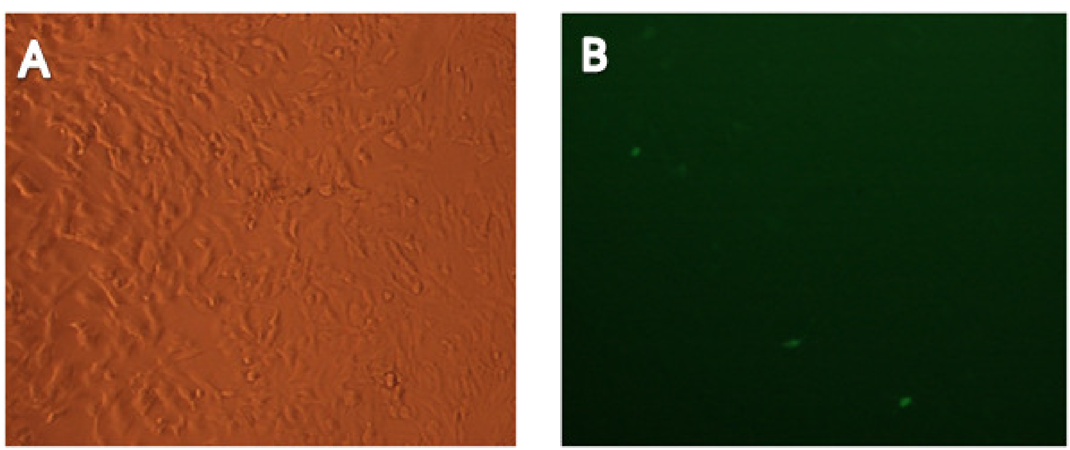


Figure 3.3 Undiffeentiated SH-SY5Y transfection. SH-SY5Ycells were incubated with 0.25 µl Lipo2000 + 0,1µg/ul gfp construct for the 6 hours. After 48 hours **A)** light microscopy image **B)** florescence microscopy images were taken.

Next for optimization I tried different ratios based on manufacturer's protocol suggestions (Table 3.1.). Besides ratio, the effect of transfection mixture incubation time on transfection efficiency of SH-SY5Y cells was tested. As a result, most of the 24 hours incubation with transfection mixtures cells died and transfection efficiency was not better than 6 hours transfected cell. When the ratios were compared, transfection of SH-SY5Y cells with 0.8 µl Lipo2000 + 0,35 µg/µl GFP construct for 6 hours was the best, and approximately half of the cells expressed GFP which suggests that transfection efficiency was 50% (Figure 3.4 A). Therefore, I decided to use this ratio for transfection of differentiated cell.

Table 3.1 The effect of different conditions on transfection efficiency. Transfection efficiency was determined by the percentage of GFP positive cells.

	Lipofectamine	GFP	Transfection mix incubation time	Transfection efficiency
	0,8	0,35	6 h	40-50%
	0,8	0,35	24 h	5%
	0,8	0,25	6 h	40%
	0,8	0,25	24 h	10%
	0,8	1,6	6h	20%
	0,8	1,6	24h	5%

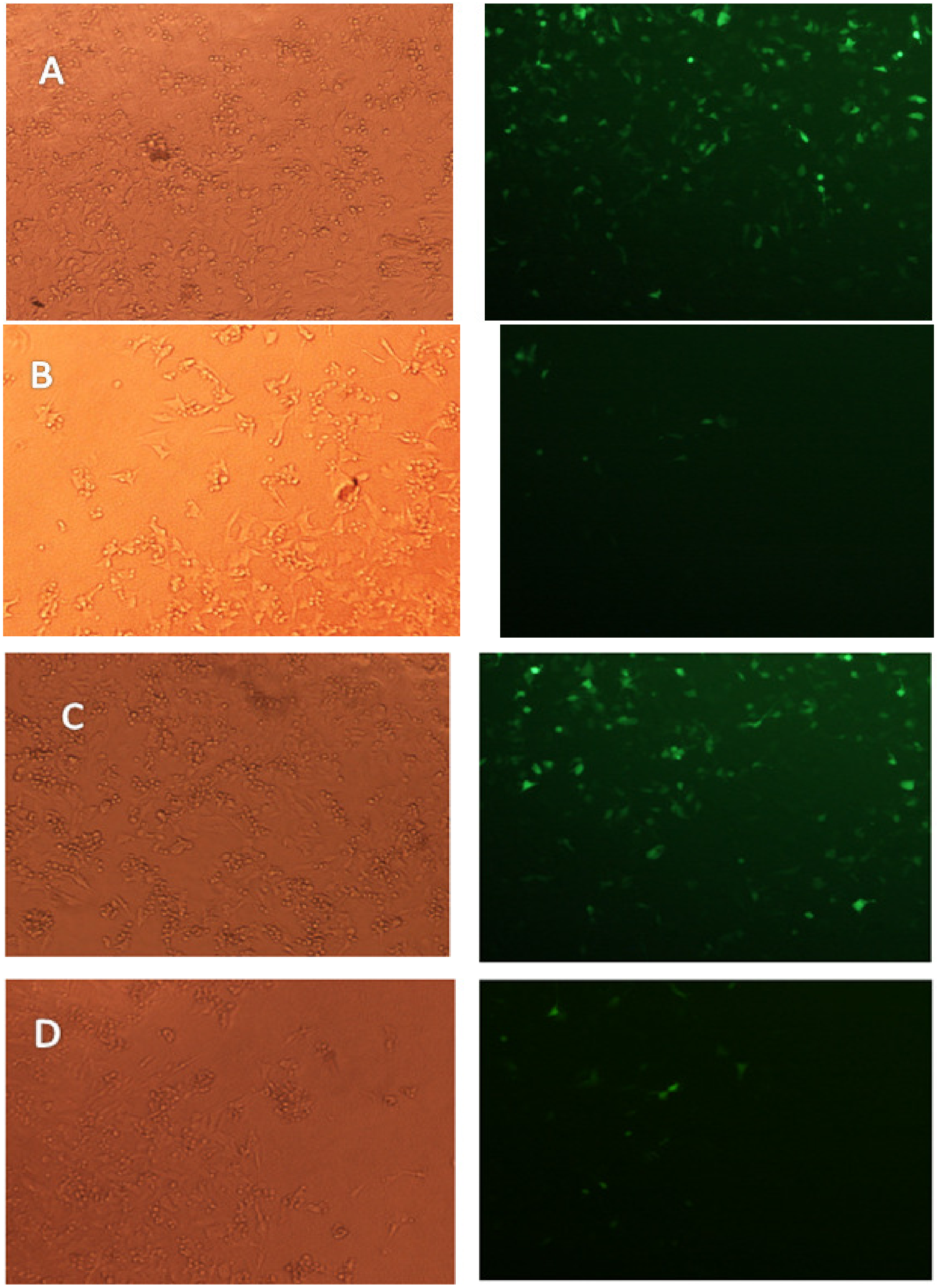


Figure 3.4 GFP Transfected cell for transfection optimization. Undifferentiated SH-SY5Y cells were incubated with 0.8 ul Lipo2000 + 0, 35 ug/ul gfp construct for **A)** 6 or B) 24 hours, with 0, 8 ul Lipo2000 + 0, 25 ug/ul gfp construct for C) 6 or D) 24 hours.

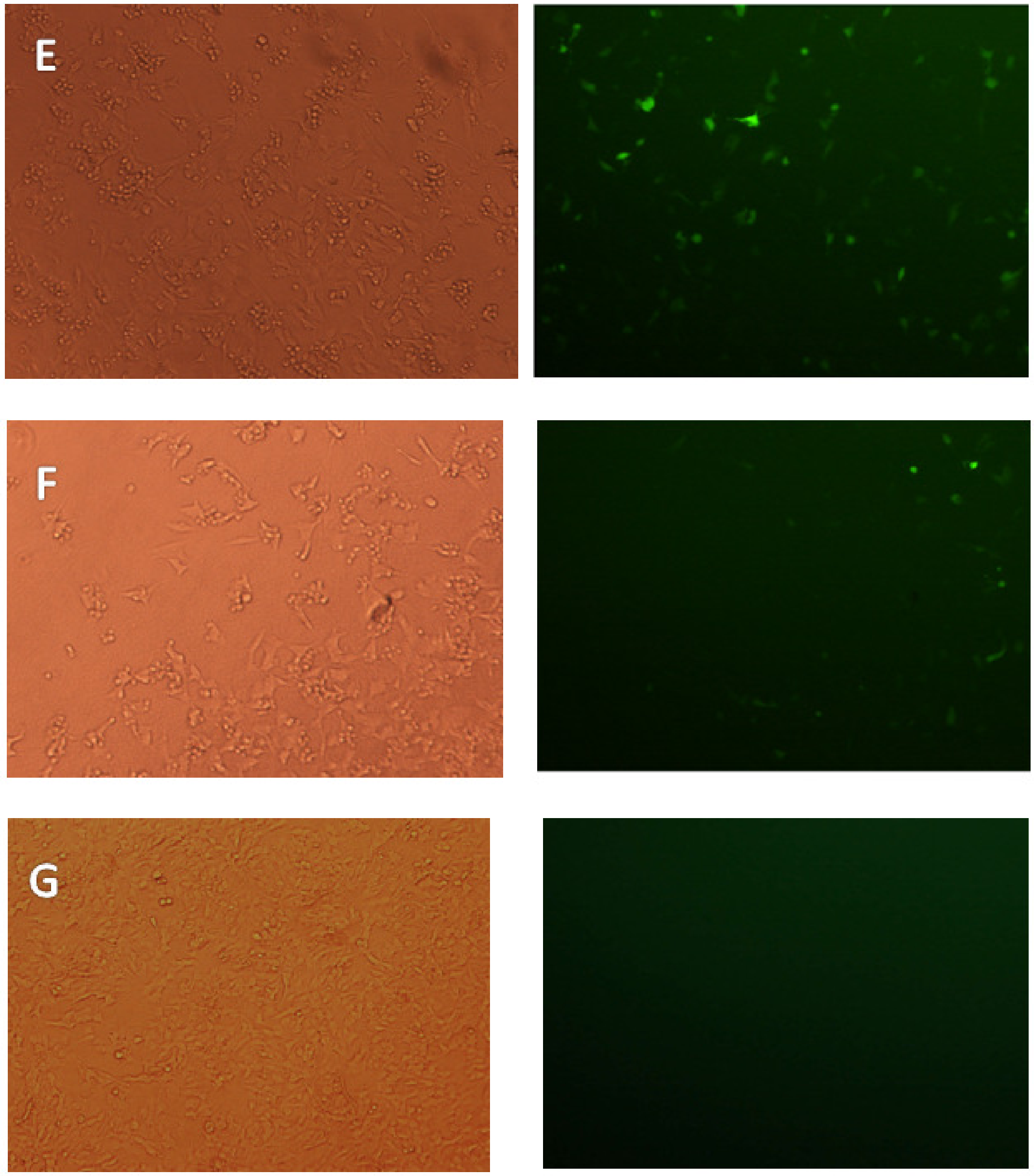


Figure 3.4 (continued) GFP Transfected cell for transfection optimization. Undifferentiated SH-SY5Y cells were incubated with 0,8 ul Lipo2000 + 1,6 ug/ul gfp construct for E)6 or F) 24 hours G) Cells that are not transfected. After 48 hours cells were observed. Left panel is light microscopy and right is florescence microscopy image of the same point.

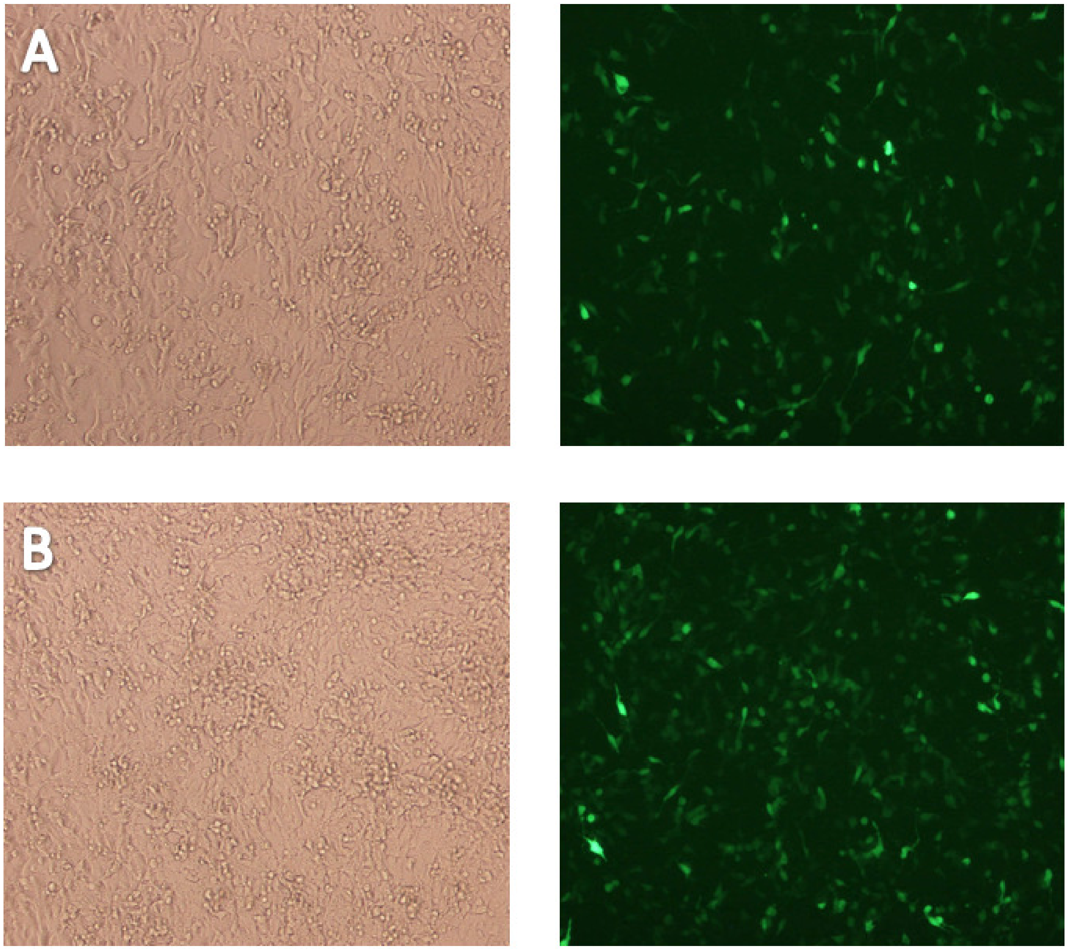


Figure 3.5 Transfection of serum starved cell. Left panel is light microscopy and right is flouresence microscopy image of the same point.

According to Wallenstine et.al., research serum starvation increase the transfection efficiency in differentiating embriyonic stem cell. (Wallenstine et.al., 2010). To increase the transfetion efficiency for SH-SY5Y cells, I tried transfection of serum starve cells. Undifferentiated SH-SY5Y cell was incubated in media lacking serum for 72 hours (Figure 3.5). As a control undifferentiated SH-SY5Y cells in normal media were used (Figure 3.5B). When serum starved cells and control cells were transfected, I observed that serum starve cell transfection efficiency is better than control cells (Figure 3. 5).

After optimizing transfetion, I checked whether my dominant negative mutant constructs can inhibit targeted cdk activities. For this purpose, I transfected undifferentied proliferating SH-SY5Y cells with cdk2dn and cdk4dn constructs to check whether the cell cycle will be inhibited. Cells were seeded as a triplicate in 96 well microplate. After the cell transfection, replicating cell was quantified by BrdU incorporation and viable cells were quantified by WST1 assay. As a control, the cells which were transfected with PCMV (empty vector) and cells which were not transfected were used. I used the optimized ratio which was lipofectamine 0.8 ul and DNA 0.35 ug for undifferentiated SH-SY5Y cells. After 48 hours BrdU and WST1 assays were performed. BrDU result was normalized based on WST1 values to assay the replicating cells in viable cell population. As a result, cdkdn and cdk2dn transfection decreased the replicating cells expected and this shows that my constructs worked very well (Figure 3.6).

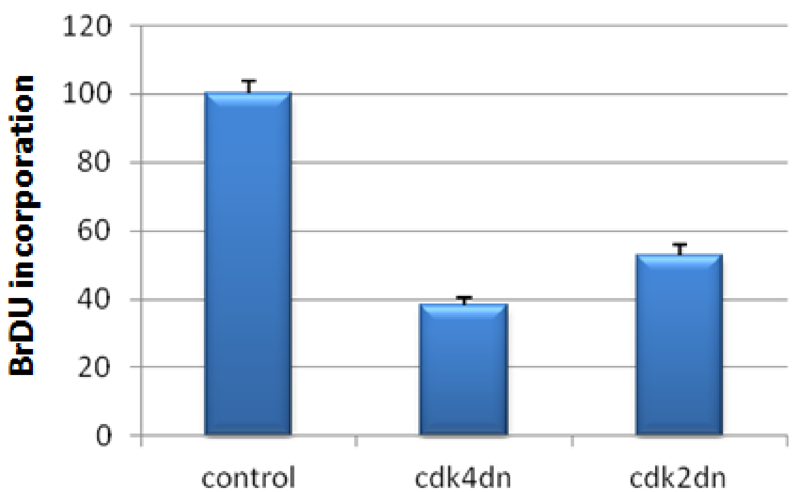


Figure 3.6 Effect cdk4dn and cdk2 dn transfection on undifferentiated proliferating SH-SY5Y cells. Brdu results were normalized to viable cell number based on WST1 results. Control was taken as 100. Standart deviations of three wells were calculated.

3.2.2 Optimization of Transfection Protocol for Differentiated SH-SY5Y Cells

Previous experiments show that the best amount of DNA and lipofectamine are subsequently 0.35µg-0.8ul, and the best transfection incubation time is 6 hours for SH-SY5Y cells. Terminally differentiated SH-SY5Y cells which were differentiated for 9 day were transfected with this ratio for 6 hours. After the transfection, media was changed with differentiation media. After 48 h, cells were observed under florescence microscopy. I couldn't detect any green cells which suggests that 9 days differentiated cells were not transfected (Figure 3.7).

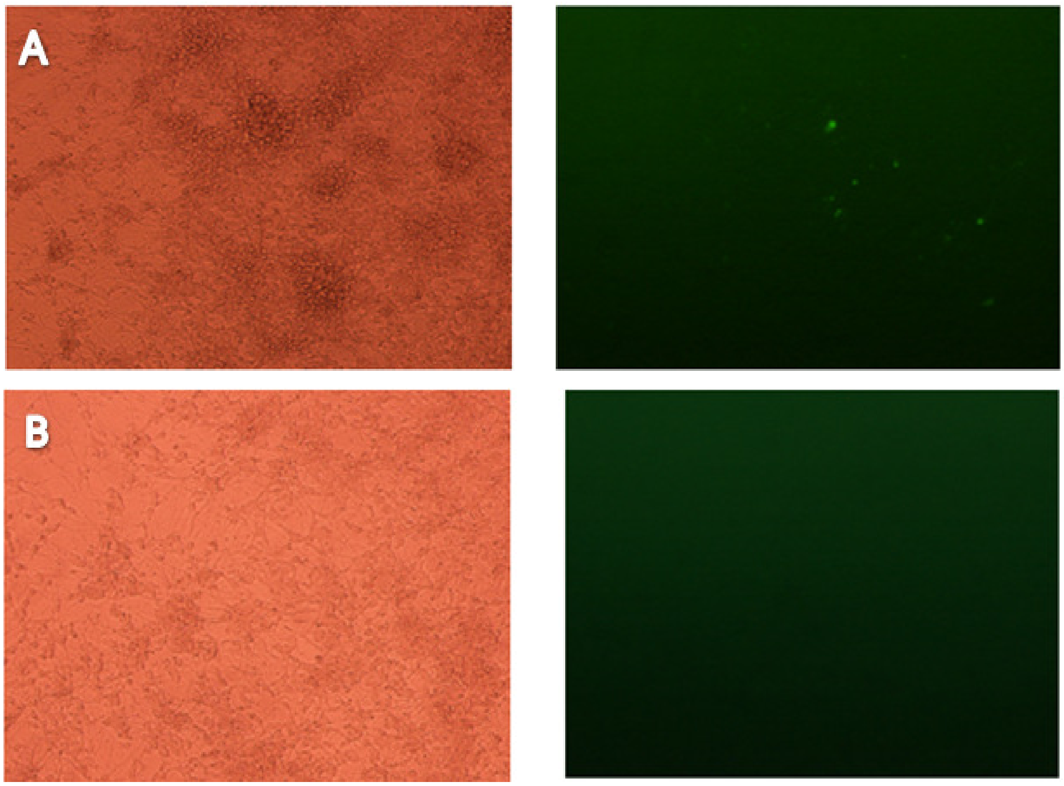


Figure 3.7 Transfection of SH-SY5Y cells which were differentiated for 9 days Differentiated cells (for 9 days) were transfected with **A)** 0.8 µl Lipo2000+ 0.35µg GFP constructs **B)** 0.8 µl Lipo2000+ 0.35µg pcmv. Left panel is light microscopy and right is florescence microscopy image of the same point.

According to the literature survey differentiated cells were resistant to plasmid transfection. Then I worked on optimization of the differentiation time. So next, SH-SY5Y cells have been differentiated for 7 days, were incubated with 0.8 µl Lipo2000 + 0, 35 µg/µl GFP construct for 6 hours. 48 hours later, cells were observed under florescence microscopy. I observed around 5% green cells which was better result than previous experiment with 9 days differentiated cells.

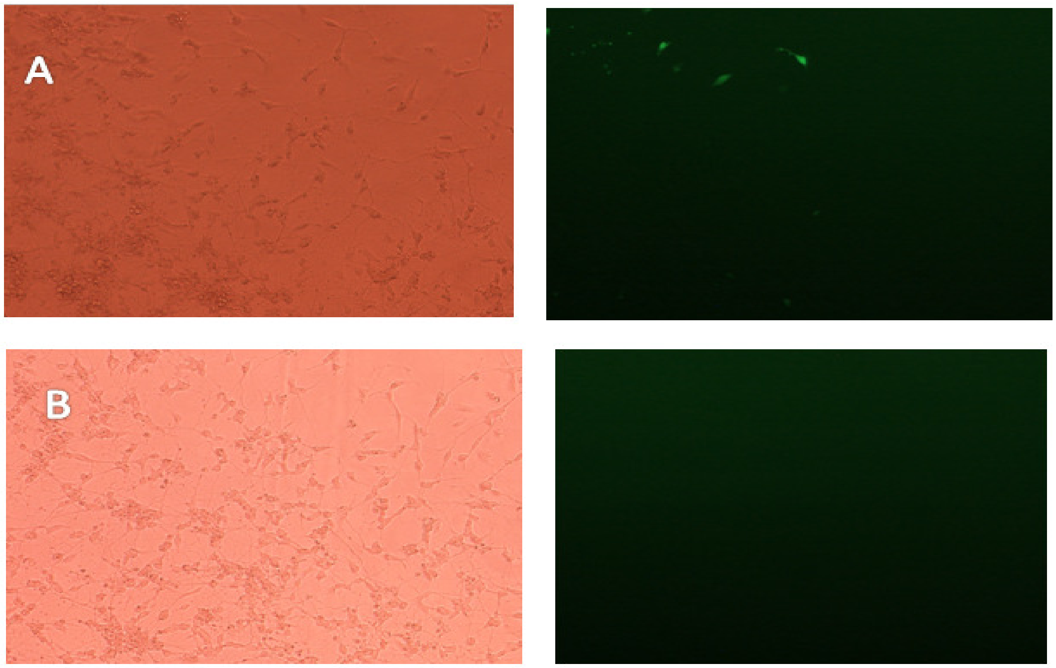


Figure 3.8 Transfection of SH-SY5Y cells which was differentiated for 7days**.** Differentiated cells (differentiate 7) were transfected. Differentiated cells were incubated with A) 0.8 ul Lipo2000+ 0.35 µg PCMV (vector) or B) Vector as negative control. After 48 hours cells were observed. Left panel is light and right is florescence microscopy image of the same point.

In order to augment the efficiency of transfection with 7 days differentiated. Different DNA:lipofectamine ratios were tried. (Table 3.1)

Table 3.2

DNA lipofectamine amounts tested for transfection of 7 days differentiated cells.

	A	**B**	**C**	**D**	**E**	**F**
GFP construct	**0,2**	**0,3**	**0,35**	**0,25**	**0,25**	**0,25**
Lipofectamine 2000	**0,8**	**0,8**	**0,8**	**0,5**	**0,8**	**1,2**

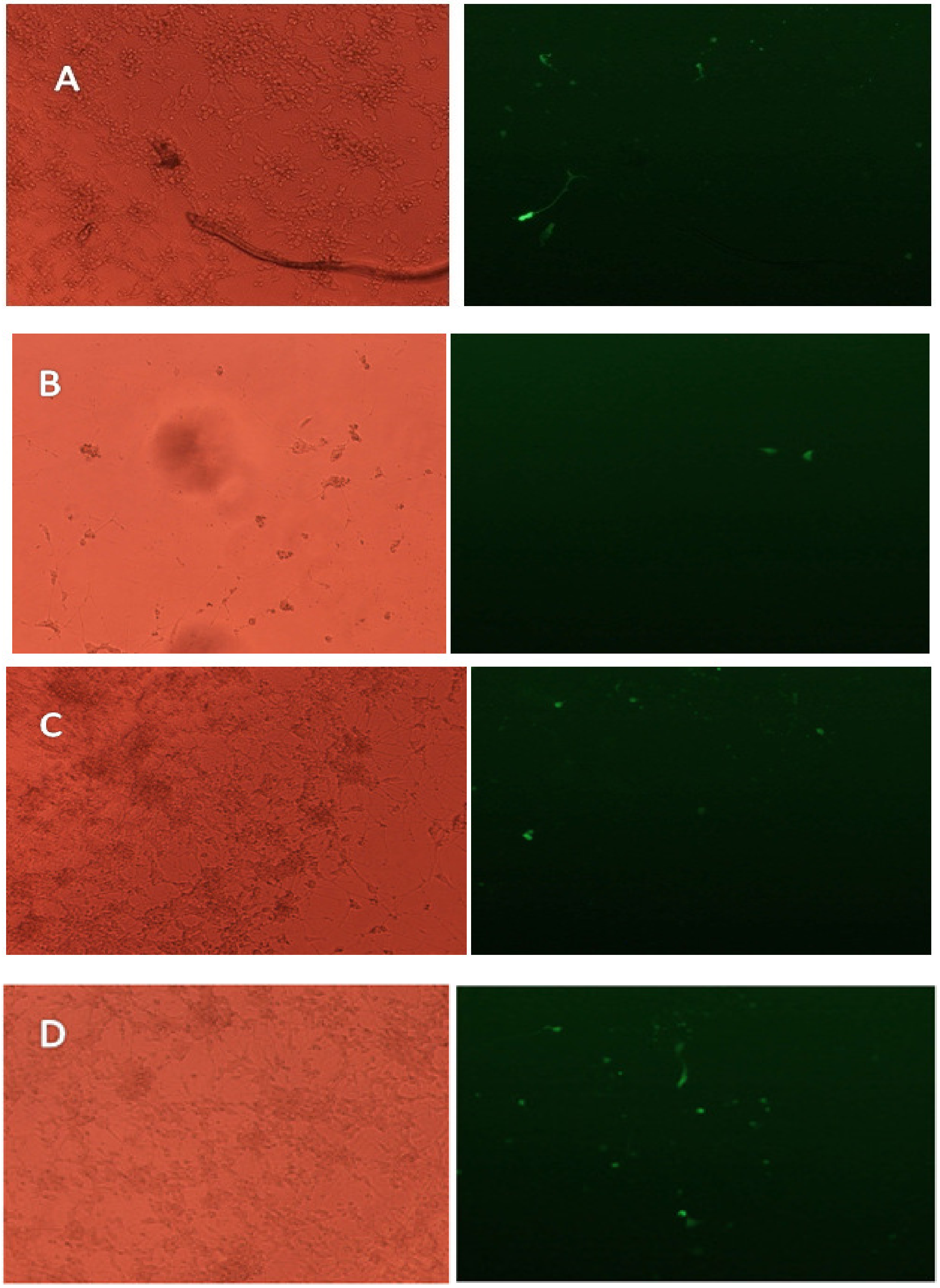


Figure 3.9 Optimization of Transfection of 7 days differentiated cell**.** Differentiated SH-SY5Y cells were incubated with A) 0.8 ul Lipo2000 + 0, 2 ug gfp construct B) 0.8 ul Lipo2000 + 0, 3 ug gfp construct for the 6 hours C) 0, 8 ul Lipo2000 + 0, 35 ug gfp construct D) 0, 5 ul Lipo2000 + 0,25 ug/ul gfp construct.

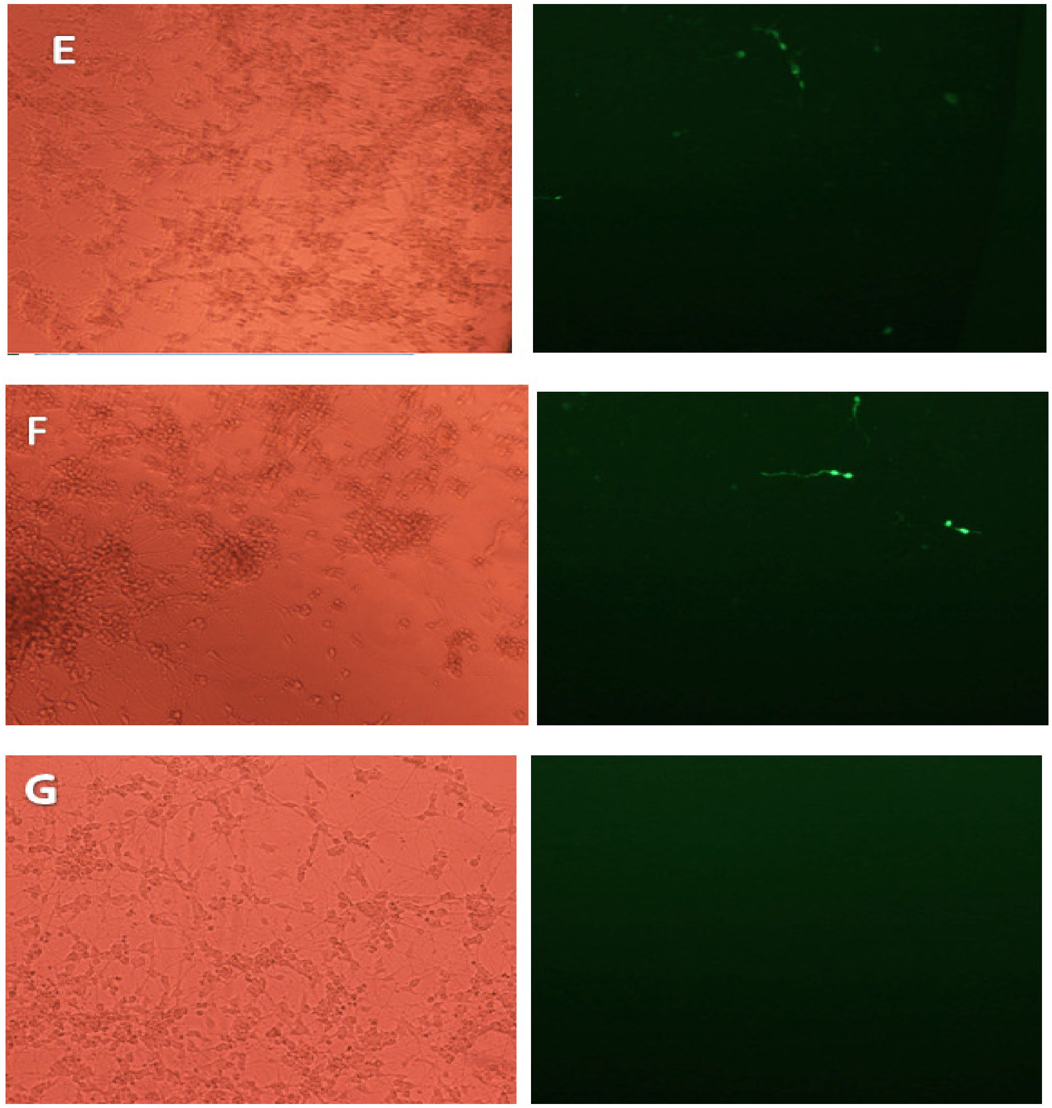


Figure 3.9 Optimization of Transfection of 7 days differentiated cell**.** Differentiated SH-SY5Y cells were incubated with E) 0, 8 ul Lipo2000 + 0,25 ug/ul gfp construct **F**) 1,2 ul Lipo2000 + 0,25 ug/ul gfp construct for 6 hours (G) no transfect. After 48 h, transfected cells were observed.

According to results, 0.25 DNA +0.5 lipo and 0.35 DNA+ 0.8 ul lipo transfection efficiency is better than the other ratios for 7days differentiated cells. Around 5-8 % of cells appeared green and this suggests that transfection efficiency was around 5-8%. Different ratios did not significantly change. 7 days differentiated cells were transfected than 9 days transfected cells but the transfection efficiency was still very low.

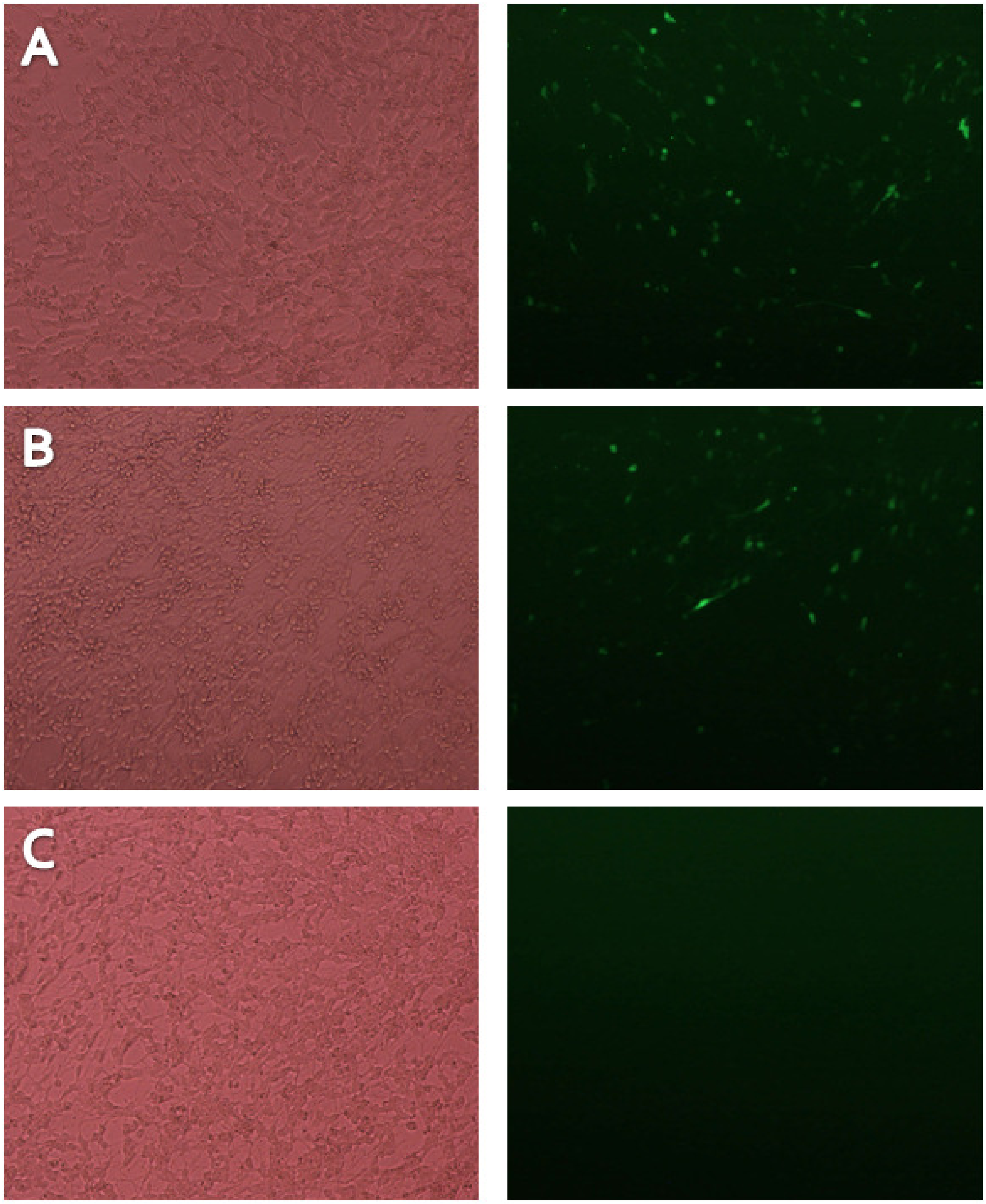


Figure 3.10 The cells were differentiated for 72 hours and then incubated with A) 0.35 DNA+ 0.8 ul lipo with B) 0.25 DNA+ 0.5 ul lipo C) as a control, cell transfected with empty vector PCMV. After the transfection cells were differentiated for two more days. Left panel is light and right panel is florescence microscopy image of the same point.

According to a studies and my results, maximal neurite length is attained in 4-5 days, so it was thought that cells differentiate in 4-5 days at retinoic acid induced protocol (Figure 3.1C) (reviewed by Chu CT, 2009). We focused on differentiation time, and SH-SY5Y cells differentiated for 3 days were transfected with 0.25 DNA +0.5 lipo or 0.35 DNA+ 0.8 ul lipofectamine. After transfection cells differentiated two more days and then GFP transfected cells were observed under florescence microscopy.

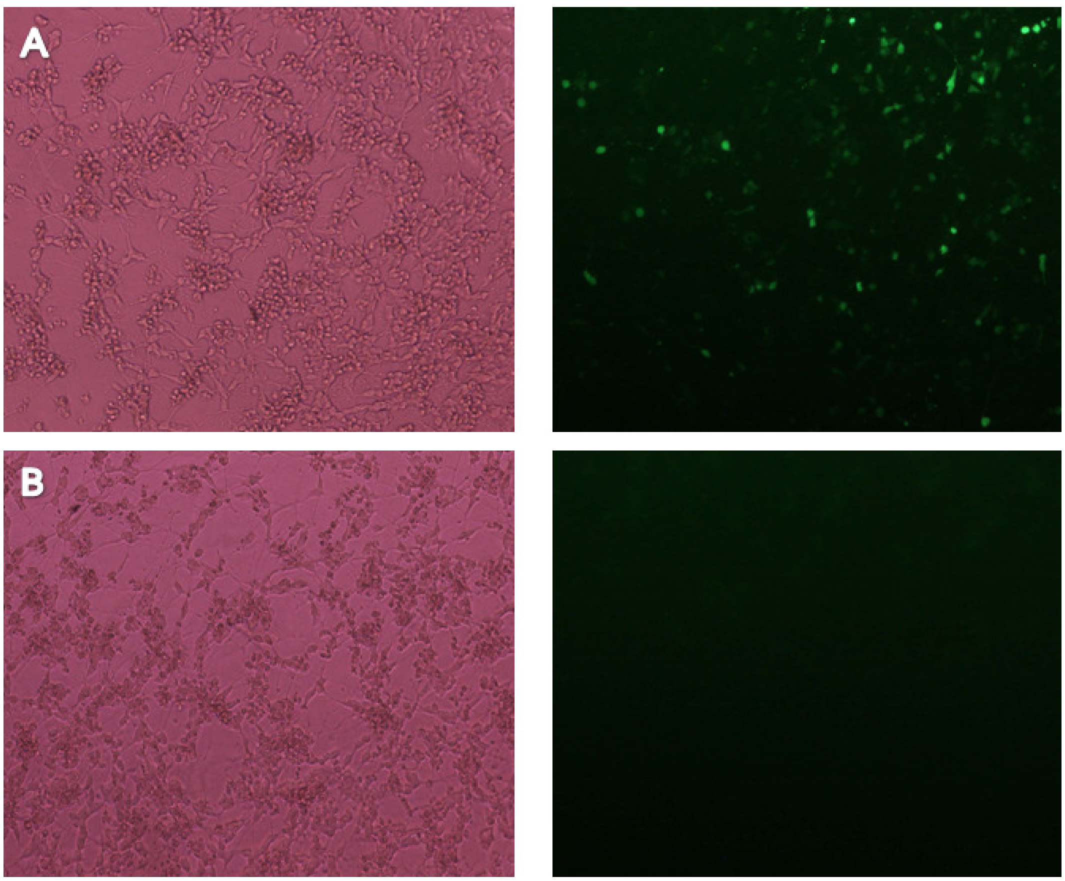


Figure 3.11 Double transfection of SH-SY5Ycells. Left panel is light microscopy image and right is florescence microscope image of the same area. Green cell population shows GFP transfected cell.

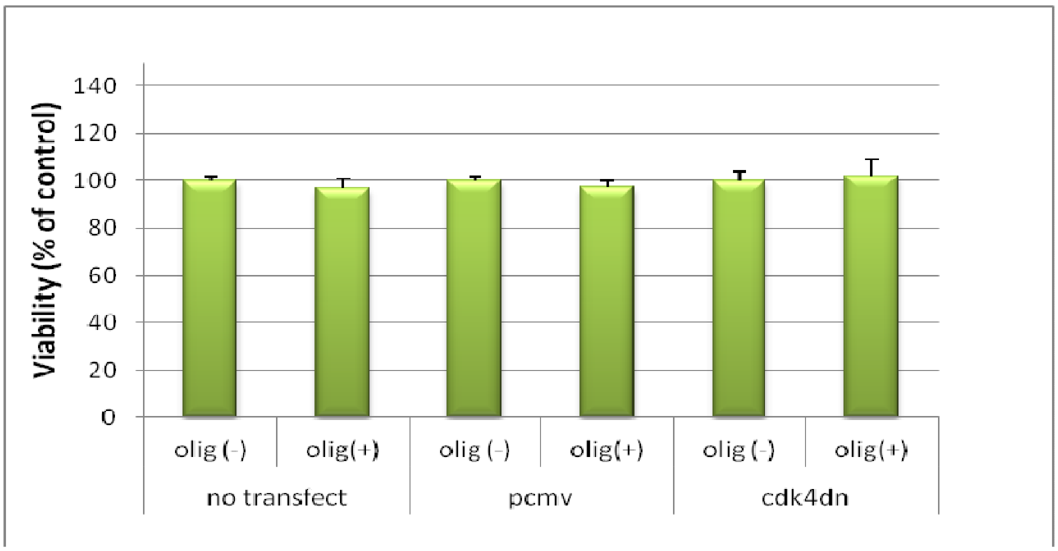
Comparing light and florescence images of the same area in Figure 3.10, 20-30% of cells appeared green and this suggests that transfection efficiency was 20-30%. The transfection ratio in the cells that were transfected with 0.35µg/µl DNA+ 0.8 µl lipo was better than the cells that were transfected with 0.25µg/µl DNA+ 0.5 µl lipo. It was concluded that the amount of 0.35 DNA+ 0.8 µl lipo was the best ratio for SH-SY5Y cell transfection. Even though I achieved to transfect the differentiated cells, the transfection ratio was not enough to detect the role of protein. Next I developed and performed double transfection protocol. It is known that undifferentiated SH-SY5Y cells are transfected with 50% ratio and SH-SY5Y cells which are differentiated for 3 days are transfecetd with 30% ratio. According to our strategy, 3 days after undifferentiated cells were transfected, the cells would be re-transfected, for this time the cells would be differentiated with RA treatment (see method 2.2.1.2). At the end of 5 days, double transfection lead to peak of protein expression in the cells (Figure 3.10). In order to test double transfection method,undifferentiated SH-SY5Y cells were culture in triplicate in 96-well microplate that would be at 95% confluency in the next day. The cells were transfected with GFP constructs for 5-6 hours. Cells were differentiated for 72 hours subsequently the cells were re-transfected with same ratio for 5-6 hours. 48 hours after the transfection cells were re-continued differentiation for 48 hours. After the transfection the cells were observed under florescence microscopy. After 48 hours image under microscope was taken. According to these observations, 50-60 % of cells seem green and this qualitative result suggests that transfection efficiency reached till 50-60%. As shown in Figure 3.12, I achieved the desired transfection ratio for differentiated SH-SY5Y cell line.

3.3 INVESTIGATING THE ROLE OF G1 AND S CDKS in AMYLOID-β OLIGOMER INDUCED NEUROTOXICITY

3.3.1 Investigating the Role of G1 Cdk, Cdk4, in Amyloid-β Oligomer Treatment

In order to reveal the role of G1 cdk, cdk4, in amyloid-β oligomer induced cell cycle, SH-SY5Y cells were transfected with cdk4dn construct as mentioned in section 3.2. As a control I used the cells which were transfected with PCMV (no insert) and cells which were not treated with transfection reagent. At the end of the transfection and differentiation protocol, Aβ oligomer was prepared freshly (mentioned in section 2.2.4and cells were exposed to oligomers for 24 hours. As negative control for oligomer treatment, cells were exposed to oligomer carrying vehicle which is 1:1 DMSO:F12 mixture. According to BrDU assay results, I have found that amyloid-β oligomer treatment triggered DNA replication in vector or untransfected cells but not in Cdk4dn transfected cells. This finding indicates that Amyloid-β oligomer induced DNA replication requires Cdk4 activity.

A



B

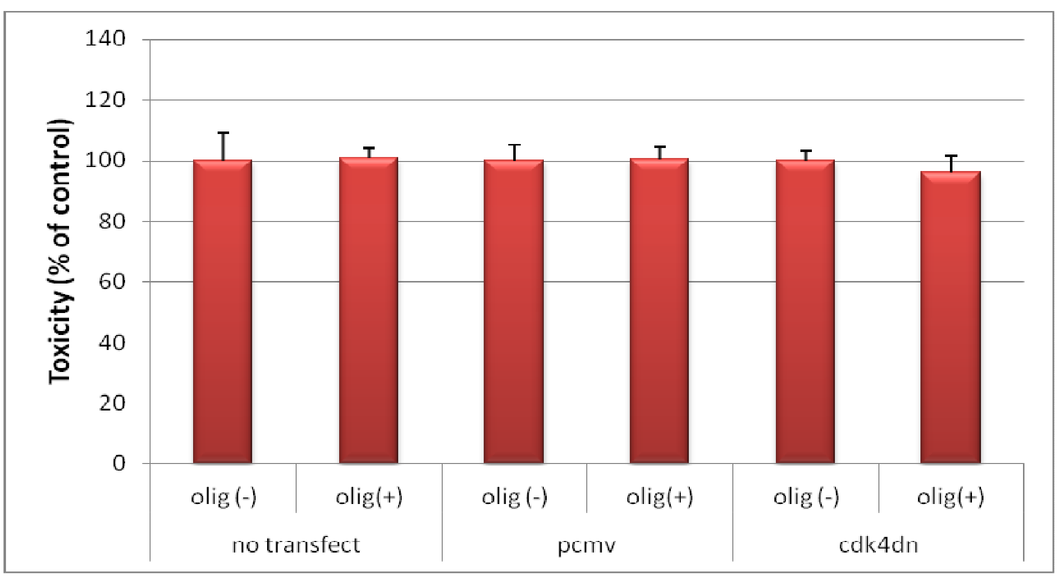


Figure 3.12 The role of cdk4 in oligomer induced cell cycle. SH-SY5Y cells were differentiated and transfected as metinoed above. Viable cell population was determined by A) WST1 viable cell assay. B) LDH cytotoxicity assay.

C

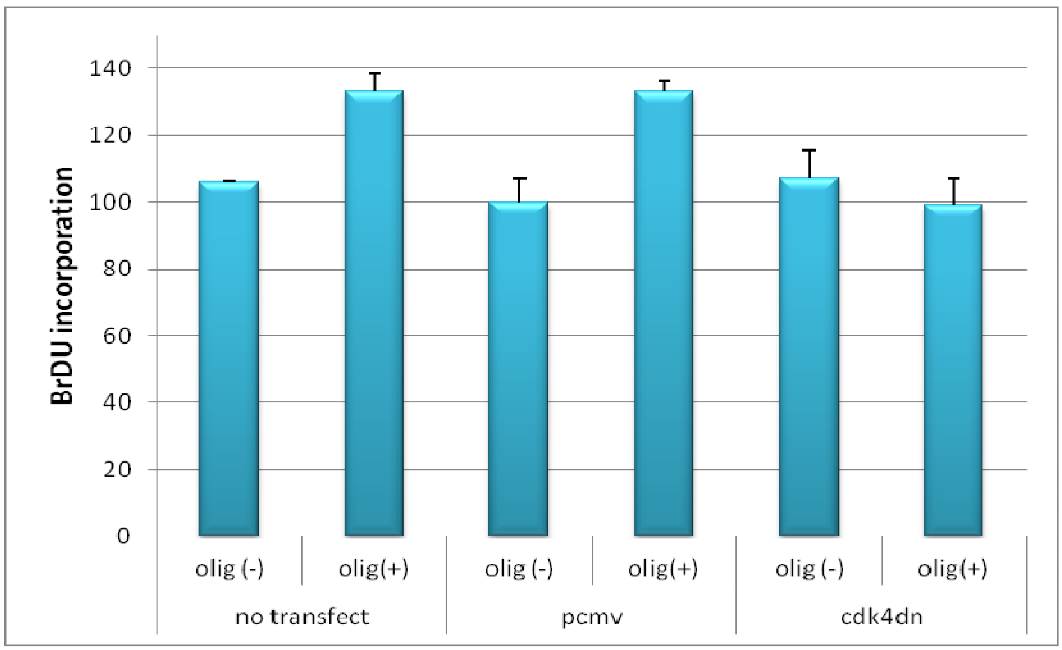
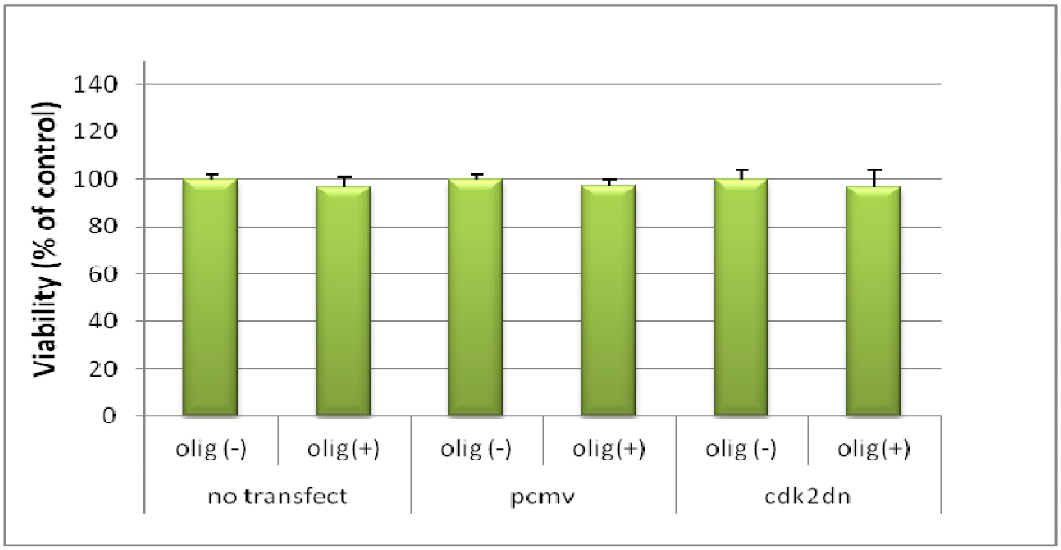


Figure 3.12 (continued) The role of cdk4 in oligomer induced cell cycle.. C) BrdU incorporation values normalized to livin cell number. Values before oligomer addition were taken as hundered. Experiments were done in triplicate and standart deviation was indicated.

3.4 Investigating the Role of Cdk2 in Amyloid-β Oligomer Treatment

Next, in order to reveal the role of cdk2 in amyloid-β oligomer induced cell cycle, I repeated the oligomer treatment experiment with Cdk2 dominant negative construct transfected cells.

A



B

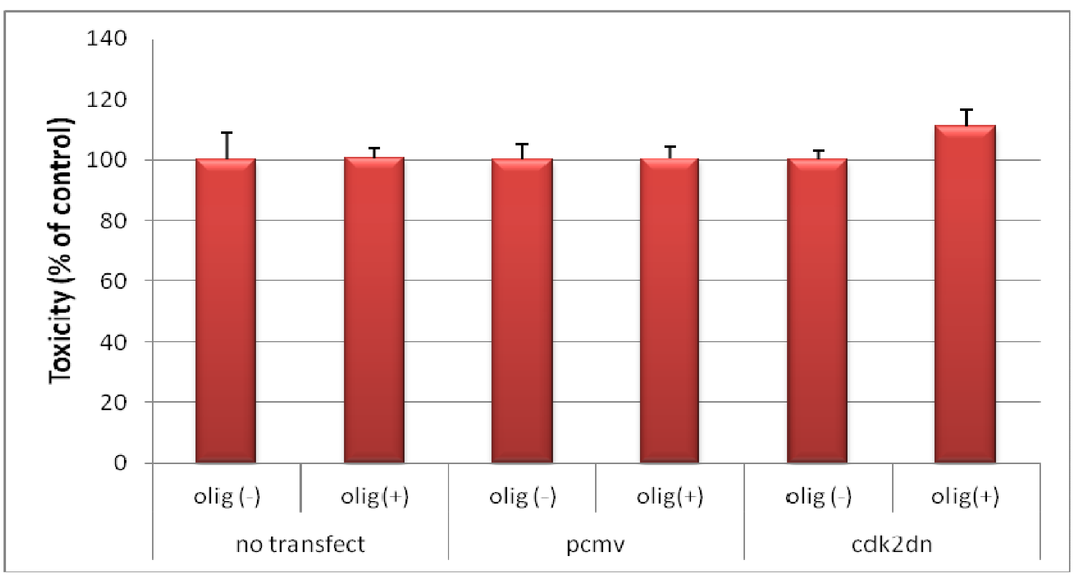


Figure 3.13 The role of Cdk2 and Cdk4 in oligomer induced cell cycle. A) WST1 viable cell assay B) LDH cytotoxicity assay

C

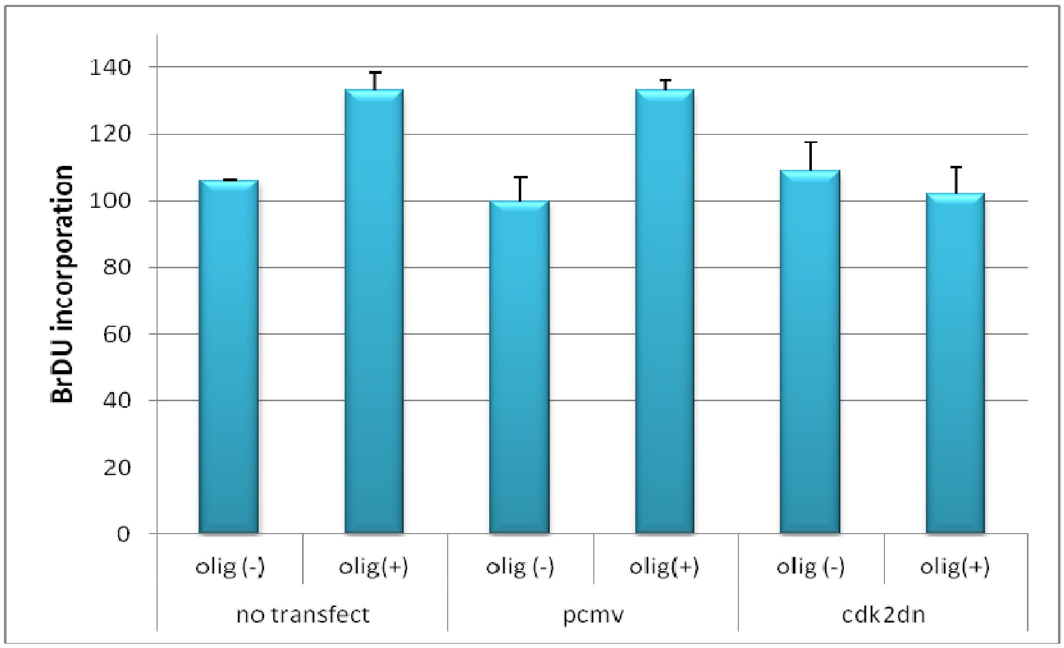


Figure 3.13 (continued) The role of Cdk2 and Cdk4 in oligomer induced cell cycle. C) BrdU incorporation values normalized to living cell number. Values before oligomer addition were taken as hundered. Experiments were done in triplicate and standart deviation was indicated.

In untransfected and vector transfected cells, there is no significant change in viable cell population and toxicity (Figure 3.13). DNA replication is increased in response to oligomer treatment didn't induce DNA replication in cdk2dn transfected cells (Figure 3.13 A-B). This result suggest that cdk2 has a role in oligomer induced cell cycle (Figure 3.13)

CHAPTER 4

DISCUSSION AND CONCLUSION

4.1 AMYLOID-β_{42} OLIGOMER INDUCED THE CELL CYCLE RE-ENTRY IN DIFFERENTIATED SH-SY5Y CELLS

Alzheimer disease (AD) is a neurodegenerative brain disorder which is also a kind of dementia marked by progressive loss of memory and impairment of cognitive ability. Alzheimer disease cause neuropathological lesions including amyloid plaques, neurofibrillary tangles, and eventual neuronal loss in brain regions associated with cognitive function (Cotman and Pike, 1994). Amyloid-β is the major component of senile plaques which are characteristic of AD and is derived by the proteolytic processing of its precursor cell surface protein, amyloid precursor protein (APP). The production of the Amyloid-β, a mainly 40- to 42-amino acid peptide, requires the action of β- and γ-secretases which process APP at its amino and carboxyl-terminus (Zheng and Koo, 2006). The amyloid-β_{40} form is the more common of the two, but amyloid-β_{42} is the more fibrollogenic and is believed to be more toxic than amyloid-β_{40}. Amyloid peptides aggregate into amyloid fibrils and plaques which are characteristics of Alzheimer disesase. The fibrils are large insoluble polymers, which are found in senile plaques. For a long time, plaques have been considered as the major cause of Alzheimer neouropathology. More recent studies have focused on the toxic effects of lower molecular weight oligomeric forms of Amyloid-β that may be more toxic than larger fibrillar aggregates (Deshpande et al., 2006). Recent studies showed that non-fibrillar soluble oligomers which are also called Amyloid-derived diffusible ligands, “ADDLs”, kills mature neurons in organotopic cultures at nanomolar concentrations (Klein, 2002 and Hsu et al., 2009).

Amyloid beta oligomers are mitogenic in vitro ((Pyo et al., 1998 and McDonald et al., 1998) and it is thought that they induce neurotoxicity by triggering cell cycle.

In this study I have investigated the pathway that mediates Amyloid-β induced cell cycle re-entry. As a cell line, I have used the SH-SY5Y cells which are also commonly used as an *in vitro* model in neurodegenerative studies (Chang et al., 2002; Xue et al., 2006; Zheng et al., 2006). SH-SY5Y cell line is thought to be a convenient *in vitro* cell line model for neuroscience (reviewed by Xie, 2010). When SH-SY5Y cell line is induced with the retionic acid and the other agents, SH-SY5Y cell line starts to differentiate and neuronal markers are displayed in differentiated SH-SY5Y cells. As a result, extension of out-neurite differentiated SH-SY5Y cells exhibit the primary neuron morphology and decrease the cell cycle like a primary neuron. I differentiated the cells with 10 µM retinoic acid (RA) and 1% N2 supplement in DMEM without FBS (Lambert et al., 1994) and neuron morphology was observed (Figure 3.1 and Figure 3.2).

When I tested the effect of the amyloid-β_{42} oligomer structure on differentiated SH-SY5Y cells, oligomers induced DNA replication which was assessed by BRdU incorporation assay (Figure 3.12 and Figure 3.13). Although the replicating cell number increased, WST1 assay showed that the viable cell number didn't increase significantly (Figure 3.12). Moreover, based on LDH results there was no increase in cell death in response to oligomers. Based on these findings I have concluded that amlyoid-β oligomers induced the cell cycle reentry but can not complete the cell cycle and divide (Figure 3.12 and Figure 3.13).

According to cumulative knowledge, neuron cells are incapable of re-entering the cell cycle. Aberrant cell cycle re-entry of neurons is deleterious because they do not divide in normal conditions in the brain. It is shown that when the cell cycle is triggered aberrantly, neuronal death is induced by apoptotic pathway (Meikrantz, 1999; Wang, 2009). However, according to LDH result there is no significant change on level of viable cell and cell cytotoxicity of amyloid-β_{42} oligomer induced differentiated SH-

SY5Y cells for 24 hours. It is possible with differentiated SHSY5Y cells, longer incubation time is needed for oligomers to kill the cells. .

4.2. CDK4 HAS A ROLE IN OLIGOMER INDUCED CELL CYCLE

The cell cycle is a highly regulated process by numerous checks and balances that ensure its proper completion. There are four phases, S phase, where DNA replication and chromosome duplication occurs; M-phase, where mitosis and cytokinesis occurs; and the gap phases that separate the two; G1 and G2. The sequential expression and activation of cyclin and cyclin dependent kinases (Cdk) complexes are the main regulators of cell cycle progression. They regulate the transition from one phase to another. Cdk4/6-cyclinD complexes control the re-entry from the resting (Go) cells to the G1 phase and Cdk2 / Cyclin E complexes are responsible for transition from G1 to S (Sher 1995).

In my thesis I am interested in the pathway that is used for oligomer induced cell cycle re-entry. Cdk4 and Cdk2 are core proteins for cell cycle pathway. If cell cycle is triggered, G1 cdks are activated by their cyclin partner and initiate the cell cycle progress. It was found that Cdk4-6 has a role Amyloid beta fibril induced neuronal death (Giovanni et al., 1999). It was also found that cdk4 mRNA level in AD brain is higher than controls (Tsujioka, 1999).

In addition to these, some studies suggest that cell cycle re-entry in AD is mediated by Cdk5. When the amyloid-β_{40} peptide is injected to rat brain, cdk4 protein level does not change but localization of cdk4 is changed depending on cdk5 (Lopes J P, 2010, 2009). Moreover, it was demonstrated that Cdk5 inhibit HDAC 1, histone deacetylase 1, by phosphorylation and cause increased expression of cell cycle proteins (Kim D et al., 2008). The other study claims that Cdk5 phosphorylates Rb and trigger neuronal cell cycle re-entry and death (Fututsagi, 2012).

In this study I have investigated the role of Cdk4 in oligomer induced cell cycle. . For this purpose, I have inhibited Cdk4 activity by transfecting cells with Cdk4 dominant negative construct and checked whether this will prevent amyloid-β oligomer induced cell cycle reentry .

As a result cdk4dn transfection of differentiated SH-SY5Y cells prevented amyloid-β oligomer induced DNA replication and I concluded that amyloid-β oligomer induced cell cycle reentry requires Cdk4 activity.

4.3. CDK2 HAS A ROLE IN OLIGOMER INDUCED CELL CYCLE

In mammalian cell cycle, Cdk2 is required for G1/S transition and triggers DNA replication initiation. Cdk2- cyclin complexes provide an accurate DNA replication in S phase (Bell and Dutta, 2002). But it was found that cdk2 knock out mice are viable (Berthet, 2003) so cdk2 is not essential for S Phase. In this survey, whether cdk2 has a role in oligomer induced cell cycle is investigated. Results showed oligomer treatment trigger DNA replication in control cells, whereas in dominant negative cdk2 transfected cells DNA replication is not induced in response to amyloid-β oligomers (Figure 3.13C). Level of viable cell and toxicity were not changed (Figure 3.13 A- B). Based on these results I have concluded that amyloid-β induced DNA replication and S phase entry is mediated by Cdk2 activity.

As conclusion, our findings indicate that similar to classic pathway, amyloid-β oligomer induced cell cycle re-entry and DNA replication is mediated by Cdk4 and Cdk2. It is known that cell cycle re-entry is deleterious for neuron cell so our findings suggest that cdk4 and cdk2 have a role in oligomer induced neurotoxicity.

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