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ASSOCIATION OF ALZHEIMER DISEASE WITH DNA TOPOISOMERASE IIβ IN PRIMARY NEURONAL

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ASSOCIATION OF ALZHEIMER DISEASE WITH DNA TOPOISOMERASE IIβ IN PRIMARY NEURONAL CELLS

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APPROVAL PAGE

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Şule TERZİOĞLU

M.S. Thesis – Biology
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ABSTRACT

In mammalian cells, DNA topoisomerase IIβ (topo IIβ) plays an important role in the initiation of selective gene transcription, neuronal differentiation and axonogenesis. Inhibition of topo IIβ activity both *in vivo* and *in vitro* results in shorter axon length and increase of DNA damage by suppressing the transcriptional induction of differentiation-related genes. However, in which pathways of axonogenesis controlled by topo IIβ has not been clarified yet. Additionally, the observed symptoms of Alzheimer`s disease such as axon shortening and increase in DNA damage give rise to thoughts about the abnormalities in the expression of topo IIβ or aberration of the enzyme in different levels. However, there is no outcome related to the effect of topo IIβ in the course of Alzheimer`s Disease (AD).

In this study, it was studied that whether or not the symptoms of Alzheimer`s disease associates with the function of topo IIβ in rat primary neuronal cell culture. For this purpose, rat cerebellar granule neurons from post-natal 9 days (P9) old rats were firstly isolated and cultured. In order to verify the establishment of the healthy primary neuronal culture, neuronal markers expression and localization were examined via western blot and immunofluorescence techniques, respectively. Then, amyloid beta (Aβ) 1-42 peptide fibrils were synthesized by the incubation of Aβ 1-42 peptide monomers at 37^0C during 24 and 48 hours, separately while shaking. These fibrils were given to primary neuronal culture on the 4^{th} day *in vitro* (DIV). To confirm the success of the establishment of *in vitro,* immunofluorescence and Congo red staining were performed. Moreover, toxicity of Aβ fibrils given to primary neuronal culture on 4^{th} DIV was observed through Real Time Cell Analysis (RTCA) system during a week. At the same time, toxicity of cytosine arabinoside (AraC) was also examined on primary neuronal culture again via RTCA. As a final step, in order to associate the function of

topo IIβ with AD, samples collected from primary neuronal culture were applied to western blot technique upon Aβ fibrils exposure to the cells during a day.

According to the results, primary neuronal culture were established. Healthy and good connections between neurons in the culture was observed morphologically. From the western blot results, Neurofilament (NF) and TAU as neuronal markers were in dominance compared to Glial Fibrillary Acidic Protein (GFAP) which is negative control of the presence of neurons in the culture. The results of immunofluorescence are also consistent with the western blot ones confirming the localization of these neuronal markers presence in the culture. From the RTCA results, the growth curve was obtained and determined that 10000 cells per 96 well plate is enough. Moreover, 10 μM of AraC was an adequate concentration to stop the division of non-neuronal cell division in the culture. To evidence establishment of *in vitro* AD, amyloid beta plaques around neurons were detected both by Aβ 1-42 antibody and Congo red stain. Besides these, the choice for the concentration of Aβ 1-42 fibril as 7 μM might have been not enough to be neurotoxic to cerebellar granule cells. It would be better to prefer 14 or 28 μM and 48 hours exposure of Aβ 1-42 peptides to primary neuronal culture rather than one day exposure.

From this aspect, the prospective contributions of topo IIβ to the diagnosis and treatment of Alzheimer`s disease will be defined.

Keywords: DNA topoisomerase IIβ, Alzheimer`s Disease, Axonogenesis, Amyloid beta 1-42 Peptide, Rat Primary Neuronal Cell Culture, Cerebellar Granule Cells.

ALZHEİMER HASTALIĞI İLE DNA TOPOIZOMERAZ IIβ'NIN PRİMER NÖRON HÜCRELERİNDE İLİŞKİLENDİRİLMESİ

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ÖZ

Memeli hücrelerinde DNA topoizomeraz IIβ'nın (topo IIβ) *in vivo* fonksiyonu hala netlik kazanmamakla beraber son yıllarda, topo IIβ'nın gen ifadesinin tetiklenmesi, başlatılması esnasında önemli bir enzim olduğu rapor edilmektedir. Topo IIβ aktivitesinin hem *in vitro* ve hem de *in vivo* durdurulması, nöronal hücre farklılaşması ile ilgili bazı genleri baskılayarak akson kısalmasına ve DNA hasarında artmaya neden olduğu bilinmektedir. Bu çalışmalar topo IIβ'nın aksonogenezde rol oynadığını ve DNA tamir mekanizmalarında gerekli önemli bir enzim olduğunu göstermektedir. Ancak, topo IIβ'nın akson oluşumunu ya da gelişimini hangi yol ile kontrol ettiği bilinmemektedir. Ayrıca, nörodejeneratif hastalıklardan Alzheimer Hastalığı'nda görülen akson kısalması, DNA hasarının artması gibi birden fazla belirtilerin olması, bir çok geni kontrol edebilen topo IIβ geninin ifadesindeki artma/azalmalar veya enzimle alakalı başka anormallikler sonucu yaşanabileceğini düşündürmektedir. Şu ana kadar yapılan çalışmalarda topo IIβ'nın bu hastalık oluşumu ya da gelişimiyle ilgisine dair bir veri bulunmamaktadır.

Bu çalışmada, Alzheimer Hastalığı'nda görülen problemlerin topo IIβ'ya bağlı gerçekleşebileceği sıçan primer hücre kültüründe araştırmıştır. Bu amaçla, primer serebellar granül hücreler 9 günlük sıçanlardan uygun methodlarla ayrıştırılmış, büyütülmüş ve yaşatılmıştır. Morfolojik olarak sağlıklı olduklarına inandığımız nöral bağlantıların, nöral belirteç ifade düzeylerini western blot ve onların lokalizasyonlarını immunfloresan teknikleri ile teyit ettik. Daha sonra, *in vitro* Alzheimer hastalığı modeli yapmak için amyloid beta 1-42 peptidi 37^0C' de 24 ve 48 saat karıştırıcı eşliğinde fibril hale getirilmiştir. Bu fibriller primer nöron kültürüne hücrelerin dördüncü gününde verilip hücreler bir gece maruz bırakılmıştır. Ertesi gün fibril oluşumunun başarısını

ölçmek adına amyloid plaklar Congo kırmızısı ve amyloid beta 1-42 antikoru ile keşfedilmiştir. Dahası, bu fibrillerin toksisiteleri gerçek zamanlı hücre analizi sistemi (RTCA) (Roche) yardımı ile hücrelerin *in vitro* ortamda ilk günlerinden ve dördüncü günlerinde aldıkları fibriller dahil bir hafta süre boyunca izlenmiştir. Aynı zamanda nöron olmayan hücrelerin büyümesini durduran cytosine arabinoside (AraC) inhibitörünün toksisitesi yine aynı sistem üzerinden bir hafta boyunca takip edilmiştir. Son olarak topo IIβ'nın Alzheimer hastalığı ile ilişkilendirilmesi adına, 24 ve 48 saat 37^0C'de inkübe edilmiş amyloid beta fibrillerinin verildiği ve bir gün süreyle maruz bırakıldığı hücrelerden protein örnekleri toplanıp western blot yöntemi ile topo IIβ'nın ifade seviyesi karşılaştırılmıştır.

Sonuç olarak, başarılı bir primer nöron kültürünün morfolojik olarak izlenmesinin ardından Neurofilament (NF-L) ve Tau gibi nöral belirteçlerin ifade düzeylerinin negatif nöral belirteç Glial Fibrillary Acidic Protein (GFAP)'ye oranla daha yüksek çıkması bizi primer nöron kültürünün standardize edildiğine kanaat ettirdi. Ayrıca RTCA sonuçlarından elde edilen büyüme eğrisi 10000 hücrenin 96 kuyucuk plaklarında yaşamasının ideal olduğunu gösterdi. Başarılı bir primer nöron kültürü eldesinin ardından, *in vitro* Alzheimer hastalığı girişiminde, nöronlar arasında dışarıdan verdiğimiz amyloid beta plaklarının varlığı umut vaad edici sonuçlardı. Bu çalışmanın son ve en önemli bir deneyi olarak yaptığımız western blot teknikleriyle amyloid beta fibrillerine bir gece maruz kalmış primer nöron hücrelerinde topo IIβ'nın ifade seviyesinin 48 saatlik ön inkübe edilmiş amyloid fibrillerin verildiği hücrelerde 24 saatlik olanlara gore daha az ekspres olduğunu gözlemledik. Diğer yandan da Tau proteinin topo IIβ'ya tam ters şekilde ifade profile göstermesi beklediğimiz bir sonuç olarak karşımıza çıktı. Fosforile Tau ifadesiyle Alzheimer modeli oluşturduğumuza bir kanıt daha koyarken topo IIβ'nun buna bağlı azalan ifade düzeyi Alzheimer hastalığı ile topo IIβ'nın nörotoksik bir ilişki içinde olduğu ispatladık.

Anahtar Kelimeler: DNA topoizomeraz IIβ, Alzheimer Hastalığı, Aksonogenez, Amyloid Beta 1-42 Peptidi, Primer Nöron Kültürü, Serebellar Granül Hücreler.

To my family…

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SYMBOL/ABBREVIATION	
Alzheimer`s Disease	AD
APP	Amyloid precursor protein
APS	Ammonium persulfate
AraC	Cytosine β-D-arabinofuranoside hydrochloride
Aβ	Amyloid beta
BM-E	Betamercaptaethanol
cTFα	C terminal fragment of alpha cleaved APP
cTFβ	C terminal fragment of beta cleaved APP
DAPI	4',6-diamidino-2-phenylindole
DIV	Day *in vitro*
DMEM	Dulbecco`s Modified Eagles Medium
EDTA	Ethylenediaminetetraacetic acid
GFAP	Glial fibrillary acidic protein
MAPs	Microtubule associated proteins
NF-L	Neurofilament-L
NFT	Neurofibrillary tangles
PBS	Phosphate Buffer Saline
PHFs	Paired helical fragments
sAPPα	N terminal fragment of alpha cleaved APP
sAPPβ	N terminal fragment of beta cleaved APP
SDS- PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
TBS	Tris Borate Saline
TEMED	Tetramethylethylenediamine
topo IIα	Topoisomerase II alpha
topo IIβ	Topoisomerase II beta
Tris HCl	Tris hydrochloric acid

CHAPTER 1

INTRODUCTION

1.1 ALZHEIMER`S DISEASE

1.1.1 General Information

Alzheimer`s Disease (AD) is an irreversible, progressive brain disease that slowly destroys cognitive functioning that thinking, remembering, and reasoning as well as behavioral abilities. These symptoms make it the most common dementia among older people. The severity of AD depends not only genetic background but also daily lives of patient.

Although it is still not known how AD process begins, lapses of memory and difficulties in finding the right words while speaking are the first signs of early stage of the disease. They mostly derive from toxic changes in AD brains such as abnormal deposition of amyloid fibrils and tau tangles. As a next moderate step of the disease, neurons lose their functions and ability to making networks between each other and eventually they die. Over time, the damage spreads along the hippocampus giving defects to memories. As more neurons die, affected brain start to shrink. At the final stage of severity, brain tissue collapse down notably (see Figure 1.1).

There are two types of AD. It can be in both genetic and sporadic forms. AD is considered familial when more than one person in a family is affected, while sporadic refers to AD cases when no other cases have been seen in close family members. AD is further

divided into early- and late-onset forms; early-onset denotes onset of the disease before age 65 years, while late-onset denotes onset after age 65 years. Almost all cases of sporadic AD are late-onset, while approximately 90% of familial AD is late-onset. Less than 10% of all AD cases are familial early-onset.2,3, but the causal factors for the vast majority of AD cases is still unknown [2].

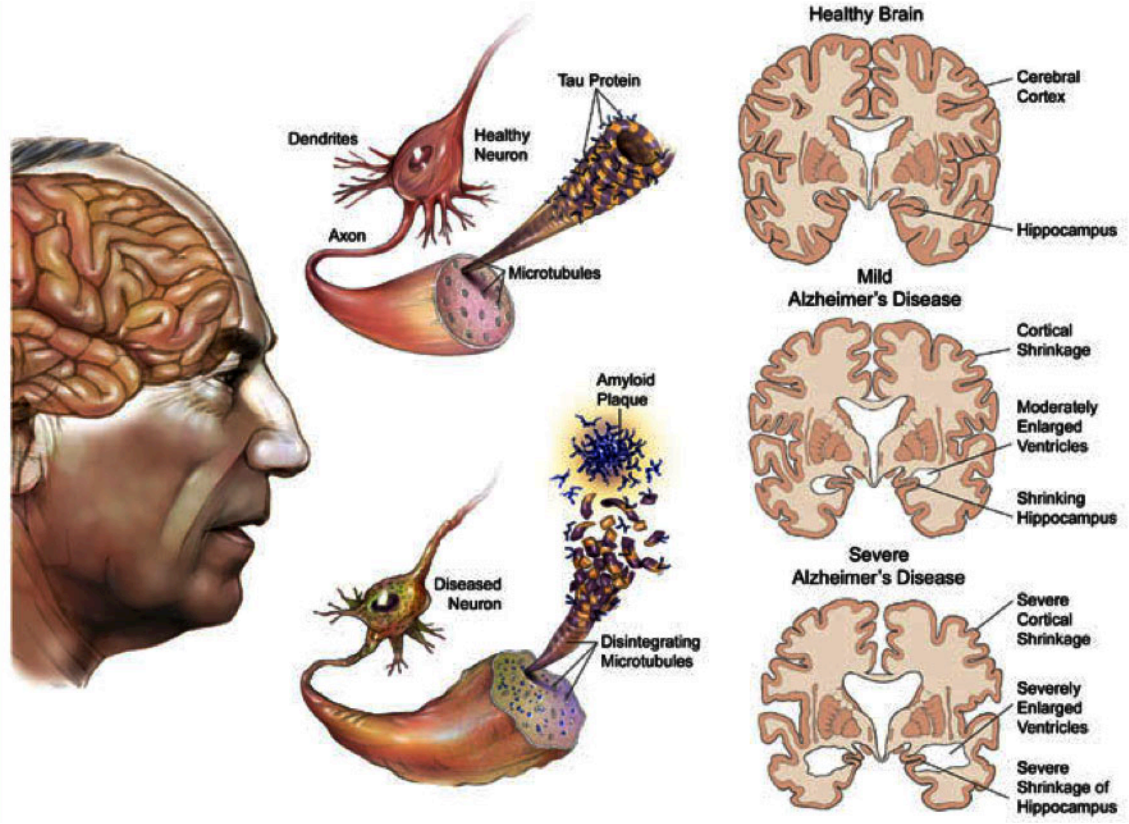

Figure 1.1 Schematic illustration of Alzheimer's disease brain [1].

1.1.2 Causes of AD

AD is a complex disease that the causes of disease are not yet fully understood. Not only genetic factors but also environmental factors contribute to the risks of disease to get it. So far, many competing hypotheses have been stated to explain the causes of diseases. One old of them is cholinergic hypothesis which says AD is caused by reduced synthesis of the neurotransmitter acetylcholine, but the hypothesis has not maintained widely because medications have not been sufficient effects [3]. Another causative hypothesis is that AD may stem from age related myelin breakdown in brain [4]. Oxidative stress may be main cause in the formation of the pathology [5].

Besides these above hypothesis, there are two hallmarks of AD that widely accepted as a reason of disease. They are amyloid plaque formation and tau tangles aggregation:

1.1.2.1 Amyloid Plaque Formation

Alzheimer's disease is characterized by distinct neuropathological hallmarks, and one of them is extracellular deposition of the amyloid β (Aβ) peptide. So, how is its mechanism? Amyloid precursor protein (APP) is an integral membrane protein expressed in many tissues and concentrated in the synapses of neurons. It participates in many crucial tasks of brain such as regulation in synapse formation, neural plasticity [6], and iron export [7]. APP can be processed in different ways, and one of them is amyloidogenic leading to amyloid plaque formation, whereas another one is non-amyloidogenic. Usually about 90% of APP enters the non-amyloidogenic pathway, and 10% the amyloidogenic one, but these ratios can change due to mutations, environmental factors, as well as the age of the individual [8] (see Figure 1.2).

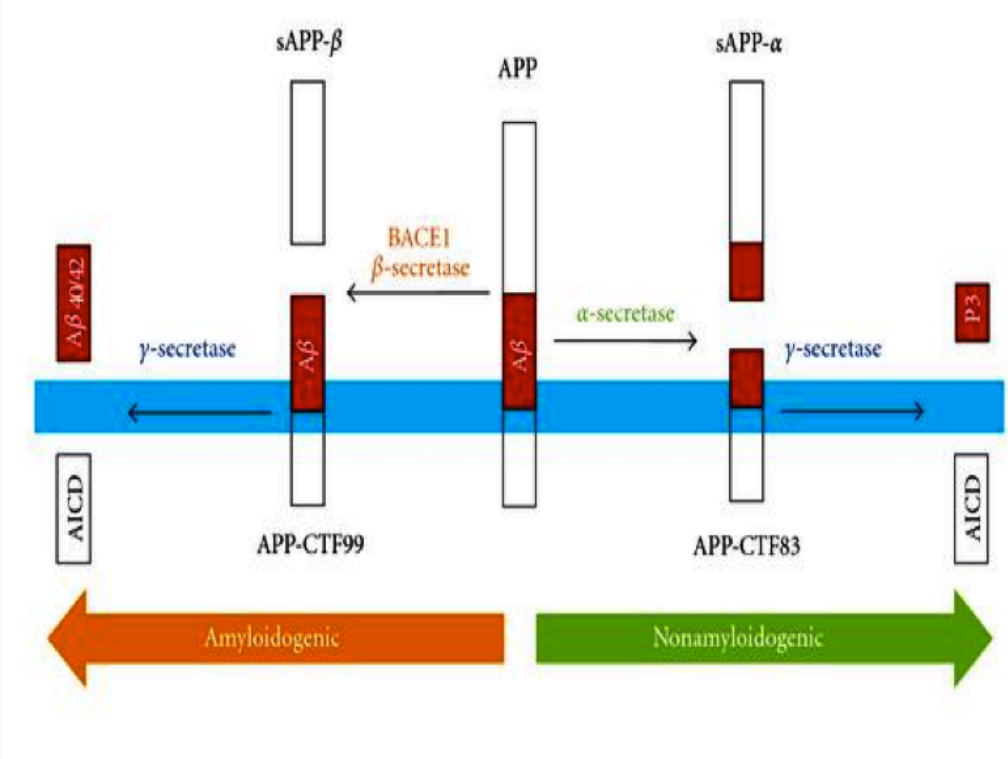

Figure 1.2 Processing of APP in both amyloidogenic and non-amyloidogenic pathways [9].

In the non-amyloidogenic pathway, APP is cleaved by one of its three secretases which is α-secretase. The cleavage of APP by α-secretase results in the generation of soluble APPs, a soluble N-terminal fragment (sAPPα) and a C-terminal fragment (CTFα). sAPPα might have biological functions in growth regulation and neuroprotection, and in blood coagulation. CTFα is retained in the membrane, where it is acted upon by presenilin-containing γ secretase to yield a soluble N-terminal fragment (p3) and a membrane-bound C-terminal fragment (AICD, or APP intracellular domain). AICD may be involved in nuclear signalling via transcriptional regulation as well as axonal transport through its ability to associate with a host of different proteins [10].

In the amyloidogenic form, APP is cleaved by β-secretases (a transmembrane aspartic protease), at the N terminus of the Aβ peptide sequence and which is a 4 kDa peptide. This cleavage yields a soluble N-terminal fragment (sAPPβ) and a membrane-bound C-terminal fragment (CTFβ). This cut is made closer to the N-terminal end of APP than with α-

secretase, making CTFβ longer than CTFα. CTFβ is then acted upon by γ-secretase (as occurred in the previous pathway), yielding a membrane-bound C-terminal fragment (AICD) the same as before, and a soluble N-terminal fragment (amyloid-β, or Aβ) that is longer than p3. Aβ is stickier than other APP fragments, and accumulates by a multi-step polymerization mechanism, with Aβ peptides aggregating into oligomers, which cluster together to form fibrils with a regular β-sheet structure. These fibrils adhere together to form plaques. Aβ plaques accumulate in the extracellular space of the brain where between brain cells by impairing points of cell-cell communication, as well as activating immune cells that trigger inflammation, which can be lethal to cells. In addition, Aβ is thought to cause oxidative damage to cells. Aβ can exert deleterious effects on neuronal and synaptic function, ultimately causing neuronal cell death [11].

All in all, Aβ is not totally bad. Soluble Aβ oligomers are required for Aβ-induced inhibition of long-term-potentiation. However, the insoluble amyloid plaques appear to disrupt neuronal function. There are many hypotheses on this process. The problem may have to do with the aggregation state of the peptide, which in turn may be influenced by the concentration, structure and length of Aβ peptide present. The concentration of Aβ in the extracellular space may affect its toxicity by influencing its aggregation. Aβ concentration appears to be influenced by several factors, including the level of neural activity and synaptic release, which seem to increase Aβ levels. Age could also play a factor, studies showing that the level of α-secretase decreases with age, while β-secretase activity appears to increase. In addition, studies indicate that cholesterol may play a role in regulating Aβ production, with high levels of cholesterol being linked with increased Aβ release and plaque formation. One possible explanation for this result is the presence of cholesterol-rich regions of membranes known as lipid rafts, which may affect the distribution of APP-cleaving enzymes. The structure of the Aβ oligomers could influence plaque formation. Whereas soluble monomeric Aβ peptide has been shown to have an unordered structure in solution, within amyloid fibrils Aβ adopts a regular β-sheet conformation. The conformational change to a β-sheet structure may trigger the formation of the abnormal fibrillary amyloid plaques found in AD patients.

Aβ peptides can differ in length from 38 to 42 amino acids. Aβ ending at amino acid 42 (Aβ42) appears to be the main species of Aβ peptide deposited in plaques. In AD patients, studies show an increase in the ratio of Aβ42 to Aβ40. In addition, genetic changes involved in familial AD result in increased amounts of Aβ42 present in the brain. Aβ peptides are metal chelators with the ability to reduce those metals. For example, *in vitro*, Aβ can reduce Cu^{2+} to Cu^{+}, and Fe^{3+} to Fe^{2+}. Aβ42 appears to be a more effective reductant than Aβ40, which may contribute to the ability of Aβ42 to activate mononuclear phagocytes in the brain and elicit an inflammatory response [12].

Therefore, it appears that there may be several contributory factors to plaque formation in AD patients, the relative importance of which still need to be deciphered.

1.1.2.1 Neurofibrillary Tau Tangles Aggregation

Besides the extracellular β-amyloid–containing plaques, another histopathological characteristic of Alzheimer's disease (AD) is intracellular tau-containing neurofibrillary tangles (NFTs). Tau is one of members of microtubule associated proteins (MAPs) that interact with microtubules of cellular cytoskeleton. They are responsible for the stabilization of microtubules which are consisted of tubulin dimers. Tau is a highly soluble MAP whose major function is specifically in axons by regulating the growing and shortening dynamics of individual microtubules in a phosphorylation- dependent manner. The structure of Tau is important for its regulation, too. Five residues (glycine, lysine, proline, serine, and threonine) make up half of its sequence, and this justifies its high solubility and the unfolded nature of the protein [13].

Neurodegenerative diseases correlated with tauopathies are linked to tau mutations and/or tau posttranslational modifications. Accordingly, tau hyperphosphorylation and cleavage are important events leading to tau intracellular accumulation, aggregation, and neuronal cell death. In AD brain all of six tau isoforms are hyperphosphorylated and aggregated into paired helical filaments (PHF) (see Figure 1.3). While conformational changes and truncation of tau following its hyperphosphorylation. The abnormally hyperphosphorylated tau purified from the oligomers is three to four fold more hyperphosphorylated as the non-hyperphosphorylated/normal tau. Tau from fetal brain

promotes microtubule assembly less efficiently than tau from adult brain and elevated levels of phosphorylated tau correlate with the presence of dynamic microtubules during periods of high plasticity in the developing mammalian brain. When tau is abnormally hyperphosphorylated, it loses its biological activity, becomes resistant to degradation, and undergoes conformational changes that render it insoluble and aggregation-prone. Abnormal hyperphosphorylation of tau leads to its aggregation into PHFs, which are the main components of NFTs [14].

The dynamics of tau phosphorylation/dephosphorylation are a main focus of attention for scientists trying to find a way to regulate this process to avoid tau hyperphosphorylation and aggregation, which impairs its clearance. However, because tau is a target for so many kinases and phosphatases, understanding this process has proven to be very difficult. Resolving the pathways that lead to tau hyperphosphorylation and aggregation will provide a basis for designing more effective therapeutic strategies for AD.

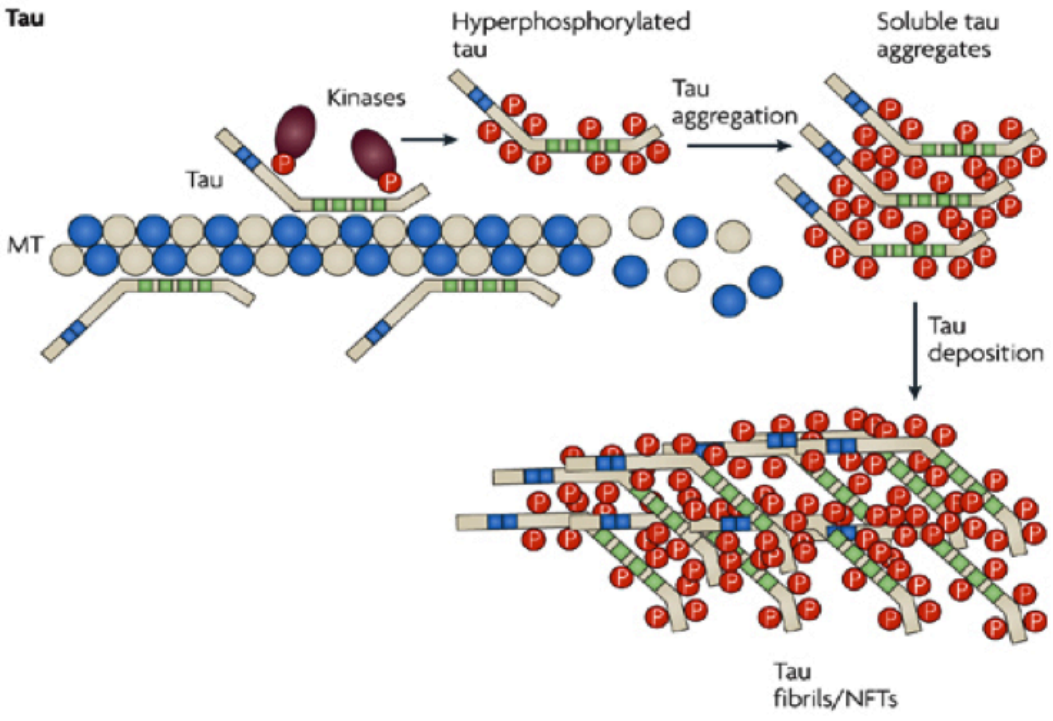

Figure 1.3 Neurofibrillary tangles as a result of hyperphosphorylation of Tau [15].

1.2 DNA TOPOISOMERASES

There are topological problems since the structure of DNA is double helix. DNA topoisomerases play crucial roles in order to solve these kinds of topological problems are used in cells. They are essential for DNA replication, transcription, recombination, as well as for chromosome compaction and segregation [16]. DNA topoisomerases form transient single or double-strand breaks in the DNA and allow DNA strands or double helices to pass through each other.

There are two types of DNA topoisomerases; type I and II, on the basis of their different architectures and mechanisms [17]. While type I enzymes introduce a single stranded break in DNA, type II enzymes introduce a break in two strands at the same time thanks to their dimeric structures. Different from type I topoisomerases, type II topoisomerases use ATP during changing DNA topology [18]. Both of them also are subcategorized into two forms: alpha and beta.

1.2.1 Type II DNA Topoisomerases

During cellular processes some topological problems occur in DNA due to the helical nature of it and its length. In case of DNA replication, recombination, chromatin remodeling, and transcription superhelical, the presence of tension in the adjacent double helical region may cause problems. Type II topoisomerases modulate the topology of DNA and relieve supercoil stress by creating double strand breaks in DNA. It provides a transient cleavage of DNA strands and allows a second double-stranded DNA segment pass through the gate. Different from other topoisomerases this transport is an ATP coupled process at type II topoisomerases. This ATP coupling is necessary for the enzyme to return its initial configuration. After the DNA transport the double-strand break is religated [19] (Figure 1.4).

There are two subclasses of type II topoisomerases, type IIA and type IIB. Type IIA topoisomerases are the enzymes DNA gyrase, eukaryotic topoisomerase II and bacterial topoisomerase IV. Type IIB topoisomerases are distinct from type IIA and found in archea and higher plants.

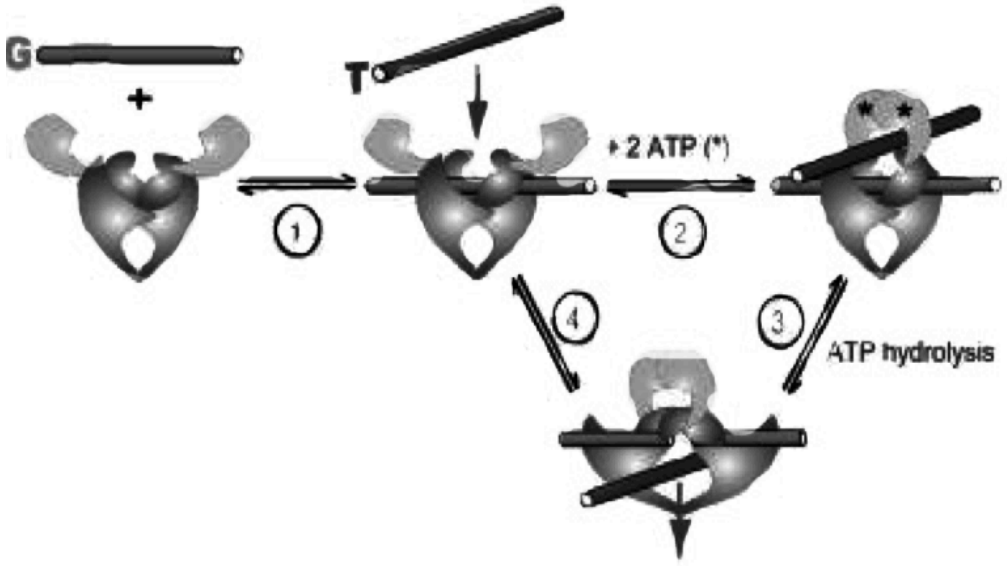

Figure 1.4 Overview the catalytic reaction of DNA topoisomerases II [19].

In mammalian cells two isoforms of topoisomerase II have been identified; 170- kDa topoisomerase IIα (topo IIα) and the 180-kDa topoisomerase IIβ (topo IIβ). Both isoforms are similar in primary structure but different in expression and catalytic activities *in vitro* . topo IIα isoform is mainly involved in mitotic processes and only present in proliferating tissues such as tumors so its expression is really high in proliferating cells especially G_2/M phase of the cell cycle indicating its role in chromosome segregation [20]. On the other hand, topo IIβ isoform is not required for normal mitotic events. It is present in all tissues including terminally differentiated tissues and its level is not changed during the cell cycle. Its presence in terminally differentiated cells indicates that it plays role in DNA metabolism especially in the transcriptional activation of some inducible genes instead of DNA replication and chromosome condensation/segregation [21]. Both the isoforms show different patterns of tissue distribution. topo IIα is shown to be higher in testes, spleen, bone marrow marrow and liver. topo IIβ is detected in high levels in differentiated tissue [22].

1.2.1.1 DNATopoisomerase IIβ

Tsutsui *et al.* showed the first clear evidences that suggested the involvement of topoisomerase IIβ in some physiological processes in post-mitotic neurons of developing brain. Topoisomerase IIβ was detected in both fetal and adult rat brain, whereas the IIα isoform was not expressed in adult brain [23].

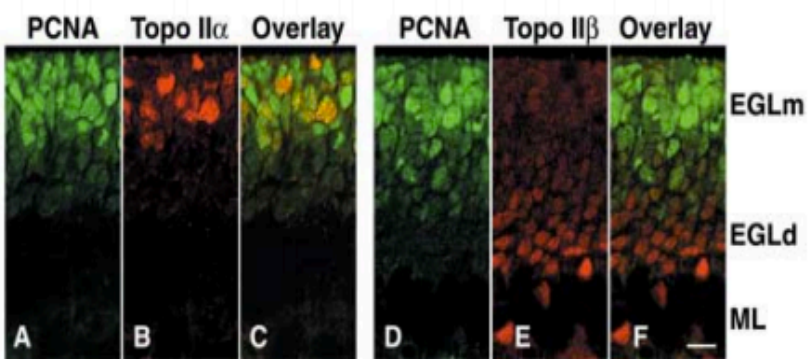

Figure 1.5 Sharp transition from the expression of topoisomerase IIα to that of IIβ in cells that had gone through the final division. Confocal micrographs of double-immunostained cerebellar cortex. Cryosections of postnatal Day 10 rat cerebellum were incubated simultaneously with isoform specific anti-topoisomerase II monoclonal antibodies and a polyclonal antibody to proliferating cell nuclear antigen (PCNA) and visualized with secondary antibodies conjugated with (B,E) Alexa Fluor 594 (anti-mouse IgG) or (A,D) Alexa Fluor 488 (anti-rabbit IgG). Overlay pictures are shown in the panels on the right (C,F). EGLm (mitotic zone of external germinal layer) EGLd (differentiating zone of EGL) ML(molecular layer) [23].

1.2.1.2 Role of Topoisomerase IIß in Neural Differentiation

As it can be seen above, the postmitotic granule cells in the external germinal layer of the developing rat cerebellum show a transition from topo IIα to topo IIβ and also blocking topo IIβ catalytic activity affects the expression of about one third of genes induced during differentiation of rat cerebellar granule neurons. In some cell lines it is not expressed suggesting that it is not required for general cellular activities but important for more specific processes *in vivo*. However, its exact function is not known yet in the embryonic stage, topo IIβ in the brain is a nucleoplasmic enzyme showing higher levels of expression in the differentiating neurons [24]. In an another study, neural defects including aberrant axonal elongation and perinatal death which was explained by the lack of innervation of the skeletal musculature in topo IIβ null mice [25] Further support for a functional role for topo IIβ in brain development comes from topo IIβ deleted mice which showed defects in corticogenesis [26]. Based on single-gene analysis, it was hypothesized that topo IIβ may regulate transcription of neuronal genes by direct binding to their regulatory regions [27]. However, this work was limited to a minor part of the rat genome, leaving open the questions as to where topo IIβ binds genome-wide and how such binding might explain the defects in brain development observed in topo IIβ deficient animals. They need further investigations. In a recent research, the role of topo IIβ in neurite outgrowth and growth cone formation was shown in cultured cerebellar granule neurons (CGNs), dorsal root ganglions (DRGs) and cortical neurons (CNs by using topo IIβ inhibitor ICRF-193 [28]. All in all, it is clear that topo IIβ can affect brain development and neuronal differentiation.

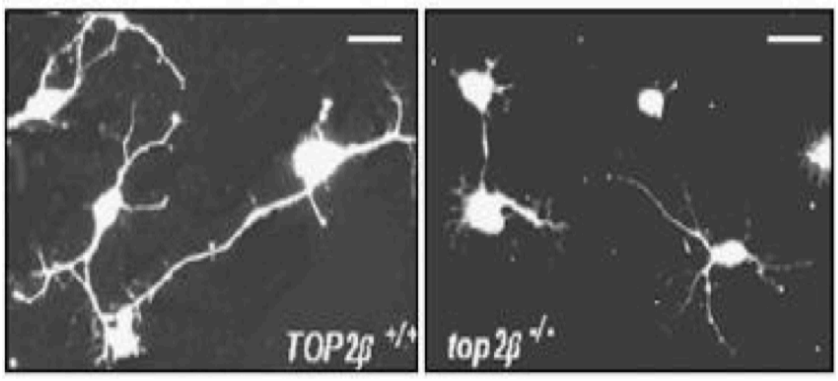

Figure 1.6 Neurons from top2β−/− mouse brains cannot extend neurites in culture [28]

1.3 PRIMARY CEREBELLAR NEURONAL CULTURE

Primary cultures of granule neurons from the post-natal rat cerebellum provide an excellent model system for molecular and cell biological studies of neuronal development and function. In addition, the cerebellum is a mostly studied and well-presented structure that provides unique opportunities for studying neuronal properties and development. Of the cerebellar neuronal types (granule cells, Purkinje cells and inhibitory interneurons), granule neurons are the most numerous and abundant type of neurons in the mammalian brain. Granule neurons are the smallest types of neurons that are found in granule cell layer of the cerebellum, innermost layer of the cerebellar cortex with the middle layer being the Purkinje cell layer, hippocampus and olfactory bulb. In rodents, cerebellar granule neurons are generated during the first two post-natal weeks from progenitor cells in the outermost layer of the cerebellar cortex, the external granule layer (EGL) [29].

One of the reasons why cerebellar granule cells have been chosen in our study is that not only their abundancy in brain but also the homogeneity of the population which are ideal for elucidating the molecular basis of neuronal development. Establishing an *in vitro* Alzheimer`s model can be achieved by obtaining a pure neuron culture is important due to the fact that amyloid fibrils only affect neurons not glial cells which are supporter of neurons, neuron like cells, being non electrically excitable.

Another reason choosing cerebellum as a source of primary neuron culture in this study is that cerebellum is a good illustration of the development of the central nervous system with ongoing neuronal differentiation strictly controlled in a stage- and spatial-specific manner where express the topoisomerase II isoforms with similar timing during development (i.e. the α isoform in proliferating cells and the β isoform in post-mitotic cells). It was shown that the abrupt increase in DNA topoisomerase IIβ expression after final mitosis during neuronal differentiation in Purkinje cells into mature neurons is the evidence of involvement of topo IIβ in the regulation of gene expression [23]. One more knowledge about highly expressed topo IIβ in cerebellum comes from the study topo II roles of the DNA repair mechanisms in ageing brain. The results of experiments showed

that topoII β is the only isoform that is significantly present in rat brain and that too predominantly in cerebellar neurons (see Figure 1.6) [20].

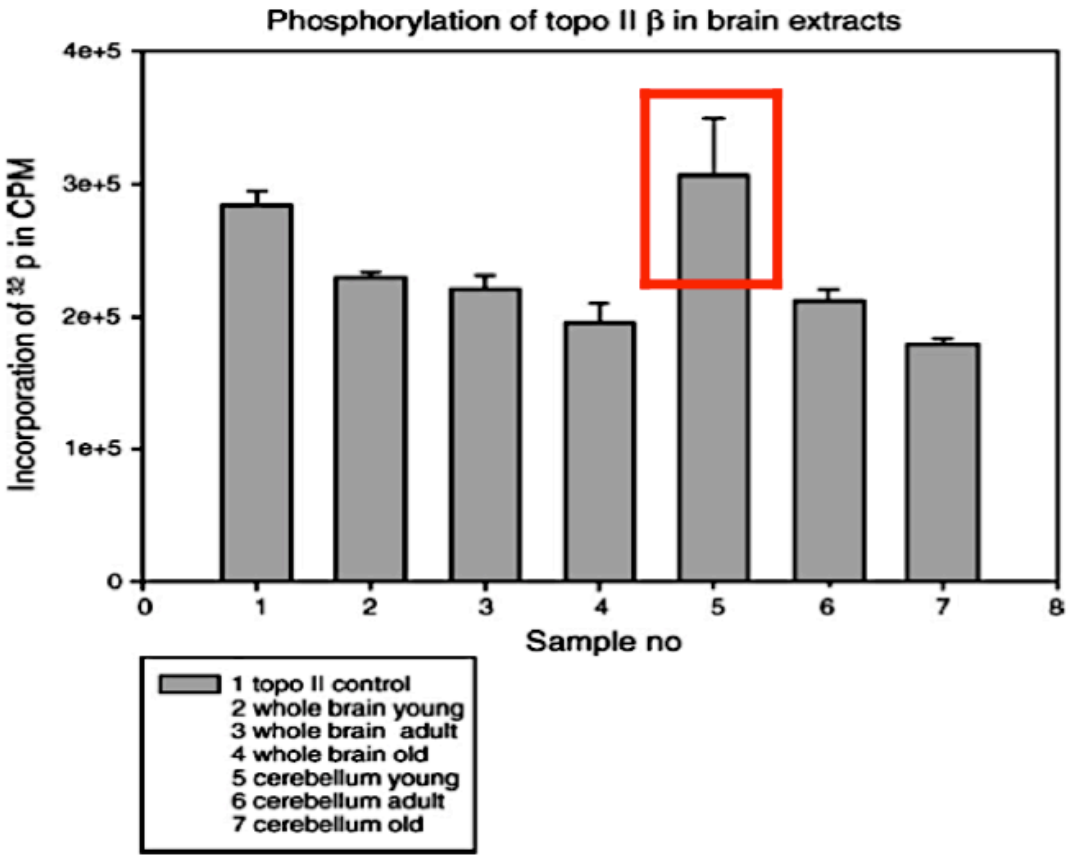

Figure 1.7 Whole brain and cerebellum region have the maximum level of phosphorylated topo IIβ and there was a decrease in this level in an age-dependent manner Cellular topo IIβ activity is known to be regulated by its phosphorylation and the phosphorylated form being the active form. [20]

All in all, the purpose of this study is that firstly establishment of primary neuronal culture from 9 days old Wistar rats and make long lived them in order to maintain *in vitro* Alzheimer`s disease model on them. With the help of the results from above trials, the role of topo IIβ expression is investigated to associate with Alzheimer`s disease in primary neuronal culture.

CHAPTER 2

MATERIALS & METHODS

2.1 ISOLATION AND EXPANSION OF RAT PRIMARY CEREBELLAR GRANULE NEURONS

2.1.1 Coating of plastic wares

Poly-l-lysin (PLL) is essential for the attachment of cells and proteins to solid surfaces in cell culture experiments. For the adherent cells, attachment factors and extracellular matrix are really crucial in the attachment, growth and development of cells. It enhances electrostatic interactions between negatively charged cell membrane and positively charged ions of attachment factors which is especially needed by neurons for their extension and axonal growth. In our lab, commercially available PLL powder (Sigma; cat.P2658) was dissolved in phosphate buffer saline (PBS) (Sigma, pH 7.4) as a stock solution concentration 2mg/ml, and stored at-20^{o}C for further use. During optimization trials of coating for primary rat cerebellum neuron culture, different concentrations of PLL have been tried for the ideal survival of neurons. In brief, all plastic wares such as 24 well-plates (TPP) and 25 cm^2 flasks (TPP) were sterilized under UV for 30 minutes. Then, they were incubated with optimized concentration of PLL as 0.1 mg/ml diluted from stock with distilled water and with the amounts of 400 µl/well and 1.5 ml/flask for 4 hours under sterile hood. After that, PLL was discarded, plastics were washed with distilled water 3 times for 5 minutes and let them air dry under sterile hood for overnight.

2.1.2 Isolation and Seeding of Rat Primary Neurons

Postnatal 9 days old Wistar rats were bought from Istanbul Bezmialem University. They sacrificed under sterile conditions at Fatih University, Cell Culture Laboratory.

Firstly, rats were euthanized with ether. This protocol was approved by Bezmialem University Ethic Committee. They were sterilized with 70% ethanol before cut off the heads. The heads were cut off with sterilized scissors in ethanol and taken into a petri dish. Then, remove skin and skull, transfer the brain into a clean Petri dish with ice cold dissecting medium including PBS with 1% Penicillin-Streptomycin. Remove meninges from each cerebellum by gently teasing with two forceps and place into clean dish with ice cold dissecting medium. Transfer pieces of cerebellum into 15 ml centrifuge tube containing 4.5 ml with hippocampus dissection medium. Add 0.5 ml Trypsin 2,5% (Gibco; cat. No. 59427C) and 0.25 ml DNase I (Sigma; cat.no. DN25) (stock solution as 10 mg/ ml in miliQwater) into tube, tube was gently mixed and incubated in 37^{o}Cwater bath for 15 minutes with shaking. Let pieces settled to the bottom of the tube. After enzymatic digestion, trypsin affect was inhibited by plating medium containing Dulbecco`s Modified Eagle Medium (DMEM) with 10% Fetal Bovine Serum (FBS). After removal of dissecting medium, add 5 ml plating medium on pieces and centrifuge them for 5 minutes at 1500 rpm 2 times. After two subsequent washing, add only 1 ml warm DMEM without FBS into the falcon tube and perform trituration 6-7 times against the side of the tube with a fire- polished Pasteur pipette. The medium is especially pushed out against the side of the tube to prevent frothing because the cells at an air-liquid interface can be lysed. After dissociation completed, and count the cells. 50 000 cells/well of 24 well plate and 750 000 cells/T25 flask were seeded in prewarmed Neurobasal (Gibco; cat.no.: 21103-49) based culture medium (NB) including 2% B-27 Serum Free supplement (Gibco; cat.no.17504-044) , 1% L-glutamine (Gibco; 2mM), and antibiotic. Incubate the cells at 37^{0} C 5% CO_2. On the following day and every 4 days, refresh the cells with the same culture media additionaly containing 2% d-Glucose (6g/L) in Neurobasal media. On the fifth day, 10µl of Cytosine β-D-arabinofuranoside hydrochloride (AraC) (Sigma; cat.no. C6645) (10µM) as a mitotic inhibitor were added to cultures in order avoid the growth of non neuronal cells like astrocytes and other glial cells.

Figure 2.1 Protocol of isolation rat cerebellum from 9 days ol Wistar rats tak, taken on April 2013, Fatih University.

2.1.3 Analysis of Neural Markers in Primary Neuron Culture

To confirm establishment of a pure neuronal culture, neuronal markers were analyzed by western blot and immunofluorescence technique.

2.1.3.1 Western Blot Technique

On the 14th day of the culture, culture mediums were discarded, the cells were washed with ice cold PBS 3 times for 5 minutes, lysed cells with 75µl/750 000 cells of 2X Lysis Buffer diluted from 4X one (see Table 2.1) with the help of a plastic scraper, taken into an eppendorf tube, sonicated them 2 cycles at minimum speed for 10 seconds, then boiled at 95^{0}C for 2 minutes.

In a next step, protein concentrations were measured via iQubit Protein Fluorometer (Invitrogen).

Equal amounts, 35 µg of proteins were loaded 7,5 % SDS PAGE gel (see Table 2.2), then run them at constant 25 mA for 90 minutes in Tris-SDS pH 8.3 buffer. Blot them via iBlot(Invitrogen) for 10 min. Then, take the membrane into blocking solution (5% skimmed milk in NewTBS+0.1% Tween20) for 1 hours while the gel were incubated in Comassie Blue solution for 0 min. and then overnight in destaining solution in order to see the efficiency of blotting. Glial fibrillary acidic protein (GFAP) (50kDa) (Millipore, Rabbit Polyclonal, cat.no.:AB5804, 1:2500), neurofilament-L (NF-L) (70kDa) (Millipore, Rabbit Polyclonal, cat.no.:AB9568, 1:1000), TAU5 (44-68kDa) (Invitrogen, Mouse Monoclonal, cat.no.:AHB0042, 1:250), and actin Clone 4 (43kDa) (Millipore, Mouse Monoclonal, cat.no.:MAB1501, 1:3000) as a positive control of primary antibodies were used with the incubation overnight at 4^{0}C in blocking solution on rocking shaker. Then, the secondary antibody horseradish peroxidase attached-goat anti mouse (1:5000) and anti rabbit (1:10000) were incubated for 1.5 hours at room temperature (RT) in blocking solution on shaker in dark room. At a final step, substrate development via LumiGlo® Chemiluminescent Substrare System (KPL, USA) and detection with film developer machine (Konica srx101) was performed.

Table 2.1 4X Sample Buffer for preparation of Western blot proteins

	4X concentration
1M Tris-HCl (pH6.8)	2 ml
0.5 M EDTA (pH 8)	160 µl
10% SDS	4 ml
B-ME	400 µl
100 % Glycerol	3.2 ml
BPB	10 mg
ddH_2O	240 µl
	10 ml

Table 2.2 The content of 7,5X SDS-PAGE gel

	Seperating Gel	Stacking Gel
Distilled water	2.875 ml	2.12 ml
1M Tris HCl	3.75 ml (pH 8.8)	0.38 ml (pH 6.8)
40% Acrylamide	1.875 ml	225 µl
2% APS	500 µl	150 µl
1% SDS	1000 µl	30 µl
TEMED	10 µl	4 µl
	10 ml	**3 ml**

2.1.3.2 Immunofluorescence staining

On the 5th day of *in vitro* cell culture, to determine the ratio of neuron population compared to non-neuronal cells, immunofluorescence method was performed. At the day of staining, culture medium was aspirated from wells. Firstly, the cells with washed PBS 3 times for 5 minutes. Then, the cells seeded on 24 well plate were fixed with 4% Paraformaldehyde / PBS (500 µl/well) for 10 minutes at RT. Cells were washed with 750 µl/well PBS (for 3 times, 5 minutes at each time on rocking shaker. 500 µl/well of 10% Normal Goat Serum (Gibco) and 10% Normal Horse Serum (Biochrome) in 0.3% TritonX / PBS (PBS-Tx) were used to block cells for 30 minutes at RT. Then, cells were

incubated with 60 µl/well spesific primary antibodies for target proteins for overnight at 4^0C. Antibodies diluted in PBS-Tx with 3% NHS. The antibodies were same with the ones used in western blot method but different concentrations. For example, GFAP is 1:500, NF-L 1:200, TAU5 is 1:100. Washing steps with PBS was repeated for three times. Cells were then treated with 50 µl/well labeled anti rabbit or anti mouse secondary antibodies (1:200) Alexa Fluor 488 and 594, respectively at RT for 1,5 hours. After this incubation, cells were washed 3 times with PBS and treated with 150 µl/well, 1/15000X DAPI (Sigma) for 15 minutes. Then washing with PBS was repeated for three times and finally cells were washed with dH_2O. A drop of Mounting Medium For Fluorescence (Vectashield) was applied on glass coverslips and they were placed on cells in the wells. Wells were observed under fluorescent microscope (Nikon), images were saved as both jpeg and tiff.

2.1.4 Establishment of *in vitro* model of Alzheimer`s disease in primary neuron culture

The plan of last trial in order to establish Alzheimer`s model on primary neuronal culture is stated in Figure 2.2.

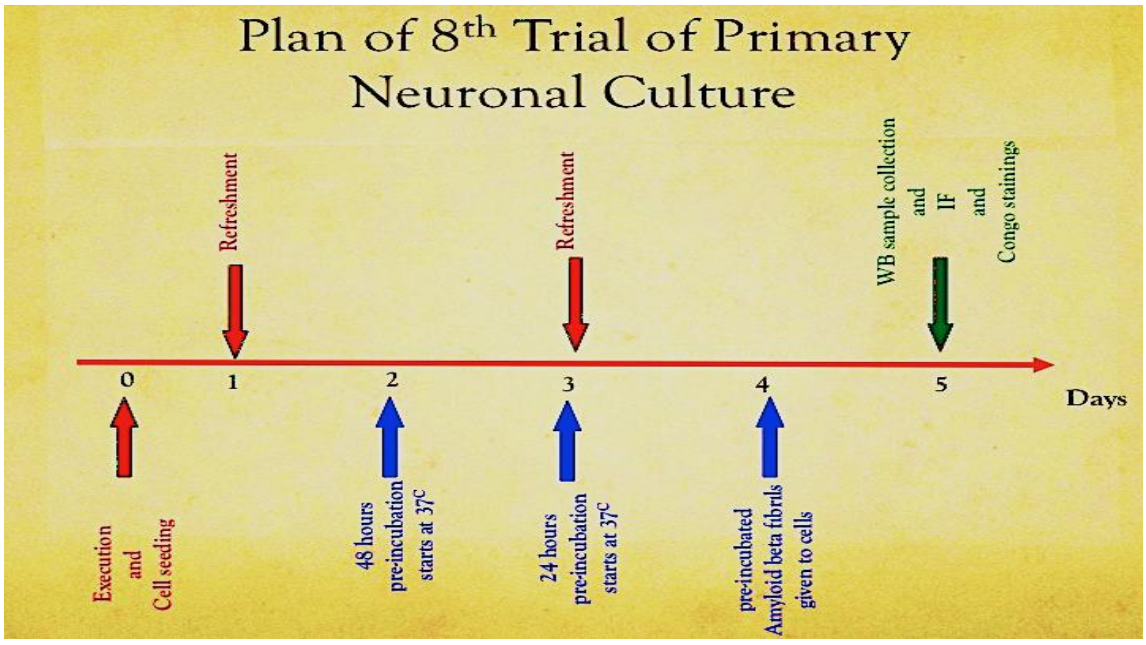

Figure 2.2. Schematic illustration of experiment design belong to last trial.

2.1.4.1 Preparation of amyloid beta fibrils

After successful survival process of primary neuronal culture during 4 day, they were exposed to amyloid beta (1-42) fragments (Sigma, cat.no.: A9810). Before incubation, lyophilized fragments (1mg) were initially dissolved by 75 µl of 1% NH_4OH, then it was immediately diluted with 1X PBS Ca^{++} and Mg^{++} free to 1 mg/ml, gently vortexed to mix. Reconstituted peptide (221µM in total) was aliquated and stored -80^0C for further applications.

To obtain fibrils and aggregates, pre-incubation is needed. Fragments were incubated at 37^0C for 24 and 48 hours, separately with gently shaking.

2.1.4.2 Analysis of the effects of amyloid beta peptides on neurons

The effects of amyloid beta fibrils to the survival rate of neurons were observed during culturing with the help of Real Time Cell Analysis System (Roche, x-Celligence RTCA), the growth curve and survival rate upon the exposure of amyloid peptides to the neuron culture were graphed.

2.1.4.3 Analysis of the amyloid beta fibril formation with Congo Red Staining

To confirm the formation of amyloid plaques, Congo Red (Bioxtra Sigma, cat.no: C6277) staining was performed. 20 mg of Congo Red stain dissolved in 10 ml of 80% ethanol including 0.1 ml of 1% sodium hydroxide. In order to stain nucleus, hemotoxylen staining was done as counterstain as well, in the following: first aliminum potassium sulfate was dissolved in dH_2O. When alum was completely dissolved, hematoxylen was added. When hematoxylen was completely dissolved, sodium iodate and glacial acetic acid were added. The solution was boiled and cooled. Finally, hematoxylen solution was filtered.

Initially, amyloid fibrils given cells after 24 hours exposure were fixed with ice old 4%PFA. Cells were stained with Congo Red for 20 min., then several quick washes with distilled water. Stain with Hematoxylen for 2 min. and again washed with distilled water. Finally, mount the cells and visualize under bright field microscope.

Table 2.3 Mayer's hematoxylen solution

Chemicals	**Amount**	**Function**
Aluminum potassium sulfate (alum)	50 g	mordant
Distilled water	1000 ml	solvent
Hematoxylen	1 g	dye
Sodium iodate	0.2 g	oxidizing agent
Glacial acetic acid	20 ml	pH control

2.1.5 Association of Topoisomerase IIβ with Alzheimer`s disease in primary neuron culture

In order to look for whether topo IIβ expression is affected or not, its expression level was checked from *in vitro* Alzheimer`s model established neuron culture by Western blot and immunofluorescence following the same protocol as mentioned above. Topo IIβ (180kDa) (3B6 from Japan, Mouse Monoclonal, 1:500) and amyloid beta 1-42 (Aβ 1-42) (6kDa) (Millipore, Rabbit Polyclonal, cat.no.: AB5078P)

The same antibodies also were used in immunofluorescence upon amyloid beta plaque exposure to primary neuron cells. For this purpose, topo IIβ 1:200, Aβ 1-42 1:100, NF-L 1:200 and TAU5 1:200 were used.

CHAPTER 3

RESULTS

3.1 OPTIMIZATION of PRIMARY CEREBELLUM NEURONAL CELL CULTURE

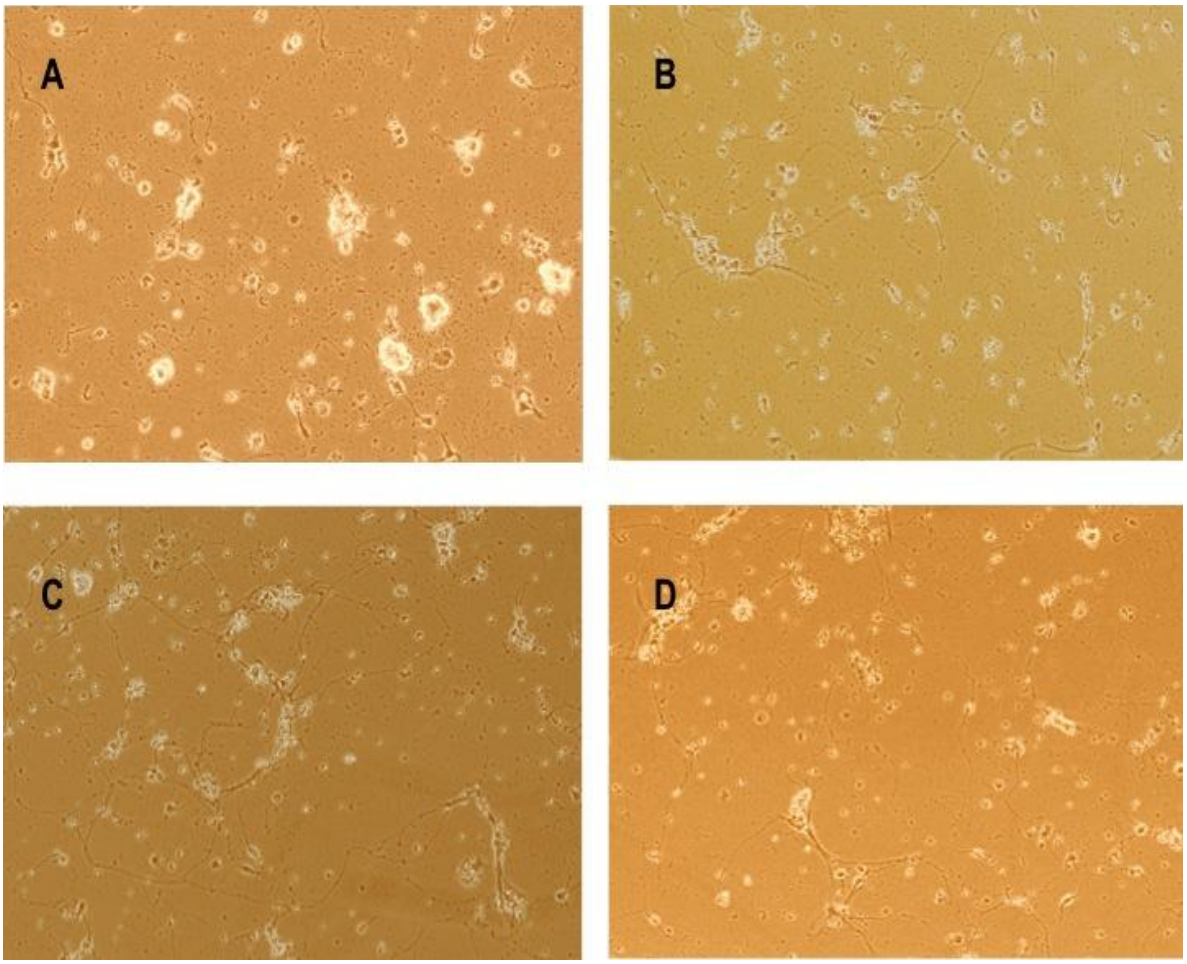

Figure 3.1 Expansion of rat primary cerebellum neuronal culture (PNC#4).

Culture media supplemented with Neurobasal (NB) media, 750 000 cells/T25 flask coated with 0.1 mg/ml PLL under 10x magnification. **A.** 1st day of culture after first refreshment with NB media supplemented with d-Glucose. **B.** 3rd day of culture after second refreshment NB media supplemented with d-Glucose **C.** 5th day after third refreshment with the same media as previous one. **D.** 7th day of culture just before massive death.

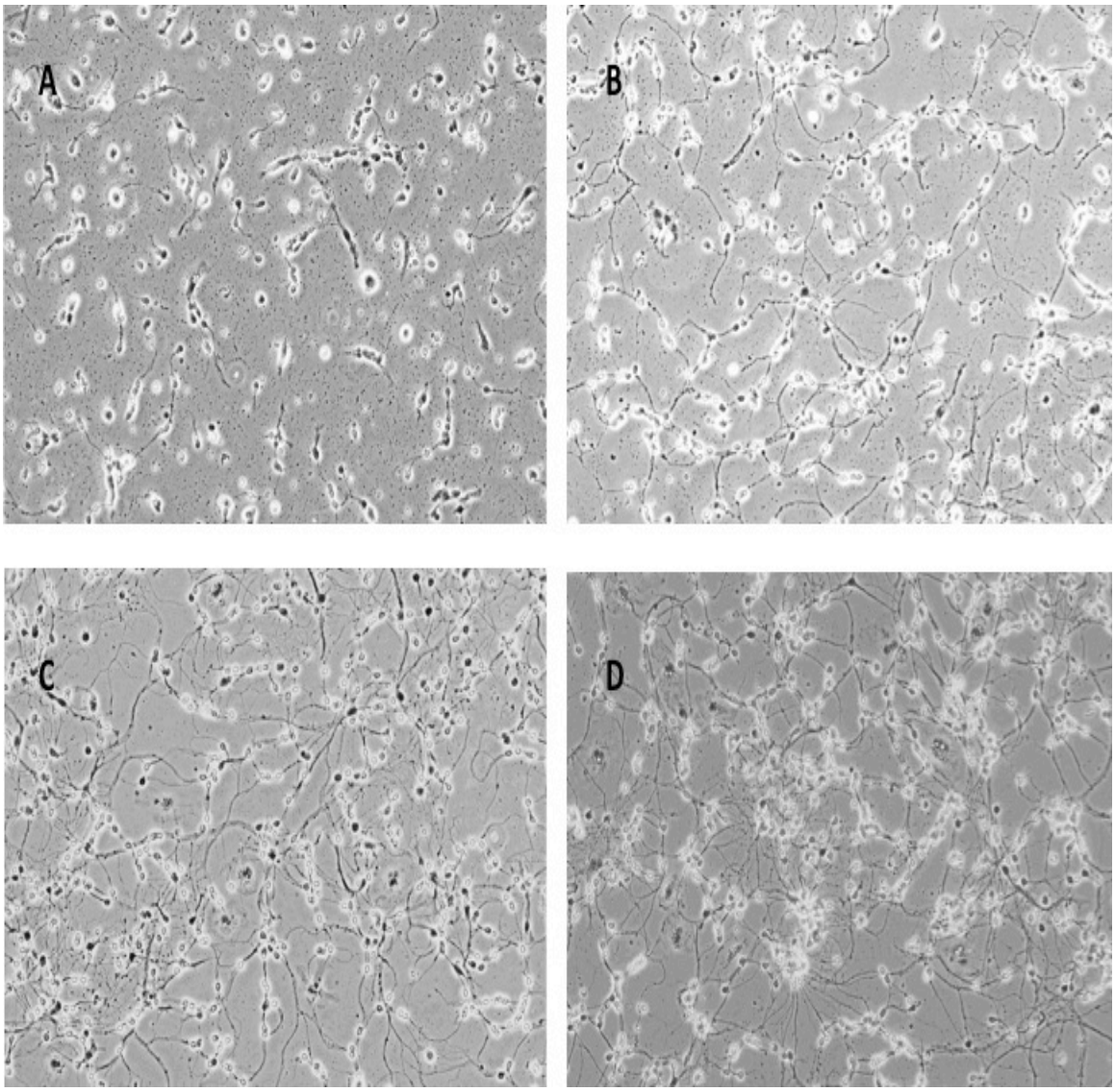

Figure 3.2 Expansion of rat primary cerebellum neuronal culture (PNC#8). Culture media supplemented with Neurobasal (NB) media, 750 000 cells/T25 flask coated with 0.1 mg/ml PLL under 10x magnification. **A.** 1st day of culture after first refreshment with NB media supplemented with d-Glucose. **B.** 3rd day of culture after second refreshment NB media supplemented with d-Glucose and added 10 μM Ara-C. **C.** 6th day after third refreshment with the same media as previous one. **D.** 9th day of culture just before massive death.

The results are from the last trials of primary neuron culture studies (PNC #8). Until the 8th trial, we sacrificed around 12 Wistar P9 rats. With continued day *in vitro* (DIV) propagation, neurons establish neuronal networks without dividing as it is known. Therefore, stabilizing the number of neurons at enough level to start an experiment was difficult for us at first. In the meantime, we noted that the importance of using fresh mediums, concentrations of PLL for coating and also the brand of plastic wares in the facilitation of neuron survival. It is clear that we achived the establishment of primary cerebellar neuronal culture.

Morphologically the presence of neuron and their networks is enough at first, so we looked for the neural marker expression profile of our cultures.

3.2 GROWTH CURVE ANALYSIS OF CEREBELLAR GRANULE CELLS via REAL TIME CELL ANALYSIS (RTCA)

All Real Time Cell Analysis results were monitored by x-Celligence (Roche) device from 8th primary neuron culture (PNC #8).

Three different cell concentrations were seeded on 96 well e-plate, observed the rate of growth during 211 hours. According to first 20 hours, high concentration cell population showed greater survival rate than the others as it is seen from Figure 3.3.

Due to the fact that, 10000 cells/wells were more consistent as a whole than other concentrations of cells on 96 well e-plate, we chose this concentration in order to perform amyloid plaque formations studies in next.

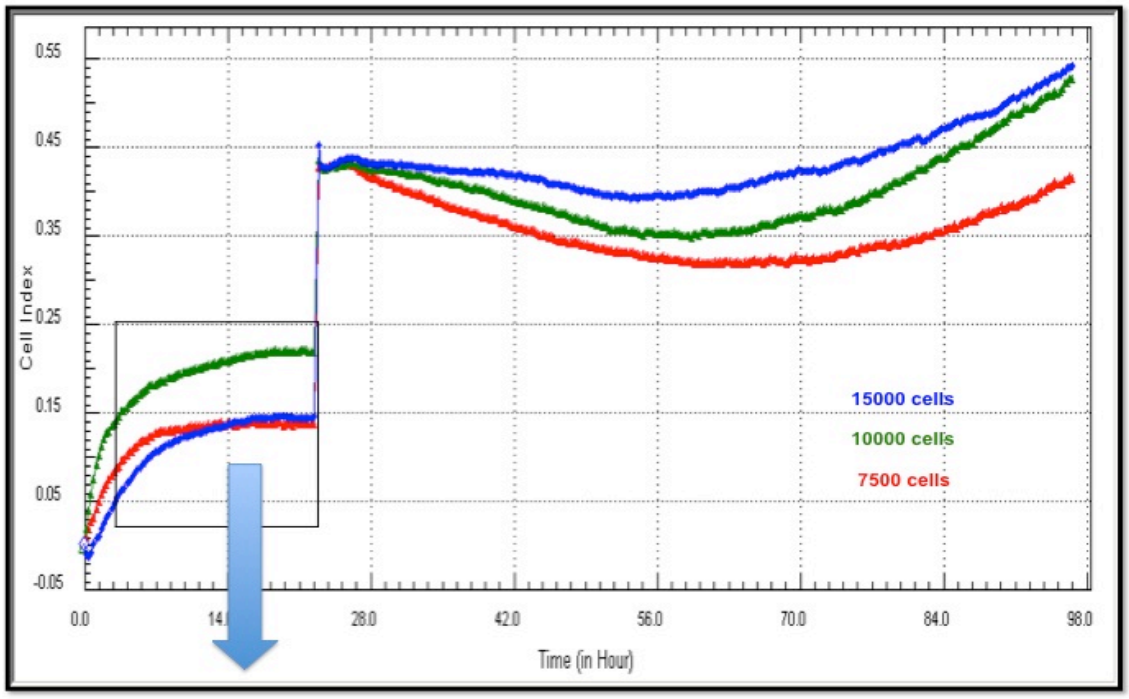

Figure 3.3 Growth curve of cerebellar granule neuron cells during 211 hours *in vitro* culture.

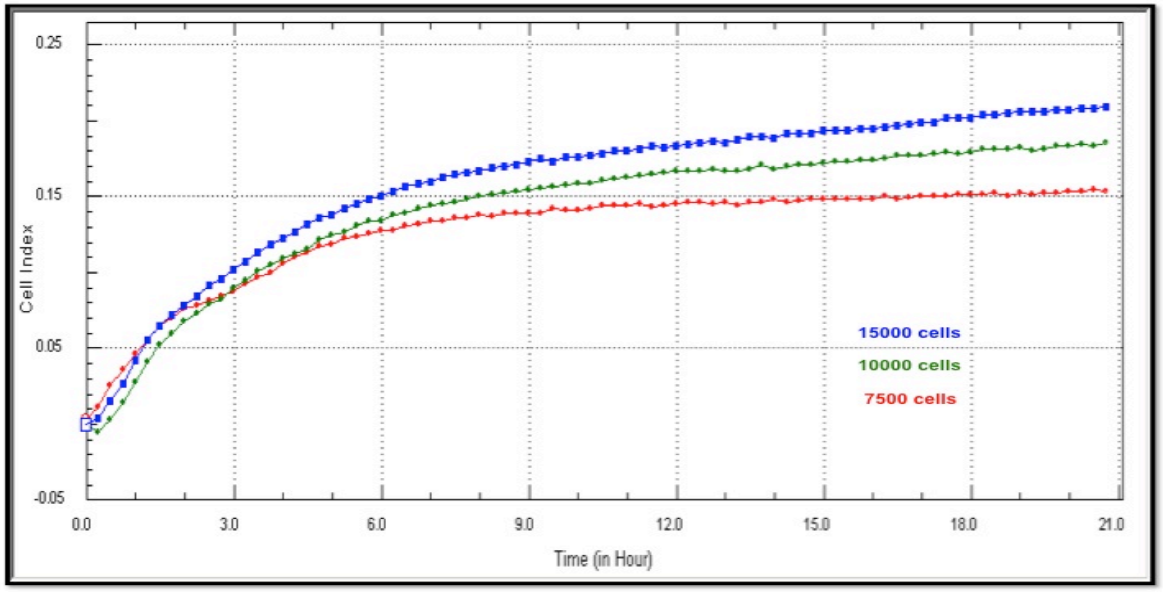

Figure 3.4 Growth curve of cerebellar granule neuron cells during first 24 hours *in vitro* culture.

3.2.1 Effect Of Cytosine β-D-Arabinofuronoside (AraC) On The Survival Of Primary Neuron Culture

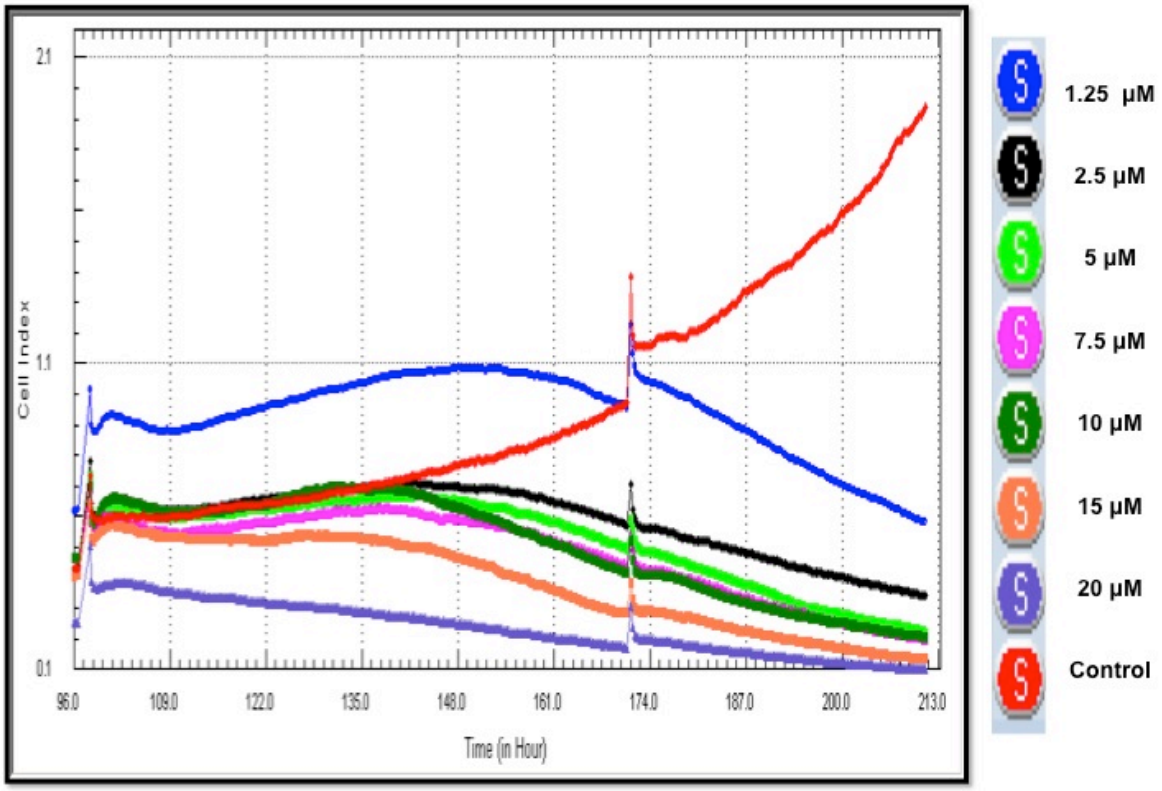

Figure 3.5 Analysis of growth curve of cerebellar granule neuron cells upon after AraC exposure on 4th DIV.

The more impulsive effect the higher concentration of AraC, our choice 10 µM pretty effective on 7500 cells/well on 96 well e-plate.

3.3 NEURAL MARKER EXPRESSION via WESTERN BLOT

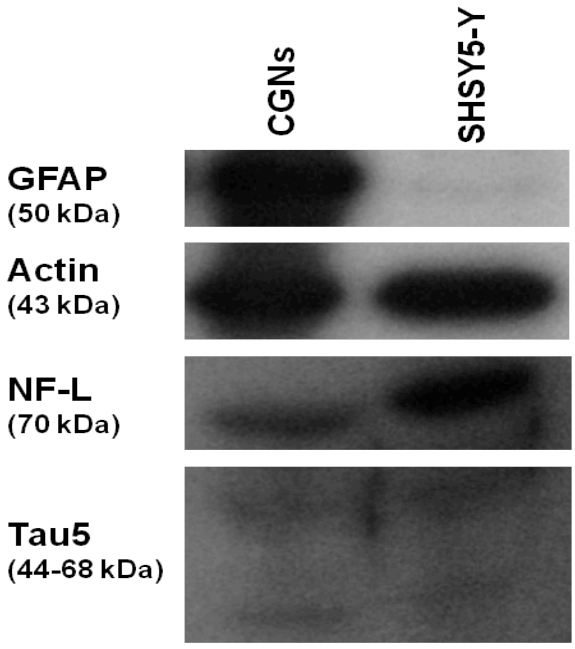

Figure 3.6 Western blot profile for neural markers in primary neuronal culture. Rat cerebellar granule neurons(CGNs) on the 6th day and neuroblastoma cell line (SHSY5Y). The samples were prepared as mentioned in chapter 2. **A.** Actin is found in all cells used as positive control. GFAP is intermediate filament of astrocytes and ependymal cells. **B.** NF-L is intermediate filaments found in neurons. TAU is a microtubule associated protein found mostly along the axons of neurons.

These samples for GFAP is only from the first trials (PNC#2) of primary neuron culture studies, others belong to the last trials. As it can be seen from the results above, pure neuron culture did not obtain yet at that time. The highest expression of GFAP means the dominance of glial cells in the culture which is an unwanted population. On the other hand, NF-L and TAU5 expression may be sufficient as in last trials.

3.4 NEURAL MARKER LOCALIZATION via IMMUNOFLUORESCENCE STAINING

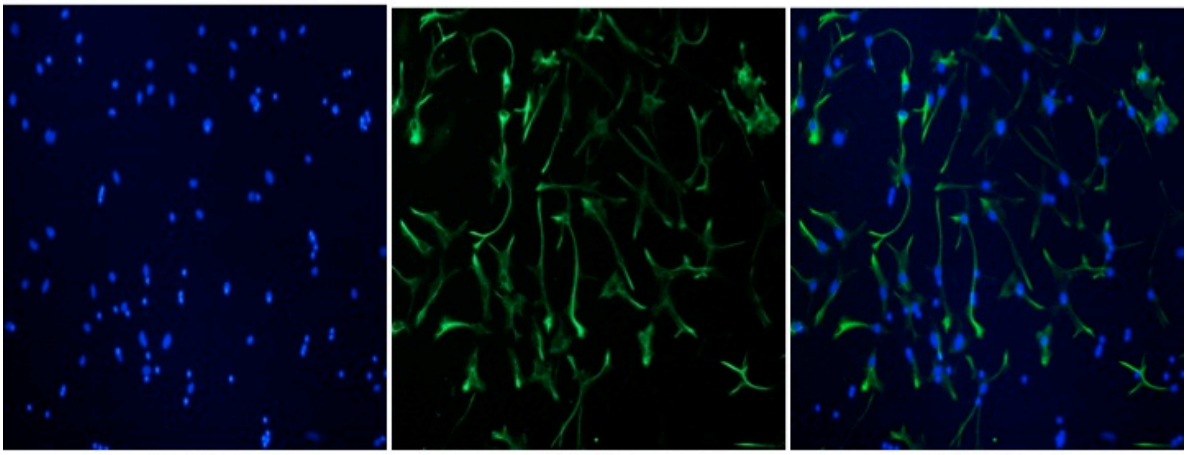

Figure 3.7 GFAP and DAPI immunofluorescence stainings. Alexa Fluor 488 goat anti rabbit polyclonal (green) as secondary antibody indicates the presence of glial cells (10x magnification).

The immunofluorescence staining for GFAP results were from our second primary neuron culture study (PNC #2). The presence of glial cell population in culture meant that pure neuron culture was not successfully isolated from P9 rat at the first trials.

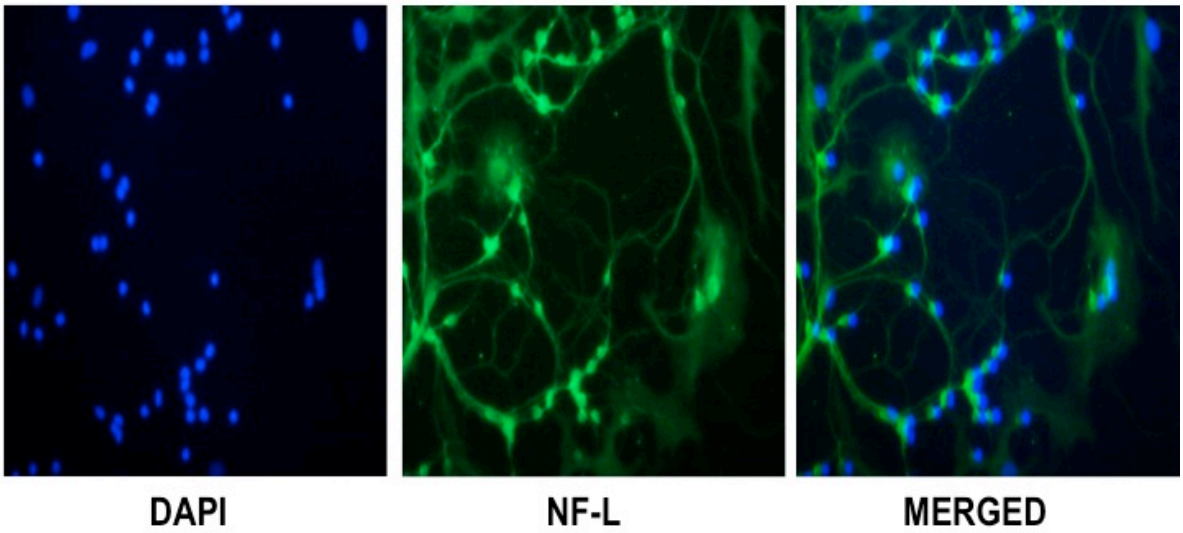

Figure 3.8 NF-L and DAPI immunofluorescencestainings. Alexa Fluor 488 goat anti rabbit polyclonal (green) as secondary antibody presents the neurons (20x magnification).

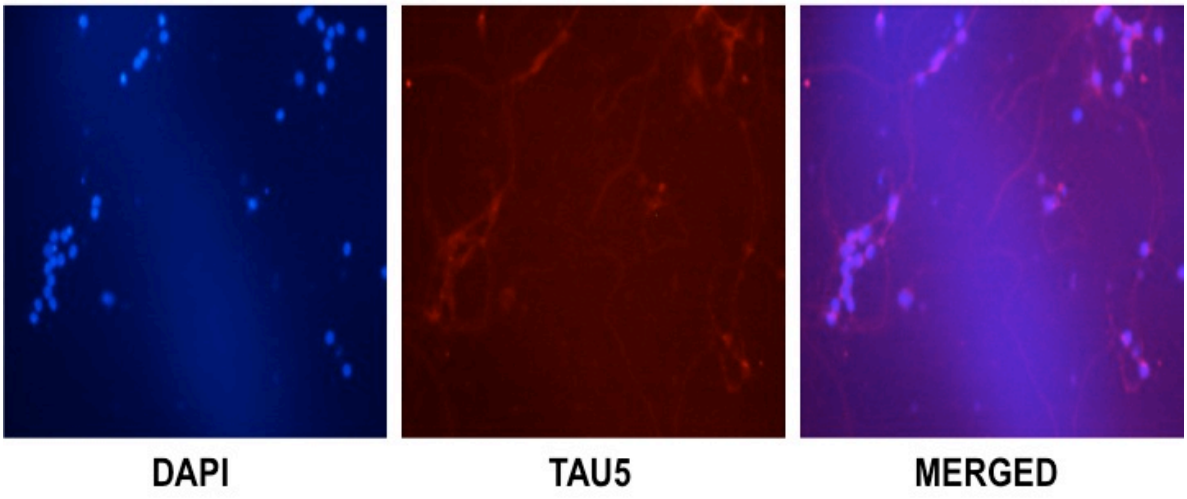

Figure 3.9 TAU5 and DAPI immunofluorescence stainings. Alexa Fluor 594 goat anti mouse monoclonal (red) as secondary antibody shows the growth of axon of neurons (20x magnification).

Both NF-L and TAU5 immunostaining pictures were from PNC#8. Although neuronal networks were established, the axons didn`t seem very healthy and looked like as broken in a way that we couldn`t explain yet.

3.5 ANALYSIS OF AMYLOID BETA (Aβ) 1-42 FIBRILS TOXICITY ON CEREBELLAR GRANULE CELLS via RTCA

All Real Time Cell Analysis results were monitored by xCelligence (Roche) device from 8th primary neuron culture (PNC #8)

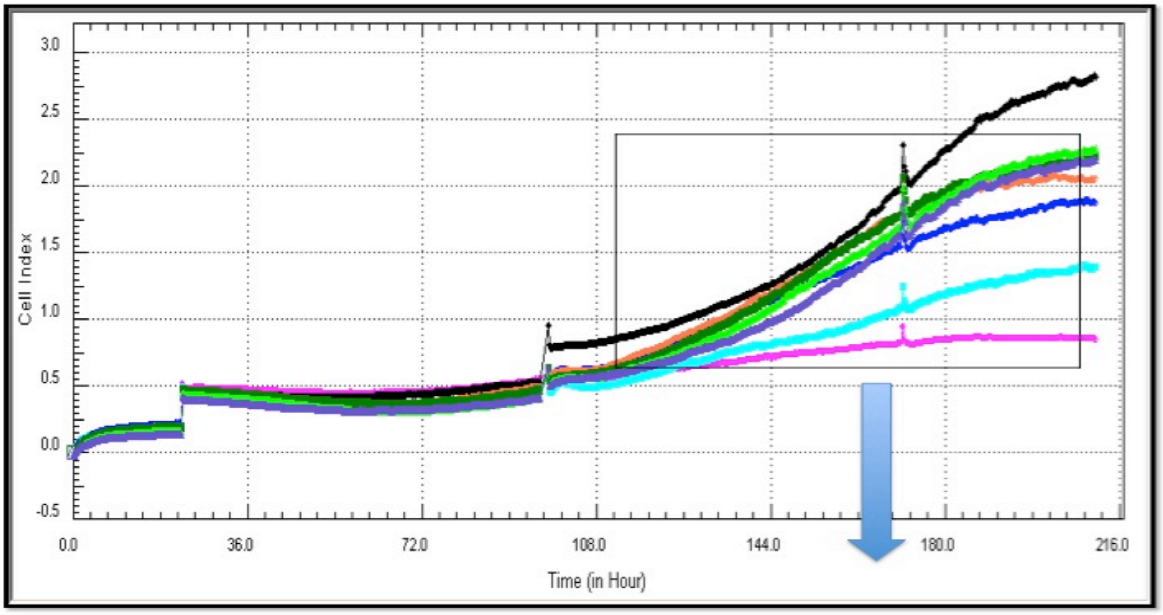

Figure 3.10 Graph of RTCA results during whole experiment time for 24 hours pre-incubation.

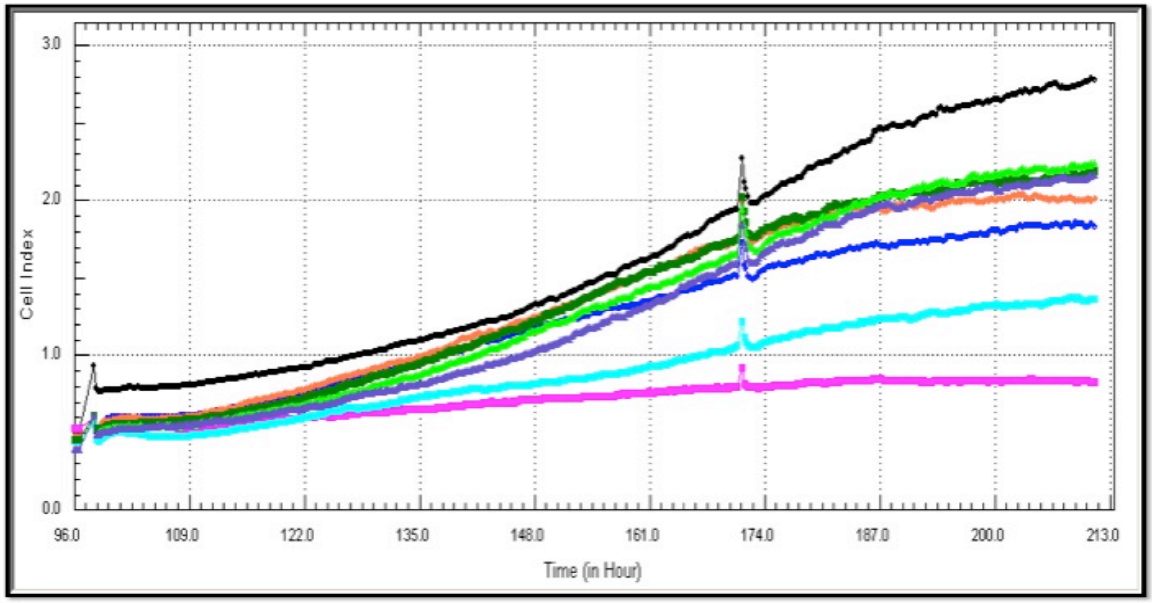

Figure 3.11 Graph of RTCA results for 24 hours pre-incubation. Different concentrations of them at 37^0C Aβ 1-42 fibrils given primary neuron culture on 4th DIV.

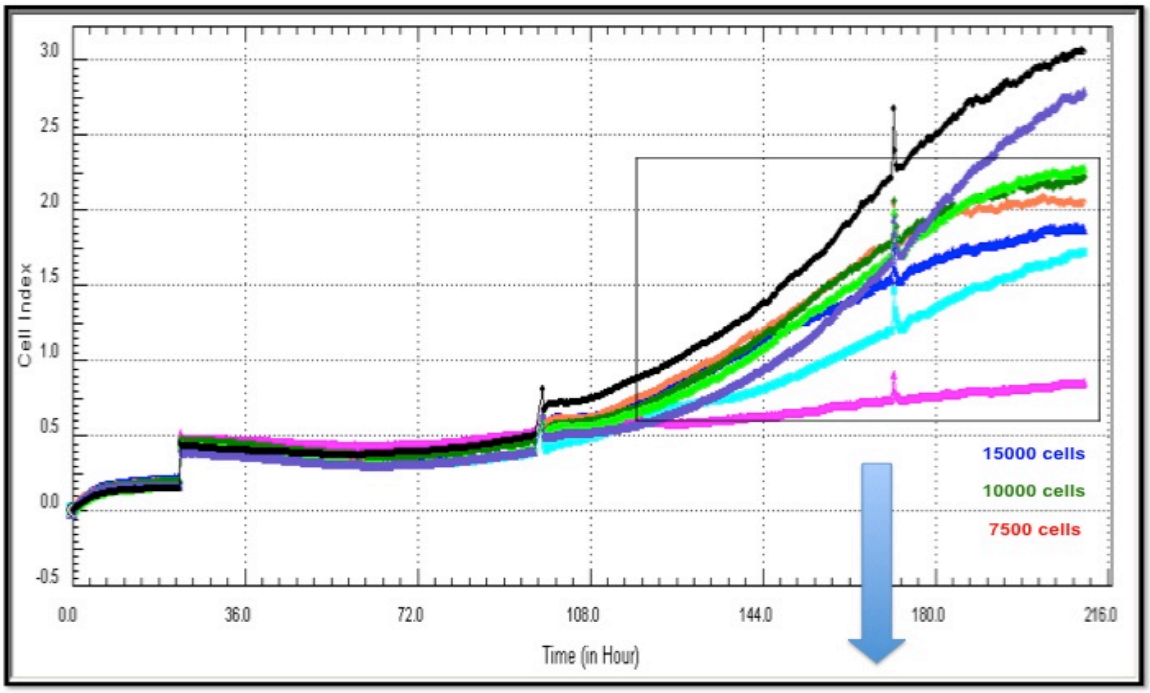

Figure 3.12 Graph of RTCA results during whole experiment time for 48 hours pre-incubation.

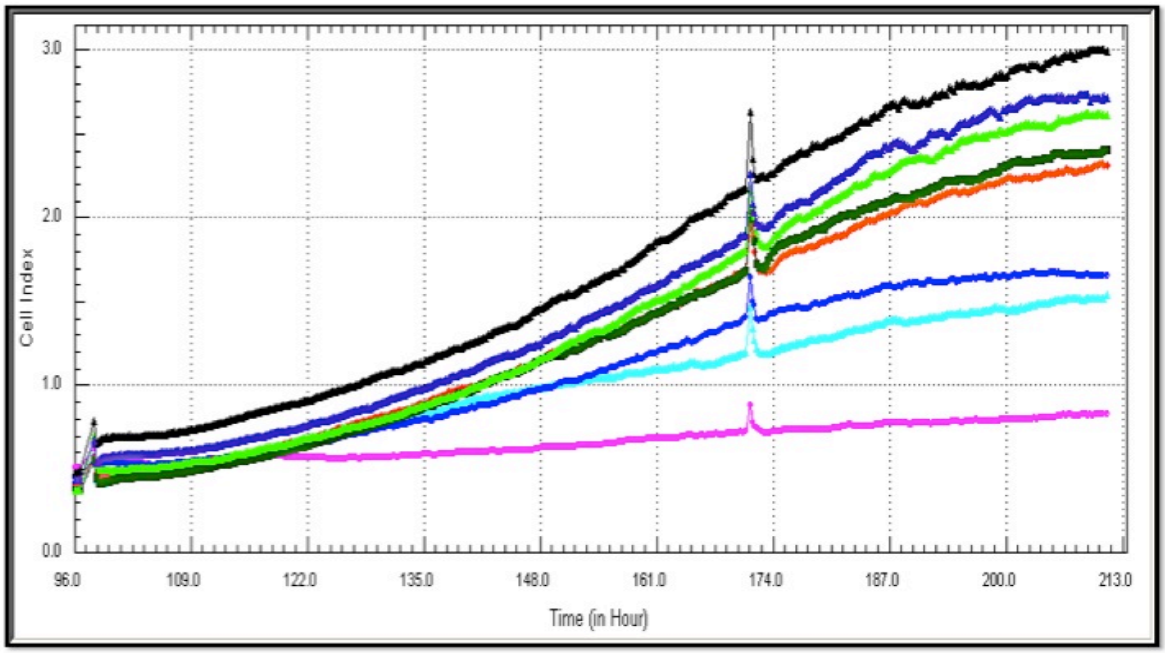

Figure 3.13 Graph of RTCA results for 48 hours pre-incubation. Different concentrations of them at 37^0C Aβ 1-42 fibrils given primary neuron culture on 4^{th} DIV

When it is carefully looked over Figure 3.10 and 13, it is clearly noticed that 48 hours pre-incubated amyloid beta 1-42 fibrils are more instrumental for each concentrations than 24 hours pre-incubated ones on primary neuron culture. For 24 hours duration at lower concentrations beginning from orange line (7 μM) to violet line (0,875 μM) have nearly same effect on cells, they cannot be analyzed separately comparing to 48 hours incubation.

Also, it can be understood that neurons show still an increasing profile even after the amyloid beta 1-42 fibrils exposure. Although they are distinguished from the control which has no amyloid beta 1-42 fibrils (black line), their rise may indicate scant amyloid fibril formation leading to inefficient effects on neurons. Besides this, we used 7 μM amyloid beta 1-42 peptide solution. This concentration seems like intermediary to be neurotoxic on primary neuronal cells. From the graphs, it can be inferred that 14 or 28 μM may give more determining toxicity on neurons.

Last but not least, growth profile for any concentrations of amyloid beta peptides is deviating from the control one at around 148th hour. However, we collected protein samples for western blot and stained cell with immunofluorescence at around 120th hour. This means that incubation with amyloid peptides for 24 hours has incompetent results to see the amyloid effect on neurons as well as for the expression profile of topo IIβ upon amyloid beta fibril exposure.

3.6 DETECTION OF AMYLOID BETA PLAQUE FORMATION IN CEREBELLAR GRANULE NEURON CULTURE via IMMUNOFLUORESCENCE and CONGO RED STAINING

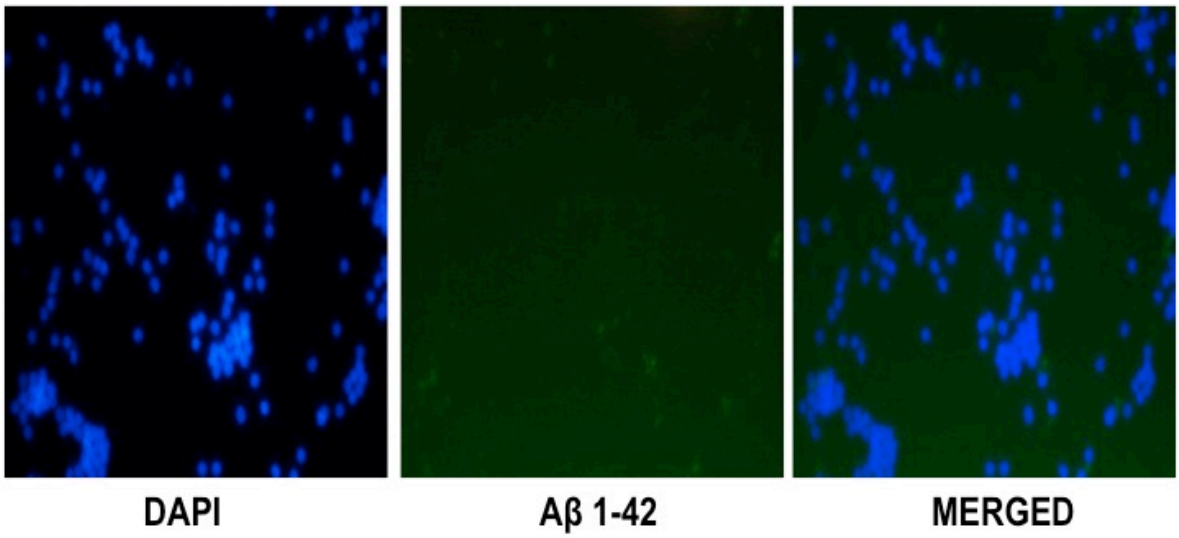

Figure 3.14 Detection of amyloid plaque formation after 24 hours later exposure of 24 hours pre-incubated amyloid 1-42 fibrils (20x magnification).

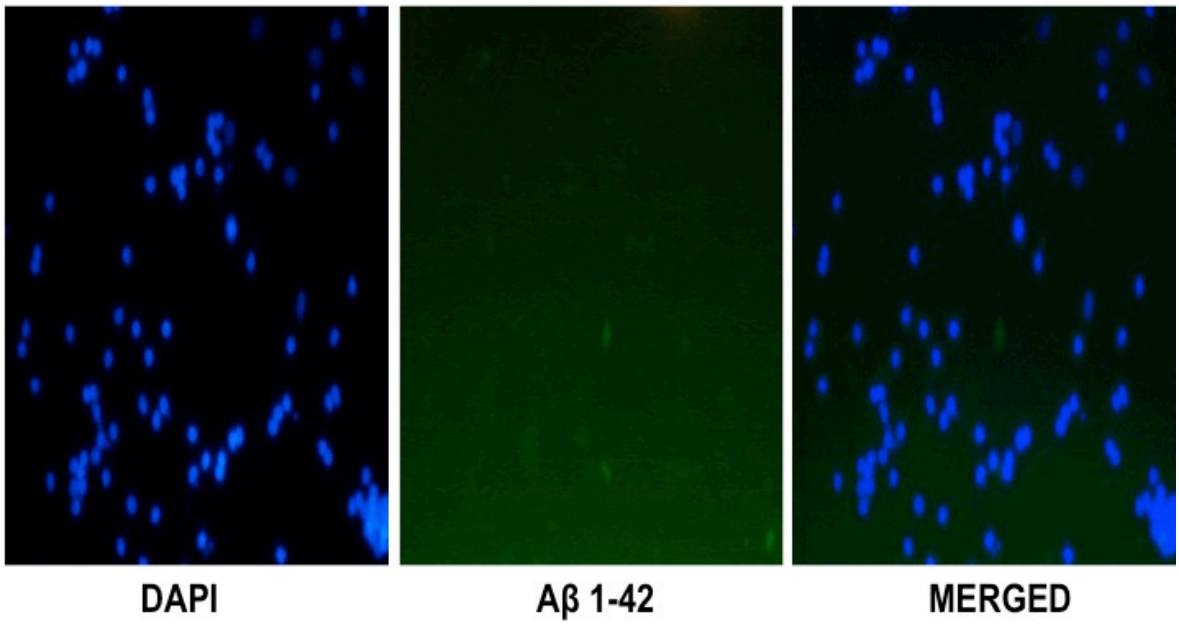

Figure 3.15 Detection of amyloid plaque formation after 24 hours later exposure of 48 hours pre-incubated amyloid 1-42 fibrils (20x magnification).

Primary cerebellar neurons (from PNC#8) were exposed to 7 µM, 24 and 48 hours pre-incubated Aβ 1-42 peptides at their 4th DIV during a day. After 24 hours from exposure, they were fixed with 4%PFA and stained with DAPI (blue) and Aβ 1-42 rabbit polyclonal first antibody (green). As seen from two figures above, localization of Aβ 1-42 between neurons is very dim in both 24 and 48 hours pre-incubated peptides, and unfortunately there seems no difference between them.

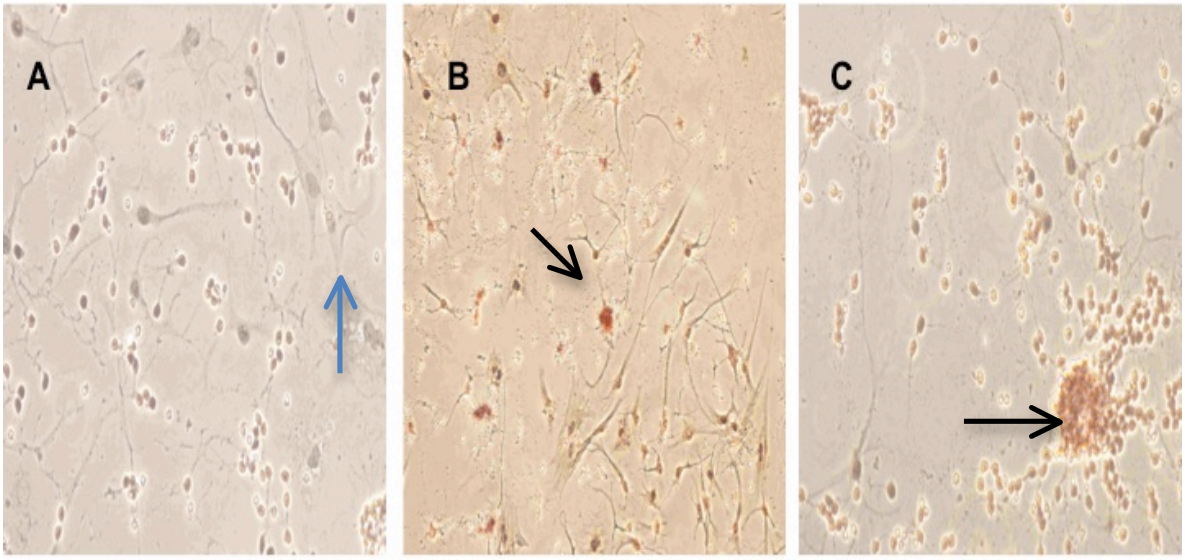

Figure 3.16 Detection of 24 hours pre-incubated amyloid fibrils in primary neuron culture. Photos were taken from different points of sample. Black arrows show amyloid plaques as pink stained by Congo Red. Blue arrow shows example of nuclei staining by Mayer`s hemotoxylin as dark red. A.control(no amyloid) (20x magnification) B.10x magnification C.20x magnification.

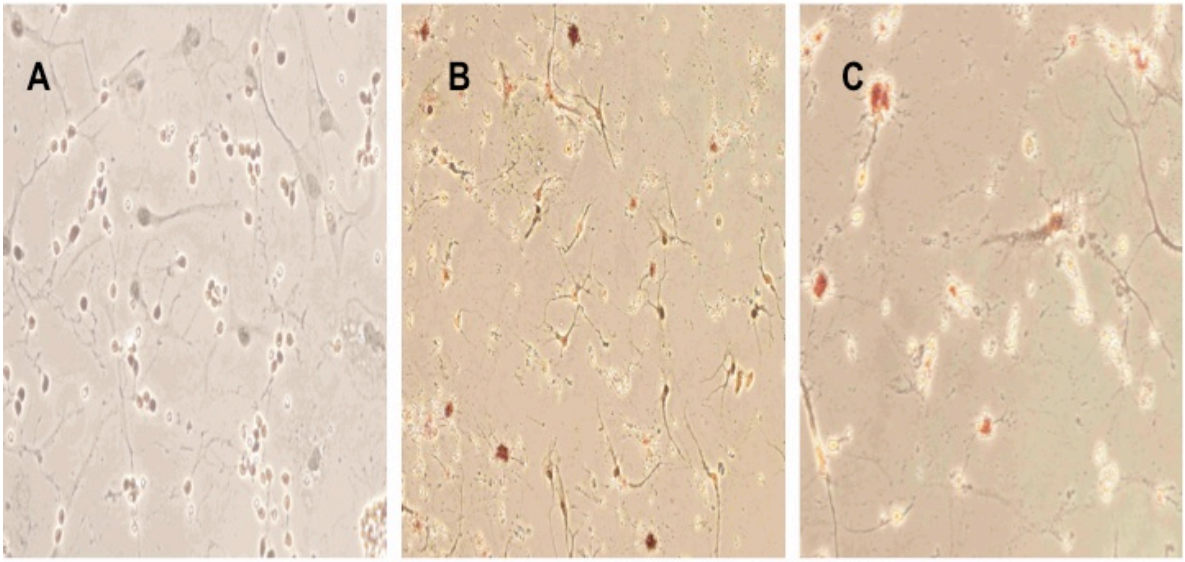

Figure 3.17 Detection of 48 hours pre-incubated amyloid fibrils in primary neuron culture. Photos were taken from different points of sample. **A.**control (no amyloid) (20x magnification) **B.**10x magnification **C.**20x magnification.

Primary cerebellar neurons (from PNC#8) were exposed to 7 μM, 24 and 48 hours pre-incubated Aβ 1-42 peptides at their 4th DIV during a day. After 24 hours from exposure, they were fixed with 4%PFA and stained by Congo red for amyloid plaque detection (pink) and by Mayer`s Hemotoxylen for nuclei stainings (dark red). As can be seen, formation of Aβ 1-42 plaques between neurons is same in both 24 and 48 hours pre-incubated peptides, and unfortunately there seems no difference between them at least morphologically.

3.7 ASSOCIATION OF AMYLOID PLAQUE FORMATION WITH THE EXPRESSION OF TOPII β via WESTERN BLOT TECHNIQUE

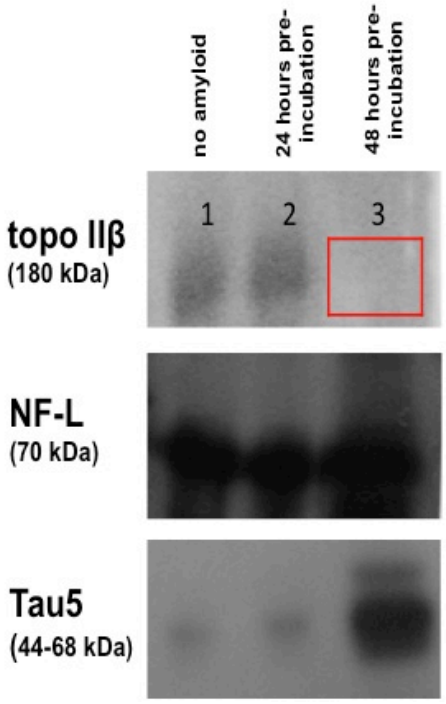

Figure 3.18 Western blot profile of rat primary cerebellum granule neurons (CGNs) (from PNC#8) upon exposure 24 and 48 hours pre-incubated Aβ 1-42 peptide. Line 1 represents primary cerebellar neuron culture containing no amyloid as negative control. Line 2 is for primary cerebellar neuron culture incubated with 24 hours pre-incubated amyloid beta 1-42 peptide for 24 hours. Line 3 is also primary cerebellar neuron culture incubated with 48 hours pre-incubated amyloid beta 1-42 peptide for 24 hours.

At the final step our study, we tried to associate the possible role of DNA topo IIβ with Alzheimer`s disease. For this purpose, primary cerebellar granule cells incubated 7 μM, 24 and 48 hours pre-incubated amyloid beta 1-42 fibrils at 37^0C during a day, then protein samples were collected as described in Chapter 2. 5μg of protein samples were loaded 7.5 % SDS gel, run and blotted. As seen from Figure 3.15, topo IIβ expression looks like same in primary neuron culture samples independently from amyloid beta fibrils exposure as in Line 1 and 2. On the other hand, almost no expression of topo IIβ in 48 hours pre-incubated amyloid beta exposed cells seems promising result, leading to think that increase in neurotoxicity causes much more injury in neurons, so expression of topo IIβ diminishes. Moreover, no change in neurofilament amount in nearly all samples is kind of thought provoking. In manual script of TAU5 from Invitrogen, it is written that it recognizes non-phosphorylated and as well as phosphorylated forms of it. TAU expression is well ahead in 48 hours pre-incubated amyloid exposed cells comparing to others. If this TAU recognized phosphorylated form, it would be really satisfactory result that phosphorylation of Tau upon amyloid toxicity is a hallmark of Alzheimer`s disease.

CHAPTER 4

DISCUSSION & CONCLUSION

Alzheimer's disease (AD) is characterized by progressive loss of memory and other cognitive functions. There are still no solutions to such a complex disease. In many distinct disciplines of science ranging from academia to pharmaceutics, researches have been tried to enlighten both the mechanism and effective treatments of AD. Besides the familial form of AD, its sporadic form contributes to multifactoriality of disease. This may hinder knowing the exact mechanism of the disease as well as its unique cure. It is critical to unravel the process of misfolding Aβ and tau protein which cause distinct histopathological changes by degenerating neurits upon accumulation, impairing neural synapticity in AD brain and leading to neuronal loss.

Topo II is an enzyme that form transient single or double-strand breaks in the DNA and allow DNA strands or double helices to pass through each other. This enzymatic activity has an important role in segregation of daughter chromosomes and thus for cell proliferation [24]. It was found that topo IIα has a role in mitotic processes and only present in proliferating tissues in the study of A.J. Schoeffler et al. [30]. Tsutsui, K. et al. demonstrated that the isoform topo IIβ. is found in terminally differentiated cells and there is no need this enzyme in general cellular activity. It was found that topo IIβ is highly expressed in differentiating cerebellar neurons [24]. Lyu, Y. showed that whole body top2β knockout mice have shown prenatal death. In addition to this it was found that topo IIβ plays a role in activation or repression of developmentally regulated genes at late stages of

neuronal differentiation [27]. Yang et al found that topo IIβ plays a role in axon growth and regulation [25]. And in the study of Nur-E-Kamal et al., it was showed that topo IIβ plays critical role in forming and maintaining the growth cone morphology and may affect neurite outgrowth through its regulation of the expression of certain neuronal genes [28].

In the previous study of ISIK`s Lab, supported by TUBITAK(106S279), it was studied the role of topo IIβ during neural differentiation in human mesenchymal stem cells by silencing it. In addition to contrubution the role of topo IIβ in neuronal differentiation, microarray results of this study elicits changes in gene expression related to Rho family and Alzheimer disease upon silencing of topo IIβ. Besides these genes, there was a remarkable decrease in axon length of neuronal differentiated topo IIβ silenced cells. From this aspect, we interrogate that whether or not there is a correlation between the function of topo IIβ and neurite shortening of Alzheimer`s disease.

In this study, we proposed to establish an *in vitro* Alzheimer model in rat primary cerebellar neuron culture and to associate topo IIβ function with the disease. With this regard, post natal 9 days old Wistar rats were taken from Bezmialem University with the ethic committee. They were sacrificed at the Fatih University, and their cerebellum was used as primary neuronal culture source. The reason why we choose cerebellum is as mentioned in both introduction and discussion parts that topo II β expression level is high at that region of the brain. Also, to obtain pure neuron culture cerebellum is a good choice because it contains more neurons than the rest of the brain put together, but it takes up only 10% of total brain volume because of its large number of tiny granule cells [29]. Primary neuronal culture is not easy to isolate, to handle, and to survive culture long time but have advantages over the immortalized cell lines which are less analogous to *in vivo* conditions. After cerebellar granule cells dissociated well from each other under sterile conditions, they were seeded on previously PLL coated plastics. Coating is straightforward but an important step to obtain healthy neurons for long time. We tried to optimize the appropriate concentration of PLL for our cerebellar culture because we needed to keep cells at least for 14 days in culture making them older to start age related *in vitro* Alzheimer model establishment trials. So far, cells have been almost kept in culture, supplied with Neurobasal media plus mitotic inhibitor (AraC) against non-neuronal cell growth, for 7

days by refreshing every 3 days. At the first trials, from immunofluorescence results the confluency of neurons were less in dominance in culture as can be seen from figures 3.6 which indicated the presence of non-neuronal glial cells. These results were confirmed also by western blot ones, they correlated with each other. Yet, we luckily improved the culture conditions with the more practice in culture. As seen from Figure 3.7 and 3.8, neuron population increased in the culture by time. During these optimization steps, we recognized the importance of using fresh mediums for cell culture in order to get happily neuronal networks. Besides that, favoring substrata for cerebellar neuronal cells is really crucial and laborious. We understood it after changed the brand of plastic wares from Greiner to TPP in use of seeding cells.

To assess which concentration for cell seeding is optimal, we applied to real time cell analysis method with the help of Roche, x-Celligence. Confluency of cells *in vitro* culture conditions is important because any contact inhibition may cause deaths in neurons. Therefore, we worked with three different cell concentrations like 7500, 10000 and 15000 cells per 96 well e-plate. As seen from Figure 3.3, their growth curves were plotted for the first 20 hours and 96 hours. The latter one shows us the progress of cells until 4^{th} in DIV just before application any other steps such as immunofluorescence, western blot and amyloid beta exposure as a next step. We assigned it as 4^{th} in DIV, because we tested many times 5^{th} day is critical for culture because that day glial cells start to arise in population which we could not control even though we use AraC as mitotic inhibitor. Meanwhile, we decided to 10000 cells/well for 96 well plate is commensurable for us, the growth in all wells were almost in consistent with each other.

In addition, we tried to optimize the concentration of AraC used for neuronal culture treatments. Cytosine arabinoside (AraC) is a pyrimidine antimetabolite that prevents cell proliferation by inhibiting DNA synthesis [31]. To study AraC effect, we chose 7500 cells/well on 96 well e-plate. As seen from Figure 3.5, Ara C has effect on our cells negatively starting from the higher concentration to the least one as expected. In fact this means the presence of glial cells besides neurons in our culture. As, AraC has a fatal effect on only non-neuronal mitotic cells at appropriate concentrations. In culture studied we used

10 μM of it, and it seems pretty adequate to limited glial cell division to preserve neuronal conformity.

After accomplishment a satisfactory *in vitro* cerebellar neuronal environment, we focused on the establishment of *in vitro* Alzheimer`s disease model on primary cerebellar granule cells. The first step in this process was the preparation of amyloid beta fibrils. As we known, neurodegeneration in Alzheimer's disease is thought to be associated with accumulation of amyloid beta which normally folded Aβ peptide in the brain [32]. There are various aggregates forms of Aβ such as monomer, oligomer, fibril and other polymer states. What make different them are their methods during formation. There are number of methods for the preparation of these form. Solvent for dissolution of lyophilized peptide, temperature and time are parameters for incubation during aggregate formation process. Furthermore, their binding capacity to different neuronal cells is also different. For example, globular amyloid beta 1-42 can not successfully bind to non aged hippocampal neurons [33]. Typically most of these preparations involve the preliminary generation of a stock of Aβ which is largely monomeric [34]. Small soluble oligomers are considered as the primary neurotoxic species, though mechanisms by which Aβ oligomers cause cell death remain unclear. It has been shown that soluble fibril-free Aβ preparations are toxic to mouse brain slice cultures following 24-h exposure [35].

In our study, we pre-incubated amyloid beta 1-42 peptide diluted with ammonium hydroxide and subsequently with miliQwater (1mg/ml) at 37^0C for 24 and 48 hours, separately. It would expect to form in fibrils forms rather than oligomer. And we applied 7 μM of them to the cerebellar granule cells on 4^{th} DIV. Based on the numerous results from immunofluorescence, western blot, Congo red stainings and RTCA analysis, it is obvious that we should focus much more on these pre incubation conditions for the amyloid beta fibrils formation. From Figure 3.10 and 11, it can be examined that almost of all concentrations of 24 hours pre incubated amyloid beta fibrils did not discriminative effect on neurons, separately. Especially, same declining profile of the cells at lower concentrations were not satisfactory compared to the case in 48 hours pre-incubated forms. Therefore, 48 hours pre incubation might be adequate for our further trials to see effectively the neurotoxicity of amyloid beta fibrils on our neurons. Moreover, we inferred from the

same results, the concentration of amyloid beta 1-42 fibrils as 7 μM seems a slim choice to establishment amyloid beta plaques. This is confirmed from not only RTCA results but also immunostainings results. According to the results, at least 14 and 28 μM indicated that amyloid beta peptides are toxic to cells. Last but not least, amyloid beta plaque were exposed to primary neuronal cells only for 24 hours, then we took the result. However, it is referred from RTCA graphs that the effects of all concentrations began to appear markedly after at 120 hours in DIV meaning that long term and continuous exposure of amyloid beta 1-42 peptides may give us much more dedicative neurotoxicity results. From the literature, it is commonly said that only after 5-7 days subsequent exposure of amyloid beta 1-42 peptide create defects in neurons such as triggering caspase activity and mitochondrial oxidation etc [36].

From immunofluorescence and Congo staining results unfortunately verify us about pre-incubations steps which have no differences for the localization of amyloid plaques between neurons in 24 and 48 hours pre-incubated fibrils.

As a last step, the expression profile of topo IIβ upon amyloid plaque treatment in primary neuronal culture was examined to give evidence its role in axonogenesis and neurite development. Despite the fact that we can not morphologically satisfied for the formation of amyloid plaques, this case is not same for western blot results. They are really expected ones. Almost no expression of topo IIβ in 48 hours pre-incubated amyloid beta exposed cells would be a promising result, leading to think that increase in neurotoxicity causes much more injury in neurons, so expression of topo IIβ diminishes as from seen Figure 3.18.

To conclude, we have shown the importance and difficulties of obtaining a pure neuron culture for Alzheimer disease studies. We are almost successful. We achieved to get pure neuronal culture as seen in the neuronal marker expression profile. Besides this, upon amyloid plaque treatment of cells, we nearly reach to see downregulation of topo IIβ expression levels leading to axon and neurite shortening as in Alzheimer disease. From the study of our lab, we saw that with the topo IIβ downregulation , the genes related to Alzheimer`s disease were affected. In this study, we evidence this association between topo IIβ and Alzheimer`s disease, too. Now, the question arises inevitably that whether topo IIβ

downregulation triggers neuronal defects in Alzheimer`s disease or Alzheimer`s disease causes such neuronal abnormalities related to topo IIβ activity. Therefore, it should be examined the key enzyme role of topo IIβ in another neurodegenerative diseases such as Parkinson or Multiple Sclerosis in order to determine the exact mechanism of topo IIβ how brings on neuronal degeneration.

REFERENCES

[1] Bird, T. D., *Alzheimer's Disease Overview,* Gene Reviews, Seattle, Wash, USA, 1998.

[2] Xiong, L., Gaspar, C., and Rouleau, G.A. , "Genetics of Alzheimer's Disease and Research Frontiers in Dementia", *Geriatrics Aging*, Vol.8, pp.31-35, 2005.

[3] Francis, P.T., Palmer, A.M., Snape, M. and Wilcock, G.K., "The Cholinergic Hypothesis of Alzheimer`s Disease; a Review of Progress", *J.Neurol. Neurosurg.Psychiatr*, Vol. 66, pp.137-147, 1999.

[4] Bartzokis, G., "Alzheimer`s Disease as Homeostatic Responses to Age-related Myelin Breakdown", *Neurobiol. Aging*, Vol. 32, pp.1341-1371, 2011.

[5] Su, B., Wang, X. and Nunomura, A., "Oxidative Stress Signaling in Alzheimer`s Disease", *Curr. Alzheimer Res.*, Vol.6, pp.525-532, 2008.

[6] Turner, P.R., O`Connor, K., and Abraham, W.C., "Roles of amyloid precursor protein and its fragments in regulating neural activity, plasticity and memory", *Prog. Neurobiol.*, Vol.70, pp.1-32, 2003.

[7] Duce, J.A., "Iron-Export Ferroxidase Activity of β- Amyloid Precursor Protein is Inhibited by Zinc in Alzheimer`s Disease", *Cell*, Vol.142, pp.857-867, 2010.

[8] Spuch, C., Ortolano, S., and Navarro, C., "New Insights in the Amyloid-Beta Interaction with Mitochondria", *J. Aging Res.*, Vol.2012, pp.2012-2021, 2012.

[9] Wang, H., Megill, A., He, K., Kirkwood, A. and Lee, H.K., "Consequences of Inhibiting Amyloid Precursor Protein Processing Enzymes on Synaptic Function and Plasticity", *Neural Plasticity,* Vol. 2012 , pp. 272374- 272398, 2012.

[10] Strooper, B.D. and Annaert, W., "Proteolytic processing and cell biological functions of the amyloid precursor protein", *J. Cell Science,* Vol. 113, pp. 1857-1870, 2000.

[11] Lakshmana, M.K., Roy, S., Mi, K. and Kang, D.E., "Amyloidogenic Processing of APP in Lipid Rafts", *The Open Biology Journal*, Vol.3, pp. 21-31, 2010.

[12] Murphy, M.P. and Vine, L.H., "Alzheimer's Disease and the Amyloid-β Peptide", *J. Alzheimer`s Disease*, Vol. 19, pp.311-323, 2010.

[13] Metcalfe, M.J. and Figueiredo-Pereira, M.E., "Relationship Between Tau Pathology and Neuroinflammation in Alzheimer's Disease", Mt Sinai J Med., Vol. 77, pp. 50-58, 2010.

[14] Igbal, K., Liu, F., Gong, C.X. and Igbal, I.G., "Tau in Alzheimer Disease and Related Tauopathies", Curr. Alzheimer Res., Vol.7, pp. 656-664, 2011.

[15] Götz, J. and Ittner, L.M., "Box 1 | APP processing and tau phosphorylation", *Nature Reviews Neuroscience,* Vol.9, pp. 532-544, 2008.

[16] Corbett, K.D. and Berger, J.M., "Structure, molecular mechanisms, and evolutionary relationships in DNA topoisomerases", *Annu. Rev. Biophys. Biomol. Struct*., Vol.3, pp. 95-118, 2004.

[17] Champoux, J.J., "DNA TOPOISOMERASES: Structure, Function, and Mechanism", *Annual Review of Biochemistry*, Vol. 70, pp. 369-413, 2001.

[18] Kabos, P., Ehtesham, M., Kabosova, A., Black, K.L. and Yu, J.S., "Generation of neural progenitor cells from whole adult bone marrow", *Exp. Neurol.,* Vol. 178, pp. 288-93, 2002.

[19] Mueller-Planitz, F. and Herschlag, D., "DNA topoisomerase II selects DNA cleavage sites based on reactivity rather than binding affinity ", *Nucleic Acids Research*, Vol. 35, pp.3764-3773, 2007.

[20] Kondapia, A.K., Mulpuria, N., Mandrajua, R.K., Sasikaranb, B., Raoa, K.S., "Analysis of age dependent changes of TopoisomeraseII α and β in rat brain", *Int. J. Devl Neuroscience,* Vol. 22, pp. 19–30, 2004.

[21] Sakaguchi A., Kikuchi A., "Functional compatibility between isoform α and β of type II DNA topoisomerase", *J Cell Science*, Vol. 117, pp. 1047- 1054, 2004.

[22] Jurenke, J.M. and Holden, J.A., "Distribution of DNA Topoisomerase II isoforms in differentiated adult mouse", *Tissue Biochem. Biophys. Acta.,* Vol. 1216, pp.191-196, 1993.

[23] Tsutsui, M.K., Sano, K., Hosoya, O. and Tsustsui, K., "Expression dynamics and functional implications of DNA topoisomerase IIβ in the brain", *Anatomical Science International,* Vol. 81, pp. 156-163, 2006.

[24] Tsutsui, K., Tsutsu, K., Sano, K., Kikuchii, A. and Tokunaga, A., "Involvement of DNA Topoisomerase IIβ in Neuronal Differentiation", *J Biological Chemistry*, Vol. 276, pp.5769-5778, 2001.

[25] Yang, X., Li, W., Prescott, E.D., Burden, S.J. and Wang. J.C., "DNA topoisomerase IIbeta and neural development", *Science,* Vol.287, pp.131–134, 2000.

[26] Lyu, Y.L., Wang, J.C., "Aberrant lamination in the cerebral cortex of mouse embryos lacking DNA topoisomerase IIbeta", *Proc Natl Acad Sci,* Vol. 100, pp.7123–7128. 2003.

[27] Lyu, Y.L., "Role of topoisomerase IIbeta in the expression of developmentally regulated genes", *Mol Cell Biol*, Vol. 26, pp. 7929–7941, 2006.

[28] Kamal A, N.E., Meiners, S., Ahmed, I., Azarova, A., Lin, C.P., Lyu, Y.L. and Liu, L.F., "Role of DNA topoisomerase IIbeta in neurite outgrowth", Brain Res, Vol. 1154, pp. 50-60, 2007.

[29] Llinas, Walton and Lang, *The Synaptic Organization of the Brain*, Oxford, 2004.

[30] Schoeffler, A.J. and Berger, J.M., " Recent advances in understanding structure– function relationships in the type II topoisomerase mechanism." *The Nucleus and Gene Expression*, Vol. 33, pp. 1465-1470, 2005.

[31] Dessi, F., Pollard. H., Moreau, J., Ben-Ari, Y. and Charriaut-Marlangue, C., " Cytosine Arabinoside Induces Apoptosis in Cerebellar Neurons in Culture." *Journal of Neurochemistry*, Vol. 64, pp. 1980-1987, 2002.

[32] Plečkaitytė M., " Alzheimer's disease: a molecular mechanism, new hypotheses, and therapeutic strategies. " *Medicina (Kaunas)*, Vol. 6, pp. 46-70, 2010.

[33] Barghorn, S. et. al., "Globular amyloid b-peptide1-42 oligomer a homogenous and stable neuropathological protein in Alzheimer's disease. " *Journal of Neurochemistry,* Vol. 95, pp. 834-847, 2005.

[34] Stine, W.B., J.r., Dahlgren, K.N., Krafft, G.A., LaDu M.J., " In vitro characterization of conditions for amyloid-beta peptide oligomerization and fibrillogenesis. " *Journal of Biological Chemistry*, Vol. 278, pp. 11612–11622, 2003.

[35] Lambert MP, Barlowa K, Chromy BA, Edwards C, Freed R, Liosatos R, et al., " Diffusible, non fibrillar ligands derived from Abeta1-42 are potent central nervous system neurotoxins." *Proc. Natl. Acad. Sci. USA,* Vol. 95, pp. 6448-53, 1998.

[36] Deshpande, A., Mina, E., Glabe, C., and Busciglio, J., "Different Conformations of Amyloid Beta Induce Neurotoxicity by Distinct Mechanisms in Human Cortical Neurons." *Journal of Neuroscience,* Vol. 26, pp. 6011-6018, 2006.