ISTANBUL TECHNICAL UNIVERSITY ★ GRADUATE SCHOOL OF SCIENCE ENGINEERING AND TECHNOLOGY

ANTI-APOPTOTIC EFFECTS OF SPEEDYRINGO IN NEURODEGENERATION

Ph.D. THESIS

Ayşegül ÜNAL

Department of Advanced Technologies

Molecular Biology - Genetics and Biotechnology Programme

NOVEMBER 2012

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İSTANBUL TEKNİK ÜNİVERSİTESİ ★ FEN BİLİMLERİ ENSTİTÜSÜ

SPEEDYRİNGO PROTEİNİNİN NÖRODEJENRASYONDAKİ ANTİ-APOPTOTİK ETKİLERİ

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To my son Kerem,

FOREWORD

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ABBREVIATIONS

UV	:Ultra-violet
CDK5	:Cylin dependent kinase 5
PN0	:Post-natal 0
HEPES	:4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
FBS	:Fetal bovine serum
DAPI	:4',6-diamidino-2-phenylindole
qRT-PCR	:Quantitative Real Time Polymerase Chain Reaction
DMSO	:Dimethyl sulfoxide
BCA	:Bicinchoninic acid
BSA	:Bovine serum albumin
RFU	:Relative fluorescence units
ACTB	:Beta-actin
GAPDH	:Glyceraldehyde 3-phosphate dehydrogenase
PBS	:Phophate Buffered Saline
TTBS	:Tris Buffered Saline-Tween
CDK2	:Cyclin dependent kinase 2
LDH	:Lactate dehydrogenase
ELISA	:Enzyme-linked immunosorbent assay
SPSS	:Statistical Package for the Social Sciences Statistical Analysis Program
pRb	:Retinoblastoma protein
E2F	:E2 promoter binding factor
CDK1	:Cyclin dependent kinase 1
RINGO	:Rapid Inducer of G_2/M progression in oocytes
DNA	:Deoxyribonucleic acid
µg	: Microgram
µl	: Microliter
µM	: Micromolar
dNTP	: Deoxyribonucleotide
DNase	:Deoxyribonuclease
ACTB	:Actin,beta
GAPD	: Glyceraldehyde-3-Phosphate Dehydrogenase
IgG	:Immunoglobulin G
HRP	:Horse Radish Peroxydase
E.coli	: *Escherichia coli*
$MgCl_2$	:Magnesium Chloride
TEMED	: Tetramethylethylenediamine
PIPES	: Piperazine-N,N′-bis(2-ethanesulfonic acid)
APS	: Ammonium persulfate
$CaCl_2$	:Calcium chloride
KCl	:Pottasium chloride
DTT	:Dithiothreitol
NaCl	:Sodium chloride
PEG	:Polyethylene glycol

RNA :Ribonucleic acid
TAE :Tris-acetate-EDTA
SDS-PAGE :Sodium dodecyl sulphate-polyacrylaimde gel electrophoresis
EDTA :Ethylenediaminetetraacetic acid
TBS :Tris buffer saline
HCl :Hydrochloride
LB :Luria Broth
SOC :Super Optimal Broth
ECMV :Encephalomyocarditis virus
IRES :Internal ribosome entry site
MCS :Multiple Cloning Site
IVS :Intervening Sequence
TUNEL :Terminal deoxynucleotidyl transferase dUTP nick end labeling

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ANTI-APOPTOTIC EFFECTS OF SPEEDYRINGO IN NEURODEGENERATION

SUMMARY

Neurons are known to be terminally differentiated non-mitotic cells. However, recent studies have shown an up-regulation of cell cycle markers causing abnormal re-activation of cell cycle in neurons in case of neurodegeneration. This unprogrammed cell cycle re-entry is indicated to be one of the earliest pathological symptoms of the most of the neurodegenerative diseases, especially that of Alzheimer's disease. In case of Alzheimer's disease, neurons have been shown to leave the resting G_0 phase and re-enter into cell cycle and even replicate their genomes, but this process cannot be successfully completed and degenerating neurons undergo apoptosis, since they do not have the mitotic machinery.

There are a number of pathological conditions causing neurodegeneration such as amyloid beta aggregation, oxidative stress and metabolic alterations. Amyloid beta aggregation, a hallmark of Alzheimer's disease, causes destabilization of calcium homeostasis, which ends up with the elevation of intracellular calcium levels leading to pathological activation of a calcium-sensitive cysteine-protease, calpain. Calpain over-activity has been shown to be very important in terms of causing cell cycle re-activation and degeneration in neurons.

Pathologic activation of calpain force neurons into apoptosis directly or indirectly. Indirect way is that overactivated calpain cleaves p35 protein into p25 and p10 fractions. Under normal conditions, p35 is the partner for CDK5, which is a non-mitotic neuron-specific kinase. They form CDK5/p35 complex that takes place in a number of cellular events in neurons. However, over-activity of calpain in neurons results in cleavage of p35 into p25 and p10 fractions, as p35 is a substrate of calpain. p25 is able to bind to CDK5 to form CDK5/p25 complex. However, this complex differs from CDK5/p35 complex in terms of localization and functions. Normally, Cyclin D-CDK4 complex is necessary for the activation of cell cycle. This complex drives cells through G_1/S transition by pRb phosphorylation, but in case of calpain induction in neurons it has been shown that CDK5/p25 complex phosphorylates the pRb protein instead of Cyclin D-CDK4 complex and re-activates cell cycle driving neurons through G_1/S transition. However, this abnormal cell cycle re-activation cannot be completed and neurons undergo apoptosis by an increase in p53 level. In addition, CDK5/p25 complex can activate p53 directly, as p53 is one of the substrates of CDK5. All these findings strongly indicate that calcium-mediated over-activation of calpain causes an increase in p53 expression and activation, and ultimately ends up with caspase-dependent apoptosis in degenerating neurons.

Direct way of calpain-mediated apoptosis is that p53 is the substrate of calpain itself and thus, calpain over-activation allows increased p53 expression and activation. Induced p53 triggers mitochondrial death signals and caspase-3 activation finally resulting in caspase-mediated apoptosis.

Although cyclins are major players that take role in activation of CDKs to regulate progression of the cell cycle, there is a novel regulatory protein, SpeedyRINGO, which can activate CDKs. In addition to its role in regulating the cell cycle, SpeedyRINGO has also been indicated to take role in preventing caspase-dependent apoptosis in mitotic cells.

Although main function of SpeedyRINGO is the regulation of cell cycle in mitotic cells, recent studies have also attributed a different role for SpeedyRINGO, such that it has an anti-apoptotic effect in DNA-damaged mitotic cells allowing cell survival. It has been shown that SpeedyRINGO prevents apoptosis in a p53-dependent manner in response to DNA damage in U2OS cells, which is a mitotic human osteosarcoma cell line. SpeedyRINGO was indicated to have strong protective effect in mitotic cells that encountered a number of extrinsic or intrinsic apoptotic factors such as UV irradiation. SpeedyRINGO fulfills this function by inhibiting caspase-3 activation and apoptosis in the presence of the gene regulatory protein p53.

Therefore, major aim of this study was to regulate cell cycle reactivation and eliminate the apoptotic effects of pathologic calpain induction by expressing SpeedyRINGO in neurons.

For this purpose, hippocampal neurons are electrophoretically transfected with SpeedyRINGO and then, calpain was induced by calcium ionophore. After that, QRT-PCR, immunofluorescence and western blotting analysis were performed to determine the changes in different cell cycle markers and in CDK5, Cyclin D1, p27, CDK2, p53 and active caspase-3 mRNA and protein levels in order to understand whether SpeedyRINGO could protect neurons against caspase-mediated apoptosis in the presence of p53. Results showed that calpain induction caused cell cycle re-activation through CDK5 instead of Cyclin D, since mRNA and protein level of CDK5 increased, while Cyclin D levels did not change. Besides that, calpain induction resulted in p53 up-regulation and subsequent caspase-3 activation, whereas SpeedyRINGO expression prevented activation of caspase-3 in the presence of p53. Furthermore, DAPI staining and TUNEL assays were performed for detection of apoptosis. Apoptotic analysis supported these findings that calpain overactivated neurons were in early apoptotic stage, while SpeedyRINGO expressing calpain overactivated neurons were not apoptotic.

In this study, it has been shown for the first time that caspase-3 activation after up-regulation of p53 caused by calpain over-activity is prevented by Speedy/RINGO in degenerating neurons. Therefore, Speedy/RINGO acts as a lifesaver for degenerating neurons by inhibiting caspase-dependent apoptosis.

SPEEDYRİNGO PROTEİNİNİN NÖRODEJENRASYONDAKİ ANTİ-APOPTOTİK ETKİLERİ

ÖZET

Nöronlar terminal olarak farklılaşmalarını tamamlamış, bu nedenle de bölünme özelliklerini kaybetmiş hücrelerdir. Fakat,yapılan son çalışmalar göstermiştir ki nörodejenerasyon durumunda, hücre siklus belirteçlerinde belirgin bir artış olmakta ve bu artış nöronlarda hücre siklusunun normal olmayan bir şekilde aktifleşmesine aktive olmasına sebep olmaktadır. Bu programsız hücre siklus aktivasyonunun birçok nörodejeneratif hastalığın, özellikle de Alzheimer hastalığının en erken belirtilerinden biri olduğu kabul edilmektedir. Alzheimer hastalığında, nöronlar G_0 adı verilen dinlenme fazından çıkarak yeniden hücre siklusuna girmekte ve hatta genomlarını replike etmektedirler. Fakat bu proses, nöronlar mitotik mekanizmaya sahip olmadıklarından, başarılı bir şekilde tamamlanamamakta ve dejenerasyona uğrayan nöronlar apoptoza gitmektedirler.

Amiloid beta birikimi, oksidatif stres, metabolik değişiklikler gibi bir çok sebep nöronları dejenerasyona sürüklemektedir. Özellikle, Alzheimer hastalığının en önemli işaretlerinden biri olan amiloid beta birikimi, nöronlardaki kalsiyum dengesinin bozulmasına ve hücre içi kalsiyum konsantrasyonunun artması sonucu bir sistein-proteaz olan kalpain enziminin patolojik olarak aşırı aktive olmasına sebep olmaktadır. Kalpainin aşırı aktivasyonunun ise nöronlarda hücre siklus aktivasyonu ve apoptoza sebep olan en önemli etkenlerden biri olduğu belirtilmektedir.

Kalpainin patolojik olarak aktive olması nöronları direkt ya da dolaylı yoldan apoptoza sürüklemektedir. Dolaylı yol, kalpainin p35 adlı proteini p25 ve p10 olarak iki parçaya kesmesidir. Normal şartlarda, p35, CDK5 adı verilen ve nörona spesifik olup mitotik olmayan bir proteinin partneridir ve ikisi CDK5/p35 kompleksini oluşturarak nöronlarda farklılaşma ve göç gibi bir çok farklı görevde yer alırlar. Fakat, kalpainin aşırı aktivasyonu durumunda p35, kalpain tarafından p25 ve p10 parçalarına ayrılır. p25 proteini de, p35 gibi CDK5 ile kompleks oluşturma özelliğine sahiptir. Fakat CDK5/p25 kompleksi, CDK5/p35 kompleksinden lokalizasyon ve fonksiyon bakımından farklılık gösterir. Normal şartlarda, hücre siklus aktivasyonu için gerekli olan kompleks Siklin D-CDK4 kompleksidir ve bu kompleks pRb proteinini fosforlayarak hücreleri G_1/S geçişine hazırlar. Kalpain aşırı aktivasyonu durumunda ise, nöronlarda hücre siklus aktivasyonuna sebep olarak pRb proteinini fosforlayan kompleksin siklin D-CDK4 yerine CDK5/p25 kompleksi olduğu gösterilmiştir. Fakat bu hücre siklusuna giriş çabası başarısızlıkla sonuçlanmakta ve nöronlar p53 seviyesinde ve aktivitesindeki bir artış sonucu apoptoza gitmektedirler. Bunlara ek olarak, CDK5/p25 kompleksi p53 proteinini substrat olarak kullanıp doğrudan aktifleştirebilmektedir. Bütün bu veriler, kalsiyum ile aşırı aktive olan kalpainin nöronlarda hücre siklusunun yeniden aktivasyonuna sebep olduğunu,

ayrıca p53'ün ekspresyonunda ve aktivasyonunda bir artışa sebep olarak dejenere olan nöronları apoptoza sürüklediğini göstermektedir.

İndirekt apoptotik etkisinin yanısıra, kalpain aynı zamanda p53'ü substrat olarak kullanıp direkt olarak indükleyebilmektedir. İndüklenen p53 mitokondriyal apoptotik sinyalleri ve kaspaz-3 aktivasyonunu tetikleyerek sonuçta kaspaz bağımlı apoptoza sebep olmaktadır.

Siklinler, hücre siklusunun kontrolünde CDK'leri aktive eden ana elemanlar olmakla birlikte, SpeedyRINGO adı verilen başka bir protein de CDK'leri aktive edebilme özelliğine sahiptir. SpeedyRINGO'nun hücre siklusunun kontrolündeki rolünün yanısıra, bazı mitotik hücrelerde kaspaz-bağımlı apoptozu önlediğine dair çalışmalar da mevcuttur.

SpeedyRINGO'nun temel görevi hücre siklusunun belirli noktalarda kontrolü olmakla birlikte, son yapılan çalışmalar bu proteine farklı bir görev yükleyerek, DNA hasarına uğramış mitotik hücrelerde anti-apoptotik olarak etkiye sahip olduğunu söylemektedirler. Bu çalışmalarda, SpeedyRINGO'nun, DNA hasarına uğramış U2OS adı verilen mitotik hücrelerde, p53 proteininin varlığına bağlı bir şekilde apoptozu engellediği, ayrıca UV ışıması gibi bir çok dış ve iç apoptotik faktöre karşı, kaspaz-3 aktivasyonunu engelleyerek, mitotik hücreleri koruduğu gösterilmiştir.

Bütün bu verilerden yola çıkılarak, bu çalışmada esas olarak, nöronlarda kalpainin aşırı aktivasyonunun ortaya çıkardığı dejeneratif ve apoptotik etkilerinin, SpeedyRINGO proteinini nöronlarda eksprese ettirerek ortadan kaldırılması amaçlanmıştır.

Bu amaçla, iki deney grubu oluşturulmuştur. Deney gruplarının birinde hipokampal nöronlarda kalsiyum iyonoforu ile sadece kalpain aktivasyonu yapılarak kalpainin aşırı aktivasyonunun nöronlarda meydana getireceği olası değişiklikleri gözlemlemek hedeflenmiştir. Diğer deney grubunda ise nöronlar öncelikle elektroporasyon yöntemi kullanılarak SpeedyRINGO ile transfekte edilmiş, ardından kalsiyum iyonoforu ve kalsiyum içeren bir medya yardımıyla hücre içi kalpainin indüklenmesi ve aşırı aktifleşmesi sağlanarak SpeedyRINGO' nun nöronları kalpainin bu aşırı aktivasyonuna karşı koruyup koruyamayacağının araştırılması amaçlanmıştır. Daha sonra, her iki deney grubunda da farklı hücre siklus belirteçlerinin yanısıra CDK5, Siklin D1, p27, CDK2, p53 ve aktif kaspaz-3 mRNA ve protein seviyelerindeki değişimlerin karşılaştırmalı olarak araştırılması amacı ile, SpeedyRINGO' nun dejenerasyona uğrayan nöronlarda anti-apoptotik etki gösterip göstermediğini anlayabilmek için QRT-PCR, floresan boyama ve western blotlama teknikleri uygulandı.

Çalışmanın sonuçlarına göre, kalpainin indüklendiği nöronlarda CDK5' in mRNA ve protein seviyelerinde artış görülürken, Siklin D' nin mRNA ve protein seviyelerinde belirgin bir artış bulunamamıştır. Bu sonuç da kalpain indüksiyonunun Siklin D yerine CDK5 üzerinden nöronlarda hücre siklus aktivasyonuna yol açtığını görüşünü desteklemektedir. Ayrıca, kalpain indüksiyonu sonucu nöronlarda p53 ün de indüklendiği ve miktarında artış olduğu ve sonrasında aktif kaspaz-3 seviyesinin de arttığı görülürken, öncelikle SpeedyRINGO' nun eksprese edilip daha sonra kalpainin indüklendiği nöronlarda p53 artışı sabit kalırken, aktif kaspaz-3 artışının engellendiği görülmüştür. p53 proetininin varlığı, SpeedyRINGO proteininin kaspaz-3 aktivasyonunu engellemesi için gerekli olduğundan bu, beklenen bir sonuçtur. Bütün bu bulgulara ek olarak, destekleyici bir diğer bulgu da hem DAPI boyaması hem de TUNEL analizi ile elde edilen ve nöronlardaki apoptoz seviyesini gösteren

sonuçlarından gelmektedir. Apoptoz analizleri göstermiştir ki, Kalpainin aşırı aktive edildiği nöronlar erken-apoptotik evrede bulunurken, önce SpeedyRINGO ekspresyonunun sağlandığı ve daha sonra kalpainin aşırı aktivasyonunun uyarıldığı nöronlarda hiç bir apoptotik belirti gözlemlenmemiştir.

Sonuç olarak, nöronlarda kalpainin aşırı aktivasyonu sonrası p53 seviyesindeki artışa bağlı olarak ortaya çıkan kaspaz-3 aktivasyonunun SpeedyRINGO proteini tarafından engellenebildiği ve böylece nöronların kalpainin apoptotik etkisinden korunabildiği ilk kez bu çalışma ile gösterilmiştir. Bu çalışma, ileride SpeedyRINGO' nun nörodejeneratif hastalıkların tedavisinde kullanılabilmesi için araştırmacılara yeni bakış açıları kazandırabilme potansiyeline sahiptir.

1. INTRODUCTION

1.1 Neurodegeneration

The term “neurodegeneration” defines the progressive loss of function and structure of neurons that finally ends up with death of neurons.

Neurons are terminally differentiated cells that have no mitotic activity. They are born far away from their area of function and they take a long journey migrating to their final destination in the nervous system. After reaching their target site, they complete their differentiation process and stop dividing by resting in quiescent (G_0) phase (Lee, 2009) in order to use their microtubule based mitotic apparatus for axonal and dendritic branching rather than division (Baas, 1999). For this reason, under neurodegenerative conditions, neurons do not have the ability to regenerate themselves and go through programmed cell death. It implies that neurodegeneration is not a reversible process.

There is a number of neurodegenerative diseases such as Alzheimer ’s disease, Parkinson ’s disease and Huntington ’s disease, which seriously affect millions of elderly population in the world.

Although neurons are known to be non-dividing cells, recent studies have shown that under neurodegenerative conditions, there is an up-regulation of cell cycle regulatory proteins causing aberrant cell cycle re-entry in neurons (Herrup, 2010) which is accepted as one of the earliest pathological symptoms of most of the neurodegenerative diseases especially that of Alzheimer’s disease (Lee, 2009). In Alzheimer’s disease, degenerating neurons re-enter into cell cycle and even replicate their genomes, but they cannot complete mitosis and undergo apoptosis, since they do not have the mitotic machinery (Zhu, 2008; Herrup, 2010) (Figure 1.1).

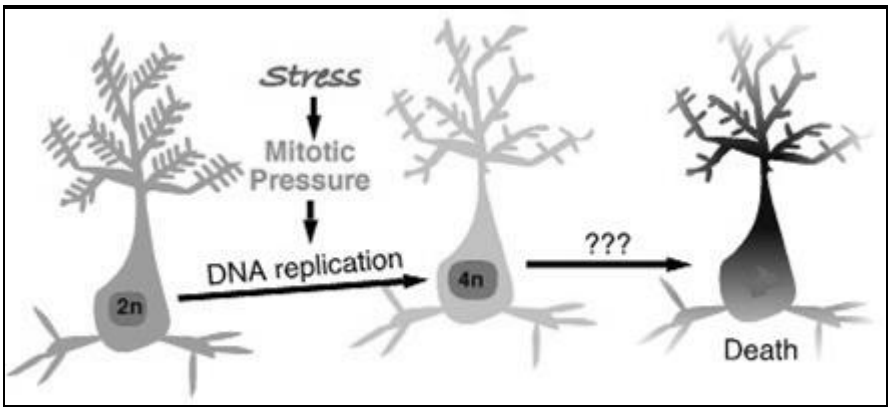


Figure 1.1 : Schematic presentation of neuronal death (Herrup, 2010).

1.1.1 Cell cycle re-activation in neurodegeneration

Cell cycle is strictly controlled at numerous checkpoints allowing normal cell proliferation in mitotic cells when necessary. There are four phases of cell cycle: G_1 (Gap 1), S (DNA replication), G_2 (Gap 2) and M (mitosis). G_1 phase is known as "growing phase" in which a large number of proteins are synthesized that are necessary for DNA replication. S phase is the "DNA replication" phase and all the chromosomes are duplicated at the end of this phase. G_2 is the phase in which especially microtubules that are required during mitosis are synthesized. Finally, M phase is the mitosis and cytokinesis phase in which duplicated chromosomes are separated into two identical sets in the nucleus. Then cytokinesis occurs and two daughter cells are generated (Cooper, 2000).

Cylins and cyclin-dependent kinases (CDKs) are the key players of the mitotic cell cycle. Various cyclin/CDK complexes are sequentially activated in particular cell cycle phases to regulate the transition from one phase to the other. In response to extracellular mitotic signals, firstly Cyclin D is expressed and binds to CDK4 and forms active CyclinD-CDK4 complex. Active Cyclin D-CDK4 complex phosphorylates retinoblastoma protein (pRb). When pRb is hyper-phosphorylated (ppRb), it dissociates from E2F/DP1/Rb complex and activates E2F. Activated E2F causes transcription of numerous genes such as Cyclin A and Cyclin E. Then Cyclin E binds to CDK2 and forms active Cyclin E-CDK2 complex, which is responsible for G_1/S transition of the cell. After that, Cyclin A forms an active complex with CDK2 that is required during S phase. In G2 phase of the cycle, Cyclin A binds to CDK1 in order to activate some transcription factors for the synthesis of necessary proteins for M phase. Finally, Cyclin B binds to and forms an active complex with

CDK1 initiating the G2/M transition. This complex also takes role in nuclear envelope breakdown at the beginning of prophase (Cooper, 2000; Dehay, 2007) (Figure 1.2).

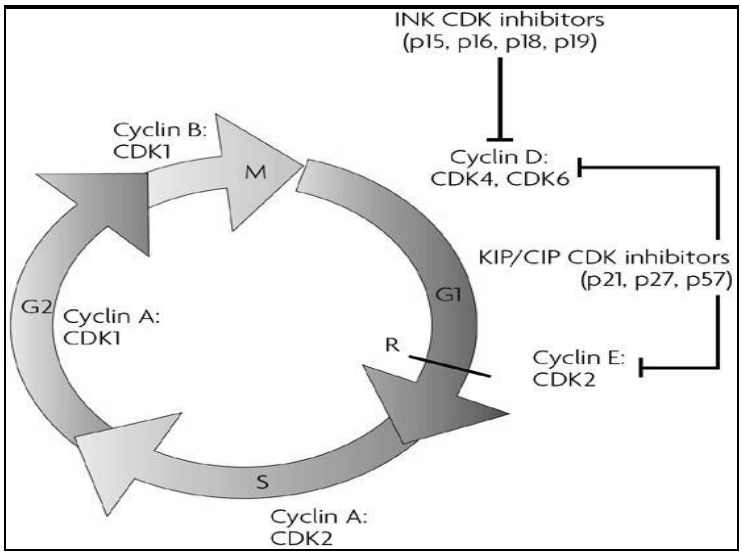


Figure 1.2 : Different phases of the cell cycle (Dehay, 2007).

On the other hand, arrest of cell cycle event is highly controlled by an increase in the concentration of CDK inhibitors in neurons, since they are post-mitotic cells. However, there are studies indicating that mitotic cyclins and CDKs are still present in adult neurons. These finding is contradictory to the widely known feature of neurons that they do not have the mitotic ability. Compared to mitotic cells, only difference is that localization of mitotic proteins is switched from nucleus to the cytoplasm in adult neurons (Schmetsdorf, 2005). Thus, these results support the idea that the functions of mitotic regulators are switched to neuronal survival in the post-mitotic neurons. It is believed that all mitotic players have alternative functions in neurons such as neuronal plasticity (Frank, 2009; Schmetsdorf, 2005).

In addition, there are some studies showing the inactivation of CDK inhibitors in case of Alzheimer's disease indicating that if necessary, these CDK inhibitors could not function to stop any cell cycle re-activation (Nagy, 2001).

Although the classic view still holds that terminally differentiated neurons, which remain in G_0 phase, are unable to re-enter into the cell cycle, a growing body of evidence has demonstrated the up-regulation of mitotic cell cycle regulatory proteins in case of neurodegeneration especially in that of Alzheimer 's disease brain (Nagy, 2001; Yang, 2007).

The exact mechanism and starting point of neurodegeneration in case of Alzheimer's disease are not well known, but some of the studies showed that cell cycle re-entry precedes some other pathological hallmarks of Alzheimer's disease such as tau hyper-phosphorylation. Thus, it is thought that cell cycle re-entry could be the starting point for development and progression of Alzheimer's disease pathology (Vincent, 1998).

Neurons have been shown to activate their cell cycle leaving G_0 phase and going into G_1 due to a number of neurotoxic conditions such as amyloid beta aggregation, metabolic alterations, oxidative stress (Herrup, 2004) (Figure 1.3). However, there is no evidence that neurons successfully complete mitosis. This indicates that activated cell cycle is arrested at one of the checkpoints before passing through mitosis. Therefore, this amateur cell cycle activation attempt leads neurons into apoptosis (Park, 2007; Federsen, 1992).

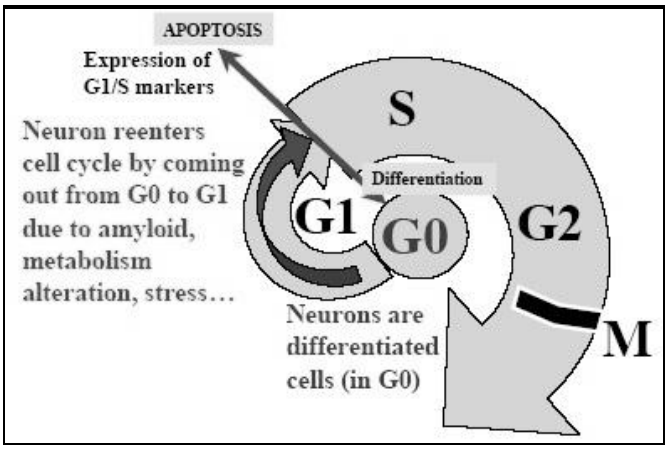


Figure 1.3 : Illustration of cell cycle re-activation in degenerating neurons (Herrup, 2004).

Amyloid beta aggregation, a hallmark of Alzheimer's disease, causes destabilization of calcium homeostasis and results in the elevation of intracellular calcium levels, which finally leads to pathological activation of a calcium-sensitive cysteine-protease, calpain. Calpain over-activity is known to be one of the most important factors causing cell cycle re-activation and degeneration in neurons (Nakamura, 1997; Araujo, 2004).

1.2 Calpain

Calpains are known as calcium-activated proteolytic enzymes (Harris, 2004). There are almost 15 types of calpains identified, but two isoforms, ì-calpain (calpain I) and m-calpain (calpain II), are especially found in mammalian tissues (Yadavalli, 2004). The difference between two isoforms is the calcium sensitivity that ì-calpain is activated at micromolar per liter levels, while m-calpain is activated at milimolar per liter levels of calcium. In its inactive proenzyme form, calpain is found in the cytoplasm, and is activated in the presence of high levels of calcium (Camins, 2006).

1.2.1 Structure and activation of calpain

Both isoforms of calpain are heterodimers, which are composed of a large 80 kDa catalytic subunit and a small 30 kDa regulatory subunit. 30 kDa regulatory subunit is identical in the same tissue type or animal species but 80 kDa subunit is specific and not identical. The large 80 kDa subunit is composed of four domains. Domain I is the autolytic cleavage site, domain II has the cysteine protease activity and interacts with the substrates. Domain III does not have a known function. Domain IV contains E-helix-loop-F-helix motifs (EF hands) on which are calcium binding sites (Neumar, 2003) (Figure 1.4).

The small 30 kDa subunit contains two domains: V and VI. Domain V is the terminal domain and possesses glycine residues, while domain VI has five EF-hand calcium binding motifs (Yang, 2004; Swapan, 2003) (Figure 1.4).

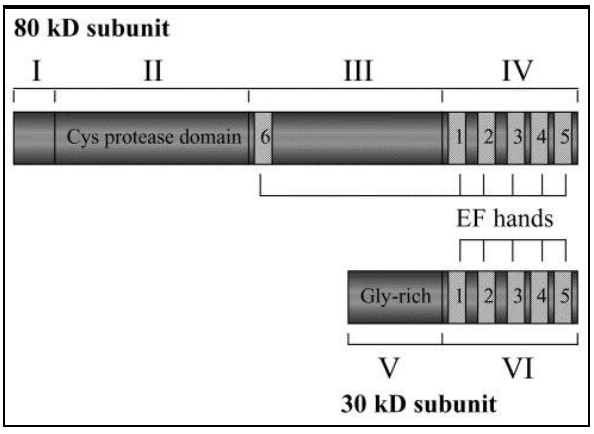


Figure 1.4 : Structural domains of calpain subunits (Swapan, 2003).

Calpain is localized in the cytosol in its inactive form in the absence of calcium. When there is an increase in intracellular calcium levels, calpain is translocated to the membrane and there, it becomes activated with calcium and phospholipids. Calpain activation occurs mainly in two stages. In inactive form, two subdomains of protease domain II (IIa and IIb) are separated by structural constraints. In the first stage of activation, this structural constraint is released in the presence of calcium ions, which is a necessity to activate the calpain and to form the active catalytic site (Moldveanu, 2002). Calcium binds to EF-hand motifs in domains III, IV and VI and this binding separates domain II from domain III. Therefore, 30 kDa subunit dissociates from 80 kDa subunit at the end of first stage. At the second stage, active site on the protease domain II is rearranged to be able to interact with its substrates (Reverter, 2001) (Figure 1.5).

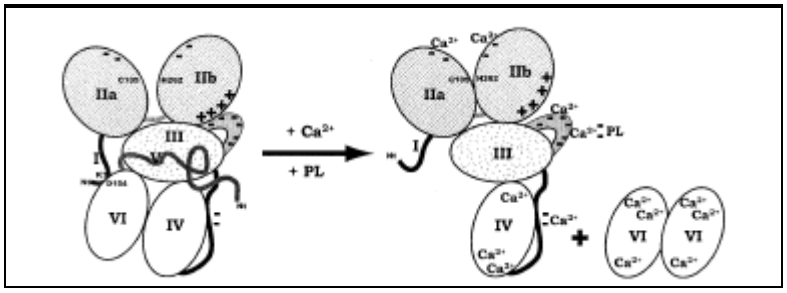


Figure 1.5 : Calpain activation mechanism by calcium (Suzuki, 2004).

Activated calpain has a number of substrates such as growth factor receptors, cytoskeletal proteins, microtubule- associated proteins and mitochondria to be able to play crucial roles in different cellular mechanisms such as progression of cell cycle (Jánossy, 2004), differentiation, apoptosis and long-term potentiation in neurons (Vanderklish, 1996; Sorimachi, 1997).

Calpain activity is strictly regulated mainly by calcium, since it has many vital cellular functions and hence deregulation of calpain activity can cause serious pathological conditions ending up with the death of the cell (Suzuki,2004).

On the other hand, there is an endogenous inhibitor of calpain, namely calpastatin which regulates the activity of ì- and m-calpain (Polster, 2005) It reversibly binds to the active site and inhibits calpain only in the presence of calcium ions (Hanna, 2008).

Recent studies have indicated that calpains play a significant role in the cell cycle re-activation and the apoptotic processes in case of neurodegeneration (Liou, 2005). Deregulation of calcium homeostasis results in calcium overload and subsequent calpain over-activation can force neurons to re-enter into cell cycle and ultimately undergo apoptosis.

1.2.2 Role of calpain in neurodegeneration

Calpain is known to be one of the most effective proteins of which deregulation leads neurons into apoptosis through different pathways (Liou, 2005).

Calpain induction and over-activation drive neurons into apoptosis directly or indirectly. Indirect way is the cleavage of p35 protein into p25 and p10 fractions by calpain. Under normal conditions, p35 is the partner for CDK5, which is a non-mitotic neuron-specific kinase, and by forming CDK5/p35 complex, they take place in important cellular events such as neuronal development and maturation (Dhariwala, 2008; Yip, 2007; Fu, 2006). However, in case of neuronal calpain over-activation, calpain cleaves p35 into p25 and p10 fractions, since p35 is a substrate of calpain. p25 has binding ability to CDK5 to form CDK5/p25 complex. However, this complex differs from CDK5/p35 complex in terms of localization and functions (Cruz, 2004). Normally, Cyclin D-CDK4 complex is required for cell cycle activation and it is responsible for G1/S transition by phosphorylating pRb protein, but in case of calpain over-activation in neurons, it was shown that CDK5/p25 complex phosphorylates the pRb protein instead of Cyclin D-CDK4 complex and re-activates cell cycle driving neurons through G1/S transition (Hamdane, 2005; Alvira, 2008) (Figure 1.6). However, this mitotic re-entry cannot be successfully completed and neurons undergo apoptosis by an increase in p53 levels. Another important finding is that CDK5/p25 complex can directly activate p53, since p53 is a substrate for CDK5 (Zhang, 2002). All these findings strongly indicate that calcium-mediated calpain activation ends up with an increase in p53 expression and activation, and therefore subsequent caspase-dependent apoptosis in degenerating neurons.

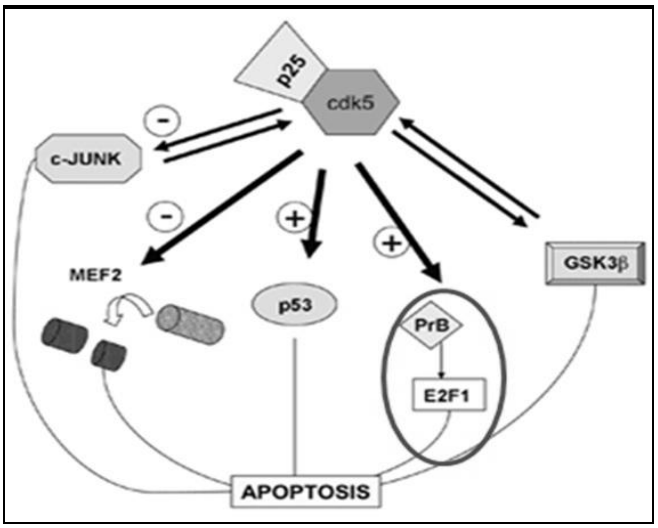


Figure 1.6 : Schematic representation of apoptotic pathways generated by CDK5/p25 complex (Camins, 2006).

Direct way for calpain forcing neurons go through apoptosis is that p53 is the substrate of calpain itself and hence, calpain over-activation directly leads to increased p53 expression and activation. Induced p53 triggers mitochondrial death signals and caspase-3 activation ultimately resulting in caspase-mediated apoptosis (Sedarous, 2003).

In addition, experimental data suggest some neuroprotective effects for inhibitors of calpain and caspase in neurons (O'Hare, 2005; Raghupati, 2004). An increased body of evidence indicates an interaction between calpain and caspase proteolytic systems. Calpain can lead to activation of caspase-3 by cleaving pro-caspase-3. Caspases can also take role in degradation process of the specific endogenous calpain inhibitor, calpastatin accelerating calpain activation. Moreover, in an experiment using ultraviolet radiation to trigger apoptosis, it was shown that calpain activity is required for caspase-3 activation (Maccioni, 2001). These findings obviously indicate that both calpain and caspase proteolytic systems are involved in progression of neuronal death (Sharma, 2004).

Since calpain over-activity is one of the most important neurodegeneration factors causing both cell cycle re-activation and the activation of apoptotic machinery as explained in detail above, it was crucial to find a way to regulate this cell cycle re-activation attempt and to prevent calpain-mediated apoptosis in degenerating neurons.

As neurons left their dividing ability, cell cycle re-activation is a rather chaotic attempt and ultimately leads neurons to apoptosis. For this reason, it was important to identify how SpeedyRINGO, a novel cell cycle regulatory protein, would be effective on positive regulation of this chaotic cell cycle re-activation in calpain over-activated neurons.

Cell cycle progression is strictly controlled by CDKs, which form complexes with distinct cyclins according to the different phases of the cell cycle. Although, cyclins are major players, which take role in activation of CDKs, there is another protein, SpeedyRINGO that can activate CDKs. Besides its role in cell cycle regulation, SpeedyRINGO has also been shown to have another important function in preventing caspase-dependent apoptosis in mitotic cells (McAndrew, 2009).

1.3 SpeedyRINGO Protein

Cell cycle progress is strictly controlled by CDKs in eukaryotic cells. Activities of CDKs are regulated mainly by cyclins. Cyclins bind, phosphorylate and activate different CDKs during different phases of the cell cycle. Although cyclins are main players in regulating CDKs activity, there is a novel protein, named SpeedyRINGO (**R**apid **In**ducer of **G**2/M progression in **O**ocytes) that can bind and regulate the activity of CDKs in many types of eukaryotic cells (Cheng, 2005).

SpeedyRINGO is a novel cell cycle regulatory protein that was first identified in Xenopus oocytes as a regulator of the meiotic cell cycle that accelerates G_2/M progression during maturation of oocytes (Nebreda, 2006). Resting oocytes are found at the G2/M of the first meiotic division. Progesterone is the stimulating factor to overcome this G2/M arrest and allow oocytes go through meiosis by inducing *mos* protein expression which is a proto-oncogene product that takes role on G2/M progression (Lenormand, 1999). However, it has been reported that a novel gene product of *Xenopus*, named SpeedyRINGO, is sufficient to stimulate and accelerate G2/M progression and meiotic maturation. It has also been shown that the rate of SpeedyRINGO- mediated meiotic maturation is faster than that of progesterone and *mos*. SpeedyRINGO has been shown to induce meiotic maturation independent of *mos* (Lenormand, 1999).

SpeedyRINGO binds and activates CDKs, although it has no sequence similarity to cyclins (Cheng, 2005). Unlike cyclins, SpeedyRINGO activates CDKs by a phosphorylation-free mechanism, which is not well known yet.

There are mainly three branches of SpeedyRINGO family: SpeedyRINGO A, B, C and a suspected fourth SpeedyRINGO D. The most conserved branch is SpeedyRINGO A, which appears to be the most slowly evolving and ancient branch of SpeedyRINGO family. It has been shown to be found in fish, chicken, sea urchin and mammals in nearly all types of cells (Cheng, 2005). In mammals, SpeedyRINGO A has been shown to have higher expression levels in testis and lower expression levels in other tissues such as brain, heart, lung, placenta, prostate, small intestine etc.

SpeedyRINGO B is the least conserved branch of SpeedyRINGO family. It has been shown to be expressed in testis of only mammals (Cheng, 2005).

SpeedyRINGO C is structurally similar to SpeedyRINGO A and it has been shown to be especially expressed in certain cell types belonging to such as liver, bone marrow and placenta tissues implying that it could be responsible for polyploidization in these tissues (Cheng, 2005).

All SpeedyRINGO family members have a central region called SpeedyRINGO box, which is conserved among all SpeedyRINGO proteins. This SpeedyRINGO box has been shown to be required for CDK binding, but not for activating (Figure 1.7, Figure 1.8) (Cheng, 2005).

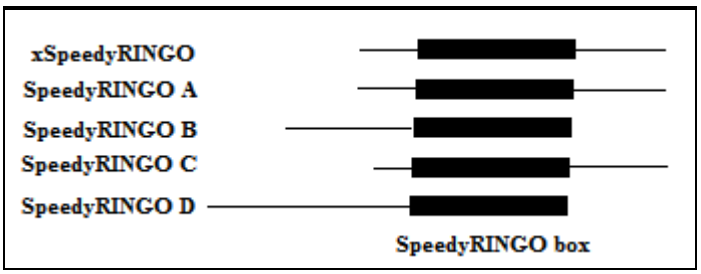


Figure 1.7 : Structural domains of SpeedyRINGO homologues, Xenopus SpeedyRINGO (xSpeedyRINGO), SpeedyRINGO A, SpeedyRINGO B, SpeedyRINGO C and SpeedyRINGO D showing the conserved SpeedyRINGO box region.

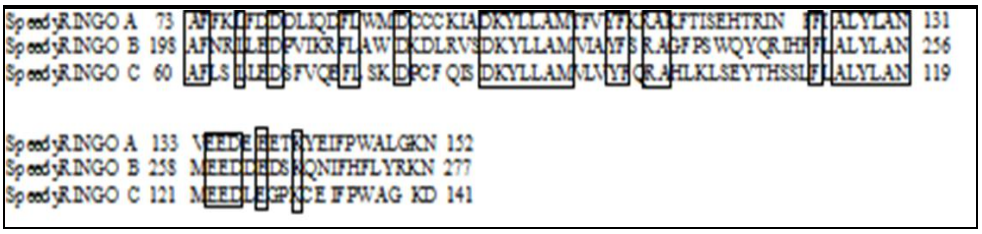


Figure 1.8 : Alignment of the conserved SpeedyRINGO boxes of human homologues SpeedyRINGO A, SpeedyRINGO B and SpeedyRINGO C using Basic Local Alignment Search Tool (BLAST) of National Center for Biotechnology Information (NCBI).

In this study, human SpeedyRINGO homologue, Spy1 was used. It is a SpeedyRINGO A family member and located on chromosome 2 at location 2p23.2. There are three identified regions of Spy1: a CDK binding site, which is, known as SpeedyRINGO box, a C-terminal CDK activation site and an N-terminal site affects the expression of Spy1. SpeedyRINGO box is required for CDK binding and alanine scanning mutagenesis studies showed that conserved feature of the box is essential for CDK binding (Cheng, 2005). However, SpeedyRINGO box is not sufficient for CDK activation on its own. C-terminal region has been shown to be essential for CDK activation. The function of N-terminal is not well known, but it has been shown to negatively regulate the expression of SpeedyRINGO itself. This negative regulation may keep SpeedyRINGO under strict control to avoid disruption of CDK-regulatory pathways, since SpeedyRINGO could activate different CDKs prematurely (Cheng, 2005).

Spy1 has been shown to be widely expressed in mitotic cells and cell lines and it has been shown to be required for S phase entry in somatic cells (Porter, 2002). CDK2 is the specific partner of Spy1 indicating that its activity is CDK2-dependent (Porter, 2002). In mitotic cells, over-expression of SpeedyRINGO binds and activates CDK2 leading to phosphorylation and degradation of $p27^{kip}$ and, consequently, accelerating G1/S phase progression and cell division. However, SpeedyRINGO-CDK2 complex has been shown to have low enzymatic activity against conventional CDK2 substrates implying that SpeedyRINGO-CDK2 is unlikely to regulate cell cycle progression on its own (McAndrew, 2007; Gastwirt, 2006).

1.3.1 Role of speedyRINGO in the inhibition of apoptosis

When there is any damage to DNA as a result of an apoptotic action during cell cycle, cells initiate cell cycle arrest by activating checkpoint responses to provide

enough time to cell for repairing the genome (Bartek, 2007). In case of encountering un-repairable DNA, cells activate their apoptotic machinery, which ultimately results in the death of cells. p53 is the key player that is induced in response to any DNA damage. Induction of p53 expression and activation results in DNA damage checkpoint response activation (Attardi, 2005).

Although SpeedyRINGO mainly functions in cell cycle regulation in mitotic cells, recent studies have also attributed a different role for SpeedyRINGO that it has an anti-apoptotic effect in DNA-damaged mitotic cells and hence allow cell survival (McAndrew, 2009). It has been shown that SpeedyRINGO prevents apoptosis in a p53-dependent manner in response to DNA damage in U2OS cells, which is a mitotic human osteosarcoma cell line (McAndrew, 2009). SpeedyRINGO was indicated to have strong survival effect in mitotic cells that encountered a number of extrinsic or intrinsic apoptotic factors such as UV irradiation. SpeedyRINGO fulfills this function by inhibiting caspase-3 activation and apoptosis in the presence of the gene regulatory protein p53 (McAndrew, 2009; Gastwirt, 2006).

Although exact mechanism of this anti-apoptotic effect is not known, it is thought that SpeedyRINGO expression allows the cells to bypass the S-phase/replication checkpoint and the G2/M checkpoint. As a result of bypass of these checkpoints, cells cannot sense the DNA damage accumulation and hence do not arrest replication (Gastwirt, 2006).

1.4 Aim of the Study

Calpains are known as calcium-activated proteolytic enzymes. Activated calpain has a number of substrates such as growth factor receptors, cytoskeletal proteins, microtubule- associated proteins and mitochondria to be able to play crucial roles in different cellular mechanisms such as progression of cell cycle, differentiation, apoptosis and long-term potentiation in neurons.

Although there are some intrinsic mechanisms regulating calcium influx in neurons, a number of neurotoxic conditions such as oxidative stress and amyloid beta aggregation cause deregulation of calcium homeostasis by increasing calcium influx into neurons. This abnormal level of calcium increase induces calpain and results in pathologic calpain over-activation. Over-activation of calpain has been known to be

one of the most important neurodegenerative factors both causing cell cycle re-activation through CDK5 and apoptosis by inducing p53 and caspase-3 activation.

SpeedyRINGO, a novel cell cycle regulatory protein, has been shown to bind and activate CDKs with a phosphorylation-free mechanism. In addition to its regulatory function, there is also another attributed function for SpeedyRINGO in preventing apoptosis by inhibiting caspase-3 activation in the presence of p53 in mitotic cells. Thus, utilizing SpeedyRINGO in non-mitotic neurons for its cell cycle regulatory and anti-apoptotic feature could be helpful for preventing calpain-based - caspase-dependent apoptosis.

Therefore, the aim of this study was to regulate chaotic cell cycle re-activation attempt with SpeedyRINGO, a novel cell cycle regulator, in calpain-based neurodegeneration, and also to prevent the apoptotic effect of calpain in degenerating neurons using SpeedyRINGO as an inhibitor of caspase-3 activation. For this purpose, hippocampal neurons are transfected with SpeedyRINGO and then, calpain was induced by calcium ionophore. After that, analysis was performed to see if SpeedyRINGO may have a regulatory effect on calpain-mediated cell cycle re-activation attempt and also to identify if SpeedyRINGO may protect neurons against caspase-dependent apoptosis in the presence of p53. Results of this study may contribute towards understanding the survival effort of neurons against calpain-based neurodegeneration.

2. MATERIALS AND METHODS

2.1 Materials

2.1.1 Equipment

The equipment used in the study is given in the Table 2.1:

Table 2.1 : Equipment.

Equipment	Supplier Company
Electrophoresis Gel System	E-C Apparatus Corporation, EC250-90 Minicell Primo
Power supply	EC250-90 Apparatus Corporation
Thermal Cycler	Applied Biosystems, GeneAmp PCR System 2700
Microcentrifuge	Beckman Coultier
Water Bath	Memmert
UV Transilluminator	ioRad UV Transilluminator 2000
UVIPhotoMW Version 99.05 for Windows 95 & 98	UVItec Ltd.
Shaker	Forma
pH Meter	Mettler Toledo MP220
Precision weigher	Precisa 620C SCS
Vortex	Heidolph, Reaxtop
Centrifuges	Biolab SIGMA 6K15, Beckman Coulter Microfuge®18, Beckman Coulter AvantiTM J-30 I, IECCL10 Centrifuge, Thermo Electron Corporation, Labnet, Labnet International C1301-230V
Magnetic stirrer	Labworld Standard Unit
Microwave	Arçelik MD582
Ice machine	Scotsman AF 10
Laminar air flow cabinets	FASTER BH-EN 2003
Incubator with CO2	Biolab SHEL LAB
Inverted light microscope	Olympus CK40
SDS-PAGE gel electrophoresis system	BIO-RAD MiniProtean
Power supply	Thermo Electron Corporation EC250- 90
Freezers	UĞUR (-20oC), UĞUR (+4 ^{o}C), New Brunswick Scientific (-80 ^{o}C)
Tissue culture dishes	34 mm, TPP
Cell scraper	30cm, TPP
Hemacytometer	Fisher Scientific
Serological pipette	TPP
Nitrocellulose membrane	Santa Cruz
3MM Whatman filter paper	Whatman
QRT-PCR equipment	Roche Light Cycler 480
Confocal microscope	Leica TCP SP2 SE
Electroporator	Amaxa Nucleofector® Device, Lonza

2.1.2 Chemicals and enzymes

Chemicals and enzymes used in this study are given in the Table 2.2:

Table 2.2 : Chemicals and enzymes.

Material	Supplier Company
Hank's Balanced Salt Solution 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid trypsin 2.5 % Neurobasal Medium B27 Supplement Alexa Fluor® 647 goat anti-rabbit IgG Alexa Fluor® 488 goat anti-mouse IgG Alexa Fluor® 647 donkey anti-rabbit SeeBlue Plus2 Prestained Protein Ladder	Invitrogen
Penicillin/Streptomycin 45% D-Glucose DNase poly-L-Lysine A23187 Calcium ionophore Methanol Donkey serum Bovine Serum Albumin Phophate Buffered Saline Tween20 (P9416) Ampicillin	Sigma-Aldrich
L-Glutamine Fetal bovine serum Goat serum	Biological Industries
CytoBuster™ Protein Extraction Reagent	Calbiochem
Rattus norvegicus Cdk5 qRT-PCR probe Rattus norvegicus CyclinD1 qRT-PCR probe Rattus norvegicus Cyclin-dependent kinase inhibitor 1B ($p27^{kip}$) qRT-PCR probe Rat ACTB Gene Assay Rat GAPD Gene Assay T4 DNA Ligase 10X T4 Ligase buffer	Roche
pIRES expression vector	Clontech Lab.
Rabbit polyclonal IgG anti-CyclinD1 antibody Mouse monoclonal IgG anti-CDK5 antibody Mouse monoclonal anti-actin antibody Mouse monoclonal anti- $p27^{kip}$ antibody	Santa Cruz
Mouse monoclonal IgG anti- beta- III- tubulin antibody Anti-HRP secondary antibodies	Promega
Rabbit polyclonal IgG anti-SpeedyRINGO antibody	Affinity Bioreagents
Mouse monoclonal anti-CDK2 antibody	Biosource
Mouse monoclonal anti-p53 antibody Rabbit monoclonal anti-caspase-3 antibody	Cell Signaling Tech.

Table 2.2 (continued) : Chemicals and enzymes.

Material	Supplier Company
Taq polymerase dNTP Mix 10x PCR buffer Nhe I restriction enzyme EcoR I restriction enzyme MassRuler™ DNA Ladder (Mix, 80bp-10Kb) 10X Tango Y buffer 25 Mm MgCl2 6X Loading dye	Fermentas
Low melting agarose TEMED	AppliChem
Tryptone	Lab M TM
PIPES Tris Base Acrylamide	BDH Laboratory
DMSO Acetone Absolute ethanol	Riedel- de Haën
Diethyl ether	LACHEMA
Glysin APS CaCl2.2H2O EDTA SDS Glucose Ethidium bromide Yeast Extract Agar KCl DTT HCl	Merck
MgCl2.6H2O NaCl	Carlo Erba
Isopopanol Glycerol PEG 8000 Glacial Acid	Fluka

2.1.3 Commercial kits

Commercial kits used in this study are given in the Table 2.3:

Table 2.3 : Commercial kits.

Kit	Supplier Company
Fluorogenic Calpain Activity Assay Kit	Calbiochem
Bicinchoninic acid (BCA) protein assay kit	Pierce
High Pure RNA Isolation Kit Light Cycler® 480 Probes Master qRT-PCR Kit Cytotoxicity Detection Kit	Roche
Quant-iT™ RNA, BR Assay Kit	Invitrogen
RevertAid™ H Minus First Strand cDNA Synthesis Kit	Fermentas
Mammalian Cell Extraction Kit	Biovision
ECL Plus Western Blotting Detection Kit	GE Healthcare
QIAquick PCR purification kit	Qiagen

2.1.4 Buffers and solutions

2.1.4.1 TAE buffer (50X)

TAE Buffer (50X) was prepared as a mixture of 20 mM glacial acetic acid, 40 mM Tris base and 1 mM EDTA (pH 8.0) in dH_2O.

2.1.4.2 2X sample buffer for SDS-PAGE

2X sample buffer was used to denature protein samples before loading on SDS polyacrylamide gel. 2X sample buffer was prepared by dissolving 2 ml (100%) glycerol, 5 mg bromophenol blue, 4 ml (10%) SDS, 2.5 ml (0.5 M) tris-HCL and 231 mg DTT in 10 ml ddH_2O.

2.1.4.3 Tris buffer saline (TBS)

Tris buffer saline (TBS) was prepared as 10X stock solution by dissolving 12.1 g Tris and 87.66 g NaCl in 1 lt ddH_2O and then pH was adjusted to 8.0. 1X Working solution was prepared by diluting 10X TBS by 10 times.

2.1.4.4 Tris buffer saline – Tween20 (TBS-T)

Tris buffer saline – Tween20 (TBS-T) is the washing buffer used in all western blots in this study. TBS-T was prepared by dissolving 0.1% (v/v) Tween20 in 1X TBS.

2.1.4.5 Towbin buffer

Towbin buffer is the transfer buffer that was used in all western blots in this study. Towbin was prepared by dissolving 20% (v/v) Methanol, 7.5g (192 mM) Glycine and 1.5 g (25 mM) Tris in 500 mL ddH_2O.

2.1.4.6 Blocking buffer for immunofluorescence analysis

Blocking buffer is used to avoid non-specific binding of the antibody. To prepare 1 mL blocking buffer 10 mg (10 mg/mL) BSA and 100 µl (10%) Goat or Donkey, (depending on the type of secondary antibody) serum were mixed in 1X PBS and filtered with 0.8µm Nalgene filter in order to prepare 1 mL blocking buffer.

2.1.4.7 Blocking buffer for western blotting

5 g (5%) skim milk and 1 g (1%) BSA were dissolved in 1X TBS in order to prepare 100 mL western blocking buffer.

2.1.4.8 Stripping buffer for western blotting

200 mM Glycine was dissolved in ddH_2O and then pH was adjusted to 2.5 with HCl in order to prepare 100 mL western stripping buffer. 0.5% Tween20 was added into glycine solution before use.

2.1.4.9 Calcium-containing medium

Calcium-containing medium was used for calpain activation study. It was prepared by dissolving 5.6 mg KCl, 1.215 mg $CaCl_2$, 250 µl HEPES, 128,4 mg Sucrose, 203 mg NaCl and 50 µl Glucose in 25 ml dH_2O.

2.1.4.10 Calcium ionophore

Calcium ionophore (1mg/ MW=523.6) was dissolved in 19.1 µl DMSO to prepare 100 Mm stock solution. Then 200 µM working solution was prepared from the stock solution in dH_2O.

2.1.5 Bacterial strain

Escherichia coli (E.coli) DH5α strain [F-, φ80d*lacZ*_M15, _(*lacZYA-argF*)U169, *deoR*, *recA*1, *endA*1, *hsdR*17(rk-, mk+), *phoA*, *supE*44, λ-, *thi*-1, *gyrA*96, *relA*1], Invitrogen.

2.1.6 Bacterial culture media

2.1.6.1 LB medium

To prepare 1 L of LB medium, 10 gr tryptone, 5 gr yeast extract and 10 gram NaCl were dissolved in ddH_2O. Then LB medium was sterilized by 15 minutes autoclaving. A final concentration of 50 µg/ml of ampicillin was added to the LB medium to make the selective media for pIRES expression vector that was used in the study. Ampicillin containing LB was stored at 4ºC.

2.1.6.2 LB-agar plate

15 gram/L of agar was mixed with LB medium and 15 minutes-sterilized by autoclaving. After cooling of LB-agar, it was poured into several plates.

2.1.6.3 SOC medium

To prepare 100 mL SOC medium, 5 gr of yeast extract, 2 gr of tryptone, 0.0186 gr of KCl, 0.058 gr of NaCl, 0.095 gr of MgCl2, 0.36 gr of glucose and 0.24 gr of MgSO4 were dissolved in ddH_2O. Then prepared SOC medium was autoclaved for 15 minutes at 120°C. Before use, antibiotic was added to make the medium selective. SOC medium was used to cultivate *E.coli* after temperature shocking during transformation.

2.1.7 pIRES expression vector

pIRES is a mammalian expression vector, which enables high level expression of two genes of interest from the same mRNA. pIRES is comprised of the encephalomyocarditis virus (ECMV) internal ribosome entry site (IRES) flanked by two multiple cloning sites (MCS A and B). pIRES also contains a partially disabled IRES sequence that decreases the translational rate of the gene, which is cloned into MCS B relative to that of MCS A.

The constitutively active cytomegalovirus immediate early promoter (*P*CMV IE), located upstream of MCS A, regulates the transcriptional expression of the desired gene. In between *P*CMV IE and MCS A is an intervening sequence (IVS) that promotes the stability of mRNA. IVS is spliced out after transcription. SV40 polyadenylation signals downstream of MCS B control proper processing of the 3' end of the mRNA. Bacteriophage T3 and T7 promoters are located downstream of MCS B and upstream of MCS A, respectively. In the vector, there is also an ampicillin resistance gene (Ampr), and a ColE1 origin of replication for selection and propagation in *E. coli*, and an f1 origin for single-stranded DNA production.

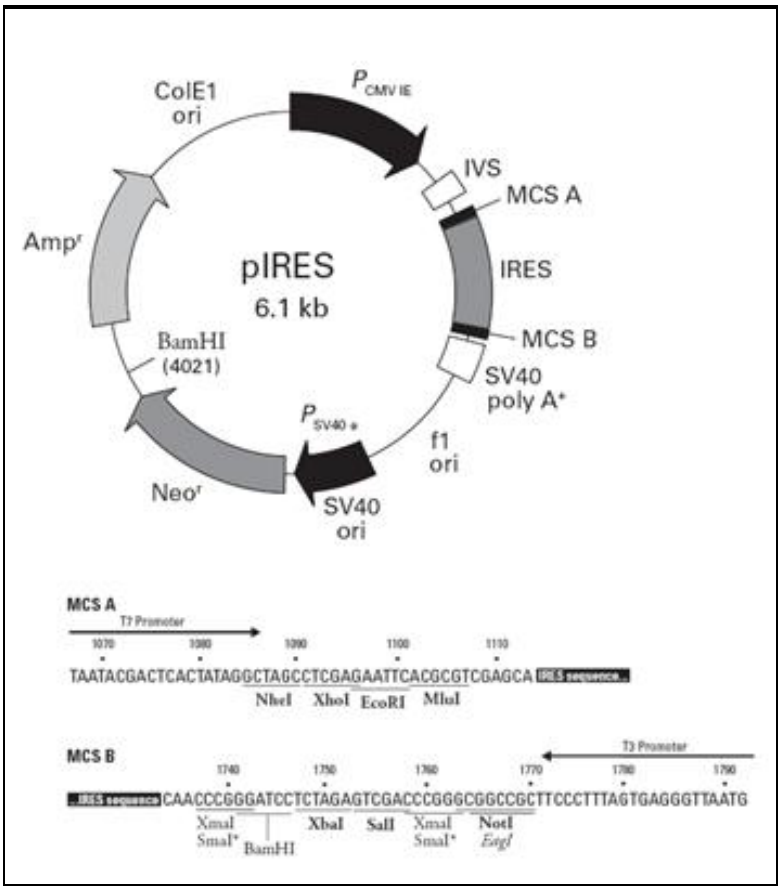


Figure 2.1 : pIRES vector map (A) and Multiple cloning site of pIRES (B).

2.1.8 Primary hippocampal neuron culture media

2.1.8.1 Hippocampus dissection medium

In order to prepare 100 mL hippocampus dissection medium, 10 mL HBSS, 1 mL 1 M HEPES and 1 mL Penicillin-Streptomycin were dissolved in 88 mL ddH_2O. Medium was sterilized with 0.2 μm filter (Nalgene).

2.1.8.2 Hippocampal plating medium

Hippocampal medium was used for washing hippocampi pieces during dissection procedure. In order to prepare 50 mL hippocampus plating medium, 1 mL B27 Supplement, 0.33 mL 45% D-Glucose, 0.25 mL L-Glutamine and 2.5 mL FBS were mixed in 45.92 mL Neurobasal medium. Medium was sterilized with 0.2 μm filter (Nalgene).

2.1.8.3 Hippocampal serum free plating medium

In order to prepare 50 mL hippocampus serum free plating medium, 1 mL B27 Supplement, 0.33 mL 45% D-Glucose and 0.25 mL L-Glutamine were dissolved in 48.42 mL Neurobasal medium. Medium was sterilized with 0.2 μm filter (Nalgene).

2.1.9 Experimental animals

Post-natal 0 (PN0) Sprague-Dawley rats were used for hippocampal neuron culturing. All the animals were handled according to appropriate animal care and utilization procedures. Ethical approval was obtained from the local scientific ethical committee. The procedures involving experimentation on animal subjects are done in accordance with both the guide of the local authorities and with the National Research Council's guide for the care and use of laboratory animals.

2.2 Methods

2.2.1 Subcloning of speedyRINGO into pIRES expression vector

2.2.1.1 Primer design

First, SpeedyRINGO was subcloned from pCS3 vector into pIRES expression vector. In order to subclone SpeedyRINGO (AY820303 / *Homo sapiens* speedy A isoform 1 complete cDNA) from pCS3 vector into pIRES, first, SpeedyRINGO was cloned out from pCS3 by PCR using primers that were specifically designed for SpeedyRINGO (Figure 2.2). All the primers were designed by adding restriction sites on their 5' ends. These restriction sites were then used for pIRES-SpeedyRINGO ligation studies. Designed primers are as in the Table 2.4:

Table 2.4 : Designed primers for PCR reaction.

Primer	Sequence
Forward primer (Nhe I restriction site added)	5' AAAAAGCTAGCATGAGCCACAATCAGATGTG 3' Primer conct.: 116,865 nmole, Tm: 60,9 ^{0}C
Reverse primer (EcoR I restriction site added)	5' CCGGAATTCCGGCTAAATCATACCTTCAGAACCA 3' Primer conct.: 82,349 nmole, Tm: 63,9 ^{0}C

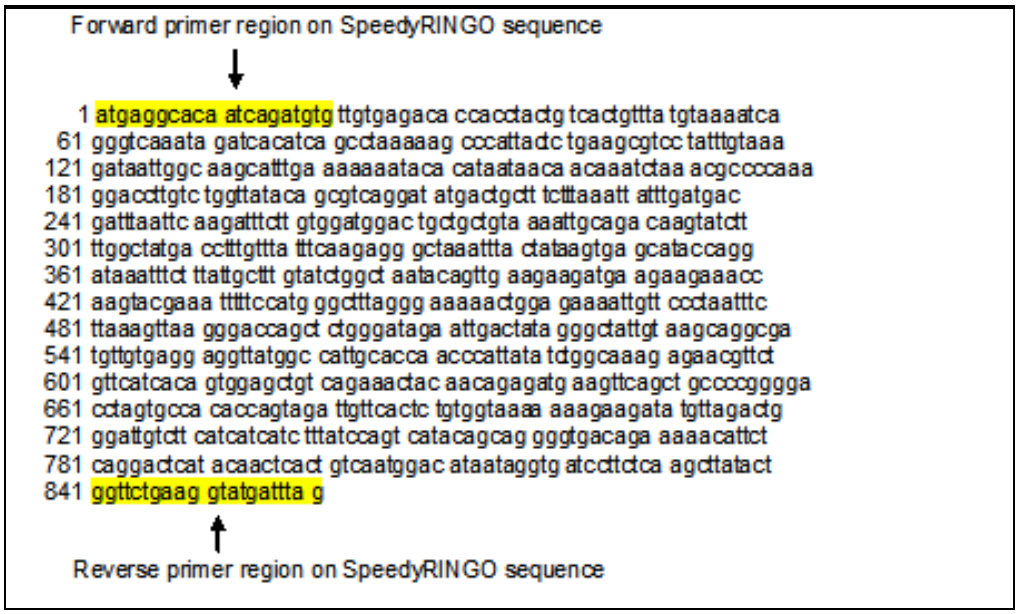


Figure 2.2 : Demonstration of primer sequences on SpeedyRINGO cDNA sequence.

2.2.1.2 Polymerase chain reaction (PCR)

Polymerase chain reaction (PCR) is a technique that is used to amplify a DNA region easily. This technique allows producing millions of copies of a DNA strand using enzymatic replication. Primers which are short oligonucleotides that are identical of nucleotide sequences on both side of the DNA strand of interest are required for PCR These primers provide DNA binding site for DNA polymerases.

There are three main steps in a PCR cycle: denaturation, annealing and DNA synthesis. In the first step, the target DNA is denatured by heating to 90-96°C to unwind the strands. In annealing step, primers bind to their complementary sequences on the target DNA according to the specified annealing temperature of primers. The last step is the synthesis of DNA by DNA polymerase at a particular extension temperature. The polymerase reads the DNA strand starting from the primer sequences and couples it with complementary bases very easily. As a result, there are two new helices instead of one, each one is the copy of the original DNA strand. After designing primers, SpeedyRINGO was cloned out from pCS3 vector by using these primers. PCR Reaction Mixture was prepared as in the Table 2.5:

Table 2.5 : PCR reaction mixture.

Ingredient	Final Concentration
pCS3-SpeedyRINGO construct(template)(1mg/ml)	1µg
Forward primer (20 µM stock)	0,4 µM
Reverse primer (20 µM stock)	0,4 µM
Taq polymerase (5 U/µl)	2.5 U
dNTP Mix (10 mM)	5 mM
10x PCR buffer	1x
Water	up to 25 µl

PCR reaction was performed according to the following program in the Table 2.6 :

Table 2.6 : PCR reaction conditions.

	Temperature (^{0}C)	Time	
İnitial denaturation	94	2 min.	
Denaturation	94	45 sec.	35 cycles
Annealing	59	30 sec.	
Elongation	72	1 min	
Final extension	72	10 min	
Final hold	4	+∞	

2.2.1.3 Agarose gel electrophoresis

Electrophoresis is used to separate molecules based on their certain properties, for instance, their size for DNA molecules. Since DNA is negatively charged, in electric field, it would migrate towards the positive pole through agarose-made sieve of molecular proportions. As a result, large DNA fragments move slower than the smaller ones. If the gel is stained with ethidium bromide after running process, migrated DNA can be visualized under ultraviolet light.

To prepare 1% agarose gel, 0.4 g of low melting point agarose was dissolved in 40 ml 1x TAE (Tris-acetate-EDTA) buffer. The agarose was then solubilized in a microwave oven until the agarose was completely dissolved. After that, agarose gel was cooled to 45ºC and ethidium bromide was added to a final concentration of 0.5 g/ml and mixed gently. The agarose gel was then poured into a horizontal gel tray to load the sample DNA and kept for 30 minutes for gel to be solidified. Then, it was replaced into the electrophoresis tank and tank filled with 1x TAE buffer.

The DNA samples were mixed with loading dye and then loaded into the agarose gel. MassRuler™ DNA Ladder, Mix (80bp-10kb) was used for size control. Electrophoretic separation was performed at a constant current for 40 minutes at 90 mV.

Finally, agarose gel was stained with ethidium bromide and then visualized under UV illumuminator that emits UV light at 302 nm. Agarose gel was photographed with a camera connected to a computer and images were stored with UVIPhotoMW Version 99.05 for Windows 95 & 98, UVItec Ltd. The size of the DNA was determined by comparing with the MassRuler DNA marker.

2.2.1.4 DNA fragment isolation from agarose

In the presence of a chaotropic salt, DNA specifically binds to the silica or glass surfaces since chaotropic salt disrupts the water molecules' intact structure, and it also disrupts its interaction with DNA. As the binding to silica/glass surfaces is specific for DNA, impurities are washed away during the process, and the pure DNA is eluted with water.

"QIAquick Gel Extraction Kit" was used to elute DNA fragments from agarose gels. The QIAquick system utilizes the spin-column technology and a silica-gel membrane with selective binding properties. Elution was performed by following instructions of the manufacturer.

The DNA fragment was excised from the agarose gel under UV illuminator using a scalpel. The gel slice was weighed in a colorless tube. Solubilization buffer that is 3 volumes of the weight of agarose was added for 1 volume of gel (100 mg ~ 100 µl). It was incubated for 10 minutes at 50°C. After the gel slice was completely dissolved, the color of the mixture was checked to be yellow. The sample was mixed with one volume of isopropanol. A QIAquick spin column was replaced in a 2 ml collection tube. The sample was applied to the QIAquick column to bind DNA, and subjected to 1 minute centrifugation. Flow-through was discarded and QIAquick column was placed back in the same collection tube. 0.5 ml of solubilization buffer was added to QIAquick column and 1 minute centrifugation was applied. 0.75 ml of washing buffer was added to QIAquick column and 1 minute centrifugation was applied. The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 minute at 10,000 x g (~13,000 rpm). QIAquick column was placed into a clean 1.5 ml microcentrifuge tube. 50 µl of H_2O was added to the QIAquick membrane to elute DNA and the column was centrifuged at maximum speed for 1 minute.

2.2.1.5 Restriction reactions for SpeedyRINGO and pIRES

Restriction enzymes are known to be vehicles of bacterial mechanism to survive against viral attacks. A restriction enzyme only recognizes and cuts specific nucleotide sequences, named “recognition sequence”. A restriction enzyme binds to its recognition sequence and cuts each of the 2 sugar-phosphate backbones of the DNA fragment. Most of the enzymes make staggered incisions, generating “sticky ends”, while a small portion of the enzymes make immediately opposite incisions on the DNA strand, generating “blunt ends”.

By PCR reaction, SpeedyRINGO was obtained with Nhe I and EcoR I restriction sites on its 5’ and 3’ ends, respectively. After that, both SpeedyRINGO gene and pIRES vector were cut using Nhe I and EcoR I enzymes.

Restriction reactions for pIRES vector and SpeedyRINGO were performed as in the Table 2.7 and Table 2.8, respectively:

Table 2.7 : Restriction reaction for pIRES.

Ingredient	Final Concentration
pIRES (0,5 µg/ µl)	1 µg
Nhe I (10 U/ µl)	10 U
EcoR I (10 U/ µl)	10 U
10x Tango Buffer	2x
Water	up to 30 µl

Table 2.8 : Restriction reaction for SpeedyRINGO.

Ingredient	Final Concentration
SpeedyRINGO (0,1 µg/ µl)	1 µg
Nhe I (10 U/ µl)	10 U
EcoR I (10 U/ µl)	10 U
10x Tango Buffer	2x
Water	up to 30 µl

Restriction reactions were incubated for 3 hours at 37 ^{0}C. Then they were loaded on 1% agarose gel and purified from there with gel extraction method.

2.2.1.6 SpeedyRINGO-pIRES ligation reaction

Ligation process covalently binds linearized DNA fragments together. In other words, DNA ligation creates a phosphodiester bond between the 3' hydroxyl of one nucleotide and the 5' phosphate of another. T4 DNA ligase is used for ligation of

DNA fragments. Ligation mixture was prepared by adding compounds given in the Table 2.9 and ligation mixture was incubated at room temperature for overnight:

Table 2.9 : Ligation reaction for SpeedyRINGO-pIRES.

Ingredient	Final Concentration
pIRES (0,4 µg/µl)	0,4 µg
SpeedyRINGO (0,03 µg/µl)	0,45 µg
10x Ligase buffer	1x
Ligase (100 U/ ml)	0,2 U
PEG 8000	2,5 µl
Water	up to 25 µl

2.2.1.7 Colony PCR

In order to determine if a colony on a plate bears the desired DNA sequence, colony PCR technique is used. Universal or sequence specific primers can be used for colony PCR reaction. In this study, both M13 F/R Universal primers (Invitrogen) and SpeedyRINGO-sequence specific primers were used. A short procedure of colony PCR is as follows;

First, cells were transformed and incubated overnight at 37°C. Then, 10 white colonies, which were expected to have the DNA of interest, were selected and picked. Each of them was resuspended in 50 µl sterile water. Then resuspended cells were heated at 100°C for 5 minutes to disrupt the bacterial cells. A PCR master mix was prepared by adding the compounds in the Table 2.10:

Table 2.10 : Colony PCR reaction mixture.

Ingredient	Final Concentration
10x PCR buffer	1x
MgCl2 (25mM)	3 mM
10x GC Melt	1x
dNTP(10mM each)	0,2 mM each
T7 forward primer	1 mM
T3 reverse primer	1 mM
Taq DNA Polymerase	1,25u/50 µl
ddH_2O	up to 240 µl

The master mix was equally distributed into PCR tubes. Following this, 1 µl of resuspended cells from each colony was added to PCR mixture. Colony PCR reaction was performed with the program in Table 2.11 using a thermal cycler and PCR reaction result was visualized on %1 agarose gel:

Table 2.11 : Colony PCR reaction conditions.

Cycles	Temperature, ºC	Time
1	94	2 minutes
	94	30 seconds
3	67	30 seconds
	68	3 minutes
	94	30 seconds
3	66	30 seconds
	68	3 minutes
	94	30 seconds
6	65	30 seconds
	68	3.5 minutes
	94	30 seconds
6	64	30 seconds
	68	4 minutes
	94	30 seconds
6	64	30 seconds
	68	4.5 minutes
	94	30 seconds
5	64	30 seconds
	68	5 minutes
	94	30 seconds
5	64	30 seconds
	68	5.5 minutes
	94	30 seconds
6	64	30 seconds
	68	6 minutes
Final extention	68	10 minutes
Final hold	4	----------

2.2.2 Hippocampal neuron culturing

Hippocampi of PN0 Spraque-Dawley rats were dissected using stereo microscopy and then were collected in dissection medium comprised of 1% 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES, 15630-080; Invitrogen), 1% Penicillin/Streptomycin (P4333; Sigma-Aldrich, St Louis, MO, USA) and 10% Hank's Balanced Salt Solution (14185; Invitrogen, Carlsbad, CA,USA). The disruption of the hippocampi was performed both mechanically and enzymatically, for which trypsin 2.5 % (15090-046, Invitrogen) and DNase (AMPD1, Sigma-Aldrich) were added. Following this, the hippocampi were incubated at 37°C for a 30 minute period.

Pieces of hippocampi were then triturated 6-7 times using fire polished Pasteur pipettes in a plating medium of Neurobasal Medium (12349-015; Invitrogen), 2% B27 Supplement (17504-044; Invitrogen), 45% D-Glucose (G8769, Sigma-Aldrich), 0.5% L-Glutamine (03-020-1, Biological Industries) and 10% fetal bovine serum (FBS) (04-007-1, Biological Industries) with the purpose of obtaining single cell suspension. Following this, the suspension was centrifuged at 130 x g for a 5 minute period at room temperature. The number of cells in the suspension was determined using a hemacytometer. The ratio of glial cells to neurons was measured and found out to be 10% in all conditions. To do this, 4',6-diamidino-2-phenylindole (DAPI) was used to label the nuclei of all the cells in the culture for the purpose of counting all the cells (neuronal, glial) in the culture and neuron-specific beta-III-tubulin antibody was used to label the neurons so that only neurons would be counted in the culture and obtain a glial-cell ratio. 1x 10^6 neurons were plated onto each 34 mm culture dishes coated with poly-L-lysine for Quantitative Polymerase Chain Reaction (qRT-PCR), 3x10^6 neurons were plated onto 25 cm^2 flasks for Western blotting and 3 x 10^4 neurons were plated onto each 18 mm cover slip coated with poly-L-Lysine (P8920, Sigma-Aldrich) for immunofluorescence analysis. A plating medium without FBS at 5% CO_2 and 37°C was used to house all cultured cells.

2.2.3 Design of the experiment

The experimental design was based on three conditions due to the fact that the primary goal of the study was to find out the survival effect of SpeedyRINGO on specifically calpain-based apoptosis in neurons. One such condition was to induce calpain without expressing SpeedyRINGO in neurons in order to determine if over-activity of calpain would indeed result in a change in related protein levels, such as caspase-3, p53 and CDK5. The second condition was transfection and expression of SpeedyRINGO followed by calpain induction for the purpose of observing preventative effect of SpeedyRINGO on calpain-based caspase-3 activation in hippocampal neurons. The last was the control condition, which served to compare the results of treated cells to non-treated cells.

Homo sapiens Speedy A isoform 1 of SpeedyRINGO (http://www.ncbi.nlm.nih.gov/nuccore/AY820303.1, Last updated February 21, 2007.) was a kind gift from Prof. Daniel J. Donoghue (Department of Chemistry and

Biochemistry, University of California San Diego) as myc-tagged Speedy A - pCS3 construct. Before starting the experiment, subcloning of Speedy A into pIRES expression vector (631605, Clontech Laboratories) was carried out.

After hippocampal cultures were prepared, with an Amaxa Nucleofector® Device (AAD-1001, Lonza), Speedy A - pIRES construct (7 μg) was electroporatically transfected into neurons for the "SpeedyRINGO expressing calpain induced neurons" experimental group and was incubated for a 30-hour period. After this, calpain was induced and an additional 18 hours of incubation was carried in order to complete a total 48 hours of SpeedyRINGO expression, as a 48 hour- incubation period was the minimum requirement for the expression of an appropriate amount of SpeedyRINGO within low-copy number pIRES expression vector in neurons. Cultures were kept in a 5% CO_2 environment at 37°C. To induce the calpain activation, a 3 μM A23187 Calcium ionophore (C7522, Sigma-Aldrich) solution was prepared using 0.1% Dimethyl sulfoxide (DMSO) and this solution was added to the culture and incubated at 37°C for 15 minutes. Then, a calcium medium (3mM KCl, 0,25 mM $CaCl_2$, 10 mM Hepes, 15 mM Sucrose, 139 mM NaCl, 5 mM Glucose) was replaced by the ionophore-containing medium and cultures were maintained at room temperature for 10 minutes.

In the other experimental group (calpain activation without SpeedyRINGO expression) hippocampi were prepared and then electroporatically transfected with empty pIRES vector (1 μg) in order normalized against possible procedural effects and allowed to incubate for 30 hours to attain identical conditions to the experimental group with SpeedyRINGO expression followed by calpain induction. Following this, calpain was induced using A23187 Calcium ionophore and Calcium medium in the last 18 hours of the total 48 hours of expression of empty pIRES vector.

In the control group, the empty pIRES vector (1 μg) was used to transfect the neurons and the neurons were then incubated for a 30-hour period to mimic SpeedyRINGO-pIRES expression. After this, 0.1% DMSO was applied to these control neurons, since the calpain was induced using Calcium ionophore in 0.1% DMSO. Then, control neurons were incubated at 37°C for 18 hours in 5% CO_2 .

2.2.4 Measuring calpain activity

In all groups, the activity of calpain was measured with Fluorogenic Calpain Activity Assay Kit (QIA120, Calbiochem) using manufacturer instructions. To summarize, proteins from ionophore-treated and diluent-treated control cells were lysed using CytoBuster^{TM} Protein Extraction Reagent (71009, Calbiochem). A BCA protein assay kit (23227, Pierce) was used to measure protein concentrations and 20 µg of total protein was used in calpain activity assay for all groups. The reported data are in relative fluorescence units (RFU), which are defined by manufacturer instructions. The value of the blank was subtracted from the values of fluorescence obtained from all samples to normalize the results. Then, duplicate readings were used to determine mean fluorescent value.

2.2.5 Analysis of quantitative real time polymerase chain reaction

Probes and primers used in this study were all purchased from Roche Universal Probe Library and are listed in Table 2.12. To use as references for qRT-PCR studies, Rat ACTB Gene Assay (Beta-actin) (05046203001, Roche Universal ProbeLibrary) and Rat GAPD Gene Assay (Glyceraldehyde 3-phosphate dehydrogenase / GAPDH) (05046220001, Roche Universal ProbeLibrary**)** were purchased.

Table 2.12 : Primer and probe sequences for qRT-PCR.

Gene name	Accession number	Primer sequence	Probe number
Cyclin D1	NM_171992.4	gcacaacgcactttctttcc tccagaagggcttcaatctg	16
p27	NM_031762.3	agacagtccggctgggtta ttctgttctgttggcccttt	130
CDK5	NM_080885.1	ctgcgatgcagaaatacgag tggccttgaacacagttcc	60
Cyclin A	NM_001011949.1	gagagggaaattgcagcttg gggcgggtatatctcttcgt	2
Cyclin B	NM_171991.2	tgtgaaagatatctatgcttacctcag cccagtaggtattttggtctaactg	65
Cyclin E	NM_001100821.1	ctgagagatgagcactttctgc tggagcttatagacttcacacacc	108
CDK4	NM_053593.2	gtcagtggtgccggagat ggattaaaggtcagcatttcca	50

For preparing stock solution for all the primers, they were diluted as a concentration of 200 μM. After that, 2 μM working solution was prepared from the stock solution for each of the primers.

All the probes were in 10 μM concentration. Each of them was 1:10 diluted to prepare 1 μM working solution.

RNA was isolated by High Pure RNA Isolation Kit (11828665001, Roche). RNA concentration measurements were performed by Qubit® Fluorometer (Q32857, Invitrogen) using Quant-iT™ RNA, BR Assay Kit (Q10210, Invitrogen). cDNA synthesis was done with Fermentas RevertAid™ H Minus First Strand cDNA Synthesis Kit (K1631, Fermentas, Glen Burnie, MD, USA).

qRT-PCR reactions were performed using a Light Cycler® 480 Probes Master qRT-PCR Kit (04902343001, Roche) in a Roche Light Cycler 480 following the program: 1 cycle of 10 minutes initial denaturation at 95°C, followed by 45 cycles of, 10 seconds of denaturation at 95°C, 30 seconds of amplification at 60°C, and 1 second of extension at 72°C.

All experiment groups were analyzed by the described qRT-PCR program 3 times with duplicates in order to exactly determine the changes in their mRNA level profiles.

2.2.5.1 Determining efficiency and error rate

In this study, it was aimed to use ΔΔCt method to analyze our RT-PCR results (Schmittgen, 2008). First of all, as a requirement of this method, error rate and efficiency values for each gene were determined. According to the literature, in order to be able to use this method, efficiency rate for each gene should be between 1.8-2.2 and error rate should be below 0.2.

For this reason, primary hippocampal neuron culture was prepared by using newborn rats. Then RNA isolation was performed and by using obtained RNA, cDNA was synthesized with the help of Reverse Transcriptase. After that, 4 different dilutions (undiluted, 1.10, 1:100, 1:1000) of cDNA templates were prepared in order to use in RT-PCR reactions and by using Roche TaqMan RT-PCR kit and Roche Light Cycler 2.0 four separate reaction was performed for each gene. Results were analyzed by using Roche Light Cycler 2.0 software.

2.2.6 Analysis of immunofluorescence

Phosphate Buffered Saline (PBS, P4417, Sigma-Aldrich) was used to wash all the neurons, which were then fixed using 100% methanol (494437, Sigma-Aldrich) for 10 minutes at -20°C. Following this, neurons were washed with PBS for 3 times and incubated with a blocking solution made of 10% goat (04-009-1, Biological Industries) or donkey serum (D9663, Sigma-Aldrich), 10 mg/ml Bovine Serum Albumin (A9418, Sigma-Aldrich) in PBS at room temperature for 1 hour. Then, the cells used in the experiment were incubated overnight with primary antibodies against CyclinD1 (rabbit polyclonal IgG, sc-717, Santa Cruz Biotechnology), CDK5 (mouse monoclonal IgG, sc-6247, Santa Cruz Biotechnology), Beta-III- Tubulin (mouse monoclonal IgG, G7121, Promega) in a dilution of 1:1000, and with primary antibody against SpeedyRINGO (rabbit polyclonal IgG, PA1-16959, Affinity Bioreagents) in a dilution of 1:500. Cells were again washed in PBS 3 times and incubated in blocking solution at room temperature for 1 hour. Following the blocking step, cell incubation was carried out at a dilution of 1:1000 of secondary antibodies, Alexa Fluor® 488 goat anti-mouse IgG (A11029, Invitrogen), Alexa Fluor® 647 goat anti-rabbit IgG (A21245, Invitrogen), Alexa Fluor® 647 donkey anti-rabbit (A31573, Invitrogen) at 37°C for 1 hour in dark. Finally, cells were washed in PBS 3 times and were analyzed at 63x oil objective under Leica TCP SP2 SE Confocal microscope (Leica Microsystems GmbH, Wetzlar, Germany) and the pixels were analyzed integratively to calculate the expressional values of the studied proteins.

2.2.7 Western blotting

Mammalian Cell Extraction Kit (K269-500, BioVision, Mountain View, CA, USA) was used to extract total protein from $3x10^6$ neurons for each experimental group using a cocktail of protease inhibitors provided with the extraction kit. The measurements for the protein concentration were done with the BCA protein assay kit (Pierce). Equal amounts (10 μg protein/lane) of samples were loaded on and separated by SDS-PAGE (12,5% or 10% sodium dodecyl sulfate-polyacrylamide electrophoresis based on the molecular weight of the proteins) and then transferred to nitrocellulose membrane (sc-3724, Santa Cruz Biotechnology) by a semi-dry blotting system (170-3940, BioRad). 5% BSA/Tris Buffered Saline-Tween (TTBS)

was used to block the membranes at room temperature for 2 hours. The membranes were stained overnight with the primary antibody at 4°C and with the secondary antibody at room temperature for 1 hour. The primary antibodies used at 1:500 dilutions were the following; mouse monoclonal anti-actin (sc-8432, Santa Cruz Biotechnology), mouse monoclonal anti-CDK5 (sc-6247, Santa Cruz Biotechnology), mouse monoclonal anti- $p27^{kip}$ (sc-1641, Santa Cruz Biotechnology), mouse monoclonal anti-p53 (2524, Cell Signalling Tech.), mouse monoclonal anti-CDK2 (AHZ0142, Biosource), and rabbit monoclonal anti-caspase-3 (9665S, Cell Signaling Tech.) in 5% BSA/TTBS. After being incubated with primary antibodies, TTBS buffer was used to wash the membranes 6 times for 5 minutes each at room temperature. After this, they were incubated with solutions of differing anti-HRP (W4011, W4021, V8051, Promega) secondary antibodies at 1:5000 dilution at room temperature for 1 hour. Blots were analyzed with ECL Plus Western Blotting Detection Reagents (RPN2132, GE Healthcare, Munich, Germany). Blots were scanned and the measurements of specific band densities were quantified and normalized based on actin as loading control.

2.2.8 Analysis of apoptosis by DAPI staining and TUNEL assay

DAPI staining was used to analyze nuclear condensation as an indicator of apoptosis. Cells were washed in PBS 3 times, then permeabilized, and finally incubated with l μg/ml of DAPI in dark for 5 minutes for nuclear staining. Cells were then visualized under confocal microscopy at 63x magnification.

TUNEL Assay was also performed to evaluate apoptosis for both experimental conditions and for a set of untreated neurons as a negative control with APO-BrdU™ TUNEL Assay Kit (A35125, Invitrogen, Molecular Probes) following the manufacturer's instructions using confocal microscopy imaging. Imaging analysis was performed at 63x magnification with 100 cells/slide. The results were represented as % of TUNEL positive neurons.

2.2.9 Analysis of lactate dehydrogenase (LDH) cytotoxicity

Measurement of LDH release for all conditions was made with Roche Cytotoxicity Detection Kit (11644793001, Roche), in accordance with the instructions of the manufacturer. First, primary neurons were plated as 5.000 cells/well onto 96 well-plates. Coupled with experimental conditions, low control was used to gather

information about activity of LDH released from the control cells, and a high control was used to gather information about maximum possible LDH release in the cells and finally a background control was used for information about the assay medium's LDH activity. These measurements were done with an ELISA reader at 492 nm. In order to calculate the cytotoxicity, the triplicates' average absorbance was calculated and each of these absorbance was subtracted from the background control absorbance.

2.2.10 Statistical analysis

Statistical Package for the Social Sciences (SPSS) Statistical Analysis Program was used to analyze the results using "paired-2 tailed student's t test". A value of P below 0.05 was deemed significant and a value below 0.001 was deemed very significant across all results. Error bars in the graphs were generated using ± s.e.m. values for qRT-PCR and ± s.d. values for immunofluorescence, Western blotting and calpain activity analysis.

2.2.11 Integrative pixel analysis

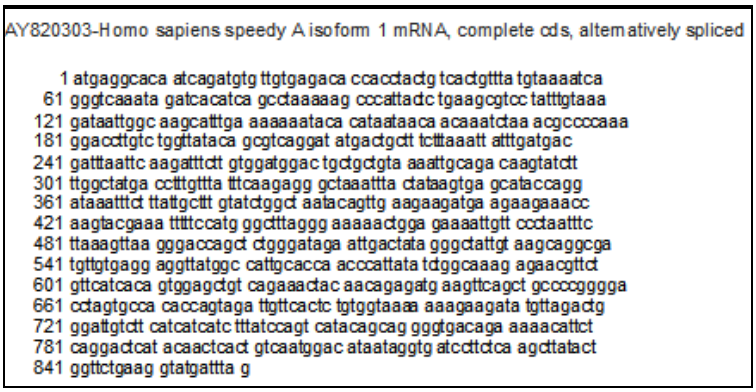
Immunofluorescence results were pixel-analyzed using ImageJ Software. For each of the experimental conditions, in excess of 100 cells were analyzed. When images were taken with the same brightness-contrast, exposure and gain settings, background subtraction was done to get total fluorescence and normalized integrated densities. For Western blot images, Photoshop CS4 Software was utilized for relative intensity analysis of the protein bands. First, for untreated cells the obtained expressional value was set to '1', and then based on this, the relative expression changes of the two other experimental conditions were calculated.

3. RESULTS

3.1 Subcloning of SpeedyRINGO into pIRES Expression Vector

3.1.1 PCR cloning of SpeedyRINGO

In order to subclone SpeedyRINGO (Figure 3.1) from pCS3 vector into pIRES, first, SpeedyRINGO was cloned out from pCS3 by PCR using primers that were specifically designed for SpeedyRINGO. All the primers were designed by adding restriction sites on their 5' ends. These restriction sites were then used for pIRES-SpeedyRINGO ligation studies.


AY820303-Homo sapiens speedy A isoform 1 mRNA, complete cds, alternatively spliced

```
   1 atgaggcaca atcagatgtg ttgtgagaca ccacctactg tcactgttta tgtaaaatca
  61 gggtcaaata gatcacatca gcctaaaaag cccattactc tgaagcgtcc tatttgtaaa
 121 gataattggc aagcatttga aaaaaataca cataataaca acaaatctaa acgccccaaa
 181 ggaccttgtc tggttataca gcgtcaggat atgactgctt tctttaaatt atttgatgac
 241 gatttaattc aagatttctt gtggatggac tgctgctgta aaattgcaga caagtatctt
 301 ttggctatga cctttgttta tttcaagagg gctaaattta ctataagtga gcataccagg
 361 ataaatttct ttattgcttt gtatctggct aatacagttg aagaagatga agaagaaacc
 421 aagtacgaaa tttttccatg ggctttaggg aaaaactgga gaaaattgtt ccctaatttc
 481 ttaaagttaa gggaccagct ctgggataga attgactata gggctattgt aagcaggcga
 541 tgttgtgagg aggttatggc cattgcacca acccattata tctggcaaag agaacgttct
 601 gttcatcaca gtggagctgt cagaaactac aacagagatg aagttcagct gccccgggga
 661 cctagtgcca caccagtaga ttgttcactc tgtggtaaaa aaagaagata tgttagactg
 721 ggattgtctt catcatcatc tttatccagt catacagcag gggtgacaga aaaacattct
 781 caggactcat acaactcact gtcaatggac ataataggtg atccttctca agcttatact
 841 ggttctgaag gtatgattta g
```

Figure 3.1 : Homo sapiens SpeedyRINGO DNA sequence.

Result of PCR reaction was then visualized with 1% agarose gel electrophoresis and it was observed that 861 bp SpeedyRINGO was successfully cloned out from pCS3 vector (Figure 3.2).

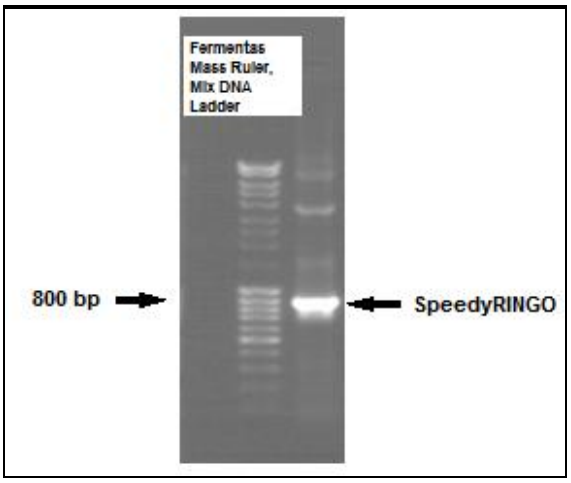


Figure 3.2 : Electrophoresis result of SpeedyRINGO PCR reaction showing successful cloning of SpeedyRINGO out from pCS3 vector. 1% agarose gel was used for electrophoresis and Fermentas Mass Ruler, Mix DNA Ladder was used for size determination of SpeedyRINGO.

After that, all PCR products are loaded on agarose gel and then purified from the gel by gel extraction method (Figure 3.3).

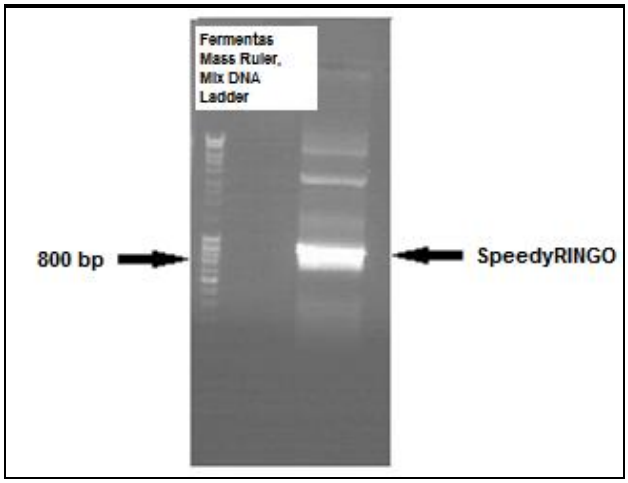


Figure 3.3 : Gel purification result of SpeedyRINGO DNA showing successful isolation of SpeedyRINGO from agarose gel. DNA fragment from agarose gel using " QIAquick Gel Extraction Kit" and Fermentas Mass Ruler, Mix DNA Ladder was used for size determination of SpeedyRINGO.

3.1.2 Restriction reactions for SpeedyRINGO and pIRES

By this PCR reaction, SpeedyRINGO was obtained with Nhe I and EcoR I restriction sites on its 5' and 3' ends, respectively. After that, both SpeedyRINGO gene and pIRES vector were cut using Nhe I and EcoR I enzymes.

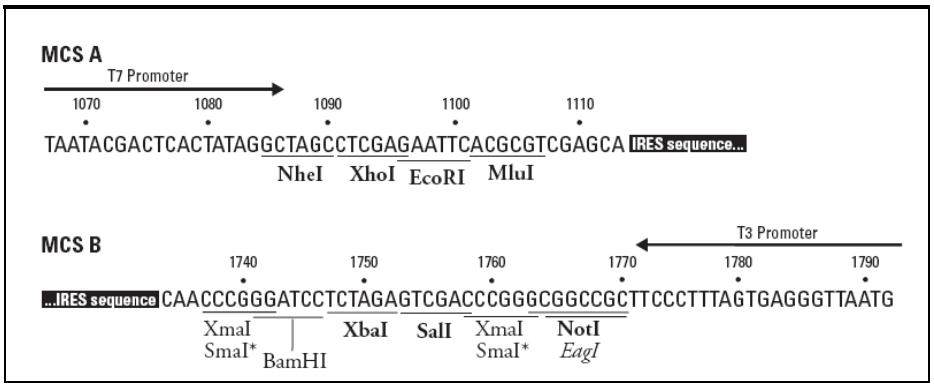


Figure 3.4 : pIRES vector multiple cloning sites (MCS A and MCS B).

As shown in the Figure 3.4, pIRES was cut and linearized by Nhe I and EcoR I enzymes, which had restriction sites in MCS A.

As the same with pIRES restriction, SpeedyRINGO gene was cut with Nhe I and EcoR I enzymes by using restriction sites added to its 5' and 3' ends for further ligation study.

Restriction reactions were incubated for 3 hours at 37 ^{0}C. Then they were loaded on 1% agarose gel and purified from there with gel extraction method (Figure 3.5).

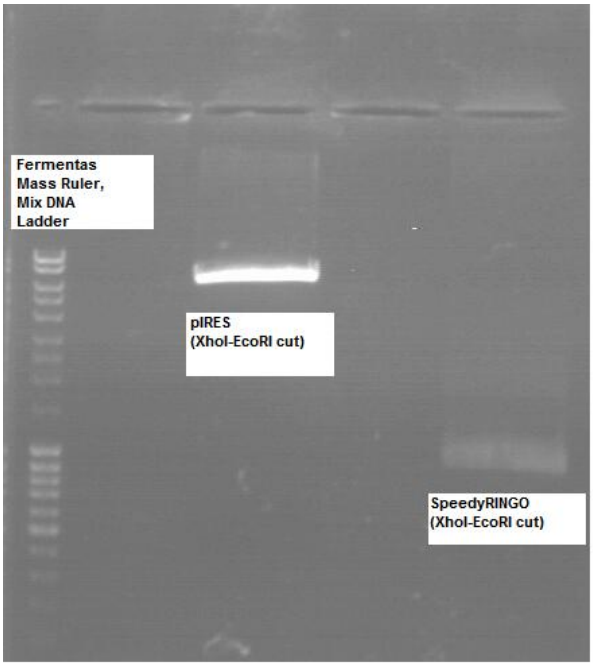


Figure 3.5 : Electrophoresis result of restriction reactions. pIRES and SpeedyRINGO were both successfully cut by Nhe I and EcoR I enzymes. Restriction fractions were isolated form agarose gel using "QIAquick Gel Extraction Kit" and Fermentas Mass Ruler, Mix DNA Ladder was used for size determination of pIRES and SpeedyRINGO.

3.1.3 SpeedyRINGO-pIRES ligation reaction

After restriction reactions were completed, ligation reaction was performed to insert SpeedyRINGO into pIRES vector.

Ligation reaction was incubated overnight at room temperature. After that, ligation mixture was transformed into DH5α strain of *E.coli*. Since pIRES is ampicillin resistant, in order to obtain only transformed cells, cells were plated into ampicillin containing LB-agar plates and incubated overnight at 37 ^{0}C. After transformation, several numbers of colonies were grown on the plate.

3.1.4 Colony PCR reaction

In order to confirm that these colonies contained SpeedyRINGO-pIRES construct, colony PCR was performed. For this reason, colonies were picked up from the plate and each of them was put into water containing eppendorf tubes. Then, all the tubes were incubated at 100 ^{0}C for 10 minutes in order to lyse the cells. With this method, DNA content of the cells were obtained and then used as template for colony PCR reaction. After colony PCR, reaction results were visualized on 1% agarose gel electrophoresis and it was observed that all the colonies had the SpeedyRINGO-pIRES construct (Figure 3.6).

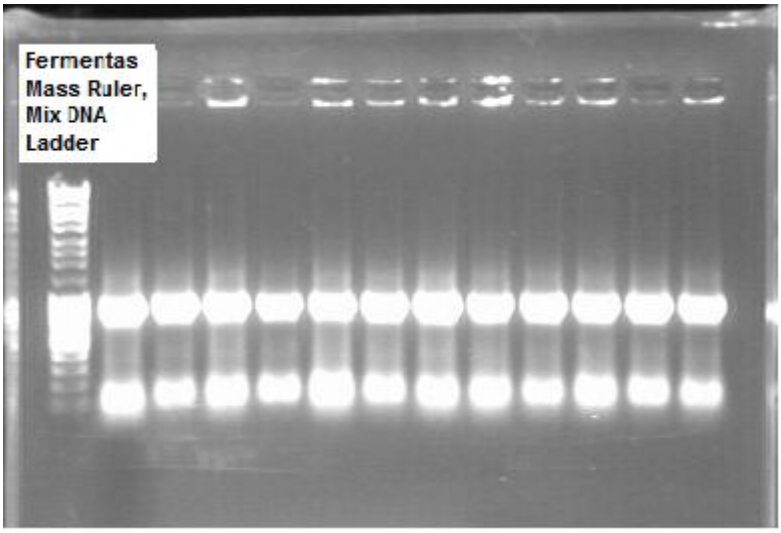


Figure 3.6 : Colony PCR results showing successful ligation and cloning of pIRES-SpeedyRINGO construct. *Escherichia coli (E.coli)* DH5α cells were transformed with pIRES-SpeedyRINGO and 12 separate colonies picked up to detect whether or not colonies had pIRES-SpeedyRINGO sequence. Fermentas Mass Ruler, Mix DNA Ladder was used for size determination of pIRES-SpeedyRINGO construct.

3.2 Calpain Activity Measurement

Calpain activity measurement was carried out to understand whether induction of intrinsic calpain by calcium ionophore was successful and sufficient and also to ensure that the observed effects in the experiments would specific to calpain over-activity. Measurements showed a significant %10 increase (*p= 0.02*) in the activity of calpain in calcium ionophore-treated neurons compared to control neurons (Figure 3.7).

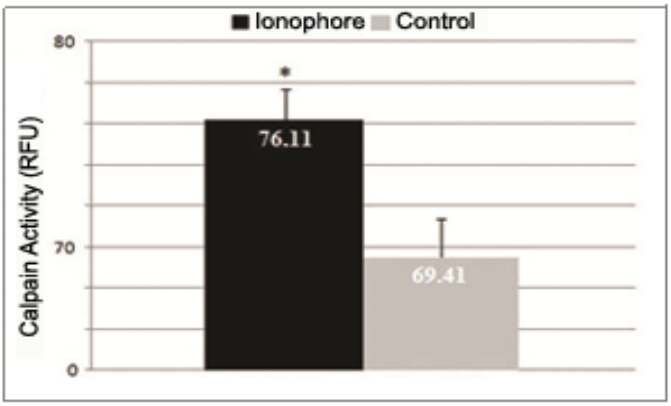


Figure 3.7 : Results of calpain activity measurement in ionophore-treated and in control neurons. Data are given as relative fluorescent units (RFU).Error bars represent mean values ± SD. n=3, * p<0,05.

3.3 Expression of Transfected SpeedyRINGO in Hippocampal Neurons

SpeedyRINGO was transfected to hippocampal neurons in order to understand whether SpeedyRINGO would successfully expressed in neurons. Results of immunofluorescence analysis indicated a 3.83 fold significant (*p = 0.0007*) increase in the expression level of SpeedyRINGO in transfected neurons showing the successful transfection and expression of SpeedyRINGO (Figure 3.8). Western blotting was also performed to confirm the results of immunofluorescence analysis and results showed that there was a 4.6 fold increase in SpeedyRINGO-transfected hippocampal neurons (Figure 3.8).

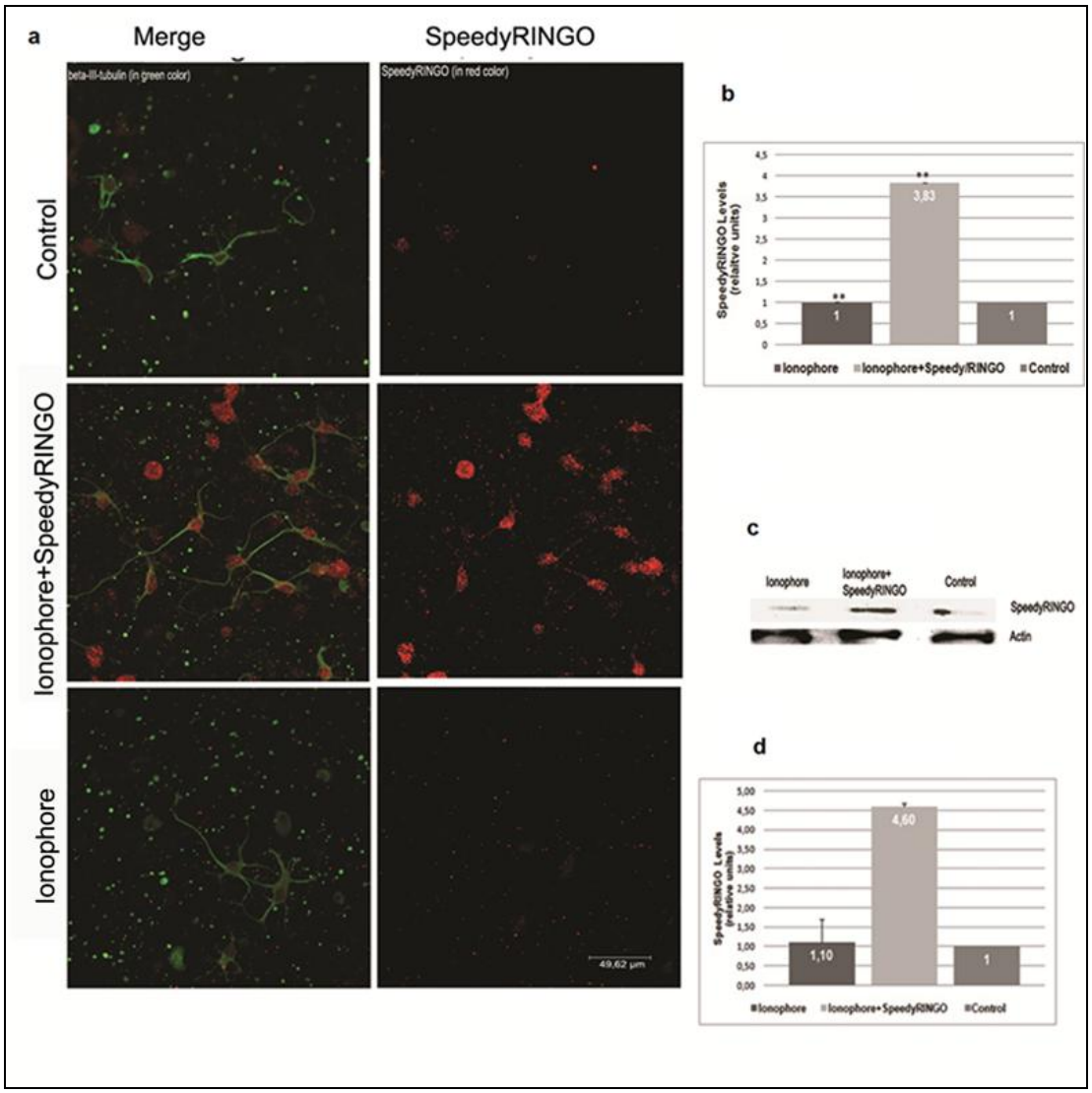


Figure 3.8 : Successful expression of SpeedyRINGO in transfected neurons. a: beta-III-Tubulin and SpeedyRINGO expression in control (upper panel), SpeedyRINGO expressing ionophore-treated (middle panel) and only ionophore-treated (lower panel) neurons. Size bar: 49.62 µm b: Relative fluorescent intensities according to the experimental/control ratio of SpeedyRINGO expression. Results were represented as fold change. Bar represents mean values ± SD. n=100, ** $p < 0.001$ c: Western blotting membrane probed with SpeedyRINGO antibody and actin antibody. d: Graph indicating quantitative analysis of signals in blots. Results were represented as fold change. Bar represents mean values ± SD.

3.4 Efficiency and Error Rate Values of GAPDH, ACTB, CDK5, Cyclin D1 and p27 Genes for qRT-PCR Analysis

Efficiency and error rates were analyzed for each gene of interest. In order to use ΔΔCT method to analyze qRT-PCR results, efficiency rate for each gene should be between 1.8-2.2 and error rate should be below 0.2. Efficiency and error rates of each gene were in expected ranges and results were given in the Table 3.1:

Table 3.1 : Efficiency and error rate values.

Gene	Efficiency	Error Rate
GAPDH	1.968	0.00875
ACTB	1.899	0.0547
CDK5	1.859	0.06
Cyclin D1	1.918	0.009
p27	1.889	0.00218
Cylin A	1.973	0.0154
Cyclin B	1.810	0.0141
Cyclin E	1.859	0.009
CDK4	1.934	0.00980

3.5 Changes in Different Cell Cycle Markers' mRNA Levels in the Presence / Absence of SpeedyRINGO in Calpain-Induced Hippocampal Neurons

Since SpeedyRINGO is a meiotic / mitotic cell specific cell cycle regulator, all of the existing studies about SpeedyRINGO were performed in mitotic or meiotic cells. For this reason, it was first aimed to identify if SpeedyRINGO would be effective on a chaotic cell cycle reactivation attempt in calpain-induced post-mitotic neurons. For this purpose, different cell cycle markers were first analyzed in mRNA level in the absence or presence of SpeedyRINGO in calpain-induced neurons to see a possible upregulation of these markers in the presence of SpeedyRINGO, which would indicate a positive regulatory function for SpeedyRINGO on cell cycle reactivation attempt in neurons. mRNA analysis results showed that calpain over-activity resulted in chaotic up- and down-regulation of mRNA levels of different cell cycle markers (Cylin A, Cyclin B, Cyclin E, CDK4). SpeedyRINGO was shown to prevent this chaotic up- and down-regulation and kept mRNA levels similar to that of control neurons, as it was shown that there was no upregulation of cell cycle markers (Figure 3.9).

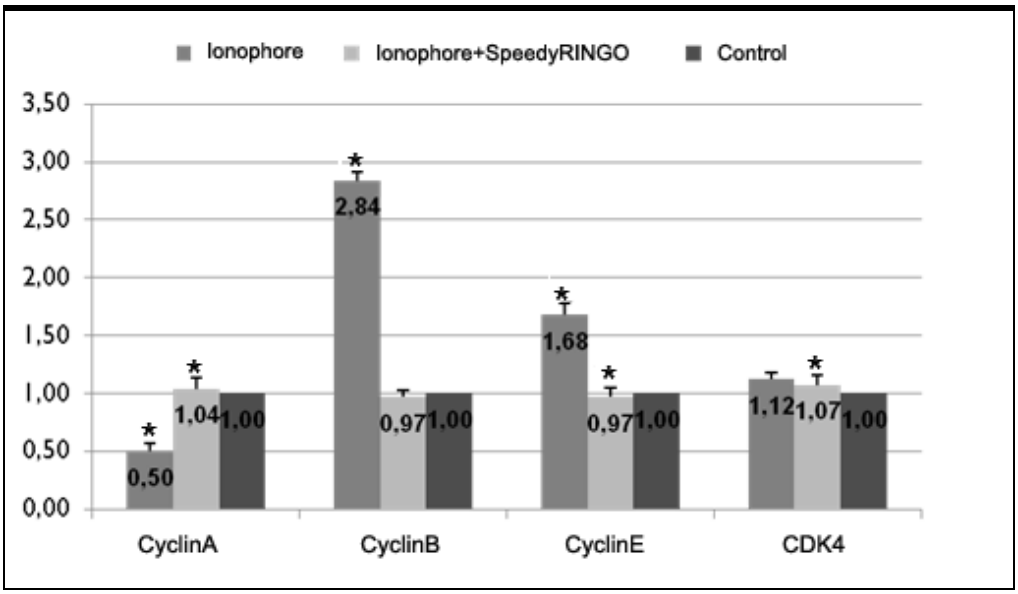


Figure 3.9 : Changes in mRNA levels of Cyclin A, Cyclin B, Cyclin E and CDK4 in ionophore- treated and in SpeedyRINGO expressing ionophore-treated neurons. Data represent CT value of treated cell / CT value of untreated cells. Bar represents mean values ± SEM. n=3, * $p<0.05$.

3.6 Changes in CDK5 and Cyclin D1 mRNA and Protein Levels After Calpain Induction

Calpain over-activation forces neurons to exit from G_0 and undergo apoptosis through CDK5 as the key factor, instead of CyclinD1, which is contrary to the conventional idea that CyclinD1 is required for G_0 exit. Pathologic activation of calpain has also been shown to lead neurons to undergo apoptosis indirectly, through CDK5 by increasing p53 and caspase-3 activation. Therefore, mRNA and protein level changes of CDK5 and CyclinD1 were determined. Results of Q-PCR indicated a 1.27 fold significant increase *($p = 0,03$)* in mRNA level of CDK5 in calpain-induced hippocampal neurons, while mRNA level of CyclinD1 significantly decreased *($p = 0,04$)* (Figure 3.9). However, in SpeedyRINGO expressing calpain-induced neurons, both CDK5 and CyclinD1 mRNA levels were decreased significantly *($p= 0,01$* and *$p= 0,04$*, respectively) (Figure 3.10).

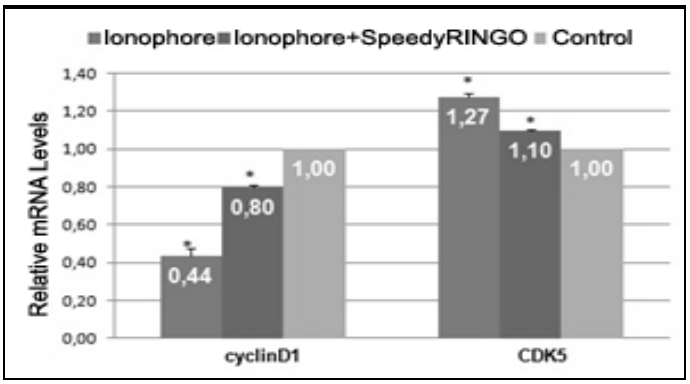


Figure 3.10 : Changes in mRNA levels of CDK5 and Cyclin D1 in ionophore-treated and in SpeedyRINGO expressing ionophore-treated neurons. Data represent CT value of treated cell / CT value of untreated cells. Bar represents mean values ± SEM. n=3, * $p<0.05$.

Besides changes in mRNA level, protein level changes were also determined with immunofluorescence. Immunofluorescence results indicated no detectable expression of CyclinD1 in either calpain-induced or control neurons (Figure 3.11). However, in calpain-induced neurons, there was a significant 1.64 fold increase in CDK5 expression (Figure 3.12) which is in accordance with the change in mRNA level.

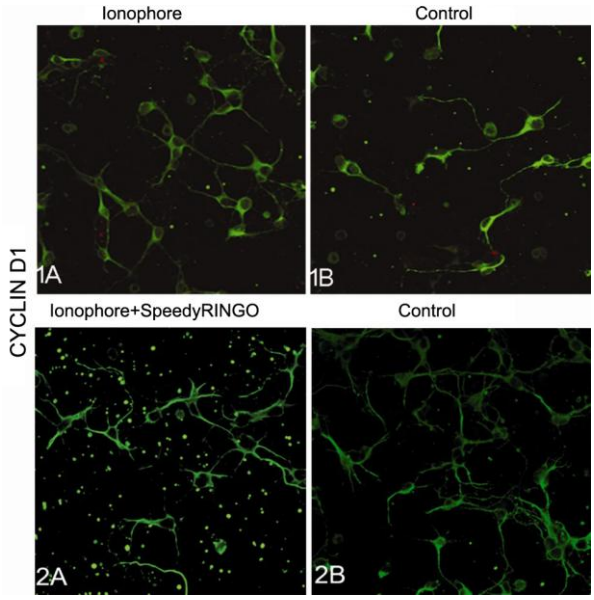


Figure 3.11 : Changes in Cyclin D1 expression in ionophore-treated, SpeedyRINGO expressing ionophore-treated and in control neurons. 1A: Cyclin D1 (red) and beta tubulin (green) expression in neurons. 1B: Cyclin D1 (red*) and beta tubulin (green) expression in control neurons. 2A: Cyclin D1(red) and beta tubulin (green) expression in SpeedyRINGO expressing ionophore-treated neurons. 2B: Cyclin D1 (red*) and beta tubulin (green) expression in control neurons. *Cyclin D1 was fluorescently labeled in red, however due to absence in neurons, it could not be detected.

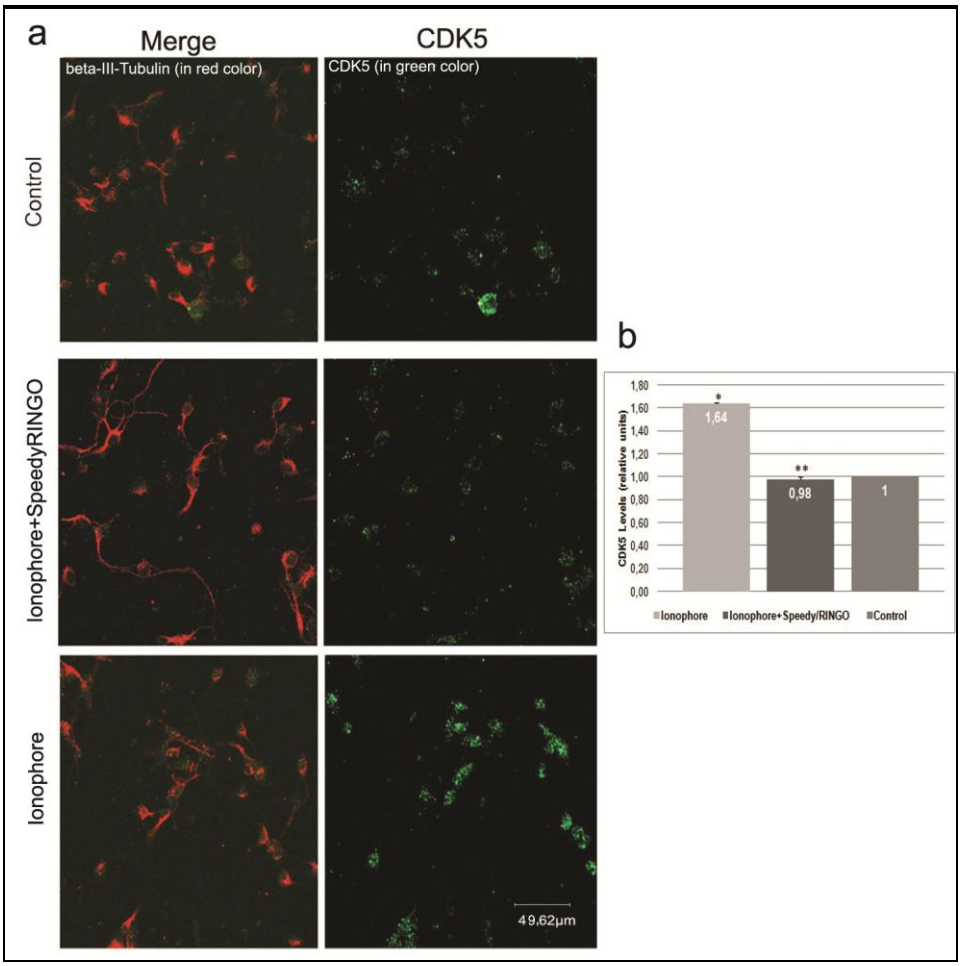


Figure 3.12 : Changes in CDK5 protein level in three experimental conditions. a: beta-III-Tubulin and CDK5 expression in control (upper panel), SpeedyRINGO expressing ionophore-treated (middle panel) and only ionophore-treated (lower panel) neurons. Size bar: 49.62 µm b: Relative fluorescent intensities according to the experimental/control ratio of SpeedyRINGO expression. Results were represented as fold changes. Bar represents mean values ± SD. n=100, ** $p < 0.001$.

In order to support the results of immunofluorescence, western blotting was also carried out. Results showed a significant, 1.31 fold increase in CDK5 protein level in ionophore-treated neurons compared to control (Figure 3.13) which was also in accordance with the changes in CDK5 mRNA level. Moreover, in SpeedyRINGO expressing calpain over-activated neurons, CDK5 protein level was almost same with the control, supporting CDK5 mRNA results, CDK5 was also decreased in protein expression (Figure 3.13).

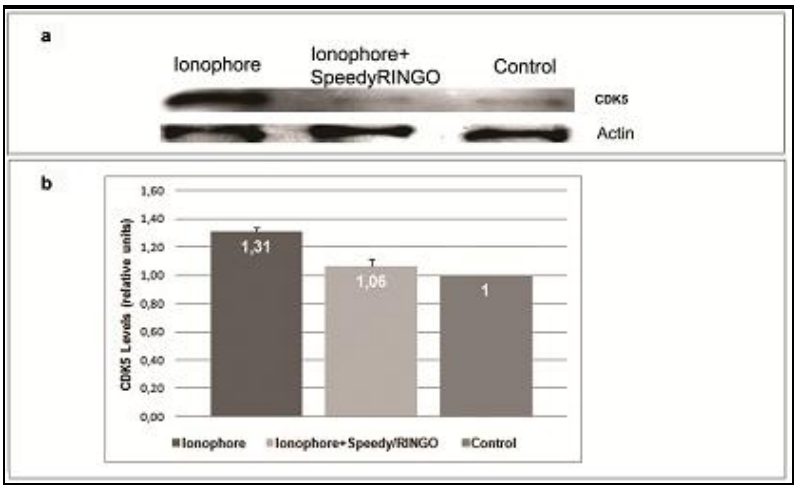


Figure 3.13 : Result of western blotting for CDK5. a: Changes in CDK5 protein level in ionophore-treated, in SpeedyRINGO expressing ionophore-treated and in control hippocampal neurons. b: Graph indicating quantitative analysis of signals in blots. Results were represented as fold changes. Bar represents mean values ± SD.

3.7 Upregulation of $p27^{kip}$ mRNA and Protein Levels After Only Calpain Induction

$p27^{kip}$ was also analyzed for the reason that $p27^{kip}$ is a general inhibitor of the different phases of cell cycle regulators and calpain over-activation has been shown to upregulate p27, which in turn results in apoptosis (Noguchi, 2003). Q-PCR results of calpain activation experiments indicated that mRNA level of common CDK inhibitor $p27^{kip}$ was increased; but upon SpeedyRINGO expression, mRNA level of $p27^{kip}$ was significantly reduced *($p = 0,048$)* (Figure 3.14a). Besides that, western blotting analysis showed a 1.58 fold increase in protein expression of $p27^{kip}$ after ionophore-treatment, while $p27^{kip}$ expression was decreased after SpeedyRINGO expression followed by ionophore-treatment compared to control neurons (Figure 3.14b).

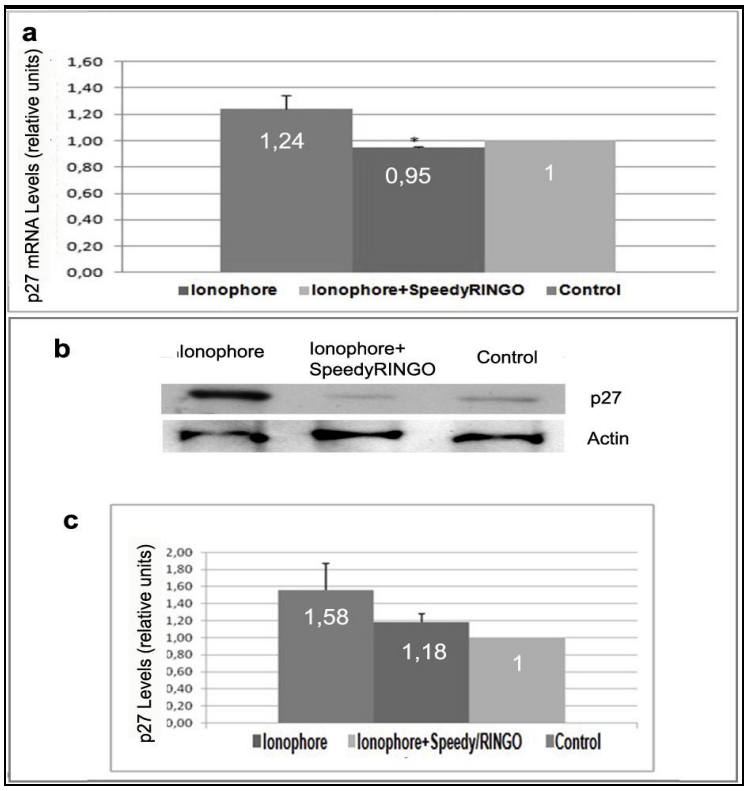


Figure 3.14 : Results of mRNA and protein level changes of p27. a: mRNA level changes in ionophore-treated, SpeedyRINGO expressing ionophore-treated and in control neurons. b: Changes in p27 protein level in ionophore-treated, in SpeedyRINGO expressing ionophore-treated and in control hippocampal neurons. c: Graph indicating quantitative analysis of signals in blots. Results were represented as fold changes. Bar represents mean values ± SD.

3.8 Over-expression of CDK2 Protein After SpeedyRINGO Expression

As CDK2 is the specific partner of SpeedyRINGO and it cannot properly function in the absence of CDK2, CDK2 protein level was also analyzed in expectation of an increase in CDK2 level in SpeedyRINGO-transfected neurons. For this purpose, western blotting was performed and results indicated that there was a 1.52 fold increase in protein level of CDK2 in SpeedyRINGO expressing neurons, whereas there was not an obvious change in ionophore-treated neurons (Figure 3.15).

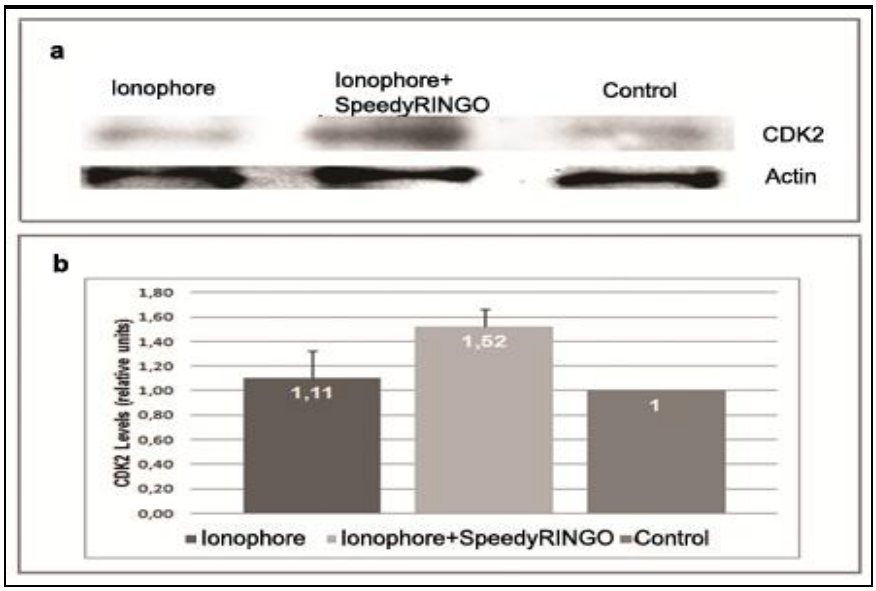


Figure 3.15 : Results of western blotting for CDK2. a: Change in CDK2 protein level in SpeedyRINGO expressing neurons. b: Graph indicating quantitative analysis of signals in Panel A. Results were represented as fold changes. Bar represents mean values ± SD.

3.9 Up-regulation of p53 Protein After Calpain Over-activation in the Absence or Presence of SpeedyRINGO

Since p53 expression is a necessity for SpeedyRINGO to prevent calpain-mediated apoptosis by inhibiting caspase-3 activation, it was crucial to determine the p53 protein level in ionophore-treated, SpeedyRINGO expressing ionophore-treated and in control neurons. For this reason, western blotting was performed and a 1.24 fold increase in p53 protein level in ionophore-treated neurons, and a 1.33 fold increase in SpeedyRINGO expressing ionophore-treated neurons were observed according to western blotting results (Figure 3.16).

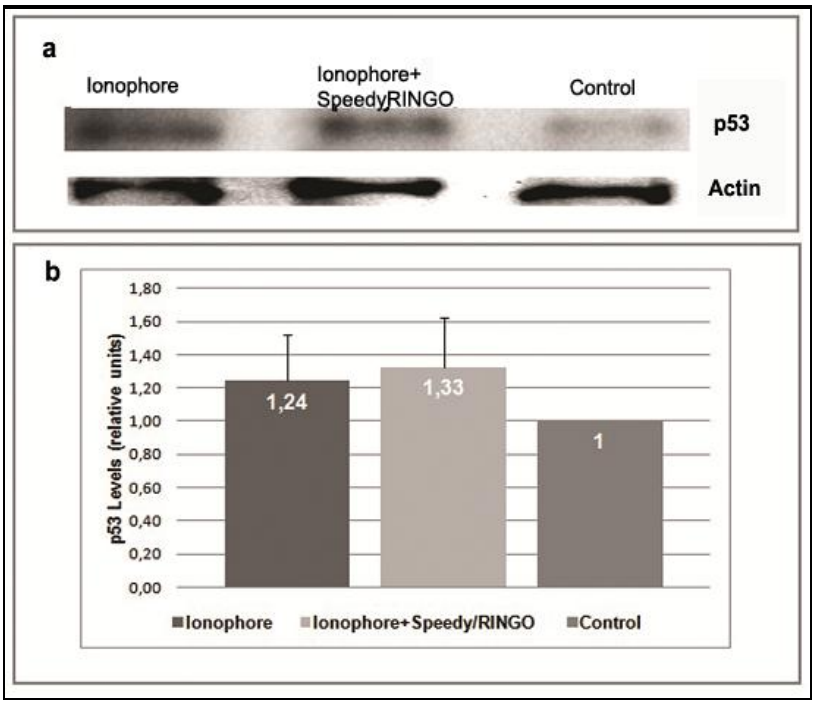


Figure 3.16 : Results of western blotting for p53. a: Change in p53 protein level in SpeedyRINGO expressing neurons. b: Graph indicating quantitative analysis of signals in Panel A. Results were represented as fold change. Bar represents mean values ± SD.

3.10 Prevention of Calpain-directed Caspase-3 Activation by SpeedyRINGO

One of the major points in this study was to determine whether SpeedyRINGO had protective effects against caspase-3 activation caused by calpain over-activity in neurons. Thus, western blotting was carried out for all experimental groups to show if there was an increase in active caspase-3 levels in calpain-overactivated neurons and also to show if there was an inhibition of this caspase-3 activation by SpeedyRINGO. Western blotting results showed a 1.37 fold increase in active caspase-3 level as a result of calpain activation in ionophore-treated neurons compared to control neurons, whereas level of active caspase-3 was decreased in SpeedyRINGO expressing ionophore-treated neurons compared to control indicating inhibition of caspase-3 activation by SpeedyRINGO (Figure 3.17).

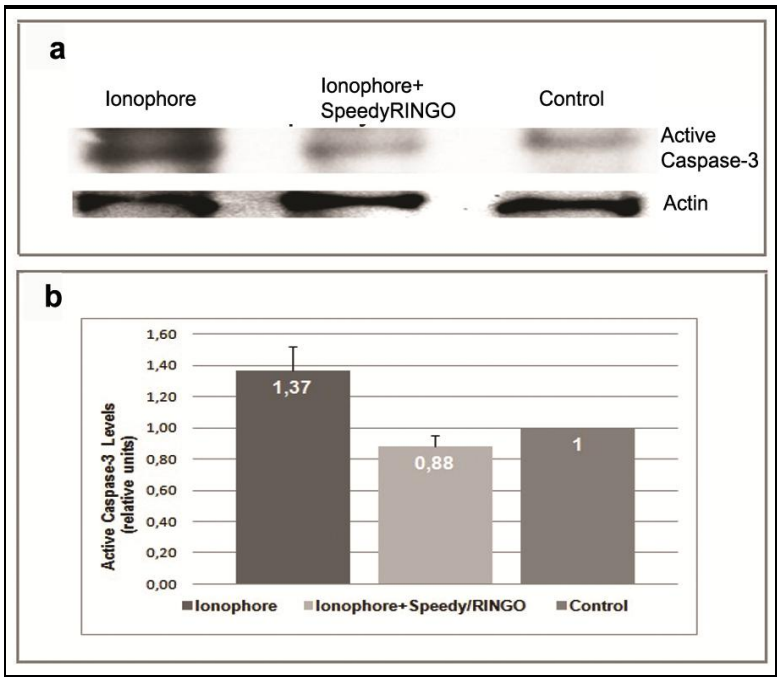


Figure 3.17 : Results of western blotting for active caspase-3. a: Change in active caspase-3 protein level in SpeedyRINGO expressing neurons. b: Graph indicating quantitative analysis of signals in Panel A. Results were represented as fold change. Bar represents mean values ± SD.

3.11 Detection of Apoptosis by DAPI Staining in Ionophore-treated and SpeedyRINGO Expressing Ionophore-treated Neurons

DAPI staining was performed to analyze DNA condensation in calpain over-activated neurons as a hallmark of apoptosis and to determine whether SpeedyRINGO would inhibit the progression of apoptosis. Results of DAPI staining were analyzed with confocal microscopy and it was shown that there was a marked DNA condensation in the nuclei of ionophore-treated neurons, whereas SpeedyRINGO-expressing ionophore-treated neurons had intact and uniformly stained nuclei (Figure 3.18). It was also detected that there was an enlargement in the nuclei of apoptotic neurons as a result of initiation of DNA fragmentation (Figure 3.18, lower left panel) which indicates early stages of apoptosis (Yang, 2007).

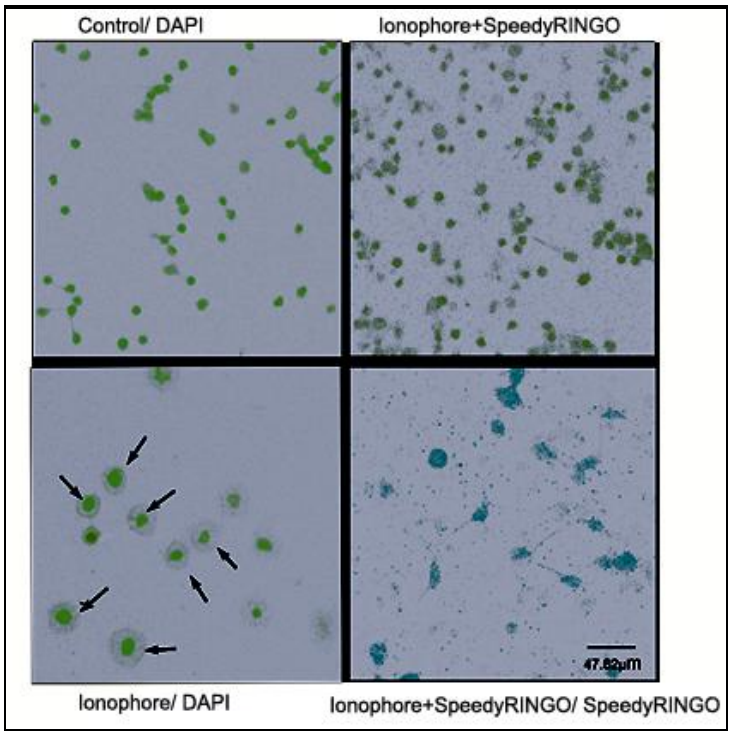


Figure 3.18 : Results of DAPI staining. DAPI staining (blue) in control (upper left panel), in calpain-induced (lower left panel), in SpeedyRINGO expressing ionophore-treated (upper right panel) neurons and SpeedyRINGO expression (red) in SpeedyRINGO expressing ionophore-treated (lower right panel) neurons. Size bar: 47.62µm.

3.12 Detection of Apoptosis by TUNEL Assay in Only Calpain-induced and SpeedyRINGO Expressing Calpain-induced Neurons

In order to detect nuclear DNA-fragmentation as a hallmark of apoptosis which could be caused by calpain induction, and also to detect if SpeedyRINGO could prevent this progress, TUNEL assay was performed and results were analyzed by confocal microscopy imaging. TUNEL assay indicated that only calpain induction caused 65% apoptotic cells in the primary hippocampal neurons (Figure 3.19), while SpeedyRINGO-expressed calpain-induced neurons did not give any apoptotic signals with TUNEL analysis.

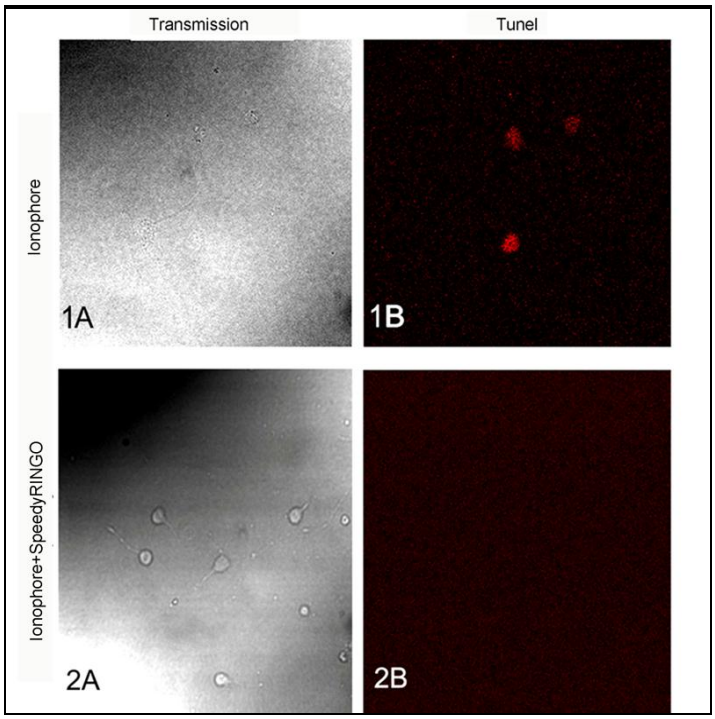


Figure 3.19 : TUNEL analysis of DNA damage in calpain-induced neurons. 1A: Transmission image of calpain-induced neurons. 1B: TUNEL-positive neurons (red) in calpain-induced neuron culture. 2A: Transmission image of SpeedyRINGO expressing calpain-induced neurons. 2B: TUNEL-positive neurons (red) in SpeedyRINGO expressing calpain-induced neuron culture.

3.13 Results of Cytotoxicity Analysis

Cytotoxicity analysis was performed for all experimental conditions as a function of released LDH from neurons to be able to identify SpeedyRINGO's recovery effort against calpain-based cytotoxicty in neurons. LDH cytotoxicity measurements indicated relatively more toxicity for calpain-induced neurons (29.53%) compared to SpeedyRINGO expressing calpain-induced neurons (10.91%). It means that SpeedyRINGO caused an approximately 3 fold decrease in the calpain cytotoxicity when expressed before inducing calpain in neurons (Figure 3.20).

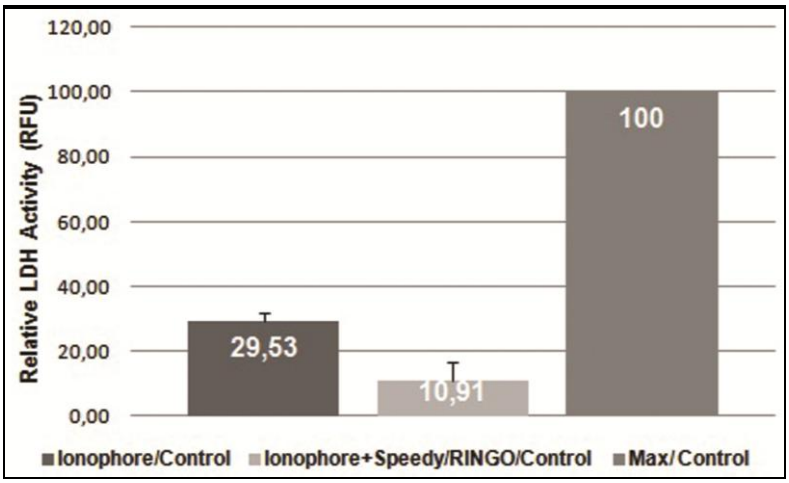


Figure 3.20 : Results of LDH cytotoxicity analysis. Data are given as relative LDH activity (%) in ionophore-treated and in SpeedyRINGO expressing ionophore-treated neurons. Bar represents mean values ± SD.

4. DISCUSSION

Calpain over-activity has been shown to directly (through p53 induction) (Hamdane, 2005; Alvira, 2008; Sedarous, 2003) or indirectly (by up-regulating CDK5 which would in turn re-activate cell cycle and also upregulate p53 expression) lead neurons into apoptosis. Thus, one of the main aims of this study was to regulate chaotic cell cycle re-activation attempt using SpeedyRINGO, a novel cell cycle regulator, to tip the balance in favor of division rather than apoptosis in degenerating neurons. Another major target of the study was to prevent the apoptotic effect of calpain in degenerating neurons utilizing anti-apoptotic function of SpeedyRINGO in calpain-induced neurons.

For these purposes, first calpain was induced by calcium ionophore in neurons. In order to ensure that calpain induction was successful and also to ensure that the observed effects in the experiments were indeed calpain-specific, calpain activity was measured after induction by calcium ionophore. Measurement results indicated 10% increase in calpain activity in calpain-induced neurons compared to control neurons. Since visualizing the gradual progression of calpain-mediated apoptotic and neurodegenerative pathway was crucial in this study, low concentration of calcium ionophore and a short incubation period (18 hours) were preferred not to cause sudden and rapid apoptotic death of neurons. Thus, a significant 10% increase in calpain activity was sufficient in this context.

In addition, efficient transfection and expression of SpeedyRINGO in neurons was also analyzed and the results showed that a mitotic protein, SpeedyRINGO, could be effectively expressed in non-mitotic neurons.

After ensuring the success of calpain induction and SpeedyRINGO expression in neurons, in order to identify regulatory effect of SpeedyRINGO on cell cycle re-activation in neurons, mRNA levels of different cell cycle markers were first analyzed in the absence and presence of SpeedyRINGO in calpain-induced neurons to see a possible upregulation of these markers in the presence of SpeedyRINGO which would indicate a possible regulatory effect for SpeedyRINGO on calpain-

based cell cycle re-activation in neurons. Results showed that there was no upregulation of these markers indicating that SpeedyRINGO did not have an inducing effect on cell cycle markers but it may have a protective effect against calpain-mediated cell cycle re-activation in neurons by keeping cell cycle marker levels similar to that of control neurons.

Cell cycle re-activation in calpain- induced neurodegeneration has been shown to be through CDK5 protein, rather than conventional Cyclin D1 protein. CDK5 is a non-mitotic kinase that is normally found in neurons, having role in development and maturation (Dhariwala, 2008;Yip, 2007; Fu, 2006). However, CDK5 has been shown to be a contributing factor in some neurodegenerative diseases that accelerate apoptosis.

In calpain-induced neurons, it has been shown that pRb protein is a substrate for CDK5 and CDK5 phosphorylates and inactivates pRb so that cell cycle progresses through G1/S transition in degenerating neurons (Hamdane, 2005; Alvira, 2008). Thus, if CDK5 level abnormally increases, it would cause an increase in pRB phosphorylation and result in aberrant cell cycle activation in neurons. This aberrant cell cycle activation ends up with apoptosis instead of division.

In addition to its role in calpain-mediated cell cycle re-activation in neurons, CDK5 has also been shown to upregulate p53, since p53 has been shown to be one of the substrates of CDK5 so that it can modulate both activity and expression of p53. Thus, increased CDK5 level results in upregulation of p53 protein due to the upregulation of its transcriptional activity (Zhang, 2002) which would finally end up with apoptosis.

Therefore, analyzing the changes in CDK5 mRNA and protein levels upon calpain activation was essential in revealing the initiation of the apoptotic process. In addition, it was crucial to determine a possible increase in CDK5 mRNA and protein levels, which could indicate a different function for it, such as re-activation of cell cycle.

The analysis results showed that over-activation of calpain increased CDK5 mRNA level significantly, which was followed by a corresponding fold increase in CDK5 protein level which may indicate re-activation of cell cycle and also initiation of apoptotic process in neurons.

While there were an increase in CDK5 mRNA and protein levels, there was a decrease in Cyclin D1 mRNA, and there was no detectable Cyclin D1 protein upon calpain activation. All these results were consistent with the findings that calpain-mediated G_0 exit is caused by CDK5 rather than CyclinD1. In addition, it is possible that Cyclin D1 may still be contributing to the G_0 to G_1 transitions of neurons. Yet, decreased levels of CyclinD1 in calpain-induced neurons may indicate that CDK5 could bring neurons to further in G_1 phase, rather than only G_0 exit, as it is known that CyclinD1 levels must be high at the beginning of G_1 phase for a cell to initiate DNA synthesis, but then must be down-regulated towards the end of G_1 phase to allow for subsequent efficient DNA synthesis. This down-regulation is regulated only by the phase of the cell cycle and occurs during each cell cycle (Yang, 2006).

Contrary to the results in the calpain-induced condition, after SpeedyRINGO expression followed by calpain induction, CDK5 upregulation was significantly inhibited in both mRNA and protein expression levels indicating efficient prevention of CDK5 upregulation by SpeedyRINGO. Inhibition of CDK5 upregulation in those neurons may also be the reason of SpeedyRINGO for preventing chaotic up- and down-regulation of cell cycle markers and keeping these markers at control levels in calpain-induced neurons (Figure 3.9). This finding is significant in that SpeedyRINGO, which regulates cell cycle progression only in some specific types of mitotic cells, is shown to be able to inhibit attempts of degenerative cell cycle re-entry in post-mitotic neurons.

In addition, based on the apoptotic role of calpain over-activation through CDK5, these results indicate that calpain over-activity indeed upregulated CDK5 which is expected to cause subsequent p53 upregulation / apoptosis and SpeedyRINGO acts as an anti-apoptotic factor in those neurons by inhibiting CDK5 upregulation.

Although there was no upregulation of mitotic markers in SpeedyRINGO expressing neurons, it was determined that only CDK2 protein expression was upregulated in SpeedyRINGO expressing neurons. It was actually expected because CDK2 is the partner of SpeedyRINGO, and over expression of SpeedyRINGO induced its partner's expression so that it can properly function when necessary.

In addition to its role in cell cycle re-activation attempt, calpain has also been shown to activate apoptotic machinery by increasing p53 level in degenerating neurons.

Western blotting studies showed that both calpain activation indeed lead to up-regulation of p53 in the absence / presence of SpeedyRINGO. As explained previously, calpain up-regulates p53 either directly (through regulating its transcription) or indirectly (via CDK5).

In this study, it has also been shown that SpeedyRINGO maintains CDK5 in its regular levels when it is expressed in neurons. In spite of this, it appears that SpeedyRINGO was not able to act on the activity of calpain since in neurons that were expressing SpeedyRINGO, p53 was still up-regulated. While this up-regulation results from the activation of calpain, which in turn, causes apoptosis, p53 presence is also needed by SpeedyRINGO for inhibiting the activity of caspase-3. Hence, in both situations, the p53 level is in favor of SpeedyRINGO since SpeedyRINGO can be effective in the inhibition of caspase-3 activation only when p53 is present, as shown before in mitotic cells (McAndrew, 2009; Gastwirt, 2006). Therefore, for SpeedyRINGO to further inhibit caspase-3, an increase in p53 in apoptosis is required, consistent with the critical role of p53 at the pendulum of cell cycle arrest and apoptosis.

p27 was also analyzed in mRNA and protein level for the reason that it is a general cell cycle inhibitor and also known as tumor-suppressor protein. Moreover, calpain over-activation has been shown to upregulate p27 levels, which has been accepted as a marker for apoptosis. Our results showing the elevation of mRNA and protein levels of $p27^{kip}$ in only calpain-induced case may indicate a possible reactive effort of neurons to inhibit cell cycle re-activation and may also indicate the presence of apoptosis in those neurons. $p27^{kip}$ level was decreased 20% in SpeedyRINGO expressing calpain-induced neurons compared to only calpain-induced neurons. All these results may also imply that SpeedyRINGO keeps neurons under stable conditions by avoiding chaotic cell cycle re-entry upon calpain induction, and therefore, there is no need to put a $p27^{kip}$ brake on this cell cycle activation. The presence of SpeedyRINGO may also have a pro-active effect on $p27^{kip}$ by preventing its up-regulation.

One of the main goals of this study was to analyze protective and recovery effect of SpeedyRINGO on calpain-based apoptosis in degenerating neurons. Therefore, the key question in this study was whether SpeedyRINGO could prevent calpain-mediated caspase-3 activation in adult neurons. Results of western blotting indicated

a 1.37 fold increase in active caspase-3 levels in calpain-induced neurons indicating the activation of apoptotic machinery. This caspase-3 level increase was contributive that calpain induction could lead neurons re-activate their cell cycle and force them undergo apoptosis through caspase-3 activation. In contrast with the apoptotic effect of calpain, SpeedyRINGO was shown to be anti-apoptotic by inhibiting caspase-3 activation in calpain-overactivated neurons. These results evidently point out that SpeedyRINGO impedes the progress of calpain-mediated apoptosis by preventing the activation of caspase-3 in degenerating neurons.

In addition to all these findings, It was noteworthy that fold changes in both transcriptional and translational level of analyzed proteins were all significant but not too high. Since a low concentration of calcium ionophore and a short time period of incubation were chosen for calpain over-activity experiments in order to prevent sudden and rapid apoptotic death of neurons. It was noteworthy to see how significant the results were even in case low level of fold changes in cyclins. This would enable visualizing the gradual progression of calpain-mediated apoptotic and neurodegenerative pathway and also the effect of SpeedyRINGO on this progression.

Together with these results, TUNEL analysis also indicated that there was 65% TUNEL-positive neurons in only calpain-induced neuron population, whereas no TUNEL-positive neuron was detected in the SpeedyRINGO expressing calpain-induced neuron population, and this supports the idea that SpeedyRINGO could inhibit caspase-dependent apoptosis in degenerating neurons.

Besides TUNEL analysis, DAPI staining also showed that over-activation of calpain resulted in condensation of the DNA and it also caused the enlargement of the nuclei, which is an indicator of the starting of DNA fragmentation indicating the early-apoptotic process in neurons. However, SpeedyRINGO transfected calpain-overactivated neurons had intact nuclei that did not show any sign of DNA condensation and nuclear enlargement. This findings support the hypothesis that SpeedyRINGO may prevent calpain-mediated apoptosis in neurons.

Moreover, cytotoxicity analysis also supported other findings that calpain induction and over-activation caused relatively more cytotoxicity in neurons (29.53%), while SpeedyRINGO expression drastically recover the cytotoxic effect of calpain induction by decreasing the cytotoxicity level to 10.91%.

To summarize, calpain over-activation was indeed an important neurodegenerative and apoptotic factor for neurons and results of this study showed for the first time that SpeedyRINGO, a specific cell cycle regulator, prevented this adverse effect of calpain by inhibiting caspase-mediated apoptosis in post-mitotic neurons.

5. CONCLUSIONS

It was shown for the first time that, mitotic cell specific regulator, SpeedyRINGO was protective against calpain-mediated apoptosis in terminally differentiated post-mitotic neurons. The results indicate that calpain induction forces neurons to re-enter cell cycle and drive them into apoptosis by increasing p53 expression and activating caspase-3, which is a landmark of caspase-dependent apoptosis. Conversely, presence of SpeedyRINGO in calpain-induced neurons prevented caspase-3 activation in a p53-dependent manner.

It was of primary importance to find a way to protect neurons against apoptotic effect of calpain over-activity, as pathological induction of intrinsic calpain has been shown to be a serious cause of cell cycle re-activation and subsequent apoptosis in degenerating neurons. It would also be worthwhile in terms of giving researchers new therapeutic insights to overcome the deadly progression of neurodegenerative diseases.

Although, the exact mechanism for the protective effect of SpeedyRINGO in calpain mediated apoptosis requires further analysis, based on the maintained presence of p53 in SpeedyRINGO expressing neurons, it could be speculated that the effects are not based on the direct inhibition of calpain activity as calpain causes p53 increase, it is rather at the downstream of this pathway. The most direct effects of SpeedyRINGO might be related with the activity of caspase-3.

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- **Yıldız Ünal A**., Korulu Ş., Karabay A. 2012: SpeedyRINGO inhibits calpain-directed apoptosis in neurons, *J Alzheimers Dis.*, 31, 779-791.
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