EFFECTS OF ENHANCED TrkA SIGNALING IN MOUSE EMBRYONIC
STEM CELL-DERIVED BASAL FOREBRAIN CHOLINERGIC NEURONS
AGAINST THE IN VITRO NEURODEGENERATIVE PROCESS OF
ALZHEIMER'S DISEASE

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ABSTRACT

EFFECTS OF ENHANCED TrkA SIGNALING IN MOUSE EMBRYONIC STEM CELL-DERIVED BASAL FOREBRAIN CHOLINERGIC NEURONS AGAINST THE IN VITRO NEURODEGENERATIVE PROCESS OF ALZHEIMER'S DISEASE

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Alzheimer's Disease (AD) is the most common neurodegenerative disease affecting more than 50 million people worldwide, and there is still no cure for AD despite extensive research efforts. The underlying cause of the disease is not well understood. Abnormal levels of neurotrophins and their receptors, and defects in neurotrophin signaling have long been correlated with AD. The neurotrophin family has four members, which are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5), and these factors mediate neuronal development, differentiation, cell survival, and synaptic plasticity through their high-affinity Tropomyosin receptor kinase (Trk) receptors. Modulation of neurotrophin signaling has been considered a potential therapeutic option against the neurodegenerative process of AD; however, there has been limited progress. A better understanding of critical residues in Trk receptors controlling neurotrophin signaling in neurons can potentially pave the road for manipulating the neurotrophin signaling in the AD context. To this end, a previous study from our group demonstrated that deletion of a conserved 3 amino acid domain (KFG domain) increases the TrkA receptor levels and enhances its

signaling. NGF-mediated TrkA signaling play crucial roles in basal forebrain cholinergic neurons (BFCNs), which are among the most affected cell types in AD. Therefore, in this thesis, we focused on examining the effects of enhanced TrkA receptor signaling in BFCNs against the *in vitro* neurodegenerative process of AD, utilizing the KFG system established previously by our group. Towards this goal, we utilized WT and TrkA-KFG in which the KFG domain is deleted in TrkA mouse embryonic stem cells (mESCs), and differentiated the lines to BFCNs. We then characterized the differentiated neurons for their BFCN identity and established *in vitro* AD model utilizing amyloid-beta (Aβ(1-42)) toxicity. Our results demonstrated that KFG removal leads to increased TrkA levels and TrkA-KFG neurons are more resistant to Aβ(1-42)-mediated neuronal death compared to WT BFCNs. Our work further suggested that enhanced TrkA signaling can protect the synaptic density of BFCNs from Aβ(1-42)-mediated toxicity through the MAPK/Erk pathway. Our approach focusing on the enhanced NGF-TrkA signaling in the concept of *in vitro* AD differs from other studies in the literature because the aim here is to make the TrkA receptors more sensitive and active in specific neuron types instead of altering neurotrophin levels or influencing their receptors with small molecules.

Keywords: Alzheimer's Disease, Basal Forebrain Cholinergic Neurons (BFCNs), Neurotrophin Signaling, Nerve Growth Factor, Amyloid-Beta (Aβ)

ÖZ

FARE EMBRYONİK KÖK HÜCRELERİNDEN BAŞKALAŞTIRILMIŞ BAZAL ÖN BEYİN KOLİNERJİK NÖRONLARDA TrkA SİNYALİNİN UPREGÜLE EDİLMESİNİN IN VITRO ALZHEIMER HASTALIĞI NÖRODEJENERATİF SÜRECİNE KARŞI ETKİSİNİN ARAŞTIRILMASI

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Alzheimer Hastalığı (AH) dünya üzerinde 50 milyondan fazla insanı etkilemekte olan bir nörodejeneratif hastalıktır. Yoğun çalışmalara rağmen, günümüzde halen AH'ye karşı etkili bir tedavi yöntemi geliştirilememiştir. Sinir sistemi regülasyonunda önemli roller üstlenen nörotrofin ve reseptörlerinin seviyelerindeki anormallikler ve nörotrofin sinyalizasyonundaki fonksiyonel bozukluluklar uzun zamandır AH ile ilişkilendirilmektedir. Nörotrofin ailesinin dört adet üyesi bulunmakta olup; bunlar Sinir Büyüme Faktörü (NGF- Nerve Growth Factor), Beyinde Türetilmiş Sinir Hücresi Büyüme Faktörü (BDNF-Brain-derived neurotrophic factor), Nörotrofin-3 (NT3-Neurotrophin-3) ve Nörotrofin- 4/5 (NT4/5-Neurotrophin- 4/5)' tir. Nörotrofinler, yüksek bağlanım ile kendilerine ait Tropomiyozin reseptör kinaz (Trk) reseptörleri ile etkileşime girerek nöron gelişimini ve farklılaşmasını, hücre hayatta kalımını ve sinaptik plastisiteyi etkileyen sinyal yolaklarını tetiklerler. Bu sıralanan özellikler, Trk sinyalizasyonunun modülasyonunu AH nörodejerasyonuna karşı kullanılabilinecek potensiyel bir tedavi yaklaşımı olarak görülmesini sağlamaktadır. Bu sebeple, nörotrofin sinyalizasyonunu kontrol eden önemli reseptör bölgelerinin moleküler

olarak detaylı tanımlanması, nörotrofin regülasyonunun AH'deki rolünün daha iyi anlaşılmasını sağlayacaktır. Önceki çalışmamızda, Trk reseptörlerinde korunmuş olan KFG bölgesinin (3 amino asit) TrkA reseptöründen çıkarılmasının hem reseptör seviyesini hem de sinyalizasyonunu arttırdığı gösterilmiştir. NGF aracılı TrkA sinyalizasyonu ve TrkA reseptörlerinin yüksek ekspresyonu, AH'de en çok etkilenen hücre tipleri arasında olan bazal ön beyin kolinerjik nöronlarında (BFCN) görülür ve çok önemli roller oynar, bu da TrkA reseptörünün modifikasyonunun BFCN'deki nörodejenerasyon için potansiyel bir yaklaşım olabileceğine işaret etmektedir. Bu sebeplele, bu tez çalışmasında BFCN'lerde upregüle edilmiş TrkA sinyalizasyonunun *in vitro* AH'ye karşı olabilecek etkileri anlamlandırılmaya çalışılmıştır. Bu amaç doğrultusunda, fare embryonik kök hücre (FEKH) temelli nöron farklılaştırma modellemesi kullanılmış olup doğal fenotip (WT) ve KFG bölgesi CRISPR-Cas9 gen düzenleme yöntemi ile çıkarılmış (TrkA-KFG) iki farklı genotipteki FEKH'ler direkt olarak BFCN'ye farklılaştırılmıştır. Farklılaştırılan nöronlar önce karakterize edilmiş ve sonuçları takriben *in vitro* amiloid-beta (1-42) (Aβ(1-42)) toksisitesi yaratılarak AH modeli sağlanmıştır. Çalışmamız sonunda elde edilen sonuçlar göstermektedir ki KFG bölgesinin çıkarılması TrkA reseptör seviyesinin artışına sebep olurken, bu bölgenin çıkarıldığı nöronlar Aβ(1-42)' nin sebep olduğu nöron ölümüne karşı daha dirençlidir. Bu bulgumuza ek olarak, upregüle edilmiş olan TrkA reseptörünün BFCN'lerin sinaptik yoğunluğundaki Aβ(1-42)'nin sebebiyet verdiği bozulmaya karşı koruduğu gözlemlenmiştir. Araştırmalarımız bu korunmanın MAPK/ERK sinyal yolağı üzerinden gerçekleştiğini göstermiştir. Bu araştırmada değerlendirilen KFG modeli, nörotrofin seviyelerini değiştirmek veya Trk reseptörlerini küçük moleküller ile etkilemek yerine, Trk resetörlerini spesifik nöron tiplerinde daha duyarlı ve aktif hale getirdiği için literatürdeki diğer *in vitro* AH modellerinden farklılık göstermektedir.

Anahtar Kelimeler: Alzheimer Hastalığı, Bazal Önbeyin Kolinerjik Nöron (BFCN), Nörotrofin Sinyalizasyonu, Sinir Büyüme Hormonu, Amiloid Beta (Aβ)

To the infinity and beyond!

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ABBREVIATIONS

AD	Alzheimer's Disease
NGF	Nerve Growth Factor
Trk	Tyropomyosin Receptor Kinase
Aβ	Amyloid-beta
pMEF	Primary Mouse Embryonic Fibroblast
mito-MEF	Mitomycin-C treated Mouse Embryonic Fibroblast
mESCs	Mouse Embryonic Stem Cells
BFCNs	Basal Forebrain Cholinergic Neurons
ADFNK	Advanced-DMEM-Fetal bovine serum-Neuro Medium- KSR
KSR	KnockOut™ Serum Replacement
RA	Retinoic Acid
Shh	Sonic-hedgehog protein Hh-Ag.1.5
BMP-9	Bone Morphogenetic Protein 9
EBs	Embryonic Bodies
SDS-PAGE	Sodium dodecyl sulfate Polyacrylamide gel electrophoresis
PVDF	Polyvinylidene difluoride
PBS(+,+)	Phosphate-buffered saline with Ca^{+2} and Mg^{+2}
DPBS	Phosphate-buffered saline without Ca^{+2} and Mg^{+2}
TBS-T	Tris-buffered saline and Tween-20

iPSCs   Induced Pluripotent Stem Cells

HFIP   Hexafluoro-2-propanol

CHAPTER 1

INTRODUCTION

1.1   Alzheimer's Disease

Alzheimer's Disease (AD) is an irreversible neurodegenerative disease that is the most common mediator of dementia, leading to cognitive impairments and motor dysfunction (Di Carlo, 2012; Dugger & Dickson, 2017; Weller & Budson, 2018). The disease is characterized by the accumulation of extracellular amyloid beta plaque (Aβ) deposits and intracellular neurofibrillary tangles (NFT) composed of phosphorylated tau protein within the cholinergic network of the brain (Butterfield & Boyd-Kimball, 2004; Ittner et al., 2010). Despite extensive research, the etiology of AD is primarily unknown (Mayeux & Stern, 2012). However, the literature suggests that both genetic and environmental factors contribute to the disease's progression (Zhang et al., 2021).

Currently, more than 50 million people around the world are affected by AD, and the number is expected to rise to 139 million in 2050 due to increasing life expectancy and living conditions, according to Alzheimer's Disease International's survey (G. B. D. Collaborators, 2021; G. B. D. D. F. Collaborators, 2022; Nordberg, 2008). This global disease increase is expected to be even higher in developing countries, where the new AD case rate will also rise from 58% to 68% in 2050 (Chavez-Fumagalli et al., 2021). The such expected increase is potentially due to growth in the elder-age population, poor diagnosis, and undeveloped health care systems (International, 2019). Therefore, the current known AD diagnosis rate is lower than the actual scenario in both high-income and low-and middle-income countries with devastating numbers, so it is highly possible to face higher numbers in the following decades than expected (Lang et al., 2017). From a national perspective, nearly 600.000 patients fight against AD in Turkey, according to the

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2014-2018 report of the Turkish Alzheimer's Disease Association, and the prevalence will rise due to the increase in the old-age population and life expectancy. This increasing population of AD patients all around the world is an economic burden for both developed and developing countries due to the high-cost healthcare treatments (Mattap et al., 2022; Wimo, Jönsson, Bond, Prince, & Winblad, 2013). In addition to the economic view, AD has also an emotional burden on society and families caring for patients fighting against the currently uncured disease (Wong, 2020). All these increasing patient numbers and burdens are signs of an urgent call for understanding the molecular mechanism of AD and developing effective therapeutic options. To develop effective therapies against AD, it is crucial to better understand the underlying cause or contributors of the disease (Heneka et al., 2015; Hou et al., 2019; Luo, Warmlander, Graslund, & Abrahams, 2016; R, 2019; Tramutola, Lanzillotta, Perluigi, & Butterfield, 2017).

1.1.1 The known molecular mechanisms of Alzheimer's Disease

Many important hypotheses, such as amyloid-beta cascade, tau cascade, oxidative stress, inflammation, and gut microbiota hypotheses, have been made for the origin and development of AD by carrying out intensive scientific studies (Butterfield & Boyd-Kimball, 2018; Cline, Bicca, Viola, & Klein, 2018; Du, Wang, & Geng, 2018; Gezen-Ak, Yilmazer, & Dursun, 2014; Kametani & Hasegawa, 2018). Despite these studies and huge investments, the main reason behind this complex disease and its molecular mechanism for neurodegeneration are not fully understood (Dubois et al., 2016).

Beyond the mysteries of AD, it is known that there are two neuropathologic types of AD named familial (FAD) and sporadic (SAD). The frequency of FAD is rare because it occurs due to autosomal mutation running within the families (N. S. Ryan & Rossor, 2010; L. Wu et al., 2014). This type of AD affects individuals below the age of 65, so it is also called early-onset AD (L. Wu et al., 2014). However, most of the AD cases are late-onset named SAD, where potentially

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environmental factors are a contributor in addition to the genetics (Piaceri, 2013). Both AD types are characterized by the presence of amyloid-beta (Aβ) plaque deposits and neurofibrillary tangles (NFT) (Arriagada, Growdon, Hedley-Whyte, & Hyman, 1992; Calabro, Rinaldi, Santoro, & Crisafulli, 2021; Grundke-Iqbal et al., 1986). The amyloid plaques consist of Aβ fragments, which are truncated parts of the amyloid precursor protein (APP), and the NFTs include the hyperphosphorylated tau protein fibrils. These aggregates have been proposed to lead to neuronal death, synaptic degeneration, neuroinflammation, imbalanced neurotransmitter, and dendritic changes (Joachim HR Lübke, 2021). Not surprisingly, most AD studies focus on the hypotheses involving the aggregation of Aβ and tau proteins as the causative processes in the disease (Glenner & Wong, 1984; Grundke-Iqbal et al., 1986). The most studied hypothesis to reveal the neurodegeneration process in AD focuses on amyloid plaque accumulation named Amyloid Cascade Hypothesis (Hardy & Higgins, 1992; Selkoe & Hardy, 2016). From a molecular perspective, it has been demonstrated that the APP cleavage leads to truncated forms of amyloid-beta in different lengths, such as Aβ(1-40) and Aβ(1-42) (Ricciarelli & Fedele, 2017). However, the amount of the more hydrophobic form, Aβ(1-42), correlates well with the total accumulated Aβ levels, and the level of this form is significantly related to the cell toxicity (Tiwari & Kepp, 2015). After the truncated Aβ formation, they accumulate within the surface of neurons in specific brain parts and around blood vessels. Such accumulation results in neuronal death, which in turn causes dementia over time (Stavljenic-Rukavina, 2004). Another leading hypothesis suggests that the accumulated Aβ(1-42) proteins disrupt the microtubule integrity, which is essential to neuronal maintenance, by diminishing the tau protein binding capacity through its over-phosphorylation. Indeed research shows that the amyloid-beta plaque-mediated NFT formation can cause microtubule death-mediated neuronal loss (Stavljenic-Rukavina, 2004). The Amyloid Cascade Hypothesis has also played a central role in clinical experiments to find therapeutic options for AD (Selkoe & Hardy, 2016). However, among the more than 500 key candidates therapeutically evaluated

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within 15 years, just the amyloid-beta clearer Aducanumab has passed the later clinical phases (Bachurin, Bovina, & Ustyugov, 2017; J. L. Cummings, Morstorf, & Zhong, 2014). With all this effort and interest, much scientific evidence shows that Aβ can be an important mediator in the AD pathology (Campion, Pottier, Nicolas, Le Guennec, & Rovelet-Lecrux, 2016).

However, recent studies suggest that Amyloid Cascade Hypothesis may not be sufficient to explain the neurobiology of the AD (De Strooper & Karran, 2016; Herrup, 2015). Therefore, several other leading hypotheses focusing on different neuronal mechanisms have been put forward. Such mechanisms include functional deficiencies in the cholesterol metabolism (Wood, Li, Muller, & Eckert, 2014), neuroinflammation (Heneka et al., 2015), oxidative damage, and mitochondrial dysfunction (Johri & Beal, 2012; Swerdlow, Burns, & Khan, 2014). One of such highly attractive hypothesis is the Neurotrophic Factor Hypothesis, which suggests that the loss of neurotrophic support could be one of the critical underlying molecular mechanisms of the AD (Chen, Sawa, & Mobley, 2018; Horacio Uri Saragovi, 2019). Neurotrophin signaling regulates neuronal survival and synaptic plasticity, and it has long been suggested that the problems in synaptic plasticity and the progressive loss in synaptic integrity within the neurons could lead to AD (de Wilde, Overk, Sijben, & Masliah, 2016; Herrup, 2015; Overk & Masliah, 2014). This is plausible as synaptic integrity changes are the early signs of the disease, present way before amyloid accumulation. As a critical example, the study on postmortem brain samples revealed that the changes in the brain start many years before the AD diagnosis that could be highly related to a decrease in one of the neurotrophin family members called nerve growth factor, which is an essential factor for neuronal survival and synaptic integrity (Pentz et al., 2021). Therefore, understanding the neurotrophic mechanism in the AD pathology can open new doors for prevention of synaptic integrity loss or activate synaptic regeneration/strengthening, which could provide effective therapeutical approaches (Mitra, Behbahani, & Eriksdotter, 2019; Sampaio, Savall, Gutierrez, & Pinton, 2017).

1.1.2 The therapeutic approaches against Alzheimer's Disease

Although AD was described more than a century ago, the avaliable approved drugs only help to relieve the symptoms rather than cure the disease (Atri, 2019) (Scheltens et al., 2016). Despite massive investments in studies focusing on AD treatment, 99% of clinical trials end up in failure (L. K. Huang, Chao, & Hu, 2020) (Aldewachi, Al-Zidan, Conner, & Salman, 2021) (J. Cummings, Lee, Ritter, & Zhong, 2018).

There are just two drugs approved within the 17 years for AD. The first one is GV-971, approved in China (Syed, 2020), and other countries have recently begun investigating its efficacy. GV-971, also known as sodium oligomannate, is a marine-derived oligosaccharide (T. Wang, 2017). After its ingestion, most of it restained in the gut proposing in the animal models that it has positive effects on neuroinflammation, the infiltration of immune cells to the brain, and the gut microbiota (X. Wang et al., 2019). Also, it is shown that Aβ deposition can be reduced in Aβ-transgenic mouse models upon treatment GV-971 (Fan et al., 2005; Hu et al., 2004; R.-w. Jiang et al., 2013; Kong et al., 2005). This drug promises cognitive function improvement by reducing both the Aβ deposition and neuroinflammation decline (Xiao et al., 2021). However, further studies and trials are needed to assess the GV-971 as a globally approved drug for AD.

 The second drug, called Aducanumab, was approved by FDA in the USA on the 8th of July, 2021 (Padda & Parmar, 2022). It is a human immunoglobulin gamma 1 (IgG1) monoclonal antibody targetting the 3-7 amino acids of Aβ (Arndt et al., 2018). It can pass the blood-brain barrier and bind specifically to Aβ plaques (Kastanenka et al., 2016; Jeff Sevigny et al., 2016). Several studies show Aducanumab-mediated reduction in the brain Aβ plaques and phosphorylated tau levels (J. Cummings et al., 2021; J. Sevigny et al., 2017; Jeff Sevigny et al., 2016). However, there is a controversy about the effectiveness of the Aducanumab (Karlawish, 2021), where The European Medicines Agency decided on the 17th of December, 2021 not to approve Aducanumab for its usage in Europe.

In a nutshell, these two drug studies are not yet accepted globally as effective AD treatment options. There could be many factors that lead to failure in the development of effective treatments against AD within 100 years or so. One potential factor is that therapeutic targets have been selected without a strong scientific foundation, and we still do not understand the molecular mechanisms may be involved in the disease initiation and progression. Due to being a complex disease, it is vital to understand the underlying molecular mechanisms of AD to assess the effective and targeted treatment. Happily, awareness of therapeutic target diversity is increasing, and drug development studies to treat AD rise with different approaches (van Bokhoven et al., 2021). Among them, neurotrophin-based strategies against AD have been proposed as highly promising methodologies based on recent work (Gauthier et al., 2020). It is currently not clear whether the abnormalities in neurotrophin levels and signaling cascades are the initial cause of AD, and whether the enhancement of neurotrophin signaling have an early beneficial effect against AD (Allen, Watson, & Dawbarn, 2011). Therefore, to efficiently and safely manipulate neurotrophin signaling in a disease context, it is essential to better understand the molecular mechanisms controlling neurotrophin signaling in neurons.

1.2   Neurotrophins and Alzheimer's Disease

1.2.1   The roles of neurotrophins in health and disease

There are four members of neurotrophins in mammalians, vital for optimum functioning and development of the nervous system which are nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3), and neurotrophin-4/5 (NT4/5) (Chao, Rajagopal, & Lee, 2006; Tessarollo, 1998). The formation of mature neurotrophins occurs by furin or other protease-mediated cleavages of the pro-neurotrophins (Chao, 2003; Chao & Bothwell, 2002). The pro-neurotrophins such as pro-BDNF and pro-NGF can induce apoptotic death within a specific cellular context, unlike mature neurotrophins (Chao & Bothwell, 2002;

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Nykjaer et al., 2004; Volosin et al., 2006; Volosin et al., 2008). The mature neurotrophins bind tropomyosin-related tyrosine kinase family (Trk) of receptor tyrosine kinases with high-affinity and activate the cell survival-related signaling cascades (Chao, 2003). Also, they can trigger the signaling pathways related to the p75NTR receptor, a member of the tumor necrosis factor receptor superfamily, by binding with a low-affinity (Chao & Bothwell, 2002). The ligand-receptor relationship between neurotrophins and their receptors has been known for many years, showing us that NGF binds the TrkA receptor; in contrast, BDNF and NT4/5 bind TrkB and activate the signaling cascades (Chao, 2003; E. J. Huang & Reichardt, 2003; Tessarollo, 1998). Besides, NT-3 mainly binds the TrkC receptor, but it also has the potential to bind TrkA and TrkB with lower affinity as compared to TrkC (Barbacid, 1994). By binding to its corresponding Trk receptor, neurotrophins activate and regulate many cellular signaling pathways, including Ras/MAPK(ERK) signaling cascade, phosphoinositide 3 kinase (PI3K), and phospholipase Cy (PLCy) pathways which are related to neuronal survival, differentiation, and synaptic plasticity (Hausott, Glueckert, Schrott-Fischer, & Klimaschewski, 2022; Kiss Bimbova et al., 2022; L. F. Reichardt, 2006).
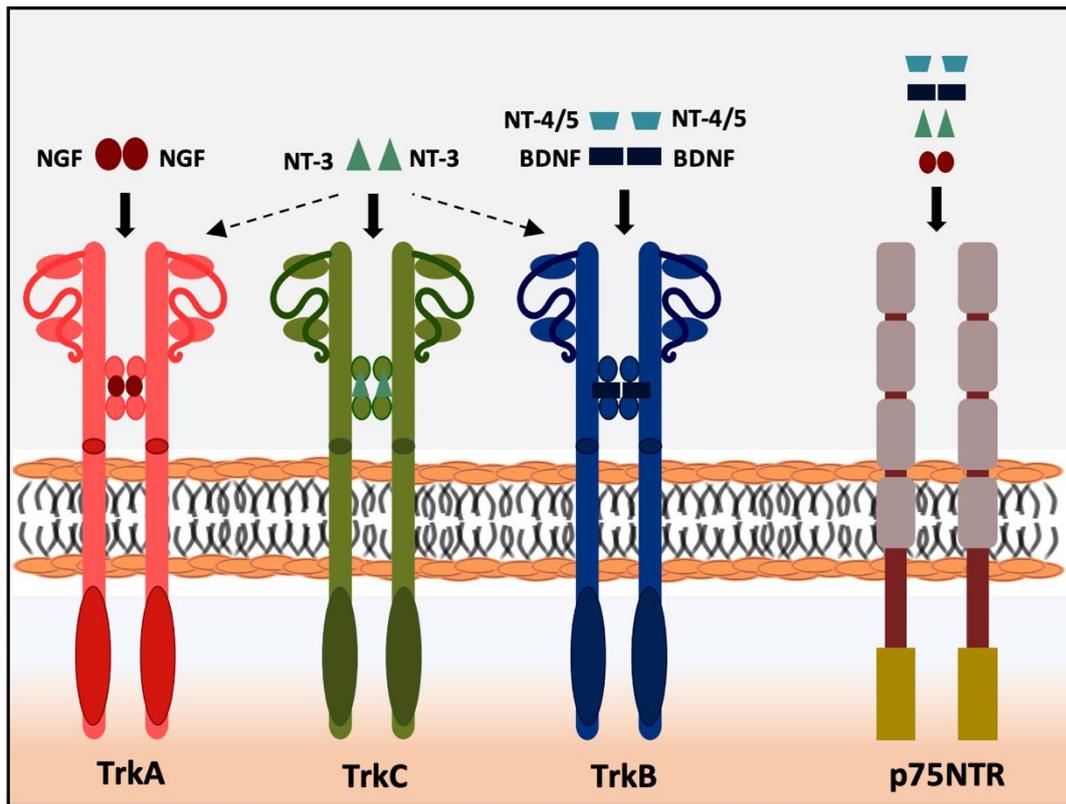
Figure 1.1 The neurotrophin signaling

The NGF is a ligand of the TrkA receptor; BDNF and NT-4/5 activate the TrkB receptor, while NT-3 binds all Trk receptors with different affinities. The interaction of neurotrophins and their specific high-affinity Trk receptors initiates cell survival, differentiation, and synaptic plasticity-related signaling pathways. Also, all neurotrophins can bind p75NTR receptors with low-affinity to initiate cell death pathways.

Previous literature well established that there are changes in neurotrophins and their receptors levels in various neurodegenerative conditions, including AD, Parkinson's Disease, and Amyotrophic Lateral Sclerosis (Chao et al., 2006). In addition to their levels, neurotrophin signaling defects have also been correlated to neurodegenerations, especially in neuronal types affected by specific neurodegenerative diseases. Therefore, neurotrophins are widely studied as therapeutic targets in various neurodegenerative conditions (Horacio Uri Saragovi,

2019). In addition to neurodegeneration, modulation of neurotrophin-related signaling pathways is the focus of cancer research because increased Trk receptor levels are seen in various types of cancer (Mitre, Mariga, & Chao, 2017). Although there have been significant efforts, past experiences demonstrated that the modulation of the neurotrophins is not as easy as thought. Most clinical trials focusing on preventing neurodegeneration using neurotrophin-based approaches failed or achieved a partial effect. The reasons behind unsuccessful trials could be unexpected side effects or poor pharmacokinetics of developed chemicals as mimetics. However, targeting neurotrophin-activated signaling in cancer or neuropathic pain seems more promising. For example, two oral pan-Trk inhibitors named Larotrectinib and Entrectinib received an FDA approval as cancer drugs in 2018 and 2019, respectively (Harada, Santini, Wilhelm, & Drilon, 2021). Also, a monoclonal antibody against NGF called Tanezumab, developed for pain treatment, has shown significant pain relief and improved physical function in patients with painful hip osteoarthritic conditions. Despite its positive effects in Phase 3 (Berenbaum et al., 2020), the FDA did not approve the drug on the 25th of March, 2021, due to concerns about the side effects. The scientific and clinical research findings show that the studies on activating or inhibiting the neurotrophin-mediated signaling cascades will continue as a therapeutical approach in many diseases, including neurodegenerative diseases and AD. To achieve successful clinical trials, it is important to understand molecular mechanisms and physiological roles of neurotrophin receptors to better design neurotrophin-based therapeutics.

1.2.2 Neurotrophins as therapeutic targets against Alzheimer's Disease

There has been a significant amount of research efforts to develop neurotrophin-based therapeutics within the context of AD for several reasons. First, it is demonstrated that neurotrophin signaling can decrease neurodegeneration in AD by increasing synaptic repair and stimulating the synaptic plasticity (Cuestas Torres & Cardenas, 2020). This relationship is very important because there is a shown

correlation between the cognitive decline and gradual deterioration of synaptic plasticity, which is presented as an early AD progression marker (Scheff, Price, Schmitt, DeKosky, & Mufson, 2007; Spires-Jones et al., 2007; Zhou et al., 2019) Therefore, the focus of the AD treatment studies turns to the role of neurotrophin signaling pathways in synaptic plasticity and its protection through the AD progression (Chao, 2003; Kazim & Iqbal, 2016; Mu & Gage, 2011). Second, it is shown that the neurotrophin and their receptor levels change in neurodegeneration, where their optimum levels are crucial for the normal functioning of the nervous system, including the regulation of neuronal communication. So, efforting to find ways to keep the neurotrophins and their receptor levels at the physiological levels could decrease the AD-mediated neurodegeneration (Sampaio et al., 2017). The third therapeutic approach is based on neurotrophin-mediated neuroprotection against the cellular toxicity of Aβ, which is a primary AD hallmark (Kitiyanant, Kitiyanant, Svendsen, & Thangnipon, 2012; L. Tapia-Arancibia, E. Aliaga, M. Silhol, & S. Arancibia, 2008; Tong, Balazs, Thornton, & Cotman, 2004; Zeng et al., 2011). To asses the neuroprotection against AD-mediated toxicity, the methodologies related to increasing the activation of neurotrophin signaling pathways are one of the focused area by researchers.

More specifically, among the neurotrophin signaling cascades, the NGF-TrkA and BDNF-TrkB signaling pathways have been gathering the main focus of the concept of therapeutical approaches against AD for many years (Caffino, Mottarlini, & Fumagalli, 2020; Sampaio et al., 2017). The effect of these neurotrophin signaling partners is mainly investigated in hippocampal and cholinergic nuclei of the basal forebrain (ChBF) (Allen et al., 2011), which are the two of the most affected brain parts in the AD (Pearson, Esiri, Hiorns, Wilcock, & Powell, 1985; Saper, German, & White, 1985). TrkA, a high-affinity NGF receptor as mentioned above, is highly synthesized in basal forebrain cholinergic neurons (BFCN) in the central nervous system (Sanchez-Ortiz et al., 2012), where this neurotrophin signaling is critical for synaptic integrity between the BFCNs and hippocampal and cortical neurons (Isaev, Stelmashook, & Genrikhs, 2017). The levels of TrkA and NGF decrease in

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BFCNs due to AD pathology, which leads to cognitive decline and a decrease in ChAT levels (Boissiere, Faucheux, Ruberg, Agid, & Hirsch, 1997; Salehi, Delcroix, & Swaab, 2004). It is hypothesized that the decrease in NGF levels could be associated with defects in retrograde transport of NGF from the hippocampus and cerebral cortex (Allen et al., 2011) due to unchanged levels of NGF in these two cholinergic regions (Allen et al., 1991; Crutcher, Scott, Liang, Everson, & Weingartner, 1993). The NGF depletion can affect cholinergic innervation in the hippocampus, which leads to spatial memory impairment (Eu et al., 2021). Also, the importance of NGF in AD is demonstrated in one of the AD mouse models, AD11, which exhibits a neurodegenerative process seen similar to AD as a result of neutralization of the NGF via transgenic antibodies (Houeland et al., 2010; Ruberti et al., 2000). Taken together, understanding how NGF-TrkA signaling can be controlled is essential to creating more specific and innovative therapeutic approaches focusing on both AD-related neuroregeneration and memory deficits (Braun, Kalinin, & Feinstein, 2017; Josephy-Hernandez, Jmaeff, Pirvulescu, Aboulkassim, & Saragovi, 2017; Nagahara et al., 2009; M. H. Tuszynski et al., 2015).

An important approach to controlling NGF-TrkA signaling may involve the manipulation of the receptor. However, until recently, factors regulating TrkA level and activity were not well-understood. A study from our group demonstrated that a 3 amino acid long conserved KFG domain within the Trk receptors negatively controls the TrkA receptor signaling (Kiris et al., 2014). After our group's initial publication, another research group confirmed that this domain is also critical in the process of Trk receptor ubiquitination by using the mass spectrometry-based analyses (Emdal et al., 2015). Our group's work also demonstrated for the first time that ubiquitination is an important mechanism that regulates the TrkA level and its *in vivo* function. In this context, our molecular mechanism studies have revealed that the KFG region is important for the receptor ubiquitination, membrane uptake, and recycling processes of the TrkA receptor, which affects the receptor level and activity (Kiris et al., 2014). The following studies analyzed

whether the TrkA protein levels increased in the central nervous system similar to the peripheral nervous system with the removal of the KFG domain of the TrkA receptor in mice models. The Western Blot analysis of BFCN samples from wild-type (WT) and KFG region removed (TrkA-KFG) mice showed that the TrkA protein level is higher in the TrkA-KFG BFCNs. Therefore, the KFG model system, in which only 3 amino acid domains are removed from the receptor, provides a highly useful tool to examine the importance of enhanced TrkA signaling in BFCN neurons within a disease context.

1.3   The aim of the study

Modulation of neurotrophin signaling is considered a critical approach to counter the neurodegenerative process of AD, as neurotrophin signaling is crucial for neuroprotection. It has been shown that the NGF-TrkA signaling pathway plays an indispensable role in BFCN neurons, which are highly affected neuronal types in AD. Importantly, defects in TrkA signaling has been correlated with AD; therefore, modulation of NGF-Trk signaling in BFCN neurons may have a neuroprotective effect against AD. However, modulation of TrkA signaling in an efficient manner is a highly challenging task. A previous study from our group demonstrated that a 3-amino acid domain in the TrkA receptor (KFG amino acids) is a critical domain regulating the receptor levels and function (Kiris et al., 2014). Deleting this domain in TrkA receptor leads to an increase in receptor level and activity, as the KFG domain is critical for ubiquitination and turnover of the receptor. To examine the effects of enhanced TrkA signaling in BFCN neurons against the neurodegenerative process of AD, we utilized WT and TrkA-KFG (KFG domains are deleted in TrkA receptor) mouse embryonic stem cell lines (kind gift by Dr. Lino Tesarallo, National Cancer Institute, Frederick, NIH, USA) and generated BFCNs from both lines via directed differentiation. We then sought to characterize the neurons and establish an *in vitro* AD model by utilizing amyloid-beta (1-42) (Aβ(1-42)) fibrillar and oligomer treatments. Our goal was to measure the potential

effects of enhanced TrkA signaling in TrkA-KFG BFCNs compared to WT BFCNs in terms of Aβ(1-42)-mediated neuronal cell death and synaptic changes.
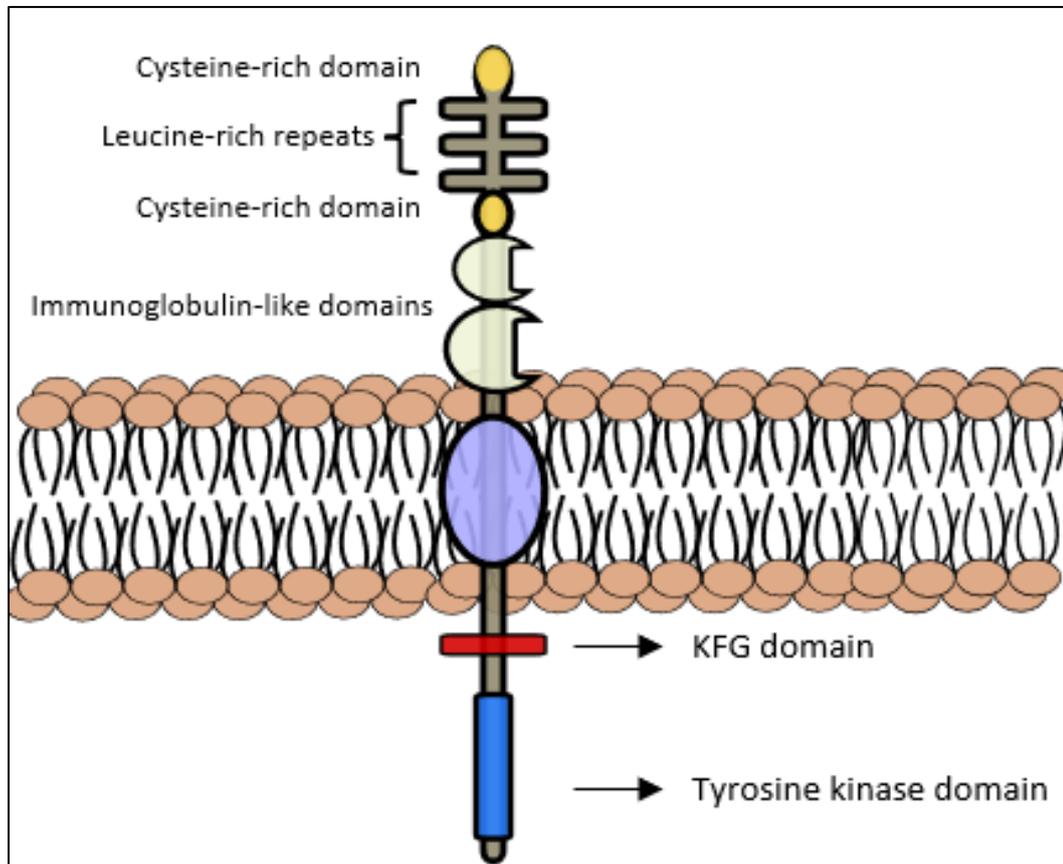
Figure 1.2 Representation of Trk receptor domains

Trk receptors consist of two cysteine-rich domains, whereas between these two domains, there are leucine-rich repeats. Below the second cysteine-rich domain, extracellular immunoglobin-like domains are present. The KFG domain is in an intracellular place which places top of the tyrosine kinase domain.

CHAPTER 2

MATERIALS AND METHODS

2.1 Preparation of mitotically inactivated Mouse Embryonic Fibroblast (MEF) cells by using Mitomycin-C treatment

Using feeder cells in stem cell (ES) culture is an extensively utilized methodology to keep ES pluripotent (Desai, Rambhia, & Gishto, 2015; Eiselleova et al., 2008; Lee et al., 2009; Talbot, Sparks, Powell, Kahl, & Caperna, 2011; Villa-Diaz, Ross, Lahann, & Krebsbach, 2013). MEFs are highly popular for pluripotent cell culture due to various advantages, including the cost. We utilized primary MEFs (pMEFs) (harvested from D13.5 mouse embryos) (a kind gift from Dr. Lino Tesarrollo (NIH, ABD)) to generate Mitomycin-C treated MEF cells (mito-MEFs) as described previously by Dr. Tessarollo's laboratory (Southon & Tessarollo, 2009).

As a first step, pMEFs were cultured in the MEF medium described in ***Appendices B*** at 37ºC, 5% CO_2. After the confluency reached approximately 80% for 15 plates (150 mm cell culture treated dishes), the Mitomycin-C treatment was started depending on our optimized protocol. The Mitomycin-C reagent was prepared and diluted as described in ***Appendices A,*** where 4 ml 0.5 mg/ml solution was filtered through a 0.22 µm syringe filter into MEF medium to get a 0.01 mg/ml final concentration. Each plate was treated with 15 ml of Mitomycin-C solution for 3 hours at 37ºC, 5% CO_2 for the inactivation of the mitotic cycle. After the incubation time, the Mitomycin-C containing medium was aspirated, and each plate was washed with PBS (+,+) three times to eliminate the remaining toxic reagent. When the washing steps were done, mito-MEFs were detached from the culture plates by treating 0.05% Trypsin solution (Gibco, Cat. No: 25300054) until they were fully detached. To inactivate the Trypsin solution, a MEF medium was added to each plate. After collecting cell suspension, it was centrifuged at 200g for

10 minutes. To count the cells, the pellet was resuspended in a lower volume of MEF medium. The suspension was centrifuged at 200g for 5 minutes to freeze the cells in the proper number. The pellet was suspended in the freezing medium described in *Appendices B* and split into cryovials. The cryovials were put into a - 80 ºC freezer with a freezing container called Mr. Frosty overnight. The following day, the cells were put into nitrogen tanks for long-term storage.

2.2 Cultivation of V6.4 mouse embryonic stem cells (mESCs)

In the project, two different types of V6.4 mESCs were used. Where one of them has a wild-type (WT) genotype, the other type has CRISPR-Cas9 edited V6.4 cells, in which the KFG domain in TrkA is homozygously deleted (TrkA-KFG). The modified mESCs were generated in Dr. Lino Tesarallo's laboratory in the National Cancer Institute, Frederick, NIH, USA, and were a kind gift to our laboratory.

Prior to the cultivation process, the 150 mm cell culture treated plates were coated with 0.01% gelatin solution, where the coating was done at room temperature under UV light and for 30 minutes. Prepared mito-MEF stocks were opened on 12 gelatin-coated plates in a MEF medium. On the following day, both wild-type (WT) and TrkA-KFG mESCs were opened on mito-MEF plates as half in the V6.4 mESC medium described in *Appendices B.* Until the mESC colonies reached 80% confluency to start the differentiation process, they were checked daily under the microscope, and medium change was performed to keep pluripotency straight. For each type of mESCs, one plate was used as a stock plate to provide continuous mESCs culture, while the remaining plates were used for the differentiation process.

2.3 Differentiation of V6.4 mESCs into basal forebrain cholinergic neurons (BFCNs)

The directed differentiation of mESCs into BFCNs includes embryoid body formation, different chemical stimulations on different days, and dissociation of

embryoid bodies, which takes 7 days and extra 5 days for the maturation of the neurons. The protocol has been followed and optimized according to well-known studies (Bissonnette et al., 2011; Yue et al., 2015).

The differentiation procedure started when the confluency of mESC plates reached up to 80%, where the mESC colonies are removed from their mito-MEF feeder cells by using the Dispase dissociation enzyme with incubating the plates at 5% CO_2, 37°C until most of the colonies detached. The detached colonies were collected with a V6.4 mESC medium, and the cell suspension was centrifuged for 5 minutes at 1000 rpm. The pellet was suspended with DMEM to remove excess Dispase enzyme and the FBS found in the V6.4 mESC medium. The cell suspension was centrifuged again at 1000 rpm for 5 minutes; then, the cell pellet was suspended with the ADFNK medium *(Appendices B)*, and the cell suspension was seeded on a 120 mm no-attachment petri dish for embryoid body (EB) induction. The day when the embryoid body induction is initiated is annotated as **Day0.** To provide continuous mESC culture, the stock plate was passaged, and plates were checked daily under the microscope to start a new set of embryoid body induction.

The treatments for the neuronal induction were started on **Day2** with the addition of 1.0 μM Retinoic Acid (RA) (Sigma, #2625) containing the ADFNK medium. To do so, the embryoid bodies were collected, mini-centrifuged, and the pellet was suspended with the medium supplemented with RA. Suspended embryoid bodies were transferred into a new petri dish to eliminate the attached cells in the old plate. For proper neuronal induction, the embryoid bodies were treated with the medium for 24 hours. After 24 hours (on **Day3**), the embryoid bodies were collected in the middle of the petri dish, and the medium containing RA was aspirated to prevent the toxicity of RA. Then, the fresh medium supplemented with 2.0 μM Smo receptor agonist and sonic hedgehog signaling activator, which is sonic-hedgehog protein Hh-Ag.1.5 (Shh) **(**Cellagen Technology, Cat.No: C4412-2s) to increase the neurogenesis and direct the differentiation into BFCNs by acting

with RA. On **Day4**, 10 ng/ml synthetic BMP-9 peptide (GenScript, Sample ID:U163XFB230-1) was added directly into the EB plate to stimulate the BMP-signaling pathway for directed differentiation (Lauzon & Faucheux, 2018; Lauzon, Marcos, & Faucheux, 2018). The optimum BMP-9 dosage for succesful differentiation was validated in a research project granted by METU Scientific Research Projects Coordination Unit *(grant number: TEZ-YL-108-2020-10291).* On **Day5**, the ADFNK medium was aspirated from the EB plate, and a more nutritious medium for neuronal growth, Neuronal Medium-1*(Appendices B),* supplemented with 10 ng/ml synthetic BMP-9 peptide was added. There was no chemical treatment on **Day6**. On **Day7**, EBs were dissociated with StemPro Accutase (Gibco, A111501) according to the manufacturer's recommended protocol. The dissociated bodies were suspended with Neuronal Medium-1 and plated on poly-D-lysine-laminin pre-coated 24-well,96-well, or 4 chamber slides, depending on the procedure will be followed. The medium was changed into a more nutritious medium containing growth factors called Neural Medium-2 *(Appendices B)* supplemented with 10 ng/ml synthetic BMP-9 peptide on **Day8.** The neuronal growth and maturation were provided by cultivating them at 5% CO_2, 37ºC, until **Day10** and **Day13.** The following characterization and amyloid-beta experiments were started after the maturation was completed.

Day-0	Day-2	Day-3	Day-4	Day-5	Day-6	Day-7	Maturation Days
Removal from MEF feeder cells	1.0 µM Retinoic Acid	2.0 µM Hh-Ag1.5	10 ng/mL BMP9	10 ng/mL BMP9	No Treatment	Dissociation	**D10:** Cell lysate **D13:** Cell lysate,ICC, treatments and tests

ADNFK MEDIUM   NEURONAL MEDIUM-1   NEURONAL MEDIUM-2

Formation of embryonic bodies on 120 mm petri dishes   Seeding on Poly-D-lysine-laminin coated 60 mm, 24-well or 96-well plates and chamber slides

Figure 2.1 The timeline for directed differentiation of mESCs into BFCNs

The 13-day differentiation protocol starts when the embryoid body formation is started by removing the mESCs from mito-MEFs, and this day is annotated as Day0. On Day2, Retinoic Acid (RA) treatment is essential for roading the

differentiation into a neuronal lineage. Day3- sonic-hedgehog protein Hh-Ag1.5 (Shh) treatment is crucial to enhance neuronal differentiation by acting together with RA. On Day4, the differentiation way turned into BFCN specific by triggering BMP signaling. To provide a more nutritious environment, the medium is changed, and BMP induction is continued on Day5. There was not any treatment on Day6, where the prepared neurons were dissociated and seeded on the plates depending on the assay type will be followed on Day7. Day10 and Day13 were the maturation days of neurons where they grow the axons and connections.

2.4   Characterization of differentiated basal forebrain cholinergic neurons (BFCNs)

2.4.1   Western Blot Characterizations

2.4.1.1   Preparation of Human Embryonic Kidney 293 (HEK293) controls

The HEK293 cell line is commonly used in transfection experiments because HEK293 cultivation is easy, the cells can grow fast, and it has high transfection efficiency. (Baldi, Hacker, Adam, & Wurm, 2007; Dalton & Barton, 2014; Thomas & Smart, 2005; Wurm, 2004). As a result, they provide the researchers with a high protein yield by comparing other mammalian cells (Backliwal et al., 2008). So, this cell line was also a good candidate for being a positive control in Western Blot experiments of this project because they have no endogenous expression of Trk receptors.

 To generate positive controls in Western Blot experiments, the HEK293 cell line was transfected with plasmids encoding TrkA, TrkB full length, and TrkB short frame (TrkB T1). Additionally, empty vector transfections were conducted to generate a negative control as these cells do not endogenously express Trk receptors. All the plasmids were generated and checked in Dr. Lino Tessarollo's laboratory (NIH, USA) and were kind gifts. HEK293 cells were cultivated at 5% CO_2, 37ºC in HEK293 medium *(Appendices B)*. When enough cell confluency was

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reached, cells were counted and seeded on pre-coated poly-D-lysine/laminin 24-well plates. After overnight incubation, cells were transfected with the counted plasmids above with the Turbofect transfection reagent (ThermoFisher, Cat.No: R0534) for 48 hours. Cell lysates were taken when the incubation time ended with phosphatase and protease inhibitor (ThermoFisher, Cat.No: A32957) (ThermoFisher, Cat.No: A32955) supplemented NP-40 cell lysis buffer (Described in *Appendicies A*). The samples were processed by centrifugation at 12000g for 20 minutes. The cell lysates were kept at -80 ºC for further use.

2.4.1.2   Western Blotting

For the Western Blot characterization experiments, five different time points in the differentiation process are considered for both WT and TrkA-KFG cell types. The cell lysates were taken on **Day0, Day2, and Day5** as indicators of pluripotency days, where **Day10** and **Day13** cell lysates were used as fully mature neuronal cultures. All cell lysates were taken with phosphatase (ThermoFisher, Cat.No: A32957) and protease (ThermoFisher, Cat.No: A32955) inhibitor-containing NP-40 cell lysis buffer and processed by centrifugation at 12000g for 20 minutes. They were diluted with 6X sample buffer after equalizing the protein concentrations with Bradford Assay. The cell lysates were kept at -80 ºC for further use.

The samples were heated at 95 ºC for 5 minutes for denaturation and loaded on 10% SDS-PAGE gels with a protein ladder *(Appendices D)*. The running was performed at 120 V for 2 hours; then, the gels were transferred on the PVDF membrane with the wet-transfer technique at 30 V for 2 hours. For the characterization part, the 5% skimmed milk in 0.01% TBS-T was used as a blocking reagent, and membranes were incubated with the blocking reagent for an hour after the transfer. After the blocking, membranes were blotted with primary antibodies listed in *Table 1* overnight at +4ºC. On the next day, membranes were washed with 0.01% TBS-T, and incubated with a secondary antibody solution for 1 hour after 20 minutes of blocking. When the incubation was done, membranes

were washed with 0.01% TBS-T solution. The visualization was developed with a chemiluminescence reagent (WesternBright ECL HRP substrate, Advansta, K-12045-D50) and done by using SynGene GeneGnome Chemiluminescence Imaging System.

Table 1 List of antibodies used in characterization studies

Antibody	Catalog Number
SSEA-1	Santa Cruz, sc-21702
Sox-2	Santa Cruz, sc-36558223
Oct3/4	Santa Cruz, sc-365509
TrkA	ATS Bio, AB-N03
TrkB	Millipore, 07-225
β- III Tubulin	R&D Systems, BAM1195
β- Actin HRP	Santa Cruz, sc-47778
SNAP-25	Biolegend, SMI 81
VAMP2	Santa Cruz, sc-69706
NKX2.1	Abcam, ab76013
Tau5	Thermo Fisher, MS-247-P

2.4.2 Immunocytochemistry Characterizations

When the embryoid bodies were dissociated on **Day7**, they were seeded on poly-D-lysine/laminin-coated 4-well chambers for ICC experiments. On the maturation of differentiated BFCNs (**Day13)**, the ICC procedure was started with a fixation step which is achieved by incubating slides for 10 minutes at 37°C with 4% Paraformaldehyde solution after PBS (+,+) washes. To permeabilize the cells, chambers were incubated at RT for 15 minutes with 0.1% TritonX-100 (Serva). After permeabilization, chambers were incubated at RT for 1 hour with a 2% BSA

solution. For immunostaining, antibodies were diluted in 2% BSA solution, and amounts were adjusted according to manufacturers' recommendations. After 3-hour RT incubation, primary antibodies were removed, and chambers were washed three times with PBS (+,+) solution for 10 minutes each. The suitable fluorescent dye-labeled secondary antibodies (AlexaFluor and Cy3) were used, and chambers were incubated for 2 hours at RT. After rising the chambers, mounting was achieved with DAPI containing ProLong Gold Antifade Reagent (Invitrogen). The imaging was performed with ZEISS confocal microscope. The markers used in the experiment can be seen in *Table 1*.

2.5 Amyloid-beta (Aβ) experiments to initiate *in vitro* Alzheimer's Disease for investigation of the KFG effect

2.5.1 Preparation of oligomeric and fibrillar amyloid-beta (1-42)

The HFIP-treated amyloid-beta (1-42) (Aβ(1-42)) purchased from AnaSpec (AS-64129-1) and PeptiTeam prepared 24 hours before the cell experiments were started. Prior to the reconstitution process, the peptide was chilled at RT for half an hour. According to the well-known protocols in the literature (Park et al., 2018; W. B. Stine, Jr., K. N. Dahlgren, G. A. Krafft, & M. J. LaDu, 2003), the peptide film was first reconstituted with DMSO to 5 mM concentration. After 30 seconds of vortexing, the reconstituted peptide was diluted with PBS (+,+) to get a 100 µM stock concentration. The prepared peptide was incubated at +4 ºC for 24 hours for oligomerization. To get fibrillar Aβ(1-42), 5 mM DMSO reconstituted peptide film was diluted with 10 mM HCl to get a 100 µM stock concentration, and the reconstituted peptide was incubated at 37 ºC for 24 hours to initiate fibrillization (Stine, Jungbauer, Yu, & LaDu, 2011). Further dilutions were done with Neuro Medium (Miltenyi Biotech, Cat. No: 130-093-570) to get four different dilutions 1.0 µM, 2.5 µM, 5.0 µM, and 10 µM. Also, the HFIP- treated scrambled Aβ(1-42) (AnaSpec, Cat.No: AS-25383), which is modified Aβ(1-42) with a scrambled sequence used as a negative control peptide for the oligomerization process. The

same oligomerization procedure mentioned above is also valid for the preparation of scrambled Aβ(1-42), where the oligomerization and aggregation were not expected because it could not form the necessary tertiary structure due to its mixed sequence.

2.5.2   Validation of prepared oligomeric and fibrillar amyloid-beta (1-42)

To show the Aβ(1-42) oligomerization was achieved, the Western Blotting was performed by using 10 μg prepared Aβ(1-42) peptide solution and scrambled Aβ(1-42) as a negative control (Cerf et al., 2009; W. B. Stine, Jr. et al., 2003; Stine et al., 2011). The samples were loaded on 12% SDS-PAGE gels being not heated at 95 ºC for 5 minutes, and they were run at 125 V until the dye front reached the bottom. Then, the proteins on the gel were wet-transferred on the PVDF membrane at 100 V for an hour. The membrane was blocked with 5% skimmed milk solution prepared in 0.01% TBS-T for 1 hour and incubated with an anti-β-Amyloid antibody (Biolegend, 6E10) at +4 ºC overnight. The next day, the membrane was washed with 0.01% TBS-T and incubated with a secondary antibody for 1 hour after 20 minutes of blocking. The visualization was enhanced with a chemiluminescence reagent (WesternBright ECL HRP substrate, Advansta, K-12045-D50) and done using SynGene GeneGnome Chemiluminescence Imaging System.

To confirm the fibrillar Aβ(1-42) preparation, Thioflavin-T (ThT) analysis was performed as it is described in the literature (Nilsson, 2004; Xue, Lin, Chang, & Guo, 2017). ThT is a benzothiazole dye that can bind to the β-sheets of amyloid-beta fibrils and gives the fluorescence upon the binding. The fibril-specific binding of ThT dye was investigated in the literature by monitoring the amyloid fibril formation in hen egg white lysozyme via fluorescence detection by dynamic light scattering (DLS) and atomic force microscopy (AFM), where it was suggested that ThT dye is unsuitable for detecting oligomer and protofibril forms of amyloid-beta

(Hill, Robinson, Matthews, & Muschol, 2009; Persichilli, Hill, Mast, & Muschol, 2011).

The ThT analysis is based on assessing the binding capacity of ThT by calculating the binding fold-change of the constant concentration of fibrillar Aβ(1-42) samples under the increasing concentrations of ThT as compared to controls. To show the bold fibrillation, the 10 µM fibrillar Aβ(1-42) was aligned on a 96-well plate where the other wells were the controls containing only Neuro Medium. After preparing the 10 mM stock ThT solution as it is described in ***Appendices A,*** the eight different concentrations (1 µM, 2 µM, 5 µM, 10 µM, 50 µM, 100 µM, 200 µM, 500 µM) were diluted. The diluted ThT solutions were given to both control and fibrillar Aβ(1-42) samples; then, the absorbance values were checked under 450 nm and 490 nm (for background). After subtracting the background values, the fold-change was calculated by proportioning the fibrillar Aβ(1-42) sample values to the control values. By using the calculated values, the ThT dose versus binding fold-change graph was created.

2.5.3   Cell Viability and Cell Death Assays

Cell viability and cell death assays were conducted to understand the effect of TrkA signaling upregulation on neuronal survival. On **Day7** of the differentiation process, the dissociated neurons were seeded on three different poly-D-lysine/laminin-coated 96-well plates by lying half the plate with WT and the other half with TrkA-KFG BFCNs. A day before the maturation, the oligomeric Aβ(1-42) was prepared as described in ***Chapter 2.5.1***. Mature neurons were treated with four different concentrations (1.0 µM, 2.5 µM, 5.0 µM, and 10 µM) of pre-prepared oligomeric Aβ(1-42) except the control wells after 12-hour starvation. Three different plates were used for three different time points to see Aβ(1-42) toxicity in 24 hours, 48 hours, and 96 hours, meaning that the plates were incubated with prepared oligomeric Aβ(1-42) for indicated time periods at 5% CO_2, 37ºC. After desired incubation times following assays were started.

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2.5.3.1   MTT Cell Viability Assay

When the Aβ(1-42) treatment time was ended, a pre-prepared MTT reagent *(Appendices A)* was added to 10 µl/well, and incubated for 4 hours at 37°C, 5% CO_2 to let the viable cells form a formazan structure with the interaction of MTT reagent. After 4 hours of incubation, the formed formazan structures were solved with MTT solubilization solution *(Appendices A)*, and the plate was incubated for 18 hours for complete solubilization. After incubation, the read-out was taken in an ELISA reader (Thermo Fisher, USA) at 570 nm.

To calculate the cell viability, the absorbance of the blank containing just medium was subtracted from the absorbances of all wells. To find the viable cell percentage sample, the sample absorbance was divided by the absorbance of control cells and multiplied by 100. The formula version of the cell viability calculation can be seen below.

$$Cell\ Viability\ \% = \frac{(Abs_{sample} - Abs_{blank})}{(Abs_{control} - Abs_{blank})}\ X\ 100$$

2.5.3.2   LDH Cell Death Assay

The LDH Assay was performed using CyQUANT™ LDH Cytotoxicity Assay as recommended by the manufacturer (Thermofisher, C20300). This kit includes negative and positive control, where the cells were treated with sterile water as a negative control and named Spontaneous LDH activity. For positive control, the Maximum LDH activity was used where the cells were treated with Lysis Buffer as a kit component. After 45-minute incubation at 37°C, 5% CO_2 with indicated control chemicals, 50 µl of each well were transferred into a new 96-well flat bottom plate. Then, the Reaction Mixture of the kit was added to each well, and after 30-minutes of incubation at 37°C, 5% CO_2 was achieved. After the incubation, the wells received the Stop Solution of the LDH kit, and the absorbances at 490 nm and 680 nm (to get background value) were measured in an

ELISA reader (Thermo Fisher, USA). To calculate the cell cytotoxicity, the following formula was used.

$$Cell\ toxicity\ \% = \frac{(Abs\text{sample} - Abs\text{spontaneous LDH})}{(Abs\text{maximum LDH} - Abs\text{spontaneous LDH})}\ X\ 100$$

2.5.4 Investigation of oligomeric Aβ(1-42)-mediated neuronal changes, focusing on synaptic density

To evaluate the importance of enhanced TrkA signaling on the synaptic density, WT and TrkA-KFG BFCNs upon Aβ(1-42) insult were analyzed by both Western Blotting and immunocytochemistry. In addition to synaptic changes, we have evaluated changes in MAPK/ERK pathway in our model systems as the alterations in this pathway has been associated with AD processes and separately with synaptic density.

2.5.4.1 Western Blot Analysis

For Western Blot analysis, neurons dissociated on differentiation day **Day7** were seeded on poly-D-lysine/laminin-coated 24-well plates. One day before the maturation day, oligomeric Aβ(1-42) was prepared as described in ***Chapter 2.5.1***. On maturation day, the wells, except for the controls and just will be treated with 10 µM oligomeric Aβ(1-42), were treated with 50 ng/ml NGF, which is an activator ligand of TrkA receptors, for 4 hours at 37°C, 5% CO_2 after 12-hours starvation. After the 4-hour compound incubation, the corresponding wells were treated with 10 µM oligomeric Aβ(1-42) and incubated for 24 hours at 37°C with 5% CO_2. When the Aβ(1-42) treatment ended, the cell lysates were taken with NP-40 lysis buffer, including phosphatase and protease inhibitors.

After the protein isolation and calculation of protein concentrations with Bradford Assay, the Western Blot procedure was conducted as described in ***Chapter 2.4.1.2***. The antibodies used in the study are provided in ***Table 2*** below.

Table 2 The antibodies used in the investigation of oligomeric Aβ(1-42)-mediated neuronal changes

Antibody	Catalog Number
Total Erk	Cell Signaling, 9102
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10)	Cell Signaling, 9106
Pan-Trk (C-15)	Santa Cruz, sc-139
Synaptophysin	Santa Cruz, sc-17750
Syntaxin (SP8)	Biolegend, 8270002
Tau5	Thermo Fisher, MS-247-P
VAMP2	Abcam, ab3347
NeuN	Millipore, MAB377B
β- III Tubulin	R&D Systems, BAM1195
β- Actin HRP	Santa Cruz, sc-47778

2.5.4.2 Immunocytochemistry Analysis

Oligomeric Aβ(1-42) toxicity-derived synaptic density change differences between WT and TrkA-KFG BFCNs were also verified with the ICC technique. The dissociated neurons were seeded on 4-well chamber slides coated with poly-D-lysine/laminin. One day before the maturation day, oligomeric Aβ(1-42) was prepared as described in ***Chapter 2.5.1***. On maturation day, the corresponding wells were treated with pre-prepared oligomeric Aβ(1-42) for 24 hours after 12 hours of starvation. When the treatment was ended, the ICC procedure proceeded as described in ***Chapter 2.4.2***. The prepared slides were imaged FLoid Imaging Station (ThermoFisher). The antibodies used for the immocytochemistry analyses are provided in ***Table 2***.

2.6 Analysis of neurotrophin signaling and Alzheimer's Disease-related signaling cascades with *in silico* models

AD is a complex neurodegenerative disease in which many signaling cascades have different roles in disease progression. Therefore, it is vital to understand the relationship between these signaling cascades in the concept of AD, where the bioinformatic analysis is the key to connecting all the dots in the light of our biological data. Among the seventy different ways to analyze the pathways, the Kyoto Encyclopedia of Genes and Genomes (KEGG), Reactome, Pathway Interaction Database, and PANTHER are the most preferred ones. In this study, the oligomeric Aβ(1-42) and TrkA-NGF signaling cascades were retrieved from the KEGG database seperately. To do so, the neurotrophin signaling [hsa47022] cascade-related pathways and Alzheimer's disease-related pathways [hsa05010] were integrated into Cytoscape 3.9 program with the KEGGScape, CytoKEGG, and CyKEGGParser plug-ins. By searching the KO KEGG code for NGF (K02582) in the KEGG Mapper-Search tool, the NGF-TrkA signaling-related pathways were additionaly retrieved. Then, the common pathways were annotated to see the molecular relationship clearly, which would help to give meaning to our data.

2.7 Statistical Analyses

The cell viability and toxicity data proceeded using the *Student t-test* including ±SD in GraphPad Prism 9.0 software. The immunocytochemistry data were analyzed in CellProfiler 4.2.1 (https://cellprofiler.org/) and statistical analyzes made in GraphPad Prism 9.0 software. In all data analyses, $p < 0.05$ was assigned as statistically significant, and ***, **, * indicate that the values are at 99.9%, 99% and 95% confidence levels, respectively. The number of biological replicates for each experiment is indicated in the figure legends.

3.1 Differentiation of V6.4 mESCs into basal forebrain cholinergic (BFCNs)

Prior to the differentiation process, mitotically inactivated MEF cells (called mito-MEF) were generated to be used in stem cell propagations as described in **Chapter 2.1.** mESCs, which grow as colonies, were co-cultured with the mitoMEFs, shown in **Figure 3.1.**
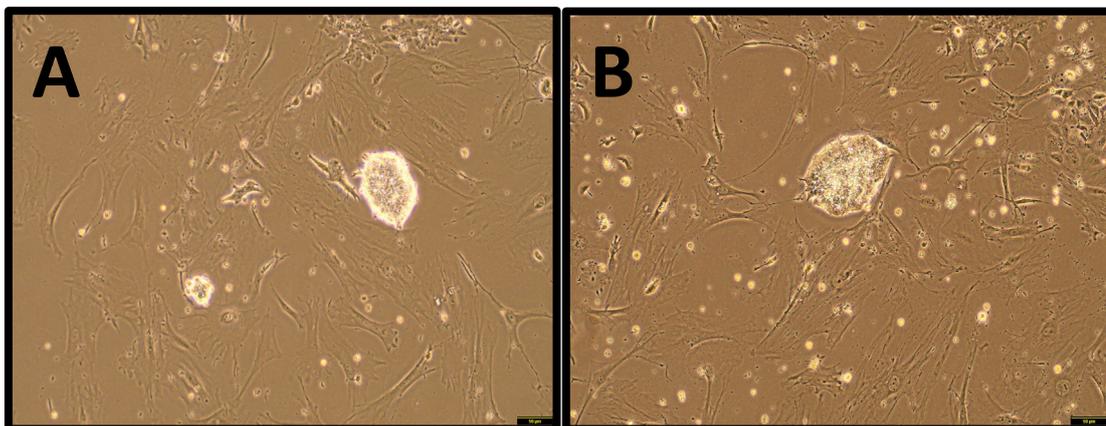
Figure 3.1 The image of (A) WT V6.4 and (B) TrkA-KFG V6.4 mouse embryonic stem cells

Both mESC lines were grown on mitotically inactivated MEF cells on gelatin-coated 150 mm cell culture treated plates. The images were taken using a BAB camera system implemented on Olympus CKX-53 under the 10X objective. Scale bar, 50μm.

For the differentiation process, we first extensively studied avaliable protocols for their suitability and efficiency to be utilized in our project. This was important as there are various protocols in the literature for directed differentiation of mESCs into BFCNs, and those protocols differ significantly from each other (Bissonnette

et al., 2011; Crompton et al., 2013; Ihnatovych et al., 2018; McComish & Caldwell, 2018; Yue et al., 2015). Therefore, we experimentally tested the available protocols and extensively evaluated conditions to optimize and establish an ideal protocol in our hands. Based on our optimizations, we selected the protocol of BFCN differentiation from pluripotent stem cells published by the Kessler and Jing groups (Bissonnette et al., 2011; Yue et al., 2015). To utilize it in our study, we further optimized the chosen protocol. *Figure 3.2* demonstrates representative cell images from various days reflecting the differentiation progress. The technical details of the protocol are provided in *Chapter 2.3*.
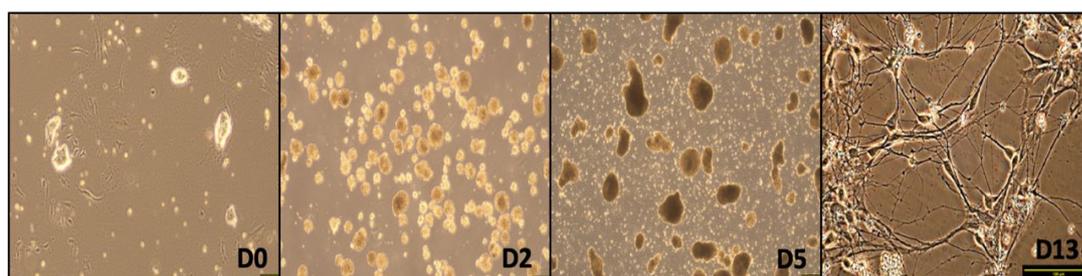
Figure 3.2 Representative images of cells/embryoid bodies on selected days of the BFCN differentiation protocol

*(A) A representation of TrkA-KFG mESCs prior to differentiation process, which is annotated as **Day0**, (B) Embryoid bodies treated with RA on **Day2** for neuronal programing (C) Embryoid bodies on **Day5** supplemented with 10 ng/ml BMP-9 in Neuronal Medium-1 to give BFCN identity to the neurons (D) The dissociated mature neurons on their **Day13** of the differentiation protocol. The images were taken using a BAB camera system implemented on Olympus CKX-53 under the 10X objective for D0 to D5(Scale bar, 50μm), and under 40X objective for D13 (Scale bar, 100μm).*

3.2 Characterization of differentiated basal forebrain cholinergic neurons (BFCNs)

3.2.1 Western Blot Characterizations

To examine the success of directed differentiation, the cell lysates were collected on different days of the differentiation process and evaluated by the Western Blot for specific markers. More specifically, cell lysates of both WT and TrkA-KFG samples were collected during the differentiation process on Days 0, 2, 5, 10, and 13, and loaded on SDS-PAGE gel in equal amounts after total protein isolation and concentration measurement by Bradford Assay. In the light of the literature, Western Blot analyzes were carried out by examining the changes in levels of specific markers, including the pluripotency markers SSEA-1, Sox-2, and Oct4, and neuronal markers Tau5 and β-III Tubulin. In addition, the change in SNAP-25 and VAMP2 protein levels were evaluated, as both are SNARE proteins known to be highly expressed in BFCNs (Bissonnette et al., 2011; Crompton et al., 2013; Ihnatovych et al., 2018; McComish & Caldwell, 2018; Yue et al., 2015). Our data demonstrated that the levels of pluripotency markers remained high at the beginning of the differentiation process, and their levels decreased towards the end of the differentiation process. So, as expected, pluripotency markers were not detected on neuronal maturation days (Day10 and Day13) while an increase in neuronal markers Tau5 and β-III Tubulin was observed on Day10 and Day13. A similar pattern is evident for SNAP-25 and VAMP2 upon neuronal maturation (See *Figure3.2*, A). In addition to these specific markers, the levels of Trk receptors were compared in WT and TrkA-KFG BFCNs. A higher level of Trk receptors was observed in KFG-removed TrkA neurons. This finding is consistent with our previous study in which an increase in TrkA receptor was detected in TrkA-KFG primary neurons. To further evaluate Trk receptor changes and ensure the Western Blot bands we observe are indeed TrkA bands, we generated control samples to run on Western Blots. For this goal, we utilized HEK293 cells transient transfected with TrkA or empty vector plasmids (See *Chapter 2.4.1.1*). The maturation day

samples (Day10 and Day13) of both WT and TrkA-KFG BFCNs were loaded on SDS-PAGE gels with negative (empty vector transfected) and positive (TrkA plasmid transfected) HEK293 control lysates. The results demonstrated is an increase in TrkA receptor levels when the KFG domain is removed from the receptor (*Figure 3.3*, B). The levels of SNAP-25 were used as a control group, which shows the similar pattern seen in *Figure 3.2*, panel A. Previous literature well established that transcription factor NKX2.1 can be utilized as a marker for BFCN identity (Bissonnette et al., 2011; Yue et al., 2015). Therefore, we examined NKX2.1 expression, and our results demonstrated both WT and TrkA-KFG BFCNs express NKX2.1 while the levels are higher in TrkA-KFG neurons. Overall, the Western Blot analyses represented in *Figure 3.3*, panel A and panel B suggest successful directed differentiation of mESCs into BFCNs. In all blots, β-Actin was used as a loading control.

In addition to TrkA receptors, the levels of TrkB receptor levels were also evaluated in both WT and TrkA-KFG BFCNs with proper controls. This is important as the KFG manipulation in the mESC line was conducted in TrkA; however, it is crucial to evaluate whether there was any effect on TrkB, another highly important neurotrophin receptor. The control samples included HEK293 cells transiently transfected with full-length TrkB (TrkB FL) or truncated TrkB isoform (TrkB T1) or the empty vector. The controls and lysates of mature BFCNs were analyzed with Western Blotting by comparing the levels of TrkB, and the neuronal marker β-III Tubulin in two types of BFCNs. β-Actin was used as a loading control (See *Figure 3.3*, panel C). Our data suggest that there are not any significant TrkB level differences between the WT and TrkA-KFG BFCNs. Despite the high levels of TrkA in BFCNs, it is known that the TrkB shows a more general expression within the brain. Besides, the modification of KFG in TrkA-KFG just includes the TrkA receptor without changing any functionalities in the TrkB receptor. Therefore, it is not surprising to see no difference in TrkB levels between WT and TrkA-KFG BFCNs with a moderate expression of TrkB in both lines. To sum up, these results suggested that the used antibodies are highly

specific to related Trk receptors, and there is a noticeable difference in TrkA levels, not in TrkB, when the KFG domain of TrkA is removed as compared to WT BFCNs.
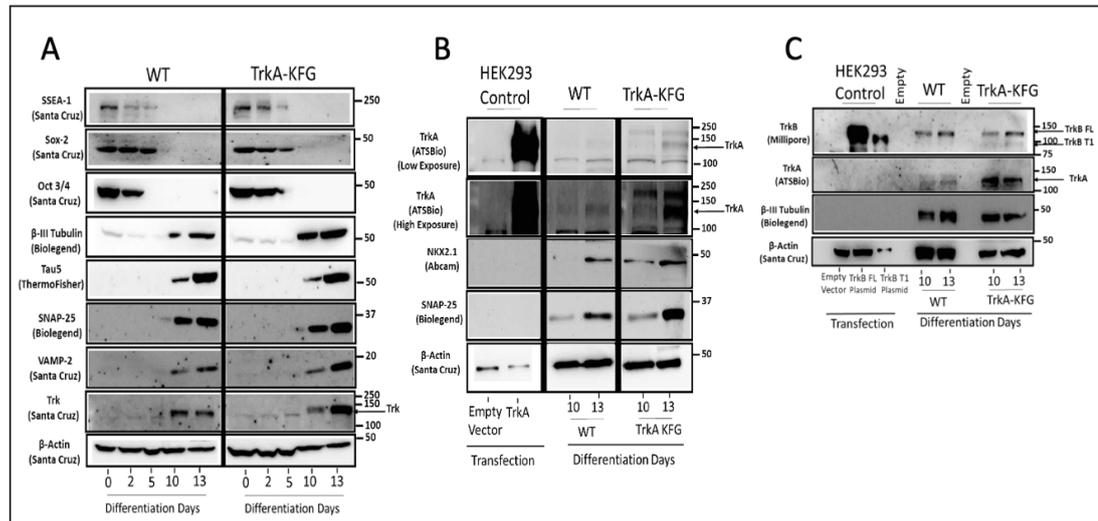
Figure 3.3 Western Blot characterization of WT and TrkA-KFG BFCNs directly differentiated from mESCs

(A)Characterization of differentiated WT and TrkA-KFG BFCNs by examining pluripotency and neuronal markers from top to bottom, (B) panel shows he comparison of TrkA levels in WT and TrkA-KFG BFCNs with HEK293 controls, and (C) presents the investigation of the TrkB levels in WT and TrkA-KFG BFCNs with HEK293 controls. β-Actin was used as a loading control. All representative images of characterization experiments were obtained from at least 3 biological replicates performed with different sets of neurons.

3.2.2   Immunocytochemistry Characterizations

As a second step for the characterization of differentiated BFCNs, immunocytochemistry (ICC) was used to analyze the differentiation at the cellular level. The details of the ICC protocol are given in ***Chapter 2.4.2***. Tau5 and β-III Tubulin are well-established neuronal markers and our ICC analyses demonstrated that neuronal differentiation was successful for both WT and TrkA-KFG (***Figure***

3.4) In these BFCNs, both presence of TrkA and colocalization of Tau5 and β-III Tubulin were observed, which is consisted with our previous work demonstrating the high expression of TrkA in BFCNs. Also, the presence of the BFCN marker, NKX2.1 (TTF1), suggested that the neurons differentiated from mESCs have BFCN characteristics, as also validated with Western Blotting in *Chapter 3.2.1*. In summary, our results in *Chapter 3.2.1* and *Chapter 3.2.2* suggest that the directed differentiation protocol was successfully optimized and characterized for the following experiments.

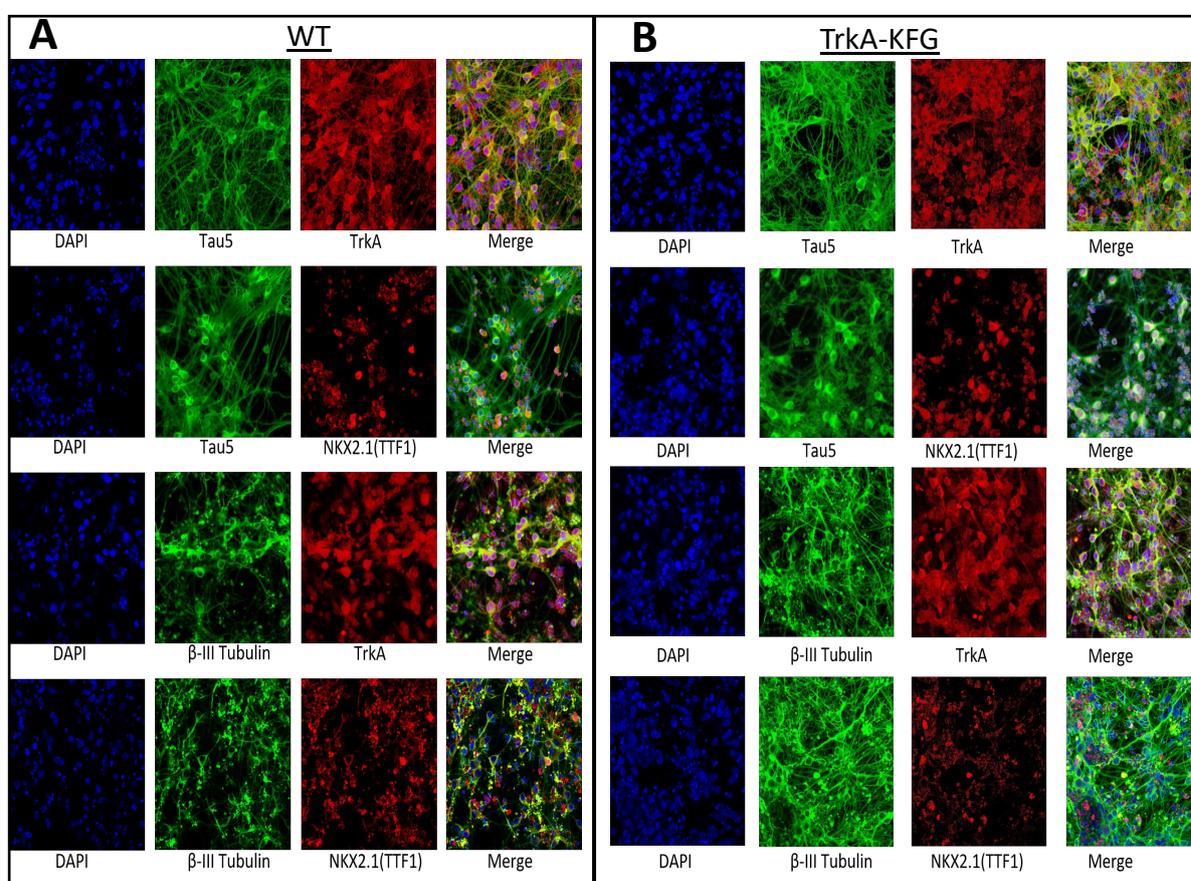***Figure 3.4*** Immunocytochemisrty characterization of WT and TrkA-KFG BFCNs directly differentiated from mESCs

The immunocytochemistry characterization of WT and TrkA-KFG BFCNs was performed by observing the presence of TrkA-colocolized neuronal markers, Tau5, β-III Tubulin, and BFCN identity marker NKX2.1. The (A) representation of WT

BFCNs and (B) representation of TrkA-KFG BFCNs are suggesting that there is the successful presence of neuronal markers which are colocalized with TrkA receptor and the BFCN marker NKX2.1. In both lines of BFCNs, DAPI was used as nuclear counterstain. The representative images were obtained from 5 biological replicates performed with different sets of neurons.

3.3 Amyloid-beta (Aβ) experiments to initiate *in vitro* Alzheimer's Disease for investigation of the KFG effect

3.3.1 Validation of prepared oligomeric and fibrillar amyloid-beta (1-42)

As described in ***Chapter 2.5.1***, oligomeric and fibrillar Aβ(1-42) were formed to assess the best neurotoxicity for *in vitro* AD modeling as described in the literature. Experimental verifications were conducted based on well-established protocols to confirm the success of the preparation (See ***Chapter 2.5.2***). A Western Blot analysis was used to confirm the oligomeric Aβ(1-42) formation (Cerf et al., 2009; W. B. Stine, Jr. et al., 2003; Stine et al., 2011). Following the literature-based optimized protocol, the prepared oligomeric Aβ(1-42) and Scrambled Aβ(1-42), a negative control that should not oligomerize, were loaded on 12% SDS-PAGE gels without pre-heating the samples at 95°C for 5 minutes and run at 125V. The wet transfer of SDS-PAGE gel on the PVDF membrane was performed at 100V for 1 hour at 4°C. To detect Aβ(1-42) oligomers, the purified anti-amyloid- beta antibody (Biolegend, 6E10) was used. The findings are consistent with the literature showing a successful presence in Aβ(1-42) oligomers, and the negative control Scrambled Aβ(1-42) did not oligomerize (***Figure 3.5***, panel A).
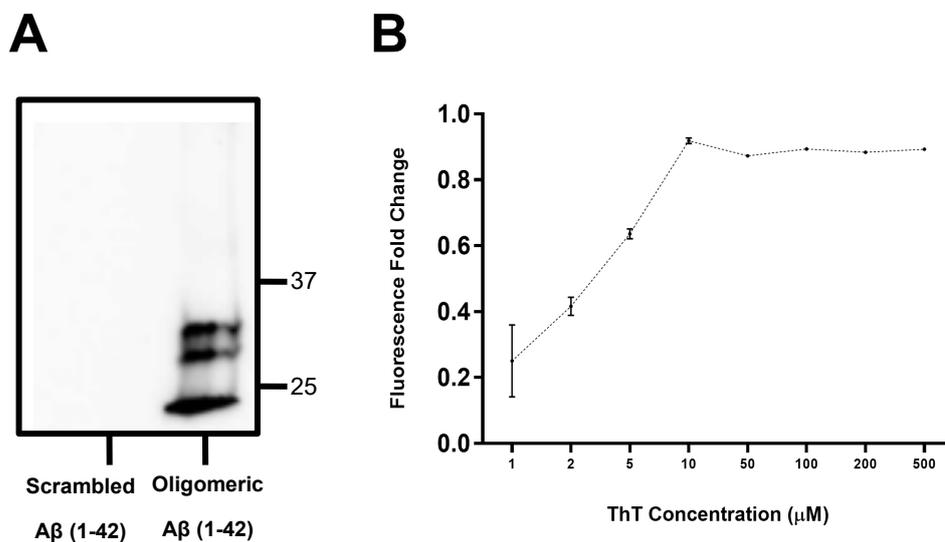
Figure 3.5 Analysis results of validated oligomeric and fibrillar Aβ(1-42)

(A) Western Blot analyses of oligomeric Aβ(1-42) and scrambled Aβ(1-42) serving as a negative control. The representative blot image represents data from 5 biological replicates performed at 5 different times. (B) A graph showing the binding capacity of 10 μM fibrillar Aβ(1-42) over increasing concentrations of ThT dye. This graph is generated from a data of 3 biological replicates performed at different times.

Thioflavin T (ThT) analysis was used to assess fibrillar Aβ (1-42) formation. It has been shown in the literature that fibrillar structures can be confirmed by examining the binding pattern of ThT dye at different concentrations of peptides (For detailed protocol, see **Chapter 2.5.2**) (Nilsson, 2004; Xue et al., 2017). This method is based on detecting fluorescence generated upon binding of ThT dye to beta-sheet structures in the amyloid-beta fibrils (Gade Malmos et al., 2017). By assessing the pattern in fold-change between the samples with and without peptide under the increasing concentration of ThT dye, the confirmation of fibril formation can be shown. In our experimental design, the binding capacity of a constant 10 μM peptide was analyzed under the 1μM-500μM range of increasing ThT concentrations. After seeing a peak in the binding pattern between 20-50μM of ThT concentration, the dye became saturated, and binding capacity remained constant. This binding pattern is compatible with literature suggesting that the fibrillar formation was conducted successfully (**Figure 3.5**, panel B). In short, our

analyses showed that both oligomeric and fibrillar Aβ (1-42) were prepared and formed successfully.

3.3.2   Cell Viability and Cell Death Assays

It is well-established in the literature by many study groups that the neurotoxicity effect of Aβ(1-42) treatments lead to neuronal death (Badshah, Kim, & Kim, 2015; Ebenezer et al., 2010; Kam et al., 2019; C. C. Liu et al., 2016). Also, it has been shown that TrkA signaling can provide neuroprotection against Aβ(1-42)-related neurotoxicity (Kitiyanant et al., 2012; Lucia Tapia-Arancibia, Esteban Aliaga, Michelle Silhol, & Sandor Arancibia, 2008). To assess the effect of enhanced TrkA signaling on Aβ(1-42)-mediated neuronal death, the MTT assay, one of the most common methods to show cell viability, was first utilized (Badshah et al., 2015; Ebenezer et al., 2010; Kam et al., 2019; C. C. Liu et al., 2016). To do so, the neurons grown on a 96-well plate were treated with five different concentrations (1, 2.5, 5 ve 10μM) of oligomeric Aβ(1-42) under three different treatment time conditions (24,48 and 96 hours). Consistent with the literature, our data demonstrated that there is a correlation between the decrease in cell viability with increasing concentrations of oligomeric Aβ(1-42). Excitingly, the cell viability was higher in TrkA-KFG BFCNs than WT under identical experimental conditions. For example, while the cell viability of TrkA-KFG BFCNs is 71% in 24 hours of 10μM Aβ(1-42) treatment condition, the viability of WT is only 31% under the same condition (The difference is statistically significant, $p<0.01$). Our control condition results are consistent with the literature, where the 10μM Aβ(1-42) leads to approximately 30% neuronal death within 24 hours of treatment (Ebenezer et al., 2010). Also, our analysis showed that Aβ(1-42) treatment increases neuron death when the treatment time frame is increased (24, 48, and 96 hours). When the general trends in the time-dependent experiment were examined, it was concluded that Aβ(1-42)-induced death is distinguishably more in WT BFCNs. As a control condition, 10μM Scrambled Aβ(1-42) negative control group did not lead to

neurotoxicity, and also did not affect the cell viability, consistent with the literature (See *Figure 3.6*, panel D).
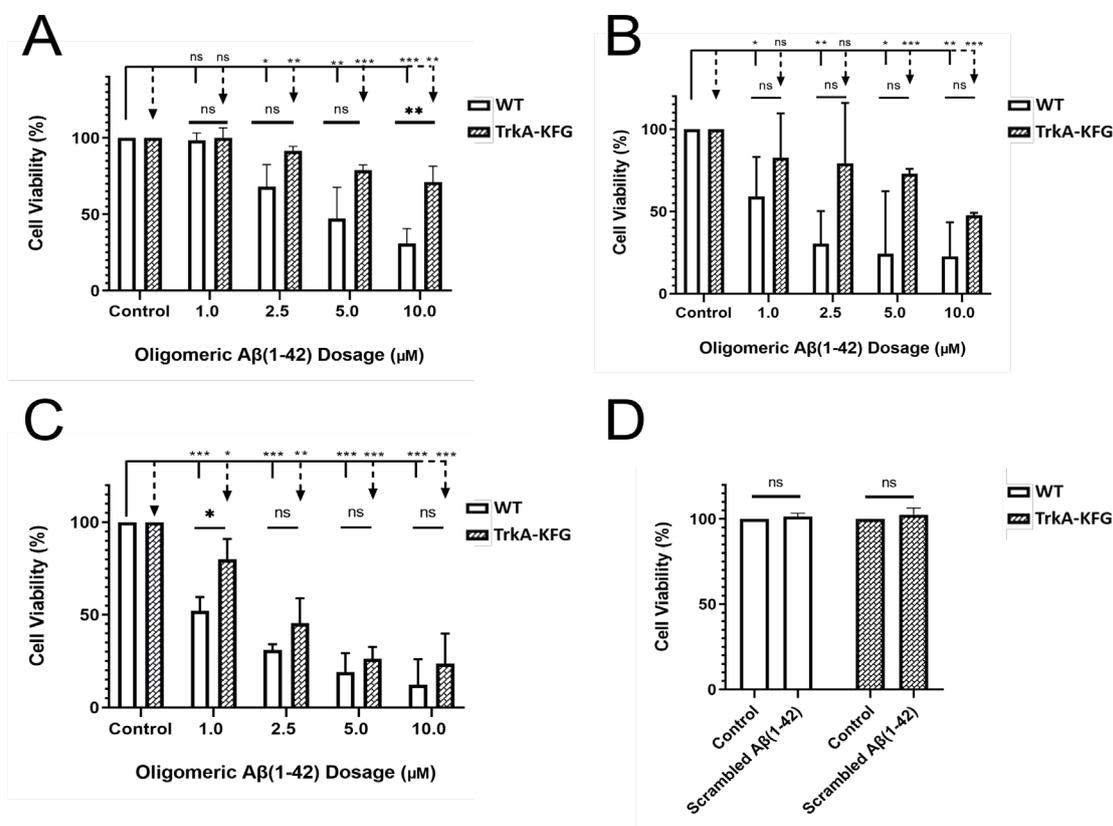
Figure 3.6 MTT test results to compare *in vitro* oligomeric Aβ(1-42)-mediated cell viability differences in WT and TrkA-KFG BFCNs.

(A)(B)(C)Results of oligomeric Aβ(1-42)-mediated toxic effect on cell viability under different treatment time points (24,48, and 96H) and four different Aβ(1-42) dosages. (D) Scrambled control, which is a mixed amino acid sequence of Aβ(1-42, did not lead to significant cell viability changes. The presented results reflect the data from 3 different biological replicates performed at different times.

Results are given as mean ± SD, and statistical analyzes were performed with Student's t-test using GraphPad Prism. ***, **, * indicate that the values are at 99.9%, 99% and 95% confidence levels, respectively.

In addition to the MTT cell viability assay, the Lactate Dehydrogenase (LDH) cytotoxicity assay was conducted to examine and compare the oligomeric Aβ(1-42)-mediated neuronal death in WT and TrkA-KFG BFCN neurons. In this analysis, neurons were treated with the highest oligomeric Aβ(1-42) dosage of our experimental set-up (10 µM) for 24, 48, and 96 hours to observe the differences better. The results demonstrated an increased cell death with prolonged treatment time. When the cell death percentages in WT and TrkA-KFG BFCNs are compared, it is evident that TrkA-KFG BFCNs are more resistant to oligomeric Aβ(1-42)-mediated cell death (***Figure 3.7***). This assay suggested that the ideal cellular condition to check the differences between WT and TrkA-KFG BFCNs is 24H ($p < 0.05$), which is line with our MTT cell viability results.
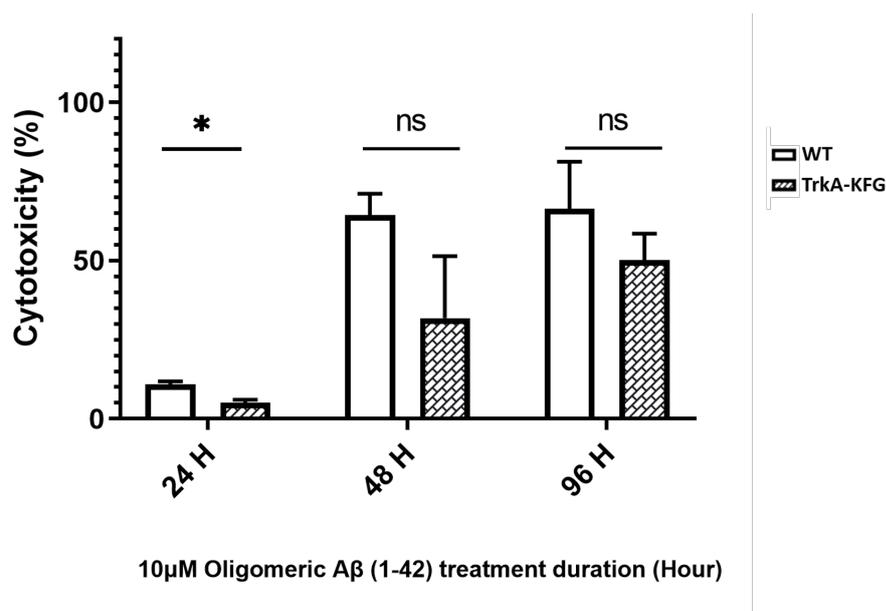
Figure 3.7 The LDH cytotoxicity assay results to compare *in vitro* oligomeric Aβ(1-42)-mediated cell death differences in WT and TrkA-KFG under different time points

The assay was conducted to measure cell death differences between TrkA-KFG and

WT BFCNs upon 10 µM oligomeric Aβ(1-42) treatment at increasing time points

(24H,48H,96H) by analyzing the cytotoxicity percentages. The presented results reflect the data from 3 different biological replicates performed at different times.

*Results are given as mean ± SD, and statistical analyzes were performed with Student's t-test using GraphPad Prism. ***, **, * indicate that the values are at 99.9%, 99% and 95% confidence levels, respectively.*

In our following analysis, fibrillar Aβ(1-42)-mediated changes in the cell viability was analyzed under five different concentrations of fibrillar Aβ(1-42) (1, 2.5, 5 ve 10 µM) at three different treatment time points (24, 48 and 96 hours). The results suggest that the decrease in cell viability is less in TrkA-KFG BFCNs as compared to WT neurons in each treatment time (See ***Figure 3.8***). Although there not statistically significant, the trend is evident that TrkA-KFG neurons are more resistant. In other words, the cell viability decreases with increasing concentrations of fibrillar Aβ(1-42) and the treatment time, similar to oligomeric Aβ(1-42) treatments. However, oligomeric Aβ(1-42) leads to higher toxicity compared to fibrillar form, which is consistent with the literature.

Figure 3.8 MTT test results to compare fibrillar Aβ(1-42)-mediated cell viability differences in WT and TrkA-KFG BFCNs

(A)(B)(C)Results of fibrillar Aβ(1-42)-mediated neurotoxicity effect on cell viability at different times (24,48, and 96H) and under four different Aβ(1-42) concentrations. The presented results reflect the data from 3 different biological replicates performed at different times.

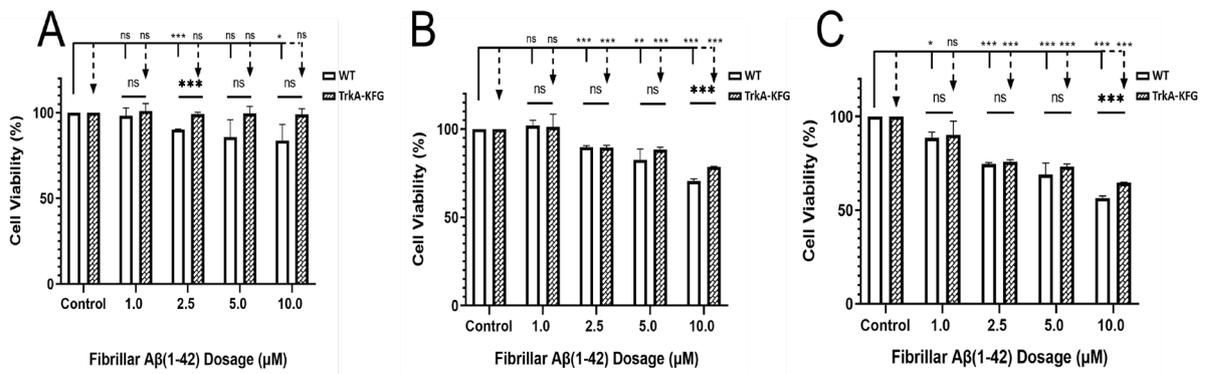*Results are given as mean ± SD, and statistical analyzes were performed with Student's t-test using GraphPad Prism. ***, **, * indicate that the values are at 99.9%, 99% and 95% confidence levels, respectively.*

In short, our results overall suggested that TrkA-KFG BFCNs have resistance toward oligomeric and fibrillar Aβ(1-42)-mediated neurotoxicity compared to WT BFCNs. As the oligomeric Aβ(1-42) is the most toxic Aβ form, in the light of our MTT and LDH results, we chose to utilize 24 hours treatment of 10 μM oligomeric Aβ(1-42) in the following experiments in our study .

3.4 Investigation of oligomeric Aβ (1-42)- mediated neuronal changes, focusing on synaptic density

3.4.1.1 Western Blot Analysis

Previous studies show that oligomeric Aβ(1-42) affects synaptic protein levels, integrity, and density both *in vitro* and *in vivo* (C.-C. Liu et al., 2014; Long et al., 2022; Rajmohan & Reddy, 2017). For example, it has been shown that Aβ(1-42) treatment leads to a decrease in the protein levels of synaptic markers VAMP2, MAP2, Synaptophysin, Syntaxin, and SNAP-25 (Ferreira et al., 2012). Therefore, we first utilized Western Blot analyses to measure the protein levels of selected synaptic markers. The blot presented in ***Figure 3.9***, panel A blotted together with a loading marker β-III Tubulin and Synaptophysin antibodies shows a distinguishable difference in the levels of Syntaxin and Synaptophysin in general, which are higher in TrkA-KFG neurons. This suggests that the synaptic density is higher in TrkA-KFG BFCNs as compared to WT BFCNs. Our data demonstrate that the levels of Synaptophysin and Syntaxin are dramatically decreased in WT BFCNs upon oligomeric Aβ(1-42) treatment; however, we did not detect a similar decrease in TrkA-KFG neurons (***Figure 3.9***, panel A). Treatment of TrkA ligand, 50 ng/ml NGF, did not rescue the protein levels of the tested synaptic markers upon Aβ(1-42) treatment in WT BFCN neurons. However, the treatment of Aβ(1-42)

with or without NGF supplementation did not appear to make an evident change in the protein levels of the two markers analyzed, especially in Synaptophysin levels. In conclusion, our Western Blot analyses with two synaptic markers suggest that TrkA-KFG BFCNs exhibit resistance against oligomeric Aβ(1-42) treatment-mediated synaptic density changes.

We then sought to shed light on molecular mechanisms regulated by TrkA signaling that may have roles in the synaptic density of neurons. It has been suggested in the literature that MAPK/ERK pathway is critical for synaptic functioning (Miningou & Blackwell, 2020), and it is critically regulated by NGF-TrkA signaling (Begni, Riva, & Cattaneo, 2016). Importantly, the alteration of MAPK/ERK pathway has been implicated in AD progression (Angulo et al., 2006; Dineley et al., 2001; Ferrer et al., 2006), where it has been shown that the ERK phosphorylation (p42/44 -MAPK on Thr202/Tyr204) is increased upon oligomeric Aβ(1-42) treatment (E. K. Kim & Choi, 2010; Kirouac, Rajic, Cribbs, & Padmanabhan, 2017; Young, Pasternak, & Rylett, 2009). It is also known that NGF treatment leads to increased ERK phosphorylation by activating its upstream kinases (L. Jiang, Ye, Wang, Yu, & Xu, 2019; Xing, Kornhauser, Xia, Thiele, & Greenberg, 1998); however it is unknown whether the oligomeric Aβ(1-42)-mediated ERK phosphorylation increase can be modulated by the enhanced NGF-TrkA signaling. Considering the importance of this relationship, we aimed to evaluate changes in MAPK/ERK pathway as a potential link to synaptic density. To do so, we utilized Western Botting with well-characterized antibodies to measure ERK phosphorylation in both WT and TrkA-KFG BFCNs with and without Aβ(1-42) treatments, and in the presence and absence of NGF. Observing the total ERK levels compared to phospho-ERK levels provides an estimation of relative ERK activity under different treatment conditions. A higher level of ERK phosphorylation was detected in TrkA-KFG BFCNs as compared to WT neurons in general, potentially due to the presence of more sensitive TrkA receptors. Consistent with the literature (Kirouac et al., 2017; Palavicini et al., 2017; Y. Wang et al., 2020; Young et al., 2009), our results demonstrated an increase in ERK

phosphorylation when the neurons were treated with oligomeric Aβ(1-42). Upon 50 ng/ml NGF treatment, the increased ERK activity was observed in both WT and TrkA-KFG BFCNs. However, interestingly, when NGF and Aβ(1-42) treatments were provided together, phospho-ERK level was less than the NGF treatment only control. To the extent of our knowledge, this effect is unfocused study, and warrants further investigation as it is currently not clear how NGF and Aβ(1-42) treatments combinatorially affect phospho-ERK levels and how such combined effect has physiological roles. However, it is clear from our data that there is upregulated ERK signaling in TrkA-KFG BFCNs, which may potentially positively affect the synaptic density. In addition to the MAPK/ERK pathway, the levels of total Trk receptors were compared in this condition and as expected, the TrkA-KFG BFCNs have higher levels of Trk receptors (**Figure 3.9,** panel B), similar to our previous characterization experiments. This work provides us guidance for further studies to elucidate how MAPK/ERK pathway might be important in AD in the context of NGF-TrkA signaling and synaptic density. For example, it would be important to focus on the literature-suggested Ras-mediated activation of ERK signaling through NGF-TrkA signaling to light the blank connections (Krapivinsky et al., 2003; Peng, Zhang, Zhang, Wang, & Ren, 2010). For the Western Blot analyses presented in the **Figure 3.9,** experiments were performed in at least 3 different biological replicates under each condition at different times, and at least 2 technical replicates were run for each biological replicate. β-Actin was used as a loading control in all blots.
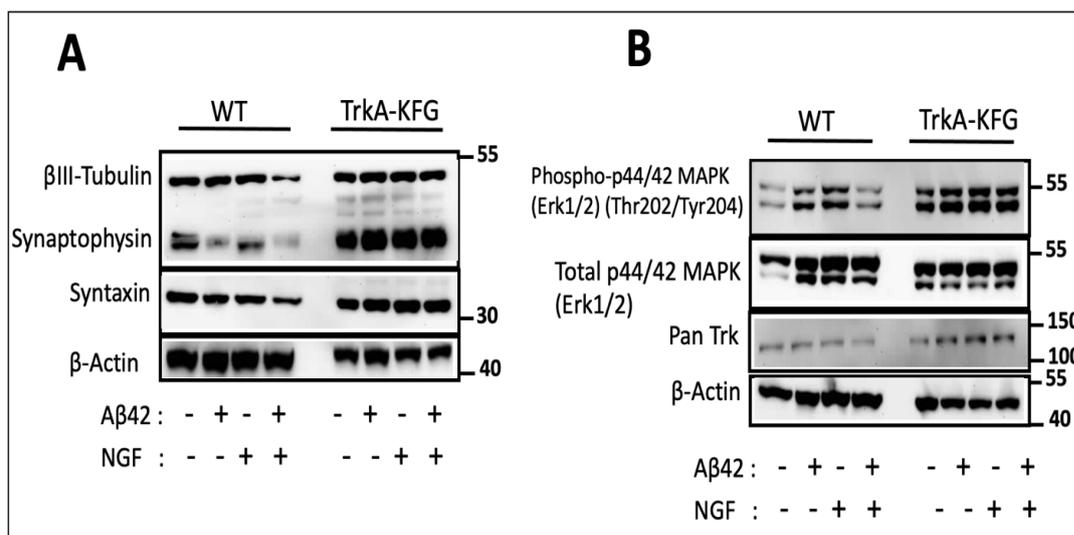
Figure 3.9 Investigation of oligomeric Aβ(1-42)-mediated neuronal changes in WT and TrkA-KFG BFCNs

(A) Analysis of synaptic density changes upon enhanced TrkA signaling. Neuronal marker, β-III Tubulin, and a synaptic marker Synaptophysin were applied to the same blot. Also, the levels of another synaptic protein, Syntaxin, was observed. (B) Investigation of NGF-TrkA-mediated cell survival signaling pathway and the level of Trk receptors. β-Actin is used as a loading control. The presented Western Blot images represent the data from at least 3 different biological replicates performed at different times.

3.4.1.2   Immunocytochemistry Analysis

As mentioned above, oligomeric Aβ(1-42) leads to neuronal degeneration and decreased synaptic density. In such conditions, morphological changes can be detected, as demonstrated by many groups in the literature. It has been shown that the detection of general morphological changes in a population of neurons provides meaningful information, especially when specific synaptic markers are utilized (Li & Gotz, 2017; Soto-Mercado, Mendivil-Perez, Velez-Pardo, & Jimenez-Del-Rio, 2021). The neuronal density can be determined by immunofluorescence staining of

β-III Tubulin, which is a marker extensively used to analyze structural defects and neurite fragmentation in both axons and dendrites (Nogueras-Ortiz et al., 2020; Sadleir et al., 2016). To asses the changes in synaptic density, we utilized specific neuronal and synaptic markers β-III Tubulin, Tau5 and VAMP2. Additionally, we used NeuN as a marker to measure neuronal loss upon oligomeric Aβ(1-42) treatments. The experimental conditions and analysis are provided in ***Chapter 2.5.5***. The representative images and bar graphs of the analyses are presented in ***Figure 3.10***.

Figure 3.10 The immunocytochemistry comparison of oligomeric Aβ(1-42)-mediated synaptic density change in TrkA-KFG and WT BFCNs with controls

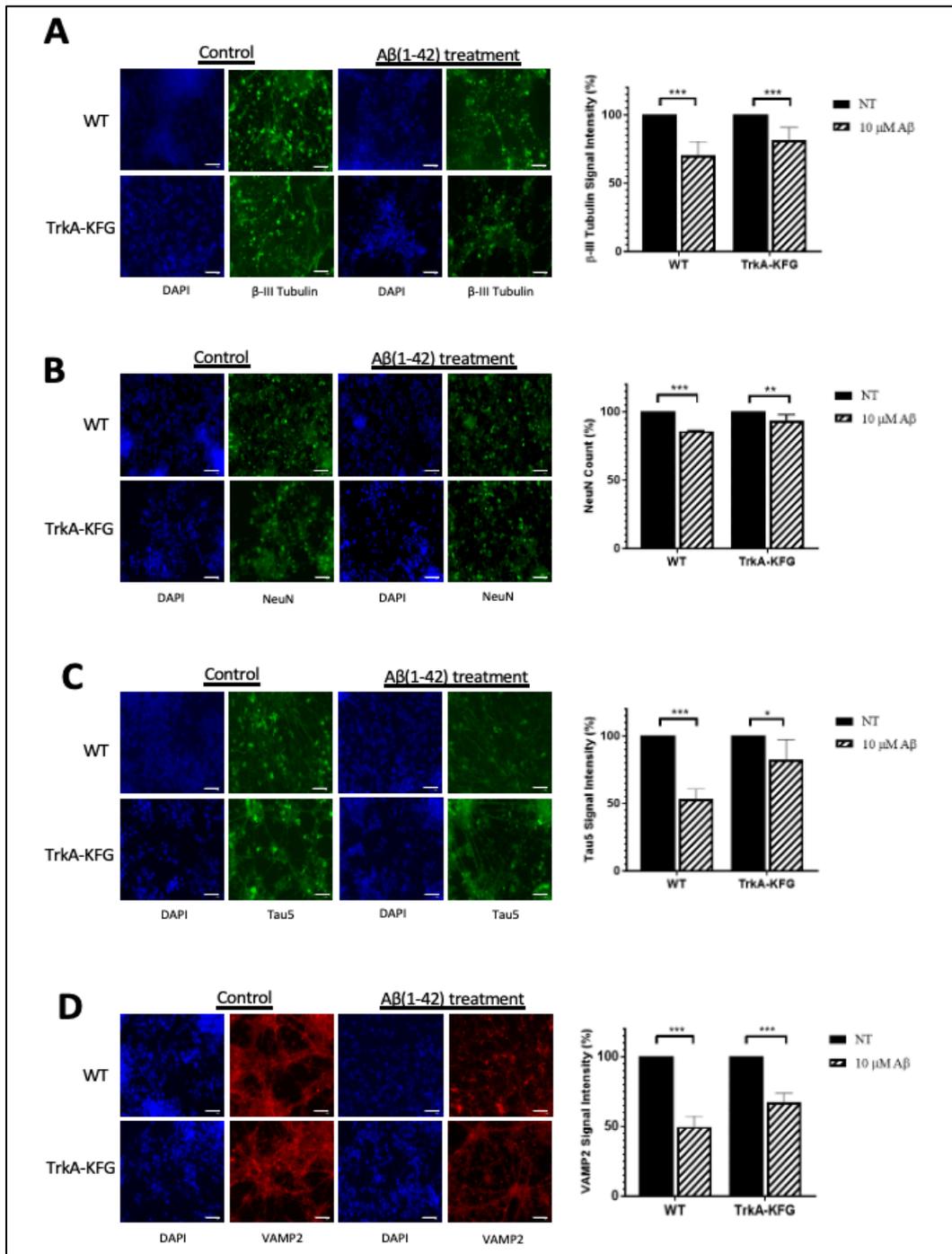*To see the difference in synaptic density, the neuron markers (A) β-III Tubulin and (C) Tau5, and (D) the synaptic marker VAMP2 were examined, (B) the neuronal*

marker NeuN was observed to asses the neuron number. DAPI was used as nuclear counterstain. Analyzes were performed using representative images obtained from 6 different biological replicates performed at different times.

Scale bar, 50 μm. Results are given as mean ± SD, and statistical analyzes were performed with Student's t-test using GraphPad Prism. ***, **, * indicate that the values are at 99.9%, 99% and 95% confidence levels, respectively.

We first compared the neuronal marker β-III Tubulin levels upon Aβ(1-42) treatments in WT and TrkA-KFg BFCNs under the same parameters. Our data demonstrated that the oligomeric Aβ(1-42) treatment caused a statistically significant decrease in β-III Tubulin level in WT BFCN neurons compared to the control sample, which is also the case for the TrkA-KFG neurons. However, while this decrease was approximately 31% in WT BFCN neurons, it remained at approximately 19% in TrkA-KFG BFCN neurons. The statistically significant change in another neuronal density marker, Tau5, is evident in both WT BFCNs (a decrease of 47% compared to controls) and TrkA-KFG neurons (a decrease of 18% compared to controls), where the drop is more distinguishable and more significant in WT BFCNs. Consistent with the literature, oligomeric Aβ(1-42) treatment caused a statistically significant reduction of VAMP2 signal in WT BFCN neurons (about 50% decrease) compared to control; in contrast, the decrease is 33% in TrkA-BFCN neurons compared to control. These results suggested that the TrkA-KFG BFCNs are less prone to oligomeric Aβ(1-42)-mediated synaptic density changes. In addition, to observe the changes in neuron population upon oligomeric Aβ(1-42) treatment, the changes in the level of NeuN were investigated. Although there was a statistically significant decrease in both neuron types, the reduction in WT BFCNs is 14% compared to the control, while the amount of NeuN decline remained at 7% in TrkA-KFG BFCN neurons, which suggests that the KFG modulation is also important for neuronal integrity and number. In summary, TrkA-KFG BFCN neurons were evaluated to be more resistant to neuronal and synaptic marker level changes caused by oligomeric Aβ(1-42) treatment compared

to WT neurons, suggesting enhanced NGF-TrkA-mediated protection of synaptic integrity and density.

3.5 Analysis of neurotrophin signaling and Alzheimer's Disease-related signaling cascades with *in silico* models

In order to evaluate the role(s) of neurotrophin signaling in AD and assess the effect of upregulated TrkA signaling in the concept of AD, we analyzed the signaling pathways associated with neurotrophin signaling and oligomeric Aβ(1-42) using *in silico* methodologies. The related pathways were retrieved from Kyoto Encyclopedia of Genes and Genomes (KEGG), which is a well-establish database for cellular functions, as described in ***Chapter 2.6***. It is shown that the common pathways modulated by both oligomeric Aβ(1-42) and TrkA-NGF are MAPK [hsa04010] signaling, ubiquitin-mediated proteolysis [hsa04120], apoptosis [hsa04210], long-term potentiation [hsa04720], regulation of the actin cytoskeleton [hsa04810], and PI3K/Akt signaling pathway [hsa0g151]. This analysis and the finding from the literature strongly suggest MAPK/ERK signaling as a mediator of synaptic regulation. This analysis will be useful for future studies as other potential pathways such as PI3K/Akt and GSK3β are implicated in synaptic integrity, which should be experimentally evaluated (***see Figure 3.11.2***).
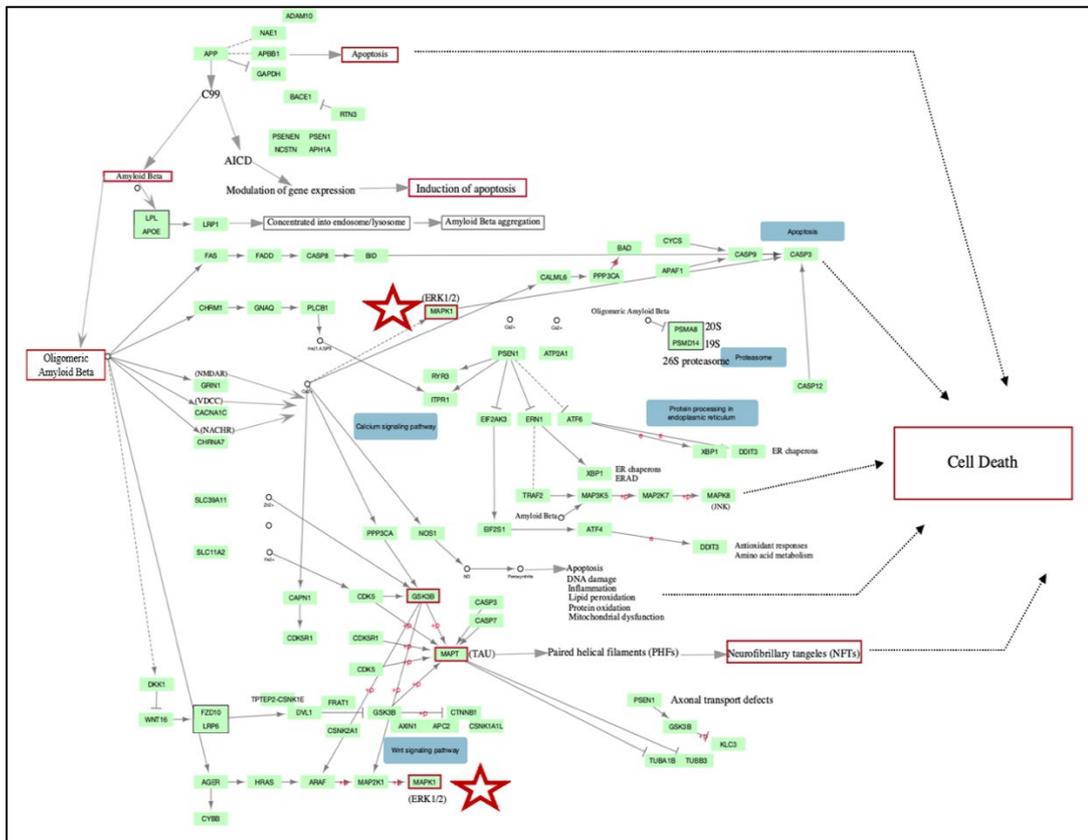
Figure 3.111.1 The KEGG analysis for oligomeric Aβ(1-42)-mediated signaling cascades

The oligomeric Aβ(1-42)- mediated pathways were retrieved from the KEGG

database in order to observe the common pathways seen in neurotrophin signaling.
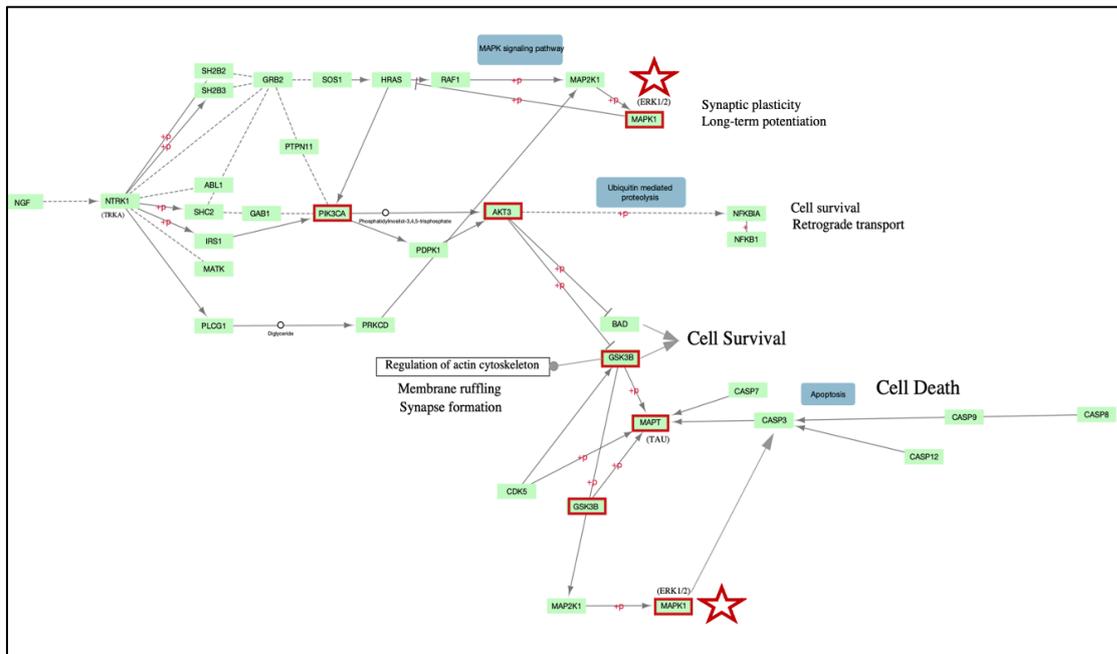
Figure 3.112.2 The KEGG analysis for neurotrophin signaling, where the NGF-TrkA signaling is retrieved

The NGF-TrkA signaling- related pathways were retrieved from the KEGG database in order to observe the common pathways seen in oligomeric Aβ(1-42)-mediated cascades in order to asses the affect of KFG removal in molecular perspective.

CHAPTER 4

DISCUSSION

Alzheimer's Disease (AD) is the most common cause of dementia, and the worldwide number of patients is expected to be doubled by 2050 (Sloane et al., 2002). Despite considerable investments in drug and clinical research, there is still no cure for AD (Deuschl et al., 2020), and the treatment aspects only depend on decreasing the symptoms of the disease. A large body of literature suggests that there is a correlation between AD progression and the decreases in levels of neurotrophins and their receptors in addition to reduction and functional abnormalities in the neurotrophin signaling (Mitre et al., 2017). Neurotrophin signaling plays a critical role in synaptic plasticity, cell survival, and neuronal differentiation, making the pathways one AD therapeutic target. However, the vast majority of the studies focusing on neurotrophin signaling enhancement against the neurodegenerative process of AD mainly aim to either increase the neurotrophin levels or develop small molecules acting as neurotrophin receptor agonists (Josephy-Hernandez, Jmaeff, Pirvulescu, Aboulkassim, & Saragovi, 2017; Kazim & Iqbal, 2016). Past experience demonstrated that both approaches are quite difficult as small molecules may have significant unwanted side effects, and peptide-based approaches suffer from availability and distribution issues, partly due to low blood-brain barrier penetration capacities (Sun, Wurzelmann, & Romeika, 2017). Therefore, novel approaches are needed. A previous study from our group demonstrated that the KFG domain is critical for ubiquitination-mediated degradation of TrkA receptor, which can regulate its levels and function (Kiris et al., 2014). Another group also validated the importance of the KFG domain by using mass spectrophotometry-based analyses (Emdal et al., 2015). As a follow-up study, this thesis research focused on the effect of enhanced neurotrophin signaling against *in vitro* AD using the KFG model system for the first time in literature. The

main question of this research was whether creating more sensitive and active TrkA receptors by modifying the conserved KFG domain could make neurons more resistant to AD neurodegenerative processes.

Towards this goal, the directed differentiation of mouse embryonic stem cells (mESCs) into basal forebrain cholinergic neurons (BFCNs), which are specific neuronal types mostly affected by AD, was used as the model system. To examine whether TrkA signaling enhancement have any effect on *in vitro* AD-related neurodegeneration, we utilized WT and TrkA-KFG mESCs to generate BFCNs and modeled AD using *in vitro* Aβ(1-42) treatments. Our approach is relevant because Aβ(1-42) treatments are well-established and characterized *in vitro* systems to model AD (Benseny-Cases, Klementieva, & Cladera, 2012; W. B. Stine, K. N. Dahlgren, G. A. Krafft, & M. J. LaDu, 2003; Tiiman, Krishtal, Palumaa, & Tõugu, 2015; Z.-Y. Wu et al., 2014). Additionally, BFCNs are relevant to AD research as these are one of the highly affected cell types in the disease. Importantly, these cells express high levels of TrkA and are therefore relevant to our research questions. In this thesis study, the differences in Trk level, cell viability and cytotoxicity, amyloid-beta (1-42)-mediated synaptic density changes, and the abundant cell survival signaling pathway were examined to investigate the neuronal effects of KFG-based TrkA signaling enhancement in BFCNs. The results showed the *in vitro* effect of KFG-removal on neurotrophin signaling enhancement, and TrkA receptor activation in BFCNs in the concept of AD. In addition to this finding, it is demonstrated that there is neuroprotection in BFCNs against *in vitro* Aβ(1-42)-related neurotoxicity and neuronal processes when TrkA signaling is enhanced. These two findings are valid with the literature and promise a neuroprotective therapeutic approach toward the decline in levels of neurotrophins and their receptors in AD (Counts et al., 2004; Josephy-Hernandez et al., 2017).

Specifically, it has been known for many years that oligomeric Aβ(1-42) decreases the cell viability and leads to the cell death by increasing the neurotoxicity (Yankner, 1996). Also, the NGF-TrkA signaling pathway is known to be critically

important for neuronal survival, neurogenesis, and neuroplasticity (Ahmad, Rizvi, Fatima, & Mondal, 2021; Levy et al., 2018), which is supported by our MTT cell viability and LDH cytotoxicity results showing that there is a critical resistance in upregulated TrkA having BFCNs against both oligomeric and fibrillar Aβ(1-42)-induced neuronal death even there is no statistically significant difference in fibrillar Aβ(1-42).

The role of synaptic functioning in memory maintenance by long-term potentiation (LTP) has been suggested in the literature for more than a decade (Martin, Grimwood, & Morris, 2000; Neves, Cooke, & Bliss, 2008; Rioult-Pedotti, Friedman, & Donoghue, 2000; T. J. Ryan, Roy, Pignatelli, Arons, & Tonegawa, 2015), which makes synaptic protection a critical approach against memory deficiency diseases including AD. Also, many studies show that the modulation of NGF-Trk signaling can fight against AD-mediated neurodegeneration and decrease synaptic dysfunction and learning/memory deficiencies, especially in BFCNs (Braun, Kalinin, & Feinstein, 2017; Hartikka & Hefti, 1988a, 1988b; Josephy-Hernandez et al., 2017; Nagahara et al., 2009; Tuszynski et al., 2015). In our KFG model system, the investigation of synaptic marker levels by Western Blot and immunocytochemistry methodologies suggested synaptic density decline upon Aβ(1-42)-mediated toxicity, where this defect is seen less in TrkA-KFG neurons as compared to WT BFCNs. This result is promising that enhanced TrkA signaling protects against AD-related degeneration. In addition, observing the changes in one of the neuronal marker (NeuN) by immunocytochemistry suggested that the KFG removal also protect the neuronal integrity and number from AD-related neurotoxicity.

The literature demonstrates that the neurodegenerative process of oligomeric Aβ(1-42) affects MAPK/ERK and PI3K signaling pathways (M.-S. Kim et al., 2014). Despite of being known for regulating the cell survival and proliferation cascades, the inhibition experiments of MAPK/ERK signaling show that this pathway is also critical for regulation of both synapses and long-term potentiation (Davis,

Vanhoutte, Pagès, Caboche, & Laroche, 2000; Silingardi et al., 2011). In addition to the importance of PI3K in the synaptic plasticity, it is known that one of the members of this signaling pathway named Akt is also critical for many processes such as memory, learning and synaptic plasticity (Datta et al., 1997). Also, it is known that NGF-TrkA signaling activates the MAPK/ERK and PI3K signaling cascades both locally and via signaling endosomes, and this activation is important for cell growth, differentiation, neuronal survival, and regulation of apoptosis, respectively (Howe, Valletta, Rusnak, & Mobley, 2001; Louis F. Reichardt & Mobley, 2004; Segal & Greenberg, 1996). Therefore, it would be suggested that enhanced NGF-TrkA signaling acts against Aβ(1-42)-induced degeneration through these two main signaling pathways. To demostrate this relationship, this thesis project focused on the TrkA-mediated activation of ERK signaling pathway, which is a highly conserved and abundant signaling cascade within the species (Guo et al., 2020). In the light of these knowledge, our results suggest that there is an upregulation of ERK signaling upon TrkA enchancement, which possible protects the synaptic density from Aβ(1-42)-induced toxicity. Also, our results concluded an unfocused finding showing that there is a decrease in phospho-ERK levels upon dual stimulation of Aβ(1-42) and NGF, which needs a further investigation. In addition to ERK signaling, according to our *in silico* analyzes, focusing on PI3K/Akt pathway to asses the whole connection would be important because the duration of ERK signaling activation is important for its leading cellular response, which is regulated by different signaling cascades (including PI3K/Akt pathway) and feedback loops (Miningou & Blackwell, 2020). Also, as mentioned above, the PI3K/Akt pathway is important in synaptic functioning and cell survival. Therefore, our ongoing experiments including PI3K/Akt pathway regulation will conclude whole image of molecular mechanisim of TrkA upregulation-mediated synaptic protection.
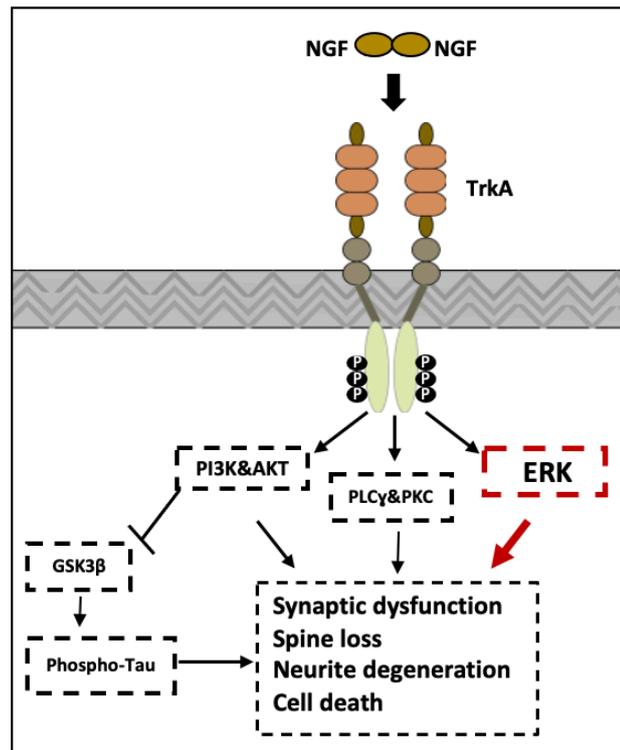
Figure 4.1 The schematic representation of the possible relationship between TrkA-mediated signaling and synaptic integrity. *Adapted from Simmons, Yang, Massa, and Longo (2016).*

Besides, our *in silico* analyses suggest that MAPK/ERK pathway can directly interact with the Glycogen synthase kinase-3 beta (GSK3β) pathway in addition to PI3K/Akt pathway. This signaling pathway is inhibited by PI3K/Akt pathway, which regulates neuronal survival and synaptic plasticity (Grimes & Jope, 2001). The Aβ-mediated inhibitory phosphorylation of GSK3β pathway induces tau phosphorylation negatively affecting the synaptic integrity. GSK3β pathway is also regulated by neurotrophin signaling, which together suggests that there could also be PI3K/Akt-regulated GSK3β-mediated neuroprotection (Beaulieu, Gainetdinov, & Caron, 2009). Therefore, our future studies will also focus on the relationship of GSK3β signaling in ERK-mediated synaptic protection, and whether the TrkA upregulation has an effect on tau hyperphosphorylation.

Rather than our findings, another critical issue to be discussed is how removing the KFG region protects against the oligomeric Aβ(1-42) toxicity by providing NGF-

TrkA signaling pathway upregulation. This could be associated with monoubiquitylation of the TrkA receptor after binding NGF, where this monoubiquitylation is prevented in our KFG model system by removing its target site, which leads long-term presence of the TrkA receptor on the neuron membrane (Kiris et al., 2014). This long-lasting placement of the enhanced receptor would promise an enhanced neurotrophin signaling upon transactivation of them, even absence of NGF. This may be an important approach in future studies developing novel methodologies against AD because this method has a potential to remove the obstacles coming from the existing neurotrophin-mimetic approaches. In addition to its therapeutical novelity, the finding of this study could be a source for regenerative cell therapies that use induced pluripotent stem cell-derived (iPSCs) neurons and are seen as a glimmer of hope for many neurodegenerative diseases, including AD (Kwak, Lee, Yang, & Park, 2018). These studies primarily focus on the transplantation of new neurons to replace the degenerated ones or on providing neuroprotection by the paracrine effect (Bali, Lahiri, Banik, Nehru, & Anand, 2017). To do so, embryonic stem cells (ESCs), iPSCs and adult stem cells are considered as important cell sources, where the importance of neurotrophins is also shown in the transplantation studies originating from these cells (Barker, Götz, & Parmar, 2018). Despite the positive signs of regenerative cell transplantation studies in experimental animals against AD, some recent studies have been unsuccessful. For example, the study published in 2015 showed the positive effects of stem cell therapies on AD for a month (Ager et al., 2015); however, other group showed that there is no improvement observed in synaptic density and cognitive functions, and the occurrence of some harmful structure which could affect the brain is detected in the long-term observation (Marsh et al., 2018). This is strongly showing that it is vital to understand and characterize the molecular mechanisms before switching into human clinical studies (Temple & Studer, 2017). In the light of this thesis project, possible preclinical studies can be considered by generating BFCNs from patients' own iPSCs after editing them to have TrkA-KFG a with CRISPR/Cas9 genome engineering. To use this technique in clinics, it is essential

to generate precise genome editing where there should not be any non-specific editing rather than in the KFG domain (Kosicki, Tomberg, & Bradley, 2018). In further studies, it will be able to generate CRISPR/Cas9-edited specific neurons with more sensitive Trk receptors (Trk-KFG) from AD patients own cells via iPSCs methodology. The generated cells would be also evaluated for patient-to-patient cell transplantation, which will promise an ultimate neurotrophin-based threapy against AD.

To sum up, there has been little progress for neurotrophin signaling-based therapeutic approaches in AD due to various issues, including the fact that regulation of neurotrophin signaling mechanisms is not well-understood. Therefore, innovative approaches could be developed to control neurotrophin signaling in neurons, which may open new avenues for neuroprotection in AD and other neurodegenerative conditions. As a step of this mission, this thesis project demonstrates for the first time in the literature that the removal of the domain of TrkA receptor could make BFCNs more resistant to *in vitro* Aβ(1-42)-mediated neurotoxicity in terms of neuronal survival and synaptic density.

CHAPTER 5

CONCLUSIONS AND FUTURE STUDIES

This study evaluated the effects of enhanced TrkA signaling in BFCNs against the *in vitro* neurodegenerative process of AD, using a model previously established by our group. The model system involved the removal a conserved domain, KFG, in TrkA, as the domain negatively regulates TrkA level and signaling. Although enhancement of TrkA signaling against AD has been studied using various approaches, to the best of our knowledge, this is the first study evaluating the enhancement of the signaling pathway against AD by manipulating specific domains of the TrkA receptor. The significant findings of this study are listed below.

1. TrkA-KFG BFCNs are resistant to *in vitro* Aβ(1-42)-mediated neurotoxicity compared to WT BFCNs.

2. Aβ(1-42)-mediated effects on neuronal synaptic density is less in TrkA-KFG BFCNs as compared to that of WT BFCNs.

3. Our study suggests that the protection of synaptic density upon TrkA upregulation may be mediated through MAPK/ERK pathway.

4. Our *in silico* analysis suggests that there could be other players in TrkA-mediated synaptic protection, in conjunction with the MAPK/ERK pathway, which should be the focus of further studies.

5. Importantly, this study suggests that making TrkA receptor more sensitive and active in neurons may have a therapeutic role against AD, which should be the focus of further studies.

As mentioned above, there are many unanswered questions, and future work can potentially expand significantly on our findings. For example, uncovering precise molecular mechanisms involved in TrkA mediated phospho-ERK regulation, and

neuronal synaptic dynamics would be fundamental. However, importantly, this study provides significant support to an attractive approach focusing on human induced pluripotent stem cell-based neuronal transplantation against AD. The possibility of making the patient's own neurons more resistant against Aβ may have therapeutic value. The results of this thesis research are promising that the KFG model has a possibility of paving the way for preclinical studies for personalized treatment. This can be achieved potentially by generating BFCNs derived from human induced pluripotent stem cells carrying genome-edited TrkA receptors.

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APPENDICES

A. Cell Culture Materials & Reagents

0.1% Gelatin (Serva, 22151.02): 0.5 g of gelatin is dissolved in 500 ml ddH$_2$O. The mixture is microwaved to dissolve gelatin completely. After filtering the solution through a 0.22 μm vacuum filter, it was autoclaved. The solution is kept at room temperature.

0.1 mg/ml Poly-D-Lysine Solution (Sigma, P0899): 100 mg stock is dissolved in 100 ml dH$_2$O to get a 1 mg/ml stock solution. The stock is aliquoted as 1 ml per aliquot and kept at -20°C. Prior to the coating procedure, the stock is 1:10 diluted with dH$_2$O to get a 0.1 mg/ml working concentration.

Laminin (Thermo Fisher, Cat.No: 23017015): To make the working concentration 2.5 μg/ μl, 1.16 mg/ml laminin stock solution was diluted with 6.5 ml DPBS prior to coating.

20 mg/ml Mitomycin-C (Cayman, Cat.No: 11435): 50 mg powder is dissolved in 2.5 ml DMSO to get 20 mg/ml stock solution. The 100 μl aliquots were kept at -20°C. For each treatment (per 15 plates), one aliquot was added into 3.9 ml PBS with Ca^{+2} and Mg^{+2}; then, this solution was added to 206 ml MEF medium to make the final concentration 0.01 mg/ml.

Retinoic Acid (Sigma, #2625): 50 mg powder was dissolved in 16.6 ml DMSO (Serva, Lot No. 171097) to get 10 mM stock concentration. The aliquots were kept at -20°C.

Hh-Ag 1.5 (Cellagen Technology, Cat.No: C4412-2s): The reagent was purchased as 10mM in DMSO. Aliquots were made and kept at -20°C.

BMP-9 Synthetic Peptide (GenScript, Sample ID:U163XFB230-1): The 98.7% pure synthetic peptide in 4.2 mg was dissolved in 17.8 ml ultrapure water

(Biological Industries, Ref. No. 01-866-1A) to get 10 nM stock. Aliquots were kept at -20°C.

Accutase (Biolegend, Cat.No: 423201): Ready to use reagent was aliquoted as 1ml in each tube and kept at -20°C.

10 mg/ml Dispase II (Gibco, Cat.No: 17105041): The powder was dissolved in PBS without Mg^{+2} and Ca^{+2} to get a 10 mg/ml stock solution.

1X Phosphate- Buffered Saline with Ca^{+2} and Mg^{+2} (PBS (+,+)):

The 1 liter of 20X A and B Solutions were prepared with dH_2O by following the recipe given below. To get 1X PBS (+,+), 50 milliliters of both Solution A and B were poured into 800 ml of deionized water. After making the pH at 7.4 with HCl, the solution is completed to 1 liter with deionized water.

Solution A (20X)   Solution B (20X)

NaH_2PO_4 : 28.8 g   NaCl   :   160 g

K_2HPO_4 : 4.8 g   KCl   :   4 g

                              $CaCl_2.2H_2O$: 2.66 g

                              $MgCl_2.6H_2O$: 2 g

1X Phosphate- Buffered Saline without Ca^{+2} and Mg^{+2} (DPBS):

Ingredient	Volume
NaH_2PO_4	28.8 g
K_2HPO_4	4.8 g
NaCl	160 g
KCl	4 g

NP-40 Lysis Buffer:

Ingredient	Volume
5M NaCl	30 ml
10% NP-40	100 ml
1 M Tris pH: 8.0	50 ml
dH$_2$O	820 ml

All reagents were combined to get 1 liter lysis buffer. The prepared solution was kept at -20°C.

Amyloid Beta (Aβ(1-42)) (AnaSpec, Cat.No: AS-64129-1 / PeptiTeam): To get oligomeric Aβ(1-42), the 1mg film was reconstituted with 44 µl DMSO. After vortexing 30 seconds, 2.2 ml DBPS was added to get 100 mM stock solution. For oligomerization, the prepared solution is incubated at +4°C for 24-hours. To get fibrillar Aβ(1-42), 5 mM DMSO-reconstituted peptide film was diluted with 10 mM HCl to get a 100 µM stock concentration, and the reconstituted peptide was incubated at 37 ºC for 24 hours to initiate fibrillization.

Thioflavin-T (ThT) (Sigma, Cat.No: T3516): The 5 g of Thioflavin-T powder is reconstituted with DPBS to make the stock concentration 10 mM, and filtered through 0.22 µm syringe filter. The further working dilutions are made with DPBS just before the use.

MTT Solution (Sigma, Cat.No: M2003): 5 mg MTT powder was dissolved in 10 ml DPBS. The solution was kept at +4°C for a month by protecting from light.

MTT Solubilization Reagent: 1 g Sodium Dodecyl Sulfate (SDS) was dissolved in 10 ml 0.01 M HCl solution.

LDH (Thermo Fisher, Cat.No: C20300): The kit contains five different reagents/supplements, which are one vial of lyophilizated Substrate Mix, 600 µl

Assay Buffer, 2.5 ml Lysis Buffer, 12 ml Stop Solution, 6 μl LDH Positive Control. All components are kept at -20°C, and chill at RT prior to use. Reaction mixture is prepared by combining the 600 μl Assay Buffer Stock Solution with dissolved Substrate Mixture in 11.4 ml dH$_2$O, and kept at -20°C for further use. The preparation of an 1X LDH Positive Control is achieved by diluting the 1.5 μl of LDH Positive Control with 1 ml of 1% BSA in PBS solution, and the prepared solution is kept at -20°C.

B. Cell Culture Media Compositions

MEF Medium and HEK Medium: It contains 88% DMEM (Gibco, 1195092), 10% Fetal Bovine Serum (FBS) (Gibco, 10270106), 1% Penicillin – Streptomycin (PenStrep) (Gibco, 15140122), 1% Glutamax (Gibco, 35050061)

V6.4 mESC Medium: It composed of 82% DMEM (Gibco, 1195092), 15% Fetal Bovine Serum (FBS) (Gibco, 10270106), 1% Penicillin – Streptomycin (PenStrep) (Gibco, 15140122), 1% Glutamax (Gibco, 35050061), 1% Non-essential amino acids (Gibco, 1140050), 0.1 mM β-mercaptoethanol (VWR, Cat. No: M131), 1000 Unit LIF (Sigma, Cat. No: L5283).

ADFNK Medium: This medium contains 1:1 Advanced DMEM/F12 medium (Gibco, 12634010) and Neuro Medium (Miltenyi Biotech, Cat. No: 130-093-570), 10% KnockOut Serum Replacement (KSR) (Gibco, 10828028), 1% PenStrep (Gibco, 15140122), 1% Glutamax (Gibco, 35050061), 0.1 mM β-mercaptoethanol (VWR, Cat. No: M131).

Neuronal Medium-1: Prepared by mixing 96% Advanced DMEM/F12 medium (Gibco, 12634010) with 1% PenStrep (Gibco, 15140122), 1% Glutamax (Gibco, 35050061) and 2% B-27 supplement (Gibco, Ref.No: 12587-010).

Neuronal Medium-2: This medium is composed of 97.5% Neuro Medium (Miltenyi Biotech, Cat. No: 130-093-570), 1% PenStrep (Gibco, 15140122), 0.5% Glutamax (Gibco, 35050061) and 1% B-27 supplement (Gibco, Ref.No: 12587-010)

Freezing Medium: The medium contains 90% of FBS (Gibco, 10270106), and 10% DMSO (Serva, 39757.01).

C. Western Blot Solutions

6X Sample Buffer Recipe:

Ingredient	Volume
12% SDS	1.2 g
60% Glycerol	6 ml
0.375 M Tris-HCl pH: 6.8	3.75 ml
0.012% Bromophenol Blue	0.0012 g
30% β-mercaptoethanol	3 ml

10% Ammonium Persulfate (APS): 1 g Ammonium Persulfate was dissolved in 10 ml dH_2O. Aliquots were kept at -20°C.

10% SDS-PAGE Separating Gel Recipe for 1 gel:

Ingredient	Volume
dH$_2$O	2.88 ml
40% Acrylamide:Bisacrylamide	1.5 ml
1.5M Tris-HCl pH: 8.8	1.5 ml
10% SDS	60 μl
10% APS	60 μl
TEMED	2.4 μl

5% SDS-PAGE Stacking Gel Recipe for 1 gel:

Ingredient	Volume
dH$_2$O	1.815 ml
40% Acrylamide:Bisacrylamide	375 μl
0.5M Tris-HCl pH: 6.8	750 μl
10% SDS	30 μl
10% APS	30 μl
TEMED	3 μl

10X Running Buffer:

Ingredient	Volume
Tris Base	30 g
Glycine	144 g
SDS	10 g

All reagents listed above were added into 900 ml dH$_2$O. After the solution became homogenate, the final volume was adjusted as 1 liter without requiring any pH adjustment. Before use, the solution is diluted to 1X with dH$_2$O.

10X Wet Transfer Buffer:

Ingredient	Volume
Tris Base	30.3 g
Glycine	144.1 g

The reagents were added to 800 ml dH_2O in portions. The final volume was adjusted to 1 liter after all reagents were dissolved in dH_2O.

To get a 1X Transfer Buffer; 700 ml dH_2O and 200 ml methanol were added to 100 ml of 10X Transfer Buffer, orderly.

10X TBS:

Ingredient	Volume
Tris Base	24 g
NaCl	88 g

All reagents listed above were dissolved in 800 ml dH_2O. After adjusting the pH to 7.6, the volume was brought up to 1 liter with dH_2O.

To get 1X TBS-T (TBS with Tween20): The 100 ml 10X TBS was diluted with 900 ml dH_2O. After that 1 ml, Tween20 (Sigma, Cat. No: P1379) was added to the solution.

Mild-Stripping Buffer:

Ingredient	Volume
Glycine	24 g
SDS	1 g
Tween20	10 ml

adjusting the pH to 2.2 with HCl, the volume was brought up to 1 liter.

Blocking Solutions:

All reagents except Tween-20 were added to 800 ml of deionized water. After

For milk solution, 2.5 g skimmed milk powder was dissolved in 50 ml 1X TBS-T by vortexing.

For BSA solution, 2.5 g BSA powder was dissolved in 50 ml 1X TBS-T by slowly shaking.

D. Immunocytochemistry Solutions

Fixation Solution: The fixation solution is 4% paraformaldehyde which is diluted 1:10 from 37% stock paraformaldehyde solution (Sigma, Cat.No: 15512).

Permeabilization Solution: This solution is 0.1% Triton X-100 which was prepared by diluting the 100X Triton X-100 in 1:1000 by using 1X PBS (+,+).

Blocking Solution: 2% BSA in 1X PBS was used as blocking solution where 1 g of BSA powder was dissolved in 50 ml of PBS (+,+) by gently shaking.

Immunostaining Solution: All primary and secondary antibodies were diluted according to manufacturer's recommendation in 0.1% BSA solution which was prepared adding 0.05 g BSA powder in 50 ml PBS (+,+).

Mounting Reagent: ProLong Gold antifade reagent with DAPI (Invitrogen, Cat.No: P36931) mounting reagent is used to preserve the chamber slides with coverslip.

E. Protein Ladders
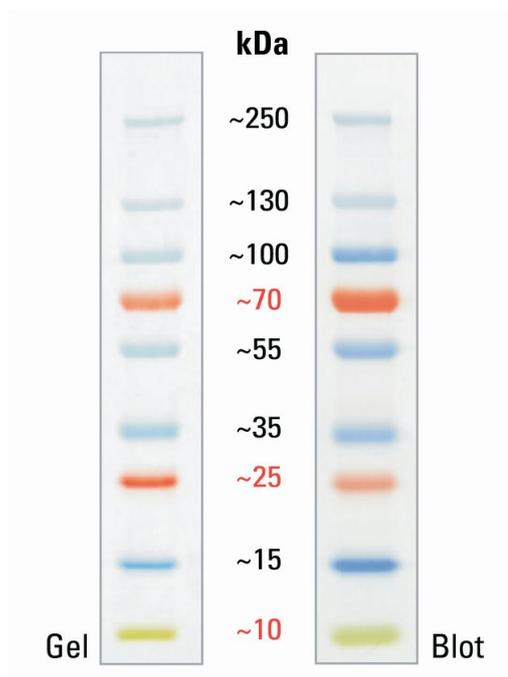
Thermo Scientific™ PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa
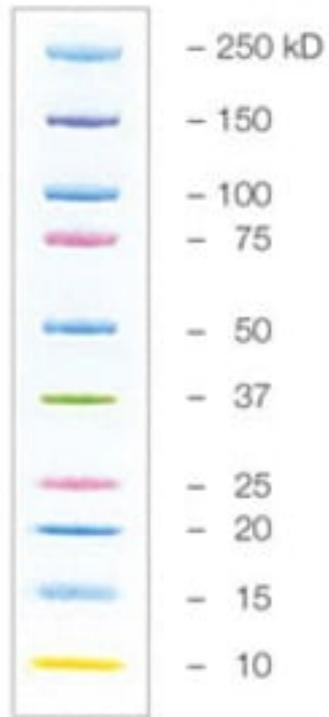
Bio-Rad, Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standard