EFFECTS OF ENHANCED TRKB SIGNALING IN MOUSE EMBRYONIC STEM CELL-DERIVED CORTICAL NEURONS AGAINST AMYLOID BETA 42-MEDIATED CELLULAR EVENTS

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EFFECTS OF ENHANCED TRKB SIGNALING IN MOUSE EMBRYONIC STEM CELL DERIVED-CORTICAL NEURONS AGAINST AMYLOID BETA 42-DERIVED CELLULAR EVENTS

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ABSTRACT

EFFECTS OF ENHANCED TRKB SIGNALING IN MOUSE EMBRYONIC STEM CELL-DERIVED CORTICAL NEURONS AGAINST AMYLOID BETA 42-MEDIATED CELLULAR EVENTS

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Alzheimer's Disease (AD) is the sixth most common cause of death globally. The disease causes memory loss and cognitive decline, eventually preventing the individual from performing simple actions in daily life. 2014-2018 Report of The Turkish Association of Alzheimer's indicates that there are at least 600,000 AD patients in Turkey and this number is expected to increase due to the rapidly aging population since AD is highly age-dependent. Despite extensive studies, there is currently no disease-modifying cure for AD. One of the therapeutic targets for AD is neurotrophin (NT) signaling as imbalances in the NTs and/or their receptor levels have been demonstrated in AD. Among the NT family of growth factors, brain-derived neurotrophic factor (BDNF) is extensively studied in AD as i) it is critical for the normal functioning of the adult nervous system, ii) BDNF and its receptor TrkB is downregulated in AD, and iii) *in vitro* and *in vivo* studies demonstrate the neuroprotective effects of BDNF against the AD process. BDNF activates its high-affinity receptor TrkB to regulate many vital processes in neurons, including synaptic plasticity, neurogenesis, and neuroprotection. Although BDNF's potential benefits against AD are well appreciated, enhancing its signaling in the brain has been challenging for a number of reasons. Our group previously established that a highly

conserved 3-aminoacid domain (KFG) of Trk receptors negatively impacts their signaling. The deletion of the KFG domain leads to an enhanced Trk activation and its downstream signaling. In this thesis, the aim was to utilize this TrkB-KFG model system to evaluate its effect against an *in vitro* AD model. To do so, WT and TrkB-KFG (KFG domain deletion) mouse embryonic stem cells (mESCs) were differentiated into cortical neurons, one of the most affected cell types in AD. Upon the generation and characterization of cortical neurons, an *in vitro* AD model was established using a well-characterized oligomeric Amyloid β(1-42) peptide (Aβ42). WT and TrkB-KFG cortical neurons were tested for viability, synaptic density changes, as well as for changes in BDNF-TrkB signaling pathways. We found that the KFG deletion leads to higher levels of TrkB receptors in TrkB-KFG cortical neurons. Importantly, TrkB-KFG cortical neurons exhibited a higher viability under Aβ42 insult compared to WT cortical neurons. Additionally, TrkB-KFG neurons exhibited resistance against synaptic density changes caused by oligomeric Aβ42 treatments as compared to WT cortical neurons. The novelty of this study lies in the TrkB-specific KFG modification which directly interferes with the TrkB receptor and does not have a known effect on anything other than TrkB, potentially creating a specific effect. To the best of our knowledge, this is the first study demonstrating the effects of enhanced TrkB signaling against AD, achieved by direct modulation of the receptor domains. This approach may be highly useful for future studies focusing on cell transplantation using modified cell sources.

Keywords: Alzheimer’s Disease, Amyloid Beta, Cortical Neurons, TrkB Receptor, Brain-Derived Neurotrophic Factor.

ÖZ

GÜÇLENDİRİLMİŞ TRKB SİNYAL YOLAĞININ FARE KÖK HÜCRELERİNDEN DÖNÜŞTÜRÜLMÜŞ KORTİKAL NÖRONLARDA AMİLOİD BETA 42 MUAMELESİNİN NEDEN OLDUĞU HÜCRESEL OLAYLARA KARŞI ETKİLERİ

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Alzheimer hastalığı (AH) küresel olarak en yaygın altıncı ölüm sebebidir. Bu hastalık hafıza kaybı ve bilişsel gerilemeye sebep olarak kişinin günlük hayatın basit eylemlerini gerçekleştirmesini engeller. Türkiye Alzheimer Derneği'nin 2014-2018 Faaliyet Raporu'na göre ülkemizde 600.000 AH hastası vardır ve bu sayının hızla yaşlanan popülasyon sebebiyle artması beklenmektedir, çünkü AH yaşa bağlı bir hastalıktır. Tedavi için yürütülen birçok bilimsel çalışma olmasına rağmen AH için henüz hiçbir hastalık önleyici tedavi bulunamamıştır. Tedavi yaklaşımlarından biri olan nörotrofin (NT) ailesinin AH için kritik olduğu bilinmektedir çünkü NT ve/veya reseptörlerindeki seviye dengesizlikleri AH'de bilinmektedir. Büyüme faktörlerinden NT ailesinin bir üyesi olan BDNF (brain-derived neurotrophic factor veya beyin-türevli nörotrofik faktör), AH kapsamında derinlemesine araştırılmaktadır çünkü i) yetişkin sinir sisteminin normal fonksiyonları için kritiktir, ii) BDNF ve reseptörlerinin seviyeleri AH koşullarında azalır ve iii) *in vitro* ve *in vivo* araştırmalar BDNF'nin AH sürecine karşı nöroprotektif etkisini göstermektedir. BDNF yüksek afiniteye sahip olduğu reseptörü TrkB'yi aktive eder ve böylelikle sinaptik plastisite, nöron oluşumu ve nöronların korunması dahil hayati süreçleri

düzenler. BDNF'nin AH'ye karşı potansiyel yararı bilinse de, beyinde sinyalizasyonunu artırmak konusunda bazı sorunlarla karşılaşılmıştır. Araştırma grubumuz Trk reseptörlerinde korunmuş olan 3 amino asitlik KFG bölgesinin sinyalizasyonu negatif etkilediğini ortaya koymuştur. KFG bölgesinin çıkarılması, Trk aktivasyonunu ve tetiklediği diğer sinyalzasyonları artırmaktadır. Bu tez çalışmasında amacımız bu TrkB-KFG model sistemini kullanarak *in vitro* AH modeli üzerindeki etkisini tespit etmektir. Bunun için, WT ve TrkB-KFG (KFG bölgesi çıkarılmış) fare embryonik kök hücrelerden (FEKH) farklılaştırılmış kortikal nöronlar, AH'den en çok etkilenen hücre tiplerinden biri, elde edilmiştir. Kortikal nöronların elde edilmesi ve karakterizasyonunun ardından, iyi karakterize edilmiş oligomerik Amiloid β (1-42) (Aβ42) muamelesi ile *in vitro* AH modeli oluşturulmuştur. WT ve TrkB-KFG kortikal nöronlar hücre canlılığı, sinaptik yoğunluk değişimleri ve BDNF-TrkB ile ilişkili sinyalizasyonlar için test edilmiştir. KFG modifikasyonunun TrkB-KFG kortikal nöronlarında daha yüksek seviyelerde TrkB reseptörüne yol açtığını tespit ettik. Önemli olarak, TrkB-KFG kortikal nöronları Aβ42 muamelesi sonrası WT kortikal nöronlara göre daha yüksek canlılık göstermiştir. Ek olarak, TrkB-KFG nöronları, WT kortikal nöronlara kıyasla, Aβ42 muamelesinin sebep olduğu sinaptik yoğunluk değişimlerine karşı direnç göstermiştir. Çalışmamızın özgün değeri direkt olarak TrkB reseptörüne etki eden ve TrkB dışında herhangi bir bilinen etkiye sahip olmayan TrkB-spesifik KFG modifikasyonuna yer vermesidir. Bildiğimiz kadarıyla, bu çalışma direkt olarak reseptör bölgesinin değiştirilmesi ile AH'de artırılmış TrkB sinyalizasyonunun etkilerini gösteren ilk çalışmadır. Bu yaklaşım gelecek çalışmalarda değiştirilmiş hücre kaynakları kullanılarak yapılan hücre transplantasyonu çalışmaları için oldukça faydalı olabilecektir.

Anahtar Kelimeler: Alzheimer Hastalığı, Amiloid Beta, Kortikal Nöronlar, TrkB Reseptörü, Beyin Türevli Nörotrofik Faktör.

To Alzheimer's Disease patients and their families

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ABBREVIATIONS

AD	Alzheimer's Disease
NTR	Neurotrophin Receptors
Trk	Tropomyosin receptor kinase
BDNF	Brain-derived neurotrophic factor
NGF	Nerve growth factor
Aβ	Amyloid β
Aβ42	Amyloid β (1-42)
MEF	Mouse embryonic fibroblast
MitoC	Mitomycin C
mESC	Mouse embryonic stem cell
RA	Retinoic Acid
MTT	3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
HEK293	Human embryonic kidney 293
Erk1/2	Extracellular signal-regulated kinase 1/2
ADFNK	Advanced DMEM-Fetal Bovine Serum-Neuro Medium-KSR
KSR	Knockout Serum Replacement
SDS-PAGE	Sodium dodecyl sulfate-Polyacrylamide gel electrophoresis
iPSC	Induced pluripotent stem cell

CHAPTER 1

INTRODUCTION

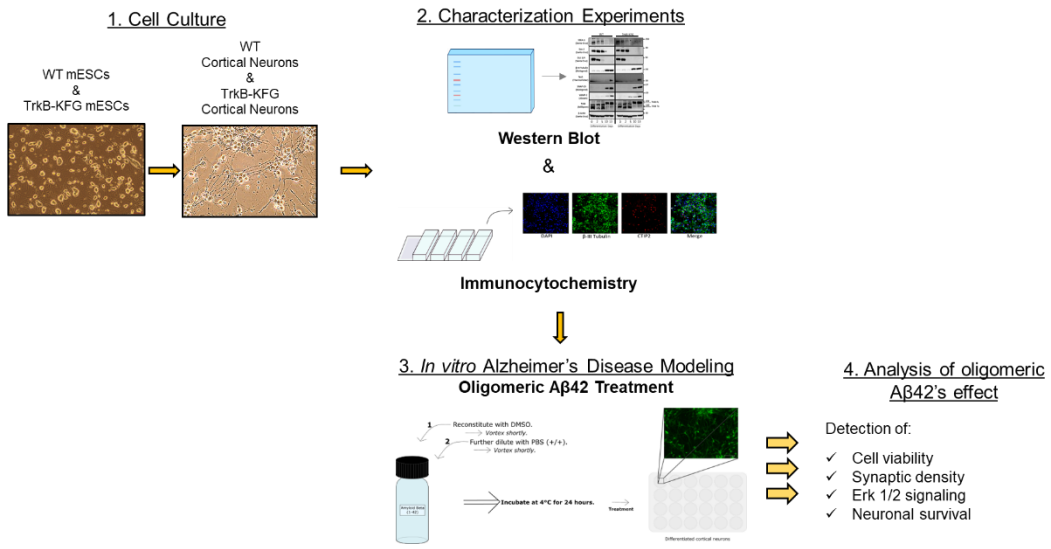

Figure 1.1. Summary of this thesis study.

1.1 Alzheimer's Disease

Alzheimer's Disease (AD) is a neurodegenerative disease (X. Du, Wang, & Geng, 2018), generally defined by the extracellular amyloid plaques formed from Amyloid β (Aβ) accumulation and intracellular neurofibrillary tangles (NFTs) that arises from hyperphosphorylated tau protein (Lane, Hardy, & Schott, 2018). AD patients demonstrate typical and atypical symptoms. Typical symptoms include memory problems and executive dysfunction, which negatively affect daily life. Atypical symptoms on the other hand include problems relating to language, vision, motion, and decision-making that are more pronounced than memory loss (Scheltens et al., 2016). Since there is no cure for AD, ameliorating these symptoms and the care of

patients is essential, both of which are both economically and emotionally costly to patients and families.

AD is a public health issue around the world, with increasing numbers every year. Globally, 55 million people were estimated to suffer from dementia (an AD-like condition of elderly people) and this number will possibly increase to 139 million in 2050, as reported by Alzheimer's Disease International's World Alzheimer Report 2022. Alzheimer's Association states in a 2022 report that, in the United States of America among people who are 65 years old or older, an estimated 6.5 million Americans had Alzheimer's dementia in 2022 (Gaugler et al., 2022). In Turkey, approximately 600.000 people suffer from AD, as reported by the Turkish Alzheimer Foundation in its 2014-2018 Report. In all these reports, old age is seen as a great risk factor, a concerning fact since the elderly population is increasing globally, which is estimated to cause a greater prevalence of AD.

Even though Aβ oligomers, plaques, and intracellular tau aggregates have been associated with the pathogenesis of AD (DeTure & Dickson, 2019), the relevance and effect of these are uncertain: it is still debated among researchers if Aβ and tau are the cause or the result of the disease (Kametani & Hasegawa, 2018). Despite many years of research, there is no consensus on the cause or mechanism of AD, and there are no, well-accepted, disease-modifying drugs as well.

While the mechanism of pathogenesis is unknown, AD is known to be either sporadic or genetic (Lane et al., 2018). Even though sporadic AD is much more common, genetically caused AD, known as familial AD (fAD), has been extensively studied demonstrating relevant mutation on specific genes (Chávez-Gutiérrez & Szaruga, 2020); Primarily *APP*, *PSEN1*, and *PSEN2* gene mutations as well as the presence of the *APOE* ε4 allele (Scheltens et al., 2016) have been determined as risk factors for AD. While the fAD cases offer much information on the genetic nature of AD, its sporadic form poses a much greater danger, as well as being more prevalent among AD patients (Chávez-Gutiérrez & Szaruga, 2020). In the rest of this text, the focus will be largely on sporadic AD.

1.2 Hallmarks of Alzheimer's Disease

As mentioned before, the hallmarks of AD are mainly amyloid accumulation and hyperphosphorylated tau (Citron, 2010). Amyloid plaques are formed out of APP (amyloid precursor protein) through its proteolytic cleavage by β-secretase (β-site APP cleaving enzyme 1, also called BACE1) and γ-secretase gives rise to toxic and soluble Aβ peptides (Brandimarti, Irollo, & Meucci, 2022). The non-amyloidogenic pathway is carried out by α-secretase and γ-secretase cleavage of APP which gives rise to non-toxic peptides that do not form plaques (T.-H. Xu et al., 2016). After the abnormal cleavage, an inability to clear the toxic form of the peptide leads to the accumulation and formation of plaques, of which the one containing 42 amino acids is the one found the most in amyloid plaques associated with AD (DeTure & Dickson, 2019), which is thought to have a more toxic property in line with the pathology.

Hyperphosphorylation of tau is suggested to be the other main hallmark of AD. Tau is a microtubule-associated protein that is abundant in axons, serving as a scaffold, and its aggregation impairs these axons, leading to neurodegeneration (X. Du et al., 2018). Some studies suggest that there's a synergy between Aβ and tau; especially, the fact that the tau pathology is downstream of Aβ (Busche & Hyman, 2020). The link between these two hallmarks of AD is proposed to be the Fyn kinase which is recruited by tau in the case that Aβ is present in the synapse (Spires-Jones & Hyman, 2014). This association is thought to emerge in synapses, also damaging this part of the neuron (Spires-Jones & Hyman, 2014). Despite this link, the exact relationship between tau and Aβ is not understood completely; some studies suggest that tau is required for Aβ pathology while other studies show a possibility that Aβ pathology is the driver of tau pathology (Bloom, 2014). With this in mind, researchers suggest a combinational therapy approach where both tau and Aβ are prevented (Sperling et al., 2018).

1.3 Hypotheses of Alzheimer's Disease

AD has been studied for decades and no efficient, well-accepted therapy or drug that changes the course of the disease has been developed. However, extensive research has led to the emergence of many hypotheses that attempt to explain the mechanism of AD pathology.

One of the most common is the Aβ hypothesis (also called amyloid cascade or amyloid hypothesis), which states that the Aβ peptide that is formed due to the cleavage of APP is the primary reason for AD. As mentioned earlier, α-, β-, and γ-secretases are involved in this process and Amyloid β (1-40) (Aβ40) or Amyloid β (1-42) (Aβ42) are formed as a result, which forms plaques that cause the pathogenesis of AD, leading to neuronal death and neurodegeneration. This hypothesis was supported heavily by the example of Down's syndrome (trisomy 21) patients who showed AD-like symptoms early (around 40 years old), this was attributed to the fact that these people have an extra copy of the *APP* gene than normal, which is on chromosome 21 (Kametani & Hasegawa, 2018). However, many studies abandoned this hypothesis as the sole reason for AD due to many shortcomings that were faced (Kepp & Muñoz, 2016). In short, it was found that the existence of Aβ peptides was not the reason for pathogenesis, Aβ production occurred in healthy brains (Kametani & Hasegawa, 2018; Paroni, Bisceglia, Seripa, & Solfrizzi, 2019); the pathogenesis was proposed to be due to an abnormal increase in Aβ42:Aβ40 ratio, which was due to a dysregulation in the clearance of the accumulation (Kepp & Muñoz, 2016). Furthermore, many candidate drugs that target Aβ peptides failed clinical trials or did not produce any clinical significance yet. Even though this hypothesis has many shortcomings, some of the new hypotheses that emerged afterward took notes from the Aβ hypothesis. While this hypothesis was not entirely discarded, its weaknesses are known and new studies attempt to add new findings to the Aβ hypothesis to reach a better one.

Another heavily studied hypothesis revolves around the other hallmark of AD: The tau hypothesis. Tau is a microtubule-associated protein that functions to stabilize

tubulin assemblies (Kametani & Hasegawa, 2018). The *tau* gene produces 6 isoforms that are the results of mRNA alternative splicing; of these isoforms, 3-repeat (3R) and 4-repeat (4R) versions of exon 10 are the pathology-causing ones (Kametani & Hasegawa, 2018). Their hyperphosphorylation leads to the formation of NFTs that cause neurons to lose their integrity, which results in neurodegeneration (X. Du et al., 2018; P.-P. Liu, Xie, Meng, & Kang, 2019). Many studies support this hypothesis; the emergence of tau lesions before Aβ peptide accumulation, many other diseases that tau pathology is linked to, and its direct correlation to AD progression are the main ones (X. Du et al., 2018; Kametani & Hasegawa, 2018).

The cholinergic hypothesis takes root from the findings that most affected regions of the brain due to AD showed a decreased concentration of acetylcholine (P.-P. Liu et al., 2019). This finding lead to the symptom-relieving drug that inhibited enzymes that work against acetylcholine, as mentioned in **Section 1.4**. On the other hand, the activity of acetylcholine relating to memory, sensory information, attention, etc. is known, which is heavily affected in AD (X. Du et al., 2018).

Another hypothesis relies heavily on the immune system to explain AD, which is the inflammation hypothesis (X. Du et al., 2018) (or, infection hypothesis, or antimicrobial protection hypothesis); according to this proposal, Aβ acts as a warning sign against pathogens, working with the innate immunity that functions in the brain (Moir, Lathe, & Tanzi, 2018). Different studies compared Aβ with antimicrobial peptides, tested its effect against pathogens, and even showed that mice that lack Aβ do not survive unsterile conditions postpartum (Moir et al., 2018). On the other hand, HSV-1 which is detected in elderly people was found to cause both abnormal processing of APP and hyperphosphorylation of tau (Fulop et al., 2018). Other studies were also done that showed the existence of microbial/pathogenic entities in postmortem brain samples (Fulop et al., 2018).

Aside from the ones that are mentioned, there are many other hypotheses on AD; such as oxidative stress hypothesis, neurovascular hypothesis, calcium hypothesis, endosomal vesicle hypothesis (X. Du et al., 2018; Fulop et al., 2018; P.-P. Liu et al.,

2019; Selkoe & Hardy, 2016). While these hypotheses confer important conclusions relating to AD, this thesis study focuses on the neurotrophins and neurotrophin receptors which are key players for neuronal health (Franco, Comaposada-Baró, & Vilar, 2020), which are affected by many processes mentioned in AD hypotheses.

1.4 Drug Candidates for Alzheimer's Disease and Therapeutic Approaches

In 2021, an antibody-based drug called aducanumab (also called Aduhelm) that targets Aβ plaques has been approved by FDA under the accelerated approval provisions which require a new clinical trial that should prove the clinical benefit of the drug, unless this is accomplished the approval may be withdrawn (FDA, 2021). Afterward, in 2022, another drug called lecanemab applied for approval (Lancet, 2022) and was granted an accelerated approval similar to aducanumab, based on Phase II clinical trial results (Reardon, 2023). These drugs aim to increase the clearance of Aβ (M. Shi, Chu, Zhu, & Zhu, 2022), accepting the theory that the main reason for pathogenesis in AD might not be the accumulation but the lack of or decrease in clearance of Aβ. However, many scientists argue that these drugs are heavily based on a hypothesis that has long been discarded as the sole explanation for AD. In addition to the scientific shortcomings of these drugs, it is argued that these drugs do not have an actual disease-modifying aspect and they could cause false hope and an unnecessary economic burden on patients (Couzin-Frankel & Piller, 2022; Walsh, Merrick, Richard, Nurock, & Brayne, 2022). Before these two drugs, GV-971 was approved for use in China, which is a marine-originated oligosaccharide that is recommended for use on mild to moderate AD to help with cognitive functions (Syed, 2020). It can reportedly reach the brain to destabilize toxic Aβ and prevent its production (Xiao et al., 2021). An additional effect is through the gut-brain axis, regulating both the gut microbiota and the neuroinflammation (Wang et al., 2020).

Aside from drugs that work towards curing AD, there are also drugs to help relieve AD symptoms. Acetyl-cholinesterase inhibitors (AchEIs) are one of the main drugs

used for symptom treatment, which stops the synaptic damage by increasing the acetylcholine amount that is available (Lane et al., 2018). N-methyl-D-aspartate (NMDA) receptor antagonist memantine is another symptom-relieving drug that decreases the neuronal damage in AD (Abeysinghe, Deshapriya, & Udawatte, 2020) by affecting glutaminergic pathways that are affected in AD pathology (Graham, Bonito-Oliva, & Sakmar, 2017). Even though these drugs help with the symptoms, the disease progression ensues and poses a great danger and discomfort for daily life which negatively affects the daily lives of patients as well as their families and carers (Lane et al., 2018). This leads to a need for special assistance for AD patients, especially in the later stages, which becomes both an emotional and an economic burden (Lane et al., 2018). Therefore, a disease-modifying drug that will stop AD pathogenesis is an urgent need.

1.5 Neurotrophin Receptors in Health and Alzheimer's Disease

Neurotrophin (NT) family is composed of nerve growth factor (NGF), brain-derived neurotrophin factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) (Chao, 2016; L. Tessarollo, 1998). Initially, NTs are secreted in an inactive form, packed inside secretory vesicles. Their activation occurs with the cleavage that results in an N-terminal prodomain peptide and an active C-terminal mature protein (Pinton, Sampaio, Savall, & Gutierrez, 2017). NTs serve as ligands for neurotrophin receptors (NTRs) which are divided into two categories, Trk receptors and p75NTR (Chao, 2016; L. Tessarollo, 1998). Trk (tyrosine receptor kinase) receptors are made up of TrkA, TrkB, and TrkC; which interact with NGF, BDNF or NT-4/5, and NT-3, respectively (Mitre, Mariga, & Chao, 2016). On the other hand, p75NTR shows a low-affinity for all NTs (Chao, 1994). While Trk receptors are associated with neuronal survival and health, p75NTR is associated with cell death (Longo et al., 2007). These interactions lead o a variety of functions: NT-Trk signalings are important for neuronal survival, growth of axons, dendrites, and synapses; (Scott-Solomon & Kuruvilla, 2018) synaptic plasticity, and cognition (Nordvall, Forsell, &

Sandin, 2022). These functions are carried out through Ras/mitogen-activated protein kinase (MAPK), phosphatidylinositol 3 (PI3K)/Akt kinase (also named as Protein Kinase B), phospholipase C (PLC) pathways (Diniz & Teixeira, 2011). Encompassing these functions and cellular pathways as well, this thesis study focuses on the BDNF-TrkB signaling pathway.

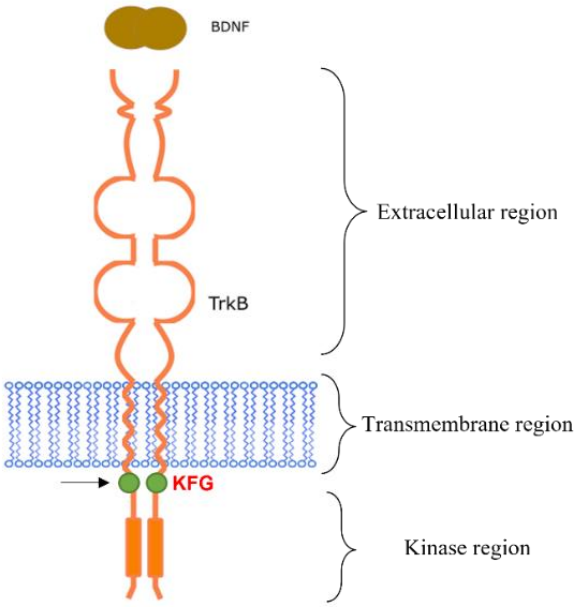

Figure 1.2. TrkB receptor and its ligand BDNF.

The 3 different regions of the TrkB receptor are shown, indicating the localization of the KFG region with respect to the receptor's placement on the cell membrane.

It has been shown that a decrease in BDNF levels is observed in the context of AD pathology (Gao, Zhang, Sterling, & Song, 2022), and in many experimental models the exogenous administration of BDNF is shown to decrease disease progression (Caffino, Mottarlini, & Fumagalli, 2020), demonstrating an inverse relationship between BDNF levels and AD pathology. Other studies show reduced levels of BDNF and TrkB in specific brain regions, one of which is the cortex (Mariga, Mitre, & Chao, 2017). These effects might occur in a few ways such as; degradation of BDNF via lysosomes, epigenetic regulation of BDNF, its release through platelets,

cleavage of proBDNF from its inactive form into the active BDNF form, alteration of mRNA *BDNF* levels, and downregulation of its upstream pathways such as CAMP responsive element binding protein (CREB) (Ng, Ho, Tam, Kua, & Ho, 2019). Relating to these processes, a study conducted on mice with TrkB insufficiency and AD pathology symptoms has resulted in findings that deficiency in BDNF-TrkB signaling and its downstream Akt/GSK-3β pathways are contributing to AD pathology, although independent of Aβ pathways (Devi & Ohno, 2015).

Due to their importance in the nervous system, NTs are being tested as neurodegenerative disease therapeutic targets (Nordvall et al., 2022; Yanpallewar, Fulgenzi, Tomassoni-Ardori, Barrick, & Tessarollo, 2021), as well as for cancer therapy (Chopin, Lagadec, Toillon, & Le Bourhis, 2016). However, the modulation of NTs and NTRs through drugs proved to have some roadblocks in this regard, which are related to pharmacokinetics, blood-brain barrier penetration, and unpredictable interference from already existing NTs in the system (Kazim & Iqbal, 2016; Yanpallewar et al., 2021). Despite these, Pan Trk inhibitors were developed and approved for cancer treatment (Harada & Drilon, 2022), as well as the highly favored 7,8-dihydroxyflavone (7,8-DHF) which binds to TrkB, mimicking BDNF (Caffino et al., 2020).

Aiming to regulate BDNF activity and the BDNF-TrkB signaling pathway, other studies were also conducted to find a candidate treatment for diseases (Akagi et al., 2015; Caffino et al., 2020; Ng et al., 2019). Small molecules appeared to be a better choice in terms of exogenous treatment, where these roadblocks were partially overcome (Caffino et al., 2020). However, another therapeutic path involves gene modulation, instead of exogenous drug treatment (Gao et al., 2022). Different ways of gene delivery have been tested, using polymer-based scaffolds, liposomes, viral vectors, and cell-based approaches (Gao et al., 2022). These approaches affected the symptoms but they did not have a direct effect on Aβ deposits and tau hyperphosphorylation (Gao et al., 2022). BDNF gene therapy was also reported to help with synaptic health, learning, and memory associated with AD, however, these effects were also independent of Aβ clearance (Mariga et al., 2017). Therefore,

although the development of NT-derived drugs against neurodegeneration has been proven for years to be a difficult approach, however, it is a long-proven fact that NTs and NTRs are ideal therapeutic targets (Baazaoui & Iqbal, 2022; Nordvall et al., 2022).

1.6 Aim of the Study: Cortical Neurons, Trk Receptors, and Alzheimer's Disease - A Potential Therapeutic Path

Neurodegeneration in AD is linked to several cellular processes (Calabrò, Rinaldi, Santoro, & Crisafulli, 2021), which makes it challenging to pharmaceutically target with a specific effect. An ideal approach against the neurodegenerative process of AD should involve both neuroprotection and interference with the disease progression. BDNF-TrkB signaling has a general and holistic effect on neuronal death, in health and disease conditions. Previous literature suggested that BDNF-TrkB signaling can be considered a therapeutic target in AD, based on several lines of evidence from various groups (J.-H. Song, Yu, & Tan, 2014). First, it has been shown that the enhancement of BDNF-TrkB activation can lead to neuroprotection (Caffino et al., 2020), which is also supported by the fact that BDNF and TrkB are important players of adult neurogenesis, memory formation, and synaptic plasticity in cortical regions of the brain (Begni, Riva, & Cattaneo, 2016). Second, in AD brains, BDNF and TrkB receptor levels were reported to be decreased (Devi & Ohno, 2015; Mariga et al., 2017). Lastly, *in vivo* (including monkey studies) and *in vitro* model studies provide compelling evidence that BDNF-TrkB signaling activation is beneficial against AD (Caffino et al., 2020; Mathivanan & Minabe, 2016).

Although the positive effects of BDNF-TrkB signaling against AD are well-recognized, it is highly challenging to increase BDNF-TrkB signaling in the brain due to several reasons. Among the main reasons, the first one is that BDNF cannot pass through the blood-brain barrier (Caffino et al., 2020; Wen et al., 2017). Secondly, viral-based BDNF delivery to the brain has proven to be difficult (Nagahara & Tuszynski, 2011). Third, the utilization of small molecule BDNF

mimetics may have non-specific effects that affect the system as a whole (Pinton et al., 2017). There have been efforts to develop BDNF-TrkB signaling-based therapies and unfortunately, no significant development has been achieved. Well-controlled systems leading to enhanced BDNF-TrkB signaling may open new doors for BDNF-TrkB signaling-based therapeutic options. To this end, our group has previously shown that the removal of the KFG domain from the Trk receptor leads to enhanced signaling (Kiris et al., 2014). Therefore, this system provides a straightforward approach to test the importance of increased BDNF-TrkB signaling against AD. This study aimed to evaluate the effects of enhanced BDNF-TrkB signaling in cortical neurons against the *in vitro* AD neurodegenerative process as compared to control conditions. To do so, we utilized WT and TrkB-KFG mouse embryonic stem cells (mESCs) to generate cortical neurons and utilized 42 amino acid long, oligomeric Aβ42 treatments to model the disease *in vitro*. In these conditions, cortical neurons were compared with controls to reveal the effects of KFG modification and AD-like conditions, in terms of synaptic proteins and neuronal death. The cortical region is one of the most affected and studied regions regarding AD, which is the focus of the thesis study (De Jager et al., 2018; Michalski & Fahnestock, 2003). However, this thesis is a part of a more comprehensive project where basal forebrain cholinergic neurons were also studied in the same setting, using a similar technical approach (Ezgin, 2022). However, BFCN neurons were evaluated for NGF-TrkA signaling as these neurons depend on this signaling pathway. The use of cortical neurons in this BDNF-TrkB-focused approach is essential since the effect of Aβ42 on cortical regions of the brain is well-documented (Sepulcre et al., 2018), and some studies place the cortical regions in the center of AD pathology (Khan et al., 2013).

CHAPTER 2

MATERIALS AND METHODS

In this section, the techniques and materials that were utilized in this project will be explained in detail. Before a lengthy description, the methodology will be summarized in chronological order to explain the experimental flow of this thesis study. First, feeder cells were cultured and mitotically inactivated to aid in the culture of mouse embryonic stem cells (mESCs). Then, mESCs were cultured and differentiated into cortical neurons by embryoid body formation followed by chemical induction. The differentiation process was characterized utilizing Western Blot and immunocytochemistry techniques, with the aid of cortical neuron-specific and neuron-specific markers, as well as stem cell markers. Afterward, the Amyloid β (1-42) (Aβ) peptide was prepared to have an oligomeric form, and it was treated to mESC-derived differentiated cortical neurons, to induce an *in vitro* AD model. Cortical neurons were also treated with a commercially obtained BDNF. To observe the effects of these treatments, the MTT cell viability assay was used. Then, synaptic density and signaling pathways were observed with Western Blot and immunocytochemistry, using antibodies specific to synaptic proteins and specific signaling pathways. Finally, bioinformatics tools were utilized to reach a holistic view of signaling pathways and to reach a meaningful conclusion.

2.1 Cell Culture for Mouse Embryonic Stem Cells and Cortical Differentiation

2.1.1 Culture and Inactivation of Feeder Cells

As a preparation for mESC culture, primary mouse embryonic fibroblasts (pMEFs) were utilized. These cells were used as feeder cells in stem cell cultures to provide the necessary cytokines and factors that help maintain the pluripotency of mESCs (Southon & Tessarollo, 2009). These cells were a kind gift from Dr. Lino Tesarrollo (NIH, USA), and have been stocked inside liquid nitrogen tanks in our department.

Before their inactivation, pMEFs were cultured with MEF Medium (contents explained in **Appendices B**) on 150 mm cell culture sterile plates, at 37°C and 5% CO_2 conditions in a cell culture incubator. In order to obtain large amounts of these cells, MEFs were cultured and passaged until 20 plates were obtained with approximately 80% confluency. Once these cells were ready, Mitomycin C (Cayman Chemicals) was treated to mitotically inactivate MEFs. This chemical is used as a cancer drug (Bradner, 2001) and is also repurposed as a way to prevent cell division of feeder cell lines. The working mechanism of Mitomycin C involves its binding to the DNA, subsequently preventing replication and ultimately, stopping mitosis (Chugh, Chaturvedi, & Yerneni, 2016).

Primary MEFs were treated with 10 μg/ml Mitomycin C. To do this, Mitomycin C was diluted first inside PBS (+/+) and filtered through a 0.22 μm filter. Diluted to its final concentration inside MEF Medium, Mitomycin C was treated gently on each plate, and cells were incubated for 3 hours. After incubation, to generate cell stocks and get rid of the Mitomycin C present in the medium, cells were gently washed 3 times with PBS (+/+). Then, 0.05% Trypsin was used to detach MEFs treated with Mitomycin C (MitoMEFs). After centrifuging twice, cells were resuspended inside the freezing medium and distributed to cryovials, adding approximately 1 ml to each

vial. Afterward, cells were stored inside MrFrosty at -80°C overnight and then transferred inside nitrogen tanks, for long-term storage.

2.1.2 Mouse Embryonic Stem Cell Culture

The mouse embryonic stem cell line (mESC) used in this thesis project was the V6.4 cell line, which was isolated from mice (Lino Tessarollo, Palko, Akagi, & Coppola, 2009; You et al., 1998). Two types of V6.4 cells were used, either the wild type (WT) which no modification has been done and TrkB-KFG, in which the KFG domain on the TrkB receptor (mentioned in **Section 1.6**) has been deleted via CRISPR-Cas9-based approach. The genome editing process on TrkB-KFG V6.4 mESCs was performed in Dr. Lino Tesarollo's laboratory (as well as the verification of the technique) and both WT and TrkB-KFG V6.4 cells were sent to our laboratory as a kind gift.

WT V6.4 and TrkB-KFG V6.4 mESCs were cultured in the same conditions, inside separate 150 mm plates. Before seeding the mESCs, 0.1% gelatin was used to coat the surface of the 150 mm plates. After 30 minutes of UV exposure, gelatin was aspirated and mESCs were seeded with V6.4 mESC Medium (contents explained in **Appendices B**). The cells were cultured inside a Cell Culture CO_2 Incubator (Esco Lifesciences) at 37°C and 5% CO_2 conditions. For the stem cell culture, the medium was changed every day to maintain pluripotency. The mESCs were observed every day with Olympus CX45 Microscope in our laboratory and once a specific colony size and number was reached, 0.05% Trypsin enzyme was used to passage the cells. To take stocks, mESCs were trypsinized and a freezing medium was used to store the cells. Cells were transferred to -80°C inside Mr. Frosty which was used to gradually decrease the temperature, and eventually, cells were stored in the gaseous phase in nitrogen tanks.

2.1.3 Cortical Differentiation of Mouse Embryonic Stem Cells

To start cortical differentiation, mESCs were cultured as explained above. When a certain colony size and confluency were met (as shown in **Figure 3.2**), the Dispase enzyme was used to separate mESCs from Mito-MEFs. When applied in a specific manner, this enzyme leaves MitoMEFs attached to the plate while detaching the mESCs. To explain the application, during roughly 6 minutes of incubation with dispase enzyme, cells were observed every 2 minutes and when necessary, a mechanical force was used upon the plates to detach mESCs. While mechanical force is not mandatory, it is needed to ensure that cells are not exposed to the Dispase enzyme for too long, which can detach MitoMEFs from the plate. Afterward, mESCs were collected with V6.4 mESC Medium, which inactivates the Dispase enzyme due to its FBS content. Then, the cell suspension was centrifuged for 5 minutes at 1000 RPM. After this first centrifuge, cells were washed once with DMEM and centrifuged for a second time with the same settings. This double-centrifuge process is important for the removal of LIF-containing medium which could disrupt the differentiation process if it remained in the culture. After the second centrifuge, cells were resuspended with ADFNK Medium (contents explained in **Appendices B**). Then, mESCs that were resuspended with ADFNK Medium were seeded inside bacteriological 120 mm plates. These plates that have low-attachment properties, in combination with the contents of ADFNK Medium, induce embryoid body formation. The process of mESC detachment from MitoMEFs and seeding on bacteriological plates with ADFNK for the first time is designated as “day 0” of the differentiation process. The directed differentiation was conducted using widely used methods described in the literature (Chambers et al., 2009; Fasano, Chambers, Lee, Tomishima, & Studer, 2010; Saurat, Livesey, & Moore, 2016; Y. Shi, Kirwan, Smith, Robinson, & Livesey, 2012).

For differentiation day 1, no treatments were done. However, on day 2, 1.5 μM retinoic acid (RA) treatment (Sigma, Cat. No. 2625) as performed, which triggers neuronal differentiation. The administration of retinoic acid was done by diluting

this factor inside the ADFNK medium, freshly just before use. Medium change was done using this ADFNK-RA mixture.

Factor treatment to low-attachment embryoid body cultures as done in a specific manner, using either one of two ways, depending on the situation of the embryoid bodies. If the embryoid bodies were ideal in size and their collection to the center of the plate occurs without any hardship, the medium was aspirated from the sides of the plate, avoiding the aspiration of embryoid bodies along with the medium. Alternatively, if the embryoid bodies differed in size and their collection to the very center of the plate was not accomplished efficiently, embryoid bodies were collected inside a 15 ml falcon tube along with the medium. After either waiting for a few minutes (if the embryoid bodies are large enough) or a quick and gentle spin down, embryoid bodies are collected at the bottom of the falcon tube, and the medium was aspirated without touching any embryoid bodies or aspirating them along with the medium. After aspiration of the medium, the fresh medium was added directly to the 120 mm bacteriological plate, or the 15 ml falcon tube where embryoid bodies can be gently resuspended and seeded on a new 120 mm bacteriological plate. Embryoid bodies were handled very gently, avoiding any unnecessary pipetting to keep them healthy. After the medium-factor mixture was given, the embryoid bodies were swirled inside the 120 mm plate to prevent clumping. Both RA treatment and the other factor treatments that will be mentioned in the next stages of the protocol were done in this way.

After RA treatment on day 2, a combination of two factors diluted in ADFNK was used to induce cortical neuron formation: 50 nM SB431542 (Cayman Chemicals, Cat. No. 13031) and 25 nM LDN193189 (Sigma, Cat. No. SML0559). These factors are used to inhibit TGF-β/Activin/NODAL and BMP signaling to give rise to a cortical neuron characteristic. While there was no treatment done on day 4, the next day (day 5) the final factor treatment was done. Again the combination of 50 nM SB431542 and 25 nM LDN193189 was used but this time, the medium was switched to Maintenance ADFNK Medium (contents explained in **Appendices B**). After this treatment on day 5, there was no treatment done on day 6, and on day 7, dissociation

was done. The dissociation process was executed by the use of the Accutase enzyme (StemPro Accutase (Gibco, A1110501) or Accutase (Biolegend, 423201). Initially, embryonic bodies were collected in a 15 ml falcon tube and centrifuged for 2 minutes at 200g. After aspiration of the supernatant, the cell pellet consisting of embryoid bodies was washed with DPBS and this cell suspension was centrifuged again for 2 minutes at 200g. After these washing steps, the Accutase enzyme was given to the embryoid bodies and incubated for 10 minutes. During this incubation, the precipitated embryoid bodies were gently pipetted approximately every 2 minutes. This allowed for all the embryoid bodies to be exposed to the enzyme equally. When the incubation time was met, embryoid bodies were gently pipetted using a 1000 µl pipette tip. This pipetting aids the dissociation done by the Accutase enzyme. When embryoid bodies are pipetted enough, their spheroid shapes were lost. However, it's also very important that pipetting was not harsh and overdone; these could damage the dissociated embryoid bodies, leading to death or unhealthy neuron formation. When the dissociation process was complete, the Accutase enzyme was inhibited using an appropriate serum-supplemented medium and the enzyme was removed from the culture. Afterward, cells were resuspended in Maintenance ADFNK and cultured in this medium. After dissociation, cells were either cultured inside poly-D-lysine/laminin-coated 96-well plates (for MTT assay), 4 well chamber slides (for immunocytochemistry), or 24 well plates (for Western Blot lysates). On day 8, medium change was done using Maintenance ADFNK. If there is a possibility of non-neuronal cell formation, AraC treatment was conducted, based on similar studies (Kiris et al., 2011).

2.2 Characterization of mESC-derived cortical neurons

2.2.1 Generation of HEK293 transfected controls

The characterization of mESC-derived cortical neurons included observation of initial stemness and gradual change in character into a neuron, and more specifically,

a cortical neuron. In addition to this, Trk receptor presence and level were characterized. In order to show that the TrkB receptor was present in both WT and TrkB-KFG-derived cortical neurons, Western Blot with HEK293 transfected controls were used. These cells were transfected with either empty vector plasmid, TrkB full-length plasmid, TrkB truncated (T1) plasmid, or TrkA plasmid. All the plasmids were kind gifts from Dr. Lino Tessarollo (NIH, USA). The transfection gives rise to the overexpression of each receptor, to be used as a positive control.

Routinely used in our laboratory due to its easy culture conditions, HEK293 cells were stored in nitrogen tanks. Before transfection, they were cultured with HEK293 Medium (contents explained in **Appendices B**) and cultivated at 5% CO_2 and 37°C conditions. For transfection, HEK293 cells were seeded on 24-well plates coated with poly-D-laminin/lysine. Using Turbofect transfection reagent (ThermoFisher, Cat. No. R0534) for 48 hours, each plasmid was individually transfected to HEK293 cells, and lysates were collected using NP-40 lysis buffer containing protease and phosphatase inhibitors and processed as described below for Western Blot analysis.

2.2.2 Western Blot

To characterize the cortical neuron differentiation process, protein lysates from day 0, day 2, day 5, day 10, and day 13 were collected and analyzed via the Western Blot technique. Appropriate antibodies were chosen that reflect either stem cell character (SSEA-1, Sox-2, and Oct3/4), neuronal character (β-III tubulin and Tau5), or synaptic markers (SNAP-25 and VAMP2). β-actin was the loading control. In addition, TrkB and TrkA levels were observed, using HEK293 transfected controls. These controls were: empty vector plasmid transfected, TrkA plasmid transfected, TrkB full-length plasmid transfected, and truncated TrkB (T1) plasmid transfected HEK293 cells. They were used to pinpoint both TrkA, full-length TrkB, and truncated TrkB levels separately.

Table 2.1 Antibodies used in Western Blot and Immunocytochemistry Analysis

Antibody	Catalog Number
SSEA-1	Santa Cruz, sc-21702
Sox-2	Santa Cruz, sc-36558223
Oct 3/4	Santa Cruz, sc-365509
Tau5	Thermo Fisher, MS-247-P
TrkA	ATS Bio, AB-N03
TrkB	Millipore, 07-225
TrkB	BD Biosciences, 610101
Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) (E10)	Cell Signaling, 9106
Total Erk	Cell Signaling, 9102
β-III Tubulin	R&D Systems, BAM1195
β-Actin HRP	Santa Cruz, sc-47778
SNAP25	Biolegend, SMI 81
Tau5	Thermo Fisher, MS-247-P
Syntaxin (SP8)	Biolegend, 827002
VAMP-2	Abcam, ab3347
Pan-Trk (C-15)	Santa Cruz, sc-139
MAP2	Millipore, MAB3418
NeuN	Millipore, MAB377B
BRN2	Abcam, ab243045
SATB2	Abcam, ab34735
CTIP2	Abcam, ab28448

To summarize the Western Blot method used in this thesis, firstly lysates were collected using NP-40-based lysis buffer (contents explained in **Appendices C**) containing protease and phosphatase inhibitors. These lysates were stored inside a -80°C freezer. Afterward, lysates were thawed on ice and centrifuged at 12000 RPM for 20 minutes at 4°C temperature. Supernatants were transferred to properly labeled

new tubes. These protein extracts were analyzed for their protein concentration using Bradford Assay. Equal amounts of protein were loaded on 10% SDS-PAGE gels and ran using 1X Running Buffer (contents explained in **Appendices C**). The proteins were then transferred to the PVDF membrane using 1X Transfer Buffer (contents explained in **Appendices C**). The transfer process lasts for 2 hours, with a 40 Volt setting. After transfer, the PVDF membrane containing the transferred proteins was incubated either in 5% TBS-T Milk solution or 5% TBS-T BSA solution (contents explained in **Appendices C**) for 1 hour, matching the solution used for the primary antibody dilution. Primary antibody incubation was done overnight at 4°C. The next day, the primary antibody was collected and appropriate washing steps with 1X TBS-T buffer were done, HRP-conjugated secondary antibody suitable to the primary antibody was added to the membrane and incubated for 1 hour. Then, more washing steps were done with TBS-T, to get rid of excess and unbound secondary antibodies. Lastly, the PVDF membrane was imaged using ECL (Enhanced Chemiluminescence) solution and SynGene Western Blot Imaging Station.

2.2.3 Immunocytochemistry

After the dissociation of cortical neurons, cells were seeded on 4-well chamber slides for characterization experiments. For immunocytochemical analysis of cortical character, CTIP2, SATB2, and BRN2 were used, which show cortical neuron character. In addition, general neuronal antibody β-III Tubulin and nuclei stain DAPI were used.

The immunocytochemistry protocol followed for the experiments conducted for the thesis can be summarized as such: Cortical neurons seeded on poly-D-lysine/laminin-coated 4-well chamber slides were initially washed with PBS (+/+) solution, and then treated with 4% formaldehyde solution for 10 minutes at 37°C. Afterward, permeabilization was achieved with 0.1% Triton-X 100 solution (**Appendices C**), which was treated at room temperature for 15 minutes. Blocking was done before primary antibody incubation, with 2% BSA solution (**Appendices**

C) for 1 hour at room temperature. Primary antibodies were diluted with 2% BSA solution and incubated for 3 hours at room temperature. Afterward, the antibody was collected and PBS (+/+) washes were done at 10-minute intervals. For secondary antibodies, either AlexaFlour or Cy3 were used, chosen according to the properties of the primary antibody. Secondary antibody incubation was done for 2 hours at room temperature and afterward, the staining procedure was complete. The chambers were removed and the cells were mounted with ProLong Gold Antifade Reagent, which includes a DAPI stain. Imaging of immunocytochemistry slides was done via ZEISS confocal microscope for characterization experiments, and FLoid Imaging Station was used for Aβ treatment experiments.

2.3 Preparation of Oligomeric Amyloid Beta (1-42) Peptide

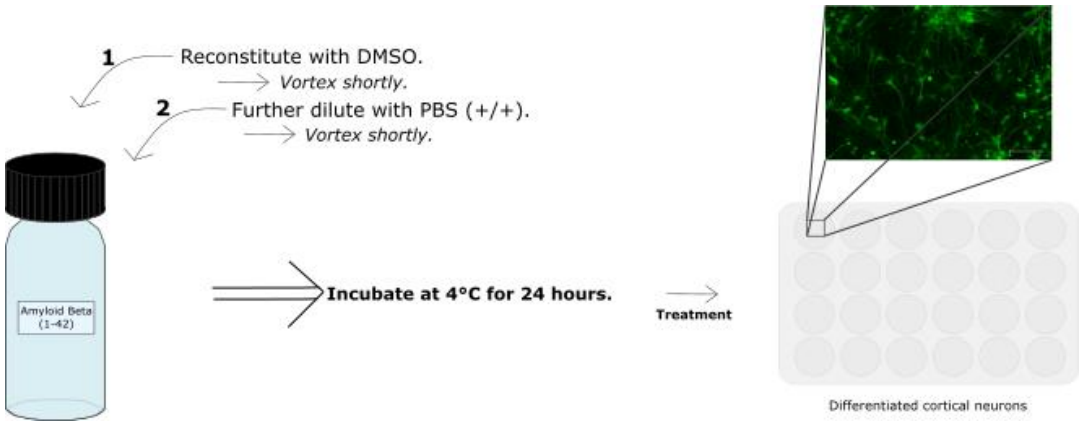

Figure 2.1. Summary of oligomeric Amyloid β (1-42) preparation.

The protocol for the preparation and use of the oligomeric peptide is depicted.

The AD model used in this thesis was obtained by treatment of oligomeric Aβ42 peptide (Millipore, Cat. No. AG968 and PeptiTeam). The HFIP (1,1,1,3,3,3-hexafluoro-2-propanol) treated lyophilized peptide was prepared according to the heavily cited and validated protocols (Stine, Dahlgren, Krafft, & LaDu, 2003; Stine, Jungbauer, Yu, & LaDu, 2010). Before every experiment, the Aβ peptide was prepared 24 hours before its treatment in order to use the most stable version of the

peptide. To begin, the lyophilized peptide was brought to room temperature and then DMSO was added in order to attain a 5 mM concentration. The peptide was vortexed shortly and afterward, an appropriate amount of DPBS was added to bring the concentration to 100 μM. Vortexing the peptide for the last time, it was incubated at 4C for 24 hours to induce oligomer formation. To validate oligomeric peptide formation, Western Blot without heat block was used. As shown in our previous work (Ezgin, 2022), this protocol leads to successful oligomeric Aβ peptide formation.

2.4 Amyloid Beta (1-42) and BDNF Treatment on Cortical Neurons

After cortical neuron differentiation was completed successfully, the experiments were planned, which required a carefully timed treatment schedule. Initially, cortical neuron cultures that will be experimented on were exposed to serum starvation for 12 hours before any treatment began. This was done by replacing the medium with Neurobasal Medium which did not include any serum supplements. Each peptide was treated after dilution with Neurobasal Medium. When 12 hours for serum starvation was complete, 50 ng/ml BDNF (PeproTech, Cat. No. 450-02) treatment was done for 4 hours. After BDNF treatment was complete, the neurotrophin-containing medium was replaced and only Aβ that was treated 24 hours beforehand was treated to the cortical neurons. For Western Blot lysates, 10 μM concentration was used for 24 hours. However, for MTT assays done on 96 well plates, 24-hour treatment was done with 1 μM, 2.5 μM, 5 μM, and 10 μM concentrations of Aβ peptide. These two treatments did not overlap, they were done separately. After 24 hours of Aβ42 treatment on cortical neurons, either (1) MTT experiments began as described below, (2) Western Blot lysates were taken as summarized above, or (3) immunocytochemistry incubations began as described above.

2.5 MTT Assay

The cell viability assay used in this study was 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Assay. Differentiated cortical neurons were seeded on poly-D-lysine/laminin-coated 96-well plates for this assay. There were also additional wells prepared for MTT Assay's blank controls, which only included Neurobasal Medium, with no cells seeded. After the treatments mentioned in **Section 2.4** were complete, the medium was removed from the wells and MTT Solution (**Appendices C**) was added for a treatment of 4 hours at 37°C and 5% CO_2 conditions. This treatment allows the metabolically active neurons to metabolize tetrazolium crystals and turn them into formazan which will give the absorbance levels at the end of the experiment. After this, 0.01 M SDS-HCl solution (**Appendices C**) was added to each well, solubilizing the formazan crystals. 18 hours of incubation at room temperature was done, preventing any light exposure to the plate, so that the colorimetric results of the assay were not affected. After 18 hours, an ELISA reader was used at 570 nm absorbance to measure the absorbance of each well. These absorbance numbers were used to calculate the cell viability percentage.

2.6 CellProfiler Analysis

Immunocytochemistry assays were conducted as explained above and after these, FLoid Imaging Station was used to obtain images. To quantify, the CellProfiler program (https://cellprofiler.org) was used. This program is an automated image editing and quantification tool. In this program, pipelines are used to automate the work done in each image. Pipelines are made up of modules, which are simply a group of specific prompts that either alter or quantify the images concerning specific adjustable parameters. In this study, the CellProfiler Analysis can be divided into two purposes: either for quantifying signal intensity or counting the number of cells that show signal. For both purposes, the DAPI stain was counted and divided with either intensity or count of a specific antibody signal, to obtain a result that reflects

the signal amount per cell. In addition, every signal was quantified with images that only include that specific signal's channel, in other words, the images that were quantified were never multichannel images. To count cells that are positive for a specific antibody, the "Identify Primary Objects" module was used with a minimum of 10-pixel units and a maximum of 40-pixel units. Each image contained roughly 200-600 cells according to this analysis. On the other hand, the intensity quantification was done with the "Measure Image Intensity" module, using "Total Intensity" data. Data obtained both ways were normalized and processed to reflect a percentage where the control groups were set to the baseline of 100%. WT and TrkB-KFG data were compared with Two-way ANOVA.

2.7 Statistical Analysis

The statistical analyses that are shown throughout this study were done using GraphPad Prism. Data are shown as Mean ± SD. The statistical test of choice was Student's t-test or Two-way ANOVA (only for ICC quantifications) where $p \leq 0.05$ was statistically significant and ***, **, * respectively mean 99.9%, 99%, and 95% confidence levels.

2.8 KEGG Pathway Analysis

The Kyoto Encyclopedia of Genes and Genomes (KEGG) database was used to obtain AD (hsa05010) and BDNF-TrkB (hsa04722) pathways, which were then merged and edited into a more relevant representation. Cytoscape 3.9.1 version was used along with CytoKEGG, and CyKEGGParser plug-ins (apps). Using the KEGG map, the pathways that were relevant for both AD and BDNF-TrkB were annotated to give more meaning to the presented pathway image.

CHAPTER 3

RESULTS

In this study, we differentiated cortical neurons from both WT and TrkB-KFG mESCs and fully characterized the resulting neuron identity. Following this, we utilized standard protocols to generate oligomeric Aβ42 (Stine et al., 2003; Stine et al., 2010) and treated the WT and TrkB-KFG cortical neurons with oligomeric Aβ42 to establish an AD model on a cellular level, similar to the literature (Kirouac, Rajic, Cribbs, & Padmanabhan, 2017; Qosa, LeVine, Keller, & Kaddoumi, 2014; L. Song, Yao, Zhang, Piao, & Lu, 2020). Using this system, we tested WT and TrkB-KFG cortical neurons for their response to the oligomeric Aβ42 treatments; in terms of cell viability, synaptic density, and pathways relating to survival. In this section, the results of these experiments are presented, as well as the preparations leading up to these experiments.

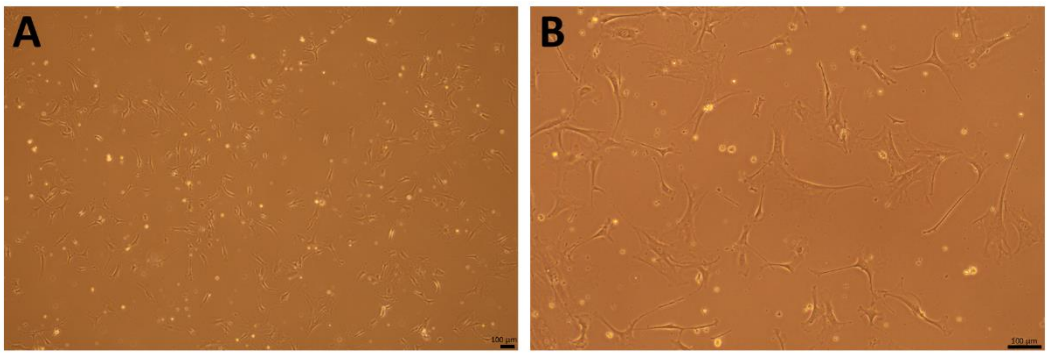

Figure 3.1. Primary mouse embryonic fibroblasts (MEF) were used to obtain the feeder cell line, MitoMEFs.

Primary MEFs are as depicted through (A) 4X and (B) 10X objectives using the Olympus CX45 microscope and BAB Camera System. These cells were cultured in 150 mm cell culture plates and mitotically inactivated using Mitomycin C to be used as feeder cells in mESC culture.

3.1 Stem Cell Culture and Directed Differentiation of Cortical Neurons

To culture mESCs, we first generated large quantities of feeder cells, mouse embryonic fibroblasts (MEFs), which were required for our mESC culture (Southon & Tessarollo, 2009). MEFs are commonly utilized in stem cell culture to keep the mESC lines pluripotent (E. Michalska, 2007; Southon & Tessarollo, 2009). We generated MEF stocks by cultivating primary MEFs, which were then mitotically inactivated using Mitomycin C (MitoC), using protocols that were validated in the literature (Southon & Tessarollo, 2009) as described in Materials and Methods. **Figure 3.1** depicts the primary MEF cells before their mitotic inactivation with Mitomycin C.

MitoC is a commercially available and efficient way of inactivating mitotic division without harming cells (E. Michalska, 2007). Mitotic inactivation of the feeder cells is vital since these cells will dominate the stem cell culture if their division continues. After their inactivation, it's also important to maintain the health of MitoMEFs since the factors they release are one of the ways the pluripotency is maintained (E. Michalska, 2007; Southon & Tessarollo, 2009). MitoMEFs are depicted in **Figure 3.2** along with mESCs.

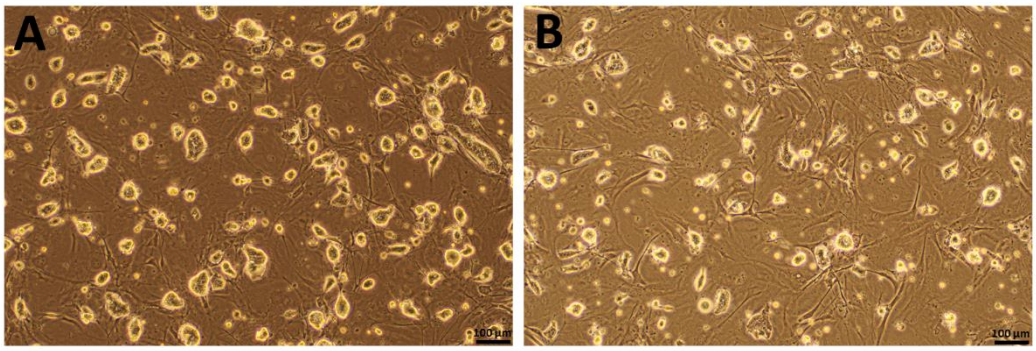

Figure 3.2. WT and TrkB-KFG mESCs under the microscope.

Two types of mESCs were used in this study, (A) WT V6.4, and (B) TrkB-KFG V6.4 colonies. Both mESC lines were co-cultured with MitoMEFs (feeder cells) and

imaged with a 10X objective using Olympus CX45 Microscope and BAB Camera System.

Upon preparing the feeder cell stocks, we then cultured our mESC lines with a LIF-containing medium (V6.4 mESC Medium, mentioned in **Appendices B**), to help preserve their pluripotency, as shown in **Figure 3.2**. Both WT and TrkB-KFG cells (KFG domains were deleted in both TrkB copies) were cultured under the same conditions. As described in Materials and Methods, these cells were generated and characterized by Dr. Lino Tessarollo (NIH, USA), and were provided to us as a kind gift. As conducted in stem cell studies, we regularly checked our cultures for colony morphologies under the microscope for potentially unwanted differentiation as summarized in **Section 2.1.2**. We have determined the pluripotency potential of both lines (**Figure 3.4**), generated cell stocks at different passages, and stored them in liquid nitrogen tanks.

For the differentiation process, we cultured both mESC lines, and upon the growth of stem cell colonies into an ideal size, as well as an ideal number, we initiated the directed differentiation to cortical cells, which is schematized in **Figure 3.3**. The figure demonstrates key steps and experimental details that are also provided in detail in Materials and Methods, **Section 2.1.3**. This protocol was established after testing many protocols available in the literature (Chambers et al., 2009; Fasano et al., 2010; Saurat et al., 2016; Y. Shi et al., 2012), followed by thorough optimization processes.

3.2 Characterization of Differentiated Cortical Neurons

The identity of the differentiated cells was evaluated by using Western Blot and immunocytochemistry, utilizing relevant markers.

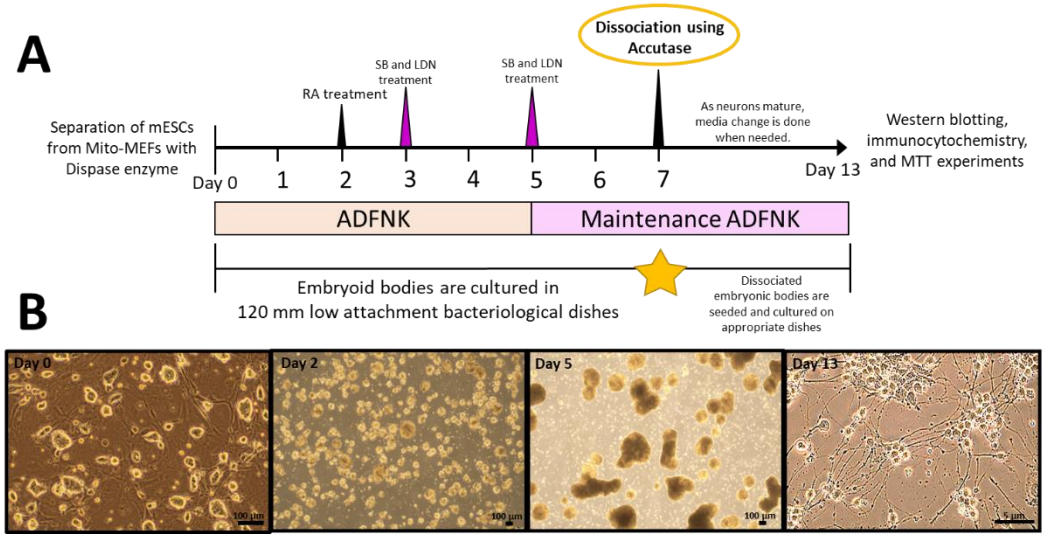

Figure 3.3. Directed differentiation of WT and TrkB-KFG mESCs into cortical neurons.

(A) The differentiation protocol used to obtain cortical neurons is summarized. The factor treatments involved in the 13-day process are emphasized. (B) Important days regarding the differentiation protocol are shown via microscope images taken on day 0, day 2, day 5, and day 13, respectively. On day 0, the ideal stem cell morphology needed to start the differentiation process is depicted, where the stem cells were still co-cultured on MitoMEFs. On day 2, embryoid body formation was observed and on day 5, the embryoid bodies became larger in size and darker in color. Lastly on day 13, the differentiation process reached its end after dissociation on day 7. Cortical neurons were showing their fully formed extensions, which is a distinct neuron morphology.

3.2.1 Cortical Neuron Differentiation Process and Trk Levels are Verified in Protein Level

We first utilized Western Blot to determine the protein levels of specific marker expression changes during the directed differentiation. To do so, cell lysates were collected at various stages of the cortical neuron differentiation process. As shown in **Figure 3.4**, the stem cell markers SSEA-1, Sox-2, and Oct3/4 were observed at day 0 and they disappear towards to end of the differentiation process. This is an expected result as stem cell marker downregulation is well-documented in the literature during differentiation (Chambers et al., 2009; Kiris et al., 2011). A neuron marker, β-III Tubulin (Tuj1), was expressed mainly on day 10 and day 13, suggesting successful neuronal differentiation. Similarly, another neuronal marker Tau5 (total tau) was also expressed toward the end of the differentiation. These data suggest successful neuronal differentiation. We have also examined the expressions of synaptic markers SNAP-25 and VAMP-2, and our data demonstrate that both were present in neuronal cultures (towards the end of the differentiation) as expected (Gomes, Hampton, El-Sabeawy, Sabo, & McAllister, 2006; Nazir et al., 2018). Lastly, the TrkB receptor levels were investigated to check whether the receptor is present in these cortical neuron cultures. We have utilized a well-characterized TrkB antibody and located the TrkB bands based on the band sizes in light of our experience and the literature. Our data suggested the presence of TrkB in both WT and TrkB-KFG cultures although further investigation (shown in **Figure 3.5**) was needed to conclude the TrkB existence in these cultures due to non-specific bands. All in all, our initial analyses suggest that stem cells were differentiated towards the neural lineage.

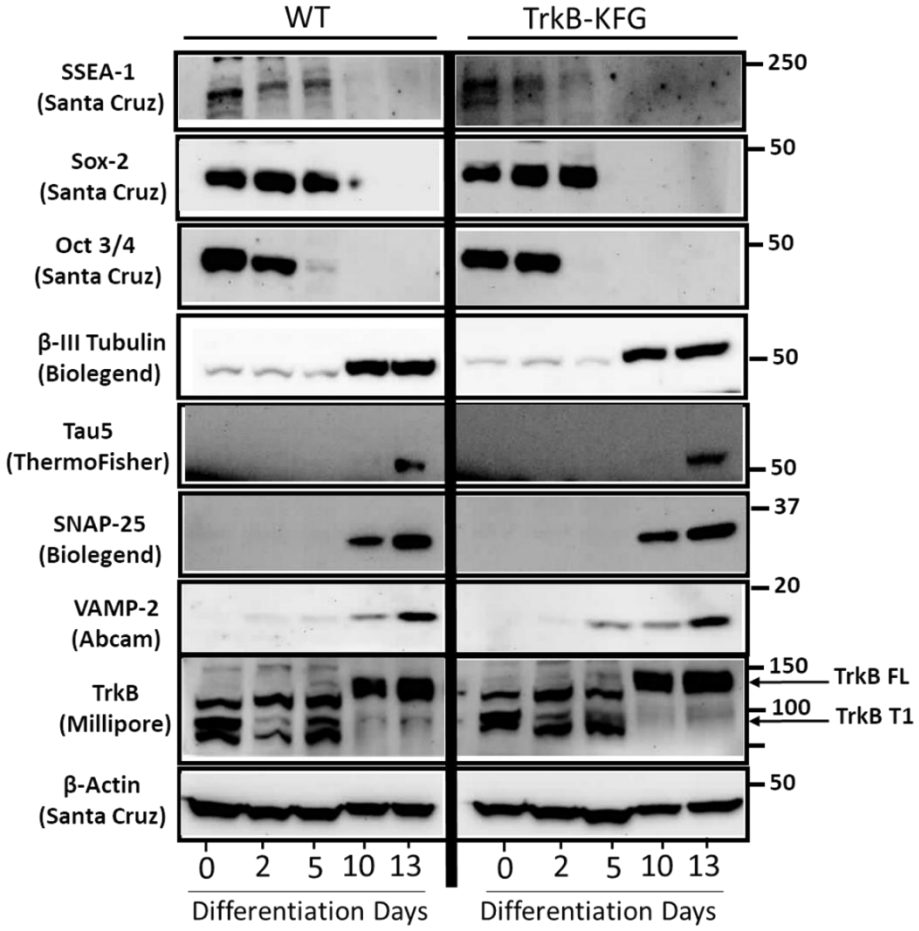

Figure 3.4. Cortical neurons are characterized using Western Blot.

The differentiation process was examined using protein lysates that were collected on selected days of the differentiation process, which are days 0, 2, 5, 10, and 13. WT and TrkB-KFG derived differentiation process was investigated individually, using identical markers. Western blot for stem cell markers (SSEA-1, Sox-2, and Oct3/4), general neuronal markers (β-III tubulin and Tau5), and synaptic markers (SNAP-25 and VAMP2) were used. Additionally, TrkB receptor level changes through the differentiation process were observed, comparing WT and TrkB-KFG cells. β-actin was the loading control. The image for either marker is representative of at least 3 different biological replicas obtained from separate differentiation sets. The images were taken via SynGene Imaging System.

While the investigation of differentiation progress is crucial, determining whether TrkB is present in these cortical neurons was critical for his study as the TrkB is in a central role in the questions asked in this work. Therefore we have specifically focused on the last portions of the differentiation process and generated additional controls to be used in Western Blot analyses to evaluate TrkB existence in these cultures, as presented in **Figure 3.5**.

TrkB receptor, BDNF, and the interactions of this receptor-ligand pair are well known to have vital functions in the nervous system (Pinton et al., 2017). The TrkB gene (named *Ntrk2*) has 24 exons that lead to many different isoforms due to mRNA splicing (Dorsey et al., 2006; Tomassoni-Ardori et al., 2019). Of these isoforms, two are known to have important functions in neurons: the full length (TrkB.FL) and the truncated one (TrkB.T1) (Dorsey et al., 2006). While TrkB contains a kinase domain that takes a central role in neuronal survival and normal neuron function, TrkB.T1 is also shown to be important for BDNF-related pathways (Saragovi, Galan, & Levin, 2019). These two isoforms are known to coexist in neurons, and even have competitive interactions that alter their function (Cao et al., 2020). Due to these, the investigation of TrkB and TrkB.T1 in cortical neurons is important.

To examine the Trk receptor presence, we generated positive and negative controls utilizing HEK293 cells, in which no endogenous TrkB expression exists. To generate the controls, HEK293 cells were transfected with either empty plasmid (negative control), TrkB.FL plasmid, or TrkB.T1 plasmid. Compared to controls, WT and TrkB-KFG cortical neurons exhibited both the full length and the T1 isoform. These data also suggested that TrkB levels are higher in TrkB-KFG cortical neurons than in WT cortical neurons. This difference was not surprising, given that the KFG modification causes an activation of the TrkB receptor (Kiris et al., 2014). β-III Tubulin was used to show neuron presence, which showed signal for WT and TrkB-KFG, but not for HEK293 cells, as expected. Lastly, the β-actin antibody was a loading control.

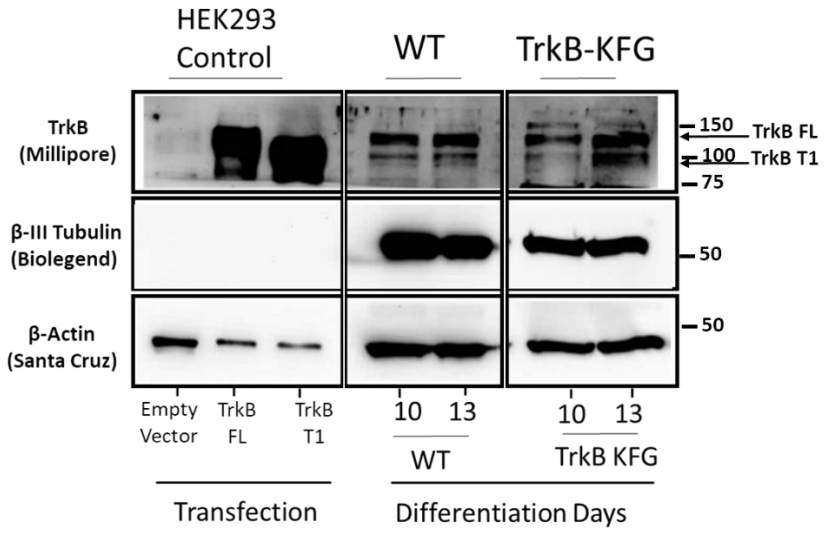

Figure 3.5. TrkB receptor levels in WT and TrkB-KFG mESC-derived differentiated cortical neurons.

Transfected HEK293 cells were used as controls, in order to detect TrkB receptor protein levels via Western Blot. HEK293 controls were transfected with either empty vector plasmid, full-length TrkB receptor plasmid (TrkB FL), or truncated TrkB receptor plasmid (TrkB T1), respectively. Both WT V6.4 and TrkB-KFG V6.4 cortical neurons at day 10 and day 13 were compared to the controls. While β-Actin is used as a loading control, β-III Tubulin was used to reveal neuron presence in the protein lysates. These results are a representation of at least 3 different biological replicas obtained from separate differentiation sets. The images were captured with SynGene Imaging System.

Similar to TrkB, TrkA levels were also investigated, shown in **Figure 3.6**, where TrkA plasmid-transfected HEK293 cell lysates were used as a control to observe TrkA levels in differentiated cortical neurons. TrkA is known to be mainly present in basal forebrain cholinergic neurons (Sanchez-Ortiz et al., 2012). While this receptor is also important for neuronal survival and functioning, similar to TrkB, it is known that cortical neurons mainly depend on BDNF-TrkB signaling among the neurotrophin family (Mu, Li, Yao, & Zhou, 1999). Our data suggested that there was no detectable level of TrkA in our cortical neuron cultures. This result is in

agreement with the literature, TrkA is not present in cortical neurons. However, TrkB levels again seem to be higher in TrkB-KFG cortical neurons (**Figure 3.6**), consistent with our previous finding, suggesting again that KFG deletion leads to increased TrkB levels in cortical neurons.

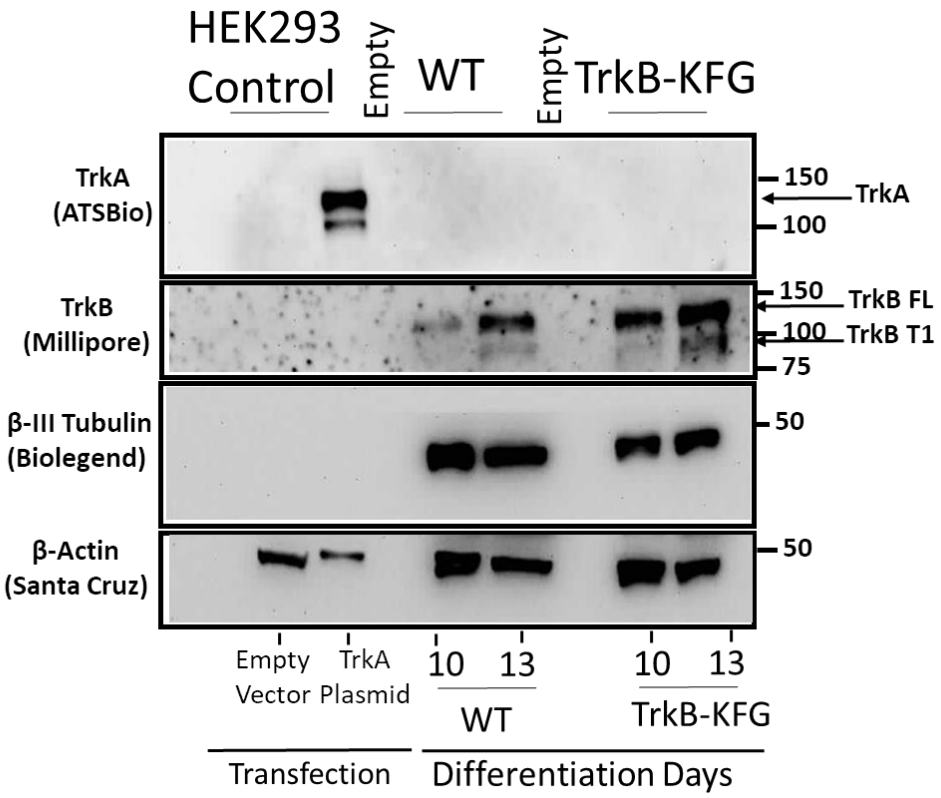

Figure 3.6. TrkA receptor levels in WT and TrkB-KFG mESC-derived differentiated cortical neurons.

HEK293 controls transfected with empty vector plasmid and TrkA plasmid were used in order to detect TrkA levels in differentiated cortical neurons on day 10 and day 13. Using the same gel setup, TrkB levels were also shown in both WT and TrkB-KFG cortical neurons. While the β-III Tubulin marker was used to indicate neurons, β-Actin served as the loading control. The images are representative of at least 3 different biological replicas produced from separate differentiation sets. The images were captured using SynGene Imaging System.

3.2.2 Cortical Neuron-Specific Characteristic is Demonstrated in Mature Neurons

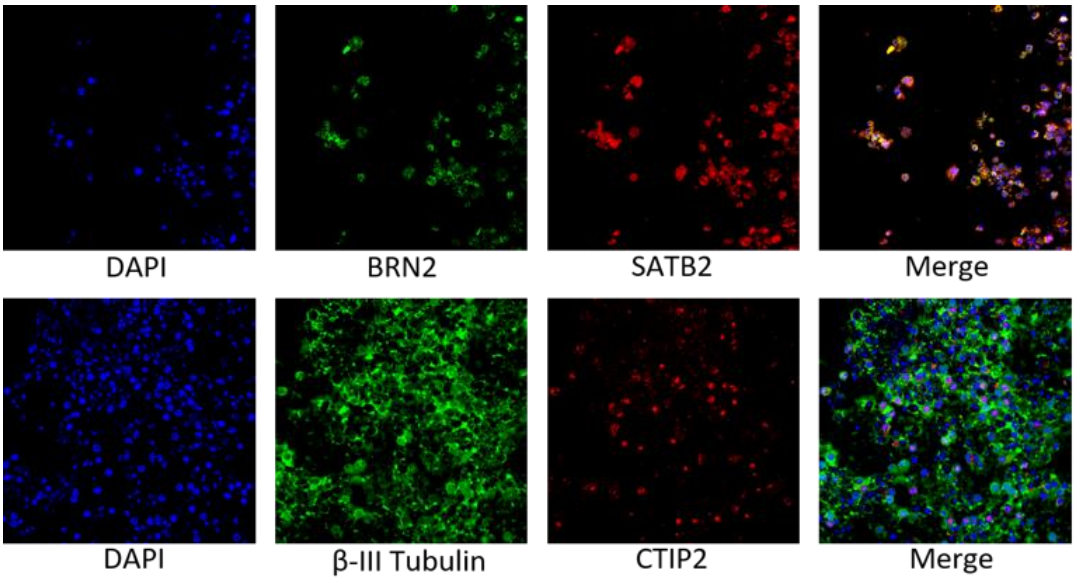

Figure 3.7. Cortical characterization of WT mESCs via immunocytochemistry.

BRN2, CTIP2, and SATB2 were used to label cortical neurons while β-III Tubulin was used as a general neuronal marker. DAPI staining shows the nucleus. Images were taken using ZEISS confocal microscope with a 40X objective, using water as the immersion medium. The images are representative of at least 5 different biological replicates that were obtained from separate differentiation sets.

In addition to determining certain protein marker expression changes during the differentiation process by Western Blot, we also performed immunocytochemistry to determine the expression of specific markers in individual cells, at the fully differentiated state. For these analyses, we have utilized well-characterized cortical markers specific cortical markers; BRN2, SATB2, and CTIP2 (Espuny-Camacho et al., 2018; Gaspard et al., 2009; Sloan, Andersen, Paşca, Birey, & Paşca, 2018; Yin et al., 2019). The results of the immunostaining were presented in **Figure 3.7** for WT, and in **Figure 3.8** for TrkB-KFG cortical neurons. The co-localization of

SATB2 and BRN2 in cortical neurons was previously shown in the literature (Eiraku et al., 2008) and in line with the literature, our results also suggest the expression and co-localization of these markers in fully differentiated cortical neurons. Our results also demonstrated that CTIP2, a well-characterized cortical marker, was present in the differentiated neurons. Neuron-specific marker β-III Tubulin was used to identify the neurons. These analyses demonstrate successful neuronal differentiation, the presence of cortical-specific markers in a subset of neurons suggests cortical identity.

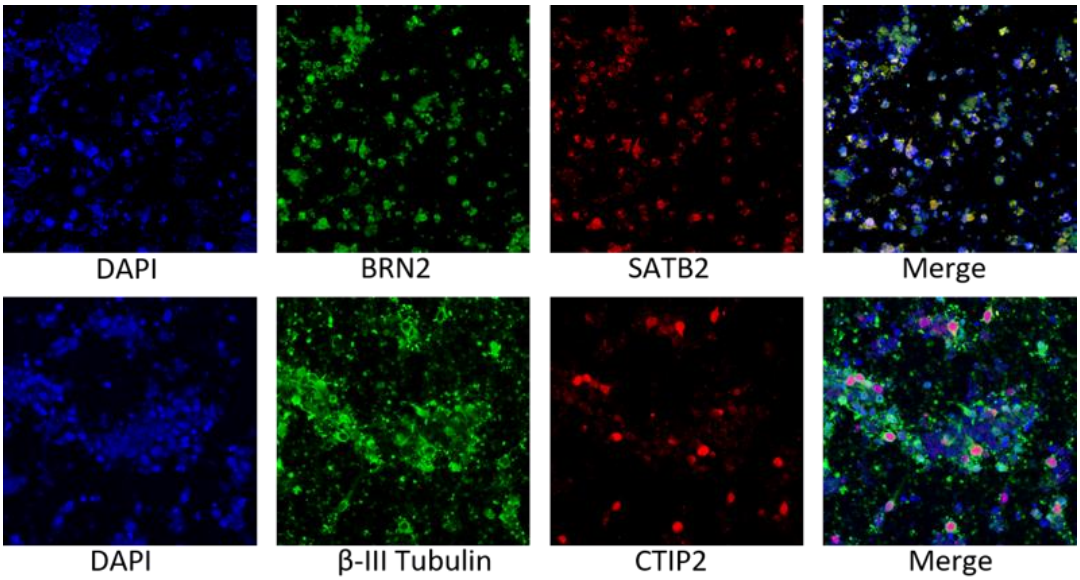

Figure 3.8. Cortical characterization of TrkB-KFG mESCs via immunocytochemistry.

BRN2, CTIP2, and SATB2 were utilized as cortical markers. β-III Tubulin was used as a general neuronal marker, and DAPI staining shows the nucleus. Images were taken via ZEISS confocal microscope with a 40X objective, using water as the immersion medium. The images are representative of at least 5 different biological replicates that were obtained from separate differentiation sets.

3.3 The Effect of Amyloid Beta (1-42) Treatments on Cortical Neurons

3.3.1 TrkB-KFG Neurons Exhibit Resistance Against Amyloid β (1-42) Mediated Neuronal Toxicity

To measure the effects of KFG domain removal in TrkB receptors on neuronal viability, we treated both WT and TrkB-KFG cortical neurons with oligomeric Aβ42. The oligomeric Aβ42 peptide was prepared, as described in **Section 2.3**, to establish an *in vitro* AD model. This model system is commonly utilized in the AD field and it is well-established that oligomeric Aβ42 is the most toxic form (Ferreira, Lourenco, Oliveira, & De Felice, 2015; Hillen, 2019; Viola & Klein, 2015). The fibrillar form of Aβ42 can be also utilized, however, our group previously showed toxicity caused by the fibrillar form is very low (Ezgin, 2022), and therefore the treatment of fibrillar Aβ42 was not included in this study. As mentioned above, the use of Aβ42 peptides as an AD-inducing agent is a widely used technique as its toxic effect is demonstrated in the literature. More specifically, Aβ42 treatment leads to cell death (Badshah, Kim, & Kim, 2015; Ebenezer et al., 2010; Kam et al., 2019; Zhao et al., 2016), alterations in cellular pathways (Badshah et al., 2015; Kam et al., 2019), and tau abnormalities (Busche & Hyman, 2020). We have utilized a well-established and commonly utilized protocol to prepare the oligomeric Aβ42 (Stine et al., 2003; Stine et al., 2010). After the preparation of the oligomeric Aβ42 peptide and its treatment on mESC-derived cortical neurons (explained in **Section 2.4**), an MTT assay was used to test cell viability, which is one of the most common techniques used to analyze the viability of neurons (Badshah et al., 2015; Kam et al., 2019).

We have utilized a 24-hour treatment as the time point, mainly because we sought to generate a model system as the experimental condition in which there is not too much cell death so that possible modest effects can be assayed, based on our previous work (Ezgin, 2022). As presented in **Figure 3.9**, the increasing doses (1 μM, 2.5 μM, 5 μM, and 10 μM) of the oligomeric Aβ42 peptide lead to a decrease in cell viability

in WT cortical neurons. However, TrkB-KFG cortical neurons showed higher cell viability upon oligomeric Aβ42 treatment, in comparison to WT cortical neurons. For example, for the highest concentration of treatment, which was 10 μM, WT cortical neurons showed 23% cell death while TrkB-KFG cortical neurons in the same condition only resulted in 9% death in the MTT assay. Although comparison at certain concentrations was not statistically significant and the differences are subtle, the overall trend was obvious. These results are supported by similar research presented in the literature where Aβ42 peptide treatment showed similar cell death (Ebenezer et al., 2010; Kam et al., 2019), as well as the neuroprotection of activated Trk signaling (Arancibia et al., 2008; Kitiyanant, Kitiyanant, Svendsen, & Thangnipon, 2011). Although it has been shown in the literature that TrkB signaling protects Aβ42-mediated neuronal toxicity (Arancibia et al., 2008; Kitiyanant et al., 2011), this piece of data demonstrates for the first time in the literature that modulation of the receptor itself by removing a specific domain can lead to resistance against Aβ42-mediated neuronal toxicity.

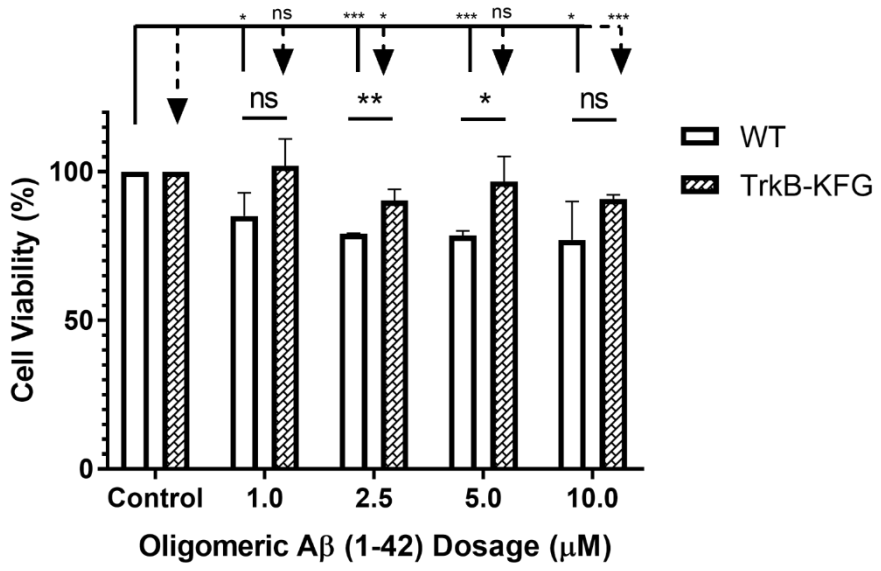

Figure 3.9. Analysis of cell viability on Amyloid β (1-42) treated WT and TrkB-KFG cortical neurons using MTT assay.

*Neurons were exposed to various concentrations of oligomeric Amyloid β (1-42) for 24 hours. Data are given as Mean ± SD. WT cortical neurons are statistically compared with TrkB-KFG cortical neurons using Student's t-test via GraphPad Prism. ***, **, and * respectively mean 99.9%, 99%, and 95% confidence levels. The bar graph is a representation of 3 different biological replicates.*

3.3.2 Treatment of Amyloid β (1-42) Leads to Changes in Erk1/2 Phosphorylation and Synaptic Protein Levels in WT and TrkB-KFG Cortical Neurons

After the determination of cell viability, in the next set of experiments, we analyzed the phosphorylation profiles of cellular survival pathways and the changes in synaptic protein levels, as presented in **Figure 3.10**. MAPK/ERK pathway is known to be affected by TrkB signaling, as well as having a role in AD pathology (Tong, 2004) and specifically, Erk1/2 phosphorylation is shown to be increased upon oligomeric Aβ peptide treatment (Kirouac et al., 2017). Consistent with the literature, our data also showed that in WT cortical neurons, Aβ42 treatment leads to increased Erk1/2 phosphorylation. On the other hand, the TrkB-KFG cortical neurons did not show an obvious increase in Erk1/2 phosphorylation upon only Aβ42 treatment. BDNF treatment only condition leads to enhanced Erk1/2 phosphorylation in both WT and TrkB-KFG neurons. This is expected as it is well established that BDNF-TrkB signaling regulates Erk1/2 phosphorylation. Aβ42 treatment slightly increased Erk1/2 phosphorylation both with and without peptide treatment in WT cortical neurons. Meanwhile, in TrkB-KFG neurons, the effect of BDNF treatment was much more drastic, with a sharp increase in only BDNF-treated cortical neurons and a noticeable decrease in TrkB-KFG cortical neurons where both BDNF and Aβ42 were treated. Although there are a few studies suggesting a combination of BDNF and Aβ treatment decreases the positive effect of BDNF (Tong, 2004), future work is needed to further explore the mechanistic basis of these observed differences. In these analyses, a Pan Trk antibody was utilized to determine general Trk receptor levels

and the data suggests slightly higher protein Trk levels in TrkB-KFG cortical neurons, which is expected, as observed in **Figure 3.5** and **Figure 3.6** with a specific TrkB antibody, TrkB-KFG neurons exhibit a higher level of TrkB receptor.

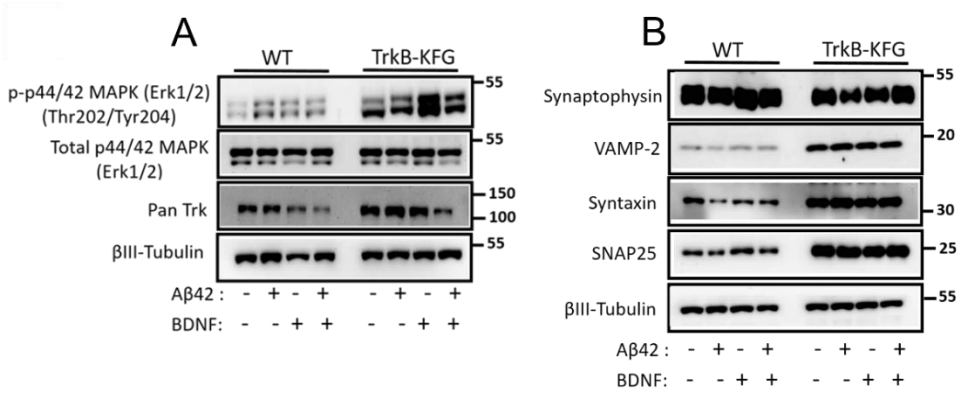

Figure 3.10. Effects of Amyloid β (1-42) treatment on WT and TrkB-KFG cortical neurons, focusing on Erk1/2 phosphorylation and synaptic protein level changes.

WT and TrkB-KFG cortical neurons were treated with either 10 μM Amyloid β (1-42), 50 ng/ml BDNF, or both for 24 hours. After treatment, protein lysates were obtained for Western Blot and analyzed for their (A) Erk1/2 phosphorylation and (B) synaptic markers. β-III Tubulin was used as a loading control. The images were captured using SynGene Imaging System and are representatives of at least 3 different biological replicas produced from separate differentiation sets.

Oligomeric Aβ42 is also known to disrupt synaptic integrity, observed through decreased synaptic protein expression (J. Liu et al., 2010), and synaptic density (Ferreira et al., 2012; D. Xu et al., 2021). Therefore, we have evaluated some key synaptic markers in both WT and TrkB-KFG neurons with and without oligomeric Aβ42 treatments. The effect of upregulated TrkB signaling on synaptic density is shown in **Figure 3.10B**, depicting synaptic protein levels of WT and TrkB-KFG cortical neurons using the synaptic markers Synaptophysin, VAMP-2, Syntaxin, and SNAP25, as well as the loading control β-III Tubulin. Overall, all these markers

except Synaptophysin were showing higher protein expression in TrkB-KFG cortical neurons regardless of treatments. In regards to Aβ42 treatment, WT cortical neurons all show a decrease in synaptic proteins, which is consistent with the literature as it has been shown that Aβ treatment disrupts synaptic density (Begni et al., 2016) and BDNF has a role in maintaining synaptic health (J.-H. Song et al., 2014). However, the combination of Aβ42 and BDNF demonstrates only a slight positive effect on the synaptic proteins in WT cortical neurons. In the case of TrkB-KFG cortical neurons, the synaptic markers show almost no change except for Synaptophysin, which decreases due to Aβ42 treatment and increases upon BDNF treatment; however, the combination of these two treatments creates an even higher level of Synaptophysin.

In the literature, one of the highly relevant downstream pathways of both AD and BDNF-TrkB is Erk1/2, which is also linked to the synaptic integrity of a neuron (Parihar & Brewer, 2010; Thomas & Huganir, 2004). The results presented above show the co-occurrence of these two cellular events, also presenting a piece of additional evidence to the positive effect of TrkB-KFG-induced TrkB upregulation since it affects both synaptic density and Erk1/2-related cellular pathways.

3.3.3 Imaging analyses of WT and TrkB-KFG neurons with and without Aβ42 treatment suggest resistance of TrkB-KFG neurons against Aβ42-mediated neuron death and synaptic degeneration

In the next stage of our investigation on the effects of upregulated TrkB signaling in *in vitro* AD conditions, we utilized quantitative immunocytochemistry-based analyses to measure neuronal death and synaptic marker level changes in the light of the literature (Decker, Lo, Unger, Ferreira, & Silverman, 2010; Knowles et al., 2009; Schedin-Weiss et al., 2017). To quantify the ICC images, software such as Image J (https://imagej.nih.gov), Image J Fiji (https://imagej.net/software/fiji/), and CellProfiler (https://cellprofiler.org) are the most frequently used programs for image-based analysis in the literature. Of these, CellProfiler (Stirling et al., 2021) was deemed the ideal one for our study due to its ability to process multiple images

in the same manner based on set parameters. The methodology followed for CellProfiler was detailed in **Section 2.6**. Other studies similar to this thesis study in the literature also utilize this program for the quantification of ICC images (Gee et al., 2020; Kuhlmann, Wagner Valladolid, Quesada-Ramírez, Farrer, & Milnerwood, 2021).

In these analyses, the effect of oligomeric Aβ42 treatment on neuronal survival was first tested using neuronal markers, β-III Tubulin (Tuj-1), NeuN, and Tau5 (for total tau) (**Figures 3.11 A, B, and C**, respectively). Comparison of WT and TrkB-KFG cortical neurons under identical conditions are presented in **Figure 3.11**, with representative images and bar graphs side-by-side. As shown in **Figure 3.11A**, Aβ42 treatment leads to a decrease in the levels of a neuronal marker, β-III Tubulin (Tuj-1), however, the negative effect was greater for WT cortical neurons as compared to TrkB-KFG cortical neurons. More specifically, TrkB-KFG cortical neurons show higher signal intensity (80.4%) upon Aβ42 treatment when compared to WT cortical neurons (62.2%). Similarly, Aβ42 –mediated signal intensity decline of other tested neuronal markers NeuN and Tau5 were also less in TrkB-KFG cortical neurons, 87.6% and 92.4% respectively; while WT cortical neurons were 66.3% and 77.1%, respectively. These results are in line with the literature since Aβ is shown to affect neuron health, observed via imaging-based techniques through neuronal markers (Calkins & Reddy, 2011). The higher neuronal marker intensities of TrkB-KFG cortical neurons, compared to WT cortical neurons indicate the preserved neuron survival, which suggests neuroprotection due to upregulated BDNF-TrkB signaling, and these results are consistent with our cell viability data described above.

Using a similar imaging-based approach, we have also measured synaptophysin and VAMP2 to gain more insight into synaptic density to complement our findings based on Western Blot analyses. Our results demonstrate that Aβ42 treatment leads to a decrease in synaptophysin levels in both WT and TrkB-KFG neurons, with signal intensities of 48.6% and 78%, respectively. However, VAMP2 did not confer a statistically significant result, yet showed a similar trend of higher signal intensity in TrkB-KFG cortical neurons in comparison to WT cortical neurons, with 87% and

75%, respectively. These findings are supported by the literature since Aβ presence is known to affect synapse structure determined via synaptic antibodies using imaging-based analyses (Calkins & Reddy, 2011; Tomiyama et al., 2010). Overall our data suggest that TrkB upregulation by KFG removal in cortical neurons has a beneficial effect against the detrimental effects of Aβ42 treatment on synaptic density and neuronal survival.

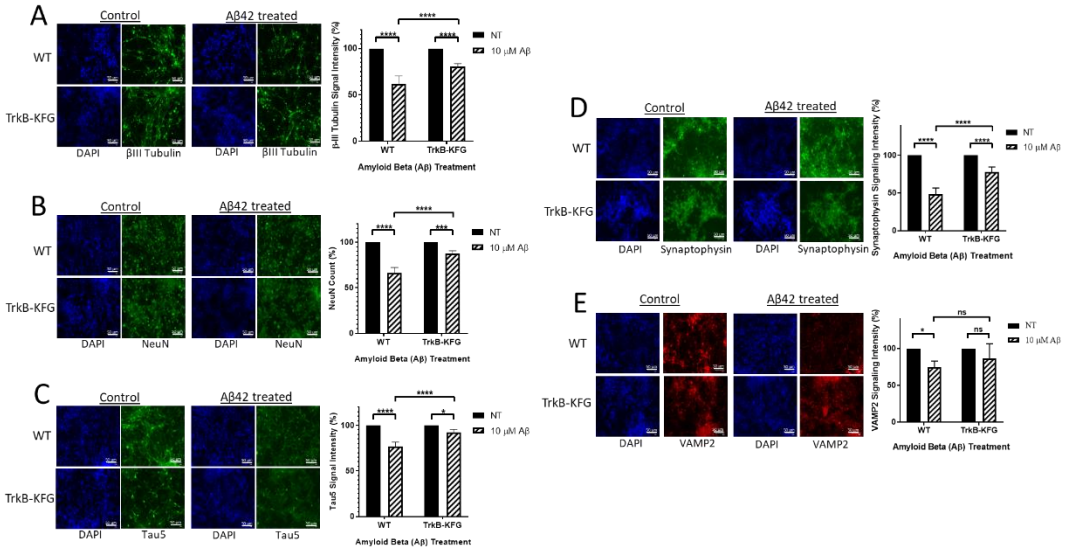

Figure 3.11. Analysis of neuronal surival and synaptic density upon Amyloid β (1-42) treatment on WT and TrkB-KFG cortical neurons using immunocytochemistry quantification via CellProfiler.

*Images obtained from FLoid Imaging Station (Scale bar, 50 μM) after Amyloid β (1-42) treatment are shown, with neuronal markers on the left and synaptic markers on the right side of the figure. Besides the images, the corresponding bar graph was placed. Images are a representation of at least 4 different biological replicas. (A) βIII Tubulin, (B) NeuN, (C) Tau5, and (D) Synaptophysin (E) VAMP2 markers were analyzed using at least 4 images. For statistical analysis, GraphPad Prism was used. Data was obtained with CellProfiler and are shown as Mean ± SD and compared with Two-way ANOVA; ***, **, * respectively mean 99.9%, 99% and 95% confidence levels.*

To summarize, in this part of the study the established *in vitro* AD model was tested for its effect against neuronal survival, changes in synaptic marker levels, Erk1/2 pathway phosphorylation changes, as well as neuronal survival and synaptic density in WT and TrkB-KFG cortical neurons. We observed a significant difference between WT and TrkB-KFG cortical neurons through the tested parameters, which implies resistance against AD-like conditions due to the upregulated BDNF-TrkB signaling which acts to prevent decrease in cell viability, synaptic damage, neuronal death, and adverse regulation of Erk1/2 pathway.

3.4 Bioinformatics Analysis Shows The Relevance of Erk1/2 and BDNF-TrkB Signaling in Alzheimer's Disease Context

AD is a multifactorial disease studied by many different fields through a variety of perspectives; hence, years of research came up with many different pathways that are related to both each other and AD pathology (Morroni, Sita, Tarozzi, Rimondini, & Hrelia, 2016). These relationships have been forming different pathway maps that are made by bioinformaticians, using data obtained by AD researchers. Using these, the relevance of BDNF-TrkB signaling and Aβ-related AD mechanisms were summarized in **Figure 3.12**. Using the widely utilized Kyoto Encyclopedia of Genes and Genomes (KEGG) (Kanehisa & Goto, 2000), two maps were merged, which are Alzheimer's disease (hsa05010) and Neurotrophin signaling pathway (hsa04722) maps.

Based on our cell viability assays, detection of synaptic protein levels as well as Erk1/2 phosphorylation levels, and lastly the imaging-based-quantification analyses, these findings were combined with cumulative data obtained from KEGG analysis for signaling pathways. Especially focusing on BDNF-TrkB signaling, AD-related pathways were considered. KEGG analysis shown in **Figure 3.12** suggests Erk1/2, as well as, phosphoinositide 3-kinase (PI3K), protein kinase B (Akt), and Glycogen synthase kinase-3 β (GSK3β) to be important pathways relating to our hypothesis, summing up the findings.

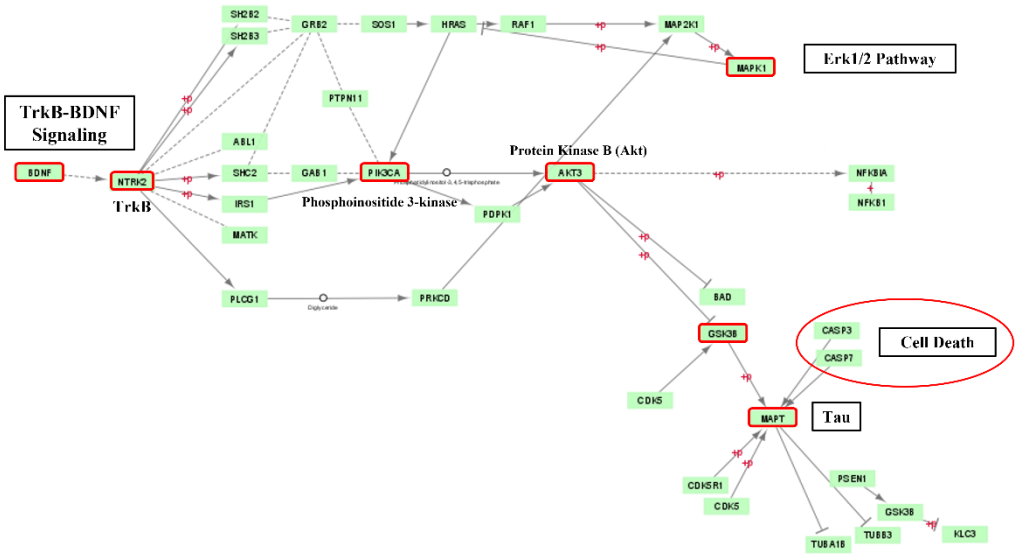

Figure 3.12. KEGG Analysis of TrkB-Signaling Pathway and its associated downstream pathways.

The represented figure was obtained using Cytoscape and its plugins (apps) KEGGScape, CytoKEGG, and CyKEGGParser. BDNF-TrkB signaling and the important downstream pathways were marked with red, as well as indications for the Erk1/2 pathway, tau, and cell death.

Starting with BDNF-TrkB signaling, PI3K and Akt pathways are triggered and their activation leads to GSK3β and tau pathways (Elliott, Atlas, Lange, & Ginzburg, 2005; Pant, Jain, Baitharu, Prasad, & Ilavazhagan, 2013; Yuan et al., 2017). As mentioned in **Section 1.2**, tau is a hallmark of AD. Ultimately, this pathway that starts with BDNF-TrkB signaling leads to caspase-mediated cell death pathways, suggesting relevance and proposing a possible effect. Another branch of the BDNF-TrkB signaling relevant to AD leads to Erk1/2, through PI3K and Akt pathways. These connections are shown in previous research from the literature as well; the Aβ-mediated effect initially starts with MAPK/ERK and PI3K pathways (Tong, 2004), which can also be activated by BDNF (J.-l. Du & Poo, 2004; Heerssen & Segal, 2002). A previous study suggested that BDNF-TrkB signaling can prevent cell death through MAPK/ERK and PI3K pathways (Hetman, Kanning, Cavanaugh,

& Xia, 1999). To sum up, our analyses suggest that MAPK/ERK and PI3K are the primary targets of the negative effect Aβ causes, which can be mediated through upregulated BDNF-TrkB signaling, leading to neuroprotection against neurodegeneration due to AD.

CHAPTER 4

DISCUSSION

Despite decades of research, there is no definitive cure for Alzheimer's Disease (AD) and its underlying disease mechanism is still unclear (Magalingam, Radhakrishnan, Ping, & Haleagrahara, 2018). Although there have been recently approved drugs (aducanumab, lecanemab, and GV-971 which are discussed in **Section 1.4**), the effectiveness of these drugs is heavily debated (Couzin-Frankel & Piller, 2022; P.-P. Liu et al., 2019; Reardon, 2023). The biggest risk factor for AD is age, and as the world's population continues to age, AD is projected to become even more prevalent. Among many hypotheses that were proposed to explain AD, the Aβ hypothesis is the most popular yet the most discredited and reinvented one (Kametani & Hasegawa, 2018; Kepp & Muñoz, 2016). While this hypothesis is still changing and evolving, being used in studies and drug development efforts, novel approaches focusing on mechanisms other than amyloid pathology may be crucial to develop effective treatments against this disease. Neurotrophins (NTs) and their receptors have been studied heavily in the context of AD, due to the changes in their levels in the pathological state (Caffino et al., 2020; Gao et al., 2022), and also their relevance to neuronal health in normal conditions (Nordvall et al., 2022; Scott-Solomon & Kuruvilla, 2018). However, despite significant efforts on their exogenous administration (Caffino et al., 2020) and several other ways of activation, such as via small molecule agonists to ameliorate AD pathogenesis, no significant advancement has yet been achieved.

4.1 Effect of KFG Deletion on TrkB Receptor

To upregulate BDNF-TrkB signaling, this study utilized a model system in which the KFG domain is deleted in TrkB receptors. In a previous study, our group

demonstrated the importance of the conserved KFG domain of Trk receptors in its ubiquitination process, which in turn has a critical role in Trk receptor levels and activation, using an *in vivo* model (Kiris et al., 2014). The importance of the KFG domain in Trk receptors was verified in another study, showing that KFG is one of the main ubiquitination sites of Trk receptors (Emdal et al., 2015). This thesis study focuses on the deletion of the KFG region from the TrkB receptor and testing its effect upon Amyloid β (1-42) (Aβ42)-mediated *in vitro* AD model. The results suggest a higher TrkB expression in TrkB-KFG-modified cortical neurons when compared to WT cortical neurons. In addition, TrkB-KFG cortical neurons demonstrated neuroprotection against Aβ42 treatments, resulting in higher cell viability (**Figure 3.9**), relatively diffused effect on cell survival pathways (Erk1/2) (**Figure 3.10A**), higher synaptic density (**Figure 3.10B**), and higher neuronal survival (**Figure 3.11A, 3.11B, 3.11C**) and synaptic density (**Figure 3.11D, 3.11E**). This effect was expected since BDNF levels are known to decrease due to AD pathology, and the increase in BDNF-related pathway activities is shown to be neuroprotective (Saragovi et al., 2019). When taken together, this information suggests that the upregulation of Trk receptors could lead to therapeutic approaches against AD; even though the NT levels or Trk activity is low in AD patients, cellular-based or NT-based treatments could still be effective. This outcome suggests that the KFG deletion is an important starting point for activating Trk receptors through modulation of the receptor's specific domains.

4.2 BDNF-TrkB Signaling Upregulation: Cell Viability, Signaling Pathways, and Synaptic Density

This thesis study suggests that the upregulated TrkB signaling protects cortical neurons against Aβ42-mediated pathology by affecting cell viability, signaling pathways, and synaptic density. The positive effect of the BDNF-TrkB signaling pathway was previously suggested and considered as a therapeutic approach against AD (Caffino et al., 2020; Pinton et al., 2017). In addition, a significant body of

research demonstrated neuroprotection due to BDNF-TrkB signaling (Arancibia et al., 2008; Kitiyanant et al., 2011; Tong, 2004). It is widely known and studied that synaptic damage, memory loss, and neurodegeneration can be overcome using BDNF-TrkB signaling (Braun, Kalinin, & Feinstein, 2017; Josephy-Hernandez, Jmaeff, Pirvulescu, Aboulkassim, & Saragovi, 2017; Nagahara et al., 2009).

The cell death caused by Aβ42 treatment was observed in *in vitro* (Kirouac et al., 2017; L. Song et al., 2020) and *in vivo* (Arancibia et al., 2008; Zhao et al., 2016) experiments. Aβ42 was also linked to AD-related pathways (Tong, 2004; Zhao et al., 2016) and cellular events (Arancibia et al., 2008; Parihar & Brewer, 2010). Meanwhile, the importance of BDNF-TrkB signaling on neuronal plasticity, neurogenesis, and neuronal survival was previously reported (Lu, Christian, & Lu, 2008; Saragovi et al., 2019). In parallel, activation of BDNF-TrkB signaling was also shown to prevent Aβ-mediated cell death (Arancibia et al., 2008). In our study, these findings from the literature were validated, as the activation of BDNF-TrkB signaling via KFG deletion caused significant protection against neuronal death, which is shown in the cell viability analysis and imaging-based-quantification that used neuronal markers.

Pathways that potentially mediate the death or the survival of the cortical neurons were tested and most importantly, changes in Erk1/2 phosphorylation were reported in this thesis. This pathway is known to be the downstream of the BDNF-TrkB pathway (**Figure 3.12**) and its relationship with Aβ toxicity in AD was also shown in the literature (Tong, 2004). Erk1/2 signaling is known to be critical for synaptic plasticity and long-term potentiation (Davis, Vanhoutte, Pagès, Caboche, & Laroche, 2000), and BDNF-TrkB signaling was also shown to mediate neuronal survival through Erk1/2, in addition to PI3K (J.-l. Du & Poo, 2004; Heerssen & Segal, 2002). Concerning Erk1/2, PI3K works together with the Akt pathway (Yoshii & Constantine-Paton, 2010). Through the PI3K/Akt pathway, inhibition of GSK3β was also reported (Grimes & Jope, 2001) which is also reported to be controlled by NT-related pathways (Hetman et al., 1999). In our data, Erk1/2 phosphorylation showed an increase in Aβ42 treated WT cortical neurons whereas in TrkB-KFG cortical

neurons there was no obvious increase. This is in agreement with the literature, as upregulated BDNF-TrkB signaling pathway can affect Erk1/2, and through this, the upregulated BDNF-TrkB can confer protection against Aβ-mediated pathology. In addition, the synaptic density of TrkB-KFG cortical neurons was preserved in the face of Aβ42 treatment, which could be argued to be through the interaction of BDNF-TrkB modulation of Erk1/2. As explained in **Section 3.4**, other pathways are related to the Erk1/2 pathway and they can be key to understanding the mechanism of AD, which should be explored as a continuation of this study.

Synaptic degeneration is one of the hallmarks of AD, after tau and Aβ-related toxicities (Selkoe & Hardy, 2016). Affected by these, the cortical region of the brain is reported to have highly degenerated upon AD pathology (A. T. Du et al., 2006). Normally expressed in high levels, TrkB levels are known to be lower in AD-affected cortical regions (Ferrer et al., 1999). On the other hand, BDNF-TrkB signaling has a distinct effect of increasing synaptic density (Park & Poo, 2012), which naturally retracts in the case of lower BDNF-TrkB signaling or levels (Genoud, 2004; Pozzo-Miller et al., 1999). In this thesis, the synaptic protein levels, as well as synaptic density were reported to be higher in TrkB-KFG cortical neurons in comparison to WT cortical neurons. Taken together, the effect of upregulated BDNF-TrkB signaling showed a parallel effect to the ones suggested in the literature in terms of cell viability, signaling pathways, and synaptic density. These results also suggest that the upregulated BDNF-TrkB signaling can also affect synaptic density and its maintenance in AD conditions.

4.3 Therapeutic Approaches and The Potential Utility of KFG Domain Modulation

Even though AD has been investigated for decades and NTs were tested as therapeutic tools in many different ways; there is still no disease-modifying cure for AD. The activation of NT signaling was trialed mainly using small molecules which failed due to their nonspecific effects, short half-lives, and roadblocks associated

with their administration since many of them failed to penetrate the blood-brain barrier. Recently a small molecule is tested in clinical trials, although other researchers pointed out its shortcomings where it could have off-target effects (Boltaev et al., 2017; Hetman et al., 2014). Other small molecule approaches were also adopted yet reasons regarding the pharmacologic properties led to failure (Hetman et al., 2019; Saragovi et al., 2019). Alternatively, viral-based or infusion methods were used to solve the problem with the blood-brain barrier, which also failed (J. Allen, J. Watson, & Dawbarn, 2011). Another study that took place for 11 years suggested that the *in vivo* gene transfer showed effects of NT in AD patients (Tuszynski et al., 2015). Even though some of these treatments are still being trialed and are showing promise for the future, to our knowledge, there are no studies that aim to increase the sensitivity of Trk receptors through their modification like KFG deletion, and ultimately pose resistance against AD pathology in neurons.

Traditional therapeutic approaches generally aim to affect the already existing cells or organs of patients, while more state-of-the-art alternatives aim for cell therapies. Regenerative medicine has been using pluripotent stem cells to treat illnesses, and the same approach could be used in AD (Kwak, Lee, Yang, & Park, 2018). Studies that involve this approach mainly focus on the introduction of new neurons to the system or neuroprotection through paracrine action (Bali, Lahiri, Banik, Nehru, & Anand, 2017). These studies utilize embryonic stem cells, induced pluripotent stem cells (iPSCs), or adult stem cells as tools for therapy (Bali et al., 2017). Through the transplantation of these cells, a healthy integration and a positive effect against AD are sought. These studies have yet to reach their final stages. Even though some initial results are reported to be promising, the long-term effects have been premature, causing damage instead of a cure (Ager et al., 2015; Marsh et al., 2017; Temple & Studer, 2017). In line with these therapy attempts, the results presented in this thesis study could be used in the future for iPSC-based therapies. A possible approach could be the use of CRISPR-Cas9 techniques on patient-derived iPSCs to generate the KFG deletion in TrkB receptors. Afterward, the generation of cortical neurons from the KFG-modified iPSCs could be done and these cells could be tested

for the possible resistance they might have. However, this methodology should be considered when CRISPR-Cas9 technology can generate specific effects in the absence of any non-specific, off-target ones. With the developing technology and techniques, patient-derived KFG-modified iPSCs could be used to replace defective neuron types to be transplanted back to the patients in possible clinical trials.

As an alternative to the regenerative approach, since many different signaling pathways are mentioned in association with BDNF and TrkB, while their activation is important, a combinational approach might be needed to tackle a disease like AD, which is thought to be caused by more than one thing. From a clinical perspective, it is known that one single drug does not efficiently ameliorate symptoms, and it is suggested in the literature that combinational treatments should be conducted. In line with this approach, as the mechanism of action of BDNF-TrkB signaling in the context of AD is uncovered, another effective activation or inhibition of a cellular event could be paired with already existing therapeutic approaches.

CHAPTER 5

CONCLUSIONS AND FUTURE STUDIES

In this thesis study, the effects of the upregulated BDNF-TrkB signaling in mESC-derived cortical neurons against the *in vitro* AD neurodegenerative process was evaluated, using oligomeric Aβ42 treatments. The upregulation of BDNF-TrkB was achieved based on previous findings from our group demonstrating that the removal of a specific TrkB receptor domain (KFG) leads to enhances in TrkB levels and activity. There have been various approaches utilized in the literature to upregulate BDNF-TrkB signaling, however, to the best of our knowledge, this is the first study to evaluate the importance of BDNF-TrkB signaling enhancement within the AD context, achieved through modulation of the receptor by removing a specific domain.

The conclusion of this study is given below;

1. WT and TrkB-KFG mESCs were successfully differentiated to cortical neurons and fully characterized.
2. TrkB-KFG cortical neurons exhibit higher levels of TrkB receptor compared to WT cortical neurons.
3. TrkB-KFG cortical neurons exhibit higher cell viability upon oligomeric Aβ42 treatments, compared to WT cortical neurons.
4. Western Blot and imaging analyses with specific markers suggest that oligomeric Aβ42 treatment-mediated detrimental synaptic density changes are less in TrkB-KFG cortical neurons.
5. Our bioinformatics analysis, in light of our results and the literature, suggests BDNF-TrkB signaling mediated MAPK/ERK pathway may be crucial for the observed positive effects of upregulated BDNF-TrkB signaling in Aβ42-mediated effects.

6. Future studies focusing on determining precise molecular mechanisms through which TrkB-KFG neurons exhibit resistance against Aβ42-mediated neuronal death and synaptic density changes may open new avenues for NT-based strategies against the neurodegenerative process of AD.

The multifaceted AD pathology has many unknown variables, as well as many known variables that could not be linked together. The extensive studies on AD create even more mysteries every day and one of the main ones seems to be the signaling pathways related to AD and its pathology. While there is much to uncover regarding the BDNF-TrkB pathway and its relevance to AD mechanisms, this thesis study lays the groundwork for further studies to test whether iPSC-derived neuron-based cell therapies can be a viable approach especially when these neurons include modified TrkB receptors.

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APPENDICES

A. Cell Culture Reagents

Mitomycin C (Cayman Chemicals, Cat. No. 11435): 50 mg powder was initially dissolved with DMSO in order to prepare a stock solution of 20 mg/ml. The stock solution was distributed to obtain 100 µl aliquotes and stored in -20°C. For treatments, the aliquot was initially diluted with PBS(+/+) and then final dilution was made in MEF Medium, where this unsterile is filtered using a syringe filter with 0.22 µm pores. The final concentration of Mitomycin C inside MEF Medium is 0.01 mg/ml.

Phosphate Buffered Saline with Ca^{+2} and Mg^{+2} (also named, PBS(+/+)): This solution was prepared in two parts, using Solution A and Solution B.

Solution A: The ingredients were 28.8 grams NaH_2PO_4 and 4.8 grams K_2HPO_4. Using deionized water, this solution was brought up to a final volume of 1 liter.

Solution B: The ingredients were 160 grams NaCl, 4 grams KCl, 2.66 grams $CaCl_2.2H_2O$, and 2 grams $MgCl_2.6H_2O$. Using deionized water, the ingredients were solved and the pH was adjusted to 7.4. Finally, the volume was brought up to 1 liter.

After the preparation of these two solutions, 50 ml of Solution A and 50 ml of Solution B were mixed. The solution was brought up to 1 liter with deionized water, making 1X PBS (+/+). No pH adjustment is needed since the pH will be 7.4 in the final solution.

Phosphate Buffered Saline without Ca^{+2} and Mg^{+2} (also named, DPBS): The ingredients for this solution were 23 grams NaH_2PO_4, 4 grams K_2HPO_4, 160 grams NaCl, and 4 grams KCl which were solved in deionized water. pH was adjusted to 7.4. The resulting solution was a 20X solution.

0.1% Gelatin (Serva, Cat. No. 22151.02): 0.5 grams of gelatin was dissolved in distilled water and heated inside a microwave in order to allow the powder's solubilization. The solution's final volume was 500 ml.

Dispase (Gibco, Cat. No. 17105041): It was in powder form upon arrival. To make aliquots, it was dissolved in PBS (-/-) to obtain a 10 mg/ml solution. It was filtered through a 0.22 μm syringe filter for sterilization and aliquoted into 1.5 ml tubes. Storage was at -20°C.

Retinoic Acid (Sigma, Cat. No. 2625): In powder form upon arrival, 50 mg was dissolved in 16.6 ml Dimethyl Sulfoxide (DMSO) (Serva, Cat. No. 171097) to obtain a stock concentration of 10 mM. Aliquoted appropriately, it was stored at -20°C.

SB431542 (Cayman Chemicals, Cat. No. 13031): Powder was dissolved in DMSO to obtain 20 mg/ml concentration, which was aliquoted appropriately and stored at -20°C. This was then diluted in either ADFNK or Maintenance ADFNK to reach a working concentration of 50 nM.

LDN193189 (Sigma, Cat. No. SML0559): Powder was dissolved in distilled water to a concentration of 5 mg/ml, which was then aliquoted and stored in a way that is protected from light exposure. Storage was at -20°C.

Accutase (Biolegend, Cat. No. 423201): The reagent was thawed once and aliquoted into 1 ml parts using fresh tubes, stored at -20°C. The aliquoted were thawed just before use.

Poly-D-Lysine (Sigma, Cat. No. P0899): 100 mg powder was dissolved with 100 ml distilled water, obtaining 1 mg/ml stock solution. These were aliquoted and stored at -20°C. Before use, the stock was diluted in a 1:10 ratio using distilled water, for a working concentration of 0.1 mg/ml.

Laminin (Thermo Fisher, Cat. No. 23017015): The stock solution was aliquoted and stored at -80°C. Before use, DPBS was used to dilute 1.16 mg/ml stock solution (there may be the batch-to-batch difference) to a working concentration of 2.5 μg/μl.

B. Cell Culture Media Compositions

MEF Medium: Inside DMEM, 10% Fetal Bovine Serum (FBS), 1% GlutaMAX, 1% Penicillin/Streptomycin (Pen/Strep).

Freezing Medium for MitoMEFs: 50% MEF Medium, 40% FBS, and 10% DMSO.

HEK293 Medium: DMEM based medium with 10% FBS, 2% Glutamax, and 2% penicillin-streptomycin.

V6.4 mESC Medium: DMEM based, 15% FBS, 1% Pen/Strep, 1% Glutamax, 1% Non-essential Amino Acids (NEAA), 0.1 mM β-mercaptoethanol, 1000 unit/ml Leukemia Inhibitory Factor (LIF).

Freezing Medium for V6.4 mESCs: 90% FBS and 10% DMSO.

ADFNK Medium: DMEM/F12 Medium, Neurobasal Medium, 10% Knockout Serum Replacement (KSR), 1% Glutamax, 1% Pen/Strep, and 0.1 mM β-mercaptoethanol

Maintenance ADFNK Medium: DMEM/F12 Medium, Neurobasal Medium, 1% Glutamax, 1% Pen/Strep, 1% B27 Supplement, and 0.1 mM β-mercaptoethanol.

C. Western Blot Buffers, MTT Reagents, and Immunocytochemistry Solutions

NP-40 Lysis Buffer: 4.4 gram NaCl, 5 ml NP-40, and 25 ml 1 M Tris-HCl (pH=8.0) were dissolved in distilled water, bringing the volume up to 500 ml. The solution was stored at 4°C.

Phosphatase Inhibitor (ThermoFisher, Cat.No: A32957): 1 tablet was dissolved in 1 ml NP-40 lysis buffer, producing 10X phosphatase inhibitor solution. This was stored at -20°C.

Protease Inhibitor (ThermoFisher, Cat.No: A32955): 1 tablet was dissolved in 1 ml NP-40 lysis buffer, producing a 10X protease inhibitor solution. This was stored at -20°C.

Lysis Buffer with Phosphatase Inhibitor and Protease Inhibitor: Prepared just before use, if 1 ml of NP-40 lysis buffer was used, 100 µl of 10X Protease Inhibitor and 100 µl of 10X Phosphatase Inhibitor was added.

6X Sample Buffer: Ingredients for the buffer were 3.75 ml 1 M Tris-HCl (pH=6.8), 1.2 gram SDS, 6 ml Glycerol, 700 µl Bromophenol Blue (prepared from 0.05 grams of powder and 10 ml distilled water), and 3 ml β-mercaptoethanol. These were distributed into fresh tubes into approx. 1 ml aliquots and stored at -20°C.

2X Sample Buffer: The buffer was prepared using 4 ml 10% SDS, 2 ml Glycerol, 2.4 ml 0.5 M Tris-HCl (pH=6.8), 1.6 ml distilled water, 700 µl Bromophenol Blue (prepared from 0.05 grams of powder and 10 ml distilled water), and 1 ml β-mercaptoethanol. The solution was stored as approximately 1 ml aliquots at -20°C.

10% Ammonium Persulfate (APS): 1 gram of Ammonium Persulfate powder was dissolved in distilled water, later bringing the volume up to 10 ml. This solution was kept away from light exposure and aliquoted. Storage was at -20°C.

SDS-PAGE Gels (ingredients for 1 gel):

10% Gel (Separating)		**5% Gel (Stacking)**	
Distilled water	2.88 ml	Distilled water	1.82 ml
40% Acryl:Bisacryl	1.5 ml	40% Acryl:Bisacryl	375 µl
1.5 Tris-HCl (pH=8.8)	1.5 ml	0.5 Tris-HCl (pH=6.8)	750 µl
10% SDS	60 µl	10% SDS	30 µl
10% APS	60 µl	10% APS	30 µl
TEMED	2.4 µl	TEMED	3 µl

These gels were prepared using BioRad's Mini-PROTEAN Tetra Cell Casting Module.

10X Running Buffer: In approx. 800 ml distilled water; 30 grams of Trizma Base, 144 grams of Glycine, and 10 grams of SDS powder were dissolved. After the contents were dissolved the volume was brought up to 1 liter.

1X Running Buffer: Before use, 10X Running Buffer was diluted to 1X Running Buffer, using 100 ml 10X Running Buffer and 900 ml distilled water.

10X Transfer Buffer: The ingredients were 30.3 grams of Trizma Base and 144.1 grams of Glycine. These were dissolved in distilled water, and the final volume was set to 1 liter.

1X Transfer Buffer: Before use, 10X Transfer Buffer was diluted using 100 ml 10X Transfer Buffer, 700 ml distilled water, and 200 ml Methanol, respectively.

10X TBS: 24 grams of Trizma Base and 88 grams of NaCl were dissolved in distilled water, pH was adjusted to 7.6, and the final volume was brought to l liter.

1X TBS-T: 100 ml 10X TBS and 900 ml distilled water were mixed and 1 ml Tween 20 was added.

Blocking Reagent for WB with Milk Powder: 2.5 grams of skimmed milk powder was dissolved with 1X TBS-T, with a final volume of 50 ml.

Blocking Reagent for WB with Bovine Serum Albumin (BSA): 2.5 grams of BSA was dissolved with 1X TBS-T, with a final volume of 50 ml. Without any vortexing, the solution was put on a rotator to be dissolved slowly.

Mild Stripping Buffer: Solving these ingredients inside deionized water, 15 gram Glycine, 1 gram SDS, and 10 ml Tween20 was used. Adjustment for pH was done to 2.2 and the final volume was 1 liter.

MTT Solution: Using 10 ml DPBS, 5 mg MTT powder was dissolved. The preparation was stored at 4°C for a month, away from light exposure.

MTT Solubilization Reagent: 1 gram of Sodium Dodecyl Sulfate (SDS) and 10 ml of 0.01 M HCl solution was dissolved, this was prepared roughly 1 hour before use.

Fixation Solution for ICC: Using a stock paraformaldehyde solution (Sigma, Cat. No. 15512) of 37%, a dilution was made to obtain a 4% paraformaldehyde solution to use for fixation.

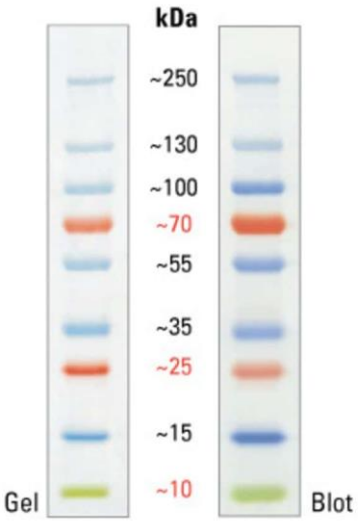
Permeabilization Solution for ICC: Diluting 100X Triton X-100 in 1X PBS (+/+), by 1:1000, a solution of 0.1% Triton X-100 was obtained for permeabilization.

Blocking Solution for ICC: 1 gram of BSA was dissolved in 50 ml of 1X PBS (+/+) buffer to achieve a 2% BSA blocking solution.

Immunostaining Solution for ICC: Secondary and primary antibodies were diluted in 0.1% BSA solution prepared with 0.05 gram BSA and 50 ml PBS(+/+). The dilutions were made according to the recommendations of antibody manufacturers.

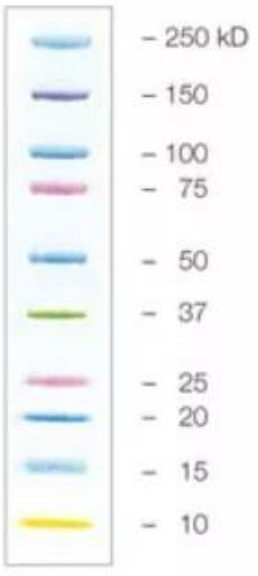
Mounting Reagent for ICC: ProLong Gold Antifade Reagent with DAPI (Thermo Fisher, Cat. No. P36931) was the mounting reagent that preserved the chamber slides closed up with a coverslip.

D. Protein Ladders



PageRuler™ Plus Prestained Protein Ladder, 10 to 250 kDa

(Thermo Fisher, Cat. No. 26619)



Precision Plus Protein™ Kaleidoscope™ Prestained Protein Standard

(BioRad, Cat. No. 1610375)

E. Supplementary Data

BAP THESIS PROJECT: Investigation of p75NTR receptor protein levels in an *in vitro* Alzheimer's Disease model established using cortical neurons with upregulated BDNF-TrkB signaling

As explained in Section 1.5, p75NTR is one of the NT receptors which is associated with inflammation, axonal damage, and cell death (Saadipour et al., 2017). This receptor has a low-affinity binding ability to all NTs (Diarra, Geetha, Potter, & Babu, 2009). In the absence of Trk receptors, NT treatment results in NT-p75NTR interaction, which leads to cell death (Cheng, Zhang, & Lian, 2015; Diarra et al., 2009). In addition to their interactions with NTs, immunoprecipitation experiments done with p75NTR suggested interactions with Trk receptors as well (Saadipour et al., 2017). In addition, the neuronal death associated with AD was suggested to be controlled by p75NTR (Murphy et al., 2015; Sotthibundhu et al., 2008), which lead to investigations on p75NTR inhibitors as a possible therapeutics (Simmons et al., 2014).

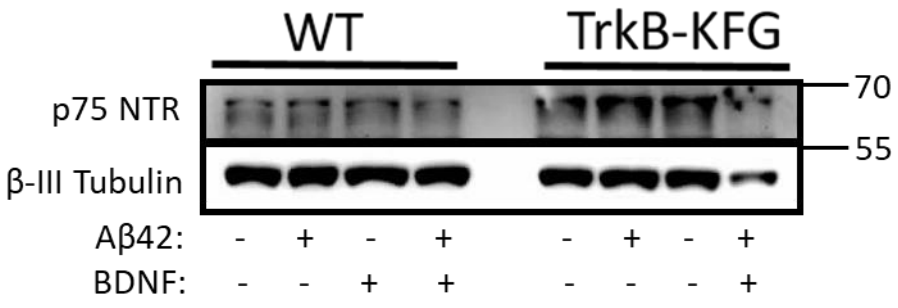


Figure 5.1 p75NTR protein expression changes in WT and TrkB-KFG neurons with and without BDNF treatment.

WT and TrkB-KFG mESC-derived differentiated cortical neurons were treated with either Aβ, BDNF, both, or none. These neurons were tested for their p75NTR expression levels, with β-III Tubulin as the loading control. Numbers on the right side of the panel indicate molecular weight in kDa.

Aim

In this part of my thesis study, the effect of upregulated BDNF-TrkB signaling in an AD context was investigated, and in this thesis project (project code: TEZ-YL-108-2022-11096) the aim was to detect p75NTR protein level changes in this experimental setting. The upregulation of BDNF-TrkB signaling, Aβ treatment, BDNF treatment, as well as a combinational approach was used to test the changes in p75NTR since it is known to have significance both in the context of AD and Trk receptors.

Results

The initial results, as shown in **Figure 5.1** as a representative, shows the increase in p75NTR in KFG-modified mESC-derived cortical neurons upon BDNF treatment. PC12 cells which endogenously express p75NTR were used to determine the correct bands for the protein, however they are not shown at this stage in our investigation. While there was no obvious increase in p75NTR levels of WT cortical neurons upon BDNF treatment, it appears that Aβ42 treatment leads to increased p75NTR levels, and the effect is more obvious in TrkB-KFG neurons. Overall, p75NTR levels were higher in TrkB-KFG cortical neurons than in WT cortical neurons. This is a surprising result that warrants further investigation. In summary, our results demonstrate that evaluation of p75NTR within the context of TrkB upregulation in AD may be critical, however, due to a limited budget, we could not further expand the research. Further studies are needed to better understand the role of p75NTR concerning TrkB-KFG mediated BDNF-TrkB signaling effect in the AD context.