# Evaluation of microneurotrophins activity on neurotrophins receptors and their role in signalling, in *in vitro* and *in vivo* neurodegenerative conditions, such as Alzheimer's Disease.

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# Αξιολόγηση της δράσης των μικρονευροτροφινών στους υποδοχείς των νευροτροφινών και ο ρόλος τους στη σηματοδότηση, σε *in vitro* και *in vivo* μοντέλα νευροεκφυλιστικών ασθενειών, όπως η νόσος Αλτσχάιμερ

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*This thesis is dedicated to my wife, my late father and his upcoming grandson.*
*I love you all very much.*

Table of Contents

## Abstract

Alzheimer's Disease is a devastating neurodegenerative disorder, which currently affects more than 50 million people worldwide. Clinically, the disease is characterized by cognitive impairment, including severe memory loss, that results in patients having difficulties in performing simple everyday tasks. Pathologically, the hallmarks of the disease include the deposition of amyloid-β plaques extracellularly and the presence of hyperphosphorylated neurofibrillary tau tangles intracellularly. Neurotrophins are a family of secreted proteins, with Nerve Growth Factor (NGF) and Brain-Derived Neurotrophin Factor (BDNF) as the most prominent family members. These control neuronal development and survival and have also been shown to modulate neuronal and synaptic function in adult brain, acting through specific high-affinity receptors, namely TrkA for NGF and TrkB for BDNF. Moreover, all neurotrophins activate the p75 pan-neurotrophin receptor although with variable affinity. Many studies have shown that neurotrophins' processing and expression levels are deregulated in AD, and this has been postulated to significantly contribute to the disease pathophysiology. Our lab has previously identified BNN27 and BNN20, two small molecules DHEA derivatives that act through TrkA, TrkB and p75 receptor in a way that resembles some of the NGF or BDNF actions, such as protecting specific neuronal populations against apoptosis. Such molecules present great potential as therapeutic agents against neurodegenerative diseases, as they can selectively mimic only specific, beneficial neurotrophic actions while avoiding undesired side effects. For the present PhD thesis, we screened and identified a series of neurotrophin analogues, which can selectively bind and activate neurotrophin receptors. Specifically, we used cell lines to identify novel small molecule DHEA derivatives that can selectively activate TrkA and can protect against cell death. These molecules, along with few more selective for TrkB were then screened using primary neuronal cells challenged with the toxic Aβ oligomers for their ability to protect cells against Aβ-mediated cell death and synapse degeneration. Lastly, we selected one potent TrkB agonist and tested it *in vivo* in the 5xFAD animal model of AD. 4-month-old animals were treated for 2 months with the drug and the efficacy of the drug to reverse the behavioural deficits was evaluated. More specifically, we showed that ENT-A061-treated 5xFAD animals showed improved ability to discriminate between environments based on context compared to placebo-treated 5xFAD littermates in the contextual fear discrimination paradigm, and similar to WT animals, a process that has been correlated with increased neurogenesis. In conclusion, ENT-A061 showed promise in improving certain aspects of AD pathology by enhancing adult hippocampal neurogenesis.

## Περίληψη

Η νόσος Αλτσχάιμερ είναι μια καταστροφική νευροεκφυλιστική διαταραχή, η οποία πλήττει σήμερα περισσότερα από 50 εκατομμύρια άτομα παγκοσμίως. Κλινικά, η νόσος χαρακτηρίζεται από σοβαρή απώλεια μνήμης και γνωστική εξασθένιση που έχει ως αποτέλεσμα οι ασθενείς να αντιμετωπίζουν δυσκολίες στην εκτέλεση απλών καθημερινών εργασιών. Παθολογικά, τα χαρακτηριστικά γνωρίσματα της νόσου περιλαμβάνουν την εναπόθεση πλακών αμυλοειδούς-β εξωκυτταρικά και την παρουσία υπερφωσφορυλιωμένων νευροϊνιδιακών δεσμών tau ενδοκυτταρικά. Οι νευροτροφίνες είναι μια οικογένεια εκκρινόμενων πρωτεϊνών, με τον Nerve Growth Factor (NGF) και τον Brain Derived Neurotrophin Factor (BDNF) ως τα πιο γνωστά μέλη της οικογένειας. Αυτές ελέγχουν την ανάπτυξη και την επιβίωση των νευρώνων και έχει επίσης δειχθεί ότι διαμορφώνουν τη νευρωνική και συναπτική λειτουργία στον ενήλικο εγκέφαλο, δρώντας μέσω ειδικών υποδοχέων υψηλής συγγένειας, του TrkA για τον NGF και του TrkB για τον BDNF. Επιπλέον, όλες οι νευροτροφίνες ενεργοποιούν τον παν-νευροτροφινικό υποδοχέα p75, αν και με χαμηλότερη συγγένεια. Πολλές μελέτες έχουν δείξει ότι τα επίπεδα επεξεργασίας και έκφρασης των νευροτροφινών απορρυθμίζονται στη ΝΑ και αυτό έχει θεωρηθεί ότι συμβάλλει σημαντικά στην παθολογία της νόσου. Το εργαστήριό μας έχει προηγουμένως εντοπίσει τα BNN27 και BNN20, δύο μικρά μόρια παραγώγων της DHEA που δρουν μέσω των υποδοχέων TrkA, TrkB και p75 και μοιάζουν με ορισμένες από τις δράσεις του NGF ή του BDNF, όπως η προστασία συγκεκριμένων νευρωνικών πληθυσμών από την απόπτωση. Τέτοια μόρια παρουσιάζουν μεγάλες δυνατότητες ως θεραπευτικοί παράγοντες κατά των νευροεκφυλιστικών ασθενειών, καθώς μπορούν να μιμηθούν επιλεκτικά μόνο συγκεκριμένες, ευεργετικές νευροτροφικές δράσεις, αποφεύγοντας τις ανεπιθύμητες παρενέργειες. Για την παρούσα διδακτορική διατριβή, ελέγξαμε και ταυτοποιήσαμε μια σειρά αναλόγων νευροτροφίνης, τα οποία μπορούν να δεσμεύουν και να ενεργοποιούν επιλεκτικά υποδοχείς νευροτροφίνης. Συγκεκριμένα, χρησιμοποιήσαμε κυτταρικές σειρές για τον εντοπισμό νέων μικρών μορίων DHEA παραγώγων που μπορούν να ενεργοποιήσουν επιλεκτικά τον TrkA και μπορούν να προστατεύσουν από τον κυτταρικό θάνατο. Τα μόρια τα οποία εντοπίστηκαν από το πρώτο αυτό έλεγχο, μαζί με άλλα ακόμη εκλεκτικά για τον TrkB, υποβλήθηκαν στη συνέχεια σε έλεγχο, χρησιμοποιώντας πρωτογενή νευρωνικά κύτταρα που προσβλήθηκαν με τα τοξικά ολιγομερή Αβ, για την ικανότητά τους να προστατεύουν τα κύτταρα από τον κυτταρικό θάνατο και τον εκφυλισμό των συνάψεων με τη μεσολάβηση του Αβ. Τέλος, επιλέξαμε έναν ισχυρό αγωνιστή TrkB και τον δοκιμάσαμε in vivo στο ζωικό μοντέλο 5xFAD

της AD. Τα ζώα ηλικίας 4 μηνών έλαβαν θεραπεία για 2 μήνες με το φάρμακο και αξιολογήθηκε η αποτελεσματικότητα του φαρμάκου για την αναστροφή των συμπεριφορικών ελλειμμάτων. Πιο συγκεκριμένα, δείξαμε ότι τα ζώα 5xFAD που έλαβαν θεραπεία με ENT-A061 παρουσίασαν βελτιωμένη ικανότητα διάκρισης μεταξύ περιβαλλόντων με βάση το πλαίσιο σε σύγκριση με τα συνομήλικά τους ζώα 5xFAD που έλαβαν εικονικό φάρμακο στο παράδειγμα διάκρισης φόβου με βάση το πλαίσιο, και παρόμοια με τα ζώα WT, μια διαδικασία που έχει συσχετιστεί με αυξημένη νευρογένεση. Το ENT-A061 έδειξε ότι υπόσχεται να βελτιώσει ορισμένες πτυχές της παθολογίας της NA ενισχύοντας την ενήλικη νευρογένεση του ιππόκαμπου. Συμπερασματικά, τα ευρήματα που παρουσιάζονται σε αυτή τη διατριβή αποκαλύπτουν την ανακάλυψη πολλών νέων ενώσεων που στοχεύουν και ενεργοποιούν ειδικά τη σηματοδότηση των νευροτροφινών, με έμφαση στους υποδοχείς TrkA και TrkB. Μέσω μιας ολοκληρωμένης αξιολόγησης αυτών των ενώσεων, προσδιορίστηκαν τα προφίλ ενεργοποίησης ενός εκτεταμένου καταλόγου ενώσεων και παρασχέθηκαν στοιχεία που υποστηρίζουν την ενεργοποίηση και την αποτελεσματικότητα ενός επιλεγμένου υποσυνόλου αυτών των ενώσεων. Τα αποτελέσματα αυτά χρησιμεύουν ως στέρεη βάση για τη συνεχή ανάπτυξη και βελτίωση αυτών των ενώσεων ως δυνητικών θεραπευτικών παραγόντων για τη θεραπεία νευροεκφυλιστικών ασθενειών. Αποδεικνύοντας την ικανότητά τους να ενεργοποιούν τη σηματοδότηση των νευροτροφινών, οι ενώσεις αυτές έχουν τη δυνατότητα να έχουν σημαντικό αντίκτυπο στη θεραπεία και τη διαχείριση αυτών των εξουθενωτικών καταστάσεων. Με περαιτέρω έρευνα και βελτιστοποίηση, αυτές οι νέες ενώσεις μπορεί να προσφέρουν ελπίδα για τα εκατομμύρια των ανθρώπων που πάσχουν από νευροεκφυλιστικές νόσους.

# 1. Introduction

## 1.1 Alzheimer's Disease

### 1.1.1 Epidemiology

Alzheimer's Disease (AD) is a devastating neurodegenerative disorder which accounts for approximately 70% of cases of dementia and is a global public health priority as recognized by the World Health Organization (Prince, 2015; Lane, Hardy and Schott, 2018). The first recorded case of Alzheimer's disease was documented by Alois Alzheimer in 1906, who observed a 51-year-old woman named Auguste Deter. Deter had symptoms of cognitive impairment, disorientation, delusions, and other behavioural changes. She died 4.5 years later and a neuropathologic evaluation of her brain showed widespread atrophy and specific changes in brain cell clusters. Alzheimer presented his findings in a lecture "on the peculiar disease process of the cerebral cortex" (Möller and Graeber, 1998). Currently more than 50 million are suffering from the disease worldwide and this number is expected to triple by 2050 (Prince, 2015). AD is widely considered as the most typical type of neurodegenerative dementia in the US, affecting minority populations more severely ('Alzheimer's disease facts and figures', 2019; Babulal *et al.*, 2019). It furthermore carries a great financial burden in societies, where the yearly cost of dementia in the US alone is expected to surpass 600 billion dollars (Prince, 2015). In England and Wales, dementia is one of the main causes of death and accounted for 11.6% of all deaths recorded in 2015. The incidence of AD increases with age, with 3% of people aged 65-75, 17% of those aged 75-84 and 32% of those over 84 years being diagnosed with the disease (Lane, Hardy and Schott, 2018).

### 1.1.2 Aetiology

Even though in most cases AD occur spontaneously, there is a rare form of familial AD (fAD), accounting for less than 0.5% of cases, which is caused by mutations in three genes - amyloid

precursor protein (*APP*), presenilin 1 (*PSEN1*), and presenilin 2 (*PSEN2*) (Bateman *et al.*, 2011; Lane, Hardy and Schott, 2018). The symptoms of fAD typically appear earlier than sporadic AD, usually between the ages of 30 and 50. On the other hand, 'typical' late-onset AD (LOAD) is thought to be the result of a combination of genetic and environmental factors, with an estimated 70% of AD risk attributed to genetics (Verghese, Castellano and Holtzman, 2011). The *APOE* gene for example, with its three variants (ε2, ε3, and ε4), is the primary risk factor for sporadic AD. E4 carriers have a three times greater risk of developing AD compared to non-ε4 carriers, and this risk increases to twelve times in homozygotes (Verghese, Castellano and Holtzman, 2011). Genome-wide association studies using large sample sizes have identified over 20 genetic risk factors, including inflammation, cholesterol metabolism, and endosomal-vesicle recycling pathways (Karch and Goate, 2015). Furthermore, microglial activation in response to amyloid deposition has been recognized as a critical factor in AD pathogenesis. Although each of these common risk genes confers only a small increased risk, a polygenic risk score can almost double the prediction of cases from chance (Escott-Price *et al.*, 2015). Advances in genetic research and next-generation sequencing have also revealed several low-frequency genes that confer a relatively high risk for AD and have provided insights into the pathogenesis of the disease (Lambert *et al.*, 2013).

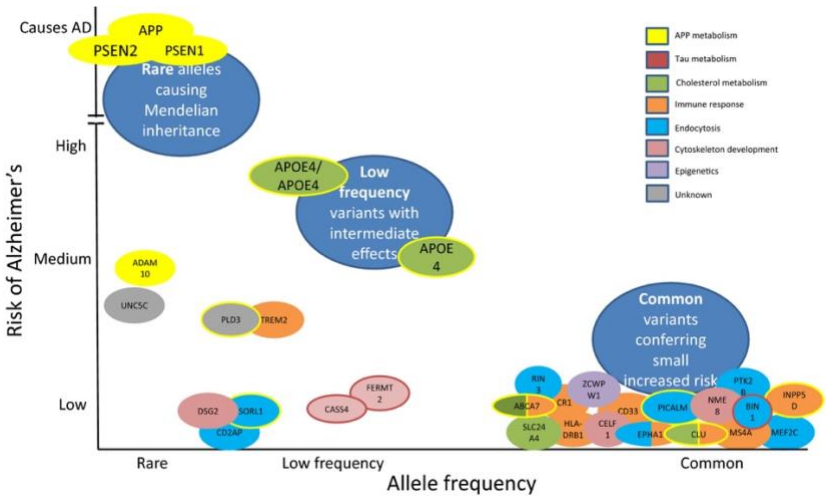


*Figure 1: Overview of genes involved in Alzheimer's Disease* (Lane, Hardy and Schott, 2018)

### 1.1.3 Pathophysiology

The defining features of AD are amyloid plaques and neurofibrillary tangles (NFTs). Other hallmarks include neuropil threads, dystrophic neurites, associated astrogliosis, and microglial activation (Serrano-Pozo *et al.*, 2011; Lane, Hardy and Schott, 2018). The downstream effects of these pathological processes are synaptic loss and neuronal death, resulting in neurodegeneration and manifested macroscopic brain atrophy (Schneider *et al.*, 2009). Amyloid plaques are primarily composed of abnormally folded Amyloid beta (Ab)40 and Ab42, both of which are by-products of APP metabolism. Aβ42 is more abundant in plaques due to its higher rate of fibrillation and insolubility (Serrano-Pozo *et al.*, 2011). The pattern of amyloid deposition is not always consistent, but it most often begins in the isocortex and only later affects subcortical structures. On the other hand, neurofibrillary tangles are made up of hyperphosphorylated tau and are primarily found in paired helical filaments. Tau pathology usually starts in the allocortex of the medial temporal lobe, such as the entorhinal cortex and hippocampus, before spreading to the associative isocortex. The loss of neurons and synapses usually occurs along with tangle formation, so the severity and symptoms of AD are more closely related to NFT pathology. Amyloid pathology, on the other hand, plateaus early in the symptomatic phase of the disease (Ingelsson *et al.*, 2004; Rother *et al.*, 2022).

#### 1.1.3.1 Synaptic changes in AD

A central aspect of AD's clinical symptoms is the loss of synaptic plasticity and synapses, which closely aligns with cognitive decline. The initial symptom in AD is a deficit in creating and retaining new memories (Weintraub, Wicklund and Salmon, 2012; Perez-Nievas *et al.*, 2013; Boros *et al.*, 2017; Arenaza-Urquijo and Vemuri, 2018). The number of synapses and postsynaptic receptors decrease early in the disease and these changes are closely tied to the decline in cognition (Koffie *et al.*, 2009; Perez-Nievas *et al.*, 2013; Boros *et al.*, 2017). More

specifically, AD is marked by the loss of synapses in the neocortex and hippocampus, which are critical to learning and memory. The loss of synapses and dendritic spines is linked to Aβ mediated removal of postsynaptic receptors, which are in turn required for Aβ impact on synapses (Alfonso *et al.*, 2014, 2016; Henley and Wilkinson, 2016; Reinders *et al.*, 2016; Guntupalli *et al.*, 2017; Zhang *et al.*, 2017; Müller *et al.*, 2018). These receptors connect structural and functional plasticity and play critical role in short-term memories formation. The selective loss of these receptors and the differing methods of storing long-term memories might explain why implicit memories (in contrast to explicit ones) are still retained even in later stages of the disease (Kopec *et al.*, 2007; Sanderson *et al.*, 2010; Kessels, Remmerswaal and Wilson, 2011; Taylor *et al.*, 2011; Bannerman *et al.*, 2018; Zhou *et al.*, 2018). This loss is linked to cognitive impairment and evidence shows that soluble Aβ oligomers are responsible for synapse dysfunction and loss by affecting multiple receptors including NMDA-type glutamate and a7-nicotinic acetylcholine (a7-nACh) receptors (Spires-Jones and Hyman, 2014; Palop and Mucke, 2016). NMDA receptors play a critical role in both long-term potentiation (LTP) and long-term depression (LTD) in diverse ways (Kandel, Dudai and Mayford, 2014). During LTP, a large influx of $Ca^{2+}$ into the synapses caused by NMDA receptors leads to the growth of dendritic spines by incorporating AMPA-type glutamate receptors. In contrast, during LTD, reduced $Ca^{2+}$ influx decreases the levels of AMPA receptors and causes spine loss (Kandel, Dudai and Mayford, 2014). It has been shown that soluble Aβ oligomers can inhibit LTP, cause spine loss, and increase LTD by downregulating NMDA receptors and removing AMPA receptors from the synapses (Cullen *et al.*, 1997; Chen *et al.*, 2000; Walsh *et al.*, 2002; Hsieh *et al.*, 2006; Shankar *et al.*, 2007). Additionally, soluble Aβ oligomers downregulate NMDA receptors, leading to decreased LTP, increased LTD and spine loss (Li *et al.*, 2009). Antagonists of NMDA receptors such as memantine and NitroMemantine can reverse Aβ-induced synaptic dysfunction and cognitive impairment (Figueiredo *et al.*, 2013; Talantova *et*

*al.*, 2013; Zhu *et al.*, 2013), thus they consist useful drugs against severe type of AD. In conclusion, Aβ-mediated LTP inhibition, LTD enhancement, and synaptic loss appear to be at the root of cognitive impairment in AD (Palop and Mucke, 2016).

1.1.3.2 Gliosis: an underestimated marker in AD.

Another cardinal feature of AD is the reactive gliosis which is reflected by the abnormal morphology and proliferation of microglia and astrocytes. While both microgliosis and astrogliosis are common in many neurodegenerative diseases with varying causes, it remains unclear whether these changes reflect a helpful, harmful, or insignificant role of glial cells in neurodegeneration (Maragakis and Rothstein, 2006; Ransohoff and Perry, 2009; Glass *et al.*, 2010; Hansen, Hanson and Sheng, 2018). Under physiological conditions, in the CNS, microglia act as resident immune cells that constantly monitor the environment and play important roles in maintaining CNS tissue, responding to injury and defending against pathogens. They also are crucial regulators of the developmental shaping of neural networks by removing undesired neurons and synapses (Nayak, Roth and McGavern, 2014; Colonna and Butovsky, 2017). Microglia have a likely significant role in AD-related synapse loss that is triggered by the complement system (Wu *et al.*, 2015; Spangenberg and Green, 2017). Microglia can cause synaptic loss and worsen tau pathology, and upon activation, they can release toxic factors that directly or indirectly damage neurons (Colonna and Butovsky, 2017; Leyns and Holtzman, 2017; Liddelow *et al.*, 2017). There is ample evidence in AD human brain tissue of complement activation, particularly in association with plaques (C1q, C3, and C4), and to a lesser extent, with neurofibrillary tangles and dystrophic neurites (C5b-C9) (Zanjani *et al.*, 2005). The expression of C1q rises in the aging brain, particularly in the hippocampus, and in mouse models of beta-amyloidosis, C1q levels are elevated and more present in synapses even before plaques form (Hong *et al.*, 2016). It has been shown that Aβ

binding to C1q can activate the classical complement cascade. Notably, elimination of C1q by genetic knockout or neutralizing antibodies prevented Aβ-mediated synapse loss in mice (Fonseca *et al.*, 2004; Hong *et al.*, 2016). Microglia can also harm neurons by releasing inflammatory mediators in response to protein aggregates and dying cells. The aggregation/fibrillation of beta-amyloid can activate Toll-like receptors (TLRs) and the NLRP3 inflammasome, leading to the production of TNFα, IL-1β, and other pro-inflammatory cytokines. Studies in amyloidosis mouse models have found that deleting NLRP3, caspase-1, and TLRs can alleviate Aβ deposition and cognitive deficits, further supporting the idea that classical inflammation contributes to the progression of AD (Heneka *et al.*, 2013; Heneka, Golenbock and Latz, 2015).

A significant discovery in AD research, and in particular related to microglia ambiguous nature, was the finding of variants of TREM2 (Triggering Receptor Expressed in Myeloid Cells 2), a cell surface protein found abundantly and exclusively in microglia in the brain and some peripheral myeloid cells, associated with AD. The *TREM2* pathogenic variant with the strongest link to AD (R47H) raises the risk of the disease by about three times and since the R47H mutation appears to impair the ability of TREM2 to activate microglia and is seen as evidence that microglia typically have a protective role against AD (Guerreiro *et al.*, 2013; Jonsson *et al.*, 2013). Importantly, TREM2 binds to apolipoproteins like ApoE and clusterin, which are highly associated with AD (Atagi *et al.*, 2015; Bailey, Devaux and Farzan, 2015; Yeh *et al.*, 2016). The TREM2 variants that increase the risk of AD appear to be loss-of-function mutations that impair the ability of TREM2 to bind to its ligands (Wang *et al.*, 2015; Kober *et al.*, 2016; Yeh *et al.*, 2016; Song *et al.*, 2017). Microglia play a crucial role in maintaining tissue homeostasis by removing debris, and TREM2 is necessary for phagocytosis of various substances, including Aβ (Kleinberger *et al.*, 2014; Atagi *et al.*, 2015; Yeh *et al.*,

2016). Microglial phagocytosis of Aβ is enhanced when it is combined with lipoproteins like LDL, ApoE, and CLU/ApoJ and studies have shown that TREM2-deficient microglia have reduced capacity to internalize Aβ-lipoprotein complexes (Terwel *et al.*, 2011; Yeh *et al.*, 2016). Evidence has also shown that microglia play a protective role in the formation of amyloid deposits. They surround the deposits, making the amyloid fibrils more tightly packed and potentially less harmful. Additionally, microglia prevent the growth of new amyloid deposits and reduce damage to nearby neuropil (Condello *et al.*, 2015). This protective "corralling" function was more prominent in small, early-stage plaques, but was found to be weakened in mice without Trem2 and in human AD tissues from TREM2 R47H carriers (Condello *et al.*, 2015; Wang *et al.*, 2015; Yuan *et al.*, 2016). The studies on TREM2 suggest that microglia can protect against the accumulation of toxic amyloid deposits and the development of Alzheimer's through various mechanisms, including the removal of soluble amyloid, phagocytosis of insoluble amyloid, activation and chemotaxis, and compaction and containment of amyloid plaques.

#### 1.1.3.3 Adult neurogenesis as a new player in AD progress

Studies using transgenic mouse models of Alzheimer's disease (AD) have provided increasing evidence of a relationship between alterations in Adult Hippocampal Neurogenesis (AHN) and AD pathology. These mouse models carry mutations in genes associated with familial AD, such as amyloid precursor protein (APP) and presenilin 1 (*PSEN1*), and the gene for the microtubule-associated protein tau (*MAPT*). Most of these studies have found decreased AHN in the early stages of AD (Disouky and Lazarov, 2021; Hanspal and Gillotin, 2022; Salta *et al.*, 2023). For example, the APPswePS1DE9 mouse model showed a significant reduction in NPC proliferation and neuronal differentiation at 2 months, before the formation of amyloid plaques and before the onset of cognitive deficits (Park *et al.*, 2006; Demars *et al.*, 2010). Similarly,

the 5xFAD mice had impaired AHN starting at 2-3 months, before amyloid deposition or cognitive decline (Choi *et al.*, 2018). The 3xTg mice showed reduced AHN starting at 2 months, before the accumulation of amyloid plaques, neurofibrillary tangles (NFTs), and the onset of memory impairment, which did not occur until 4-5 months (Billings *et al.*, 2005; Rodríguez *et al.*, 2008).

The fact that AHN impairment occurs before the onset of AD hallmark symptoms and cognitive decline suggests that AHN impairment may contribute to the progression of cognitive decline in AD. This is supported by the finding that eliminating AHN worsened cognitive dysfunction in both APPswePS1DE9 mice and 5xFAD mice (Hollands *et al.*, 2017; Choi *et al.*, 2018). Nevertheless, in other studies, targeting neurogenic populations improved synaptic plasticity and cognitive function in APP/PS1 and hAPP-J20 mice. One of the studies depleted Gfap-expressing cells, while the other eliminated all proliferating cells, including microglia and astrocytes (Hollands *et al.*, 2017; Deczkowska *et al.*, 2018; Zhang, Mei, *et al.*, 2021; Zhang, Wei, *et al.*, 2021). The apparent benefits of eliminating these disease-associated populations suggest that improving AHN function, particularly in the early stages of AD, may help increase brain reserve and be a promising strategy for AD treatment. Although most AD models still need to be tested for changes in pattern separation, as seen in AD patients, these findings demonstrate that AHN impairment itself causes cognitive deficits in rodent models of AD (Salta *et al.*, 2023).

While in mice it is well accepted that AHN is present and furthermore, declines sharply in animal models of AD, this was not the case regarding AHN in humans until very recently (Lima and Gomes-Leal, 2019). The fact whether neurogenesis persists during adulthood in humans have been a long-standing debate. Quite recently there were two contradicting studies, one

reporting that neurogenesis is practically non-existent in humans older than 7-years old, while the second reported the presence of thousands of immature neurons in the dentate gyrus, even in elderly people (Boldrini *et al.*, 2018; Sorrells *et al.*, 2018). The debate was then resolved one year later following a landmark study by Moreno-Jimenez et.al. which presented further evidence in support of the existence of AHN in humans as well as its decline in AD patients (Moreno-Jiménez *et al.*, 2019).

### 1.1.4 Pathogenesis hypotheses for AD origin

The most widely accepted theory regarding AD pathogenesis is the amyloid hypothesis. It suggests that the accumulation of abnormal forms of beta-amyloid (Ab) in the brain, produced through sequential cleavage of the amyloid precursor protein (APP) by beta- and gamma-secretase enzymes, is the primary cause of AD (Hardy and Selkoe, 2002; Lane, Hardy and Schott, 2018). The imbalance between Aβ production and Aβ clearance is thought to drive this process. The formation of neurofibrillary tangles (NFTs) and subsequent neuronal dysfunction and neurodegeneration, which may be mediated through inflammation, are believed to be secondary effects (Lane, Hardy and Schott, 2018). Overwhelming genetic evidence supports the central role of Aβ in AD, as all familial Alzheimer's disease (fAD) mutations impact either Aβ generation or processing, leading to the overproduction of toxic forms of beta-amyloid. Conversely, an *APP* missense pathogenic variant (A673T) leads to a lifelong decrease in APP cleavage by beta-secretase and a reduced risk of AD. In sporadic cases of AD, apolipoprotein E (ApoE) and many other risk genes are involved in Ab clearance (Jonsson *et al.*, 2012).

Other than the amyloid hypothesis, one of the oldest hypothesis for the pathogenesis of AD is the cholinergic one. Cholinergic neuron damage is seen as a major factor in cognitive decline in AD, leading to the development of the cholinergic hypothesis (Davies and Maloney, 1976;

Bartus *et al.*, 1982). This theory posits that a reduction of cholinergic activity in the central nervous system significantly contributes to cognitive decline that comes with aging and AD. Acetylcholine (ACh) is a crucial neurotransmitter utilized by cholinergic neurons that play a role in various critical bodily functions such as attention, learning, memory, stress response, wakefulness, sleep, and sensory perception (Bartus *et al.*, 1982; Coyle, Price and DeLong, 1983). Based on this hypothesis, several drug treatments have been developed to increase the levels of ACh in the brain, including acetylcholinesterase inhibitors (AChEIs) and cholinergic agonists (Schmitz and Nathan Spreng, 2016; Hampel *et al.*, 2018; Schmitz *et al.*, 2018; Marucci *et al.*, 2021). Acetylcholinesterase inhibitors work by blocking the enzyme that breaks down ACh in the brain, leading to an increase in the levels of synapse-located ACh. Cholinergic agonists, on the other hand, directly activate cholinergic receptors in the brain, leading to increased Ach-dependent effects (Marucci *et al.*, 2021). Common drugs used for AD treatment that belong to these categories include donepezil, rivastigmine and galantamine (AChEIs), and nicotine (a cholinergic agonist). However, it is important to note that these drugs only provide symptomatic relief and do not cure the underlying pathological mechanisms of the disease (Do Carmo, Kannel and Cuello, 2021).

## 1.2 Neurotrophins and their receptors

### 1.2.1 Neurotrophins and their receptors structure

Neurotrophins are growth factors that control multiple crucial aspects of neuronal cell function during development and adulthood. These functions include cell specification, proliferation and survival; axonal growth and remodelling; development and plasticity of synapses. There are four neurotrophins in mammals; Nerve growth factor (NGF), Brain-Derived Neurotrophic

factor (BDNF), Neurotrophin 3 (NT3) and Neurotrophin 4 (NT4). From an evolutionary point of view, neurotrophin genes are believed to have arisen via sequential duplications of genome part from an ancestral chordate (Hallböök, 1999). This notion is further supported from the fact that neurotrophin genes share homologies in their sequence as well as their structure. Their gene product is a pro form of the neurotrophin that contains a signal sequence and the pro domain followed by the mature neurotrophin. Their crystal structure reveals that they form homodimers, which are bound non-covalently, and they share profoundly homologous structures and features that are also common among other growth factors (McDonald *et al.*, 1991; Robinson *et al.*, 1995; Butte *et al.*, 1998).

Neurotrophins bind strongly to the Tropomyosin Related kinase (Trk) receptor, which belongs to the Receptor Tyrosine Kinases (RTK) superfamily, with high (at the picomolar range) affinity. Each neurotrophin shows strong selectivity for a specific receptor, namely NGF for TrkA; BDNF and NT4 for TrkB and NT3 for TrkC. The presence of each receptor is crucial for the ligand's ability to promote differentiation and survival of a neuronal cell. Additionally, it has been shown that ectopic expression of the receptor can induce cell responsiveness in the respective ligand in neuronal cells that are normally independent of that ligand (Allsopp *et al.*, 1994; Huang and Reichardt, 2003; Reichardt, 2006). Each of the Trk receptors have an extracellular domain (ECD) followed by a transmembrane region (TM) and an intracellular domain (ICD). The ECD region of each receptor contains three leucine-rich repeats surrounded by two cysteine-rich clusters followed by two immunoglobulin-like domains. The immunoglobulin-like domain adjacent to the membrane serves as the primary neurotrophin binding site as it was revealed by the crystal structure of each of the receptors as well as the complex of TrkA domain with NGF. The ICD part of the receptor embodies several tyrosine residues, which act as anchoring sites for several other proteins and enzymes, and a tyrosine

kinase domain. The receptor is activated upon neurotrophin binding that induces dimerization of the receptor and subsequent trans-phosphorylation of the tyrosine kinase domain (Urfer *et al.*, 1998; Ultsch *et al.*, 1999; Wiesmann *et al.*, 1999; Wiesmann and De Vos, 2001).

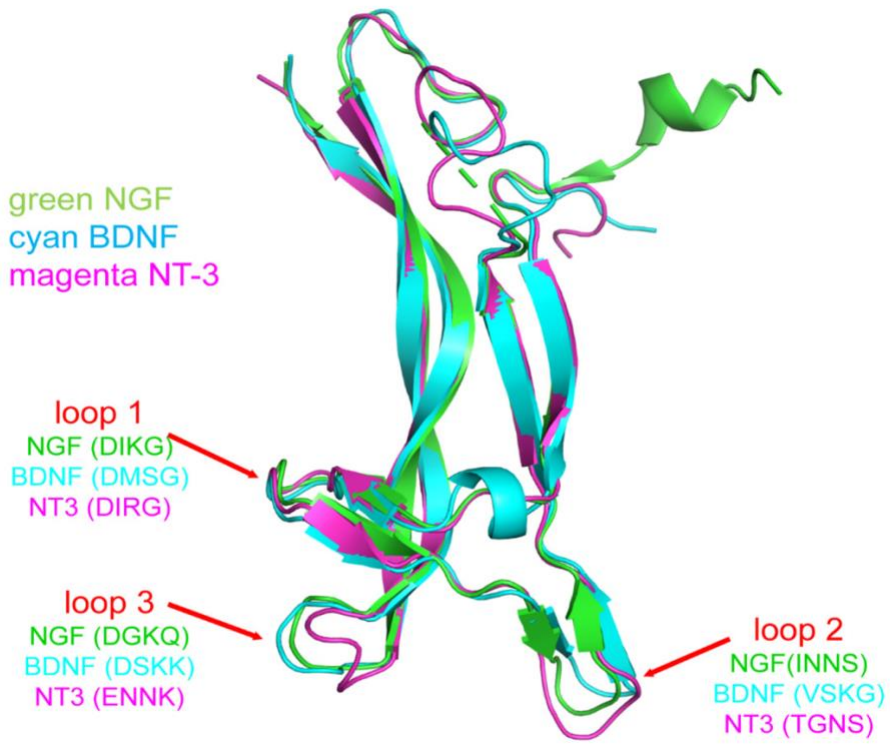


*Figure 2: Structures of NGF, BDNF and NT-3* (Gupta *et al.*, 2022)

Ligand binding and activation of Trk receptors results in the initiation of downstream signalling pathways, which in turn have profound effects on pivotal neuronal cell functions such as survival, differentiation, axonal growth and synaptic plasticity. Major pathways that are activated include the phosphatidylinositol 3-kinase (PI3K)/AKT, mitogen-activated protein kinase/extracellular signal-regulated kinase (Ras/ ERK), and phospholipase C-γ (PLC-γ). Which pathway will be activated and the downstream effect it will have is primarily influenced by the position of phosphorylated tyrosine on Trk receptor and the cell population (Reichardt, 2006; Deinhardt and Chao, 2014).

### 1.2.2 Neurotrophin signalling pathways

#### 1.2.2.1 PI3K/AKT pathway

Trk phosphorylation at tyrosine 490 results in recruitment and phosphorylation of fibroblast growth factor receptor substrate 2 (Frs2) and the Src homology domain- containing (Shc) adaptor proteins. This recruitment can then activate PI3K pathway via Ras-dependent or independent mechanisms. Ras-dependent activation of PI3K occurs following Shc and/or Frs2 phosphorylation and subsequent recruitment of Grb2 bound with Ras exchange factor son of sevenless (SOS) while Ras-independent activation requires Gab1 recruitment, which occurs after phosphorylation of Grb2, and leads to binding and activation of PI3K (Reichardt, 2006; Pramanik, Sulistio and Heese, 2017). PI3K activation results in the production of phosphatidylinositol (3,4,5)-trisphosphate (PIP3) via phosphorylation at the 3' position of phosphatidylinositol (4,5)-bisphosphate (PIP2) (Skaper, 2012; Deinhardt and Chao, 2014). Akt is then activated by PIP3, translocates to the plasma membrane and through phosphorylation of various target proteins and substrates, such as BAD, forkhead transcription factor FKHRL1, IκB and GSK3β, promotes cell survival (Hamanoue *et al.*, 1999; Brunet, Datta and Greenberg, 2001; Zhou *et al.*, 2004). In addition to cell survival signals, PI3K, acting through PIP3, has profound effects on neuronal growth and differentiation by regulating small GTPase proteins of the Rho family such as RAS- homolog family member A (RhoA) and cell division cycle 42 (Cdc42) (Yuan *et al.*, 2003; Pramanik, Sulistio and Heese, 2017). For example, PIP3 activates RAS-related protein Rap1B, which then further activates Cdc42. Subsequently, Cdc42 effects various downstream targets and proteins that are crucial for cell morphology, polarity and adhesion by regulating actin polymerization and microtubule

stabilization (Schwamborn and Püschel, 2004; Nishimura *et al.*, 2005; Watabe-Uchida *et al.*, 2006).

1.2.2.2 Ras/Erk pathway

Further to activation of PI3K/Akt pathway, Shc-Grb2 complex recruitment to Trk, which is modulated by the Frs2/SHP2 complex, also results to Erk pathway activation (Kao *et al.*, 2001; Wu, Lai and Mobley, 2001; Reichardt, 2006). Both Frs2 and Shp2 are essential for recruitment of Grb2/Sos complex, which then acts on Ras by replacing a GDP for a GTP molecule and thus constituting it active (Goldsmith and Koizumi, 1997; Easton, Royer and Middlemas, 2006; Dance *et al.*, 2008). Ras, in turn, activates Raf, a protein kinase, which ultimately leads to phosphorylation and activation of Erk1 and Erk2 following Mek1 and Mek2 phosphorylation by Raf (Deinhardt and Chao, 2014; Pramanik, Sulistio and Heese, 2017). Erk activation can also be achieved by recruitment of Crk and subsequent binding and activation of C3G, a guanine exchange factor, which results in the initiation of the Raf/MEK/Erk pathway . Association of Crk with Trk receptors can be attained by Crk binding to activated Frs2 or, alternatively, to ankyrin repeat-rich membrane spanning adaptor (ARMS) (Arévalo *et al.*, 2004). Eventually, Erk activation leads to CREB-regulated gene expression, which involves multiple genes required for neuronal survival and differentiation (Reichardt, 2006; Deinhardt and Chao, 2014).

1.2.2.3 PLC-γ pathway

PLC-γ pathway activation, on the other hand, requires phosphorylation of Trk in a distinct tyrosine position compared to PI3K and Erk pathways. Trk phosphorylation in tyrosine 785 in TrkA, and the corresponding residues in TrkB and C, creates a docking site for binding of PLC-γ to Trk receptor. Recruited PLC-γ then generates IP3 and DAG by hydrolysing PIP2, which

causes intracellular $Ca^{2+}$ to be released. Increased levels of cytoplasmic $Ca^{2+}$ result in activation of $Ca^{2+}$-dependent enzymes such as CaMK and the phosphatase calcineurin and the activation of CamK/CREB signalling pathways (Reichardt, 2006; Pramanik, Sulistio and Heese, 2017). Importantly, $Ca^{2+}$ release and DAG production drives Raf/Erk signalling via activation of PKC. In addition to that, activated PKC contributes to stimulation of the capsaicin VR1 channel and TRPC, which are involved in BDNF-evoked elevation of $Ca^{2+}$ at synapses (Skaper, 2012; Deinhardt and Chao, 2014).

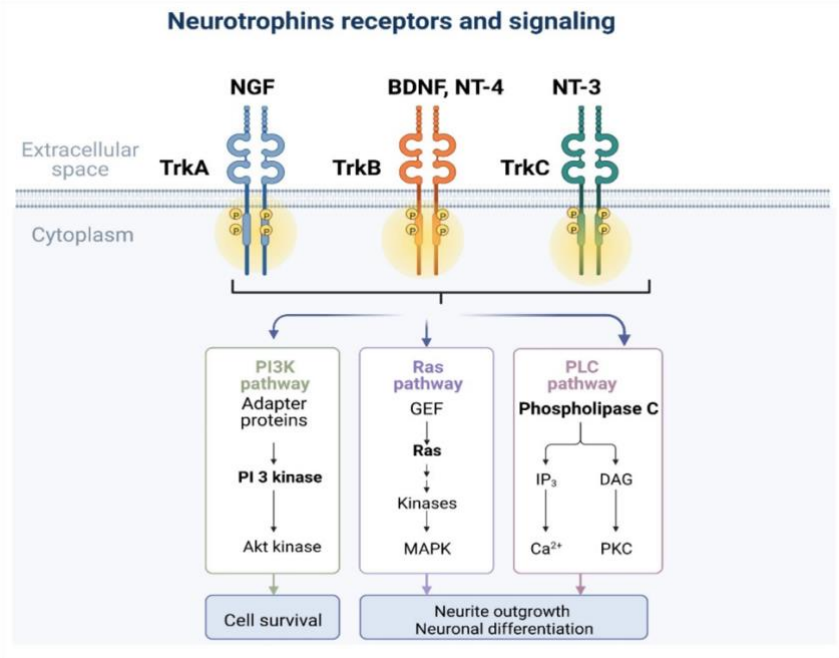


*Figure 3: Neurotrophin Signaling pathway (Gupta et al., 2022)*

## 1.2.3 p75 pan-neurotrophin receptor

On the other hand, p75 is a type 1 transmembrane protein and was initially identified as the NGF receptor but is now considered the 16th member of the Tumor Necrosis Factor Receptor (TNFR) family (Skeldal and Coulson, 2016). Its extracellular portion consists of four cysteine-rich domains (CRD1 to CRD4) and sites for N- and O-linked glycosylation (Underwood and Coulson, 2008). The extracellular domain is connected to the intracellular domain of p75 (p75ICD) via an alpha-helical transmembrane segment, and the p75ICD is notable for

possessing a death domain (Liepinsh *et al.*, 1997). p75 can bind all neurotrophins, albeit with lower affinity compared to that of their high affinity Trk receptors (Conroy and Coulson, 2022; Xiong *et al.*, 2022). Remarkably, p75 can act contradictory depending on the context, the cell type and its ligand. For instance, it can promote cell growth, survival and differentiation when it is bind by mature neurotrophins NGF, BDNF and NT-3, while it can have the exact opposite effects, resulting in cell death when activated by their pro-form, using sortilin as co-receptor (Becker *et al.*, 2018). Furthermore, this can be influenced by the availability of the respective neurotrophin receptor at any given time. Specifically, it has been shown that in cells expressing p75 but at the same time lacking the high affinity Trk receptor, even mature neurotrophins can indeed induce cell death (Casaccia-Bonnefil *et al.*, 1996; Bamji *et al.*, 1998; Wiese *et al.*, 1999; Troy, Friedman and Friedman, 2002; Domeniconi, Hempstead and Chao, 2007; Kenchappa *et al.*, 2010; Matusica *et al.*, 2015). p75NTR does not have intrinsic enzymatic activity, so its signal transduction is achieved through the recruitment of adaptor proteins in the cytoplasm (Reichardt, 2006). It also interacts with various co-receptors, such as Trk receptors, sortilin, and NogoR, allowing it to perform a range of complex functions (Huang and Reichardt, 2003; Barker, 2004). Upon ligand activation, p75NTR is cleaved by γ-secretase to generate the intracellular domain (ICD), which is responsible for specific signalling (Zampieri *et al.*, 2005; Forsyth *et al.*, 2014). The ICD of p75NTR has several interactors that mediate a diverse set of biological functions. As a member of the TNF receptor super-family, p75NTR participates in various signalling pathways through protein-protein interactions facilitated by its intracellular death domains. These death domains are globular six-helix bundles that are essential for signalling in the TNF receptor super-family (Xiong *et al.*, 2022). Like other members of the TNF receptor superfamily, p75NTR lacks enzymatic activity, and its signalling occurs through the recruitment and release of cytoplasmic effectors to and from its intracellular domain (ICD) in response to ligand binding (Gentry, Barker and Carter, 2004). Numerous intracellular

proteins have been identified as interactors of p75NTR, including the p75NTR-associated cell death executor (NADE), neurotrophin receptor interacting factors 1 and 2 (NRIF1 and 2), and neurotrophin receptor–interacting melanoma antigen homolog (NRAGE), which enhance p75NTR-mediated apoptosis (Casademunt *et al.*, 1999; Mukai *et al.*, 2000; Salehi *et al.*, 2000; Benzel, Barde and Casademunt, 2001). On the other hand, co-expression of Trk receptors is crucial for preventing p75NTR-mediated apoptosis (Xiong *et al.*, 2022).

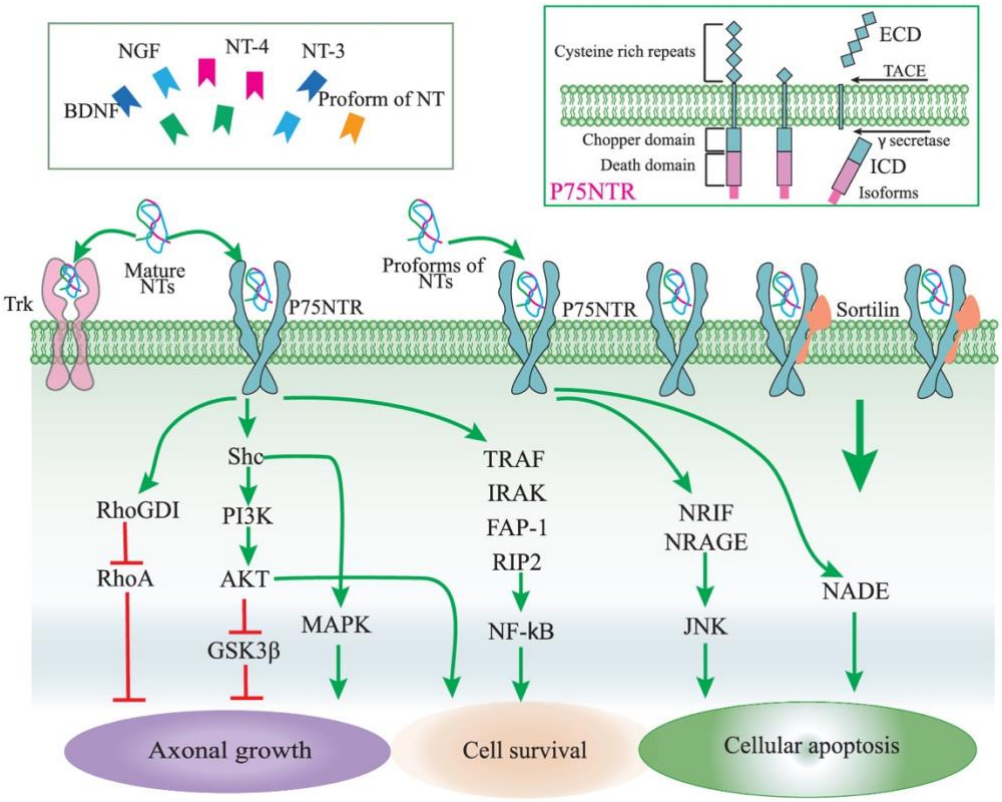


*Figure 4: p75$^{NTR}$ signalling pathway (Xiong et al., 2022)*

## 1.2.4 Neurotrophin function in neurons

Neurotrophins regulate crucial and multiple aspects of neuronal function, such as survival and differentiation by modulating different signalling pathways as described above. Whether a signal will translate into axonal growth or expression of genes relating to survival largely depends on the signalling pathway that is activated as well as the neuron type and the Trk receptor expression. For example, NGF induced activation of PI3K/Akt pathway leads to

increased neuronal survival of basal forebrain cholinergic, primary septal, dorsal root ganglion, motor and hippocampal neurons (Kuruvilla, Ye and Ginty, 2000; Namikawa *et al.*, 2000; Culmsee *et al.*, 2002; Markus, Zhong and Snider, 2002; Madziar *et al.*, 2008). On the other hand, Ras/Mek/Erk pathway activation by NGF is required for sensory neurons elongation and innervation as well as for cholinergic neurons differentiation in the basal forebrain during development (Li *et al.*, 1995; Markus, Zhong and Snider, 2002; Finegan *et al.*, 2009; Newbern *et al.*, 2011). Similarly, BDNF induces survival of cerebellar granule cells and retinal ganglion cells via activation of the PI3K/Akt pathway (Bonni *et al.*, 1999; Nakazawa, Tamai and Mori, 2002), while Ras/Mek/Erk induction by BDNF in hippocampal CA1 neurons regulates synaptic density and plasticity via CREB (Alonso, Medina and Pozzo-Miller, 2004; Fritsch *et al.*, 2010). Importantly, modulation of synaptic plasticity and long term potentiation (LTP) in those neurons by BDNF can also be achieved via activation of the PLC-γ pathway and subsequent activation of the CamK/CREB mediated gene expression (Minichiello *et al.*, 2002; Yamada *et al.*, 2002; Numakawa *et al.*, 2009). Notably, both NGF and BDNF have been implicated to have important roles regarding synaptic plasticity, a neuronal function that is crucial for learning and memory and its impairment has been shown to be an early feature of Alzheimer's Disease. Specifically, NGF has been shown to convert HFS-induced LTP into LTD in low concentrations and, conversely, greatly increase LTP at higher concentrations (Conner *et al.*, 2009; Arias *et al.*, 2014). BDNF, on the other hand, has been shown to be necessary and sufficient for LTP induction in experiments where BDNF treatment of hippocampal slices resulted in Theta Burst Stimulation-induced LTP, while experiments in KO or heterozygous mice for BDNF revealed substantial reduction of hippocampal LTP (Korte *et al.*, 1995; Figurov *et al.*, 1996; Patterson *et al.*, 1996). Similar to NGF and BDNF, NT3-TrkC signalling has also been shown to be important regulator of neuronal survival and function, but it has been comparatively less studied. Remarkably, NT3 has been shown to be stronger

activator of the PI3K/Akt pathway, compared to NGF, resulting in increased survival of cortical neurons (Markus, Zhong and Snider, 2002; Liot *et al.*, 2004).

## 1.3 Neurotrophins and Alzheimer's Disease

For more than three decades, the degeneration of cholinergic basal forebrain (CBF) neurons has been studied as a key neurotransmitter system affected early in AD. CBF neurons, which connect to the entire neocortex and hippocampus, may be a target for AD treatment (Hampel *et al.*, 2018). The idea of the "cholinergic hypothesis" of AD gained traction after it was found that acetylcholinesterase inhibitors (AChEIs) had significant effects on AD patients, leading to the creation of a larger family of AChEIs (Bartus *et al.*, 1982; Hampel *et al.*, 2018; Mufson *et al.*, 2019). Currently, AChEIs, such as donepezil, are one of the few FDA-approved drug classes for the treatment of AD. In fact, evidence has shown that donepezil can reduce atrophy within the nucleus basalis of Meynert and the medial septum/diagonal band in prodromal AD, providing both structural effects and symptom relief (Johannsen *et al.*, 2006; Mangialasche *et al.*, 2010; Cavedo *et al.*, 2017; Hampel *et al.*, 2018). The function of CBF on the other hand depends on the binding of NGF to its receptors TrkA and $p75^{NTR}$. This supports the idea that disruption of NGF signalling might be a major cause of cholinergic dysfunction in AD (Schwab *et al.*, 1979; Mufson *et al.*, 2019). CBF neurons express TrkA receptors and p75NTR and transport them to NGF production sites in the cortex and hippocampus. Upon binding, mature NGF activates signalling pathways in CBF neurons that control neuronal survival (Kaplan and Miller, 2004). These cholinergic neurons rely on NGF to preserve their biochemical and morphological characteristics (Nordvall, Forsell and Sandin, 2022). In AD, NGF levels decrease while the levels of its precursor, pro-NGF, increase, leading to an imbalance that is believed to play a significant role in the degeneration of BFCNs (Peng *et al.*, 2004). BFCNs

also express TrkB and respond to Brain-Derived Neurotrophic Factor (BDNF), which is essential for their postnatal maturation and function (Fryer *et al.*, 1996; Ward and Hagg, 2000). BDNF enhances BFCN survival, increases the levels of Choline Acetyltransferase (ChAT) and Acetylcholinesterase (AChE), and plays a crucial role in synaptogenesis and synaptic plasticity, important mechanisms for learning and memory in the adult central nervous system (CNS) (Nonomura and Hatanaka, 1992; Ward and Hagg, 2000). Furthermore, it positively modulates hippocampal Long-Term Potentiation (LTP), a cellular mechanism believed to underlie memory formation (Rex *et al.*, 2007). Similar to NGF, BDNF is also implicated in the pathophysiology of AD.

### 1.3.1 NGF and its receptors, TrkA and p75NTR, in AD

The potential of NGF as a therapeutic agent for neurodegenerative diseases has been suggested by many studies (Chan *et al.*, 2004; Aloe *et al.*, 2015; Mitra, Behbahani and Eriksdotter, 2019; Wang *et al.*, 2020). A decrease in the levels of NGF in the nervous system and cerebrospinal fluid (CSF) has been observed in AD patients (Budni *et al.*, 2015). Cholinergic degeneration is a well-known feature of AD, linking NGF and AD (Francis *et al.*, 1999). Studies have shown that mice lacking NGF and TrkA display a reduction in ChAT and loss of cholinesterase activity in the basal forebrain and hippocampus (Crowley *et al.*, 1994). The restoration of long-term potentiation in aged, cognitively impaired rats by chronic NGF infusion provides further evidence of NGF's role as a crucial neurotrophin in the CNS (Villoslada *et al.*, 2000; Lübke *et al.*, 2021). The dysregulation of NGF has been linked to various neurodegenerative diseases including AD and MS (Biernacki *et al.*, 2005; Cattaneo and Calissano, 2012). In addition to that, the degeneration of the basal forebrain cholinergic system and resulting NGF deficits have also been linked to cognitive decline and dementia in AD patients (Iulita and Cuello, 2014). Furthermore, studies have shown that NGF enhances amyloid precursor protein and promotes

the non-amyloidogenic cleavage pathway, reducing the generation of Aβ in the brain of mice (Yang *et al.*, 2014). It has also been reported that the levels of NGF in the CSF and dentate gyrus of AD patients are higher than those in a control group (Faria *et al.*, 2014; Budni *et al.*, 2015). In some trials, NGF treatment in AD patients resulted in lower levels of Aβ1-42 in their CSF (Andreasen *et al.*, 1999; Ferreira *et al.*, 2015). Studies by Tuszynski et al. (2015) found that after NGF gene transfer therapy in early stage AD patients, a response to NGF was seen with axonal extensions towards the NGF source and activation of the markers CREB and c-Fos (Tuszynski *et al.*, 2015). Furthermore, NGF treatment has been shown to improve brain activity in an electroencephalogram, glucose metabolism, and cognition in AD patients, as well as to lower brain shrinkage, improve clinical status, and elevated levels of CSF Aβ1-42 (Ferreira *et al.*, 2015).

Reduced TrkA levels in the cortex have been found to be correlated with lower cognitive performance, as measured by the Mini-Mental State Exam (MMSE), in AD patients (Counts *et al.*, 2004). This suggests that decreased levels of NGF in the cortex and nucleus basalis of Meynert (nbM) may provide an indication of the early onset of AD (Mufson *et al.*, 2019). Further, proNGF, the precursor protein of NGF, has been extensively studied in AD and its alterations have been linked to cognitive dysfunction during the progression of the disease in key areas such as the frontal cortex, posterior cingulate, precuneus, superior temporal cortex, and hippocampus (Mufson *et al.*, 2012; Sperling, Mormino and Johnson, 2014; Perez *et al.*, 2015). Indeed, isolated proNGF from AD cortex triggers neuronal cell death through its interaction with p75NTR, in a process dependent on γ-secretase shedding of the receptor, while proNGF from healthy brain does not cause apoptosis (Pedraza *et al.*, 2005). Moreover, the levels of proNGF are higher in the lateral parietal cortex of patients diagnosed with MCI or mild AD compared to those with normal cognitive function (Peng *et al.*, 2004). On the other

hand, proNGF levels in the precuneus remain unchanged until the advanced stages of AD, similar to what has been observed in the frontal cortex and hippocampus, suggesting that alterations in ProNGF might be critical in the AD brain (Fahnestock *et al.*, 2001, 2004; Podlesniy *et al.*, 2006; Al-Shawi *et al.*, 2008; Mufson *et al.*, 2012; Perez *et al.*, 2015). ProNGF has a stronger binding affinity with p75NTR, which is enhanced in the presence of sortilin and leads to apoptosis (Lee *et al.*, 2001; Nykjaer *et al.*, 2004; Pedraza *et al.*, 2005; Al-Shawi *et al.*, 2008). The homeostatic regulation of NGF receptors and proNGF's binding with TrkA, although with less affinity than mature NGF, activate pathways that impact CBF neuron function and induce neuroprotective effects through its weaker binding with the TrkA receptor (Fahnestock *et al.*, 2001, 2004).

The pro-apoptotic effect of proNGF signaling through p75NTR is dependent on the interaction of p75NTR with sortilin, a Vps10p domain trafficking protein that acts as a co-receptor on the cell surface with p75NTR to mediate proNGF-activated cell death. Sortilin triggers p75NTR-driven apoptosis after proNGF treatment, indicating its role in cell death (Mamidipudi and Wooten, 2002; Roux and Barker, 2002; Nykjaer *et al.*, 2004). Preventing this interaction hinders proNGF's binding with p75NTR and prevents cell degeneration (Bronfman and Fainzilber, 2004; Kaplan and Miller, 2004; Nykjaer *et al.*, 2004; Teng *et al.*, 2005). The response of p75NTR to proneurotrophins might depend on the identity and effectiveness of the bound co-receptor. Interestingly, the levels of sortilin and p75NTR in the cortex remain stable throughout the progression of AD. The pro-survival or pro-apoptotic signalling in CBF neurons during the early stages of AD might depend on changes in the stoichiometry of TrkA, p75NTR, the availability of specific co-receptors, and the physiological role of proNGF in different environments. A shift in the balance of these factors could alter the response that proNGF binding activates in CBF neurons during the progression of AD. Further understanding these

interactions will be crucial for the development of neurotrophic strategies for the treatment of dementia (Bruno *et al.*, 2004; Longo *et al.*, 2007).

### 1.3.2 BDNF and its high-affinity receptor TrkB in AD

Reduced levels of BDNF in the AD brain suggest that the lack of neurotrophic support from BDNF may worsen neurodegeneration. Indeed, growing evidence suggests that increasing BDNF signaling could improve cognition in AD (Arancibia *et al.*, 2008; Jiao *et al.*, 2016). In a mouse model of AD that showed amyloid and tau pathology, transplanting stem cells into the brain improved cognition (Blurton-Jones *et al.*, 2009). Interestingly, BDNF depletion negated the positive impact of the transplantation, while expressing ectopic BDNF had a similar beneficial effect on cognition (Blurton-Jones *et al.*, 2009). There is also genetic evidence linking BDNF to cognitive function. A common polymorphism in the human BDNF molecule, with a frequency of 25-32% in Caucasian populations and higher in Asian populations, replaces valine at position 66 with methionine. It has been shown that this variant impairs the internal sorting of BDNF, its transport to dendrites, and secretion, leading to weakened hippocampal function (Kuczewski, Porcher and Gaiarsa, 2010). In both sporadic and familial forms of AD, data show that the Val66Met BDNF polymorphism increases the rate of cognitive decline, affects hippocampal atrophy, and impacts CSF Tau levels (Boots *et al.*, 2017; Lim *et al.*, 2018). These findings support the idea of boosting BDNF signalling as a potential therapeutic approach to enhance synaptic plasticity and improve cognitive function in AD.

It has been observed that the BDNF mRNA levels in the hippocampus are reduced when the glutamatergic neurons are blocked by the administration of scopolamine or when the GABAergic system is stimulated (Da Penha Berzaghi *et al.*, 1993; Connor *et al.*, 1997). In addition, the cholinergic system has been found to play a role in regulating BDNF mRNA

levels in the hippocampus (Rossor *et al.*, 1982; Phillips *et al.*, 1991). The degeneration of both the glutamatergic and cholinergic systems is a common neuropathological feature of AD, lending further support to the notion that BDNF might contribute to cognitive impairment in AD (Coyle, Price and DeLong, 1983; Araujo *et al.*, 1988; Connor *et al.*, 1997; Lübke *et al.*, 2021). The reduction of BDNF mRNA levels in the hippocampus of individuals with AD suggests that BDNF may play a role in the progression of AD. As BDNF provides support to the basal forebrain cholinergic system, a decrease in BDNF levels may contribute to the progressive atrophy of these neurons in AD (Phillips *et al.*, 1991; Lübke *et al.*, 2021). Furthermore, BDNF treatment has been shown to decrease Aβ production and repair Aβ-induced damage, reduce cell death, improve cognitive function and synaptic loss, and slow down cognitive decline (Rohe *et al.*, 2009; Li *et al.*, 2012). The role of BDNF in memory and LTP has also been explored, with increased TrkB signaling and BDNF expression leading to improved memory and LTP. These findings suggest a potential role of BDNF in AD (Monteggia *et al.*, 2004; Ji *et al.*, 2010; Wan *et al.*, 2014).

Several postmortem studies have shown that the levels of BDNF mRNA and protein are reduced in the brains of AD patients (Michalski and Fahnestock, 2003; Meng, He and Xing, 2013). Increasing BDNF levels through gene transfer in the entorhinal cortex improved memory and spatial learning in APP transgenic mice, aged rats, and aged APP/PS1/tau transgenic mice following neuronal stem cell transplantation in the hippocampus (Blurton-Jones *et al.*, 2009; Nagahara *et al.*, 2009; Lattanzio *et al.*, 2014). It has furthermore been shown an increase in neurogenesis and BDNF mRNA levels in the hippocampus of APP/PS1 mice (Hsiao *et al.*, 2014). Additionally, BDNF mRNA expression was found to be lower in the post-mortem AD hippocampi when compared to normal levels. Studies have shown that the conversion of pro-BDNF to BDNF can be inhibited by the Aβ protein (Zheng, Sabirzhanov

and Keifer, 2010). Additionally, Aβ can indirectly affect BDNF levels at synapses through the hyperphosphorylation of tau, via activation of calcineurin (Ramser *et al.*, 2013). Aβ can also inhibit the retrograde transport of the BDNF-TrkB complex through the deubiquitinating enzyme, ubiquitin C-terminal hydrolase L1 (Poon *et al.*, 2013). Furthermore, *in vitro* experiments showed that oligomeric Aβ significantly decreases BDNF expression (Garzon and Fahnestock, 2007; DaRocha-Souto *et al.*, 2012; Rosa and Fahnestock, 2015). Hence, it is suggested that the interaction of Aβ with PKA activation can reduce CREB phosphorylation, leading to decreased BDNF expression, which represent a novel mechanism for Aβ-induced BDNF downregulation (Rosa and Fahnestock, 2015; Colucci-D'amato, Speranza and Volpicelli, 2020). Experiments in neuronal cell lines suggest that BDNF can shift processing of APP towards the non-amyloidogenic a-secretase pathway (Holback, Adlerz and Iverfeldt, 2005). Furthermore, BDNF-TrkB activation of the PI3K-Akt pathway can reduce the activity of GSK3β, a key tau kinase, through inhibitory phosphorylation (Elliott *et al.*, 2005). In addition, BDNF stimulation has been observed to cause de-phosphorylation of tau in neuronal cells, including the common AD-associated site AT8 (Tanila, 2017). Although the effects of BDNF on Aβ production are not fully understood, BDNF co-incubation in the hippocampus or entorhinal cortex was found to prevent Aβ1-42-induced impairment of LTP induction (Arancibia *et al.*, 2008; Kitiyanant *et al.*, 2012; Criscuolo *et al.*, 2015; Tanila, 2017; Lübke *et al.*, 2021).

## 1.4 Alzheimer's Disease Drug Development

### 1.4.1 Existing AD drugs

There has been a lot of effort and investment put into developing drugs for Alzheimer's disease, yet currently available options are limited (Cummings *et al.*, 2021). For the past 20 years, only

four drugs - donepezil, rivastigmine, galantamine, and memantine - have been approved for treating the symptoms of AD (Tatulian, 2022). The first three are acetylcholinesterase inhibitors, which enhance neurotransmission by reducing the hydrolysis of acetylcholine. Memantine is a N-methyl D-aspartate receptor antagonist that regulates calcium entry into neurons and protects against glutamate-induced excitotoxicity. It also reduces tau hyperphosphorylation by activating phosphoprotein phosphatase 2A (Chohan *et al.*, 2006; Wang *et al.*, 2021). Although these drugs are well-tolerated and can slow down the progression of symptoms such as memory loss, mood changes, attention deficit, irritability, and delusions, they do not modify the underlying disease. There is ongoing research and development by both academia and the pharmaceutical industry for an effective and disease-modifying AD drug. Amyloid beta and tau have been major therapeutic targets for AD for a long time, with the main strategies being inhibition of amyloid beta production, inhibition of aggregation, clearance by monoclonal antibodies, suppression of tau phosphorylation, and active immunotherapy. The first potentially disease-modifying AD drug, a monoclonal antibody against aggregated amyloid beta (aducanumab), was approved by the US Food and Drug Administration in June 2021 while and improved version of it approved in October 2022 (Tatulian, 2022).

### 1.4.2 Drugs targeting Aβ

Several drugs aimed at inhibiting the β- and γ-secretase enzymes in APP processing have gone through phase II and III clinical trials, but none have been approved. Small molecule inhibitors of BACE1, such as verubecestat, lanabecestat, and LY3202626, have shown promise by reducing Aβ production in both healthy individuals and people with mild to moderate AD (Egan *et al.*, 2019; Long and Holtzman, 2019; Alette M. Wessels *et al.*, 2020; Sur *et al.*, 2020). However, these inhibitors were discontinued due to their inability to slow cognitive decline

and adverse side effects, such as weight loss, hair discoloration, psychiatric problems and brain shrinkage. Some of these drugs even worsened cognitive function in people with early AD (Alette M Wessels *et al.*, 2020). Atabecestat, a non-selective BACE1 and BACE2 inhibitor, showed a dose-dependent decrease in Aβ concentration in the cerebrospinal fluid but was dropped from further trials due to liver toxicity and reversible cognitive impairment in subjects with mild memory loss (Sperling *et al.*, 2021). Drugs targeting PSEN1, avagacestat and semagacestat, failed due to skin cancer and worsened cognitive decline (Long and Holtzman, 2019; Panza *et al.*, 2019). R-flurbiprofen, a form of a nonsteroidal anti-inflammatory drug, was tested for its ability to reduce Aβ 42 production through modulation of γ-secretase and reached phase III trials, but was discontinued due to its inability to show any improvement in patients' symptoms, likely due to its poor blood brain barrier permeability (Long and Holtzman, 2019; Asher and Priefer, 2022). The reasons for the cognitive decline caused by β- and γ-secretase inhibitors are not well understood, with possible causes including the inhibition of these enzymes' actions on non-APP targets, a shift in APP processing towards the production of more harmful forms of Aβ, and the possible role of Aβ in cognition (Alette M Wessels *et al.*, 2020). The dose-dependent, biphasic action of γ-secretase inhibitors has also been considered as a factor in this shift in APP processing (Svedružić *et al.*, 2021).

Many anti- Aβ mAbs have been developed, targeting either monomeric Ab (solanezumab) or fibrillar/oligomeric forms (bapineuzumab, crenezumab, gantenerumab, lecanemab, and aducanumab). Aducanumab was initially seen as a breakthrough in treating AD by reducing Aβ load and slowing cognitive decline, as it was able to cross the BBB in both mice and humans with prodromal or mild AD (Sevigny *et al.*, 2016). However, in March 2019, the phase III trial revealed that the drug was futile due to side effects such as vasogenic edema. In October 2019, Biogen decided to continue the pursuit of approval for aducanumab after re-evaluating data

that showed a higher dose of the drug reduced cognitive decline by up to 22% compared to placebo, with side effects like edema. In June 2021, the FDA approved aducanumab for AD under the brand name Aduhelm, despite objections from the scientific advisory committee, causing significant controversy (Lalli *et al.*, 2021). The FDA later limited the use of Aduhelm to patients with mild AD or mild cognitive impairment, as clinical trials had only been conducted on those with these conditions, but not on those in earlier or later stages of AD.

The FDA's approval of Aduhelm (aducanumab) has sparked significant controversy (Mullard, 2021; Tampi, Forester and Agronin, 2021). One reason is that the committee reviewing the drug was not aware that an accelerated approval pathway was being considered. Another reason is that a post-approval confirmatory trial will not be finished before 2030. It's also unclear if reducing Aβ is a valid target for treating AD and if there is a relationship between Aβ reduction and improved cognition. Additionally, this approval has negatively impacted drug development and regulation, as pharmaceutical companies aim to approve drugs that lower Aβ or other biomarkers without clear clinical benefits. Some patients with AD have dropped out of important trials to take Aduhelm, and there have been issues with the drug's labelling, including indications and dosing. Furthermore, brain MRI scans for monitoring amyloid-related imaging abnormalities (ARIA) will add to the cost and complexity of AD treatment. The yearly cost for Aduhelm is high ($56,000), but its health benefits are estimated to only be worth $2500-$8300 per year. Finally, the approval has led to difficult conversations between clinicians, patients, and families regarding the drug's efficacy, indications, potential need for genetic testing, side-effect monitoring, and cost (Alexander and Karlawish, 2021). Despite the controversies, the FDA recently granted breakthrough status to two potential AD treatments: donanemab by Eli Lilly & Co. and lecanemab by Biogen and Eisai (Tampi, Forester and Agronin, 2021).

Regarding Lecanemab, ın a phase 3 clinical trial, it was evaluated as a treatment for early AD. The study lasted 18 months and involved 1795 participants between 50 and 90 years of age with mild cognitive impairment or mild dementia due to Alzheimer's disease, who were randomly assigned to receive either lecanemab (10 mg/kg every 2 weeks) or placebo. The primary endpoint was the change in Clinical Dementia Rating-Sum of Boxes score at 18 months, while secondary endpoints included changes in amyloid burden, cognitive function, daily living activities, and composite scores for Alzheimer's disease. Results showed that the lecanemab group had a mean decline of 1.21 in the CDR-SB score, compared to a decline of 1.66 in the placebo group, with the difference being statistically significant ($P<0.001$). In addition, lecanemab was associated with reductions in amyloid burden, as well as less decline in other measures of cognition and function compared to placebo. However, the drug was associated with adverse events, such as infusion-related reactions and amyloid-related imaging abnormalities. In conclusion, while lecanemab showed promise in reducing amyloid markers and slightly slowing decline in early Alzheimer's disease, longer trials are needed to fully assess its efficacy and safety (van Dyck *et al.*, 2022).

### 1.4.3 Drugs targeting Tau

The development of anti-tau drugs has focused on small molecules (like Antisense Oligonucleotides, ASOS) or mAbs that can inhibit tau aggregation or phosphorylation. A small-molecule called leuco-methylthioninium bis (hydromethanesulphonate) (LMTM), which inhibits tau aggregation, produced promising results in a Phase III clinical trial involving 800 patients with mild AD and is now being explored for use in individuals with more advanced AD (Wilcock *et al.*, 2018). On the other hand, the tau phosphorylation inhibitor tideglusib failed in its Phase II trial due to a lack of benefits (Elmaleh *et al.*, 2019). Despite the fact that

most of tau is intracellular, the small pool of extracellular tau is believed to play a role in the spread of tau pathology in the brain (Ayers, Giasson and Borchelt, 2018). Antibodies have been developed to clear extracellular misfolded tau, like for instance, Tilavonemab, which binds the N-terminal part of tau, was discontinued after a Phase II trial showed no benefit. Other anti-extracellular tau antibodies, including semorinemab and gosuranemab, that target the N-terminus of monomeric or aggregated tau, are in different phases of clinical trials (Ryszard and Marzena, 2020; Vaz and Silvestre, 2020; Walsh and Selkoe, 2020; Cummings *et al.*, 2021).

Over 30 drug candidates have reached phase III clinical trials, but only one, aducanumab, has been approved. Despite the large number of active trials, the success rate of developing drugs for AD is low, with 99% of candidates being discontinued due to lack of clinical benefit (Cummings, Feldman and Scheltens, 2019; Elmaleh *et al.*, 2019; Vaz and Silvestre, 2020; Cummings *et al.*, 2021; Lalli *et al.*, 2021). There are several factors contributing to this outcome, including the genomic variation and structural complexity of the Amyloid beta, the presence of pre-symptomatic damage, misdiagnosis, issues with trial design and analysis, and difficulties with the delivery of large molecular weight biologics and/or some small molecules into the brain (Cummings, Feldman and Scheltens, 2019; Pardridge, 2019, 2020). The *APP* gene, which is involved in the development of Ab, undergoes significant changes in human neurons, resulting in numerous variants of Ab, some of which are related to early onset AD. The occurrence of these variants is much higher in the brains of people with AD (Kamboh, 2018; Lee *et al.*, 2018). Treatment with mAbs targeting specific forms of Ab may be effective, but other potentially harmful forms may still exist. Additionally, if brain damage has progressed too far, clearing Ab or blocking its production may have limited impact. For instance, some AD drug candidates, such as the BACE-1 inhibitor verubecestat, have failed to

slow neuropathological changes, even in people with early stages of AD (Egan *et al.*, 2019; Nicoll *et al.*, 2019; Walsh and Selkoe, 2020).

### 1.4.4 Neurotrophins as pharmacological agents against AD

Attempts have been made to use NGF and BDNF as treatments for various human neurodegenerative diseases, including Alzheimer's Disease, but the use of recombinant proteins has so far not been successful due to their short lifespan in the bloodstream. However, a clinical trial in which NGF was delivered directly into the basal forebrain of ten Alzheimer's disease patients showed promising signs of improved cognitive function and neuronal function (Eyjolfsdottir *et al.*, 2016). Another ongoing clinical trial of gene therapy using AAV2-BDNF is being conducted for early Alzheimer's based on positive results in preclinical animal studies, which suggest that BDNF has neuroprotective effects (Nagahara *et al.*, 2013). The lack of success with recombinant neurotrophins has led to exploration of alternative therapies, such as small peptide mimetics, agonistic monoclonal antibodies, and small molecules that can activate the NGF/TrkA and BDNF/TrkB pathways (Nordvall, Forsell and Sandin, 2022).

The TrkB agonist/stimulator LM22A-4 was identified through the use of structural information from BDNF in an in silico screening process (Massa *et al.*, 2010). Another *in silico* screen resulted in the discovery of LM22B-10, which binds to both TrkB and TrkC receptors (Yang *et al.*, 2016). To improve bioavailability, structural changes were made to produce PTXBD10-2, which was found to activate TrkB and TrkC receptors and improve cholinergic neurite integrity in the basal forebrain in a mouse model of AD, as measured by the presence of choline acetyltransferase (CHAT) (Gonzalez *et al.*, 2022). Another example of a compound developed using the structure of BDNF is the dimeric dipeptide GSB-106, designed from a loop region of BDNF, which has demonstrated neuroprotective and antidepressant effects (Vakhitova *et al.*,

2021). Furthermore, the LM11A-31, a p75 modulator has been shown to have beneficial effects in mouse model of the disease during mid to late stage of the disease and a modified formulation of it is currently under clinical trial (Simmons *et al.*, 2014) (Clinical Trial Numberi: NCT03069014).

Natural products have been a valuable source of compounds, which have been found to impact Trk signaling. For example, gambogic amide, asiaticoside and sarcodonin G have been shown to activate TrkA, while deoxygedunin acts on TrkB (Jang *et al.*, 2007; Jang, Liu, Chan, *et al.*, 2010; Cao *et al.*, 2018; Nalinratana, Meksuriyen and Ongpipattanakul, 2019). Although natural products offer a diverse range of chemicals that have inspired many drugs, they can also present challenges in exploring structure-activity relationships and improving drug-like properties due to their chemical complexity and tendency for polypharmacology (Ho, Tran and Chai, 2018; Newman and Cragg, 2020). One well-studied natural product-derived compound in the neurotrophin field is 7,8-dihydroxy flavone (7,8-DHF), a TrkB agonist. 7,8-DHF has been extensively studied both in vitro and in animal models and has been shown to have effects similar to BDNF, including cognitive and antidepressant benefits, neuroprotection, neuroplasticity, and neurotrophic properties. Derivatives of 7,8-DHF have been developed, including the benzimidazole derivative (CF3CN), which has improved pharmacokinetic properties (Jang, Liu, Yepes, *et al.*, 2010; Zeng *et al.*, 2012; English *et al.*, 2013; Zhang *et al.*, 2015; Chen *et al.*, 2021).

### 1.4.5 Neurosteroidal analogs as neurotrophin mimetics: a novel approach for developing therapeutic agents against AD

Neurosteroids are hormones that are produced in the brain and nervous system, particularly in glial cells, astrocytes, and neurons. These steroids that act on the nervous system have been

shown to affect neuronal function, differentiation, and provide neuroprotection. They can cross the blood-brain barrier due to their lipophilic nature (Schumacher *et al.*, 2003). Research on the role of neurosteroids in AD has revealed a decrease in their levels in the brains of patients compared to healthy individuals, while administering neurosteroids to AD patients shows potential for neuroprotection (Compagnone and Mellon, 2000; Diaz Brinton and Ming Wang, 2006). These hormones typically work by activating receptors such as $GABA_A$, NMDA and sigma-1 (σ-1) (Compagnone and Mellon, 2000; Charalampopoulos *et al.*, 2008). Dihydroepiandrosterone (DHEA) is the most abundant steroid found in adult systemic circulation and serves as a precursor for the production of estrogens and androgens. DHEA is produced in the adrenal glands, gonads, and locally in the brain and is derived from cholesterol with the aid of the enzyme cytochrome 17. It is present in both free form and sulphur-bound (DHEAS) (Baulieu, 1998). While DHEA produced in the brain functions locally in a paracrine manner, adrenal DHEA has a systemic role. Its concentration reaches a peak in early adulthood and gradually decreases over time, particularly in neurodegenerative conditions like AD (Baulieu, 1998; Weill-Engerer *et al.*, 2002; Schumacher *et al.*, 2003; Gravanis *et al.*, 2012).

The study by Lazaridis et al. (2011) found evidence that DHEA binds to both NGF receptors, TrkA and p75NTR. This binding leads to the activation of survival kinases such as the Shc-PI3KAkt and Src-MEK-ERK pathways through autophosphorylation of TrkA. The interaction between DHEA and p75NTR also impacts the binding of p75NTR to various operators, determining the fate of cells to be either apoptosis or survival based on the ratio of TrkA and p75NTR receptors (Lazaridis *et al.*, 2011). DHEA has been found to have neuroprotective and survival effects, as well as promoting neurogenesis by increasing the number of newly formed neurons in rats and human neural stem cell cultures. It increases neural stem cell proliferation over the long term through its impact on NMDA receptors and Sigma-1 (Suzuki *et al.*, 2004;

Charalampopoulos *et al.*, 2008). Additionally, Pediaditakis et al. (2015) identified DHEA as an ancestral ligand of neurotrophin receptors. Specifically, they showed that DHEA was able to bind to all vertebrate Trk receptors and activate two of them (TrkA and TrkC) while it was also able to bind to and activate all invertebrate Trk receptors (Pediaditakis *et al.*, 2015). Nevertheless, long-term administration of DHEA has not been proved useful since it is metabolised into estrogens and androgens in humans, which have been shown to induce side adverse effects such as hormone-dependent cancer (Compagnone and Mellon, 2000; Calogeropoulou *et al.*, 2009).

Synthetic versions of DHEA that do not have endocrine effects make up a new group of molecules that can cross the blood-brain barrier and bind to neurotrophin receptors, giving a protective effect. Researchers have created 17-helix epoxy analogs of DHEA with modifications at positions C3 and C17 that cannot activate estrogen or androgen receptors and can function as agonists for neurotrophin receptors, similarly to the parent molecule. These synthetic compounds, referred to as "steroidal microneurotrophins," can also cross the blood-brain barrier, making them potential candidates for treating neurodegenerative diseases. The most studied and active of these compounds are the analogs BNN20, BNN23, and BNN27. They bind to the TrkA and p75NTR receptors, leading to TrkA phosphorylation and detachment of p75NTR from its intracellular mediator, RhoGDI (Calogeropoulou *et al.*, 2009).

BNN20 is a synthetic analog of DHEA that is modified by epoxidation of the 17th carbon hydroxyl group. It has been demonstrated to have neuroprotective effects both in vitro and in vivo (Calogeropoulou *et al.*, 2009; Panagiotakopoulou *et al.*, 2020). BNN20 has been found to almost completely protect degenerated dopaminergic neurons in the substantia nigra of a mouse model for Parkinson's disease. Furthermore, BNN20 has been shown to reduce inflammation,

protect oligodendrocytes, and promote their differentiation in a mouse model of focal demyelination induced by LPS (Kalafatakis *et al.*, 2021).

The microneurotrophin BNN27 is a derivative of DHEA, modified at position 17 with a spiroepoxy structure. It can cross the blood-brain barrier and was shown to activate TrkA receptor by phosphorylation and trigger pro-survival signaling in the cell (Pediaditakis, Efstathopoulos, *et al.*, 2016). Binding studies confirmed that BNN27 can bind to both TrkA and p75NTR receptors and prevent apoptosis through regulation of the interaction between RhoGDI, RIP6, and RIP proteins (Pediaditakis, Efstathopoulos, *et al.*, 2016; Pediaditakis, Kourgiantaki, *et al.*, 2016a). BNN27 can enhance the efficacy of low levels of NGF, by facilitating its binding to TrkA receptors and promoting axonal outgrowth. Studies have also suggested that BNN27 can improve memory by interacting with the cholinergic system, protect oligodendrocytes and myelin in demyelinating disorders, and provide therapeutic benefits for diabetic retinopathy by addressing both neurodegeneration and inflammation of the disease (Bonetto *et al.*, 2017; Pitsikas and Gravanis, 2017; Ibán-Arias *et al.*, 2018).

## 1.5 Aims of the current research project

The aims of the present research project are the following

1. To screen and identify novel small molecules mimetics of neurotrophins for their ability to activate NGF receptor TrkA, its downstream signalling kinases Akt and Erk1/2 as well as to protect cells against serum deprivation induced cell death.
2. To select the most potent TrkA and TrkB agonists and further screen them in primary neuronal cells and an *in vitro* model of amyloidosis.
3. To investigate the effect of a novel TrkB agonist in reversing the harmful phenotype in the 5xFAD animal model of Alzheimer’s Disease.

# 2. Materials & Methods

Cell lines

PC12 and HEK293T cells were obtained from LGC Promochem and cultured under specified conditions. NIH3T3 cells were stable transfected with human TrkB plasmid. PC12 cells were grown in DMEM medium (11965084, Gibco, Grand Island, New York, USA) containing 10% Horse Serum (16050122, Gibco, Grand Island, New York, USA), 5% Fetal Bovine Serum (10270106, Gibco, Grand Island, New York, USA), 100 units/ml Penicillin and 0.1mg/ml Streptomycin (15140122, Gibco, Grand Island, New York, USA) at 5% CO2 and 37oC. Cells were used between passages 5-20. HEK293T and NIH3T3 cells were grown in the same medium as PC12 cells supplemented with 10% Fetal Bovine Serum and lacking Horse Serum. For transient transfection of HEK293T cells lipofectamine was used and cells were transfected with human p75NTR and Traf6 plasmids.

Immunoprecipitation and Immunoblotting

For immunoprecipitation experiments PC12 or HEK293T cells were used when at 70-80% confluent. Cells were starved from serum for 4hrs and subsequently treated with 100ng/ml NGF or 500nM of compound ENT-A013 for 30'. Cells were then lysed in Pierce™ IP Lysis Buffer (87788, ThermoFischer Scientific, Rockford, USA) containing proteases (539138, Calbiochem, Darmstadt, Germany) and phosphatases inhibitors (524629, Calbiochem, Darmstadt, Germany). Lysates were then immunoprecipitated overnight at 4oC with TrkA antibody (1:100, 06-574, Sigma-Aldrich, St. Louis, MO, USA) or p75NTR antibody (1:100, ab6172, Abcam, Cambridge, UK) followed by 4hrs incubation with protein G-plus agarose beads (sc-2002, Santa Cruz Biotechnology, California, USA). Beads were then collected, washed 3X with lysis buffer, resuspended in SDS loading buffer and subjected to Western Blot against phosphorylated Tyrosine (1:1000, BAM1676, R&D systems, Mineapolis, USA) or Traf6 antibody (1:2000, ab33915, Abcam, Cambridge, UK). Whole cell lysates were subjected

to Western Blot against TrkA (1:1000, 06-574, Sigma-Aldrich, St. Louis, MO, USA), phosphorylated TrkB (1:1000, ABN1381, Sig-ma-Aldrich, St. Louis, MO, USA), phosphorylated TrkC (1:1000, STJ90960, St John's Laboratory, London, UK), TrkB (1:1000, 07-225-I, Sigma-Aldrich, St. Louis, MO, USA), TrkC (1:1000, C44H5, Cell Signalling Technology, Danvers, MA, USA), p75 (1:1000, 839701, Biolegend, San Diego, USA), Traf6 (1:2000, ab33915, Abcam, Cambridge, UK) phosphorylated Akt (1:1000, 9721S, Cell Signalling Technology, Danvers, MA, USA), phosphorylated Erk1/2 (1:1000, 9101S, Cell Signalling Technology, Danvers, MA, USA), total Akt (1:1000, 4691S Cell Signalling Technology, Danvers, MA, USA) and total Erk1/2 (1:1000, 4695S, Cell Signalling Technology, Danvers, MA, USA).

CellTox assay

CellTox assay (G8742, Promega, Leiden, Belgium) was used to assess survival of PC12 cells under serum deprivation conditions. PC12 cells were plated in 96-well plates, starved from serum for 4hrs and subsequently treated with NGF (100ng/ml) or ENT-A013 (500nM) in the presence or absence of TrkA inhibitor GW441756 (20uM, G-190, Alomone labs, Jerusalem, Israel) for 24hrs. CellTox assay reagents and Hoescht (1:10,000, H3570, Invi-trogen, Massachusetts, USA) were then added to each well for 30' and then cells were imaged with a Zeiss AXIO Vert A1 fluorescent microscope. CellTox positive cells were normalised to total number of cells for each image.

Primary Dorsal Root Ganglia neurons

Primary Dorsal Root Ganglia (DRG) neurons were isolated from P0-P1 mouse pups (C57BL/6J, Jackson Laboratory) as described previously [16] and cultured with Neurobasal medium (21103049 Gibco, Grand Island, New York, USA) containing 2% B27 (17504044,

Gibco, Grand Island, New York, USA), 100ng/ml NGF (N-100, Alomone Labs, Jerusalem, Israel), 1X GlutaMax (35050061, Gibco, Grand Island, New York, USA), 10mM HEPES (15630080, Gibco, Grand Island, New York, USA), 1% Penicillin/Streptomycin (15070063, Gibco, Grand Island, New York, USA) and 10uM 5-Fluoro-2'-deoxyuridine (343333, Calbiochem, Darmstadt, Germany). For TUNEL assay, cells were cultured for 14 days in vitro and then medium was deprived of NGF and ENT-A013 (500nM, every 24hrs) added along with NGF-neutralizing antibody (N8773, 1:500, Sigma-Aldrich, St. Louis, MO, USA) and cells incubated for 48hrs. Cells were then fixed with 4% PFA and labelled with TUNEL (11684795910, Roche, Hertfordshire, UK) following the manufacturer's protocol. Subsequently, cells were immunostained against Tuj1 (1:2,000, 801201, Biolegend, San Diego, USA) and anti-mouse Cy3 (1:1000, Invitrogen, A10521, Massachusetts, USA) and imaged with a Leica SP8 confocal microscope. For neurite outgrowth assay, cells were cultured for 5 days in vitro with medium deprived of NGF in the presence of ENT-A013 (500nM, every 48hrs), along with NGF-neutralizing antibody. Subsequently, cells were fixed with 4% paraformaldehyde, immunostained against TUJ1, anti-mouse Alexa fluor 488 (1:1000, A-11029, ThermoFischer Scientific, Rockford, USA) and imaged by confocal microscopy and neurite length was measured using Fiji.

Primary Hippocampal neurons

Primary hippocampal neurons were isolated from E17.5 mouse embryos (C57BL/6J, Jackson Laboratory) as previously described [17] and cultured with Neurobasal medium containing 2% B27, 1X GlutaMax, 10mM HEPES and 1% PenStrep. Cells were maintained in a humified incubator at 37oC and 5% CO2. For TUNEL assay, cells were left in culture for 16-18 days and then treated with 5uM oligomeric Aβ along with ENT-A013 (500nM, every 24hrs) for 48hrs. Cells were then fixed with 4% PFA and labelled with TUNEL (Roche, cat#

11684795910) following the manufacturer's protocol. Subsequently, cells were immunostained against Tuj1 and imaged with confocal microscopy. For synaptic plasticity assay, cells were left in culture for 16-18 days and then treated with 5uM oligomeric Aβ along with ENT-A013 (500nM) for 4hrs. Cells were then fixed with 4% PFA and im-munostained against Tuj1 and Synaptophysin (1:1000, PA1-1043, Invitrogen, Massa-chusetts, USA) and anti-mouse Alexa fluor 488 (1:1000, A-11029, Invitrogen, Massachu-setts, USA) and anti-rabbit Alexa fluor 546 (1:1000, A10040, Invitrogen, Massachusetts, USA). Images acquired using confocal microscopy and total area of synaptophysin posi-tive puncta was measured and normalized to Tuj1 total area.

Preparation of Aβ oligomers

Aβ (1-42) peptide was purchased from AnaSpec (AS-20276, AnaSpec, California, USA) and prepared according to manufacturer's instructions. For oligomeric Aβ treatment, Aβ peptide was diluted in DMEM at the specified concentrations and left for 24hrs at 37oC. It was then centrifuged for 5' at 15,000g and the supernatant collected as oligomeric Aβ as it has been previously described [18].

Electrophysiology

C57BL/6J mice were euthanized under halothane anaesthesia and the brain was quickly dissected and placed in ice-cold oxygenated artificial cerebrospinal fluid (aCSF; 95% O2/5% CO2) containing (in mM): 125 NaCl, 3.5 KCl, 26 NaHCO3, 1 MgCl2, and 10 glucose (pH = 7.4, 315 mOsm/l). The brain was then placed onto the stage of a vibratome (Leica, VT1000S, Leica Biosystems GmbH, Wetzlar, Germany) and transverse slices containing the hippocampus were obtained. The brain slices (400um) were then placed in a sub-merged chamber containing oxygenated (95% O2/5% CO2) aCSF (in mM): 125 NaCl, 3.5 KCl, 26

NaHCO3, 2 CaCl2, 1 MgCl2, and 10 glucose (pH = 7.4, 315 mOsm/l) at room temperature and left resting for at least 1hr. Following that, slices were treated for 3-5 hrs with oligomeric Aβ1-42 (5uM) in the presence or absence of compound ENT-A013 (500nM) and subsequently placed in the recording chamber. A recording electrode, containing 3M NaCl was placed in the stratum radiatum of the CA1 area of the hippocampus and a platinum/iridium metal stimulating microelectrode (Harvard apparatus, Cambridge, UK) was placed in the same layer, 300um apart. Field excitatory post-synaptic potentials (fEPSP) were amplified using an EXT-02F amplifier (National Instruments), digitized with ITC-18 (Instrutech, Inc.) and recorded in a computer running Windows 10 with IgorPro software (Wavemetrics Inc, Lake Oswego, OR, USA). Electrical stimulation was generated using a stimulator equipped with a stimulus isolation unit (npi electronic GmbH, Tamm, Germany). To induce LTP, theta-burst stimulation (TBS), consisting of 5 pulses at 100Hz, was given at theta-rhythm (every 200ms) and repeated three times with an inter-stimulus interval of 20s. The fEPSP amplitude was calculated from the minimum voltage value of the synaptic response (3-5ms following stimulation) compared to the baseline voltage prior to stimulation. fEPSP recordings were acquired every 90” for 10’ before TBS and continued for approximately 50’ following LTP induction. Recorded traces were analysed using custom made script in IgorPro software.

Animals

All animals handling and experiments were approved by the ethical committee of FORTH (20/28-11-2018). Four month old C57BL/6 male mice and their littermates heterozygotes for hAPP were administered with ENT-A061 compound or placebo for the duration of 2 months. ENT-A061 was formulated into pellets that were subcutaneously implanted in the animals and a steady dose of 10mg/kg was released daily for 2 months after the surgery. Behavioural

experimentation began when animals reached 5.5 months of age and sacrificed at 6.5 months old.

Behavioural experiments

Animals were subjected in the following tests in order: Novel Object Recognition, Object-to-Place recognition, Temporal object recognition, Morris Water Maze test, Reverse Morris Water Maze test and Contextual Fear Conditioning and Discrimination test. Before the start of the behavioural experimentation the animals were familiarised with the experimenter for 7 days. Additionally, animals received one day off between the experiments.

Novel Object Recognition test

Animals were habituated for 10’ for 3 consecutive days in an open field chamber 45 x 45 x 45 immediately prior of the experiment day. On the experiment day, two identical objects were presented to the animals and were left to examine them for 10’. After 1h one object was replaced by a novel one and the animals were left to explore for 5’. 24h later, the novel object was replaced with a third one and animals again left for 5’ to explore. The discrimination index of the test phase was calculated as: [(time exploring the novel object − time exploring the familiar object)/(time exploring the novel object + time exploring the familiar object)].

Object to place recognition test

Animals were habituated for 15’ and the following day animals were presented with two non-identical objects and left to explore for 10’. After 1h one object was displaced and animals were left to explore for 5’. The object discrimination index was calculated as previously described.

Temporal object recognition test

Animals were habituated for 15’ and the following day animals were presented twice with two non-identical objects and left to explore for 10’ each time. For the test trial, animals were exposed to one object from the first (less familiar) and one from the second trial (familiar) and were left to explore for 5’. The object discrimination index was calculated as before.

Morris Water Maze test

The maze consisted of circular pool 60cm in height and 120cm in diameter placed in a room with ambient lightning and spatial cues upon the walls. The pool was filled with 20-22 $^{o}$C water up until 40cm and a platform 8 cm wide was placed just underneath the water surface in the centre of a quadrant. During training sessions (days 1-4), mice were undergone 4 trials and in each trial they were left to swim for 60s and try to find the platform. If the animal found the platform it was allowed to stay on the platform for 3s while if not, it was guided to the platform and allowed to stay for 10s. On day 5, mice underwent a probing trial where the platform was removed, animals placed in the quadrant opposite to the platform location and were left to swim for 90s. For each training day the average latency to find the platform and the number of successful trials was recorded. For probe trial, the time and distance spent in the platform quadrant was recorded as well as the number of times the animal crossed the location where the platform had been. For the Reverse Morris Water Maze test, the same procedure was followed but the platform was placed in a different quadrant.

Y-Maze Spontaneous Alternation test

The Y-Maze apparatus consists of three 35 cm long arms (A, B and C) at 120$^{o}$ angle from each other. Animals were introduced to one arm and they left to freely explore for 8’. The number

and the sequence of arm entries was recorded and analysed, where an arm entry was considered when all four limbs had entered the arm. The alternation index was calculated as (the number of alternating triads)/(total number of triads).

Contextual Fear Conditioning and Discrimination test

For the contextual fear conditioning test day 1, animals were placed in a chamber and after 7' of freely exploring the chamber they received a mild electrical foot shock (0.5mA, 1s) and subsequently were left for additional 5' in the chamber. On day 2, they were placed back in the chamber for 3' and the freezing behaviour was recorded. One hour later, they were placed in the same chamber but with different context, consisting of an altered floor (red carton), odour (2% vinegar) and auditory (white noise), for 3' and the freezing behaviour was recorded. On day 3 the same procedure was followed as in day 2. The percentage of freezing was measured using JWatcher and calculated as [(times found not moving)/(times found moving + times found not moving)*100].

# 3. Results

**Chapter I: Biological screening of novel compounds for their neurotrophic activity in cell lines**

### 3.1.1 Screening of compounds that induce TrkA downstream signalling kinases AKT and ERK1/2 activation and promote survival of PC12 cells

The first goal of this thesis was to characterize newly synthesized compounds, DHEA analogs or others, that can induce TrkA signalling and have a protective, pro-survival effect. In order to achieve that, we used PC12 cells, which endogenously express TrkA and p75 receptors and have been routinely used for investigating NGF-TrkA signalling. Here, we deprived cells from serum for 4hrs, and then treated cells for 30' with either NGF or compound. Western Blot analysis against phosphorylated Akt and Erk1/2 revealed several compounds that could activate either Akt or Erk1/2 (Figure 5). More specifically, compounds ENT-A020 (FC 2.02 ± 0.23), ENT-A021 (FC 2.78 ± 0.83) and ENT-A023 (FC 3.30 ± 1.26) were shown to significantly increase activation of Akt, compared to negative control Serum Free (Figure 5B). Similarly, compounds ENT-A016 (FC 7.32 ± 2.46) and ENT-A023 (FC 10.44 ± 5.06) were shown to significantly increase activation of Erk1/2 compared to control (Figure 5C).

The next question was whether these compounds could protect PC12 cells from serum deprivation-induced cell death. To achieve this, we used PC12 cells that had been starved of serum in their culture medium for 24 hours. Following that, we used CellTox assay to calculate the percentage of cell death in comparison to the untreated control. Figure 6 shows a few additional compounds that demonstrated promise in protecting PC12 cells from Serum deprivation-induced cell death. PC12 cells treated with compounds ENT-A025 (10.4% ± 3.2) and ENT-A059 (11.6% ± 1.9) and bacterial extract 987 (14.2% ± 3) demonstrated a significantly lower percentage of cell death when compared to the negative Serum Free control (22.8 ± 1.9). Overall, none of the aforementioned compounds were able to activate the TrkA

signalling cascade while also protecting PC12 cells against Serum deprivation-induced cell death. Despite this, we were able to identify a few other compounds that could activate TrkA, its downstream signalling cascade, and protect PC12 cells from Serum deprivation-induced cell death.

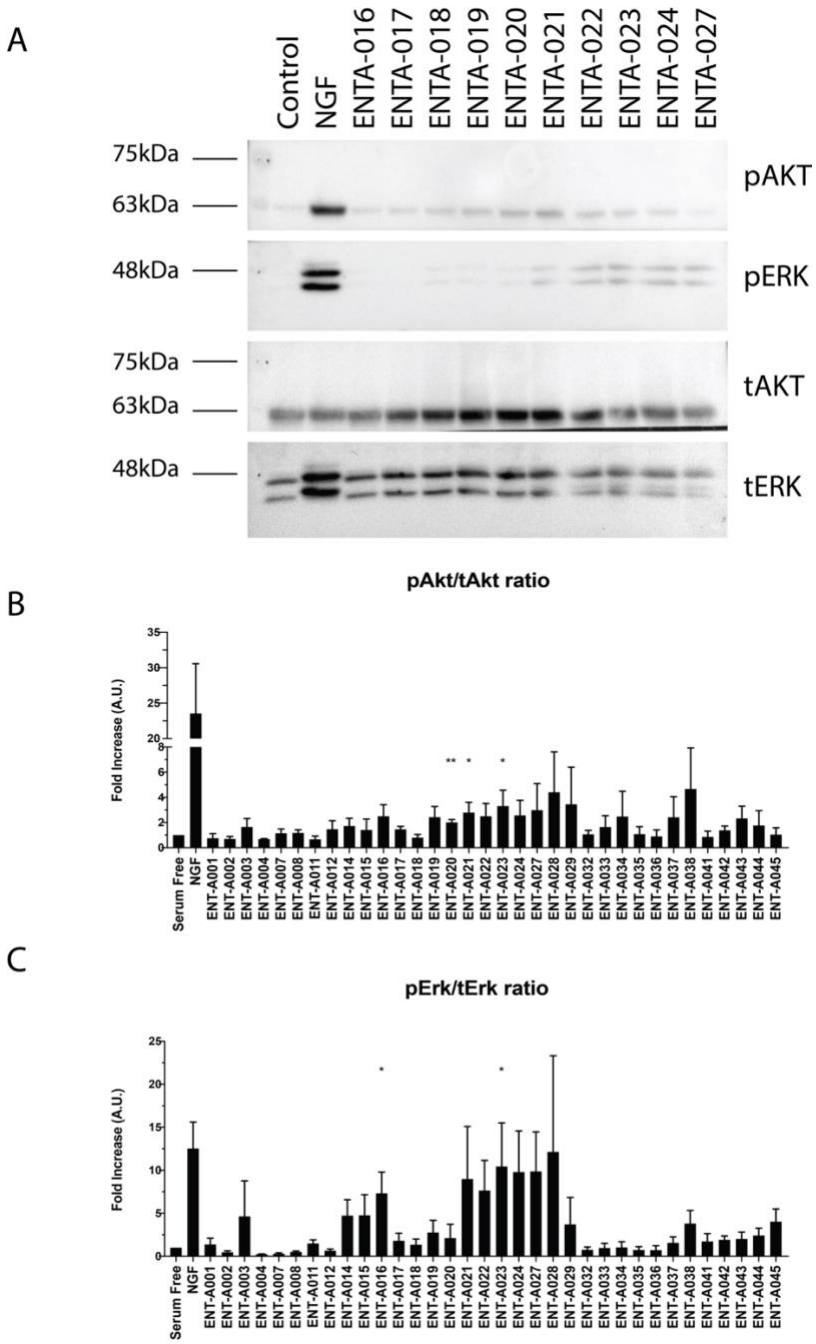


*Figure 5: Screening and identification of compounds of interest for their ability to activate TRKA downstream signaling kinases AKT and ERK1/2. (A) Example Western Blot against AKT and ERK1/2 from PC12 cells treated with respective compound or NGF for 30' and subjected. (B, C) Quantification of 2-3 independent experiments. Data are shown as mean ± SEM, *: p<0.05, **: p<0.01; Student's t-test against negative control.*

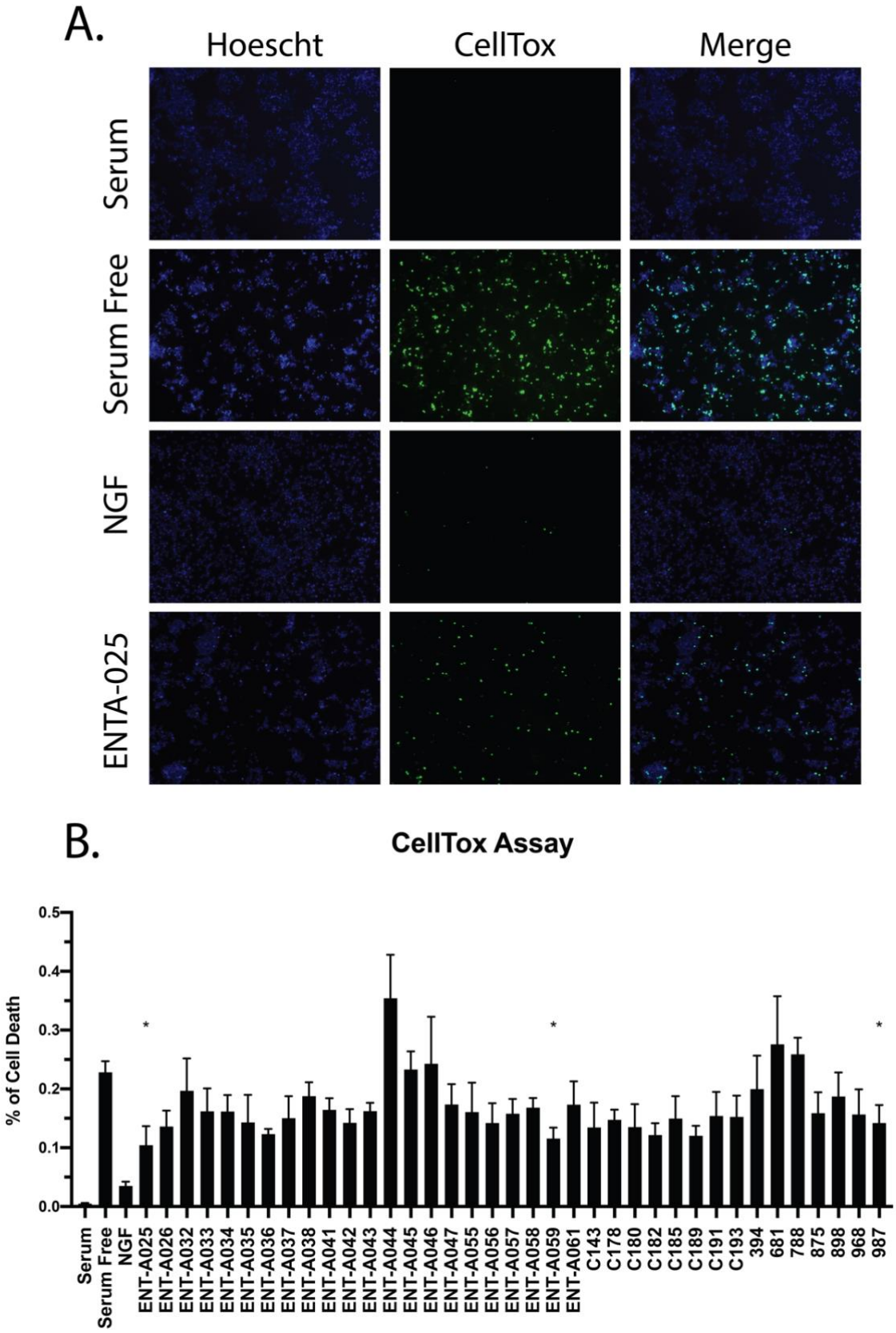


*Figure 6: Screening and identification of compounds of interest to promote PC12 cells survival. PC12 cells starved from serum and then treated with compound or NGF for 24hrs and then CellTox and Hoescht added and measurement taken. (A) Representative images and (B) Quantification. Data are presented as ± SEM, *: p<0.05; one-way ANOVA, multiple comparisons, Turkey's test correction, n=3-4 independent experiments.*

### 3.1.2 ENT-A010 activates TrkA and its downstream kinase Akt and protects PC12 cells against cell death in a TrkA-dependent manner.

ENT-A010 is one of the first generation BNN27 analogs that was shown to induce TrkA signaling. To test TrkA phosphorylation, PC12 cells were stimulated for 30 min with ENT-A010 or NGF, and proteins were immunoprecipitated for TrkA and immunoblotted against phosphorylated tyrosine. ENT-A010 induced TrkA phosphorylation (FC 2.53 ± 0.51), at levels similar to those of NGF (FC 3.6 ± 1). Additionally, ENT-A010 treatment in PC12 cells was able to induce phosphorylation of Akt, a pro-survival kinase, downstream of TrkA. Indeed, Figure 7A,B shows significantly increased Akt phosphorylation in ENT-A010-treated cells compared to control (FC 5.47 ± 1.35). We then proceeded to investigate the potential anti-apoptotic effect of ENT-A010 in PC12 cells that have been deprived from serum and treated for 24hrs in the presence of either NGF or ENT-A010. Figure 7C-D shows that indeed treatment with ENT-A010 (10.39% ± 1.14) protects PC12 cells against serum deprivation-induced apoptosis in levels comparable to NGF treatment. Furthermore, pharmacological inhibition of TrkA using the specific inhibitor GW174456 showed complete reversal of the observed rescue effect, revealing a selective action of ENT-A010 through TrkA receptor in promoting cell survival.

### 3.1.3 ENT-A040 induces TrkA phosphorylation and activates its downstream signaling kinases Akt and Erk1/2 while promoting PC12 cell survival via TrkA

ENT-A040 is a second generation BNN27 analog, which was designed based on the ENT-A010 structure to achieve an improved and more specific action on TrkA receptor. Compound ENT-A040 was tested for its ability to activate TrkA signalling and shown that it could strongly induce activation of both kinases Akt (FC 2.9 ± 0.13) and Erk1/2 (FC 3.7 ± 0.57) (Figure 8 A, B). Furthermore, immunoprecipitation experiment using TrkA antibody for precipitating total

TrkA protein and subsequent Western Blot analysis against phosphorylated Tyrosine residues, showed that synthetic compound ENT-A040 can induce TrkA phosphorylation at levels similar to those of NGF. We then proceeded to investigate any potential anti apoptotic effect of ENT-A040 in PC12 cells that have been starved from serum and left for 24hrs in the presence of either NGF or ENT-A040. Indeed, treatment with ENT-A040 protects PC12 cells against apoptosis in levels comparable to NGF treatment. Furthermore, pharmacological inhibition of TrkA using the specific inhibitor GW174456 showed reversal of the observed effect revealing a selective action of ENT-A040 through TrkA receptor in promoting cell survival (Figure 8 C, D).

### 3.1.4 ENT-A013 Activates TrkA and Its Downstream Signalling Kinases Akt and Erk1/2

In order to test whether ENT-A013 activates the TrkA neurotrophin receptor and its downstream signalling kinases, we used PC12 cells, a cell line that express TrkA and p75NTR and has been routinely used to study NGF-TrkA signalling. For our experiments, cells were starved of serum for 4 h in order to be synchronized prior to treatment with NGF (100 ng/mL) or ENT-A013 (500 nM) for 30′. Immunoprecipitation experiments with TrkA antibody and subsequent Western blot analysis against phosphorylated Tyrosine residues showed that compound ENT-A013 can induce phosphorylation of TrkA (% of control FC 2.05 ± 0.21) at levels comparable to NGF (% of control FC 2.30 ± 0.62) (Figure 9A,B). In addition to that, this interaction is specific for TrkA, since ENT-A013 did not induce phosphorylation of other neurotrophin receptors TrkB or TrkC (Data not shown). Furthermore, ENT-A013 can also induce phosphorylation of Akt (% of control FC 3.8 ± 1.15) and Erk1/2 (% of control FC 2.5 ± 0.6), two kinases that are downstream of TrkA signalling and have been associated with NGF promotion of cell survival.

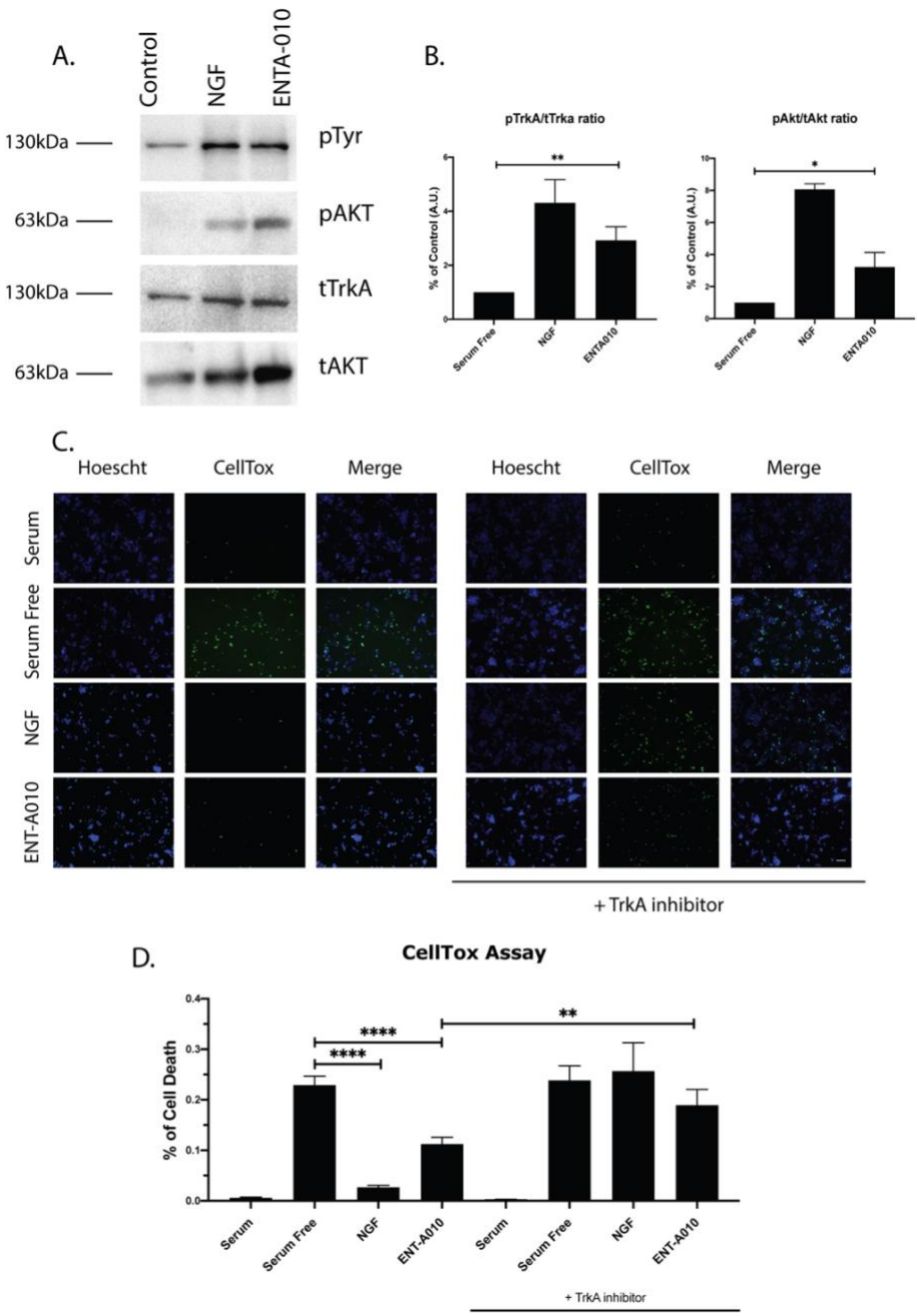


*Figure 7: ENT-A010 activates TrkA and its downstream signalling kinase Akt and protects PC12 cells against serume deprivation induced cell death. (A, B) ENT-A010 induced TrkA phosphorylation and Akt phosphorylation. PC12 cells were treated for 30 min with NGF (100 ng/ml), ENT-A010 (500 nM) or vehicle control. TrkA was immunoprecipitated and membranes were immunoblotted for phosphoTyr and whole cell lysates were analysed for TrkA. For Akt activation, whole cell lysates were subjected in Western Blot. Representative blots are shown. Data are shown as mean ± SEM, *: p<0.05, **: p<0.01; n=3-5 independent experiments. (C) ENT-A010 promoted survival of PC12 cells via TrkA. PC12 cells were treated for 24 h with NGF (100 ng/ml), ENT-A010 (500 nM) or a vehicle control, with or without the selective TrkA inhibitor GW174456 under serum starvation and stained with CellTox (green) and Hoechst (blue). Scale bar: 200 µm. Quantification of cell viability, calculated as percentage of dead cells per total number of cells in each image. Data are shown as mean ± SEM, n=8-9 and 3-4 for the conditions with GW174456, **: p<0.01, ****: p<0.0001.*

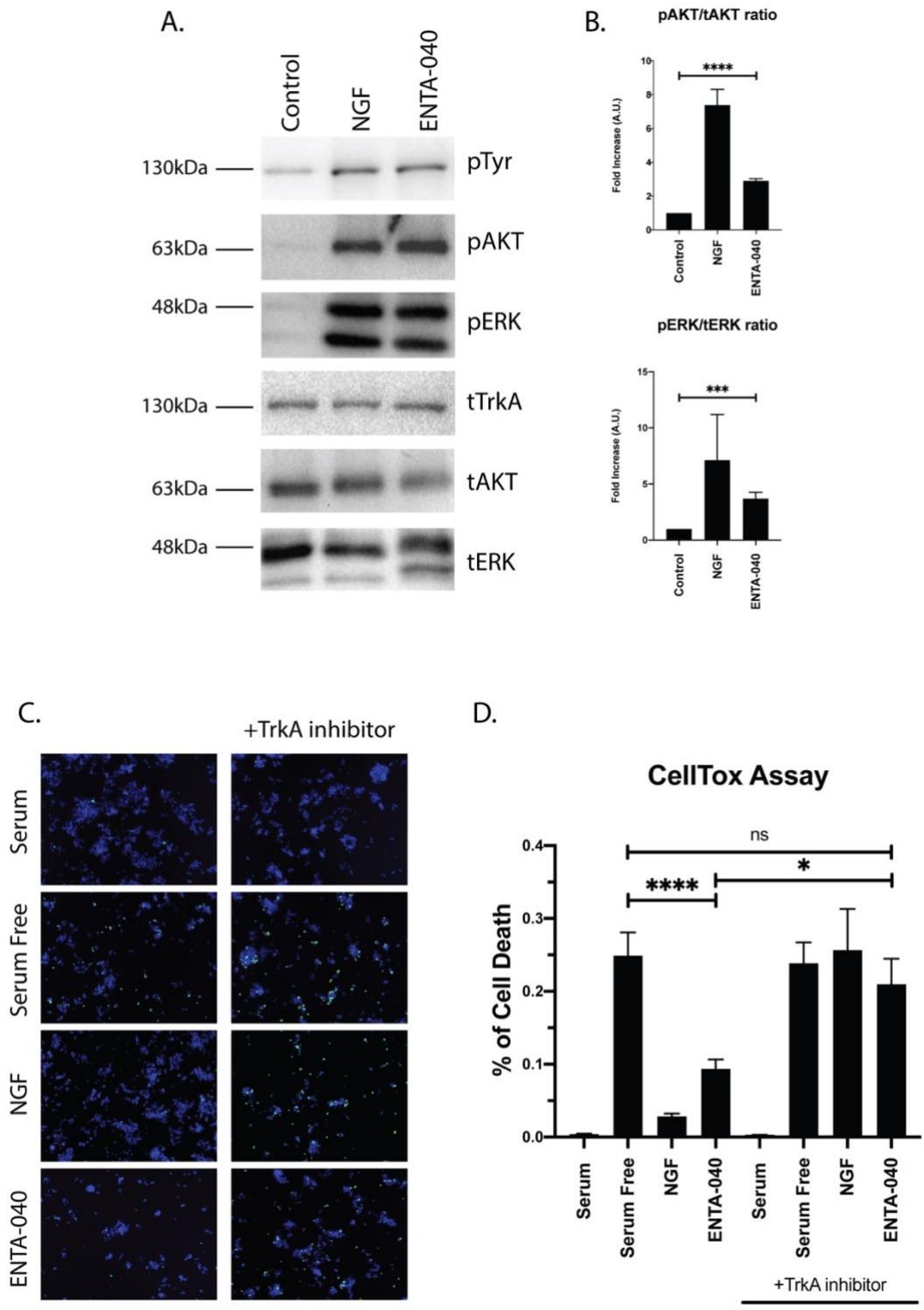


*Figure 8: ENT-A040 activates TrkA and its downstream signalling kinases Akt and Erk1/2 and protects PC12 cells against Serum-deprivation induced cell death via TrkA. (A) ENTA-040 induces TrkA phosphorylation and Akt and Erk1/2 activation in similar levels to NGF control. (B) Quantification of 3-4 independent experiments, 1 for pTyr/TrkA. Data are shown as mean ± SEM, *: p<0.05, **: p<0.01; ***: p<0.001; ****: p<0.0001; Student's t-test against negative control. (C, D) PC12 cells treated for 24hrs with NGF or ENTA-040 under serum free conditions and in the presence of the specific TrkA inhibitor GW174456. Representative images and quantification. Data are shown as mean ± SEM, *: p<0.05, ****: p<0.0001; One-way ANOVA, multiple comparisons, Turkey's test correction, n=4-8 independent experiments*

### 3.1.5 Both ENT-A013 Geometrical Isomers Activate TrkA Receptor and Its Downstream Signaling Kinase Erk1/2

Based on ENT-A013 structure, it is evident that it can be distinguished in two geometrical isomers, termed ENT-A013E and ENT-A013Z. In order to elucidate if there are any differences in the activity of each isomer on TrkA receptor activation, we treated PC12 cells with each isomer (500 nM) or NGF (100 ng/mL) for 30′ followed by immunoprecipitation with TrkA and subsequent Western blot analysis against Phosphorylated Tyrosine residues. Figure 9C, D shows that each of the isomers can indeed induce TrkA phosphorylation at similar levels with NGF treatment (% of control FC 59.4 ± 37.6 for ENT-A013E, 69.3 ± 46.14 for ENT-A013Z). Additionally, both isomers can activate downstream signaling kinase Erk1/2 (% of control FC 7.87 ± 2.23 for ENT-A013E, 4.13 ± 1.46 for ENT-A013Z), hence suggesting that both ENT-A013E and ENT-A013Z achieve NGF-mimicking action equally with regards to the ENT-A013 [E,Z (1:1) mixture of isomers].

### 3.1.6 ENT-A013, ENT-A013E and ENT-A013Z Sustain TrkA and Erk1/2 Phosphorylation Long Term and Up to 1 h after Treatment

We then investigated whether the ENT-A013-mediated activation of TrkA is a fast transient effect or can be sustained over longer periods of time. To this end, we treated PC12 cells with either compound (ENT-A013, ENT-A013E and ENT-A013Z) or NGF for 10, 30 or 60′. Figure 9E shows that indeed all three compounds can sustain TrkA phosphorylation for up to 1hr following treatment at comparable levels to NGF treatment, suggesting a different pattern of TrkA activation compared to the BNN27 compound, which shows different kinetics on TrkA-Erk1/2 activity compared to NGF.

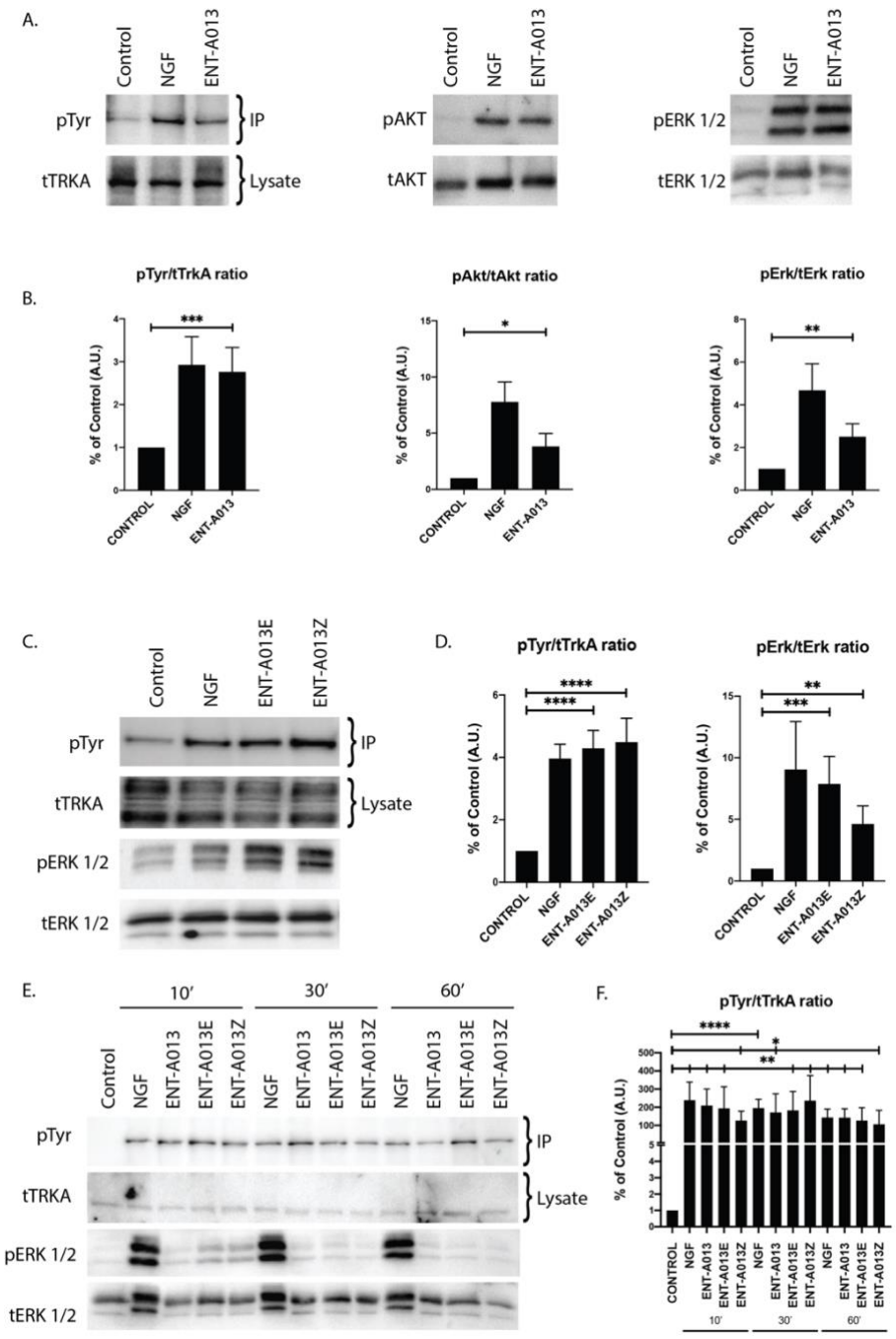


*Figure 9: ENT-A013 ([E,Z (1:1) mixture] and the pure isomers ENT-A013E & Z activate TrkA and its downstream signaling kinases. (A,B) 30' treatment of PC12 cells with ENT-A013 or vehicle was able to induce strong phosphorylation of TrkA and its downstream signaling kinases Akt and Erk1/2 as shown by immunoprecipitation or whole lysate western blots. Representative blots of 3-6 independent experiments (A) and quantification (B) is shown. Data are shown as ±SEM, Stu-dent's t-test against Control; ** p<0.01; *** p<0.001. (C,D) 30' treatment of PC12 cells with ENT-A013E & Z or vehicle was able to induce phosphorylation of TrkA and Erk1/2 as shown by immunoprecipitation or whole lysate western blot respectively. Representative blots of 4-6 inde-pendent experiments (C) and quantification (D) is shown. Data are shown as ±SEM, Student's t-test against Control; * p<0.05; ** p<0.01; *** p<0.001. (E,F) PC12 cells treated for 10', 30' or 60' with ENT-A013, ENT-A013E, ENT-A013Z or vehicle show that all compounds can induce TrkA phosphorylation which is sustained over 60' after treatment. Representative blots of 3 (IP) or 2 (Erk1/2) independent experiments (E) and quantification for pTyr activation (F) are shown. Data represent ±SEM, Student's t-test against Control; * p<0.05; ** p<0.01; **** p<0.0001.*

### 3.1.7 ENT-A013 Protects PC12 Cells from Serum Deprivation-Induced Cell Death through TrkA Receptor

Having established that compound ENT-A013 [E,Z (1:1) mixture] and its pure isomers ENT-A013E and ENT-A013Z induce sustained phosphorylation of TrkA and its downstream kinases, we then proceeded to investigate the potential anti-apoptotic effect of these compounds. We used PC12 cells treated for 24 h with our compounds or NGF in the absence of serum. Following treatment, cells were stained with CellTox reagent and Hoescht to identify apoptotic cells. Our compounds could protect cells from serum deprivation-induced cell death (0,11 ± 0,01 for ENT-A013, 0,07 ± 0,02 for ENT-A013E and 0,08 ± 0,02 for ENT-A013Z vs. 0,24 ± 0,02 for serum-deprived cells). Furthermore, we show that this anti-apoptotic effect is dependent upon the TrkA receptor's activity since analysis of cells treated for 24 h with the compounds in the presence of a specific TrkA inhibitor revealed no protective effect (Figure 10 A, B). Lastly, we performed a dose–response experiment to identify the $IC_{50}$ and the optimal effective concentration of these compounds. All compounds showed strong anti-apoptotic effect at 500nM and up to 100 μM while no toxicity effects were observed. The $IC_{50}$ for ENT-A013, ENT-A013E and ENT-A013Z anti-apoptotic function was calculated at 166.72 nM, 281.19 nM and 272.89 nM, respectively (Figure 10C).

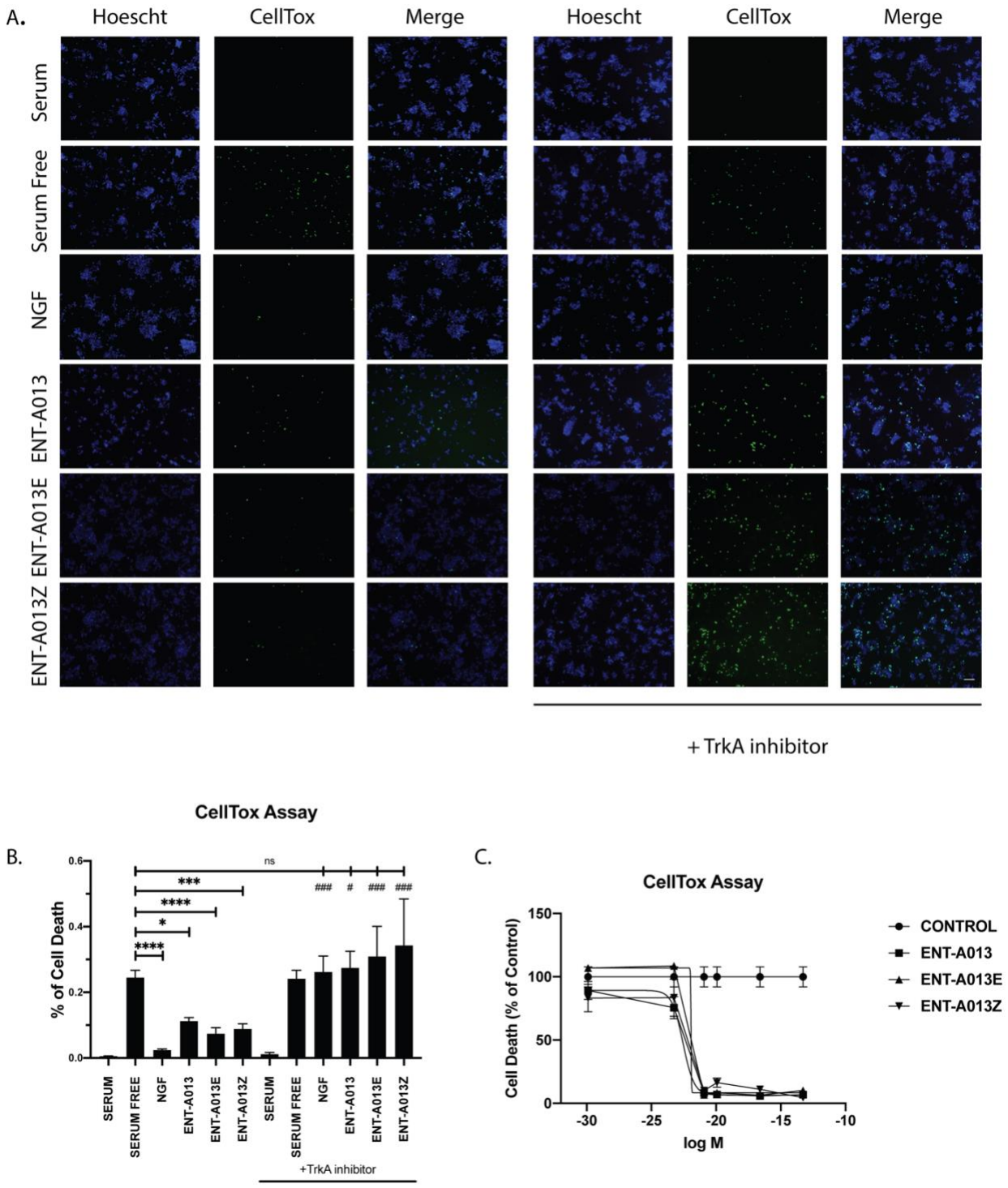


*Figure 10: ENT-A013, ENT-A013E and ENT-A013Z protect PC12 cells against serum deprivation induced cell death in a TrkA-dependent manner. PC12 cells were starved from serum and treated with NGF or ENT-A013 or ENT-A013E or ENT-A013Z or vehicle in the presence or absence of a specific TrkA inhibitor GW441756 for 24hrs and CellTox assay was used to quantify cell death. CellTox positive cell number was normalized to total Hoescht positive cell number in each image. Representative images from 4-9 independent experiments are shown (A) and quantification (B). Data represent ±SEM. One-way ANOVA, Turkey's test correction * and # $p<0.05$; ** and ## $p<0.01$; *** and ### $p<0.001$; **** $p<0.0001$. (C) Dose response curve. IC50 value of compounds ENT-A013, ENT-A013E and ENT-A013Z calculated at 166.72 nM, 281.19 nM and 272.89 nM respectively.*

**Chapter II: Deciphering the functional properties of specific neurotrophin analogs in primary neuronal cells, challenged with toxic amyloid oligomers.**

### 3.2.1 Effects of TrkA-specific compounds in promoting Dorsal Root Ganglian neurons survival in the absence of NGF

We have previously identified certain compounds that were able to activate TrkA and its downstream signalling kinases Akt and/or Ekr1/2. Additionally, they were able to promote cell survival and protect cells against Serum Deprivation-induced cell death in a TrkA-dependent manner. Specifically, these compounds were ENT-A010, ENT-A040 and ENT-A013. We have now used Dorsal Root Ganglia neurons, a neuronal population whose development and survival heavily relies on NGF signalling, to determine whether these compounds can mimic NGF activity and function in a neuronal population.

Hence, we isolated Dorsal Root Ganglia neurons from P0-P1 mouse pups and cultured them for two weeks in the presence of NGF. At 14 days *in vitro* (DIV), the neuronal medium was replaced with one lacking NGF and supplemented with the respective compound and an NGF-neutralizing antibody, in order to block the effect from endogenously produced and secreted NGF. Cells were treated for 48 h, then fixed, and cell death was assessed using TUNEL assay. Indeed, Figure 11 shows that all three compounds were effective in reversing the NGF deprivation-induced apoptosis, and notably, in similar levels with the NGF-treated neurons, indicating that these compounds could effectively mimic NGF protective effects in neuronal populations that are dependent on the neurotrophin's presence. Specifically, TUNEL staining showed that neurons treated with either ENT-A010 (25.82% ± 4.93), ENT-A040 (27.81% ± 10.93) or ENT-A013 (23.32% ± 9.7) showed significantly lower percentage of cell death compared to NGF absence (64.94% ± 8.73) and comparable levels to when NGF was present in the culture medium (20.71% ± 6.72) (Figure 11).

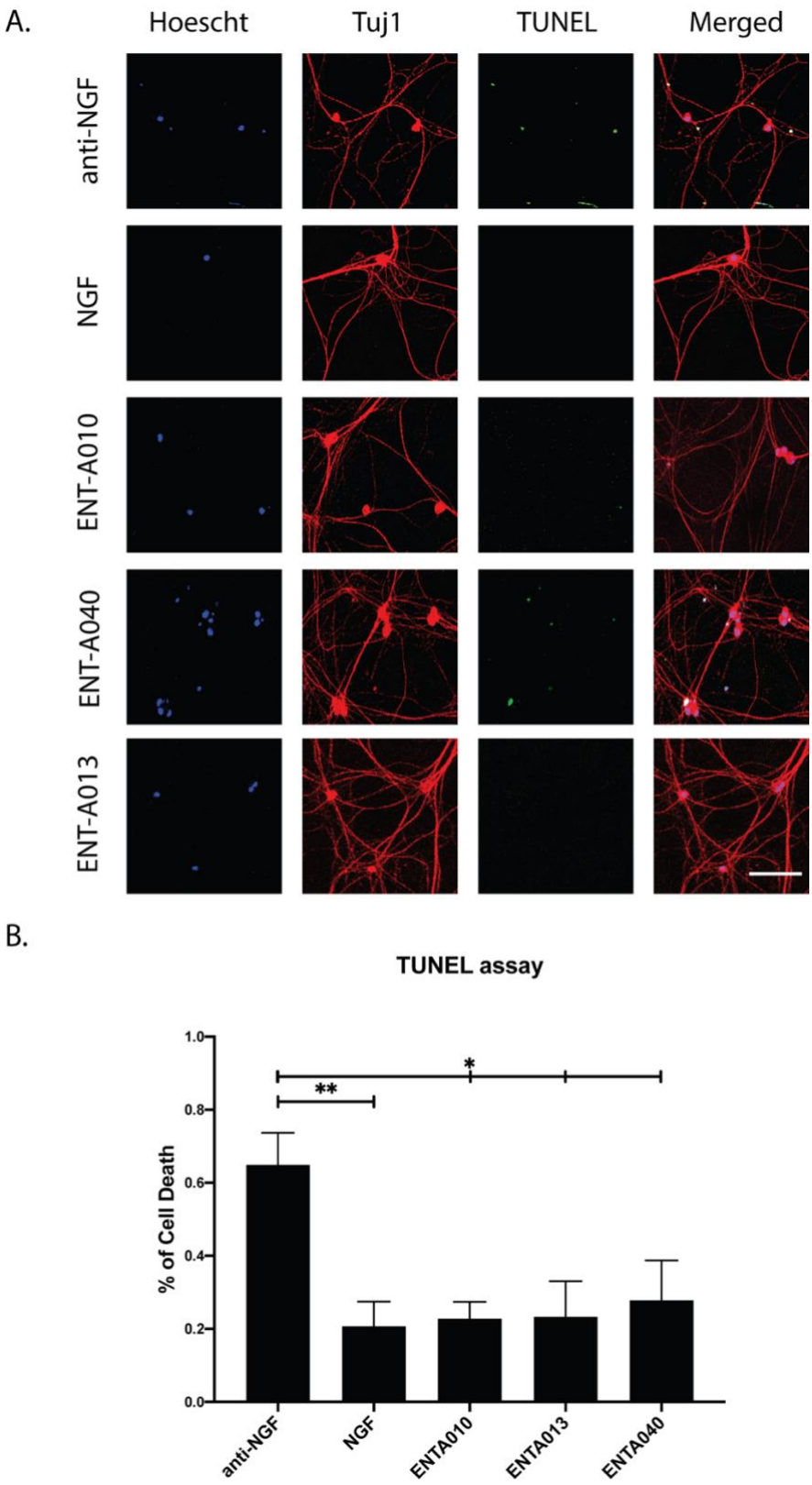


*Figure 11: ENT-A010, ENT-A013 and ENT-A040 protects primary DRG neurons against NGF-deprivation induced cell death. (A, B) Primary DRG neurons were treated for 48hrs with the respective compound in the absence of NGF and in the presence of an anti-NGF neutralizing antibody. TUNEL assay was used to assess neuronal cell death. Representative images from 3-5 independent experiments. Data are shown as ±SEM. One-way ANOVA, Turkey's Test correction. * $p<0.05$; ** $p<0.01$.*

### 3.2.2 Effects of TrkA or TrkB specific compounds in protecting primary hippocampal neurons against Aβ-induced cell death

The Alzheimer's Disease (AD) phenotype is partly attributed to the toxic effects caused by oligmeric Aβ, which results in reduced neuronal trophic support, caused by deregulation of neurotrophic signalling. It is therefore reasonable that a small molecule that can selectively activate neurotrophin signalling and promote neuronal survival could alleviate the detrimental effects caused by toxic Aβ oligomers, especially during the early phases of the disease. In order to investigate whether ENT-A013 can protect cells against Aβ-induced toxicity we isolated neurons from the hippocampus of E17.5 mouse embryos, a brain area that is heavily affected in AD. Neurons were left in culture for 16–18 DIV and then were treated with oligomeric Aβ and the respective compounds for 48 h. Subsequently, cells were fixed and cell death was assessed using TUNEL assay.

In addition to the compounds already mentioned that we showed them to be potent and specific TrkA agonists exerting neuroprotective actions, for this experiment more compounds were tested, as well that were shown to be equally potent and specific TrkB agonist. TrkB is the specific receptor for BDNF, a neurotrophin that is crucially important in regulating neuronal function, including synapse function and plasticity. ENT-A010-treated cells (25.07% ± 1%), ENT-A040-treated cells (30.4% ± 4.6%), ENT-A013-treated cells (29.8% ± 2.5%), ENT-A011-treated cells (20.6% ± 4.1%) showed significantly decreased apoptosis compared to Aβ-treated ones (41.5% ± 4.2%) and comparable to control cells (18.7% ± 2.8%) (Figure 12). On the other hand, ENT-A044-treated cells (34.3% ± 4.4%) failed to show any significant difference with regards to Aβ-induced apoptosis (data not shown). These results suggest that ENT-A010, ENT-A013, ENT-A040 and ENT-A011 can protect primary hippocampal neurons

from oligomeric Aβ-induced cell death, introducing a significant therapeutic potential of these molecule against a major pathological factor of AD.

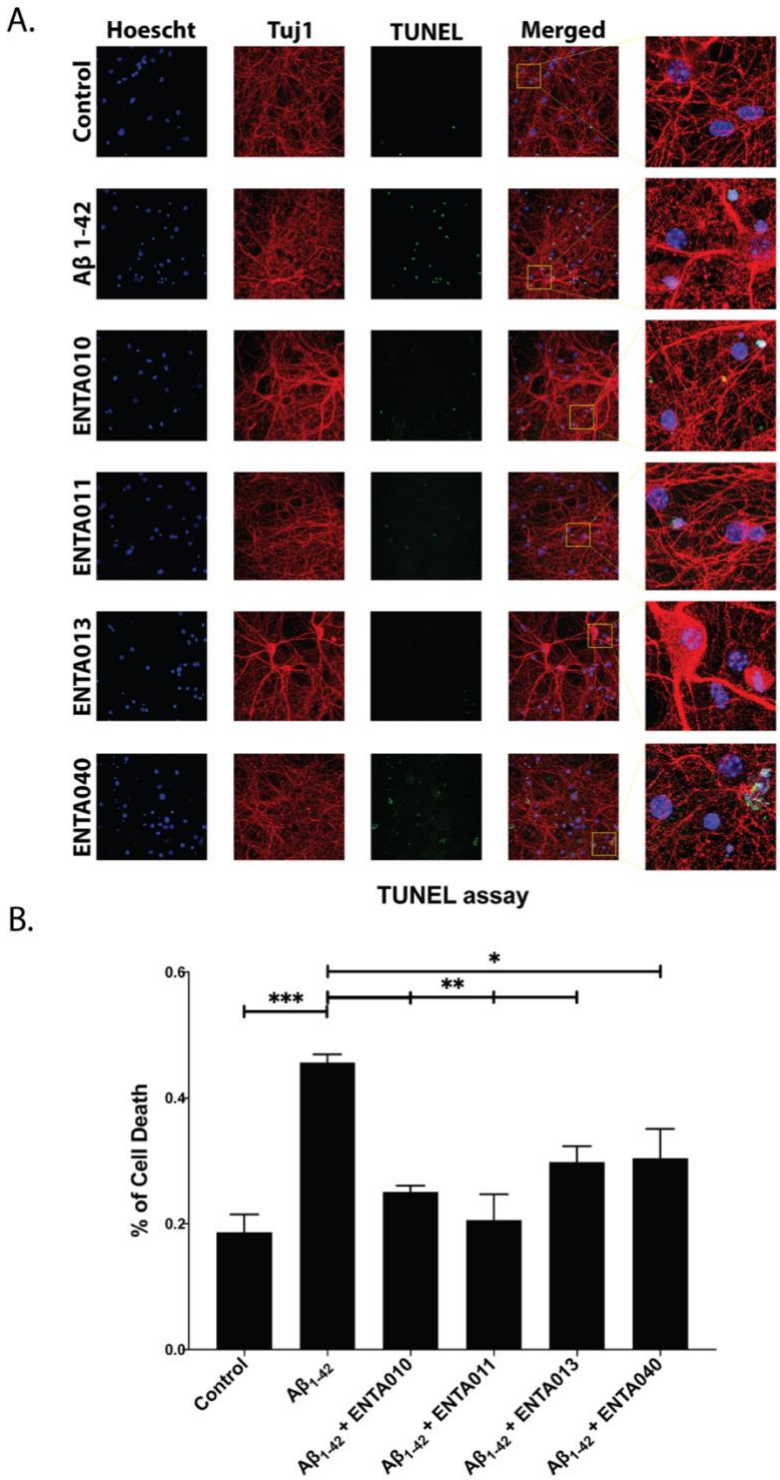


*Figure 12: ENT-A010, ENT-A011, ENT-A013 and ENT-A040 shows anti-amyloid activity. (A,B) Primary hippocampal neurons were treated for 48hrs with oligomeric Aβ in the presence of the respective compounds and TUNEL assay was used to quantify cell death. Representative images from 3 independent experiments. Data are shown as ±SEM. One-way ANOVA, Turkey's Test correction. * p<0.05; ** p<0.01; *** p<0.001.*

### 3.2.3 Effects of TrkA or TrkB specific compounds in protecting primary hippocampal neurons against Aβ-induced synapse loss

Synapse degeneration is a major hallmark of AD, occurring early in the disease's progression, and has been strongly correlated with deficiencies in learning and memory among AD patients. Additionally, it has been shown that neurotrophin signaling plays a crucial role in regulating synapse number as well as enhancing synaptic strength and plasticity. To investigate whether our compounds can protect against Aβ-induced synapse degeneration we used primary hippocampal neurons treated with Aβ and the respective compound. Cells at 14–16 DIV were treated for 4 h, and the synapse number was assessed using immunostaining against synaptophysin, a pre-synaptic marker. Cells treated with Aβ and compounds ENT-A010 (FC 0.96 ± 0.05), ENT-A040 (FC 1.08 ± 0.07), ENT-A013 (FC 1.08 ± 0.08) and ENT-A011 (FC 1.05 ± 0.06) showed significantly reduced synapse degeneration compared to cells treated only with Aβ (FC 0.78 ± 0.03) (Figure 13). Furthermore, cells treated with Aβ and ENT-A044 failed to show resilience in synapse loss (FC 0.89 ± 0.07). Collectively, these results suggest that compounds ENT-A010, ENT-A011, ENT-A013 and ENT-A040 can ameliorate several aspects of Aβ-induced pathology in primary neuronal cells.

### 3.2.4 ENT-A013 Partially Reverses Long-Term Potentiation (LTP) in Brain Slices Treated with Oligomeric Aβ

We next sought to investigate whether ENT-A013 can ameliorate the toxic effects of Aβ in impairing LTP in brain slices from wild type animals. Impaired LTP is an important feature of AD and is considered to be a cellular mechanism that underlies memory and learning deficits, especially in the hippocampal region. Brains from wild type animals were sliced transversely and treated with oligomeric Aβ (5 μM) in the presence or absence of ENT-A013 (500 nM) for 3–5 h and then subjected to LTP protocol. Field Excitatory Postsynaptic Potentials (fEPSPs)

were recorded. Figure 14 shows that control brain slices exhibited significant synaptic potentiation following Theta-Burst Stimulation (TBS). More importantly, TBS in brain slices treated with Aβ and ENT-A013 exhibited significantly increased synaptic potentiation compared to brain slices treated only with Aβ, therefore further supporting the notion that ENT-A013 can be an effective drug in counteracting the deleterious effects of Aβ in synaptic functioning, and potentially restoring cognitive performance in AD patients.

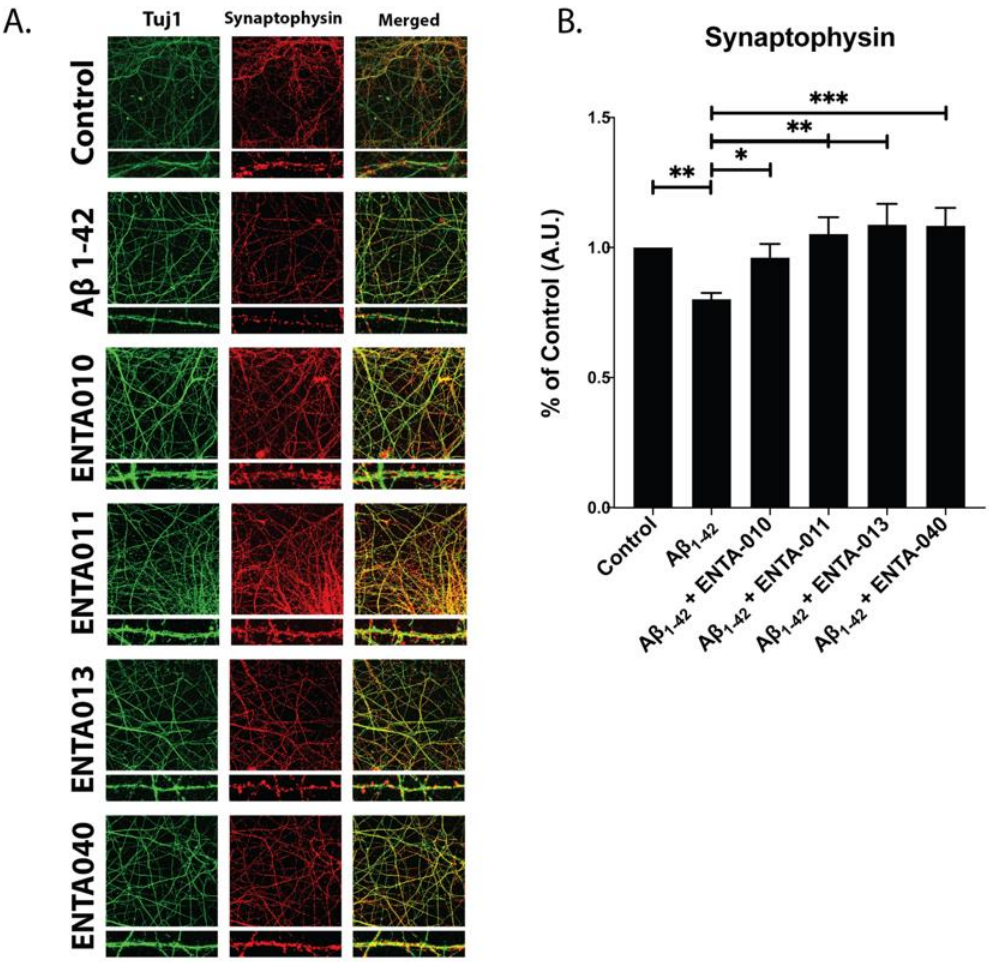


*Figure 13: ENT-A010, ENT-A011, ENT-A013 and ENT-A040 rescues Aβ-induced synapse loss. (A,B) Primary hippocampal neurons treated for 4hrs with oligomeric Aβ in the presence of the respective compounds and immunostained against synaptophysin to assess synapse number. Total Synaptophysin area was normalized to total Tuj1 area in each image. Representative images from 4-5 independent experiments. Data are shown as ±SEM. One-way ANOVA, Turkey's Test correction. ** p<0.01.*

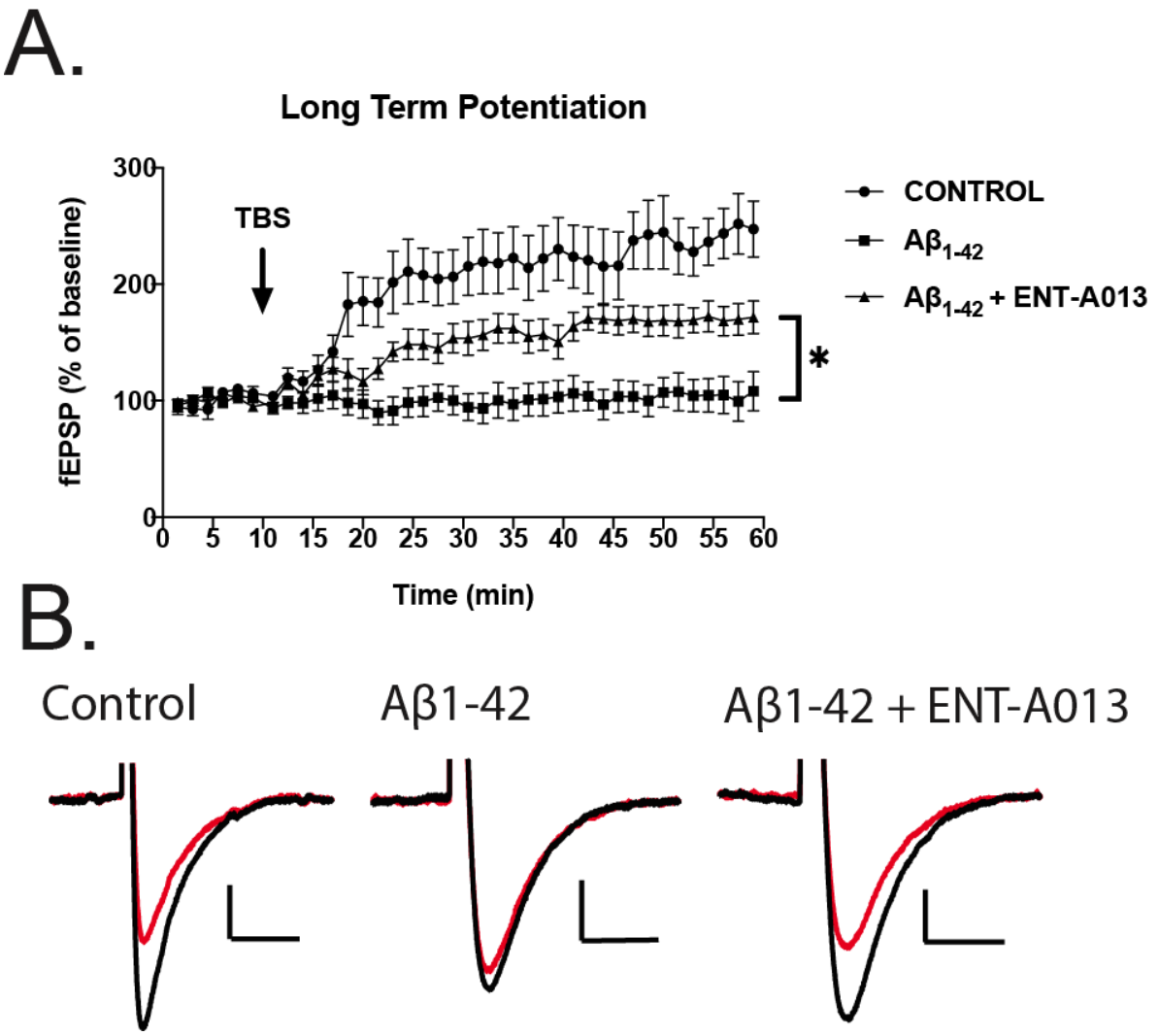


*Figure 14: LTP in hippocampal brain slices treated with with oligomeric Aβ (5uM) in the presence or ab-sence of ENT-A013 (500nM) for 3-5 h. (A) Graph showing LTP induced with TBS in control brain slices (i.e., no treatment, circles), in brain slices treated with oligomeric Aβ (5uM) and in brain slices treated with oligomeric Aβ (5uM) (squares) and ENT-A013 (500nM) (triangles). Statistical analysis ANOVA shows that ENT-A013 treated brain slices have significantly higher synaptic potentiation compared to those treated only with Aβ. Error bars represent SEM. Mixed effects multiple comparison analysis. N=7-9 slices from 3-4 animals. * p<0.05 (B) Representative traces before (red) and after (black) theta burst stimulation for the 3 different groups.*

**Chapter III: *In vivo* studies of neurotrophin analogs in mouse model of AD.**

### 3.3.1 Effects of ENT-A061 on the 5xFAD animal model of Alzheimer's Disease

We have identified the compound ENT-A061 that acts as BDNF agonist and can selectively bind and activate TrkB receptor. Upon activation of TrkB receptor, this compound induces a downstream signalling cascade protecting NIH-3T3TrkB cells from serum deprivation-induced apoptosis. Additionally, experiments in primary hippocampal neurons treated with oligomeric Aβ and ENT-A061 showed that ENT-A061 can protect primary neurons from Aβ-induced cell death and synapse degeneration. Furthermore, experiments in primary adult and embryonic neural stem cells (NSC) showed that ENT-A061 is able to induce proliferation and survival of primary NSCs. These data suggest that ENT-A061 is a promising candidate to be tested in vivo in an AD animal model, exploring its neuroprotective and neurogenic properties.

Compound ENT-A061 was chosen to be administered in 5xFAD animal model of Alzheimer's Disease and we subsequently observed its efficacy on reversing the pathological phenotype. The 5xFAD animal model is a well-established Alzheimer's Disease mouse model that harbours five familial AD mutations and manifests most of the hallmarks of the disease such as Aβ plaques, astrogliosis, synaptic loss and LTP defects, behavioural and memory deficits and neuronal loss. Additionally, it is a disease model in which the phenotype is evident starting at 1-2 months of age. Our experimental plan was to subcutaneously implant pellets (in 4 month old mice) that steadily release the drug every day at controlled concentration (10 mg/kg/day) (Figure 15). After two months of treatment, animals were subjected to the following behavioural tests. Novel Object Recognition test, Object to Place Recognition test, Temporal Object recognition test, Morris Water Maze test, Reverse Morris Water Maze test, the Y-Maze test and the Contextual Fear Conditioning and Discrimination test to assess effects on working, spatial and fear memory. In addition to that, tissue was collected for histological analysis of

astrogliosis, synapse number, neural regeneration and neuronal death, which will not be discussed in this thesis. Lastly, we used a subset of these animals to perform *in vitro* electrophysiological experiments measuring long-term potentiation.

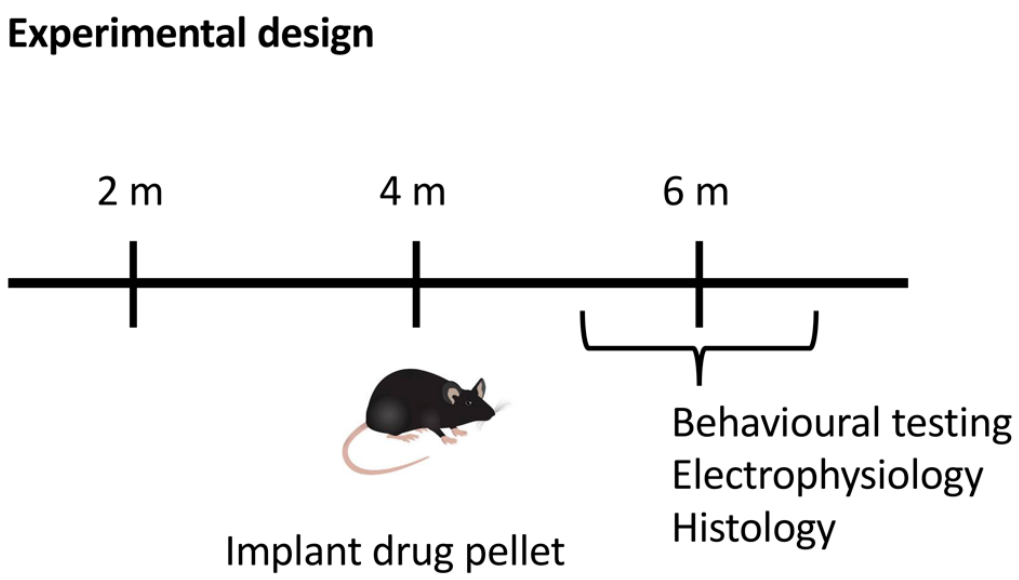


*Figure 15: Experimental Design. 4 months old 5xFAD and Wild Type animals were implanted with a drug pellet containing ENT-A061 or placebo, which was slowly released in daily dosage of 10mg/kg/day. Following 2 months from the pellet implantation, animals were subjected to behavioural experimentation and subsequently were sacrificed and their brains isolated and used for electrophysiological and histological experiments.*

### 3.3.2 Effects of ENT-A061 on the object recognition tests in the 5xFAD animal model of Alzheimer's Disease

After two months of treatment with the ENT-A061 animals were subjected to several behavioural tests. To begin with, animals were presented and accustomed with the arena and familiarised with the arena for 2 days. Following that, we proceeded with the Novel Object Recognition test, a test that is commonly used for behavioural assessment of the ability of animals to recognize and differentiate between novel and familiar objects. This test is relevant to Alzheimer's disease since the disease affects the ability of individuals to form new memories, which is a key aspect of this test. Specifically, we presented animals with two identical objects and let them explore for 10'. One hour later, one object remained the same while the other

object was replaced and animals left to explore for 5'. Finally, 24 hours later, the novel object was further replaced with a new object and animals left to explore for 5' (Figure 16A). We assessed the ability of the animal to recognise the novel object by calculating the discrimination index by counting the time the animal spent exploring the novel object in each case over the total time the animal explored both objects (Figure 16B, C). There was no difference between the experimental groups in the short-term (1 hr) test, while in the long-term (24 hrs) test even though there is not significant differences between the experimental groups, there is an evident trend of difference that the 5xFAD treated with ENT-A061 seemed to remember and discriminate better the novel object versus the familiar compared to the 5xFAD-placebo group.

Following this test, we proceeded in performing the Object to Place Recognition test, another behavioural assay that is used to assess the ability of animals to recognize and differentiate between objects and their location and assesses the ability of the animal to form spatial memories. For this test, animals were presented with two non-identical objects in the same arena as before and were left to explore for 10'. Following that, one hour later, one object was relocated to a different place in the arena and the animals were left to explore for 5' (Figure 16D). Similarly to before, we assessed the ability of the animal to differentiate between the novel location of the object by calculating the discrimination index by counting the time the animal spent exploring the object at the new location over the time the animal spent exploring both objects. Figure 16E shows that there is no significant difference between the experimental group tested.

Subsequently, we performed the Temporal Object Discrimination test. This test is used to assess the ability of animals to recognize and differentiate between objects based on the time they were presented and therefore their ability to form and consolidate temporal memories. For this test, a pair of objects was presented to the animals followed by a different pair of objects

presented to them one hour later. For each pair of objects animals were left to explore and familiarise themselves for 10'. For the testing phase of the experiment, animals were presented with one object from the first pair and one object from the second pair of objects and left to explore for 5' (Figure 16F). Similarly to before, the object discrimination index was calculated and presented. Figure 16G shows that there is no significant difference between the experimental groups tested. Conclusively, these tests showed no difference between the groups tested.

### 3.3.3 Effects of ENT-A061 on the Morris Water Maze Test in the 5xFAD animal model of Alzheimer's Disease

Following the objects test, we proceeded with the Morris Water Maze Test, a test where the spatial memory formation and consolidation is tested. For this test, animals were placed in a pool and initially were trained to learn the location of the platform. On the testing day, animals were placed in the pool with the platform removed and left exploring for 90 seconds (Figure 17A). While training, latency time to locate the platform and successful trials were recorded and presented in Figure 17B, C and for testing day, we measured the % of distance covered in the platform quadrant as well as the number of times the animals crossed the location of the removed platform (Figure 17D, E). In addition to the Morris Water Maze test, we subsequently performed the Reverse Water Maze Test, where in the same setting and immediately after the animals have acquired learning of the platform location, animals underwent the exact same procedure (training and testing) but with the platform now placed in the opposite quadrant (Figure 17F). This test was used to assess the cognitive flexibility of the animals to learn again the new location of the platform. Figure 17G - J shows that there were no significant differences between the genotype and treatment groups in these tests.

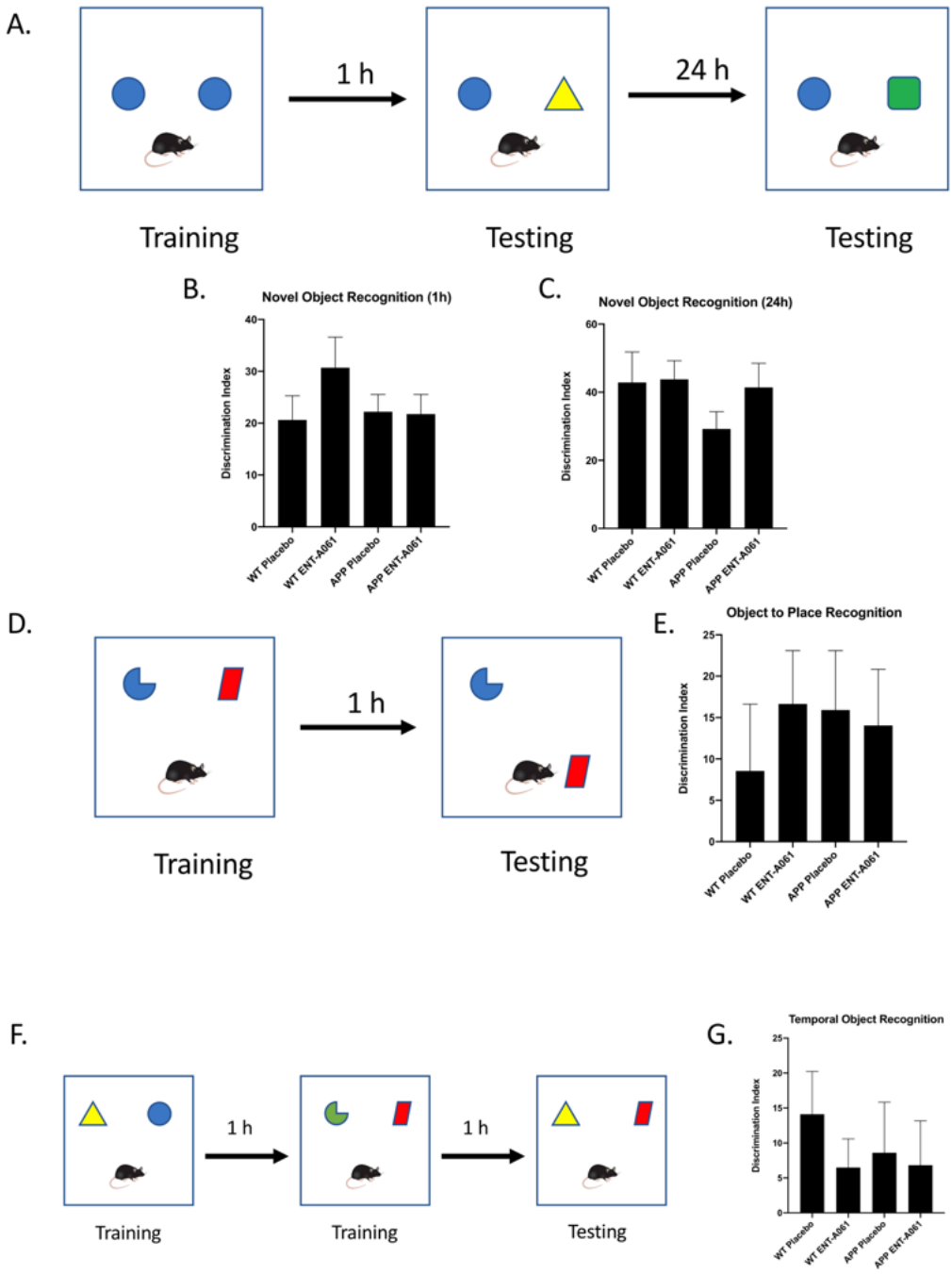


*Figure 16: Effects of ENT-A061 in the object recognition tests in 5xFAD animals. (A) Novel Object Recognition Test experimental design and quantification for short-term (B) or long-term (C) discrimination index. (D, E) Object to Place recognition test and quantification. (F, G) Temporal Object Recognition test and quantification. . Data are shown as ±SEM. One-way ANOVA, Turkey's Test correction. N=8-11 animals*

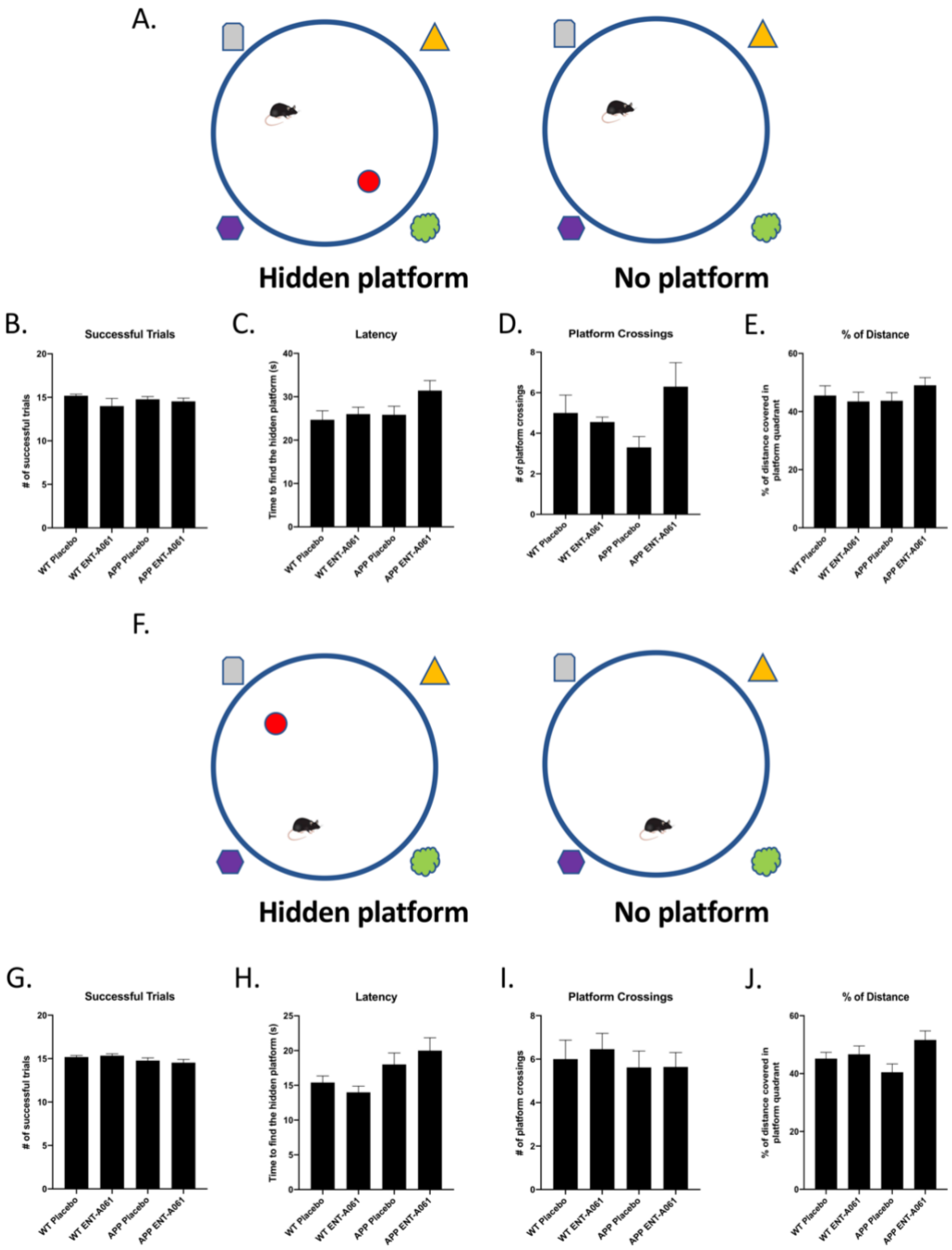


*Figure 17: Effects of ENT-A061 in the Morris Water Maze Tests in 5xFAD animals. (A-E) Morris Water Maze Test experimental design and quantification for learning (B, C) and spatial memory (D, E). (F-J) Reverse Morris Water Maze Ttest and quantification for learning (G, H) and spatial memory (I, J). Data are shown as ±SEM. One-way ANOVA, Turkey's Test correction. N=9-12 animals.*

### 3.3.4 Effects of ENT-A061 on the Y-Maze Test in the 5xFAD animal model of Alzheimer's Disease

Following the Water Maze Test, we subsequently performed the Y-Maze Test. For the Y-Maze, a construction of Y shape was used, and animals were placed in one arm of the Y maze and left to freely explore (Figure 18A). We then assess the ability of the animals to form working memories by calculating the spontaneous alternation index as a measure for working memory formation and consolidation. Figure 18B shows that there is no significant differences between the groups tested.

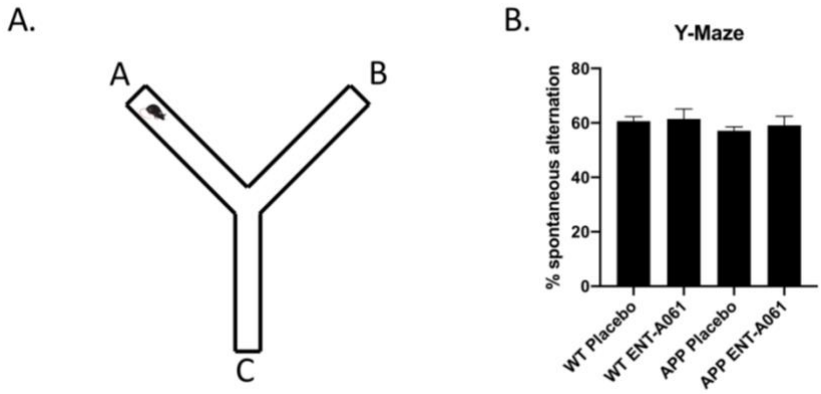


*Figure 18: Effects of ENT-A061 in the Y-Maze Test in 5xFAD animals. (A) Y-Maze experimental design and (B) quantification. Data are shown as ±SEM. One-way ANOVA, Turkey's Test correction. N= 10-12 animals.*

### 3.3.5 Effects of ENT-A061 on the Contextual Fear Conditioning and Discrimination Test in the 5xFAD animal model of Alzheimer's Disease

We then moved on to test the animals for their ability to form fear memories as well as their ability to discriminate them based on the context. To this end, animals were placed in a cage with a metal grid and after exploring for 7 minutes they received a mild foot shock and subsequently left in the same cage for additional 5 minutes. The following day, animals were placed in the same cage and left explore for 3 minutes and the freezing behaviour was calculated. One hour later, the animals were placed in the same cage but with different context.

More specifically, the cage had altered flooring (red carton), odour (2% vinegar) and sound (white noise) (Figure 19A). The same procedure was followed the next day as well, 48 hours following the initial foot shock (Figure 19D). The purpose of this test is to test for the ability of the animals to form fear association memories and to be able to discriminate between subtly different contexts. Figure 15 shows that even though there were not significant differences between groups in the fear conditioning test there are significant differences in the fear discrimination test. More specifically, the 5xFAD-placebo treated animals failed to recognize the different context and showed increased freezing behaviour compared to the WT animals as well as to the 5xFAD-ENT-A061 treated one (Figure 19C, F). This result suggests that ENT-A061 was able to influence the ability of the animals to discriminate between contexts in this fear association test.

### 3.3.6 Effects of ENT-A061 on the Long-Term Potentiation in the 5xFAD animal model of Alzheimer's Disease

Following the behavioural tests, animals were sacrificed and brain slices were used to assess Long Term Potentiation. Brains were sliced transversely and Long Term Potentiation was recorded following Theta Burst Stimulation. Figure 20 shows that there is significantly increased LTP in the WT-Placebo animals compared to the 5xFAD-Placebo ones. On the other hand, even though there is no significant difference in the 5xFAD-ENT-A061 treated group compared to the 5xFAD-Placebo one, there is a trend of increase which needs to be further explored and investigated.

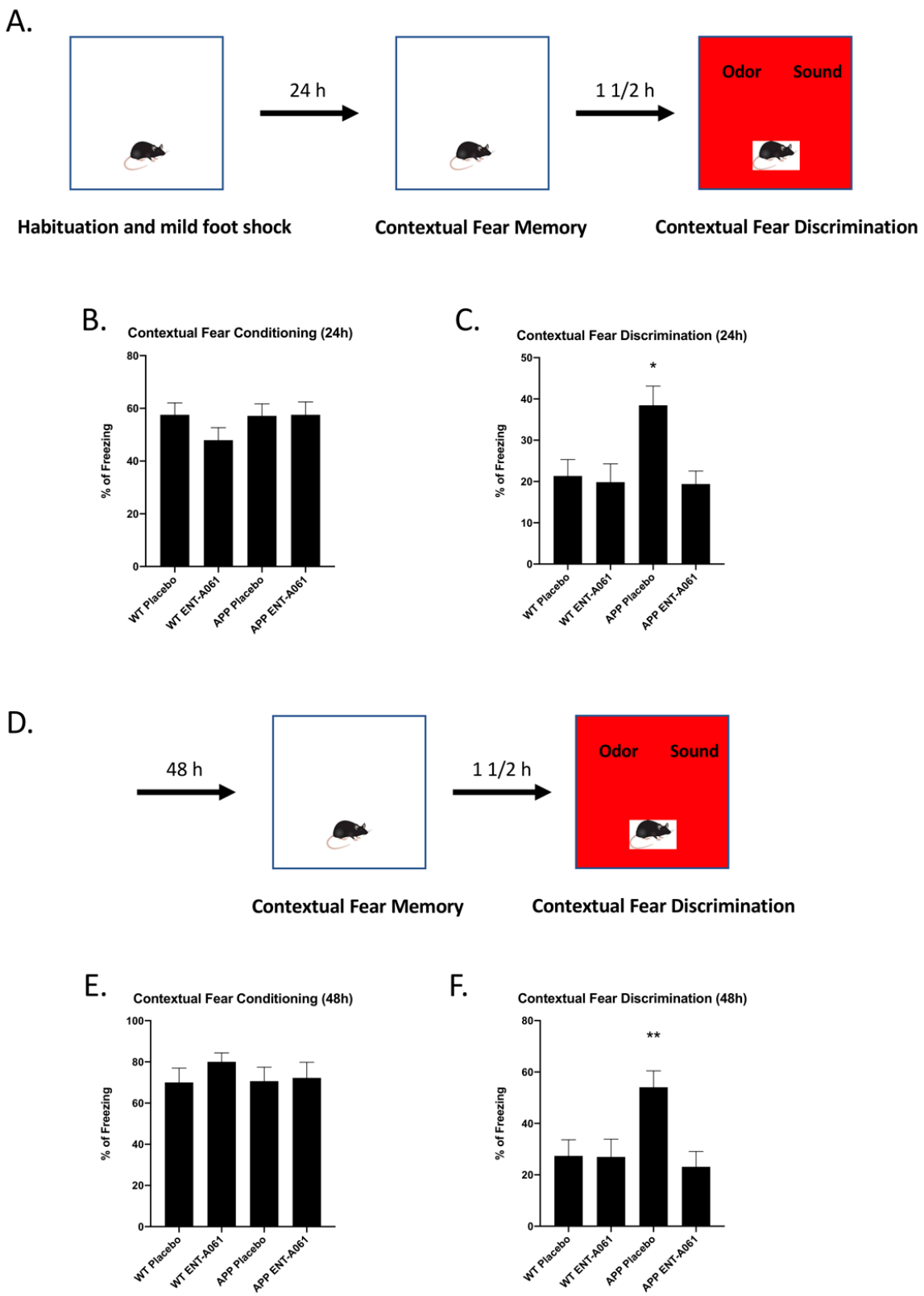


*Figure 19: Effects of ENT-A061 in the Fear Conditioning and Discrimination test in 5xFAD animals. (A-C) Fear Conditioning and Discrimination test experimental design and quantification 24 hours after the initial foot shock. (D-F) Fear Conditioning and Discrimination test experimental design and quantification 48 hours after the initial foot shock. Data are shown as ±SEM. One-way ANOVA, Turkey's Test correction. * p<0.05; ** p<0.01. N=9-12 animals/group.*

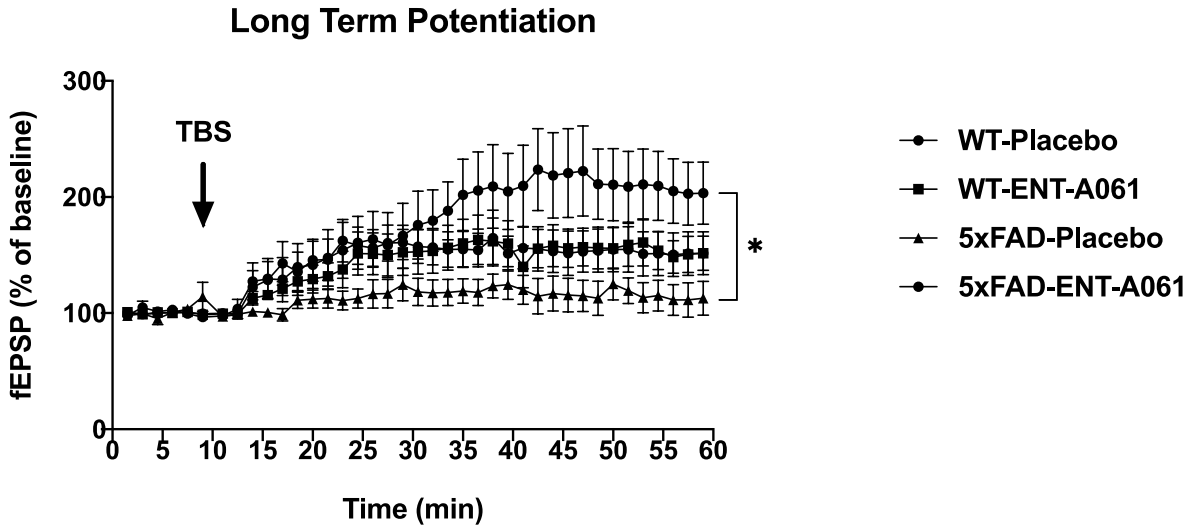


*Figure 20: LTP in hippocampal brain slices from 5xFAD and Wild Type animals treated with ENT-A061 or Placebo. Graph showing LTP induced with TBS in WT brain slices treated with ENT-A061 or Placebo and in 5xFAD brain slices treated with ENT-A061 or Placebo. Data are shown as ±SEM. One-way ANOVA, Turkey's Test correction. * $p<0.05$. n=10 brain slices, 5 brain slices for the WT-ENT-A061 group.*

# 4. Discussion

The search for a viable treatment for neurodegenerative diseases has been ongoing for many years, with neurotrophins being a promising option. Neurotrophins, such as NGF, show potential in preventing or reversing neurodegeneration, promoting neurite growth, and improving synaptic plasticity. However, their clinical application is limited due to their size, poor stability, and low ability to penetrate the blood-brain barrier. Recent studies have shown promising results using small molecules that act as ligands of neurotrophin receptors. This PhD thesis presents a series of TrkA-specific compounds that were synthesized with potential to provide protection against Alzheimer's disease. The compounds, including ENT-A010, ENT-A011, ENT-A013, and ENT-A040, act on TrkA or TrkB receptors and are shown to protect primary hippocampal neurons against the harmful effects of Aβ oligomers in an *in vitro* model of amyloidosis. Additionally, the potent and specific TrkB agonist, ENT-A061, was tested in the 5xFAD animal model of Alzheimer’s Disease and showed to significantly improve the animal's performance in the fear context discrimination task.

## 4.1 Biological characterization of TrkA specific compounds

Treating neurodegenerative diseases remains a significant challenge despite the progress made in understanding their pathophysiology (Long and Holtzman, 2019; Knopman *et al.*, 2021). Neurotrophins, like NGF, have the potential to prevent or reverse neurodegeneration, enhance neurite growth, and improve synaptic plasticity (Allen and Dawbarn, 2006; Longo and Massa, 2013). They can also exhibit anti-inflammatory effects and help clear Aβ in microglia (Rizzi *et al.*, 2018; Fodelianaki *et al.*, 2019). However, as a large protein, NGF has limited usefulness for treatment due to low stability, poor blood-brain barrier penetration, and low diffusion within the CNS (Longo and Massa, 2013). This, combined with the potential for hyperalgesia, hinders its use as a pharmacological agent (Jönhagen *et al.*, 1998). Small molecules that function as ligands of neurotrophin receptors, like oligopeptides, can regulate neurotrophin

signaling and alleviate pathological features in models of neurodegenerative diseases (Longo and Massa, 2013; Simmons *et al.*, 2013; Tep *et al.*, 2013; Xie *et al.*, 2019). Neurosteroids, such as DHEA, have similar protective and anti-inflammatory effects as neurotrophins and are being explored as potential treatments for CNS disorders involving neurodegeneration and neuroinflammation (Charalampopoulos *et al.*, 2006; Lazaridis *et al.*, 2011; Alexaki *et al.*, 2018; Fodelianaki *et al.*, 2019). Studies have shown that DHEA binds to TrkA receptor and activates downstream signaling, promoting neuronal survival and suppressing microglia-mediated neuroinflammation (Charalampopoulos *et al.*, 2008)Lazaridis et al., 2011) With the aim of creating DHEA analogs that cannot act as classical androgens or estrogens, a series of compounds were synthesized, from which, one was selected and named BNN-27. BNN-27 was found to prevent neuronal apoptosis and promote survival by binding to the NGF receptors p75NTR and TrkA, reduce microglial inflammatory responses, and increase oligodendrocyte survival in a mouse model of demyelination (Calogeropoulou *et al.*, 2009; Pediaditakis, Efstathopoulos, *et al.*, 2016; Pediaditakis, Kourgiantaki, *et al.*, 2016b; Bonetto *et al.*, 2017). A novel DHEA analog, ENT-A010, was selected from a new set of synthesized compounds (manuscript in preparation) due to its ability to induce TrkA phosphorylation. The findings presented in this thesis show that ENT-A010 provides protection comparable to NGF in serum-starved PC12 cells, NGF-starved DRG neurons, and Aβ-treated primary hippocampal neurons. Additionally, it enhances Aβ phagocytosis and partially restores the LPS-altered microglial homeostatic signature in microglia (Yilmaz *et al.*, 2022).

Recent single-cell RNA analysis has discovered a subset of microglia called disease-associated microglia (DAM) in Alzheimer's disease, ALS, multiple sclerosis, aging, or related models in humans or mice (Keren-Shaul *et al.*, 2017; Krasemann *et al.*, 2017; Deczkowska *et al.*, 2018). DAMs are characterized by decreased expression of homeostatic genes such as P2ry12,

Tmem119, and Cx3cr1 and increased expression of phagocytic genes, including Trem2 (Butovsky *et al.*, 2014; Colonna and Butovsky, 2017; Deczkowska *et al.*, 2018). The current view is that the clearing role of microglia has a protective effect in Alzheimer's disease (Deczkowska *et al.*, 2018). Trem2 facilitates the engulfment of Aβ by microglia, is essential for DAM activation, and restricts the progression of Alzheimer's disease (Yeh *et al.*, 2016; Keren-Shaul *et al.*, 2017; Ulland and Colonna, 2018; Zhao *et al.*, 2018). A TREM2 mutation has been linked to a heightened risk of Alzheimer's disease [86] (Guerreiro *et al.*, 2013). Acute inflammation caused by LPS reduced the expression of homeostatic genes and Trem2 in the hippocampus, consistent with previous findings, and that these effects were partially reversed by pretreatment with ENT-A010 administered systemically (Sousa *et al.*, 2018; Yilmaz *et al.*, 2022). In line with these findings, ENT-A010 was shown to improve phagocytosis and Aβ clearance and boost the expression of Trem2 and Mertk in microglia in vitro. These results suggest that ENT-A010 may promote a protective role in microglia. Unlike NGF or DHEA, which also activate TrkA and have anti-inflammatory effects on microglia [35,37,40,43], ENT-A010 did not change the expression of Il-1β, Il-6, Tnf, iNos, or Hk2 in LPS-stimulated microglia, potentially due to the activation of different TrkA-dependent or independent signaling pathways by ENT-A010 (Yilmaz *et al.*, 2022).

In conclusion, we have created a C17-spiro-cyclopropyl-DHEA analogue that can activate TrkA phosphorylation and AKT signaling, reach all tested brain regions upon peripheral administration, enhance neuronal survival, boost microglial phagocytic ability, and restore microglial homeostasis in the hippocampus under inflammatory conditions. These properties make ENT-A010 a promising candidate for treating neurodegenerative diseases in which neuronal survival and microglial clearance capacity are impaired and microglial homeostasis is disrupted. Further exploration of ENT-A010's effects in animal models mimicking

neurodegenerative diseases is necessary to shed light on the role of neuroactive steroid and neurotrophin mimetics in the neurodegenerative and inflammatory aspects of CNS diseases.

ENT-A013 is a specific agonist for the TrkA receptor that mimics the activity of NGF in both cell lines and primary neural populations. We now depict its neuroprotective and anti-amyloid properties. Compared to previous DHEA-based derivatives such as BNN27 and BNN20, ENT-A013 has a stronger specificity for the TrkA receptor and can effectively activate it without affecting the TrkB, TrkC, or p75NTR receptors. Molecular docking studies have shown that ENT-A013 can bind to the TrkA-NGF complex through two sites, site 1a and 1b, with site 1b providing a more stable interaction with the receptor. While ENT-A013 and BNN27 have similar interactions with the receptor, they differ in their activities. Additionally, ENT-A013 only weakly inhibits some CYP450 drug-metabolizing isoforms and does not cause biodegradation or liver metabolism. It appears to be a well-tolerated drug candidate (Rogdakis *et al.*, 2022). Data presented in the present thesis show that ENT-A013 can cause sustained phosphorylation of TrkA and Erk1/2 for up to an hour after treatment, while BNN27 can only phosphorylate TrkA for up to 6 hours and fails to activate Erk1/2 after 15 minutes. ENT-A013 was effective in protecting against cell death caused by deprivation of serum or NGF withdrawal in PC12 cells and primary DRG neurons, mimicking the neuroprotective properties of NGF. However, it did not promote neurite outgrowth. The differences in NGF effects between ENT-A013 and BNN27 may be due to differences in their structures that impact various signaling pathways and cellular phenotypes. This provides the opportunity to understand the preferred versus non-preferred effects of neurotrophins, such as neuroprotection vs hyperalgesia. The actions of ENT-A013 are mediated through NGF-TrkA signaling, as they are completely abolished by inhibiting TrkA with a specific inhibitor. Additionally, ENT-A013 demonstrated the ability to counter the negative impact of Aβ on neurons and synapses. Studies

using primary hippocampal neurons showed that ENT-A013 was effective in reversing both neuronal cell death and synapse loss, which are key features of AD. Furthermore, ENT-A013 was able to restore synaptic potentiation in brain slices treated with Aβ, a critical process that is severely impacted in AD patients and closely linked to cognitive decline. Finally, ENT-A013 was able to alter APP processing towards a non-amyloidogenic pathway, similarly to DHEA, providing a mechanism for the compound's action against the harmful effects of Aβ (Rogdakis *et al.*, 2022).

The signaling pathways of NGF play a crucial role in the development, growth, and function of neurons in the central and peripheral nervous system (Huang and Reichardt, 2003; Reichardt, 2006). In Alzheimer's disease, there is a strong connection between the disruption of NGF signaling and the manifestation of the disease's hallmarks. The NGF-TrkA receptor complex in the basal forebrain cholinergic neurons (BFCN), which is a population of neurons severely impacted early in the progression of AD, has been shown to be disturbed in various ways (Teipel *et al.*, 2011; Kilimann *et al.*, 2014; Triaca *et al.*, 2016; Triaca, Coccurello and Giacovazzo, 2018). A decrease in NGF processing leads to an increase in pro-NGF compared to mature NGF, which is accompanied by a loss of the TrkA receptor and a shift towards increased pro-NGF/p75NTR pro-apoptotic signalling (Ioannou and Fahnestock, 2017; Mufson *et al.*, 2019). Gene expression profiling and network analysis of single neurons have revealed a strong association between hindered neurotrophin signaling and AD susceptibility (Roussarie *et al.*, 2020). These findings support the convergence of two previously established hypotheses for the onset of AD, the cholinergic and neurotrophic hypotheses. The disruption of NGF signaling causes the BFCNs to decline, triggering the AD pathology, and providing NGF could potentially reverse these effects and improve the AD pathology (Davies and Maloney, 1976; Appel, 1981).

Currently, the only drugs for AD target acetylcholinesterase and only provide limited relief of symptoms. It is therefore crucial to find "disease-modifying" drugs (Zhu *et al.*, 2013; Birks and Harvey, 2018; Secnik *et al.*, 2020). The first FDA-approved drug for AD targets the Aβ peptide, but it has sparked controversy among experts (Howard and Liu, 2020; Schneider, 2020; Knopman, Jones and Greicius, 2021). Many other attempts to target Aβ plaques or peptides have either failed or are in late stage clinical trials (Cummings, Feldman and Scheltens, 2019; Liu *et al.*, 2019; Cummings *et al.*, 2020, 2021). Delivery of NGF through intracerebroventricular injections or gene therapy has also been unsuccessful due to unwanted effects or lack of improvement for patients (Jönhagen *et al.*, 1998; Capsoni *et al.*, 2000; Tuszynski *et al.*, 2005). In contrast, ENT-A013, a small, BBB-permeable molecule with good pharmacokinetic properties that can act as an NGF mimetic, has potential to counteract the harmful effects of Aβ in AD. ENT-A013 is a potent and selective TrkA agonist with neuroprotective properties and shows promise as a lead molecule for further preclinical development in animal models of the disease to fight AD (Rogdakis *et al.*, 2022).

## 4.2 TrkB specific compounds

The reduction of BDNF levels in the hippocampus of AD patients has been strongly linked to the progression of the disease. BDNF is involved in supporting the basal forebrain cholinergic system, which is known to degenerate in AD. Decreased levels of BDNF mRNA and protein have been observed in the brains of AD patients and increasing BDNF levels has been shown to improve memory and cognitive function in AD patients and mice models of AD (Ibrahim *et al.*, 2022). Furthermore, the interaction of Amyloid β with PKA activation can reduce BDNF expression, leading to decreased synaptic plasticity and cognitive function. In addition, BDNF stimulation has been observed to cause de-phosphorylation of tau, another protein associated

with AD, and shift processing of APP towards a non-amyloidogenic pathway (Jiao *et al.*, 2016; Nigam *et al.*, 2017). These findings suggest that boosting BDNF signaling could be a potential therapeutic approach for AD (Azman and Zakaria, 2022; Gao *et al.*, 2022). Moreover, BDNF-TrkB signaling has been also shown to be involved in regulating adult hippocampal neurogenesis, a process heavily affected in AD (Vilar and Mira, 2016; Colucci-D'amato, Speranza and Volpicelli, 2020; Salta *et al.*, 2023). It is therefore evident that a compound which could activate TrkB and its downstream signaling would be beneficial. In this thesis we present data that ENT-A011, a TrkB-specific compound (manuscript in preparation) was able to counteract the effects of Aβ in primary hippocampal neurons. Specifically, it was able to rescue primary neurons from cell death as well as protect them against synapse loss when treated with Aβ.

The ENT-A061, a BNN-27 derivative, was selected for *in vivo* testing in the 5xFAD animal model of Alzheimer's disease (AD). Data not shown in this thesis indicate that ENT-A061 acts as a BDNF agonist and specifically binds and activates the TrkB receptor, triggering a downstream signaling cascade. ENT-A061 also protects NIH-3T3TrkB cells from serum deprivation-induced apoptosis. Primary hippocampal neurons treated with oligomeric Aβ and ENT-A061 showed that ENT-A061 could prevent Aβ-induced cell death and synapse degeneration. Additionally, ENT-A061 was found to increase proliferation and survival of primary adult and embryonic neural stem cells (NSCs) in experiments. These findings suggest that ENT-A061 is a promising candidate for in vivo testing in an AD animal model, due to its neuroprotective and neurogenic properties. The 5xFAD animal model of AD is a well-established and well-characterized model that features 5 familial AD mutations and exhibits symptoms such as Aβ plaques, astrogliosis, synaptic loss, behavioral and memory deficits, and

neuronal loss. This model also manifests the phenotype of the disease as early as 1-2 months of age.

4-month-old 5xFAD animals treated with ENT-A061 for 2 months showed improved ability to discriminate between environments based on context compared to placebo-treated 5xFAD littermates in the contextual fear discrimination paradigm. However, the effects of ENT-A061 on other aspects of AD phenotype remain inconclusive. Tests of spatial and working memory, such as the Y-maze and Morris Water Maze, did not show any effect of the drug, and no difference was observed between the control groups of placebo-treated wild-type and placebo-treated 5xFAD animals, making these data inconclusive. This may be due to technical issues during behavioral experiments, such as temperature variations that affected the animals' performance in the tests. On the other hand, the improvement in context discrimination performance seen with ENT-A061 treatment has been linked to the neurogenesis process, which is critical in AD (Tronel *et al.*, 2012; Berdugo-Vega *et al.*, 2020; Besnard and Sahay, 2021). Adult hippocampal neurogenesis relies on BDNF-TrkB signaling, and ENT-A061 has been shown to activate this signaling in vitro, suggesting that it might improve certain aspects of AD pathology by enhancing adult hippocampal neurogenesis.

## 4.3 Conclusions

In conclusion, the findings presented in this thesis reveal the discovery of several new compounds that specifically target and activate neurotrophin signaling, with a focus on TrkA and TrkB receptors. Through a comprehensive evaluation of these compounds, the activation profiles of an extensive list of compounds were determined and evidence was provided to support the activation and effectiveness of a select subset of these compounds. These results serve as a solid foundation for the continued development and refinement of these compounds

as potential therapeutic agents for the treatment of neurodegenerative diseases. By demonstrating their ability to activate neurotrophin signaling, these compounds have the potential to have a significant impact on the treatment and management of these debilitating conditions. With further research and optimization, these novel compounds may offer hope for the millions of people affected by neurodegenerative diseases.

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# 6. Appendix

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## Development and Biological Characterization of a Novel Selective TrkA Agonist with Neuroprotective Properties against Amyloid Toxicity

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**Abstract:** Neurotrophins are growth factors that exert important neuroprotective effects by preventing neuronal death and synaptic loss. Nerve Growth Factor (NGF) acts through the activation of its high-affinity, pro-survival TrkA and low-affinity, pro-apoptotic $p75^{NTR}$ receptors. NGF has been shown to slow or prevent neurodegenerative signals in Alzheimer's Disease (AD) progression. However, its low bioavailability and its blood–brain-barrier impermeability limit the use of NGF as a potential therapeutic agent against AD. Based on our previous findings on synthetic dehydroepiandrosterone derivatives, we identified a novel NGF mimetic, named ENT-A013, which selectively activates TrkA and exerts neuroprotective, anti-amyloid-β actions. We now report the chemical synthesis, in silico modelling, metabolic stability, CYP-mediated reaction phenotyping and biological characterization of ENT-A013 under physiological and neurodegenerative conditions. We show that ENT-A013 selectively activates the TrkA receptor and its downstream kinases Akt and Erk1/2 in PC12 cells, protecting these cells from serum deprivation-induced cell death. Moreover, ENT-A013 promotes survival of primary Dorsal Root Ganglion (DRG) neurons upon NGF withdrawal and protects hippocampal neurons against Amyloid β-induced apoptosis and synaptic loss. Furthermore, this neurotrophin mimetic partially restores LTP impairment. In conclusion, ENT-A013 represents a promising new lead molecule for developing therapeutics against neurodegenerative disorders, such as Alzheimer's Disease, selectively targeting TrkA-mediated pro-survival signals.

**Keywords:** neurotrophin; TrkA neurotrophin receptor; nerve growth factor; Alzheimer disease; amyloid-beta; neurotrophin mimetic; neuronal survival; synapse; LTP

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# ENT-A010, a Novel Steroid Derivative, Displays Neuroprotective Functions and Modulates Microglial Responses

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**Abstract:** Tackling neurodegeneration and neuroinflammation is particularly challenging due to the complexity of central nervous system (CNS) disorders, as well as the limited drug accessibility to the brain. The activation of tropomyosin-related kinase A (TRKA) receptor signaling by the nerve growth factor (NGF) or the neurosteroid dehydroepiandrosterone (DHEA) may combat neurodegeneration and regulate microglial function. In the present study, we synthesized a C-17-spiro-cyclopropyl DHEA derivative (ENT-A010), which was capable of activating TRKA. ENT-A010 protected PC12 cells against serum starvation-induced cell death, dorsal root ganglia (DRG) neurons against NGF deprivation-induced apoptosis and hippocampal neurons against Aβ-induced apoptosis. In addition, ENT-A010 pretreatment partially restored homeostatic features of microglia in the hippocampus of lipopolysaccharide (LPS)-treated mice, enhanced Aβ phagocytosis, and increased *Ngf* expression in microglia in vitro. In conclusion, the small molecule ENT-A010 elicited neuroprotective effects and modulated microglial function, thereby emerging as an interesting compound, which merits further study in the treatment of CNS disorders.

**Keywords:** ENT-A010; DHEA; neuroprotection; microglia; phagocytosis; hippocampus; Aβ; TRKA

## 1. Introduction

Neurodegeneration and neuroinflammation are fundamental hallmarks of central nervous system (CNS) disorders [1]. Neurodegeneration refers to the impaired function and loss of neurons, which is often permanent due to the limited regenerative capacity of the adult neural system [2]. Neuroinflammation involves the inflammatory activation of glial cells, that is, microglia and astrocytes, and can have protective or destructive consequences for the neural system [1,3]. Microglia play a key role in the maintenance of homeostasis due to synaptic pruning and their clearing and neurotrophic function [3–5], while their inflammatory activation is a prerequisite for elimination of insults (infections