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“In Vivo Study of the p75 Neurotrophin Receptor's Neurogenic Role.”

Master Thesis

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«In vivo μελέτη του ρόλου του υποδοχέα νευροτροφινών p75 στη νευρογένεση.»

Διπλωματική εργασία

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Summary

This thesis focuses on the critical function of the p75NTR receptor in a variety of cellular processes, including neurogenesis, neural development, and possible therapeutic applications. Notably, earlier studies have demonstrated the significance of p75NTR in these processes, but this study aims to investigate p75NTR's neurogenic role both *in vivo* and *in vitro*. The choice of the appropriate mouse models was crucial in order to further investigate p75NTR's function in adult hippocampal neurogenesis and in AD. First, we demonstrated that in 2-month-old mice, p75NTR deletion reduces neural stem cell (NSCs) proliferation while increasing differentiation. These results highlight how crucial function the receptor has in controlling NSCs' proliferation and differentiation.

Recent research has connected decreased adult hippocampus neurogenesis to the progression of neurodegenerative diseases including Alzheimer's disease (AD). However, our experiments show that NSCs' proliferation is unexpectedly enhanced in 2-month-old AD mice (5xFAD). This raises the possibility that the central nervous system has a compensating mechanism to encounter neurodegeneration brought on by AD. Importantly, p75NTR deletion in 5xFAD mice reduces NSCs' proliferation to levels even lower than in wild-type mice, emphasizing the receptor's role in this process. Additionally, the project focuses on the mechanisms that lead to these alterations in proliferation and differentiation of NSCs upon p75NTR's deletion. A non-autonomous role for p75NTR is suggested by the fact that conditional deletion of p75NTR specifically in neural progenitor cells has no impact on NSCs' proliferation and differentiation. These findings shed light on the intricate connections that take place between various cell types in the nervous system.

On the other hand, this study describes ENT-A044, a synthetic analog of the natural neurosteroid DHEA, that can activate TrkB and p75NTR receptors, as a possible therapeutic agent. Although DHEA prevents neuronal cell death, its long-term use is problematic because of its hormonal side effects. So, some years ago in our laboratory, a library of synthetic analogs of DHEA that maintain its neurotropic function but lack its undesirable endocrine effects was generated. However, as seen in mouse and human neuronal cultures, the effects of ENT-A044 differ depending on the receptors expressed and the type of neuronal cells. While ENT-A044 triggers cell death predominantly through p75NTR in human NSCs, it decreases cell death in p7 mouse hippocampal NSCs, probably through TrkB activation. This demonstrates how neurotrophin receptor functions vary between species. Based on the above data, ENT-A044 could be used to target cancer cells and lead them to apoptosis through p75NTR-mediated cell death pathways. Lastly, future studies should delve deeper into the function of p75NTR in older mice with severe AD pathology and analyze alterations in gene expression linked to p75NTR signaling by single-cell transcriptomic investigations.

Περίληψη

Η παρούσα διπλωματική εργασία επικεντρώνεται στην κρίσιμη λειτουργία του υποδοχέα p75NTR σε μια ποικιλία κυτταρικών διεργασιών, συμπεριλαμβανομένης της νευρογένεσης, της νευρικής ανάπτυξης και πιθανών θεραπευτικών εφαρμογών. Ειδικότερα, προηγούμενες μελέτες έχουν αναδείξει τη σημασία του p75NTR σε αυτές τις διαδικασίες, αλλά η παρούσα μελέτη αποσκοπεί στη διερεύνηση του νευρογενετικού ρόλου του p75NTR τόσο in vivo όσο και in vitro. Η επιλογή των κατάλληλων μοντέλων ποντικιών ήταν καθοριστικής σημασίας για την περαιτέρω διερεύνηση της λειτουργίας του p75NTR στη νευρογένεση του ενήλικου ιππόκαμπου και στη νόσο του Αλτσχάιμερ (ΝΑ). Πρώτον, αποδείξαμε ότι σε ποντίκια ηλικίας 2 μηνών, η διαγραφή του p75NTR μειώνει τον πολλαπλασιασμό των νευρικών βλαστικών κυττάρων (NSCs), ενώ αυξάνει τη διαφοροποίηση. Τα αποτελέσματα αυτά αναδεικνύουν πόσο κρίσιμη λειτουργία έχει ο υποδοχέας στον έλεγχο του πολλαπλασιασμού και της διαφοροποίησης των NSCs.

Πρόσφατες έρευνες έχουν συνδέσει τη μειωμένη νευρογένεση του ενήλικου ιππόκαμπου με την εξέλιξη των νευροεκφυλιστικών ασθενειών, συμπεριλαμβανομένης της νόσου του Αλτσχάιμερ (ΝΑ). Ωστόσο, τα πειράματά μας δείχνουν ότι ο πολλαπλασιασμός των NSCs είναι απροσδόκητα ενισχυμένος σε ποντίκια ηλικίας 2 μηνών με ΝΑ (5xFAD). Αυτό εγείρει την πιθανότητα ότι το κεντρικό νευρικό σύστημα διαθέτει έναν αντισταθμιστικό μηχανισμό για να αντιμετωπίσει τη νευροεκφύλιση που επιφέρει η ΝΑ. Κάτι εξίσου σημαντικό είναι ότι η διαγραφή του p75NTR σε ποντίκια 5xFAD μειώνει τον πολλαπλασιασμό των NSCs σε επίπεδα ακόμη χαμηλότερα από εκείνα των ποντικών άγριου τύπου, τονίζοντας τον ρόλο του υποδοχέα σε αυτή τη διαδικασία. Επιπλέον, το πρότζεκτ επικεντρώνεται στους μηχανισμούς που οδηγούν σε αυτές τις μεταβολές στον πολλαπλασιασμό και τη διαφοροποίηση των NSCs κατά τη διαγραφή του p75NTR. Ένας μη αυτόνομος ρόλος του p75NTR υποδηλώνεται από το γεγονός ότι η υπό όρους διαγραφή του p75NTR ειδικά στα νευρωνικά προγονικά κύτταρα δεν έχει καμία επίδραση στον πολλαπλασιασμό και τη διαφοροποίηση των NSCs. Τα ευρήματα αυτά ρίχνουν φως στις περίπλοκες συνδέσεις που λαμβάνουν χώρα μεταξύ των διαφόρων κυτταρικών τύπων στο νευρικό σύστημα.

Από την άλλη πλευρά, η παρούσα μελέτη περιγράφει το ENT-A044, ένα συνθετικό ανάλογο του φυσικού νευροστεροειδούς DHEA, το οποίο μπορεί να ενεργοποιήσει τους υποδοχείς TrkB και p75NTR, ως πιθανό θεραπευτικό παράγοντα. Αν και η DHEA αποτρέπει τον θάνατο των νευρικών κυττάρων, η μακροχρόνια χρήση της είναι προβληματική λόγω των ορμονικών παρενεργειών της. Έτσι, πριν από μερικά χρόνια στο εργαστήριό μας, δημιουργήθηκε μια βιβλιοθήκη συνθετικών αναλόγων της DHEA που διατηρούν τη νευροτροφική της λειτουργία, αλλά στερούνται των ανεπιθύμητων ενδοκρινικών επιδράσεών της. Ωστόσο, όπως φάνηκε σε καλλιέργειες νευρώνων ποντικών και ανθρώπων, οι επιδράσεις του ENT-A044 διαφέρουν ανάλογα με τους υποδοχείς που εκφράζονται και τον τύπο των νευρικών κυττάρων. Ενώ το ENT-A044 προκαλεί κυτταρικό θάνατο κυρίως μέσω του p75NTR

στα ανθρώπινα NSCs, μειώνει τον κυτταρικό θάνατο στα p7 NSCs του ιππόκαμπου ποντικού, πιθανώς μέσω της ενεργοποίησης του TrkB. Αυτό καταδεικνύει πώς οι λειτουργίες των υποδοχέων νευροτροφινών διαφέρουν μεταξύ των ειδών. Με βάση τα παραπάνω δεδομένα, το ENT-A044 θα μπορούσε να χρησιμοποιηθεί για τη στόχευση καρκινικών κυττάρων και την οδήγησή τους σε απόπτωση μέσω μονοπατιών κυτταρικού θανάτου που διαμεσολαβούνται από τον p75NTR.

1.Introduction

1.1. Part One

1.1.1. Neurotrophins and their receptors

Neurotrophins are considered a family of proteins that are essential for the development of the nervous system of all vertebrates. This family consists of the secreted growth factors BDNF, NGF and NT3/4. Their functions extend from neuronal development to neurodegenerative and neuropsychiatric disorders (Numakawa & Odaka, 2022). Except for their common effects on neuronal survival and growth, they influence axonal and dendritic extension, synaptic structure and plasticity, neurotransmitter release, and long-term potentiation (LTP) (Huang & Reichardt, 2001). The effects of neurotrophins are directly related to two different types of transmembrane receptors, the Trk tyrosine kinases that include TrkA, TrkB, and TrkC, and the p75 neurotrophin receptor (p75NTR) which is a part of the tumor necrosis factor (TNF) receptor superfamily. Trk receptors bind neurotrophins with high affinity and more specifically NGF activates only TrkA, BDNF and NT4 activate only TrkB and NT3 activates only TrkC. NT3 has been shown to activate each of the Trk receptors in some cell types(Huang & Reichardt, 2003). p75NTR binds all different neurotrophins but with low affinity.

As mentioned before, the pan-neurotrophin receptor p75 is a member of the TNF receptor superfamily. It is widely expressed in different cell types of the central nervous system (CNS) and participates in controversial signaling pathways. p75NTR consists of three main domains: the extracellular domain (ECD), the transmembrane domain (TMD), and the intracellular domain (ICD) which is divided into the juxtamembrane domain (JTM) and the death domain (DD) *(Figure 1)*. Referring to the ECD of p75NTR consists of four cysteine-rich domains (CRDs) and through them, it seems to interact with the neurotrophins by making homodimers (Vilar, 2017). Its function is characterized by regulating programmed cell death, especially when it binds the pro-forms of neurotrophins, but it also has a major role in cellular homeostasis, cell cycle arrest, cell survival and proliferation depending on the ligand and the co-receptors that bind to p75NTR. Particularly, recent studies have shown that when p75NTR binds mature neurotrophins this interaction can lead to cell death but when p75NTR acts as a co-receptor of Trk receptors for mature neurotrophins this interaction usually leads to cell survival (Chao, 2003; Meeker & Williams, 2015)*(Figure 2).*

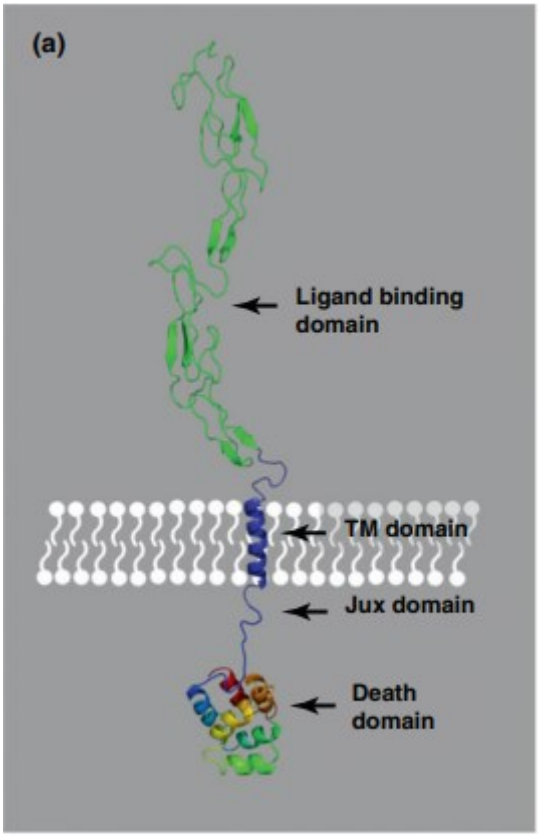


Figure 1. Structural view of a p75NTR subunit in the plasma membrane, with structural models of the extracellular (ligand-binding) domain and the death domain. TM, transmembrane. Jux, juxtamembrane (a flexible domain, unstructured in solution, linking the TM and death domains) (Ibáñez & Simi, 2012)*).*

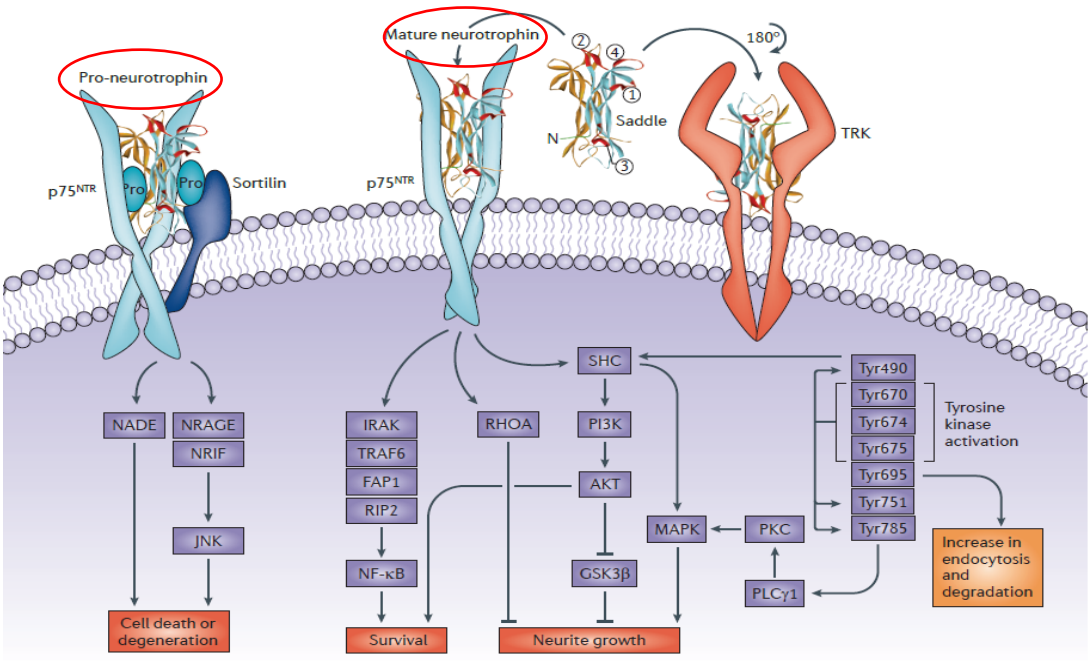


Figure 2. Neurotrophin receptor signaling. Trk ligand binding by mature neurotrophins results in the phosphorylation of an array of intracellular domain tyrosine residues, which activate kinase activity, resulting in further receptor autophosphorylation. Trks induce the signaling that leads to survival, via extracellular Ras kinase, via phosphatidylinositol kinase 3 (PI3K) and phospholipase C-γ (PLC-γ). Mature neurotrophins binding to p75NTR, depending on the context, may augment neurotrophin binding to Trk receptors, reinforce Trk signaling through AKT and MAPKs, and further promote survival through the nuclear factor-κB (NF-κB) pathway, or antagonize the actions of TRK through the activation of JUN N-terminal kinase (JNK) and RHOA pathways. Pro-neurotrophin binding in complex with sortilin selectively activates cell-death-related pathways (Longo & Massa, 2013).

1.1.2. Adult hippocampal neurogenesis

In the previous century the general opinion in the scientific community was that the generation of newly born neurons does not occur in the mature nervous system. In the late 1980s Nowakowski and his colleagues showed with bromodeoxyuridine (BrdU) labelling that the formation of new neurons does occur in the brain of adult rodents (Nowakowski et al., 1989) but until 2000s adult neurogenesis was not broadly accepted. The last twenty years a variety of studies provide evidence about new neurons can and indeed be formed in the adult mammalian brain (Gross, 2000). Furthermore, numerous studies have provided evidence that adult neurogenesis occurs in the mature nervous system of primates and humans and these studies have been reviewed by several scientific groups (Bonfanti,2016; Abbott & Nigussie, 2019). It is already known that the formation of new neurons takes place in two major regions of the adult mammalian brain: the subgranular zone (SGZ) of the dentate gyrus (DG) of the hippocampus and the subventricular zone (SVZ) which is located among the lateral walls of the lateral ventricles (Zhao & Overstreet-Wadiche, 2008) *(Figure 3)*. The new neurons developed in SVZ mostly migrate anteriorly to the olfactory bulbs where they differentiate to interneurons (Kaneko et al., 2017; Ming & Song, 2011). On the other hand, neuronal stem cells in the SGZ of the DG differentiate into neurons in the adult hippocampus (Ming & Song, 2011). These neurons mostly participate in learning, memory, and cognitive functions in general (Cameron & Glover, 2015; de Miranda et al., 2017).

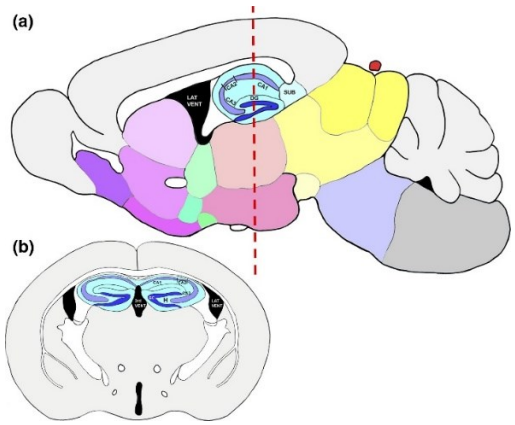


Figure 3. Diagrams of adult C57BL/6 mouse brain sections that show the hippocampus. (a) Sagittal section and (b) cross-section (Abbott & Nigussie, 2020).

Focusing on the hippocampus, the proliferation and maturation of NSCs from the SGZ is regulated by different signaling mechanisms and neurotransmitter release at local synaptic circuits. These NSCs differentiate into Type 1 and Type 2 cells which correspond to the early survival phase (X. Jin, 2016; Song et al., 2012). During the proliferative phase, Type 1 cells are in the subgranular cell layer (SGL) of the DG, express NSCs' biomarkers such as Sox2 and nestin and are positive for the presence of BrdU after its injection to animals. Afterwards, Type 1 cells give rise to intermediate progenitor cells that firstly characterized by glial and NSCs markers and called Type 2a cells. With further differentiation the Type 2a cells change and start to express immature neuronal markers such as doublecortin (DCX), a microtubule-associated protein that is related to neuronal differentiation and migration, and thus are called Type 2b cells. After the survival phase that lasts approximately 2 weeks in rodents, these cells finally lead to the formation of neuroblasts (or Type 3 cells) that are subjected to intense morphological changes and gene expression reprogramming to generate mature neurons. Type 3 cells are still characterized by DCX expression (Sánchez-Huerta et al., 2016) (Figure 4).

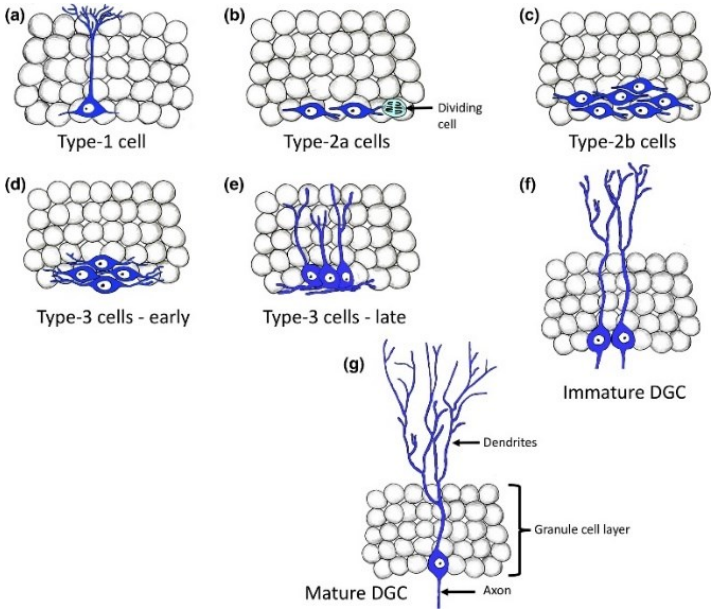


Figure 4. Diagrams of the formation of new neurons in the adult hippocampus. (a) Type-1 cell, which are radial granule cells; early progenitor cell that is relatively quiescent with respect to proliferation. (b) Type-2a cells that are a form of intermediate progenitors as indicated by the cell undergoing cell division. (c) Type-2b cells that also are a form of intermediate progenitors that have initiated fate specification. (d) Early Type-3 cells that are continuing to undergo fate specification. (e) Late Type-3 cells that are starting to differentiate into dentate granule cells (DGCs). (f) Immature DGC that is continuing to undergo morphogenesis and maturation into DGCs. (g) Mature DGC (Abbott & Nigussie, 2020).

A variety of hormones including sex, stress, and metabolic hormones, influence adult hippocampal neurogenesis. For instance, estradiol which is the major estrogen seems to promote cell proliferation and survival and to inhibit cell death in SGZ (Mahmoud et al., 2016; Triviño-Paredes et al., 2016). Cortisol which is a stress hormone seems to prevent neurogenesis (Triviño-Paredes et al., 2016). Finally, thyroid hormones positively affect hippocampal neurogenesis but only when mature neurons have fully formed and not in the survival, proliferating phase (Sánchez-Huerta et al., 2016). When considering the human brain, the situation is more complicated. Whereas several studies have shown that there is evidence of adult hippocampal neurogenesis in the human DG (Gonçalves et al., 2016; Moreno-Jiménez et al., 2021), there is a recent publication in Nature that puts this concept under investigation. This study suggests that neurogenesis occurs in children but is almost absent in adults (Sorrells et al., 2018). However, the same year Boldrini and his team showed that adult hippocampal neurogenesis occurs also in humans until the age of 80 years (Boldrini et al., 2018).

1.1.3. p75NTR and adult hippocampal neurogenesis

Almost 20 years ago, in the beginning of the 20th century, it has been demonstrated that p75NTR is expressed by precursor cells in the SVZ and when the BDNF binds to the receptor and activates it, p75NTR promotes the processing of neuronal precursor cells to become newly formed neuroblasts (Young et al., 2007). Except for that, low expression of p75NTR has been noticed in the rat hippocampus and more specifically in the DG hilus and the granule cell layer (Barrett et al., 2005). Interestingly, Catts and his colleagues showed that there is a significant decrease in neuroblasts and newly born neurons and at the same time an increased cells death of neuronal progenitors in the DG of p75NTR KO mice when compared to wild-type. Furthermore, this study revealed behavioral changes related to neurogenesis in p75NTR KO mice. However, it is yet unclear whether the altered behavior is caused by a decrease in neurogenesis or whether both phenotypes are a result of a coexisting effect of p75NTR deficiency. So, this was the first study that showed that animals lacking p75NTR exhibit abnormal behavior as well as dramatically reduced hippocampal neurogenesis, which results from a reduction in the differentiation and maturation of neurogenic progenitor cells (Catts et al., 2008).

After these first indications of p75NTR's neurogenic role, several scientific teams have tried to identify the mechanisms underlying the adult hippocampal neurogenesis and to further investigate the effects of p75NTR's signaling in this phenomenon. In 2009, it was reported that p75NTR is necessary for fluoxetine (an antidepressant drug) to trigger an increase in hippocampus neurogenesis, thus supporting its function as a regulator of basal neurogenesis (Colditz et al., 2010). A year later, it has been discovered that p75NTR is expressed by BrdU-positive cells and that newborn cells also express it when they display neuronal or glial markers. Another unexpected observation is the reduction in the number of newborn neurons and non-

neurons in p75NTR KO mice, suggesting that this receptor plays a role in the development of both neurons and non-neurons (Bernabeu & Longo, 2010). The same year, another scientific group confirmed that adult wild-type and transgenic AD mice localize the p75NTR to their primary cilia, but the localization of p75NTR is significantly lower in hippocampal CA1 and CA3 cells and cerebral cortical cells (Chakravarthy et al., 2010).

Furthermore, it is generally known that in order to structure neural networks *in vivo*, newly formed neurons send polarizing signals that specify a single axon and several dendrites. So, a study revealed that p75NTR regulates the polarity of axons, is necessary for the determination of their fate and localizes asymmetrically in developing neurons in response to neurotrophins. Local exposure to neurotrophins causes early accumulation of p75NTR into a single undifferentiated neurite to determine axon destiny, in cultured hippocampus neurons. In addition, deletion or knockdown of p75NTR prevents newborn neurons from initiating an axon during cell-cycle *in vitro*, as well as during adult hippocampus neurogenesis *in vivo*. Thus, in the adult hippocampus and cortical development, p75NTR regulates neuronal polarity, modulating pattern and assembly of neural circuits (Zuccaro et al., 2014).

As mentioned before p75NTR is widely expressed in the nervous system during development and controls axonal outgrowth, migration, and neuronal differentiation. Additionally, p75NTR controls the survival and death of developing neurons, with functional results reliant on timing and cellular context. Based on these facts, a recent study has demonstrated that in Nestin-Cre p75 floxed mice, conditional deletion of the p75NTR gene in neural progenitors causes anatomical abnormalities of the brain, including a significant reduction in brain volume with the neocortex and basal forebrain having the greatest shrinkage. Adult and developing cortical projection neurons and interneurons were decreased, which is consistent with higher levels of death of these cells' embryonic progenitors and with decreasing rates of neurogenesis. These findings imply that p75NTR influence development, differentiation, and survival of these neuronal progenitors (Meier et al., 2019).

1.1.4. Alzheimer's Disease (AD)

Alzheimer's disease (AD) is a neurodegenerative disorder that mostly causes dementia in the elderly population. It is responsible for cognitive deficits of the elderly with the main early symptom being the difficulty in remembering recent events. The progression of AD includes language impairments, disorientation and behavioral problems (Kalaria et al., 2008). It is also characterized by cognitive deficits that include learning difficulties, impaired spatial orientation, and cognitive performance (McKhann et al., 2011). The hallmarks of AD's pathology are the formation of amyloid β-plaques and the neurofibrillary tangles. Furthermore, in AD neuronal degeneration occurs mostly in the cortex and hippocampus (Braak & Braak, 1991).

The cases of AD are separated to early-onset, familial and late-onset, sporadic cases. The familial cases are usually caused by hereditary mutations in presenilin 1,2 (PSEN1, PSEN2) or amyloid precursor protein (APP) genes and they represent almost

5% of AD patients. The sporadic cases represent most of the AD population, and their exact cause is still unknown. A variety of environmental and genetic factors, such as aging, lifestyle, and APOE4 influence the risk for developing AD (Paroni et al., 2019). The two major hypothesis for the cause of AD are the cholinergic and the amyloid hypothesis. The cholinergic neurons seem to degenerate in AD and the amyloid-β blocks choline uptake and acetylcholine release, thus leading to the cognitive deficits of AD patients (Breijyeh & Karaman, 2020). The amyloid hypothesis suggests that Aβ plaques are generated by the excessive accumulation of Aβ peptide due to problematic processing of APP by β- and γ-secretase. PSEN1 and PSEN2 genes are crucial for the catalytic function of γ-secretase, so their mutations lead to amyloidosis. In AD, Aβ plaques are formed at first in entorhinal cortex and hippocampus (Lazarov & Demars, 2012). So, it is expected that in the AD brain adult hippocampal neurogenesis would be majorly affected.

1.1.5. AD and adult hippocampal neurogenesis

Recent studies from the past decade show a clear impairment of adult neurogenesis in neurodegenerative diseases such as AD, in both humans and animal models that can lead to reduction of cognitive performance (Moreno-Jiménez et al., 2019; Mu & Gage, 2011). Hippocampal neurogenesis is expected to reduce with age during normal human aging, much as it did in mice (Gage, 2000). Indeed, it was discovered that as people age, fewer DCX-positive cells are present in their brains (Moreno-Jiménez et al., 2019). Additionally, Boldrini and his team discovered that the older human brain had less neuroplasticity and lower angiogenesis, indicating diminished, but not absent, neurogenesis (Boldrini et al., 2018). Additionally, it has been hypothesized that neurodegenerative conditions like AD are aggravated by the physiological changes that come with age. As a result, adult neurogenesis is thought to deteriorate earlier and more quickly in AD patients. In contrast to that theory, a scientific team has mentioned that the hippocampus of AD patients had higher levels of neurogenic substances like DCX. They hypothesized that AHN was elevated in AD patients as a defense mechanism against the cell loss brought on by neurodegeneration (K. Jin et al., 2004). Similarly, an increase in stem cells that were Nestin-positive in AD patients also has been noted (Ziabreva et al., 2006).

On the contrary, recent investigations have led to the opposite findings. The brains of AD patients and people with normal aging were compared for DCX positive cells. In all the ages studied, a decline in the quantity of immature neurons in AD brains was demonstrated (Moreno-Jiménez et al., 2019). Additionally, another team discovered that AD patients have fewer DCX-positive cells and stem cells than healthy people. This study also suggested a link between cognitive function and neurogenesis in AD patients. This raises the prospect of using adult neurogenesis as a measure for AD patients' cognitive performance. With the help of *in vivo* imaging techniques, it would be possible to evaluate the function of newborn neurons and the changes that occur to them under pathological circumstances (Tobin et al., 2019).

Since the study of human tissue has its limits, animal models have been essential for exploring a variety of features of AD development and manifestations. There is a variety of mouse models that are used to study AD such as 5xFAD, APP/PS1 and 3xTg which all carry mutations in AD-related genes. In order to study the neurodegeneration that is caused by AD in the SGZ, immature neurons are mostly indicated by the marker DCX. Depending on when the animal is sacrificed and when the injections take place, BrdU can be also used to determine survival or proliferation. In 5xFAD mice, a reduced adult hippocampal neurogenesis has been demonstrated at a young age (2-4 months) (Moon et al., 2015). In adult 5-6 months old 3xTg and APP/PS1 mice there was a decrease in neurogenesis and more specifically in the survival of newly formed neurons (Valero et al., 2014; Verret et al., 2007). Finally, in aged (10-12 months old) mice there was a significant reduction in adult hippocampal neurogenesis and an excessive accumulation of Aβ peptide in their brains (Hamilton et al., 2010).

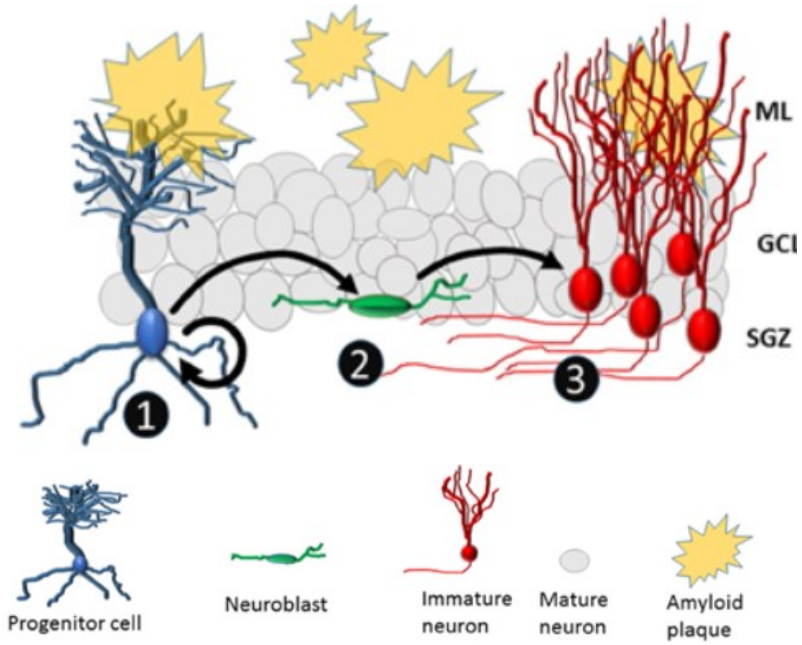


Figure 5. Amyloid plaques affect neurogenesis (Martinez-Canabal, 2014).

1.1.6. p75NTR and AD

The death of cholinergic neurons in the basal forebrain, which expresses the highest amount of p75 in the adult brain, is one of the early signs of Alzheimer's disease. Moreover, the disease results in the death of cholinergic neurons in the hippocampus, septum pellucidum, and cerebral cortex, all of which are essential for memory. Previous studies revealed that p75NTR dependence underlies the neuronal loss seen in the basal forebrain of Alzheimer's patients. TrkA inhibits the production of amyloid-β peptide by reducing the β-cleavage of the amyloid precursor protein (APP), whereas p75NTR promotes the cleavage, and Aβ was found to cause p75-mediated neurotoxicity in several cell lines. Alzheimer's patients have lower levels of TrkA in their basal forebrain cholinergic neurons, as well as higher amounts of pro-

neurotrophins in their parietal cortex. This raises the potential that neuronal death in Alzheimer's disease may also be brought on by apoptosis mediated by pro-neurotrophins via p75 signaling (Diarra et al., 2009). So, for many years it was believed that p75NTR promotes Aβ neurotoxicity in AD without exactly knowing the mechanisms behind this phenomenon.

Having the above in mind, there is an arising interest in targeting neurotrophins as potential therapeutic agents for AD by mostly focusing on the p75 neurotrophin receptor (p75NTR) since it seems to be involved in important and complex pathways (Xiong et al., 2022). Most significantly p75NTR expression is increased in case of injury or disease of the central nervous system (CNS), so it is tempting to think that regulation of its levels may have therapeutic effects in these cases (Chao, 2003; Meeker & Williams, 2015). The major problem in understanding the influence of neurotrophins is the contradictive effects of mature and pro-neurotrophins. The survival signaling of mature neurotrophins through Trk receptors is exactly the opposite of the apoptotic, neurodegenerative signaling of pro-neurotrophins through the p75NTR. In aging and in AD p75NTR expression is higher as well as the levels of pro-neurotrophins, so the apoptotic signals are stronger, and the neuronal cells are led to death (Crooks & Meeker, 2022).

Considering all the above, numerous pharmacological approaches have developed recently that focus on regulating p75NTR to promote neurotrophin survival signaling while preventing neurodegenerative effects of pro-neurotrophins-p75NTR signaling. The two main approaches that show how modulating p75NTR can be beneficial in neurodegenerative disorders are the use of LM11A-31, a small non-peptide ligand, and the regulation of the p75NTR ectodomain (ECD). LM11A-31 ligand blocks the p75NTR degenerative signaling pathways and at the same time, it promotes the survival signaling through PI3K/Akt pathway by influencing p75NTR activity as a strict agonist or antagonist (Crooks & Meeker, 2022). Referring to p75NTR ECD, a few years ago Wang and colleagues showed that p75NTR can modulate the deposition of Aβ peptide by enhancing Aβ production, but it blocks Aβ aggregation with its ECD. This was a shocking reveal since p75NTR was considered to promote Aβ neurotoxicity without knowing its exact influence on AD progression. This study demonstrated that in APP/PS1 mice, p75NTR knockout lowered the soluble Aβ levels, but promoted the aggregation of Aβ and the formation of Aβ plaques. The injection of a recombinant ECD of p75NTR inhibited fibrillation of Aβ and decreased the Aβ plaques in the hippocampus of these mice (Wang et al., 2011).

A few years later, Yao and colleagues, since the displacement of p75ECD from the plasma membrane is physiologically modified, showed that p75ECD was importantly lowered in the cerebrospinal fluid (CSF) and in the brains of AD patients and APP/PS1 transgenic mice (Yao et al., 2015). On the same page, it was demonstrated by another team that the levels of p75ECD were increased in the blood serum but decreased in the CSF of AD patients when compared with a control group of elderly individuals with no neurodegenerative pathologies. So, p75ECD can serve as

a biomarker for AD, and measuring its serum and CSF levels can be a potential strategy for following AD development (Jiao et al., 2015). It was also observed that the reinstatement of p75ECD to the physiological level by intracranial administration of the gene that encodes the human p75ECD through an AAV vector, before and after the Aβ aggregation in the brains of APP/PS1 mice led to the improvement of behavioral impairments and AD-related pathologies such as Aβ aggregation and neuroinflammation. Furthermore, p75ECD can attenuate amyloidogenesis by inhibiting the expression and function of β-secretase. So, their data established that p75ECD is a physiological neuroprotective molecule against Aβ-induced neurotoxicity in the brain of AD patients (Yao et al., 2015).

Almost a year later, members of the same team investigated if the peripheral administration of AAV-p75ECD in APP/PS1 mice can reduce Aβ deposition and related pathologies. They concluded that the intramuscular injection of AAV-p75ECD augmented p75ECD levels in the circulation, restored the behavioral deficits of APP/PS1 mice, and lowered the Aβ aggregation. So, not only the intracranial but also the peripheral administration of AAV-p75ECD can be an efficient therapeutic strategy for AD (Q.-H. Wang et al., 2016). A recent clue about how p75NTR affects AD pathology comes from a study published by Yi et al., which analyzes how p75NTR relates to Amyloid Precursor Protein (APP) internalization. They observed that in 5xFAD animals that express inactive variants of p75NTR there is higher neuroprotection against AD pathologies than in mice that do not express the receptor. More specifically, mice that are deprived of either the p75NTR death domain or a specific cysteine residue of their p75NTR transmembrane domain (Cys259), had reduced AD deficits when compared with the global knock-outs. So, this study suggests that using drugs that block the p75NTR transmembrane domain may serve as potential therapeutic agents for AD since they could block the amyloidogenic APP cleavage (Yi et al., 2021).

1.2. Part Two

1.2.1 Dehydroepiandrosterone (DHEA) and its chemical analogs

Neurosteroids are mostly generated by neuroglial cells, astrocytes, and neurons in the central and peripheral nervous system. Steroids that influence the nervous system have been proven to affect neuronal activity and differentiation as well as provide neuroprotection (Compagnone & Mellon, 2000). Previous research examining the neurosteroid physiopathology in AD have revealed a considerable decrease in neurosteroid concentrations in specific brain regions in AD patients as compared to individuals without a pathological condition. So, it was hypothesized that endogenous neurosteroid administration in AD would suggest a potential neuroprotective effect (Diaz Brinton & Ming Wang, 2006).

Dehydroepiandrosterone (DHEA) was the first neurosteroid that was researched. As a precursor molecule for the synthesis of estrogens and androgens, it is the most prevalent steroid in the systemic circulation of adult humans. It is mostly generated in the gonads, adrenal glands, and occasionally locally in the brain. While DHEA produced in the adrenal glands has a systemic function, DHEA produced in the

brain functions locally in a paracrine manner. Its daily synthesis varies with age, reaching its peak concentration in early adulthood. Thereafter, its concentration in the brain and circulation steadily diminishes, especially in situations like AD that are associated with neurodegeneration (Gravanis et al., 2012; Schumacher et al., 2003).

In terms of its biological activity, Lazaridis and his team showed that DHEA binds to NGF receptors. This was the first report to demonstrate a steroid's direct binding to neurotrophin receptors. TrkA is autophosphorylated because of DHEA binding, and a signaling pathway is initiated via survival kinases like the Shc-PI3KAkt and SrcMEK-ERK pathways. Additionally, the ratio of TrkA to p75NTR receptors regulates whether cells will undergo apoptosis or survival, and binding of DHEA to the p75NTR influences the binding of the receptor to the TRAF6, RIP2, and RhoGDI operators (Lazaridis et al., 2011) *(Figure 6).*

DHEA, except for its neuroprotective and survival effects, also exhibits action in terms of neurogenesis, as it increases the number of newly formed neurons in the rat and more specifically in the dentate gyrus of the hippocampus (Karishma & Herbert, 2002). In fact, it has been demonstrated that DHEA and its derivatives stimulate long term neural stem cell proliferation by acting through NMDA and Sigma-1 receptors, promoting neurogenesis and neuronal survival in cultures of human neural stem cells (Charalampopoulos et al., 2008).

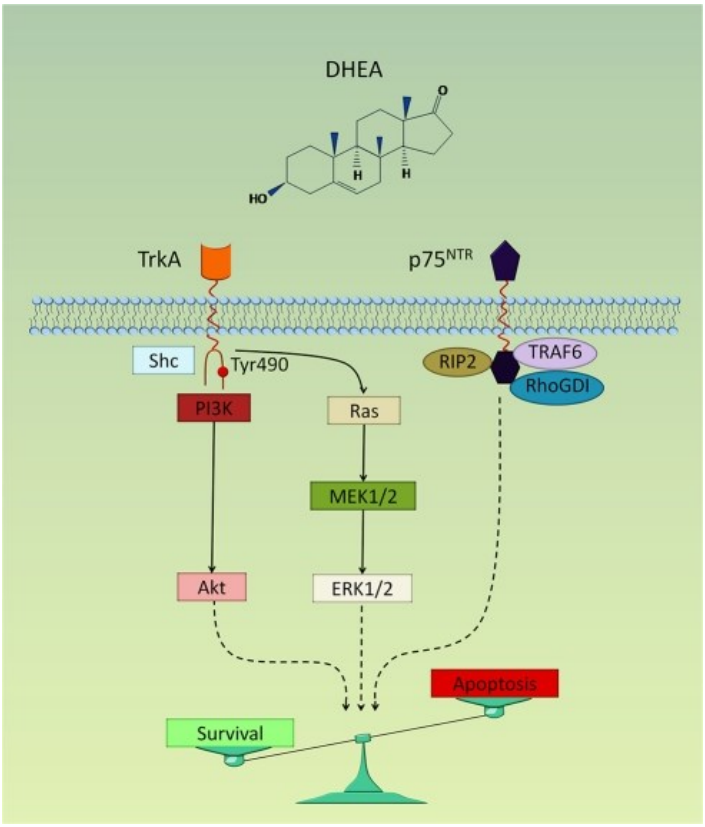


Figure 6. Hypothetical model of NGF receptor-mediated signaling pathways involved in the effects of DHEA on neuronal cell fate. DHEA binds with high affinity to TrkA and p75NTR receptors (Lazaridis et al., 2011).

As mentioned before DHEA declines with age or due to a neurodegenerative disorder so there is a need for the development of novel compounds to protect the neuronal cells and neurogenesis. In the past two decades, researchers have become increasingly interested in using DHEA as a treatment. Endogenous neurosteroids are still known to cause broad endocrine adverse effects, such as hormone-dependent neoplasms, when converted into estrogens, androgens, or progesterones, thus limiting their clinical use (Compagnone & Mellon, 2000). Small molecules—chemical analogs of DHEA—that maintain the neurotrophin benefits but lack the undesirable endocrine effects might be pharmacologically ideal to solve these adverse effects. One of the first substances to be examined was BNN27, a C-17-spiro derivative of DHEA, which has been demonstrated to work by selectively activating both p75NTR and TrkA receptors. A library of novel small molecules with notable pharmacological properties that lacked the endocrine side effects of the original molecule were recently produced based on the previously mentioned compounds (Pediaditakis et al., 2016).

1.2.2. Synthetic compound ENT-A044

After a lot of testing in several chemical analogs of DHEA our scientific team chose to focus on compound ENT-A044, which is a chemical entity derived from DHEA, with a C17 structural modification. Previous experiments in our laboratory, that included Western Blot analysis for phosphorylayted TrkB and total and phosphorylated AKT, ERK, have revealed that ENT-A044 does not activate TrkA but binds and activates TrkB. Furthermore, it was noted that treatment of HEK293T cells transfected with p75 cDNA plasmid, with ENT-A044 leads to cell death. The cell death in these cells was observed with CellTox Assay and confirmed with Western Blot analysis for total and phosphorylated JNK.

Recently it was further examined by our laboratory how ENT-A044 interacts with neurotrophin receptors, particularly p75NTR and TrkB. The effects of ENT-A044 on the p75NTR, among other DHEA synthetic analogs that were also evaluated for their capacity to activate neurotrophin receptors, made it stand out in this process. More particularly it has been demonstrated that ENT-A044 is involved in activating survival pathways in p7 mouse hippocampus neural stem cells that express the TrkB receptor and death signaling pathways in p75NTR-transfected HEK293T cells subjected to serum deprivation. Moreover, our team determined the activated signaling pathways facilitated by the p75 receptor's RIP2 interactor protein. Notably, the expression of p75NTR in neural precursor cells (NPCs) produced from human induced pluripotent stem cells (iPSCs) was described for the first time, as well as the subsequent death signaling pathways triggered by treatment with ENT-A044. TrkB receptor is also expressed by these neural precursors. The effects of ENT-044 on human neural stem cells are entirely different from those observed in mouse cell or cell lines, where both receptors were co-expressed, since treatment with the compound result in a considerable induction of cell death. Having in mind that NGF treatment also led to elevated cell death, this apoptotic action of ENT-A044 is solely mediated by p75NTR as TrkA receptor is not expressed (Papadopoulou et al., 2023).

Part of this recent publication has been conducted throughout the duration of my Master thesis.

1.3. Aim of the study

p75 neurotrophin receptor (p75NTR) is a multifunctional cell surface receptor that has been linked to the complex interactions between adult hippocampal neurogenesis and Alzheimer's disease (AD) pathology. Disrupted downstream signaling pathways and altered expression patterns are linked to dysregulated p75 receptor signaling in AD. This abnormal signaling may contribute to reduced adult hippocampal neurogenesis, a process vital for learning and memory. p75 receptor's contribution to AD-related neuroinflammation, amyloid-beta toxicity, and tau pathology, further aggravates the dysfunction of neural stem cells and impairs progenitor cell proliferation and differentiation in AD. The complicated relationship between Alzheimer's disease, the p75 neurotrophin receptor, and adult hippocampal neurogenesis needs to be understood to develop novel therapeutic approaches to mitigate neurodegeneration and cognitive loss in AD. So, as a method of intervention in the impairment of neurogenesis related to AD, targeting the p75 neurotrophin receptor shows promising. Modifying p75NTR signaling may promote the survival, differentiation, and proliferation of neural progenitor cells, promoting the growth of new neurons in the adult hippocampus. Interventions targeting the p75 receptor may provide a multimodal strategy to mitigate neurogenesis deficiencies, reduce cognitive decline, and maybe halt the course of Alzheimer's disease by treating both the neurotrophic and neuroinflammatory elements of AD pathology.

Based on the above data, this study aims to investigate p75NTR's neurogenic role both *in vivo* and *in vitro*. The choice of the appropriate mouse models was crucial in order to further investigate p75NTR's function in adult hippocampal neurogenesis and in AD. In this study p75NTR KO and p75NTR floxed/Nestin-Cre, male and female mice were used in order to elucidate the impact of p75NTR's total or conditional deletion in the adult hippocampal neurogenesis. Moreover, 5xFAD were used as an animal model of AD, and 5xFAD/p75NTR KO mice were generated in order to study the effect of p75NTR's total deletion in AD background. Proliferation assay was conducted in these mice and the adult hippocampal neurogenesis was evaluated with immunofluorescent staining with BrdU, Sox2, and Dcx primary antibodies. Except for that, p75NTR's signaling and its role in human NSCs survival was examined in neural stem cells derived from human induced pluripotent stem cells (human iPSCs-derived NSCs). In these cells it was assessed with Western Blot analysis the expression of p75NTR, as well as RIP2 which is p75NTR's interactor protein.

Furthermore, as mentioned before the naturally occurring steroid hormone DHEA can bind to the p75NTR receptor's extracellular domain, which sets off a series of intracellular signaling processes. The interaction of DHEA with p75NTR as wells as with TrkA appears to alter several cellular responses, possibly affecting cell apoptosis and survival, and activates neurotrophin signaling pathways. The connection between DHEA and p75NTR implies that this interaction could be used for therapeutic therapies

intended to improve neural resilience and cognitive function. In this study we decided to focus on the synthetic compound ENT-A044, a chemical analog of DHEA, since from previous experiments of our laboratory it was shown that ENT-A044 interacts with p75NTR and TrkB. So, we tried to test how this compound acts in a background that both p75NTR and TrkB are expressed.

2. Materials and Methods

2.1 Mice

Two and six-months old wild-type (WT), p75NTR KO, p75NTR floxed/Nestin-Cre, 5xFAD and 5xFAD/p75NTR KO male and female mice were used. p75NTR KO mice described by Lee et al. (1992) carry a targeted mutation in ExonIII of the receptor which leads to the complete deletion of the full-length receptor. However, a short isoform of the receptor is still expressed. To generate mice with conditional deletion of p75NTR in neural stem cells (NSCs), $p75^{fl/fl}$ mice were crossed to transgenic mice containing the Cre recombinase gene only in cells that express nestin (Nestin-Cre). The loxP sites of $p75^{fl/fl}$ mice targeted the ExonII of p75NTR in $Nestin^{+}$ cells kindly provided by Dr Sebastaian Thieme from TU of Dresden. 5xFAD mouse model is commonly used as an animal model for Alzheimer's Disease (AD). These mice express human APP and PSEN1 transgenes with a total of five AD-linked mutations. 5xFAD/p75NTR KO were generated by crossing 5xFAD with p75NTR heterozygous mice. All mice were maintained in a C57BL/6J background, had free access to food and water and were housed under a 12-h light-dark cycle.

2.2 Proliferation Assay

To evaluate the proliferation rate of NSCs in two and six-months old WT, p75NTR KO, 5xFAD and 5xFAD/p75NTR KO mice, the animals were injected intraperitoneally with 100mgr/kg BrdU dissolved in water for injection for five consecutive days. On day 6 they were deeply anesthetized with isoflurane and intracardially perfused with 0.9% NaCl solution. After the perfusion, brains were dissected and brain regions (hippocampus, cortex, cerebellum) from the right hemispheres were isolated and snap-frozen for further analyses and the left hemispheres were collected for histological analyses.

2.3 Immunofluorescent staining and Confocal microscopy

The left hemispheres of the animals were post-fixed with 4% paraformaldehyde (PFA) in PBS overnight at 4 °C, immersed in a 30% sucrose solution in PB and incubated overnight at 4 °C. Then, they hemispheres were transferred in a 7,5% gelatin-15% sucrose gel diluted in PB 1X. When the gels became solid, the tissues were embedded in OCT compound (Tissue-Tek), were snap-frozen in -45 to -50 degrees Celsius and stored at −80 °C. Forty-µm-thick coronal sections were cut by cryosectioning to cover the whole hippocampus and transferred onto slides. The slides were blocked with a blocking solution (1% BSA 10%, 10% Normal Donkey Serum or Goat Serum in PBSTx 0,1% or 0,3%) for 1h at RT, followed by overnight incubation with primary antibodies. For the proliferation assay the slides were stained with anti-BrdU

(sheep 1:200) and anti-Sox2 (rabbit 1:100 dilution) primary antibodies. The next day, sections were washed and incubated with donkey anti-sheep IgG-Alexa Fluor 647 and donkey anti-rabbit IgG Alexa Fluor 555 for 1h at RT. Then, the slides were mounted using Fluoromount. Additionally, other slides from the same animals were stained with anti-Dcx (rabbit 1:100) primary antibody. The next day, sections were washed and incubated with donkey anti-rabbit IgG Alexa Fluor 555 for 1h at RT or overnight at 4 °C. Then, the nuclei were counterstained for 15min with Hoechst (1:5000 in PBS 1X) and the slides were mounted using Fluoromount.

Confocal imaging was performed using an SP8 Leica System. Images were acquired with the LAS_X software. Slides stained with anti-Neun and anti-BrdU were excited with two laser lines at 555nm and 647nm and emissions were detected with hybrid detectors within a window of 571 nm-625 nm (Alexa-Fluor 555) and 660nm-710nm (Alexa-Fluor 647), respectively. Slides stained with anti-BrdU and anti-Sox2 were excited with two laser lines at 647nm and 555nm and emissions were detected with hybrid detectors within window of 660nm-710nm (Alexa-Fluor 647) and 571 nm-625 nm (Alexa-Fluor 555), respectively. An oil immersion 40×/0.8NA Plan-Apochromat was used at zoom factor 1. Pixel size corresponded to 1024 nm × 1024 nm. Laser power, pinhole size and hybrid detector gain were determined for each fluorophore at the beginning and were kept constant throughout the imaging procedure. Z-stacks were taken with a step size of 3µm, and processing was performed with the FIJI software.

2.4 Cell isolation and Culture

HEK293T cells were obtained from from LGC Promochem GmbH and frozen at -80 ˚C in a freezing medium. The cells need to be defrosted rapidly, not gradually. Thawing can be done in an incubator of 37˚C or by holding the cryovial in the hand. As soon as little ice melts, cells need to be transferred into cell culture medium in order to dilute DMSO. HEK293 cells were cultured in DMEM medium containing 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO2. They were transiently transfected with human p75NTR plasmid, as well as with TRAF6 and RIP2 plasmids, using Turbofect Transfection Reagent, according to manufacturers' instructions. The next day the cells were used for experimental procedures.

Primary hippocampal neural stem cells (NSCs) were isolated as previously described (Moutin et al., 2020). Briefly, the p7 pups were put on ice for mild sedation, their head was sprayed with 70% ethanol and quickly decapitated. The brain was gently removed under the stereoscope in cold HBSS/5% P/S medium on ice. The hippocampi were isolated and chopped in 1x1mm pieces. Then they were transferred in a 15ml tubes containing cold HBSS/5% P/S medium and sit on ice until the tissue has reached the bottom of the tubes. About the tissue processing for NSCs culture, first the excess of HBSS/5% P/S medium was removed, and the tissue was washed with fresh HBSS/5% P/S medium twice. After that 500ul warmed up accutase solution per 4 brains was added and incubated at 37˚C for a total of 30 min, with trituration of the

tissue with the 1000uL pipette 3-4 times every 5 minutes. Then the accutase solution was diluted down with 5 mL per 4 brains of warm DMEM/F12 medium and was shaked gently to wash away the accutase. A spin down for 5min at 100g followed, the supernatant was removed, and cells were resuspended in 1 mL of full DMEM/F12 medium. The full DMEM/F12 medium contains B27 supplement without vitamin A, D-glucose, Primocin, FGF, EGF and Heparin. Finally, the cells were plated in T25 flasks containing 5 mL of full DMEM/F12 medium and cultured at 37 °C and 5% CO2. The NSCs cultures were fed every 2 days by adding 1mL of warm full DMEM/F12 medium. They were checked every day for neurospheres formation and when the neurospheres were relatively large but bright in the center, a passage of the cells was necessary using accutase.

NSCs were derived from human iPSCs as previously described ((Ehrlich et al., 2017) by Dr. Chanoumidou at our laboratory in IMBB FORTH. iPSCs colonies were sectioned and enzymatically detached from mouse embryonic fibroblasts. The pieces of iPSCs colonies were collected and cultured as embryoid bodies. When confluent, cells were split by treatment with accutase. The identity of the cells was verified with immunocytochemistry for NESTIN and SOX1. Then the lysates of human iPSCs-derived NSCs were used for Western Blotting.

2.5 ImmunoPrecipitation & Western Blotting

For the ImmunoPrecipitaion Assay HEK293T cells or human iPSCs-derived NSCs were suspended in Pierce™ IP Lysis Buffer supplemented with protease inhibitors and phosphatase inhibitors. The lysates were immunoprecipitated with p75NTR antibody (1:100) overnight at 4°C. The next day protein G-plus Agarose beads were added to the samples and incubated for 4hrs on the rotor at 4°C. After centrifugation the supernatant was removed and the immunoprecipitated lysates containing the beads were stored at -20°C until the day the Western blotting was performed.

Protein lysates from HEK293T cells or human iPSCSs-derived NSCs were prepared in ice-cold RIPA buffer supplemented with protease and phosphatase inhibitors. Protein concentration was determined with the BCA assay. Protein lysates were mixed with reducing Laemmli SDS sample buffer, denatured at 95 °C for 5 min and 40-60 μg of protein were loaded on a polyacrylamide gel and separated with SDS-PAGE. Afterward, proteins were transferred onto nitrocellulose membranes and blocking was performed with 5% BSA in TBS-Tween20 buffer for 1h. Then, primary antibodies were added to the membranes and incubated overnight at 4 °C. Primary antibodies used were following: anti-phospho JNK (1:1000), anti-total JNK (1:1000), anti-p75(1:1000), anti-RIP2(1:1000), anti-TRAF6(1:2000), anti-actin (1:2000) diluted in 5% BSA in TBS-Tw. The next day the membraned were washed with TBS-Tw and anti-rabbit IgG or anti-mouse IgG (only for actin) diluted 1:5000 was added to the membranes and incubated for 1h at RT. Immunoblots were developed using ECL Western Blotting kit (Thermo Fischer Scientific). The image analysis and the quantification of band intensities were performed using the FIJI software.

2.6 CellTox Assay

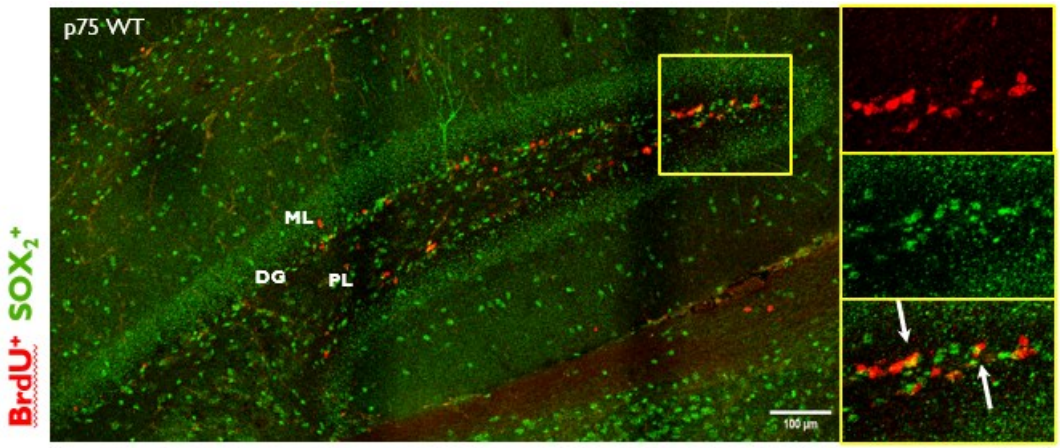
After 48hours of treatments, we use the CellTox™ Green Cytotoxicity Assay kit to assess survival of p7 hippocampal NSCs under EGF/FGF deprivation conditions. Cells were plated in 96-well plates, starved for 4hrs, and then treated with BDNF (500ng/mL) and compound ENT-A044 (500 nM). CellTox assay reagents and Hoechst (1:10.000) were added to each well for 30 minutes and cells were imaged with a fluorescent microscope. Positive cells for CellTox reagents were normalized to total number of cells for each image.

3. Results

3.1 Role of p75NTR in the proliferation of mouse NSCs

3.1.1. p75NTR is essential for the proliferation of NSCs in 2 months old mice.

Firstly, in order to evaluate if p75NTR has an impact on the proliferation rates of NSCs in two months old mice, we used WT and p75NTR KO animals. The mice were injected intraperitoneally with 100mgr/kg BrdU dissolved in water for injection for five consecutive days. On day 6 they were deeply anesthetized with isoflurane and intracardially perfused with 0.9% NaCl solution. Cryosections of their left hemisphere were immunofluorescent stained with BrdU and Sox2 primary antibodies to assess the NSCs' proliferation. As it is shown in Figure 7, the $BrdU^{+}Sox2^{+}$ cells are significantly decreased in p75NTR KO 2mo mice. This result indicates that p75NTR plays a crucial role in the proliferation of NSCs in young adult mice.



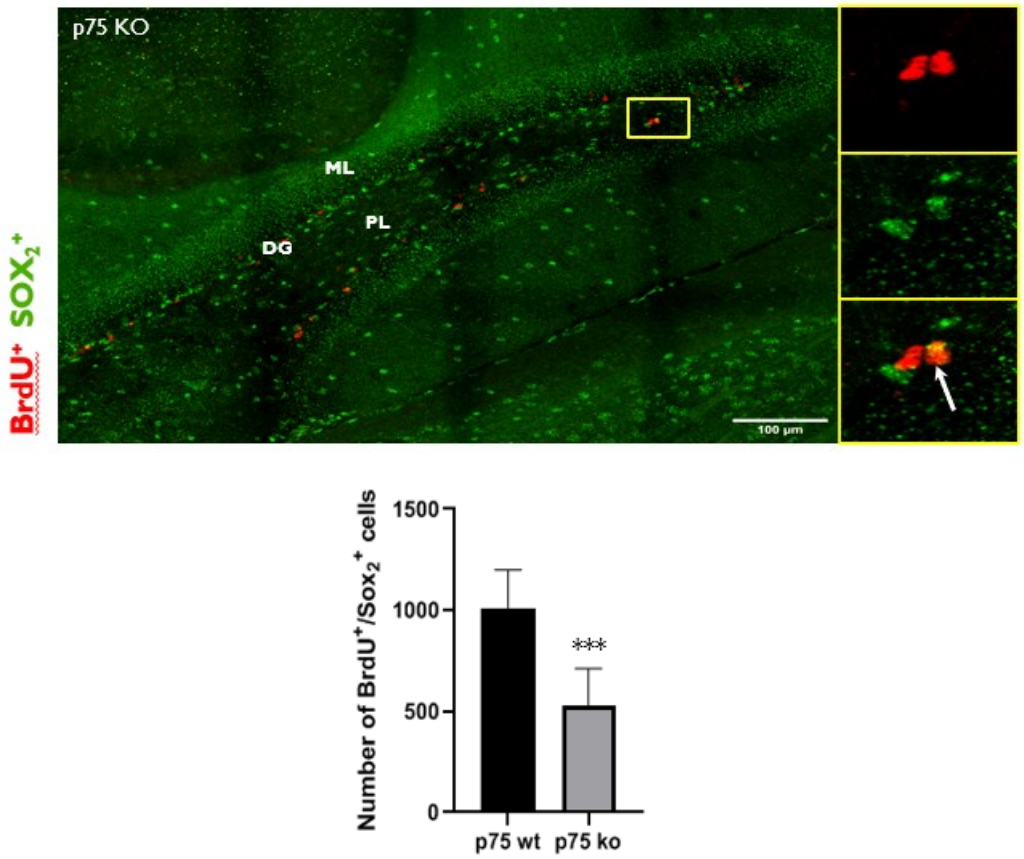


Figure 7. Hippocampal Dentate Gyrus from 2-months-old WT vs p75NTR KO, injected with BrdU. Sections were immunostained for BrdU (red) and co-immunostained with Sox2 (green). *(n = 8 WT & 8 p75NTR KO, t-student (unpaired t-test), *** p 0,0001)*

3.1.2. Increased proliferation rates of NSCs in 5xFAD heterozygous and homozygous 2 months old mice.

In order to assess the proliferation of NSCs in terms of a neurodegenerative condition such as Alzheimer's disease, we performed the same procedure with five consequent BrdU injections and immunofluorescent staining in the hippocampus of 2 months old 5xFAD heterozygous and homozygous mice. As demonstrated in Figure 8, there is a statistically significant increase in the proliferation rates of NSCs in 5xFAD heterozygous 2 months old mice when compared to WT. The elevated proliferation in these animals suggests that the central nervous system tries to develop a compensatory, homeostatic mechanism against the neurodegenerative effects of AD-like pathology and thus reverse the neuronal loss that is the result of neurodegeneration generally observed at the onset of the disease, approximately in around 4 months old. However, no significant differences between 5xFAD heterozygous and homozygous mice were observed, with the proliferation rate again being elevated when compared to WT animals. So, we decided to continue our study with 5xFAD heterozygous mice which are more commonly used in the literature.

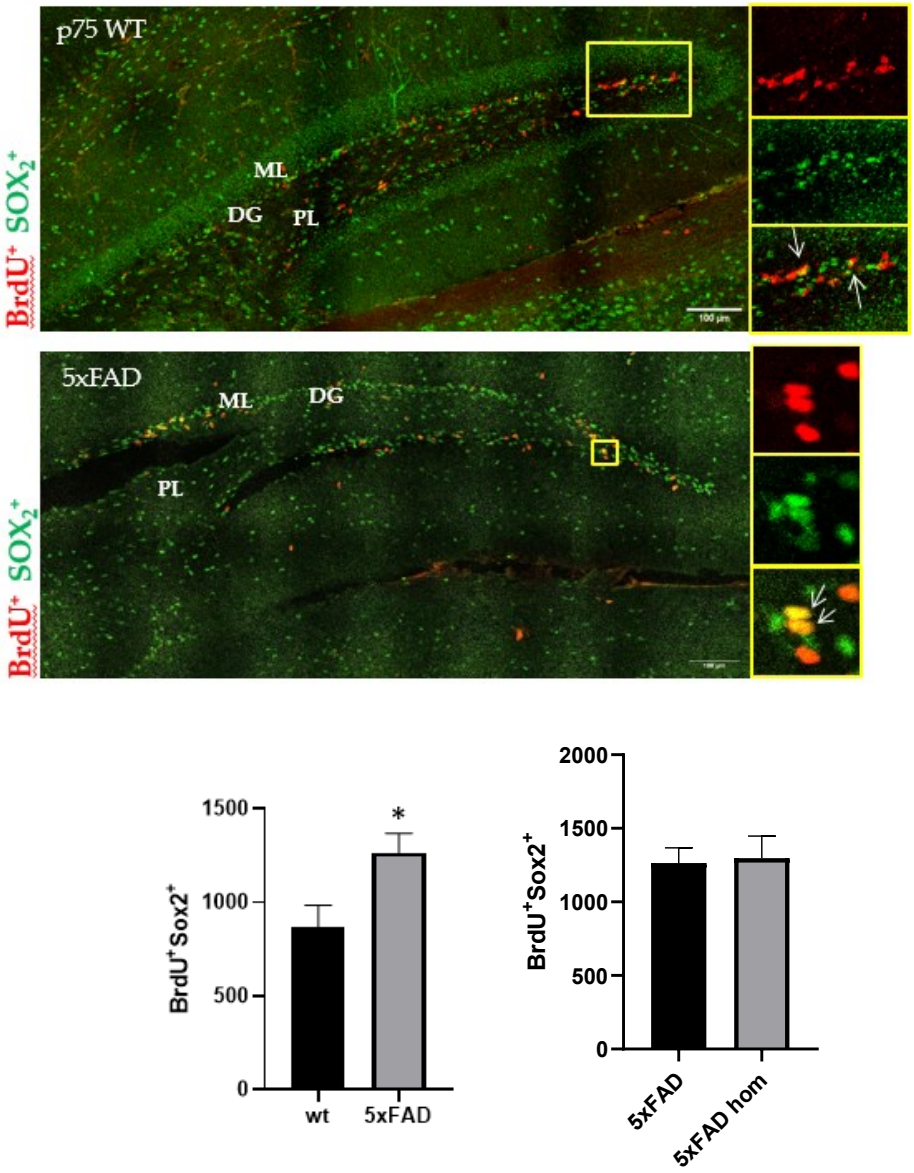


Figure 8. Hippocampal Dentate Gyrus from 2-months-old WT vs 5xFAD heterozygous (left) and 5xFAD heterozygous vs homozygous (right), injected with BrdU. Sections were immunostained for BrdU (red) and co-immunostained with Sox2 (green). *(n = 8 p75 wt, 4 5xFAD heterozygous & 4 5xFAD homozygous, t-student (unpaired t-test) *p < 0,05)*

3.1.3. Deletion of p75NTR in 5xFAD 2 months old mice reverse the increased proliferation of NSCs.

Considering the above results, we analyzed the outcome of p75NTR's deletion in 5xFAD 2mo mice. So, through numerous crossings we generated double transgenic mice which carry 5xFAD mutations as well as a total KO of p75NTR (5xFAD/p75NTR KO). We followed again the same procedure with five consequent BrdU injections and immunofluorescent staining of the hippocampus of these mice with BrdU and Sox2 primary antibodies. The measurement of $BrdU^+Sox2^+$ cells revealed that in 5xFAD/p75NTR KO mice there was a significant decrease in the proliferation rate of

NSCs when compared to WT, almost at the same levels as in p75NTR KO mice (Figure 9). This result confirms the essential role of p75NTR for the proliferation of NSCs since in the 5xFAD background where the proliferation is elevated, p75NTR's deletion is enough to abolish this elevation and drop the level of proliferation lower than this of WT mice.

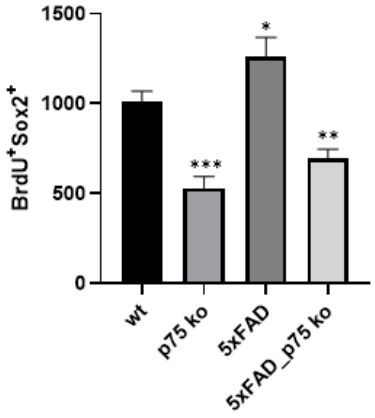


Figure 9. Measurement of $BrdU^{+}Sox2^{+}$ cells in 2 months old WT, p75NTR KO, 5xFAD & 5xFAD/p75NTR KO mice injected with BrdU. Sections were immunostained for BrdU (red) and co-immunostained with Sox2 (green). *(n= 8 p75wt, 8 p75 KO, 4 5xFAD & 4 5xFAD p75NTR KO, t-student (unpaired t-test) *p<0,05 ** p 0,0067 *** p 0,0001)*

3.1.4. p75NTR affects proliferation of NSCs in a cell non-autonomous manner.

As mentioned before, p75NTR has a wide expression in the nervous system. So, we analyzed if this reduction in the proliferation of NSCs in p75NTR KO mice was due to the loss of p75NTR's expression in NSCs or it was caused by the loss of p75NTR's expression in other cell types. In order to do so, we used 2 months old $p75^{flfl}$ Nestin-Cre^{+} mice which carry a conditional deletion of p75NTR only in the cells that express nestin which are the NSCs. The results of the analysis of $BrdU^{+}Sox2^{+}$ cells after the BrdU injections and the immunofluorescent staining demonstrated that there was no significant difference between the proliferation rate of NSCs in WT and $p75^{flfl}$ Nestin-Cre^{+} 2 months old mice. This suggests that p75NTR influences the proliferation of NSCs through cell non-autonomous signaling pathways.

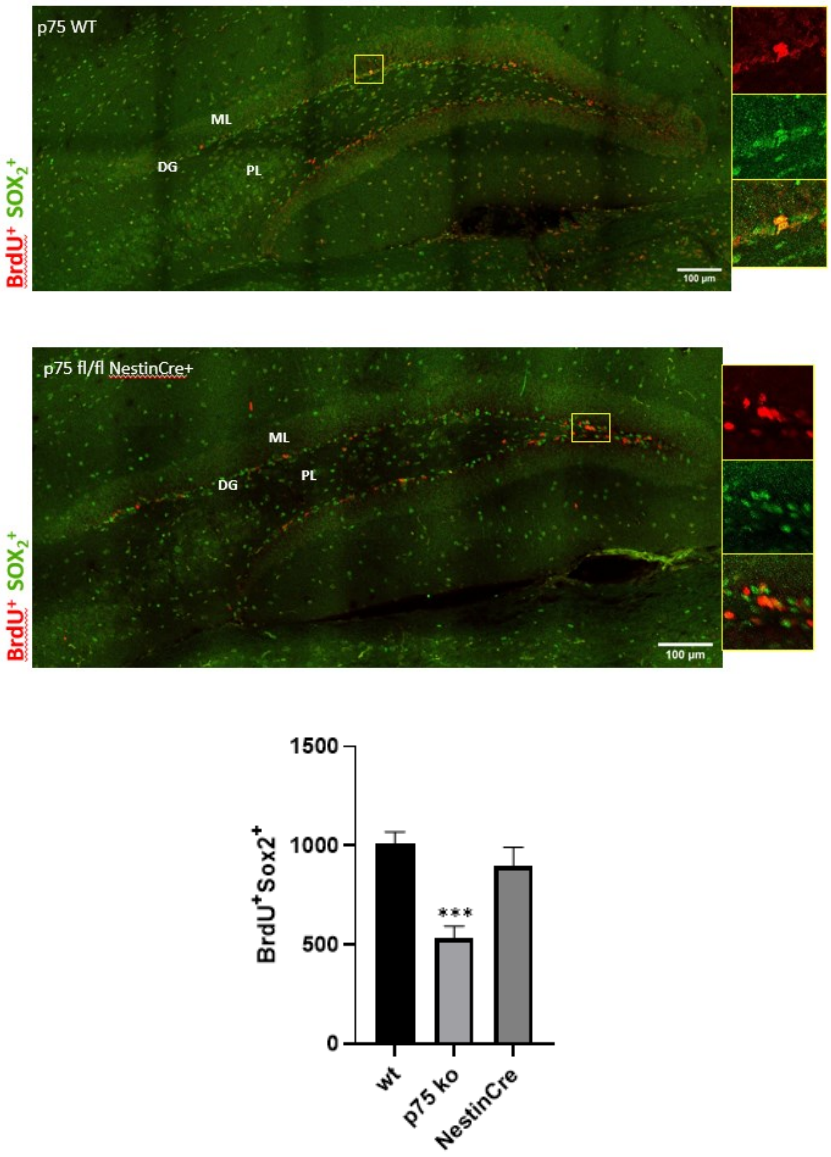


Figure 10. Hippocampal Dentate Gyrus from 2-months-old WT, p75NTR KO & p75^{flfl} Nestin-Cre^{+} mice injected with BrdU. Sections were immunostained for BrdU (red) and co-immunostained with Sox2 (green). *(n = 8 WT, 8 p75NTR KO & 6* p75^{flfl} Nestin-Cre^{+}*, t-student (unpaired t-test), *** p 0,0001)*

3.1.5. No effect of p75NTR's deletion in the proliferation of NSCs in 6 months old mice.

Finally, we studied the influence of p75NTR's deletion in NSC's proliferation in adult 6 months old mice. The immunofluorescent staining of their hippocampus with BrdU and Sox2 primary antibodies revealed no significant differences between all groups. However, the number of BrdU^{+}Sox2^{+} cells was importantly lower than this of 2 months old mice of the same groups (Figure 11). This outcome suggests that the proliferation of NSCs diminishes to such an extent with aging that the effects in proliferation caused by the p75NTR's deletion cannot be detected.

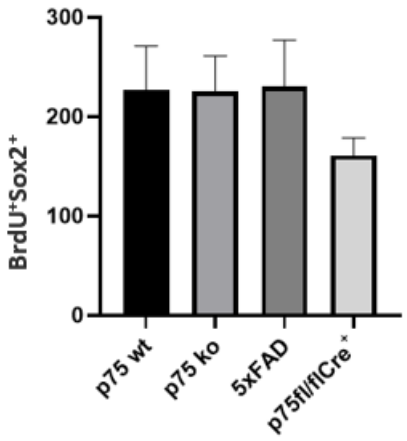
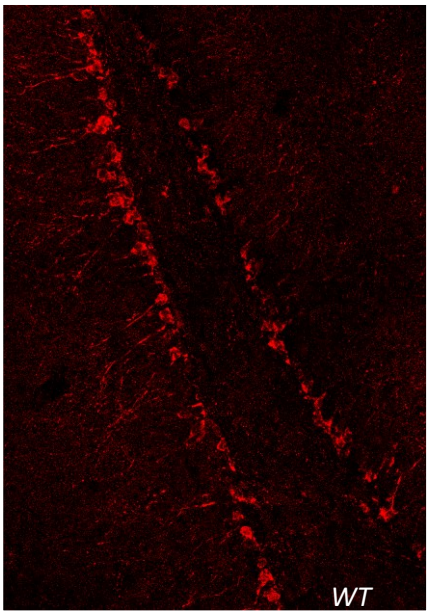


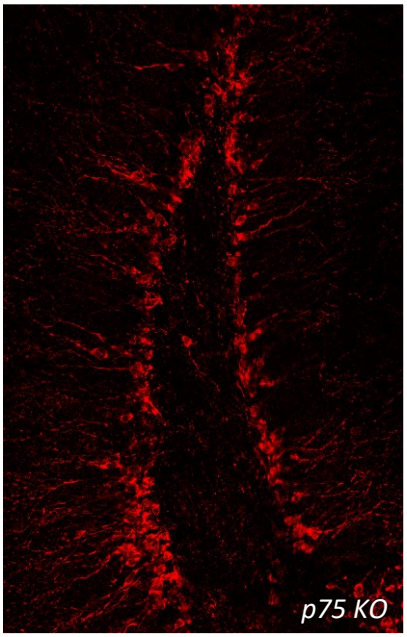
Figure 11. Measurement of $BrdU^{+}Sox2^{+}$ cells in 6 months old WT, p75NTR KO, 5xFAD & $p75^{flfl}$ Nestin-Cre^{+} mice injected with BrdU. *(n= 6 WT, 4 p75NTR KO, 4 5xFAD & 3 $p75^{flfl}$ Nestin-Cre^{+}, ordinary one-way ANOVA)*

3.2. Role of p75NTR in the production and differentiation of immature neurons

3.2.1. p75NTR promotes differentiation of immature neurons to mature neurons.

Moving on, we analyzed the impact of p75NTR in the production and differentiation of new immature neurons. To do so, we compared the number of doublecortin (DCX) positive cells in the DG of the hippocampus between WT and p75NTR KO 2 months old mice. We observed that the number of DCX^{+} cells was significantly higher in p75NTR KO than in WT animals (Figure 12). This result indicates that the newly born immature neurons cannot proceed to differentiate into mature neurons upon deletion of p75NTR. At the same time, other experiments from our laboratory have revealed that $NeuN^{+}$ cells (that correspond to neurons) are significantly decreased in p75NTR KO mice when compared with WT. Taken together, these outcomes uncovered that p75NTR is promoting the survival and differentiation of newly born cells.





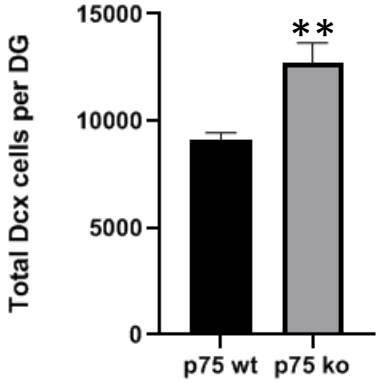
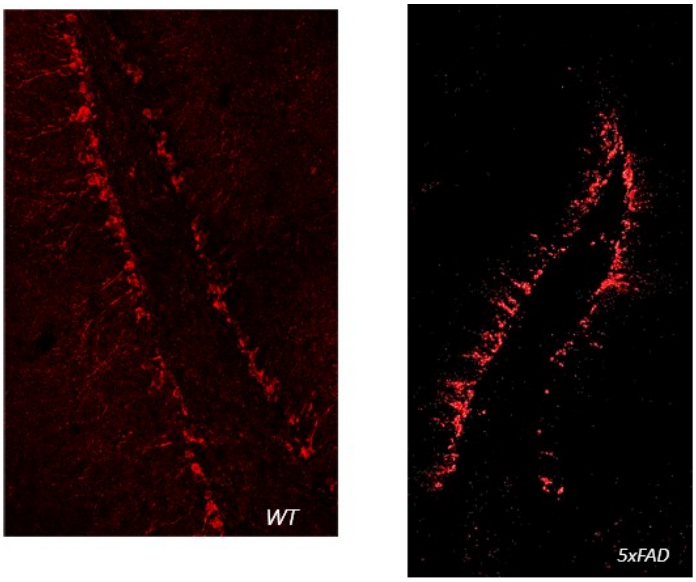


Figure 12. Hippocampal Dentate Gyrus from 2-months-old WT vs p75NTR KO. Sections were immunostained for DCX (red). *(n = 5 p75 wt & 5 p75 ko, t-student (unpaired t-test), ** p<0,005)*

3.2.2. Unaltered production of immature neurons in 5xFAD 2 months old mice.

In order to assess the production of immature neurons in terms of a neurodegenerative condition such as Alzheimer's disease, we measured the DCX^+ cells in 5xFAD 2 months old mice. By comparing the DCX^+ cells in the DG of the hippocampus of WT and 5xFAD 2 months old mice we concluded that although there is a trend for an increase, there were no significant changes between the two groups (Figure 13). So, AD background affects the proliferation of NSCs by increasing it but does not have a major impact in the production of new immature neurons, since their number is slightly higher than that of WT animals.



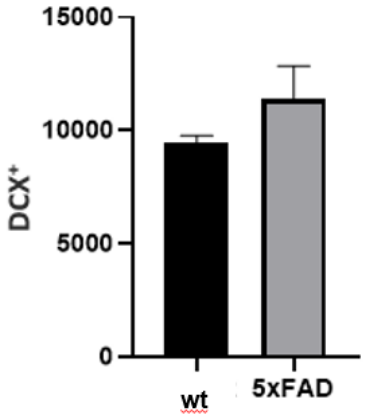
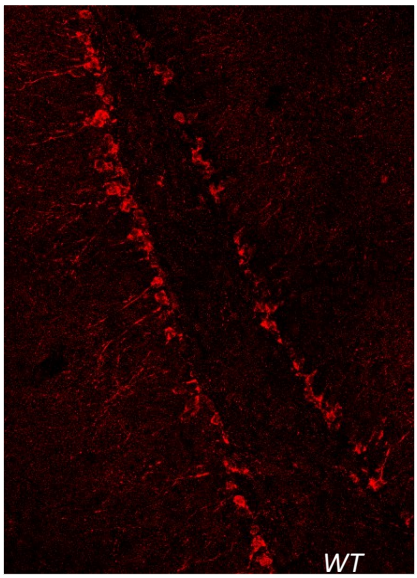
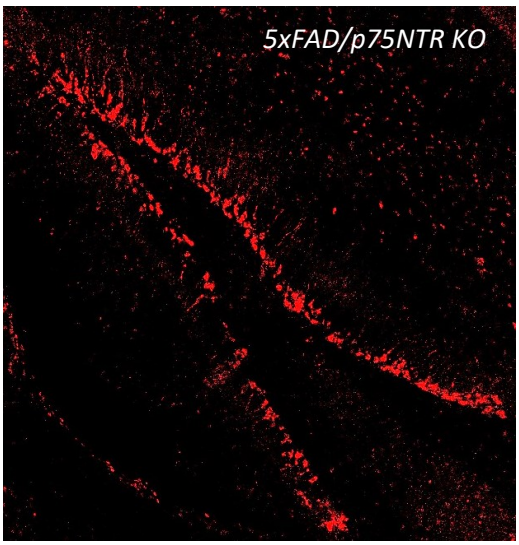


Figure 13. Hippocampal Dentate Gyrus from 2-months-old WT vs 5xFAD. Sections were immunostained for DCX (red). *(n = 5 p75 wt & 4 5xFAD, t-student (unpaired t-test), # p 0,08)*

3.2.3. Deletion of p75NTR in 5xFAD 2 months old mice does not affect the production of immature neurons.

Having the above in mind, we analyzed the number of DCX^+ cells upon p75NTR's deletion in 5xFAD 2 months old mice. The level of DCX^+ cells in the DG of the hippocampus of 5xFAD/p75NTR KO 2 months old mice was almost the same as this of the WT mice of the same age (Figure 14). So, p75NTR's deletion is enough to abolish the elevation in NSCs proliferation of 5xFAD but does not affect the production of immature neurons. This result indicated that p75NTR acts differently in WT and in AD background.





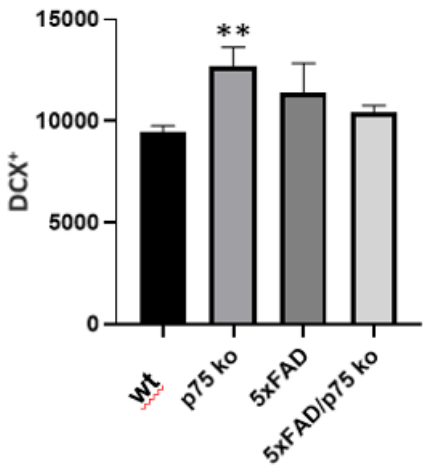
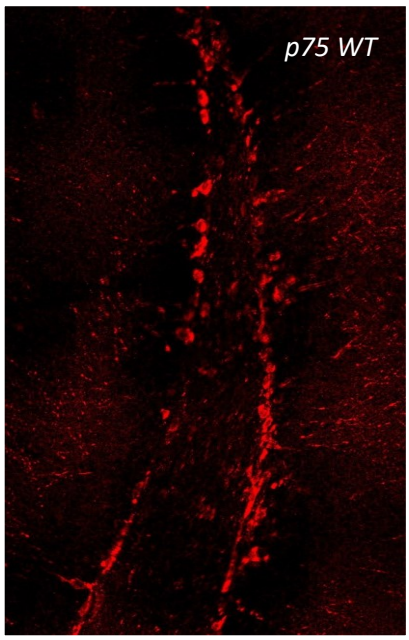
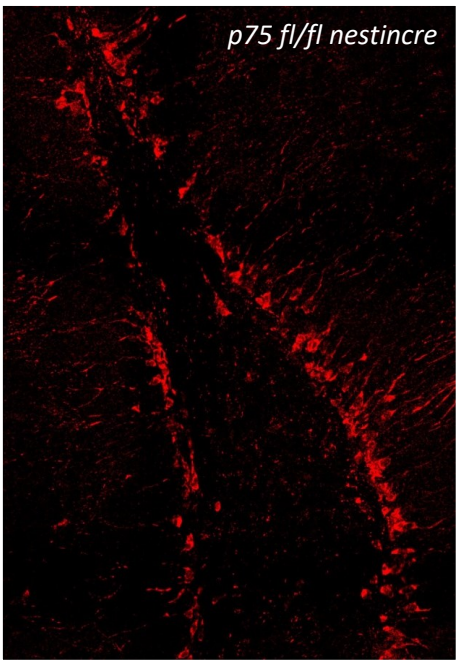


Figure 14. Hippocampal Dentate Gyrus from 2-months-old WT, p75NTR KO, 5xFAD & 5xFAD. Sections were immunostained for DCX (red). *(n = 5 WT, 5 p75NTR KO, 4 5xFAD & 4 5xFAD/p75NTR KO, t-student unpaired test)*

3.2.4. p75NTR expressed by NSCs does not affect production of immature neurons.

Here again we analyzed if p75NTR expressed by Nestin positive cells has an impact in the production of immature neurons. In order to do so, we used again 2 months old $p75^{flfl}$ Nestin-Cre^{+} mice which carry a conditional deletion of p75NTR only in the cells that express nestin. The results of the analysis of DCX^{+} cells demonstrated that there was no significant difference between the production of immature neurons in WT and $p75^{flfl}$ Nestin-Cre^{+} 2 months old mice (Figure 15). Following up with the above data about the proliferation of NSCs, this suggests that p75NTR expressed by NSCs does not influence the production of immature neurons.





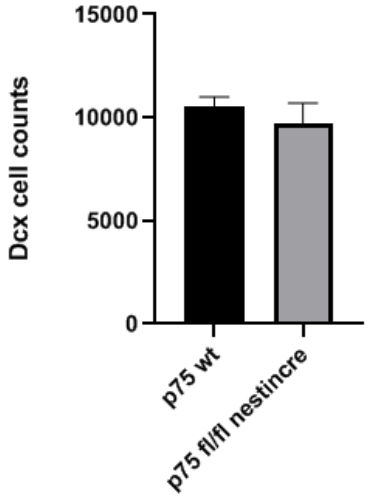


Figure 15. Hippocampal Dentate Gyrus from 2-months-old WT vs p75^{flfl} Nestin-Cre^{+}. Sections were immunostained for DCX (red). *(n = 3 WT & 3* p75^{flfl} Nestin-Cre^{+}*, t-student unpaired test)*

3.2.5. No effect of p75NTR's deletion in the production of immature neurons in 6 months old mice.

Finally, we studied the influence of p75NTR's deletion in the production of immature neurons in adult 6 months old mice. The immunofluorescent staining of their hippocampus with DCX primary antibody revealed no significant differences between all groups. However, the level of DCX^{+} cells in 6 months old animals was significantly lower than that of 2 months old mice of the same groups (Figure 16). So, this result reveals that the production of immature neurons diminishes to such an extent with aging that the influence of p75NTR's deletion cannot be detected.

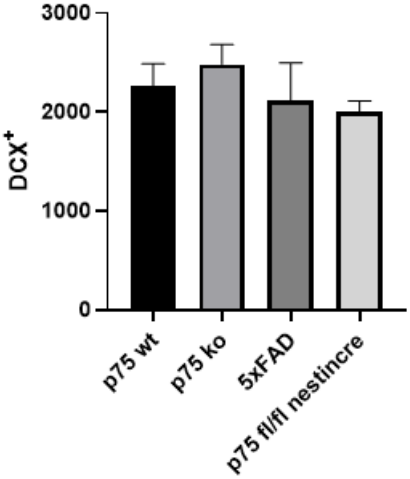
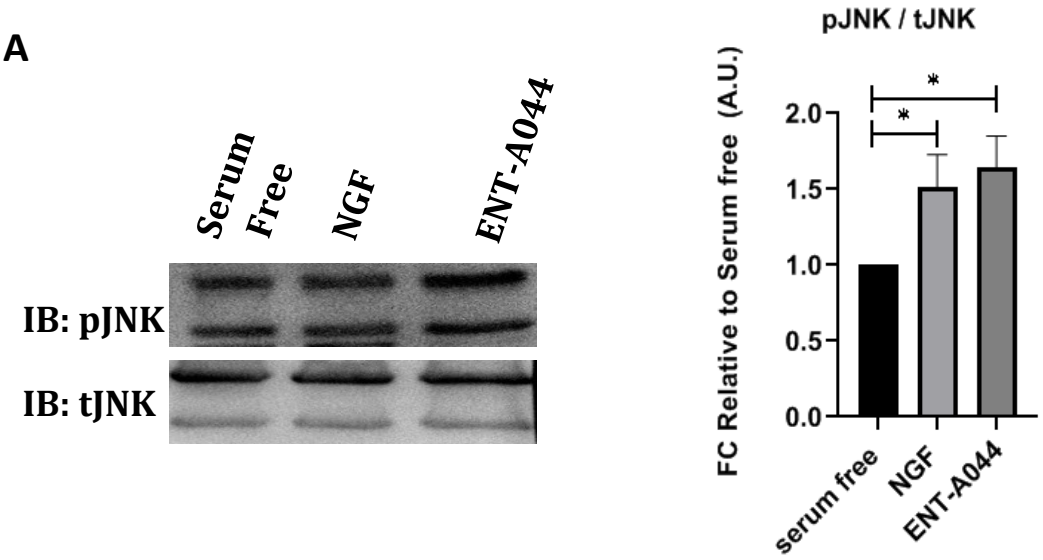


Figure 16. Measurement of DCX^{+} cells in 6 months old WT, p75NTR KO, 5xFAD & p75^{flfl} Nestin-Cre^{+} mice injected with BrdU. *(n= 3 WT, 3 p75NTR KO, 3 5xFAD & 3 p75^{flfl} Nestin-Cre^{+}, ordinary one-way ANOVA)*

3.3. p75NTR activation mediated by new synthetic analog ENT-A044.

3.3.1. p75NTR activation and increase of cell death in HEK293T cells transfected with p75NTR & RIP2 plasmids.

As mentioned above, our laboratory revealed that a new synthetic analog of DHEA, ENT-A044, acts as p75NTR's non-selective agonist. More specifically, we transfected HEK293T cell line, which does not express neurotrophin receptors endogenously, with plasmid cDNA of p75NTR and then we treated the cells with ENT-A044. After the transfection and treatment, we isolated the cell lysates and conducted a Western Blot analysis for phosphorylated and total JNK. Upon activation of the p75NTR, the mitogen-activated protein kinase JNK is triggered. JNK's phosphorylation was elevated after 30 minutes of treatment with NGF or our compound (Figure 17A). The results of this analysis indicated that HEK293T cells that express p75NTR, demonstrate an increased cell death when treated with ENT-A044 and thus this compound can promote JNK-mediated cell death through p75NTR. Moreover, we used an immunoprecipitation assay to define more thoroughly the p75NTR signaling pathways activated by ENT-A044. The signaling of p75NTR depends on interactions between proteins, such as TRAF6 and RIP2. Co-immunoprecipitation experiments for the p75NTR and its interactors TRAF6 and RIP2 revealed that, RIP2 protein exhibits a significant interaction with the p75NTR in samples treated with our compound (Figure 17B) (Papadopoulou et al., 2023).



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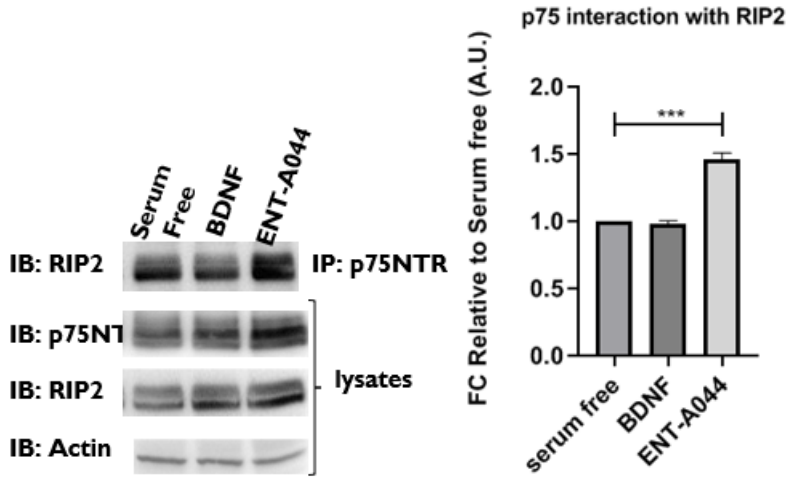

Figure 17. A. Western blot analysis on transfected HEK293T cells after 30 min of treatment with the ENT-A044 and quantification of pJNK expression *(unpaired t-test against control serum free, * p < 0.05) B*. Lysates were immunoprecipitated with p75NTR-specific antibody and immunoblotted with antibodies against RIP2. Total lysates were analyzed for p75NTR or actin expression via immunoblotting *(unpaired t-test against control serum free, *** p < 0.005)* (Papadopoulou et al., 2023).

3.3.2. ENT-A044 enhances cell survival in p7 mouse hippocampal NSCs.

Previous experiments of our laboratory using ENT-A044 have revealed that this compound activates TrkB but not TrkA (Papadopoulou et al., 2023). Since TrkB acts as a co-receptor of p75NTR we examined the effects of ENT-A044 in a system in which both TrkB and p75NTR are expressed. So, by using primary cultures of p7 mouse hippocampal NSCs which express both TrkB and p75NTR, we demonstrated via CellTox Assay that ENT-A044 administration for 48 hours can result in survival signaling when both receptors are expressed, since the levels of cell death were diminished (Figure 18) (Papadopoulou et al., 2023).

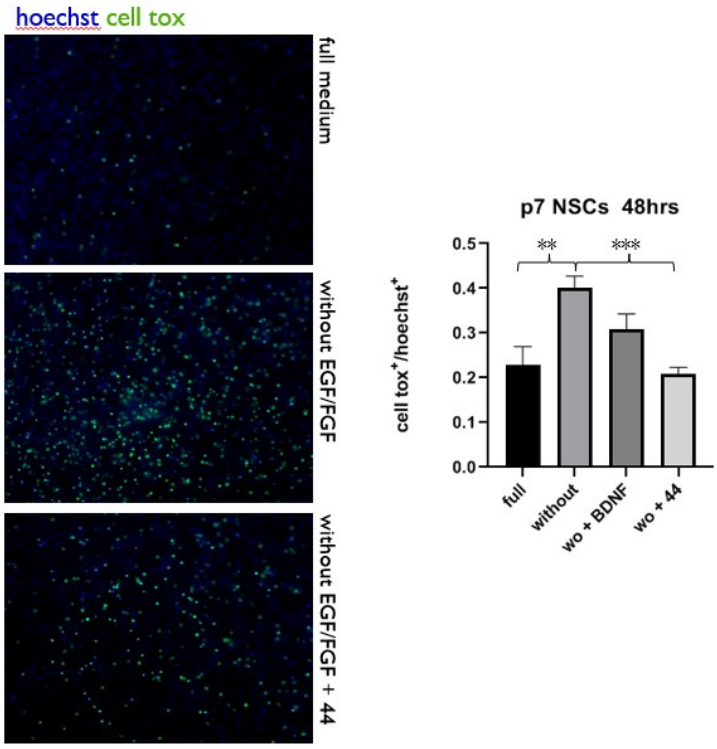

Figure 18. Cell tox assay on P7 mouse hippocampal NSCs after 48 h of treatment with the tested compound ENT-A044. Quantification of cell tox+ cells (green)/Hoechst+ cells (blue) *(N=3, one way ANOVA, **p < 0,01, ***p<0,001)* (Papadopoulou et al., 2023).

3.3.3. ENT-A044 promotes cell death in NSCs derived from human induced pluripotent stem cells (iPSCs).

Finally, we studied the effects of the compound in human iPSCs-derived NSCs which express both TrkB and p75NTR. These cells were characterized by increased phosphorylated JNK observed by Western Blotting after 24h treatment with the compound. Moreover, increased pJNK was noticed upon NGF treatment indicating that the apoptotic signaling activated is entirely related to p75NTR (Figure 19). At the same time other experiments in our laboratory showed by CellTox Assay that treatment with ENT-A044 for 48h had a detrimental impact on cell survival. Taken together, although human iPSCs-derived NSCs express both p75NTR and the TrkB receptor, ENT-A044 causes cell death mediated by p75NTR, highlighting important differences between human and mouse neuronal cell functions mediated by neurotrophin receptors (Papadopoulou et al., 2023).

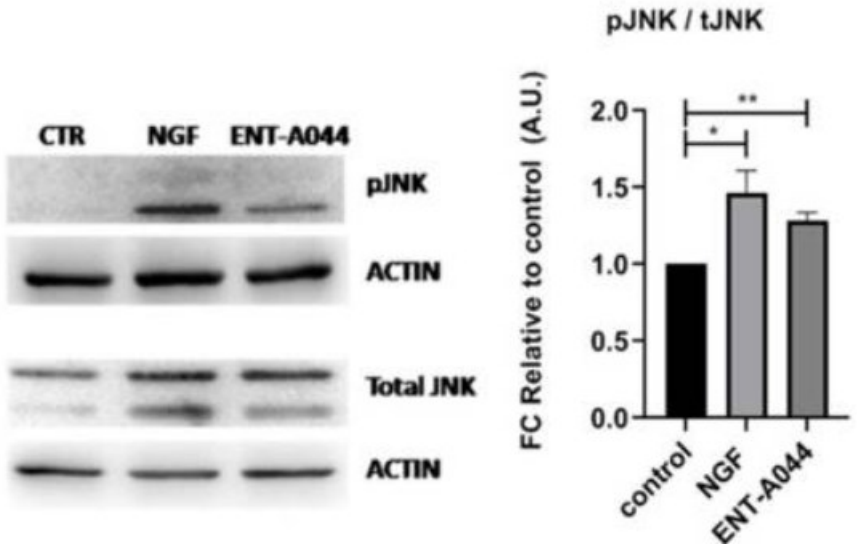

Figure 19. Quantification of Western blot analysis of phosphorylated and total JNK performed on human iPSCs-derived NSCs treated with ENT-A044 or NGF for 24 h *(unpaired t-test, * $p < 0.05$, ** $p < 0.005$)* (Papadopoulou et al., 2023).

4. Discussion

As mentioned above, it is generally known that p75NTR plays a significant role in several cellular processes depending on the ligand and the co-receptor binding. An important study almost 15 years ago revealed that, there is a significant decrease in neuroblasts and newly born neurons and at the same time an increased cells death of neuronal progenitors in the DG of p75NTR KO mice when compared to wild-type. Furthermore, this study showed behavioral changes related to neurogenesis in p75NTR KO mice (Catts et al., 2008). After this study, a lot of scientific teams have provided data that enhance the crucial role of p75NTR in neurogenesis, neural survival, and behavior, as well as in neural development and circuit assembly (Bernabeu & Longo, 2010; Meier et al., 2019; Zuccaro et al., 2014). Here in this project, we demonstrated that p75NTR's total deletion leads to a decrease in NSCs' proliferation but in an increase in their differentiation in 2 months old mice. This indicates that p75NTR plays a crucial role in proliferation and differentiation of NSCs.

In terms of neurodegenerative conditions, recent studies have associated Alzheimer's disease with reduced adult hippocampal neurogenesis. More specifically, Moreno-Jimenez and his team have shown that whereas there are thousands of immature neurons in the DG of neurologically healthy human, the number and maturation of these neurons progressively declined as AD advanced (Moreno-Jiménez et al., 2019). However, in our study the NSCs' proliferation was increased in 2 months old 5xFAD mice. The elevated proliferation in these animals suggests that the central nervous system possibly tries to develop a compensatory, homeostatic mechanism against the neurodegenerative effects of AD-like pathology and thus reverse the neuronal loss that is the result of neurodegeneration generally observed at the onset of the disease, approximately in around 4 months old. Considering the above results,

we also analyzed the outcome of p75NTR's deletion in 5xFAD 2 months old mice and we demonstrated that the proliferation of NSCs was decreased in 5xFAD/p75NTR KO mice almost at the same levels as in p75NTR KO mice, but their differentiation was unaltered. These results also indicate the essential role of p75NTR in the proliferation but milder in the differentiation of NSCs since in WT and in 5xFAD background where the proliferation is elevated, p75NTR's deletion is enough to abolish this elevation and drop the level of proliferation lower than this of WT mice.

Additionally, a recent study has revealed that the conditional deletion of p75NTR gene in neural progenitors causes anatomical abnormalities of the brain (Meier et al., 2019). In our study, we analyzed if this reduction in the proliferation of NSCs in p75NTR KO mice was due to the loss of p75NTR's expression in NSCs or it was caused by the loss of p75NTR's expression in other cell types. The conditional deletion of p75NTR only in nestin positive cells did not affect the proliferation and differentiation of NSCs in 2 months old mice. This emerges the possibility that p75NTR influences the proliferation of NSCs in a cell-non autonomous and that the receptor expressed by NSCs does not affect their differentiation. These contradictory results can be attributed to different genetic background of p75NTR-deficient animals. To conclude the above data, provide evidence for p75NTR's major neurogenic role.

In the previous years our laboratory has demonstrated that DHEA, which is a natural neurosteroid with lipophilic characteristics, can act on TrkA and p75NTR and inhibit neuronal cell death (Lazaridis et al., 2011). The long-term use of DHEA as a potential treatment for neurodegeneration is problematic, especially in patients with a genetic predisposition to hormone-dependent tumors, as it is metabolized in vivo to estrogens and androgens, which affect the endocrine system (Compagnone & Mellon, 2000). A library of novel small molecules-chemical analogs of DHEA was developed with BNN27 to be the first synthetic molecule that was described to interact with TrkA, thus activating neuronal survival signaling pathways (Pediaditakis, Efstathopoulos, et al., 2016). It also interacts with p75NTR promoting the interactions of the receptor with RIP2 and TRAF6 effector proteins (Pediaditakis, Kourgiantaki, et al., 2016).

A recent study of our research group has revealed that ENT-A044, which is a small synthetic compound of DHEA triggers both TrkB and p75NTR activation. However, its influence in the signaling pathways activated is associated with which receptors are expressed and with the species origin of the neuronal cells. Some of the experiments of this study that have been conducted during this Master Thesis have showed that ENT-A044 has different effects on murine or human neuronal cells (Papadopoulou et al., 2023). More specifically, in primary cultures of mouse p7 hippocampal NSCs we showed with CellTox Assay that treatment with the ENT-A044 diminishes the levels of cell death. So, this compound can trigger the survival signaling pathway activated by TrkB receptor. On the other hand, in NSCs derived from human iPSCs Western Blot analysis for total and phosphorylated JNK revealed that treatment with ENT-A044 leads to cell death. Having in mind that treatment with NGF also led to elevation of cell death, the apoptotic signaling activated is entirely related to p75NTR.

So, the above data highlight important differences between human and mouse neuronal cell functions mediated by neurotrophin receptors. To conclude, taking advantage of ENT-A044 mediating cell death through p75NTR, we may be able to use ENT-A044 therapeutically to treat diseases like cancer by focusing on the eradication of cancer cells.

Finally, to further investigate p75NTR's neurogenic role *in vivo* and *in vitro* it would be interesting to repeat the same *in vivo* experiments in 4 months old mice of the same groups to assess the effects of the receptor to the proliferation and differentiation of NSCs. At this age, the AD pathology has progressed and Aβ plaques have accumulated in the brain of 5xFAD mice so the results would be more representative. Furthermore, it would be a good idea to isolate RNA from lysates of hippocampal tissue of these animals and perform RNA sequencing to evaluate the differences in gene expression related with p75NTR's signaling pathways. Lastly, we can perform transcriptomic analysis at a single cell level in the presence of endogenous ligands and synthetic agonists of p75NTR, such as ENT-A044, to assess their impact in gene expression associated with p75NTR's signaling.

In conclusion, the project has significant implications for our comprehension of how the p75NTR receptor functions in a variety of cellular processes, particularly neurogenesis, neural survival, behavior, neurodegeneration, and possible therapeutic applications. In more detail, it makes a connection between p75NTR and neurodegenerative conditions like AD. The decreased proliferation of NSCs in 5xFAD/p75NTR KO mice and p75NTR KO mice suggests that the receptor plays an important role in regulating NSC function under pathological conditions. Moreover, by demonstrating that p75NTR affects NSCs proliferation in a cell-non autonomous manner, the project adds complexity to our understanding of how this receptor influences neural development. On the other hand, the study of synthetic analogs of DHEA introduces them as potential therapeutic agents to modulate p75NTR signaling. The identification of species-specific effects of compounds like ENT-A044 on neurotrophin receptors provides valuable information for future translational studies and potential drug development. Furthermore, the discovery that ENT-A044 can cause cell death via p75NTR points to a potential route for therapeutic intervention in conditions like cancer. This has the potential to expand the application of p75NTR-targeted therapies beyond neurodegenerative conditions. All in all, the findings of this study have implications for both fundamental neuroscience research and the development of strategies to address neurodegenerative diseases and other conditions influenced by p75NTR signaling.

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