PhD Thesis

«Deciphering the role of the p75 pan-neurotrophin receptor in adult hippocampal neurogenesis as a novel therapeutic approach to Alzheimer's Disease».

«Προσδιορισμός του ρόλου του παν-νευροτροφικού υποδοχέα p75 στην ενήλικη νευρογένεση του ιπποκάμπου, ως νέα θεραπευτική προσέγγιση στη Νόσο Αλζχάιμερ».

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Heraklion 2023

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The present research has been conducted at the Department of Pharmacology, School of Medicine, University of Crete and the Institute of Molecular Biology and Biotechnology, Foundation of Research and Technology-Hellas (IMBB FORTH), under the supervision of Professor Ioannis Charalampopoulos.
This research was funded from the Hellenic Foundation for Research and Innovation (H.F.R.I) under the "1st Call for H.F.R.I. Research Projects to support Faculty members and Researchers and the procurement of high-cost research equipment" (Project Number: 2301, KA10490). Moreover, Maria Anna Papadopoulou acknowledges a personal funding from Bodossaki Foundation.

Lastly, it was financed by Greece and the European Union (European Social Fund-ESF) through the Operational Programme «Human Resources Development, Education and Lifelong Learning» in the context of the Act "Enhancing Human Resources Research Potential by undertaking a Doctoral Research" Sub-action 2: IKY Scholarship Programme for PhD candidates in the Greek Universities».

ΗΡΑΚΛΕΙΟ 2023

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ACKNOWLEDGMENTS

At this point I would like to thank from the bottom of my heart the supervisor of this doctoral thesis, Professor Ioannis Charalampopoulos, for the unique opportunity that he offered me to be nurtured under his scientific, and often paternal, guidance throughout these years. His steadfast commitment to the uninterrupted, productive and above all prudent study of the role of neurotrophins in nervous system has always acted as a stimulant for me at all stages of the preparation of this thesis. I thank him warmly for teaching me that even in these difficult times, dedication to scientific knowledge and unremitting work are capable of producing meaningful science.

I would also like to thank Professor Ioannis Zaganas and Achilleas Gravanis, members of my three-member advisory committee, for their support in many stages of the execution of the present study, for their thoughts about how to continue with our study and for their crucial corrections on the text of this thesis.

I owe a great debt of gratitude to all the seven-members of the examination committee. Many of them were my professors during my Master's degree on Neurosciences and they introduced me to the beautiful world of experimental research on brain. The knowledge that I gained from all of them still remain to this day my valuable guide.

I am very thankful to my respected colleagues, Kanelina Karali, Konstantina Chanoumidou, Maria Kokkali, Thanasis Rogdakis, Despoina Charou, Ioanna Zota, Konstantina Georgelou and Alexandros Tsimpolis for their support and patience all of these years. I really appreciate all your assistance. I thank them all, warmly for filling my daily life with vitality and providing essential support during my many hours of work at the laboratory bench. Special thanks to Ioanna Kanteli and Maria Peteinareli, for working with me and for the great times we had together. I especially thank them because apart from the hours they have dedicated to help me with my project, they were valuable colleagues and above all dear friends.

To all my loved ones, my parents Dimitra and Konstantinos, my brother Mpampis and his wife Ioanna, my sister Sofia and her husband Panagiotis, as well as my two beloved nieces Efthimia and Dimitra, who have been constant values in my life, I thank them for the love, understanding and support they have generously shown me over the years. They all taught me early on, that I ought to make dreams, that I should strive hard and fairly trying to make them true and they are always going to be by my side, no matter what.

Last but not least, I am truly grateful to Panagiotis for his patience and support. All my efforts and choices were accompanied by his unconditional love and understanding, which made everything looks easier.

This Thesis is dedicated to my beloved parents Dimitra & Konstantinos

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Abstract

The pan-neurotrophin p75 receptor (p75NTR) is a member of the TNF death receptor superfamily, being widely expressed in many cell types among the neural tissue, including adult neural stem cells (aNSCs). Its remarkable up-regulation during neurodegeneration and its controversial signaling, ranging from survival to cell death, have attracted a special interest on this receptor as a potential pharmacological target. The association between p75NTR and Alzheimer's Disease (AD) is of great interest in general, as p75NTR is highly expressed in degenerative populations in the development of AD pathology. p75NTR has been also extensively linked with AD by serving as a receptor for Amyloid-beta (Aβ), the major component of the plaques, found in the brain of AD patients. Furthermore, recent revolutionary studies are highlighting the importance of this receptor on the adult hippocampal neurogenesis, but contradictory results and lack of explicit methodology rather confuse the field, than defining the role of the receptor.

Our study ranges from *in vivo* detection of adult neurogenesis in combined p75KO and AD mouse models to *in vitro* cell cultures. We reveal that the p75NTR is important to neurogenesis and the fate of aNSCs and we also explore the role of p75NTR in neurogenesis under the neuropathology of AD, as well. More specifically, this study focuses on the effects of p75NTR in adult neurogenesis of the dentate gyrus (DG) of rodent hippocampus, under physiological and neurodegenerative conditions, such as AD, as well as on examination of receptor's activity related to cell death or survival. By revealing and controlling the specific signaling pathways necessary to mediate the actions of p75NTR on stem cells, we could target the endogenous ability of neurogenesis.

In the first part of our study, we evaluated the *in vivo* adult hippocampal neurogenesis in p75 knockout mice, compared to wildtype (2 months old and 6 months old mice). Our study was based on the evaluation of two different mouse models of p75 gene deletion. The first one, is the p75 ko Ngfrtm1Jae targeted mutation 1 -developed by Dr Rudolf Jaenisch and named p75NTR/ExonIII-, which lacks the full-length receptor. The second one is a conditional knock out which has a specific deletion of exon II in Nestin expressing cells based on the recombination of a p75NTR^{flox} mouse with the Nestin/Cre mouse. Our results from the first mouse model are showing a significantly decreased number of proliferative aNSCs in p75 ko mice of 2 months old. These results are pointing out the necessity of p75NTR on adult neurogenesis. In a second step, we examined the effects of receptor's deletion on the differentiation of aNSCs, using Dcx and NeuN markers and we gained results suggesting that immature neurons cannot proceed and differentiate into mature neurons.

In parallel, we examined the mice with the specific p75 exonII deletion on Nestin$^+$ cells. This mouse line provides significant evidence for the role of p75 in neurogenesis by defining the cell autonomous signaling pathways that are controlling stem cell fate. Finally, in order to investigate the role of p75NTR in a mouse model of Alzheimer's Disease, we have crossed p75 ko exonIII with the 5xFAD mouse, an amyloid beta dependent mouse model of AD. In this mouse line, we also identify neurogenesis levels compared to wild-type as well.

Additionally, we explore p75-dependent effects on human iPSCs-derived NPCs, in order to validate the results of the existing animal models but also to explore the effects of this neurotrophin receptor in human neurons, since these kind of studies are very rare till now, underlying the novelty of our work. Study of p75NTR involvement in human neurogenesis includes various research questions. Firstly, the expression and activity of p75NTR as well as its downstream mediators are identified. Moreover, we investigate p75NTR's effects in human induced Pluripotent Stem Cells (hiPSCs)-derived NPCs, depicting receptor's signaling and its dysregulation in NPCs derived from AD patients bearing the ApoE4 mutation. Furthermore, we study the role of the receptor in combination with the toxic effects of oligomerized Amyloid-beta, showing how p75NTR regulates hiPSCs-derived NPCs in the presence of Aβ amyloid, indicating its involvement in both AD progress and neurogenesis. These results provide useful information regarding the role of p75NTR in NPCs maintenance and its putative therapeutic role.

In a pharmacological approach of our studies, we tested a chemical library of novel synthetic neurotrophin analogs in order to decipher the signaling properties of the receptor. These synthetic analogs are derived from dehydroepiandrosterone (DHEA), a previously shown activator of the receptor, and they were designed with preferable pharmacological properties that could selectively activate p75NTR and specific pathways that originate from this receptor. Despite the beneficial effects of neurotrophins on brain function, their therapeutic use is compromised due to their polypeptidic nature and blood–brain-barrier impermeability. To overcome these limitations, our previous studies have proven that small-sized, lipophilic, DHEA-derived synthetic analogs can exert neurotrophic effects, as they bind to specific neurotrophin receptors and thus they could be used as neurotrophin mimetics with favorable pharmacological properties. The present study also revealed the biological characterization of a newly synthesized analog, ENT-A044, and its role in inducing cell-specific functions of p75NTR. Treatment of cells with this compound showed a significant increase at cell death and pJNK expression, mediated by p75NTR in human iPSCs-derived NPCs as opposed to primary cell cultures derived from mice, where ENT-A044 led to survival. Thus, Neurotrophin Analog ENT-A044 Activates the p75 Neurotrophin Receptor, Regulating Neuronal Survival in a Cell Context-Dependent Manner. In conclusion, ENT-A044 is proposed as a lead molecule for the development

of novel pharmacological agents, providing new therapeutic approaches and research tools, by controlling p75NTR actions.

All the aforementioned studies offer valuable tools for selective control of p75NTR in AD, through the specific regulation of its endogenous neurogenic potential, as a novel repairing mechanism against neurodegeneration. In addition, adult neurogenesis consists a scientific field that has recently attracted special interest, although with many anticipating findings and strong arguments. The discovery of adult neurogenic potential in the brain opens new avenues and opportunities for treating neurological diseases, particularly through the exploitation of endogenous regenerative capacity. The detailed mapping of single cell properties and functions, based on the tempo-spatial expression of multifunctional regulatory receptors such as p75NTR, could provide an advantage for controlling neurogenesis and the related cognitive functions.

Περίληψη

Ο παν-νευροτροφικός υποδοχέας p75 (p75NTR) είναι μέλος της υπερ-οικογένειας των υποδοχέων θανάτου Tumor Necrosis Factor (TNF) που εκφράζεται ευρέως σε πολλούς τύπους κυττάρων του νευρικού ιστού, συμπεριλαμβανομένων των ενήλικων νευρικών βλαστικών κυττάρων (aNSCs). Η αξιοσημείωτη αύξηση της κυτταρικής του έκφρασης κατά τη διάρκεια της νευροεκφύλισης και η πολύπλοκη σηματοδότησή του, που κυμαίνεται από την επιβίωση έως τον κυτταρικό θάνατο, έχουν προσελκύσει ιδιαίτερο ενδιαφέρον για τον υποδοχέα αυτό, ως πιθανό φαρμακολογικό στόχο. Η συσχέτιση μεταξύ του p75NTR και της Νόσου Αλτσχάιμερ (ΝΑ) παρουσιάζει ιδιαίτερα μεγάλο ενδιαφέρον, καθώς ο p75NTR εκφράζεται σε μεγάλο βαθμό σε πληθυσμούς αποπτωτικών νευρικών κυττάρων κατά την εξέλιξη της παθολογίας της ΝΑ. Ο p75NTR έχει επίσης συνδεθεί εκτενώς με τη ΝΑ, καθώς χρησιμεύει ως υποδοχέας για το αμυλοειδές-β (Αβ), το κύριο συστατικό των αμυλοειδικών πλακών, που βρίσκονται στον εγκέφαλο των ασθενών με ΝΑ. Επιπλέον, πρόσφατες μελέτες αναδεικνύουν τη σημασία αυτού του υποδοχέα στην ενήλικη νευρογένεση του ιπποκάμπου, αλλά τα αντιφατικά αποτελέσματα και η έλλειψη σαφούς μεθοδολογίας δεν αποσαφηνίζουν το ρόλο του υποδοχέα.

Η παρούσα μελέτη εκτείνεται από την *in vivo* ανίχνευση της ενήλικης νευρογένεσης σε διασταυρούμενα μοντέλα ποντικών με έλλειψη του υποδοχέα (p75 knock out) και ζωϊκά μοντέλα της ΝΑ, έως την μελέτη του υποδοχέα σε *in vitro* πρωτογενείς καλλιέργειες νευρικών κυττάρων. Η μελέτη μας καταδεικνύει ότι ο p75NTR είναι σημαντικός για τη νευρογένεση και την τύχη των ενήλικων βλαστικών κυττάρων (aNSCs) και διερευνούμε τον ρόλο του p75NTR στη νευρογένεση υπό τη νευροπαθολογία της ΝΑ. Πιο συγκεκριμένα, η παρούσα μελέτη επικεντρώνεται στον έλεγχο των δράσεων του p75NTR στην ενήλικη νευρογένεση της οδοντωτής έλικας (DG) του ιπποκάμπου των τρωκτικών, υπό φυσιολογικές και νευροεκφυλιστικές συνθήκες, όπως η ΝΑ, καθώς και στην εξέταση της κυτταρικής δράσης του υποδοχέα που σχετίζεται με τον κυτταρικό θάνατο ή την επιβίωση. Αποκαλύπτοντας και ελέγχοντας τα συγκεκριμένα σηματοδοτικά μονοπάτια που είναι απαραίτητα για τη διαμεσολάβηση των δράσεων του p75NTR στα βλαστικά κύτταρα, θα μπορούσαμε να στοχεύσουμε την ενδογενή ικανότητα της νευρογένεσης ως μια καινοτόμο θεραπευτική προσέγγιση.

Στο πρώτο μέρος της μελέτης μας αξιολογήσαμε την *in vivo* ενήλικη νευρογένεση του ιππόκαμπου σε ποντίκια p75 knockout, σε σύγκριση με ποντίκια άγριου τύπου (ποντίκια ηλικίας 2 και 6 μηνών). Η μελέτη μας εξαρτάται από την αξιολόγηση δύο διαφορετικών μοντέλων ποντικών με διαγραφή του γονιδίου p75. Το πρώτο, είναι το p75 ko Ngfrtm1Jae στοχευμένης μετάλλαξης 1 -το οποίο αναπτύχθηκε από τον Δρ. Rudolf Jaenisch κι ονομάστηκε p75NTR/ExonIII-, και το οποίο στερείται του υποδοχέα πλήρους μήκους. Το δεύτερο, προέκυψε από την διασταύρωση p75flox ζώων με ζώα

που έχουν μια ειδική διαγραφή του εξονίου II σε κύτταρα που εκφράζουν νεστίνη (Nestin Cre), με βάση τη χρήση του συστήματος ρεκομπινάσης. Τα αποτελέσματά μας από το πρώτο μοντέλο ποντικού δείχνουν, μειωμένο αριθμό πολλαπλασιασμού των aNSCs σε p75 ko ποντίκια. Τα αποτελέσματα αυτά καταδεικνύουν την αναγκαιότητα του p75NTR στην ενήλικη νευρογένεση. Σε ένα δεύτερο βήμα, εξετάσαμε τις επιπτώσεις της διαγραφής στη διαφοροποίηση των NSCs, χρησιμοποιώντας τους δείκτες Dcx και NeuN και αποκτήσαμε αποτελέσματα που υποδηλώνουν ότι οι ανώριμοι νευρώνες δεν μπορούν να διαφοροποιηθούν περαιτέρω σε ώριμους νευρώνες.

Επιπλέον, εξετάσαμε ποντίκια με την ειδική διαγραφή του p75 exonII στα κύτταρα που εκφράζουν νεστίνη. Αυτή η σειρά ποντικών μας δίνει μια απάντηση για τον κυτταροειδικό ρόλο του p75 στη νευρογένεση, καθορίζοντας επίσης τα αυτόνομα ή μη, σηματοδοτικά μονοπάτια που ελέγχουν τη μοίρα των βλαστικών κυττάρων. Επιπλέον, διασταυρώσαμε τα p75 ko exonIII ζώα με τα 5xFAD, ένα μοντέλο ποντικού με ΝΑ εξαρτώμενο από το τοξικό αμυλοειδές-β. Σε αυτή τη σειρά ποντικών, προσδιορίζουμε επίσης επίπεδα νευρογένεσης σε σύγκριση με του αγρίου τύπου.

Σε μια φαρμακολογική προσέγγιση της έρευνάς μας, μελετήσαμε τις εξαρτώμενες από τον p75 υποδοχέα επιδράσεις σε ανθρώπινα νευρικά βλαστικά κύτταρα (NPCs) που προέρχονται από επαγώμενα πολυδύναμα βλαστικά κύτταρα (iPSCs), προκειμένου να ελέγξουμε την σημασία των αποτελεσμάτων που είχαμε από τα προαναφερθέντα ζωϊκά μοντέλα σε ανθρώπινα νευρικά κύτταρα. Να σημειωθεί ότι ο ρόλος του p75NTR δεν έχει μελετηθεί στο παρελθόν σε ανθρώπινα NPCs, γεγονός που αναδεικνύει την καινοτομία της εργασίας μας. Η μελέτη της συμμετοχής του p75NTR στην ανθρώπινη νευρογένεση περιέλαβε διάφορα ερευνητικά ερωτήματα. Πρώτον, προσδιορίσαμε την έκφραση και την δραστηριότητα του p75NTR καθώς και των ενδοκυττάριων διαμεσολαβητών του. Επιπλέον, διερευνήσαμε τις επιδράσεις του p75NTR σε NPCs που προέρχονται από ανθρώπινα επαγόμενα πολυδύναμα βλαστικά κύτταρα (hiPSCs), διασαφηνίζοντας τη σηματοδότηση του υποδοχέα και τη δυσλειτουργία του σε NPCs που προέρχονται από ασθενείς με ΝΑ που φέρουν τη μετάλλαξη ApoE4. Επιπλέον, δεικνύουμε τις δράσεις του p75NTR στην επιβίωση των NPCs που προέρχονται από hiPSCs υπό την παρουσία των ολιγομερών αμυλοειδούς Αβ, διερευνώντας τη συμμετοχή του υποδοχέα τόσο στην εξέλιξη της ΝΑ, όσο και στο ρόλο της ανθρώπινης νευρογένεσης στην ΝΑ. Τα αποτελέσματα αυτά παρέχουν χρήσιμες πληροφορίες σχετικά με τον ρόλο του p75NTR στη κυτταρική τύχη των NPCs και τον πιθανό θεραπευτικό του ρόλο στον άνθρωπο.

Επιπροσθέτως, για να αποκρυπτογραφήσουμε τις σηματοδοτικές ιδιότητες του υποδοχέα, δοκιμάσαμε μια βιβλιοθήκη νέων, συνθετικών αναλόγων της δευδροεπιανδροστερόνης (DHEA), με συγκεκριμένες φαρμακολογικές ιδιότητες που θα μπορούσαν να δράσουν στον p75NTR και να ενεργοποιήσουν επιλεκτικά

συγκεκριμένα μονοπάτια σηματοδότησής του. Παρά τις ευεργετικές επιδράσεις των νευροτροφινών στη λειτουργία του εγκεφάλου, η θεραπευτική τους χρήση δεν είναι εφικτή λόγω της πολυπεπτιδικής τους φύσης και της αδιαπερατότητας του αιματοεγκεφαλικού φραγμού. Για να ξεπεραστούν αυτοί οι περιορισμοί, οι προηγούμενες μελέτες μας απέδειξαν ότι συνθετικά ανάλογα που προέρχονται από τη DHEA μπορούν να δράσουν όπως οι νευροτροφίνες, καθώς ενεργοποιούν εκλεκτικά τους υποδοχείς νευροτροφινών και μιμούνται τις ενδογενείς νευροτροφίνες, στερούνται ενδοκρινικών παρενεργειών, κι επομένως έχουν προτιμητέα φαρμακολογικά χαρακτηριστικά. Η παρούσα μελέτη διερεύνησε τις βιολογικές δράσεις ενός νεοσυντιθέμενου αναλόγου, του ENT-A044, και τον ρόλο του στην επαγωγή κυτταροειδικών λειτουργιών του p75NTR. Η χορήγηση του αναλόγου καλλιέργειες ανθρώπινων NPCs που προέρχονται από iPSCs, έδειξε σημαντική αύξηση στον κυτταρικό θάνατο και αύξησε την έκφραση της pJNK μέσω του p75NTR, σε αντίθεση με πρωτογενείς κυτταρικές καλλιέργειες που προέρχονται από ποντίκια, όπου το ENT-A044 οδήγησε σε επιβίωση. Έτσι, το ανάλογο νευροτροφίνης ENT-A044 ενεργοποιεί τον υποδοχέα νευροτροφινών p75, ρυθμίζοντας τη νευρωνική επιβίωση με τρόπο εξαρτώμενο από το είδος του κυττάρου και του οργανισμού προέλευσης του. Συμπερασματικά, το ENT-A044 προτείνεται ως μόριο-οδηγός για την ανάπτυξη νέων φαρμακολογικών παραγόντων, παρέχοντας νέες θεραπευτικές προσεγγίσεις και ερευνητικά εργαλεία, μέσω του ελέγχου σηματοδότησης του p75NTR.

Τα αποτελέσματα των μελετών μας προσφέρουν πολύτιμα εργαλεία για τον επιλεκτικό έλεγχο του p75NTR στη ΝΑ, προκειμένου να ελεγχθεί το ενδογενές δυναμικό νευρογένεσης ως ένας νέος μηχανισμός νευρικής επιδιόρθωσης. Επιπλέον, η ενήλικη νευρογένεση είναι ένα νέο επιστημονικό πεδίο με πολλά σημαντικά ευρήματα αλλά κι αντικρουόμενες απόψεις. Η ανακάλυψη του δυναμικού της ενήλικης νευρογένεσης στον εγκέφαλο ανοίγει νέους δρόμους και θεραπευτικές ευκαιρίες για τη αντιμετώπιση νευρολογικών ασθενειών, ιδίως μέσω της εκμετάλλευσης της ενδογενούς αναγεννητικής ικανότητας. Η λεπτομερής χαρτογράφηση των ιδιοτήτων και των λειτουργιών σε επίπεδο ενός κυττάρου, με βάση τη χωρικο-χρονική έκφραση πολυλειτουργικών ρυθμιστικών υποδοχέων, όπως ο p75NTR, θα μπορούσε να αποτελέσει σημαντικό πλεονέκτημα για τον έλεγχο της νευρογένεσης και των σχετικών γνωστικών λειτουργιών.

1. INTRODUCTION

The brain constitutes the foundational apparatus governing cognition, perception, memory and behavior within the human organism. It serves as the facilitator for executing all actions, engagements and emotional experiences. Its profundity exceeds the boundaries of our current comprehension, as scientists persistently strive to unearth its latent capacities. Evidently, a comprehensive grasp of its potential remains elusive, thereby underscoring the imperative nature of comprehending its intricacies.

The nerve cell is the basic unit of the brain. Our understanding of the pathophysiology of neurodegenerative diseases, aging and neuro-genesis/-regeneration is influenced by studies into the architectural structure and interconnections of neural cells as well as their proliferation, differentiation and maturation. The pursuit of understanding this enigmatic and elaborate organ engrossed neuroscientists, seeking to elucidate the intricate evolution, organization, growth, and regenerative abilities of billions of nerve cells that coalesce into operational systems throughout an individual's lifespan.

The propulsion behind scientific exploration in neurosciences resides in two principal domains: the comprehension of human behavior and the discovery of new therapies for diverse neurological disorders. It is within this context that contemporary neuroscientific inquiry has been predominantly fixated upon deciphering the contributions of endogenous components, such as neurotrophins and their corresponding receptors.

Neurotrophic factors are secreted proteins that have a broader nutritional effect on neurons. Rita Levi-Montalcini's efforts led to the discovery of Nerve Growth Factor (NGF), the prototype neurotrophic factor (Levi-Montalcini & Hamburger, 1953). Then, between the years of 1980 and 1990, the research of Yves-Alain Barde and Hans Thoenen opened the door to the identification of other members of this family, including brain-derived neurotrophic factor (BDNF) (Barde et al., 1982). Other members of the neurotrophin family were found concurrently with these ones. Over the past 15 years, our understanding of the biology of neurotrophic factors has grown, and it is now evident that members of the neurotrophin family play a crucial role not only in the formation of the nervous system, but also in the maintenance of brain health. Notably, the recent decade has witnessed remarkable strides in apprehending the involvement of neurotrophin receptors across a spectrum of neurodegenerative afflictions. The ongoing scrutiny of neurotrophins continues to augur promising therapeutic prospects for the amelioration of these diseases.

In this study, we aim to investigate the effects of the pan-neurotrophin receptor, namely p75NTR, in adult neurogenesis of the dentate gyrus of rodent hippocampus, under physiological and neurodegenerative conditions, such as the case of Alzheimer’s Disease. Furthermore, we also examine the activation of the receptor, by endogenous and synthetic ligands that give rise to cell-specific functions of the receptor and offer the opportunity to take over control of its effects.

The unique cytoarchitecture of the hippocampal formation and its fundamental role in the mechanisms of memory and learning have attracted the interest of scientists, making it one of the most studied structures in the modern era of neuroscience. In the 1960s, the publication of the work of J. Altman's paper 'Autoradiographic and histological evidence of postnatal neurogenesis in rats' (Altman & Das, 1965), identified for the first time newly formed neurons in the dentate gyrus of the hippocampal formation of adult rodents, shook the then prevailing aphorism of Ramon y Cajal: "In the adult brain the neural pathways are fixed and unchangeable: all may die, but nothing can be regenerated" (Cajal & May, 1991). But it took another three decades for the neuroscientific community to accept the brain's ability to produce nerve cells throughout adult life. Nowadays, there is extensive research on a big variety of factors controlling the adult neurogenesis and on how we can manipulate their properties to promote the proliferation of neuronal cells. The p75 neurotrophic receptor is one of these factors, playing a significant role in adult neurogenesis, although its role is still controversial and under investigation. In addition, recent revolutionary studies are highlighting the importance of this receptor on the adult hippocampal neurogenesis as a novel component of the armamentarium against AD pathology.

Other category of important endogenous molecules that mediate neurological effects are the steroid hormones, which are synthesized in the nervous tissue and thus they are named neurosteroids, like dehydroepiandrosterone (DHEA). These molecules have significant neuroprotective properties, as demonstrated both *in vitro* and *in vivo*. The identification of steroidal compounds that mimic the functions of neurotrophin receptors by activating specific neurotrophin receptors, while they avoid non-preferable endocrine effects, has been resulted from the pioneering research into the mechanism of action of DHEA on neurotrophin receptors. For the treatment of many neurodegenerative illnesses, such small sized, lipophilic compounds have not only already been developed but are currently under preclinical tests.

PART 1

1.1.1. Neurotrophins and their receptors

Neurotrophins

Neurotrophins play a major role in inducing neuronal survival and growth, as well as regulating a wide range of functions of neuronal cells. They belong to the category of growth factors, which are secreted proteins with signaling properties, and they have the ultimate aim of promoting the development, differentiation and survival of

neuronal cells. They are released by the target tissue and protect the presynaptic neuron from entering to programmed cell death, allowing the neurons to survive. They can also promote the differentiation of progenitor cells into neurons (Allen & Dawbarn, 2006a; E. J. Huang & Reichardt, 2001). Their functions also include neuroplasticity (Long Term Potentiation), as well as a contribution to pain homeostasis (Eriksson et al., 1998; McAllister et al., 1999; Pezet & McMahon, 2006).

NGF is particularly critical for the survival and maintenance of sympathetic and sensory neurons in developing and mature nervous system. It binds to and activates the high-affinity receptor TrkA (Lewin & Carter, 2014) though induction of receptor's autophosphorylation and subsequent signalling cascades. Alternatively, the NGF/TrkA complex can also be transported backward into the neuron body (Harrington & Ginty, 2013). More generally, the TrkA receptor is expressed in both neuronal and non-neuronal cells. The hippocampus and cortex are targets of cholinergic innervation and express the highest levels of NGF expression in the CNS. In non-neuronal cells, the highest levels of expression are found in salivary glands and the heart (Lewin & Carter, 2014).

Next experiments in the field led to the discovery and molecular cloning of BDNF as a trophic factor (Barde et al., 1982). BDNF is a neurotrophic factor found in both the brain and the periphery. It is a protein that acts through the high-affinity TrkB receptor on certain neurons in the central and peripheral nervous system and promotes neuronal survival, as well as neuronal growth and differentiation. It is active in the hippocampus, cortex, cerebellum and forebrain, regions vital for learning and memory (Hofer et al., 1990). It also plays a major role in the process of neurogenesis and has been attributed great importance in terms of its role in neuroplasticity (Lewin & Carter, 2014).

Sequence analysis of genes expressing NGF and BDNF showed that these two proteins are structurally related (Leibrock et al., 1989). Conserved regions of the NGF and BDNF transcripts allow the design of primers for a polymerase chain reaction that will detect sequences derived from both genes. The use of these primers, however, has revealed additional members of the same gene family, such as neurotrophin-3 (NT3) and neurotrophin-4/5 (NT4/5). NT-3 is unique among the neurotrophins in the number of neurons it is able to stimulate and this is due to its ability to activate two of the neurotrophin receptors (TrkC and TrkB) (Berkemeier et al., 1991; Davies et al., 1993; Jones & Reichardt, 1990; Maisonpierre et al., 1990).

Mature neurotrophins exist as linked dimers, regarding their structural state in their active form, in a non-covalent manner between monomers of molecular weight ~13,500 Da (Bothwell & Shooter, 1977). The mature BDNF and NT-3 factors consist of 119 amino acids with NGF being 1 amino acid less. The neurotrophins display approximately 55% common sequence comprising six conserved cysteine residues

which, in NGF (Radziejewski et al., 1992), form three disulfide bridges. High-resolution structures have been determined for each of the existing neurotrophins. The highly conserved interaction of neurotrophin dimers allows the formation of heterodimers, between different neurotrophins *in vitro* (Jungbluth et al., 1994).

Neurotrophins interact with four receptors, TrkA, TrkB, TrkC and p75NTR. The function of these receptors is complex, as both p75NTR (p75 NeuroTrophin Receptor) and Trk (Tropomyosin Receptor Kinase) receptors can function independently, in neurons expressing both, but also by interacting with each other in ways that can alter the signaling characteristics of each one of them. All four neurotrophins, both as immature forms called pro-neurotrophins and as mature forms, can bind and activate signaling pathways via p75NTR, as well as Trk receptors (Figure 1) (Chao, 2003).

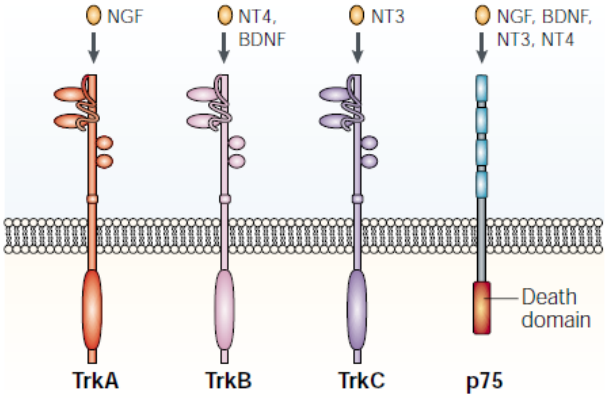

Figure 1: Models of Trks and p75NTR receptor activation by their ligands. Each neurotrophin, binds selectively to a specific receptor, while all bind to the p75NTR (Chao, 2003).

Through the simultaneous activation of Trk receptors and p75NTR, neurotrophins can coordinate the functions of the two receptors according to their spatial and temporal expression. Increased amounts of neurotrophins promote differentiation during neuronal development. When p75NTR expression is no longer required or can result in excessive cell death, Trk receptors play an anti-apoptotic role and stabilize the neuronal population (Blöchl & Blöchl, 2007). p75NTR regulates the size of neurotrophin-dependent cell populations. Therefore, the ratio of the receptors affects how many cells survive in the end.

Trk receptors

Neurotrophins bind with high affinity to their Trk receptors and induce their dimerization leading to receptor's autophosphorylation and activation of intracellular signaling. Aminoacid residues with autophosphorylation potential in the kinase domain play an important role. Additional tyrosines are then autophosphorylated in other parts of the cytoplasmic domain of most receptors. The resulting phosphor-tyrosines function as specific sites for binding signaling molecules recruited to the receptor. These cytoplasmic signaling molecules contain Src homology-2 (SH2) and phosphotyrosine binding sites (PTB) (Lemmon & Schlessinger, 2010).

Trk receptors influence neuronal development and differentiation through the activation of different signaling pathways. The three major pathways are PLCγ (phospholipase γ), Ras/MAPK (mitogen-activated protein kinase) and PI3K (phosphatidylinositol 3-kinase) (Figure 2). These signaling cascades eventually lead to the activation of transcription factors, such as CREB (cAMP response element-binding protein), which in turn activate expressed genes to induce either cell survival or death (Chao, 2003; Lemmon & Schlessinger, 2010).

The protein encoded by the TrkA gene, a transmembrane tyrosine kinase, functions as an NGF receptor. Similarly, the product of the TrkB gene is a functional receptor for the BDNF and NT-4 neurotrophic factors (Klein et al., 1991; Squinto et al., 1991). In addition, NT-3 shows affinity for the receptor encoded by the trkC gene (Lamballe et al. 1991) - (Figure 1). NT3, due to the alternative splicing that the TrkA transcript undergoes, can also efficiently activate the TrkA receptor (Clary & Reichardt, 1994). Nevertheless, NGF and NT3 affect in a different way the TrkA signaling.

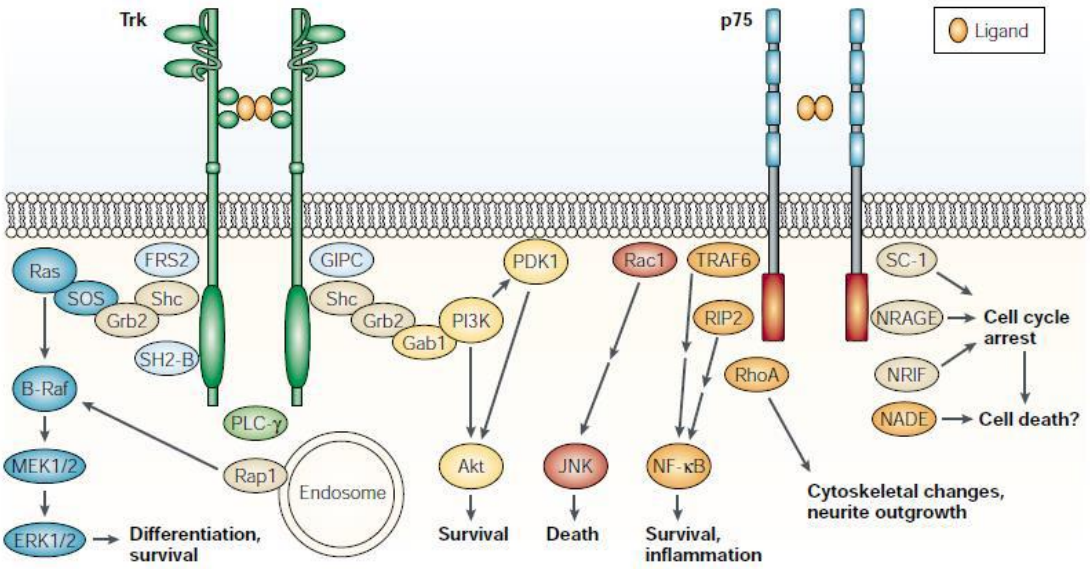

Figure 2: Neurotrophin receptor signalling. Trks induce survival-driven signalling via extracellular Ras kinase, via 3phosphatidylinositol kinase (PI3K) and phospholipase C -γ (PLC-γ). The p75NTR receptor activates NF-κB and Jun N-terminal kinase (JNK) and regulates RhoA activity. These responses are mediated through proteins that bind to the cytoplasmic domain of p75NTR (Chao, 2003).

In terms of their structure, Trks consist of an extracellular domain, which binds the ligand, a transmembrane domain and an intracellular catalytic domain, which is capable of binding and phosphorylating selected substrates (Figure 3). Binding of a ligand to the extracellular domain induces a series of structural rearrangements in the receptor leading to its enzymatic activation. After activation, the receptors undergo the process of endocytosis and the receptor-binding complex undergoes axonal transportation, and eventually either recycling or degradation (Ginty, 2002).

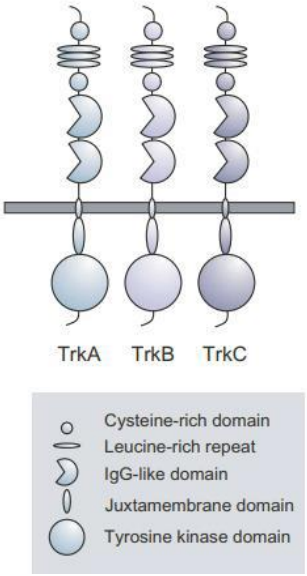

Figure 3: Schematic representation of Trk receptors. Each extracellular (glycosylated) domain comprises three leucine-rich repeats (LRR-Leucine Rich Repeat), flanked by two cysteine repeats and then by two immunoglobulin-like domains. Intracellularly, Trk receptors have the tyrosine kinase domain. Residues Y490 and Y785, serve as major binding sites for initiating signalling (Dechant & Barde, 2002).

p75 neurotrophin receptor

Unlike Trk receptors, which are enzymatic recetors and have a defined neurotrophic-protective role, p75NTR lacks any intracellular enzymatic activity and has a multimodal role, ranging from cell survival to programmed cell death, having the ability to bind all neurotrophins (Figure 1). In addition, as a high-affinity receptor is heterodimerizing with the protein Sortilin, and it selectively binds the immature forms of neurotrophins, pro-neurotrophins. It is a member of the Tumor Necrosis Factor Receptors (TNFR) super family and it was the first member of this family to be characterized (Johnson et al., 1986; Radeke et al., 1987). Its wide expression in many cells types among the neural tissue, including adult neural stem cells (NSCs), as well as its remarkable up-regulation during neurodegeneration or injury and its controversial signaling in different cell types, have increased the scientific interest on this receptor as a potential tool for prevention of cell death and/or induction of neurorepair. Its structure differs from that of Trk receptors, as it lacks enzymatic activity and mediates its action through interaction of its death domain with intracellular proteins (Figure 4). The binding of neurotrophins to the receptor is mediated through the four cysteine-rich regions in its extracellular domain (Baldwin & Shooter, 1995). Generally, it is a transmembrane protein, 75kDa in size, where it undergoes glycosylation in its extracellular domain (Johnson et al., 1986). The disulfide in the transmembrane region acts as a pivot point, so that when the extracellular domain clamps onto the neurotrophin, the intracellular domains separate (Vilar et al., 2009). Crystal structure studies, have shown that neurotrophins can interact with dimers of the p75 neurotrophin receptor and p75NTR forms disulphide-linked dimers independently of neurotrophin binding through the highly conserved Cys257 in its transmembrane domain (Vilar et al., 2009). Receptor cleavage facilitates the subsequent binding of

signaling molecules essential for cell death induced by p75NTR. The intracellular domain (ICD - "death domain") of p75NTR contains a region similar to that of TNFR (Chapman & Kuntz, 1995; Ibáñez & Simi, 2012) (Figure 4).

The most well-known function of the receptor is the ability to induce programmed cell death. One of the first indications of this, was a study by Bredesen's group showing that the expression of p75NTR in a series of neuronal cells, increased apoptosis following serum deprivation (Rabizadeh et al., 1993). The ability of p75NTR to induce programmed cell death in response to ligand binding is observed in a wide variety of neural and non-neural cell types. These studies have established this receptor as a critical regulator of developmental apoptosis.

Thus, p75NTR has been implicated in the development, propagation and execution of neuronal and glial death during development or under neurodegenerative conditions. However, in recent years, a Janus face is ascribed to this receptor: not only induces cell death, but, in a cell-specific manner, it can also rescue neuronal cells from apoptosis or even induce the proliferation of their precursors. The aforementioned properties of the p75NTR clearly indicate its importance as a novel regulator of many cellular effects on the nervous system, but also the complexity of its signaling pathways. Several studies have been initiated to describe p75NTR-dependent actions on neural stem cells and adult neurogenesis, but contradictory results and lack of explicit methodology rather confuse the field than define the role of the receptor.

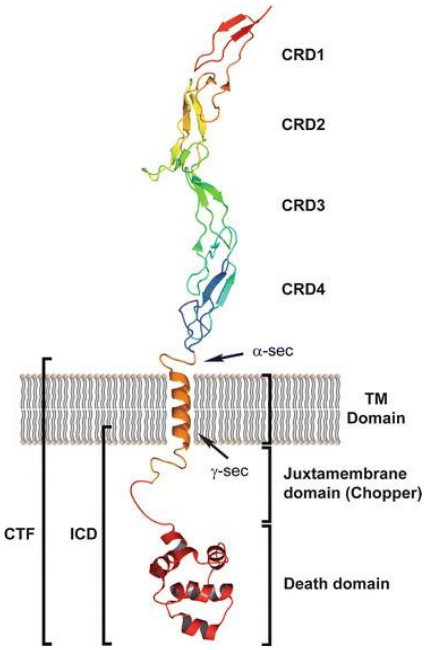

Figure 4: Structure of p75 receptor.

The developmental role of p75NTR has been particularly characterized in sympathetic neurons. These neurons express both NGF receptors, TrkA and p75NTR, which

together mediate a survival signal in response to NGF. However, Miller and colleagues showed that selective activation of p75NTR by BDNF leads to apoptosis (Bamji et al., 1998). More generally, it is expressed by many neuronal cell types, as well as by neural stem cells, astrocytes, oligodendrocyte precursors, Schwann cells and glial cells. It is widely expressed in the developing nervous system, but also selectively expressed during adulthood in the normal adult brain. However, some regions maintain p75NTR expression at lower levels including sensory neurons spinal cord motor neurons and specifically basal forebrain cholinergic neurons where it is observed a co-localization of choline acetyltransferase (ChAT) and p75NTR immunoreactivity (Allen & Dawbarn, 2006b; Friedman, 2000; Verge et al., 1992). Several non-neural tissues also express the receptor during some stages of development, such as kidneys and muscles (Ernfors et al., 1990; Wheeler & Bothwell, 1992). It has therefore been shown to regulate a variety of cellular responses, including cell survival, cell cycle, synaptic function and myelination, and plasticity. The multiplicity of these cellular functions arises from the variety of ligands that bind to it and the co-receptors that interact with p75NTR and regulate its signaling.

p75NTR and induction of specific signaling pathways

The conformational rearrangements of the transmembrane domain through the C257 residue are also the key factor for dimerization of the receptor itself and induction of specific signaling pathways, such as the RhoGDI/RhoA, cJun kinase, TRAF-6/RIP2, NFkB and apoptotic pathways (Vilar et al., 2009), while the death domain is primarily mediating the aforementioned signaling properties through the recruitment or release of specific intracellular interactors in its surface (Charalampopoulos et al., 2012).

p75NTR promotes survival through interactions with Trk receptors by regulating the ability of the ligand to bind to Trk receptors (Patapoutian & Reichardt, 2001), inhibits axonal regeneration through Nogo (Nogo-R) and Lingo-1 receptors, and promotes apoptosis through binding to Sortilin (Chao, 2003; DeFreitas et al., 2001). Its pro-apoptotic actions are largely dependent on the ligands that bind to the receptor and the expression of other receptors with which it interacts. Further signaling, involves interactions modulated through p75NTR's endomembrane proteolysis (RIP) (Lewin & Carter, 2014) (Figure 2).

More specifically, activation of the transcription factor NFkappaB, through the interaction of the RIP2 protein and p75NTR, can lead to neuronal survival (Vicario et al., 2015), while causing the release of RhoGDI from the receptor and subsequent inactivation of RhoA (Charalampopoulos et al., 2012). The binding of Rho-GDI to p75NTR can be facilitated by a complex consisting of myelin-associated glycoprotein (MAG) and by the simultaneous dimerization of NgR/Lingo1R receptors, which serve as co-receptors of p75NTR (Yamashita & Tohyama, 2003). On the other hand, p75NTR-

induced phosphorylation of cJun/JNK induces cell death. One group of factors that contribute to JNK activation, through interactions with the ICD of the p75NTR receptor, is the family of TNF receptor-related factors (TRAFs) (Khursigara et al., 1999). Cell death induced by p75NTR is also associated with its other co-receptors, such as sortilin, SorCS2 and SorL1 (Nykjaer et al., 2004; Nykjaer & Willnow, 2012), which are selectively activated through pro-neurotrophin binding. Pro-neurotrophins can be secreted to act as potent activators of p75NTR signaling (Lebrun-Julien et al., 2010). In addition, other ligands can also bind to p75NTR receptors, such as Aβ-amyloid (Yaar et al., 2002), thus determining subsequent signaling.

1.1.2. Adult Neurogenesis

For a very long time, neurogenesis was thought to occur during development and the early postnatal periods. Numerous investigations, however, have shown that neurogenesis continues in two specific locations in the adult mammalian brain: the subventricular zone (SVZ) near the lateral ventricles and the subgranular zone at the dentate gyrus of the hippocampus (SGZ). According to research by Zhao et al. (2006) and Kempermann (2016), stem cells are created in these neurogenic niches, multiply, and develop in both neurogenic niches before eventually giving rise to neuronal and non-neuronal cells that will integrate into the pre-existing networks to the olfactory bulb and the hippocampal formation (Ming & Song, 2011).

Transient amplifying cells are produced by proliferating quiescent radial glia-like cells in the adult rodent brain's SVZ, and these cells in turn produce neuroblasts. Through

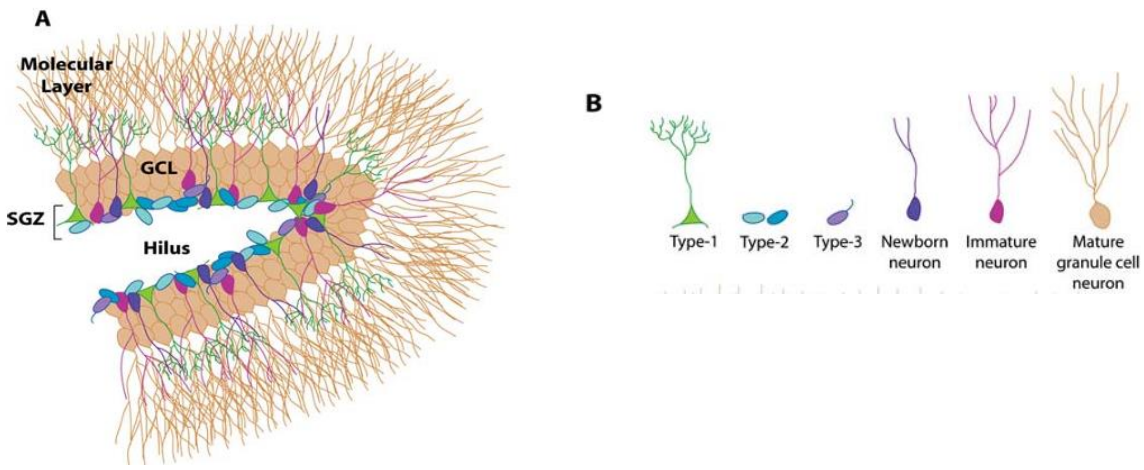

Figure 5: A) subgranular zone at the dentate gyrus of the hippocampus (SGZ), B) proliferation of radial glia-like cells and nonradial precursors (Eisch et al., 2008).

the rostral migratory stream (RMS), which is created by astrocytes, these cells are moving in the direction of the olfactory bulb (OB) (Lois et al., 1996; Ming & Song, 2011). Immature neurons begin to specialize into particular interneurons once they reach the olfactory bulb, including GABAergic granule neurons and periglomerular cells (Lledo et al., 2006). Only about 40% of the freshly formed cells will survive after reaching the OB, according to (Petreanu & Alvarez-Buylla, 2002). In the SGZ (figure 5A), radial glia-like cells and nonradial precursors proliferate to produce intermediate progenitors with glial (type 2a) and neuronal (type 2b) phenotypes, which in turn produce neuroblasts (type 3) (Figure 5B). The excitatory dentate granule cells of the dentate gyrus are formed when these growing neurons migrate to the layer of granule cells. Then, as they pass through the hilus and enter the CA3 region of the hippocampus, the newborn neurons that will survive start to lengthen their axonal and dendritic processes (Kempermann, 2016; Zhao, 2006). Hippocampal neurogenesis is very important since the hippocampus plays critical roles in processes including learning, memory and behavior(Kempermann, 2016; Yau et al., 2015).

Markers being expressed by NSCs and neurons in adult Neurogenesis

At each stage of their development, the newly generated cells express specific neuronal markers and have distinct morphology, allowing for a complete investigation of their differentiation and maturation process (Duan et al., 2008; Ehninger & Kempermann, 2008; Kempermann et al., 2004; von Bohlen und Halbach, 2011). Immunohistochemical identification (Figure 6) of adult brain NSCs demonstrates that these cells retain their capacity for self-renewal and pluripotency (Gage, 2000; Shimozaki, 2014) and continue to express the transcription factor Sox2 (sex determining region Y-box 2) (Thiel, 2013; von Bohlen und Halbach, 2011), but also express the cytoplasmic markers glial fibrillary acidic protein (GFAP) - major component of the interstitial fibrils of the cytoskeleton of mature CNS astrocytes (Eng et al., 2000) and brain lipid binding protein (BLBP) - a cytoplasmic protein that has an active role in the capture, transport and metabolism of fatty acids, being a characteristic marker of ciliated neuroglia cells (Duan et al., 2008; Kempermann et al., 2004; Schmid et al., 2006; Seri et al., 2004).

Neural precursor cells (NSCs) are derived from the asymmetric, usually mitotic division of NSCs and are also localized in the SGZ (Bonaguidi et al., 2011). The main feature of most NSCs of this stage is the expression of the protein nestin, which in the developing brain is expressed by astrocytes and ciliated neuroglia cells (Ajtai & Kálmán, 2001). Interestingly, within the SGZ, the expression of nestin is not restricted to NSCs, but is also observed in endothelial cells (Palmer et al., 2000) and in some astrocytes (Kronenberg et al., 2006). The remaining population of NSCs appears more differentiated, showing a gradual decrease in the expression of nestin and a parallel increase in the expression of markers of neuronal directionality, such as the calcium-

binding protein doublecortin (DCX) (Duan et al., 2008; Kempermann et al., 2004; Ribak et al., 2004) which is detected in the growth cones of neuraxons and dendrites of newly generated neural cells.

DCX$^+$ cells exiting the mitotic cycle begin their differentiation into new immature granular cells. The immature newly generated granular cells continue to express the antigenic markers DCX, PSA-NCAM and NeuroD, the expression of which gradually disappears, while the expression of post-mitotic neuronal markers begins. The most important of these is neuronal nuclei protein (NeuN), which is considered to be the most reliable marker of CNS neurons (Mullen et al., 1992). It is a soluble protein localized in both the nucleus and cytoplasm of post-mitotic neurons (Lind et al., 2005).

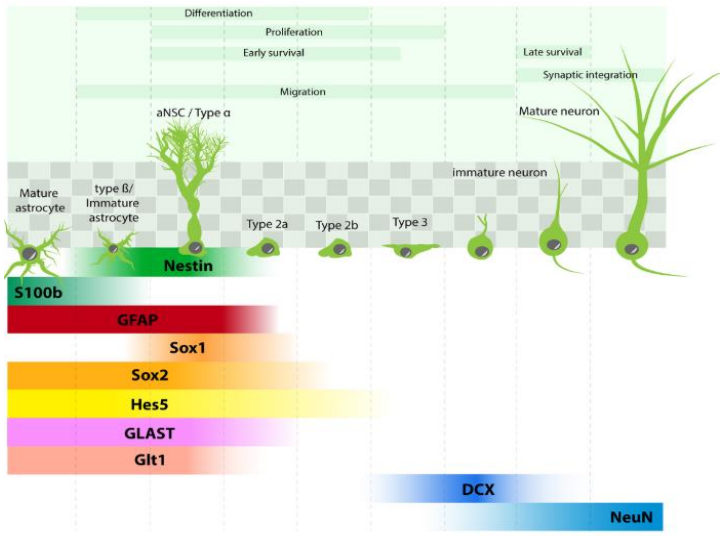

Figure 6: Immunohistochemical identification of adult brain NSCs (Cassé et al., 2018).

Human adult neurogenesis

Human adult neurogenesis is still a subject of debate since different studies report variable numbers of new neurons (Sorrells et al., 2018). Two examples of species-specific differences between humans and rats include the absence of olfactory bulb neurogenesis and the existence of striatal neurogenesis in humans (Bergmann et al., 2012, 2015; C. Wang et al., 2011). In the initial study, Eriksson et al. showed that BrdU insertion into proliferating progenitor cells allowed them to show that adult brains of cancer patients continue to generate newborn neurons (Eriksson et al., 1998). Despite those exciting preliminary findings, the use of BrdU on living human cells has significant limitations because of its nature as a mutagen and a possible carcinogen (Taupin, 2007).

Later on, it was shown that neurogenesis persists throughout adult life (about 700 new neurons per day) and that these newborn neurons have specific features in relation to brain functions, taking advantage of the elevated atmospheric 14C levels caused by above-ground nuclear bomb testing in the years 1955–1963 and by

analyzing its concentration in neuronal genomic DNA (Spalding et al., 2013), conflicting works have been published. Boldrini et al.'s analysis of data from 28 participants, ages 14 to 79, revealed that neurogenesis may continue up to the eighth decade of life (Boldrini et al., 2018). However, Sorrells and colleagues support the notion that adult neurogenesis does not take place in humans by showing a decline in the number of proliferating progenitors and immature neurons with age using brain samples from newborns and people up to 77 years old (Sorrells et al., 2018). Furthermore, the most recent research by Franjic et al. emphasizes the likelihood that neurogenesis does not take place in humans (Franjic et al., 2022). They evaluated single-nucleus transcriptomes in five hippocampal-entorhinal subregions in pigs, macaques, and humans using Dcx as a marker for all species. As a result, scientists discovered that humans do not possess immature neurons, denying adult neurogenesis (Franjic et al., 2022).

Even if the inconsistent results may be explained by technological limitations or changes in the age of the patients, this controversy highlights the necessity for more extensive research in the area.

p75NTR and its role in the Adult hippocampal Neurogenesis

Neurotrophins and neurotrophin receptors are expressed in various stem cell types and play a pivotal regulatory role with respect to stem cell differentiation, survival and migration (Pramanik et al., 2017). Neurotrophic factors control adult postnatal neurogenesis in the main neurogenic regions of the brain, the Sub-Granular Zone (SGZ) of hippocampus and the SVZ of the lateral ventricles (Barnabé-Heider & Miller, 2003; Vilar & Mira, 2016).

The aforementioned properties of the p75NTR clearly indicate its importance as a novel regulator of many cellular effects on the nervous system, but also the complexity of its signaling pathways. As a pan-neurotrophin receptor p75NTR is gaining interest as a "fate decision protein" in stem cells, modulating their potency and differentiation (Tomellini et al., 2014). Several studies have been initiated with the aim to describe p75NTR-dependent actions on neural stem cells and adult neurogenesis (Tomellini et al., 2014), but contradictory results and lack of explicit methodology rather confuse the field than define the role of the receptor. In addition, adult neurogenesis is a new scientific field with many anticipating findings and strong arguments for its physiological role, as already discussed. Those contradictory results can be attributed to different genetic backgrounds of p75NTR- deficient animals and species-specific differences (Bernabeu & Longo, 2010; Boskovic et al., 2014; Catts et al., 2008; Dokter et al., 2015; Poser et al., 2015).

Three common mouse models for studying p75NTR deficiencies that express various isoforms, or shortened variants of the p75NTR protein, have been examined. The

p75NTRExIII model, which lacks the full-length receptor but still expresses the short p75NTR isoform, the p75NTRExIV-knockout mice that lack both long and short isoforms and have a fragment with molecular weight of 26kDa that is being overexpressed and last but not least, a p75NTR deficient mouse model that has been created by Boskovic and coworkers. In this model, there is a conditional deletion of p75NTR in ChAT neurons, after taking advantage of p75NTRlox/lox animals (Boskovic et al., 2014).

It has been established that p75NTR is expressed by precursor cells in the SVZ and that when 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). The rat hippocampus, and more specifically the DG hilus and the granule cell layer, have been found to have low expression of p75NTR aside from that (Barrett et al., 2016). The first model (p75NTRExIII) is one of the most well studied and exhibits decreased hippocampal neurogenesis (Bernabeu & Longo, 2010; Catts et al., 2008; Colditz et al., 2010) or no change compared to wild type animals (Dokter et al., 2015) as demonstrated by the BrdU proliferation assay. These decreased levels of hippocampal neurogenesis have been attributed to a decrease in the proliferation and maturation of neuroblasts in the adult DG, after observing the immunostainings of specific markers such as NeuN and PSA-NCAM (Bernabeu & Longo, 2010) or to an increased neuroblasts cell death, as shown with TUNEL assay by Catts et al., 2008. More specifically, in this mouse model it seems that there is a promoted cell death, rather than survival and differentiation on the neuroblasts that are produced (Catts et al., 2008). The decreased number of developing neurons may potentially be related to the neuroblasts' impaired commitment to their neuronal fate. According to Catts et al., the p75 receptor is thought to be essential for the survival and/or differentiation of cells going through neural differentiation. As a result, p75NTR can control hippocampus neurogenesis, and its deletion can also prevent cells from differentiating or maturing as they should. Golditz et al., also showed that p75NTR serves as a regulator of basal neurogenesis and is required for fluoxetine, an antidepressant medicine, to cause an increase in hippocampal neurogenesis (Colditz et al., 2010).

Thus, p75NTRExIII-knockout mice, do not express a p75NTR protein capable of ligand binding but the truncated proteins mimic naturally occurring proteolytic fragments of p75NTR and are capable of proapoptotic signaling (Murray, 2004; Paul, 2004). That would be also a reason for the interpretation of results obtained with these p75NTR deficient models.

Generally, the location of p75NTR is much lower in cerebral cortical cells and hippocampal CA1 and CA3 cells than it is in adult wild-type and transgenic AD mice, according to research published the same year (Chakravarthy et al., 2012). Bernabeu & Longo have also shown that there is expression of p75NTR in the hippocampal NSCs

and that the p75 receptor is also important for the cells that express glial markers (Bernabeu & Longo, 2010).

On the other hand, in p75NTRExIV-knockout mice, there is an increase in the number of DCX positive cells (newborn neurons) and a decreased cell death (Poser et al., 2015) without any changes in cell proliferation. These results indicate a cell-autonomous function of p75NTR in newborn cells. Last but not least, Boskovic et al. paved the pathway for taking advantage of p75NTR lox/lox mice with a conditional deletion of the receptor only within areas of interest (Boskovic et al., 2014). As previously established, p75NTR regulates axonal outgrowth, migration, and neuronal differentiation while being broadly expressed throughout the nervous system during development. In addition, time and cellular environment play a role in how p75NTR regulates the survival and demise of growing neurons. Zuccaro's team has shown that p75NTR polarized expression is essential for the initiation of axon in newly generated neurons of the adult SGZ and p75NTR is transiently expressed by newborn neurons by using injections of lentiviruses driving shRNA against p75NTR (Zuccaro et al., 2014). Furthermore, a recent study has shown that conditional deletion of the p75NTR gene in neural progenitors causes anatomical abnormalities of the brain in Nestin-Cre p75 floxed mice. These abnormalities include a significant reduction in brain volume, with the neocortex and basal forebrain experiencing the most shrinkage. The loss of adult and developing cortical projection neurons and interneurons is consistent with increased levels of embryonic progenitor mortality and lower rates of neurogenesis. These results suggest that p75NTR affects growth, differentiation, and survival of these neuronal progenitors (Meier et al., 2019).

In conclusion, due to the diverse genetic backgrounds of p75NTR-deficient animals and species-specific variations, animal studies produce inconsistent findings regarding the role of p75NTR in adult neurogenesis. An unmet need under neurodegenerative conditions such as Alzheimer's Disease, would be addressed by targeting the pan-neurotrophin p75NTR receptor through research into the cellular and molecular underpinnings of its activation in adult neurogenesis.

1.1.3. Alzheimer's Disease

A significant risk factor for neurodegenerative diseases is aging. Age-related changes in the brain include increased neuronal loss and decreased neurogenesis (Schultz & Sinclair, 2016). Changes in adult neurogenesis seem to be a common hallmark in many neurodegenerative diseases including Alzheimer's Disease (Moreno-Jiménez et al., 2019). The most frequent cause of dementia in older people is Alzheimer's disease. According to Armstrong (2019), AD is seen as a multifaceted disease with a complicated pathophysiology that is brought on by a number of risk factors, including

genetic background and environmental factors (A. Armstrong, 2019). Aging is solely responsible for the progression of AD (Hou et al., 2019).

Alzheimer's Disease leads to neuronal death and synaptic loss in certain areas of the brain, affecting especially the hippocampal formation and at a later phase cognitive function as well. AD is characterized by accumulation of protein aggregates of αβ-amyloid and tau (Pramanik et al., 2017) and 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 and is created due to a complex combination of our genes, our environment and our lifestyle (Paroni et al., 2019).

Aβ-amyloid is biosynthesized from the transmembrane amyloid precursor protein (APP) - by proteolytic cleavage enzymes such as β-secretase and γ-secretase (Armstrong, 2009; Sadigh-Eteghad et al., 2014). The accumulation of Aβ oligomers especially Aβ40 and Aβ42, can create the amyloid plaques which are extracellular deposits found in the brains of people with AD. Eventually, this accumulation has been implicated to neurotoxicity ("the amyloid hypothesis"). The four major hypotheses for the cause of AD are the amyloid and cholinergic hypothesis, the tau phosphorylation and the mitochondrial dysfunction as well. The cholinergic neurons seem to degenerate in AD and the β-amyloid blocks choline uptake and acetylcholine release, thus leading to the cognitive deficits of AD patients (Breijyeh & Karaman, 2020). On the other hand, the hyperphosphorylated insoluble aggregates of tau proteins, which are called neurofibrillary tangles, can also lead to cell death (Armstrong, 2009; Mohandas et al., 2009). In the mitochondrial hypothesis, dysregulated mitochondrial activities result in impaired ATP synthesis, poor glucose metabolism, and elevated ROS generation, ultimately leading to synaptic dysfunction (Figure 7) (Abuelezz et al., 2021).

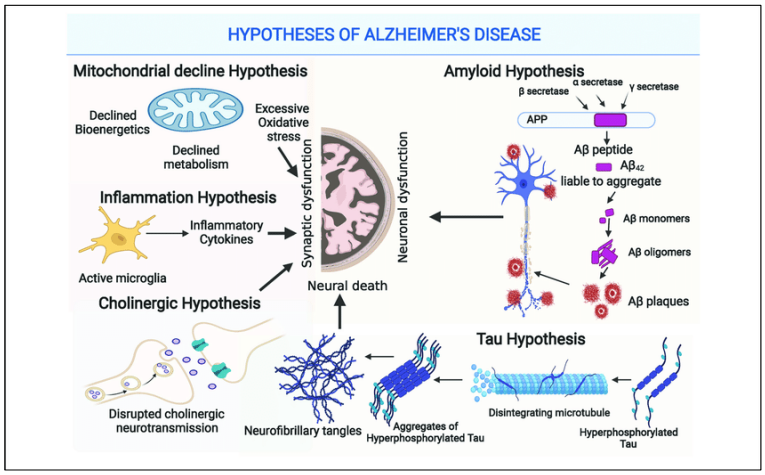

Figure 7: Figure is showing the several theories for the progression of Alzheimer's disease (AD) (Abuelezz et al., 2021)

Adult Neurogenesis in Alzheimer's Disease

Widespread brain atrophy that starts in the entorhinal cortex and progresses to the neocortical regions is a hallmark of AD. In AD patients, the hippocampal region is severely damaged and is linked to reduced adult neurogenesis and memory deficits, in both humans and animal models (Jahn, 2013; Moreno-Jiménez et al., 2019; Mu & Gage, 2011) (Figure 8).

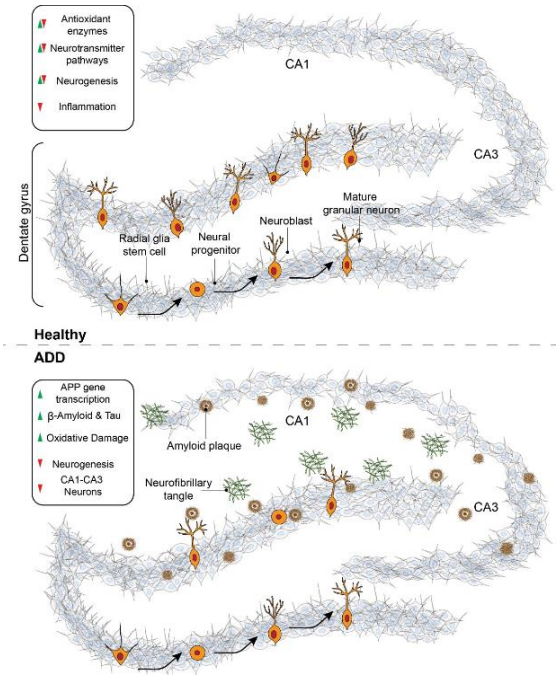

Figure 8: In AD, neurogenesis has been consistently demonstrated to be impaired, but the cause of this impairment is still unknown. The accumulation of αβ-amyloid, the intracellular neurofibrillary tangles, and the death of neurons (particularly in the dentate gyrus of the hippocampus) all play significant roles in AD. Neuronal loss in the dentate gyrus has long been linked to neurofibrillary tangles. It should be noted that αβ accumulation contributes significantly to the disruption of neurogenesis and current research has begun to clarify the impact of APP gene expression on the neurogenesis process.

In fact, it was found that as people age, their brains contain fewer DCX-positive cells (Moreno-Jiménez et al., 2019).The older human brain also had reduced angiogenesis and less neuroplasticity but still present neurogenesis, according to Boldrini and his team's findings (Boldrini et al., 2018). Demars' study suggests that impaired neurogenesis is an early critical event in the course of Alzheimer's disease that may underlie memory impairments, showing a reduced proliferation and neuronal differentiation in AD mice (Demars et al., 2010). Kuhn et al. demonstrated that adult rats experience decreased neuronal progenitor proliferation that is age related (Kuhn et al., 1996). Another study revealed a significant reduction in the amount of $BrdU^+$ cells, which are increased following an APP deletion, in mice overexpressing APP, indicating that there is a limitation on the proliferation of NSC cells (Naumann et al. 2009).

Additionally, it has been proposed that age-related physiological changes exacerbate neurodegenerative diseases like AD. Adult neurogenesis is predicted to decline earlier and more rapidly in AD patients as a result. Despite suggesting that AD exacerbates the decline of neurogenesis more than physiological aging, there are only a few limited studies that clearly showed the neurogenesis within AD. NSCs isolated from AD and

healthy postmortem tissue and stained with Ki-67 (a nuclear protein that is associated with cellular proliferation) and Musashi1 (a stem cell marker) revealed that viability of NSCs is decreased in AD subjects within the hippocampus compared to age matched healthy controls. Moreover, NSCs from AD patients reach senescence sooner than cells obtained from healthy controls (Lovell et al., 2006).

Contrary to the above research and findings, enhanced neurogenesis has been seen in a small number of people who still have an intact cognitive function (Briley et al., 2016). The temporal compensatory mechanism for the missing neurons may be responsible for this increase and also the variability of the brain samples and the challenge of preserving the tissues of postmortem brains are likely to be the causes of several case reports of AD patients who had contradictory results (Farioli-Vecchioli et al., 2022). According to a scientific team, the hippocampus of AD patients contained higher quantities of neurogenic chemicals such DCX, contradicting this idea. According to their theory (Jin et al., 2004), neurogenesis was raised in AD patients as a protective measure against the cell loss brought on by neurodegeneration. A rise in stem cells that were Nestin-positive has also been seen in AD patients (Ziabreva et al., 2006).

Animal models have been crucial for examining a number of aspects of AD development and symptoms, because human tissue research has its limitations, as discussed above. A number of mice models, including 5xFAD, APP/PS1, and 3xTg, each carrying mutations in genes associated to AD, are used to study AD. Immature neurons are primarily indicated by the marker DCX in the SGZ, which is utilized to examine the neurodegeneration derived from AD. BrdU can be used to assess survival or proliferation depending on the time of the animal's sacrifice and the injections. The 5xFAD mouse is a well-established AD animal model that resembles many of the amyloidogenic pathology of AD and the associated cognitive decline, while it has been also described to appear reduced neurogenic ability (Jawhar et al., 2012; Oakley et al., 2006; Zaletel et al., 2018).

Very recently a pioneer study at the field has shown that these mice are responding to neurotrophin BDNF and exercise by increasing their adult neurogenesis and improving their cognitive impairments (Choi et al., 2018). On the other AD mouse models, neurogenesis, and more specifically the survival of newly generated neurons, decreased in adult 5–6 months old 3xTg and APP/PS1 mice (Valero et al., 2014). Finally, adult hippocampal neurogenesis was significantly reduced and there was an abnormal buildup of APP peptide in the brains of old (10–12 months old) mice (Hamilton et al., 2010).

p75NTR and its therapeutic potential in Alzheimer's Disease

The progression of AD is largely influenced by neurotrophins (Durany et al., 2000; Ginsberg et al., 2006; Hock et al., 2000; Michalski & Fahnestock, 2003; Peng et al., 2005). Cholinergic neurons degenerate in the latter stage of AD, and NGF is a key regulator of these cells. NGF protein levels have been found to be higher in parts of the brain damaged by AD, such as the hippocampus and frontal cortex (Hock et al., 2000) but further investigations have found that proNGF is upregulated rather than NGF (Peng et al., 2005) and pro-NGF accumulation in the preclinical stage of AD has been shown to be correlated with cognitive deterioration (Peng et al., 2005). ProNGF has been found to bind to p75NTR without interacting with TrkA and to cause neuronal degeneration (Ioannou & Fahnestock, 2017). According to Al-Shawi et al. (2008), increased proNGF levels in the hippocampus of AD and elderly people are likely a clue that the proNGF/p75NTR axis contributes to neuronal degeneration (Al-Shawi et al., 2008). On the other hand, pro-BDNF and mature BDNF levels are decreased in the human postmortem brain tissues of AD patients in the hippocampus and parietal cortex (Durany et al., 2000; Michalski & Fahnestock, 2003). According to studies, BDNF stimulation may both reduce the Aβ-induced neuronal degeneration (Meng et al., 2013) and cause tau to be dephosphorylated through activation of TrkB and phosphatidylinositol 3-kinase (PI3K) signaling. Additionally, it appears that greater serum levels of BDNF are restoring cognitive function as well (Weinstein et al., 2014).

Augmentation of endogenous neurogenesis represents a promising therapeutic approach to decrease neuronal loss in neurodegenerative disorders. p75NTR seems that it can serve as a target along this line.

At this point, it is important to mention that p75NTR signaling contributes to neurodegeneration caused by many diseases, and the association between p75NTR and Alzheimer's disease (AD) has been particularly studied. In addition to Purkinje neurons in the cerebellum, p75NTR is expressed at high levels in cholinergic neurons of the adult basal forebrain, a population that undergoes severe degeneration early in the progression of AD pathology. According to previous studies it seems that p75NTR is an important player in regulating Aβ deposition in AD patients (Y.-J. Wang et al., 2011b; Yao et al., 2015) as well as mediating tau hyperphosphorylation (Shen et al., 2019a), indicating the receptor's involvement in the pathogenesis of AD (Zeng et al., 2011). Yao and colleagues 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). Another team showed 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).

In addition, several in vitro studies have shown that αβ-amyloid, the major component found in the brains of AD subjects, is a pro-apoptotic binder for p75NTR (Knowles et al., 2009; Saadipour et al., 2013; Yaar et al., 1997). Yaar et al. used NIH-3T3 cell lines expressing p75 NTR and shown that these cells undergo apoptosis in the presence of aggregated-amyloid, compared to wild type cells that do not express p75NTR. Furthermore, normal neural crest–derived melanocytes that endogenously express p75NTR, undergo apoptosis in the presence of aggregated-amyloid, but not in the presence of control peptide synthesized in reverse (Yaar et al., 1997). Some years later, Knowles et al. showed the interaction of p75NTR and αβ amyloid in neuron cultures and in chronic AD mouse model. Fluorescence resonance energy transfer analysis revealed that Aβ oligomers interact with the extracellular domain of p75NTR and in neuron cultures where the ECD of p75NTR is deleted, there is a decreased number of dead cells because of αβ treatments compared to wild type cells. Furthermore, Thy1-hAPPLond/Swe × p75NTR−/− mice exhibited significantly decreased hippocampal neuritic dystrophy and complete reversal of basal forebrain cholinergic neurite degeneration relative to those expressing wild-type p75NTR. Interestingly, the amount of Aβ levels is not changed, showing that p75NTR does not mediate all Aβ effects, but plays a significant role in enabling Aβ-induced neurodegeneration *in vitro* and *in vivo* (Knowles et al., 2009). Saadipour et al. demonstrated that sortilin expression is increased in the AD brain in human and mice and that αβ amyloid oligomer increases sortilin expression through p75NTR and RhoA signaling pathways (Saadipour et al., 2013).

However, these ideas remained controversial due to other reports where p75NTR expression is protective against Ab-induced toxicity (Bengoechea et al., 2009). Sotthibundhu et al. showed that Amyloid-β can bind to p75NTR and up-regulate the proliferation of neural progenitors as well as adult neurogenesis in SVZ precursor cells (Sotthibundhu et al., 2009). 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). 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. The recombinant ECD of p75NTR inhibited fibrillation of Aβ and decreased the Aβ plaques in the hippocampus of these mice (Y.-J. Wang et al., 2011).

Furthermore, there is also an involvement of p75NTR in tau hyperphosphorylation according to Mañucat-Tan et al., showing that in p75NTR knockout, pR5 mice (transgenic mice with the P301L human Tau mutation) the phosphorylation of human Tau was attenuated, also hypothesizing that targeting p75NTR could reduce or prevent the pathologic hyperphosphorylation (Mañucat-Tan et al., 2019). Moreover, it is hypothesized that tau hyperphosphorylation follows aβ accumulation, despite the

fact that there is yet no proof of how aβ might cause tau phosphorylation. To demonstrate that p75NTR mediates aβ-induced tau phosphorylation, Shen et al. used p75NTR knockout mice. they intraventricularly administered Aβ42 into a mouse model of human tauopathy with or without the genetic deletion of p75NTR, demonstrating that this mitigated the neuronal loss brought on by Aβ in the CA1. In addition, they have used the soluble form of the p75NTR extracellular domain (p75NTRECD-Fc), which prevents the binding of Aβ and inhibits tau hyperphosphorylation (Shen et al., 2019).

Another intriguing study by Yi et al. revealed that, in contrast to fully knockout mice, p75NTR knock-in mice lacking specifically the death domain or transmembrane Cys259 had reduced αβ amyloid content in the hippocampus of 5xFAD mice and a greater neuroprotection from AD neuropathology and memory impairment. This was due to an interaction between p75NTR and APP that promoted non-amyloidogenic APP cleavage while reduced APP internalization and colocalization with BACE1, the crucial protease for the production of neurotoxic APP fragments (Yi et al., 2021).

According to Coulson et al. 2009, p75NTR may be relevant to both AD and the observed cholinergic impairment (Coulson et al., 2009). Although its function is yet unknown, p75NTR is expressed by cholinergic basal forebrain (cBF) neurons throughout life (Yeo et al., 1997). Acetylcholine (Ach) is a neurotransmitter that can control adult neurogenesis (Mohapel et al., 2005). Aged and sick brains have lower levels of Ach than healthy brains do (Shohayeb et al., 2018). The administration of a cholinesterase inhibitor can rescue survival of newborn neurons (Winner et al., 2006), due to increased levels of Ach. p75NTR and TrkA expression in cBF neurons, is relatively high compared with other CNS cell populations (Boskovic et al., 2014; Fortress et al., 2011) and NGF, can act like a stimulator for activating choline acetyltransferase (ChAT) in cBF neurons and synthesize Ach (Mobley et al., 1986). Enhanced cholinergic innervation of the hippocampus and an increase in both the number and the size of cBF neurons have been seen in transgenic mice missing p75NTR (ChAT-cre p75in/in) (Barrett et al., 2016; Boskovic et al., 2014). In a chronic, transgenic model of Aβ overexpression, deletion of p75NTR inhibits the degradation of basal forebrain cholinergic projections (Knowles et al., 2009), which might be the cause of further Aβ deposits, cortical degeneration, and enhancement of cognitive deficits. Additionally, inhibiting p75NTR expression might lead to elevated levels of Ach, which would affect inadequate neurogenesis.

Alzheimer's disease (AD) cognitive deficiencies and memory loss are significantly impacted by impaired neurogenesis. Increasing neurogenesis by p75NTR targeting could be a potential treatment for AD.

PART 2

1.2.1. Neurosteroids and Dehydroepiandrosterone (DHEA)

Etienne Baulieu first described the ability of neurons and glia to produce steroids in the early 1980s by metabolizing hormone precursors that are transported there via the bloodstream and also by producing steroids from cholesterol on their own (Baulieu & Robel, 1990). The central and peripheral nervous systems, specifically the neurons, astrocytes, and neuroglial cells, synthesize neurosteroids. It has been demonstrated that neurosteroids impact neuronal activity and differentiation and offer neuroprotection (Compagnone & Mellon, 2000). The activation of receptors such the $GABA_A$ and NMDA receptors as well as sigma-1 (-1) is thought to be the general mechanism by which neurosteroids initiate their activities (Charalampopoulos, Margioris, et al., 2008; Compagnone & Mellon, 2000).

Dehydroepiandrosterone (DHEA) was the first neurosteroid that was characterized and further studied. It serves as a precursor molecule for the synthesis of androgens and estrogens. The cytochrome 17 enzyme (CYP17) synthesizes it from cholesterol, with the majority of its production occurring in the gonads, adrenal glands, and locally in the brain. While adrenal DHEA has a systemic function, DHEA generated in the brain operates locally in a paracrine manner. Its daily synthesis is age-dependent, reaches a peak concentration in early adulthood, and then gradually declines over time, especially in neurodegenerative diseases like AD (Baulieu & Robel, 1998; Schumacher et al., 2003; Weill-Engerer et al., 2002).

Until recently, no particular receptor for DHEA has been identified which could signal to support existing neuroprotective effects of this endogenous molecule. Early research had demonstrated that activation of DHEA's membrane binding sites resulted in transient and sequential phosphorylation of MEK/ERK kinases or PI3K/Akt, which in turn activated CREB and NFkB transcription factors. These factors in turn controlled the transcription of the anti-apoptotic Bcl-2 proteins or the phosphorylation/deactivation of the pro-apoptotic Bad protein, respectively, protecting PC12 cells from apoptosis (Charalampopoulos et al., 2004; Charalampopoulos et al., 2006; Charalampopoulos et al., 2008). Because PC12 neuroendocrine cells lack functional $GABA_A$ or NMDA receptors and are unable to metabolize DHEA into estrogens and androgens, they serve as a useful model for the experimental study of the membrane effects of DHEA on neuronal cells (Greene & Tischler, 1976). Therefore, using this model, it would be feasible to study which receptors specifically DHEA activates to execute its membrane activities in the central and peripheral nervous system. Effective apoptosis prevention is provided by both NGF and DHEA. According to Riccio et al. (1999), their apoptotic action begins at the

plasma membrane and is followed by activation of the same pathways that have an anti-apoptotic effect and mediated by DHEA (Riccio et al., 1999). Due to these similarities in the signal transduction pathways activated by DHEA and NGF, a role for NGF receptors in the anti-apoptotic action of DHEA was proposed (Lazaridis et al., 2011).

The study by Lazaridis et al. (2011) provides proof that DHEA binds to NGF receptors, which is relevant to its biological function. This was the first report to demonstrate a steroid direct binding to neurotrophin receptors. As a result, when DHEA binds to TrkA, the receptor is autophosphorylated and a signaling pathway, including the Shc-PI3KAkt and Src-MEK-ERK pathways, is initiated. Furthermore, binding of DHEA to the p75NTR receptor affects the binding of p75NTR to TRAF6, RIP2 and RhoGDI effectors, and it is the ratio between TrkA and p75NTR receptors that ultimately determines whether the cell fate will be apoptosis or survival (Lazaridis et al., 2011) (Figure 9).

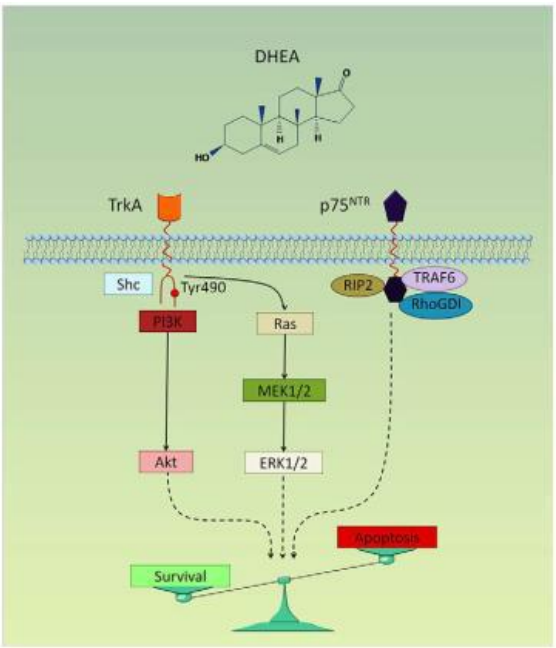

Figure 9: NGF receptor signaling pathways involved in DHEA actions. DHEA binds with high affinity to TrkA and p75NTR (Lazaridis et al., 2011)

DHEA has an additional action in terms of neurogenesis, since it increases the amount of newly generated neurons in the rat, notably in the dentate gyrus of the hippocampus, going beyond its neuroprotective and survival benefits (Karishma & Herbert, 2002). In fact, DHEA has been shown to support neurogenesis and neuronal survival in human neural stem cell cultures, and its derivatives have also been shown to promote long-term proliferation of neural stem cells by acting on NMDA and Sigma-1 receptors (Charalampopoulos, 2008; Suzuki et al., 2004).

In the human body, considerable amounts of DHEA are released throughout embryogenesis. These levels fall during the first six months after birth and start to rise again after the reticular fate of the adrenal cortex develops. The maximum levels of DHEA are seen in the blood and cerebrospinal fluid in the middle of the second decade, and they steadily decrease until they are at their lowest levels around the age

of 70 (Conley & Bird, 1997). In addition to neurodegenerative diseases and aging, physical or emotional stress is another factor in the decreasing levels of DHEA, with depression and chronic inflammation serving as prominent examples (Charalampopoulos et al., 2004).
Therefore, the creation of new compounds for neurogenesis and nerve cell protection is required. In the past two decades, researchers have become increasingly interested in using DHEA for the treatment of diseases. Endogenous neurosteroids cannot be used in clinical trials while in humans, they are converted to estrogens, androgens, or progesterones, which are known to cause generalized endocrine side effects, including hormone-dependent neoplasms (Compagnone & Mellon, 2000).

1.2.2. DHEA Synthetic Analogs

The severe lack of effective treatments for neurodegenerative diseases has sparked intense interest in the creation of neuroprotective molecules that can stop the progressive loss of nerve cells or even reverse it. Small sized agonists that bind to neurotrophin receptors and can pass the blood-brain barrier with potential therapeutic applications, are being developed by numerous research teams trying not to produce any negative side effects.

DHEA's ability to protect various types of nerve cells through its binding to neurotrophin receptors makes it a candidate for use in the treatment of neurodegenerative diseases. However, DHEA is metabolized *in vivo* to estrogens, androgens and related metabolites that affect the endocrine system, altering the hormonal microenvironment of the brain. Therefore, the long-term use of DHEA as a potential therapy is problematic, particularly in patients with a genetic predisposition to hormone-dependent tumors. On the other hand, the use of NGF or the other neurotrophins in the treatment of these conditions is also limited, due to their non-permeability across the blood-brain barrier and despite their proven beneficial effect, their therapeutic usefulness is inhibited by their polypeptide nature and their large size. Therefore, the development of small molecules, agonists of neurotrophin receptors, with therapeutic applications that can cross the blood-brain barrier seems necessary. Synthetic DHEA analogues, lacking endocrine effects, represent a new class of molecules that cross the blood-brain barrier and bind to neurotrophin receptors, having a neuroprotective effect. Recently, a library of DHEA analogues, with modifications at the C3 and C17 positions, lacking the ability to metabolize to estrogens or androgens, that may serve as agonists of neurotrophin receptors and may also cross the blood-brain barrier with potential applications in the treatment of neurodegenerative diseases, has been created by our collaborators, specifically from Professor’s Calogeropoulou Lab in the National Hellenic Research Foundation in

Athens, Greece according to EuroNeuroTrophin project and have been screened for their properties (Calogeropoulou et al., 2009a; Gravanis et al., 2012).

BNN 27

The BNN27 microneurotrophin was synthesized by epoxidation of C17 using t-BuOOH in the presence of vanadyl acetonylacetonate, resulting in a mixture of diastereomeric epoxides 27 and 23, in a 2:1 ratio and in 70% yield. Its transmembrane binding, to TrkA or p75NTR receptors, was evaluated using binding assays. After studying the ability of BNN27 to bind to both NGF receptors, its antiapoptotic efficacy was also investigated, following both TrkA and p75NTR receptors activation, through phosphorylation and interaction with RhoGDI, RIP2 and TRAF6 proteins, respectively (Pediaditakis, Efstathopoulos, et al., 2016; Pediaditakis, Kourgiantaki, et al., 2016).
Other research studies that have used the BNN27 analogue have promising results, indicating that it may enhance memory, possibly by interacting with the cholinergic system (Pitsikas & Gravanis, 2017). It may also act neuroprotectively in oligodendrocyte populations and on myelin, with potential therapeutic applications in demyelinating disorders (Bonetto et al., 2017) and finally, it shows a therapeutic effect also for diabetic retinopathy (DR), as it attacks both neurodegeneration and inflammation exhibited by the disease (Ibán-Arias et al., 2018)

ENT-A013

Recently, Rogdakis et al. showed that a new NGF mimetic, known as ENT-A013, which is a chemically stable and more potent compound than BNN27, selectively activates TrkA receptor and exerts neuroprotective and anti-amyloid actions. It protects PC12 cells against serum deprivation-induced cell death by selectively activating the TrkA receptor and its downstream kinases Akt and Erk1/2. Furthermore, ENT-A013 prevents synaptic loss and Amyloid-induced apoptosis in hippocampal neurons and promotes their survival after NGF removal in primary Dorsal Root Ganglion (DRG) neurons. To sum up, ENT-A013 is a promising new lead chemical for creating treatments for neurodegenerative diseases like Alzheimer's by specifically focusing on TrkA-mediated pro-survival signals (Rogdakis et al., 2022). ENT-A044 derives from the same library of newly synthesized compounds and is structurally similar to ENT-A013. Its biological characterization is being achieved as the aim of this PhD Thesis.

1.3 Aims of the study

p75NTR has been linked to the complex interactions between adult hippocampal neurogenesis and Alzheimer's disease (AD) pathology. This complicated relationship between Alzheimer's disease, p75 pan-neurotrophin receptor and adult hippocampal neurogenesis needs to be better understood in order to develop novel therapeutic approaches to mitigate neurodegeneration and cognitive loss in AD. Targeting p75NTR, by modifying its signaling, may promote the survival, differentiation and proliferation of neural progenitor cells, promoting the growth of new neurons in the adult hippocampus and may provide a strategy to mitigate neurogenesis deficiencies and reduce cognitive decline observed in AD pathology.

The lack of effective treatment for devastating neurodegenerative diseases has stimulated great interest in the development of neuroprotective and neuroregenerative means that can prevent and/or reverse progressive loss of neural function. The discovery of adult neurogenesis potential and neural stem cells in the adult brain opens new avenues and opportunities for treating neurological diseases and disorders, particularly through the exploitation of endogenous regenerative capacity.

This thesis is separated into two different parts. The first part of our study is dedicated to the investigation of the cellular and molecular effects of the pan-neurotrophin p75 receptor (p75NTR), in adult neurogenesis (AN) of the dentate gyrus of rodent hippocampus, under physiological and neurodegenerative conditions, such as the case of Alzheimer Disease (AD). In parallel, human induced Pluripotent Stem Cells (hiPSCs)-derived neural progenitor cells are utilized in order to investigate the role of this receptor to human neurogenic processes in physiological and neurodegenerative conditions, as well.

More specifically, in the first part of our studies, we evaluated the *in vivo* adult hippocampal neurogenesis in p75 knockout mice, in order to estimate the effects of this receptor to adult neurogenesis compared to p75 wild-type mice. Our study depends on the evaluation of neurogenesis levels on two different mouse models of p75 gene deletion. The first one, is the p75 ko Ngfrtm1Jae targeted mutation 1 (developed by Rudolf Jaenisch, p75NTR/ExonIII-), which lacks the full-length receptor but expresses the short p75NTR isoform. In the second mouse model, the deletion of p75 gene is managed by the specific deletion of exon II in Nestin expressing cells (Neural Stem Cells) based on the use of the Nestin/Cre recombinase system. The precise role of the p75 receptor in the regulation of adult hippocampal neurogenesis remains controversial. There are many studies examining the role of p75 receptor but the results are often contradictory due to different genetic backgrounds of p75NTR-deficient animals and there are also species-specific differences. These p75 knockout

lines are expressing different isoforms - truncated versions of the p75NTR protein. For that reason, we wanted to examine p75 KO mice, with a deletion of exon II mice, as well. Furthermore, we wanted to validate if p75 expression derived by NSCs is the one controlling the adult neurogenesis in SGZ or other cellular mechanisms derived from p75 expression in other cell types like astrocytes or microglia. Moreover, 5xFAD were used as an amyloid beta dependent mouse 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 all of these mice and the adult hippocampal neurogenesis was evaluated with immunofluorescent staining against BrdU – proliferation marker, Sox_2 – marker for NSCs and Dcx - marker for immature neurons.

We also explore p75-dependent effects on hiPSCs-derived NPCs, in order to validate the existing animal models but also to offer a novel platform for drug screening of new compounds with neuroprotective and neurogenic effects against AD. One of the major limitations in drug development is the discrepancy that is observed between animal models and human responses to a new compound. Thus, the validation of animal models and its correlation with human studies could importantly minimize the risk of failure.

In the second part of our study, we tested new neurotrophin analogs for their selectivity towards p75NTR. While neurotrophins have been shown to slow or prevent neurodegeneration, their reduced bioavailability and inability to penetrate the blood-brain-barrier limit their use as potential therapeutics. Using a library of novel, synthetic DHEA derivatives, we screened for molecules that have selectivity toward p75 neurotrophin receptor activation. Neurotrophin mimetics, that have been created by Dr Theodora Calogeropoulou (National Hellenic Research Foundation, Athens, Greece) and collaborators from EuroNeuroTrophin network, were tested on neurotrophin-dependent p75NTR cell lines and primary hippocampal mouse cells, investigating downstream signaling, as well as cell survival and p75 NTR mediated death.

Conclusively, we propose that the detailed characterization and knowledge of the p75NTR signaling in adult neurogenesis will provide an important benefit for developing multiple therapeutic applications against neurodegenerative diseases.

2. MATERIALS & METHODS

Plasmids, Antibodies and Proteins

Plasmids of TrkB, p75NTR, RIP2 and TRAF6 were previously described by Lazaridis et al., 2011. Normal expression of all constructs was verified by immunoblotting.

The origin of antibodies was as follows: Traf6 antibody (1:2000, ab33915, Abcam, plc., Cambridge, UK) and RIP2 (1:1000, ADI- AAP-460, Enzo Life Sciences Farmingdale). Whole cell lysates were subjected to Western blot analysis against phosphorylated TrkB (1:1000, ABN1381, Sigma-Aldrich, St. Louis, MO, USA), TrkB (1:1000, 07-225-I, Sigma-Aldrich, St. Louis, MO, USA), p75NTR (1:1000, 839701, Biolegend, Inc., San Diego, CA, USA), Traf6 (1:2000, ab33915, Abcam, PLC., Cambridge, UK), phosphorylated Akt (1:1000, 9721S, Cell Signaling Technology, Danvers, MA, USA), phosphorylated Erk1/2 (1:1000, 9101S, Cell Signaling Technology, Danvers, MA, USA), total Akt (1:1000, 4691S Cell Signaling Technology, Danvers, MA, USA), total Erk1/2 (1:1000, 4695S, Cell Signaling Technology, Danvers, MA, USA), Actin (1:2000, sc-47778 Santa Cruz Biotechnology, Inc., Dallas, TX, USA), pJNK (1:1000, 4668 Cell Signaling Technology, Danvers, MA, USA) and tJNK (1:1000, 9252 Cell Signaling Technology, Danvers, MA, USA). For Immunofluorescence, we used primary antibodies for p75NTR (1:100, Promega, G3231 and 1:100, 839701, Biolegend), DCX (1:100, abcam 207175), BrdU (1:100, Thermo Fisher Scientific, MOBU B35128), Sox_2 (1:100, cell signaling 2748) and NeuN (1:100, Millipore MAB377).

Secondary antibodies: horseradish peroxidase-conjugated anti-rabbit IgG (Cat. No. 65-6120; Invitrogen) and horseradish peroxidase-conjugated anti-mouse IgG (Cat. No. AP- 124P; Millipore). Anti-mouse Alexa Fluor 488 (Cat. No. A11029; Invitrogen), anti-rabbit Alexa Fluor 555 (Cat No. A10040; Invitrogen). NGF (Cat. No. 01-125) was purchased from Millipore and BDNF (450-02) purchased from Peprotech. TrkB inhibitor - ANA-12 was purchased from Sigma (SML0209, Sigma-Aldrich, Burlington, MA, US) and p75NTR inhibitor is a p75NTR antibody purchased from abcam (1:100 for IP and 2.5 ng/mL for acting like inhibitor, ab6172, Abcam plc., Cambridge, UK).

Mice

Two and six-months old wild-type (WT), p75NTR KO, p75NTR fl/fl NestinCre, 5xFAD and 5xFAD/p75NTR KO male and female mice were used. p75 ko mice (Ngfrtm1Jae targeted mutation 1, Rudolf Jaenisch p75NTR/ExonIII-) were obtained from the Jackson Laboratory and maintained on C57BL/6 background described by (Lee et al., 1992). This model lacks the full-length receptor but express the short p75NTR isoform. To generate mice with conditional deletion of p75NTR in neural stem cells (NSCs), $p75^{fl/fl}$ mice were crossed to transgenic mice containing the nestin gene driving expression of Cre recombinase (Nestin-cre). The loxP sites of $p75^{fl/fl}$ mice targeted the

ExonII of p75NTR in Nestin$^+$ cells kindly provided by Dr Sebastian 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 mouse models were kept in the Animal House of the Institute of Molecular Biology and Biotechnology (IMBB-FORTH, Heraklion, Greece). Animals were housed in a temperature-controlled facility on a 12 h light/dark cycle, fed by standard chow diet and water ad libitum. Genotyping was performed on tail DNA using specific primers for each animal mouse model.

BrdU injections, Tissue Processing and Immunohistochemical analysis

BrdU injections are performed for 5 days in young mice of 2 months old and mice of 6 months old of the preferred phenotype. BrdU pulse is followed by isolation and slice preparation of hippocampus (DG area) and immunostainings of the slides, with specific markers for neuronal precursors and differentiated cells. More specifically, we use two different protocols of injections. To test the effect of p75 gene deletion on the proliferation of the NSCs in the adult DG, of these different age groups we administered i.p. 100 mg kg^{-1} of the thymidine analog BrdU for 5 days to mark all proliferative cells at the S phase of cell cycle and sacrificed the mice 24 h later. In a second step, we examined the effects of p75 gene deletion on the survival rate of NSCs of the SGZ and the production of new neurons at the age group that we examine, in parallel to its effect on NSCs proliferation. To this aim, we pulsed 2-month-old mice with BrdU for the first 5 days of a 3-week period and after 21 days overall we sacrificed the mice and studied the number of BrdU$^+$ NSCs that survived.
More specifically, after the BrdU injections (10mgr/ml-Sigma, B5002), mice are being anesthetized, trans-cardially perfused with saline and the brains are being dissected. The right hemisphere is stored in -80°C for Western blot analysis and the left hemisphere is post-fixed by 4% paraformaldehyde (Sigma-Aldrich, St. Louis, MO, USA) in phosphate buffered saline (PBS) overnight at 4°C. After thorough washing in PB 0.1 M, brains were cryoprotected by being immersed in 30% sucrose solution in PB 0.1 M for 24 h at 4°C until they sunk and then are being frozen in isopentane -40°C and stored in cryoprotective medium (15% sucrose/7,5% gelatine) at − 80 °C until they processed for coronal sections. Coronal sections of 40 μM were cut in the dorsoventral axis of hippocampus (from bregma -4 mm to -1mm) and immunofluorescence protocol followed. Sections on slides were fixed with 4% paraformaldehyde solution for 15 min, washed in 0.1% Triton X-100 in PBS (PBST) for 15 min at room temperature, then washed in 0.3% Triton X-100 in PBS (PBST) for 30min at room temperature and blocked in 5% BSA before they were incubated with primary antibodies in 0.1% PBST overnight at 4°C. The day after, sections were washed three times with 0.1% PBST and incubated

with Alexa Fluor chromophore-conjugated secondary antibody (1:500) in 0.1% PBST for 1 h at room temperature. Finally, sections were washed twice again with PBS and got mounted, using antifade reagent. For detection of BrdU-labeled nuclei, specimens have been previously incubated in 2N HCl at 37°C, followed by a 10-min rinse in 0.1 M sodium tetraborate pH 8.5 and two rinses with PBS before blocking step.

Cell counts and quantification are based on a modified unbiased stereology protocol. Seven out of every 10 adjacent sections were chosen (covering the whole DG area of the hippocampus) and processed for BrdU immunohistochemistry. The number of $BrdU^{+}$ cells was then counted under × 40 magnification under a fluorescent microscope (Leica sp8-Leica Microsystems CMS, Mannheim, Germany) at the area of granular cell layer and SGZ of a total of 7 sections and the average number of cells was estimated. The mean was then multiplied with the total number of sections (75 per mouse) to estimate the total number of cells per DG.

Cell Lines

HEK293T cells were obtained from LGC Promochem GmbH (Teddington, UK) and cultured using DMEM medium (11965084, Gibco, Grand Island, NY, USA) that contained 10% Fetal Bovine Serum (10270106, Gibco, Grand Island, NY, USA) and 100 units/mL Penicillin and 0.1 mg/mL Streptomycin (15140122, Gibco, Grand Island, NY, USA) at 5% CO2 and 37 °C. They were transiently transfected with human p75NTR and TrkB plasmids, as well as TRAF6 and RIP2 plasmids, using Turbofect Transfection Reagent (R0531, Thermo Fisher Scientific, Waltham, MA, USA) based on manufacturers' instructions. Plasmids that expressed p75NTR and TrkB were previously described in Lazaridis et al. 2011. Transfected cells were typically used on the second day following transfection. NIH3T3 cells were grown in the same medium and stably transfected with human TrkB plasmid. PC12 cells were cultured under the same medium, which contained 10% Horse Serum and 5% Fetal Bovine Serum. All cells were used in the passages 5–20.

Primary Cell Cultures

Primary hippocampal NSCs were isolated from post-natal day 7 (P7) mouse pups (C57BL/6J, The Jackson Laboratory, Bar Harbor, ME, USA). These cells were grown in DMEM/F12 medium that contained B27 supplement without vitamin A, D-glucose, Primocin (0.1 mgr/mL), FGF (0.02 μgr/mL), EGF (0.02 μgr/mL) and Heparin (0.1 mgr/mL) at 5% CO2 and 37 °C. They were checked every day for neurosphere formation and their morphology—primary neurospheres observed after 5–7 days—when relatively large but bright neurospheres have formed, and a passage of the cells was necessary, which occurred using accutase. Glial cultures were isolated from the

cortex of post-natal day 2 (P2) mouse pups (C57BL/6J, The Jackson Laboratory, Bar Harbor, ME, USA). Cells were grown in high glucose DMEM medium that contained 200 U/mL penicillin, 200 µgr/mL streptomycin and 10% fetal bovine serum (FBS). At day 7, anti-mitotic agent Ara-C was added to the medium at a final concentration of 10 µM and maintained for 3 to 4 days to target the highly proliferative microglial cells. When Ara-C was removed, primary astrocytes reached a purity of 97%, and they were cultured using 5% CO2 and 37 °C. For all experiments, cells were plated on PDL/laminin, and the assays were performed after 24 h.

Generation and Culture of Human Neural Progenitor Cells (NPCs)

NPCs were generated from human-induced Pluripotent Stem Cells (iPSCs), as previously described in (Ehrlich et al., 2017). Briefly, iPSCs colonies were sectioned and enzymatically detached from mouse embryonic fibroblasts. The pieces of iPSCs colonies were collected and cultured as embryoid bodies, in suspension, in a medium that consisted of knockout DMEM (Invitrogen, Waltham, MA, USA), 20% (v/v) Knockout Serum Replacement (Invitrogen), 1 mM of β-mercaptoethanol (Invitrogen), 1% non-essential amino acids (NEAA; Invitrogen), 1% penicillin/streptomycin/glutamine (PAA) supplemented with 10 µM of SB-431542 (Ascent Scientific, Bristol, UK), 1 µM of dorsomorphin (Tocris, Bristol, UK), 3 µM of CHIR99021 (CHIR; Axon Medchem, Reston, VA, USA) and 0.5 of µM purmorphamine (Alexis, Miami, FL, USA). After two days, the medium was changed to N2B27 medium, which contained ½ DMEM-F12 (Invitrogen) and ½ Neurobasal (Invitrogen) supplemented with N2 supplement (Invitrogen), B27 supplement lacking vitamin A (Invitrogen) and 1% penicillin/streptomycin/glutamine supplemented with the aforementioned small-molecules. On day 4, SB-431542 and dorsomorphin were replaced by 150 µM of ascorbic acid (AA; Sigma, St. Louis, MO, USA). On day 6, the spheres were cut into smaller pieces and plated on Matrigel-coated plates (BD Biosciences, England, UK) in the N2B27 medium supplemented with 3 µM of CHIR, 0.5 µM of SAG (Cayman Chemical, Ann Arbor, MI, USA) and 150 of µM AA. When confluent, cells were split via treatment with accutase (Sigma, St. Louis, MO, USA). The identity of the cells was verified via immunocytochemistry for NESTIN and SOX1.

Differentiation of Human Neural Progenitor Cells (NPCs)

For the derivation of human neurons, iPSCs-derived NPCs were cultured with N2-B27 medium supplemented with 1 µM SAG (Cayman Chemical), 2 ng/mL BDNF (Peprotech), 2 ng/mL GDNF (Peprotech), and 100 µM AA (Sigma) for 6 d and afterward with N2B27 medium supplemented with 2 ng/mL BDNF (Peprotech), 2 ng/mL GDNF (Peprotech), 0.5 ng/mL TGF-β3 (Peprotech), 100 µM dbcAMP, and 100 µM AA. Additionally, 5 ng/mL Activin A (Sigma) was added to the medium from days 7–9. After 9 d of neuronal differentiation, cells were detached, singularized by treatment with

accutase, and reseeded at densities of 2×10^5 per well in 24-well plates containing glass coverslips. After 13d and 21d of differentiation, cells were used for immunofluorescence stainings.

Immunoprecipitation and Immunoblotting

Cells were suspended in Pierce™ IP Lysis Buffer (87788, Thermo Fischer Scientific, Waltham, MA, USA) supplemented with protease inhibitors (539138, Calbiochem, Darmstadt, Germany) and phosphatase inhibitors (524629, Calbiochem, Darmstadt, Germany). Lysates were pre-cleared for 1 h with protein G-plus Agarose beads (sc-2002, Santa Cruz Biotechnology, Inc., Dallas, TX, USA) and immunoprecipitated with p75NTR antibody (1:100, ab6172, Abcam plc., Cambridge, UK) overnight at 4 °C. Protein G-plus agarose beads were incubated with the lysates for 4 h at 4 °C via gentle shaking. Beads were collected via centrifugation, re-suspended in 2× SDS loading buffer and subjected to Western blot analysis against Traf6 antibody (1:2000, ab33915, Abcam, plc., Cambridge, UK) and RIP2 (1:1000, ADI- AAP-460, Enzo Life Sciences Farmingdale). For immunoblot (IB) analysis, the beads were suspended in sodium dodecyl sulfate-loading buffer and separated through SDS-PAGE. Proteins were transferred to nitrocellulose membranes and blotted with the corresponding antibodies. Immunoblots were developed using the ECL Western Blotting Kit (Thermo Fisher Scientific), and Image analysis and quantification of band intensities were performed with ImageJ Software. For the phosphorylated forms of proteins, such as Akt, Erk1/2, TrkB and JNK, the analysis was derived from the phosphorylated fraction relative to the total fraction. For the immunoprecipitation assay (activation of p75NTR according to TRAF6 and RIP2 interactions), the analysis was derived from the immunoprecipitated fraction relative to the total fraction.

Treatments with Compound ENT-A044

Cells were starved of serum for four hours so that they could be synchronized prior to treatment and then stimulated with 100 ng/mL of NGF or/and 500 ng/mL of BDNF and the examined compound ENT-A044 (500 nM). The activation of the tested receptors and the Immunoprecipitation assay treatment lasted for 20 min. For the phosphorylation of JNK protein, treatment lasted for either 30 min or 24 h, as indicated in (Charalampopoulos et al., 2012). Human NPCs were treated with 1 μM of ENT-A044 for 48h via the cell tox assay and 24h via Western blot analysis.

Cell Tox Assay

After 24h of treatments, we used the CellTox™ Green Cytotoxicity Assay kit (G8742, Promega Corporation, Maddison, WI, USA) to assess the survival of NIH-3T3, PC12, HEK293T and P7 hippocampal NSCs under conditions of serum and EGF/FGF deprivation, respectively. NSCs were studied after 48 h. Cells were plated in 96-well plates, starved for 4 h and treated with NGF (100 ng/mL) and/or BDNF (500 ng/mL) and compound ENT-A044(500 nM) in the presence or absence of p75NTR inhibitor

MC-192 (2.5 ng/mL, ab6172, Abcam, plc., Cambridge, UK) and TrkB inhibitor 100 µM of ANA-12 (SML0209, Sigma-Aldrich, Burlington, MA, US) for 24 h. CellTox assay reagents and Hoescht (1:10,000, H3570, Invitrogen, Waltham, MA, USA) were added to each well for 30 min, and cells were imaged using a fluorescent microscope (Zeiss AXIO Vert A1, Zeiss, Jena, Germany). Positive cells for cell tox reagent were normalized to reflect the total number of cells per image. We also refreshed the examined compound, as well as BDNF or/and NGF and HEK293T cells and P7 hippocampal NSCs, and they were imaged again after 48 h. AD pathology was mimicked by treating the human NPCs with αβ-amyloid peptides (10Mm of aβ1-42 oligomers) and after 24hours we used cell tox assay. Furthermore, the activity of p75NTR was prohibited by using a p75NTR specific neutralizing antibody. Dead cells were then counted with a fluorescent microscope at 485–500nmEx.

Preparation of Aβ Oligomers

Aβ (1–42) peptide was purchased from AnaSpec (AS-20276, AnaSpec, Fremont, CA, 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 24 h at 37 °C. It was then centrifuged for 5′ at 15,000× g and the supernatant collected as oligomeric Aβ as previously described in (Li et al., 2011).

BRDU assay in iPSCs-derived NPCs

iPSCs-derived NPCs were cultured on Matrigel for 24h with or without treatment of p75NTR inhibitor (abcam MC-192, ab6172, 2,5ng/ml). After 24h the cells were pulsed with 1µM BrdU for 4 hours and fixed with 4%PFA for subsequent immunostaining for BrdU and Hoechst for nuclear labeling.

Statistical Analysis

All values are expressed as the mean ± SEM. Student's t-test was used for the comparison of two groups, and one-way ANOVA was used for multiple group comparisons. A $p < 0.05$ was considered to mark statistical significance. Statistical analysis was performed using GraphPad Prism 7 (GraphPad Software Inc., San Diego, CA, USA).

3. RESULTS

3.1 PART 1 - p75NTR in adult neurogenesis of the DG of rodent hippocampus, under physiological and neurodegenerative conditions.

3.1.1. Confirmation of the lack of p75NTR expression in the p75 knock out mice

In the first part of our studies, we evaluated the *in vivo* adult hippocampal neurogenesis in p75knockout mice, in order to estimate the effects of this receptor to adult neurogenesis compared to wild-type mice. Our study depends on the evaluation of neurogenesis levels on p75 ko Ngfrtm1Jae targeted mutation 1 mice (developed by Rudolf Jaenisch, p75NTR/ExonIII-), which lack the full-length receptor but express the short p75NTR isoform. It is known, that the Basal Forebrain has an increased expression of p75NTR in wild type mice. To be sure about the deletion of p75NTR in our knock out mice models we identified the absent expression of the receptor in Basal Forebrain after immunohistochemical staining using primary antibodies for p75NTR (Figure 10).

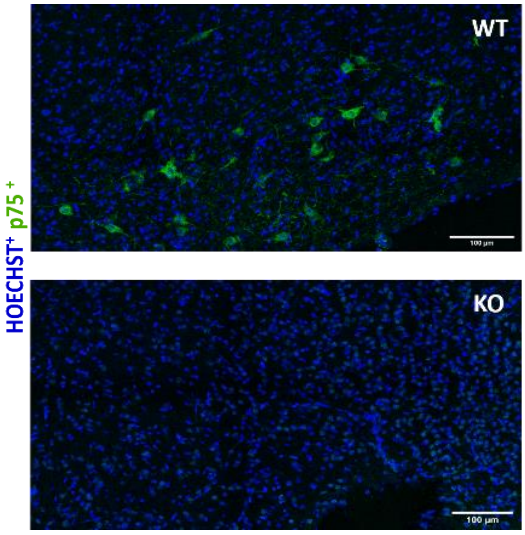

Figure 10: p75NTR expression at the basal forebrain of wild type and knock out mice (Coronal sections 40µm from 2-month-old mice after immunostaining with anti-p75 antibody (promega G323A, 1:100) and Hoechst for nuclear staining (WT, wild type; KO, knock out, scale bar: 100µm).

3.1.2. Lack of p75NTR expression in the hippocampus of 2 months old mice, but significant expression in hypothalamic formation and striatum of 2 months old wild type mice and DG of 6 months old mice

We also wanted to test the expression of p75 receptor in the DG of the adult hippocampus at the age group of 2 months old. For that reason, we have co-immunostained coronal sections of the DG, for p75NTR & NeuN (figure 11), from wild type mice. We have no expression of p75NTR at the DG of the adult hippocampus (Figure 11 a,b), but we can still observe its expression at the area of hypothalamus, as

well (figure 11a,c). Here it seems like our available antibodies cannot detect the expression of p75NTR in the DG, most probably because of a really decreased expression of the receptor in that specific area. Interestingly, there is expression in hypothalamus and striatum as well (Figure 11c,d).

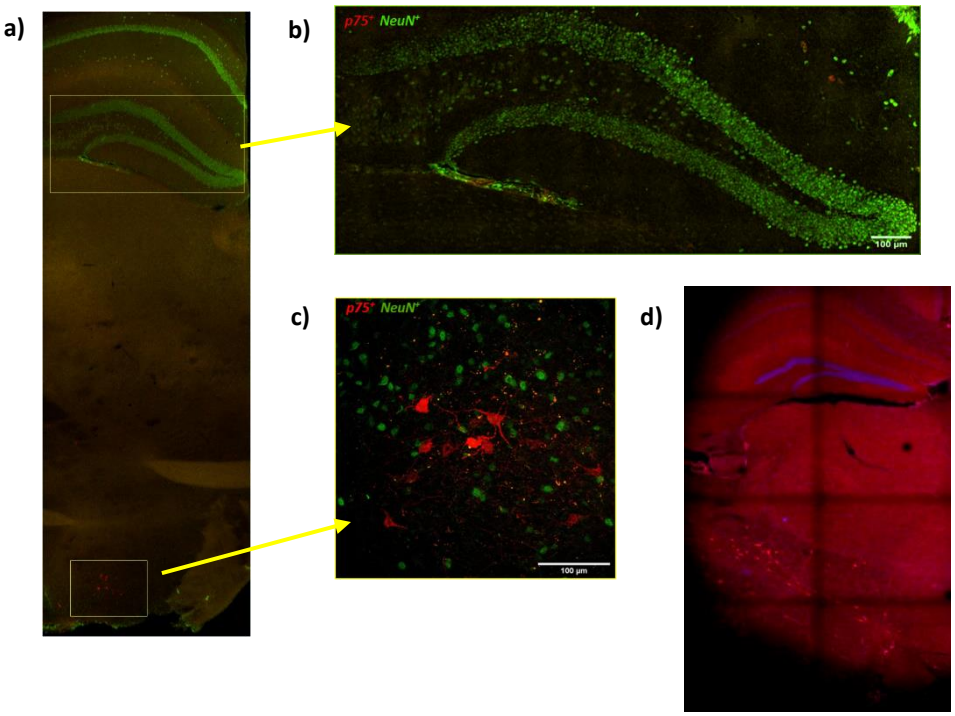

Figure 11: a) Coronal section of the whole left hemisphere from 2-months-old wild type mouse. Sections were co-immunostained for p75NTR (red) and NeuN (green). b) DG formation where p75NTR expression is absent. c) Hypothalamus formation where p75NTR is expressed (red). d) Striatum formation where p75NTR is expressed (red) (scale bar 100µm).

We also wanted to test the expression of p75 receptor in the DG of the adult hippocampus at the age group of 6 months old mice. For that reason, we have co-immunostained coronal sections of the DG, with primary antibodies for p75NTR (figure 12), from wild type mice. We can observe the expression of p75NTR at the DG of adult hippocampus, with the biggest number of p75NTR$^+$ cells to be at the hilus of hippocampal formation. Having no second marker, we assumed that p75NTR$^+$ cells here, are probably NSCs and glial cells like astrocytes. Thus, it seems like p75NTR expression is increasing, because of the age of mice.

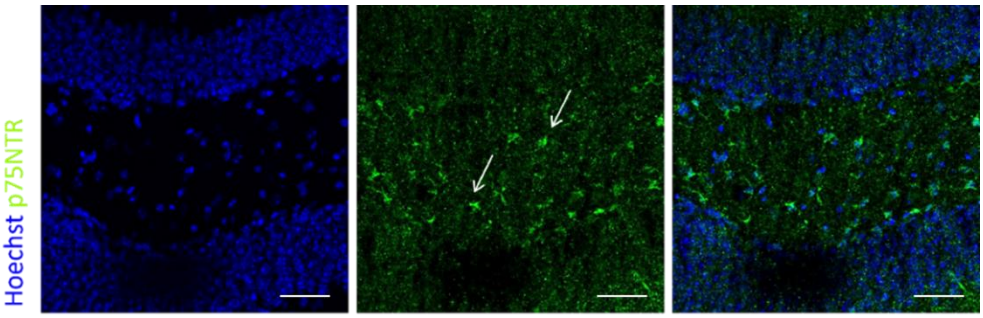

Figure 12 (previous page): Coronal section of dorsal DG from 6-months-old wild type mouse. Sections were immunostained for p75NTR. Hoechst was also used like a nuclear marker (scale bar 50µm).

3.1.3 p75NTR is crucial for the proliferation of NSCs and adult neurogenesis of two months old mice.

To test the effect of p75 deficiency on the proliferation of the NSCs in the adult DG, our aim was to count $BrdU^+$, Sox_2^+ and both $BrdU^+/Sox_2^+$ cells, at the DG of the hippocampus. Coronal sections of dorsal DG from WT and p75ko mice, were co-immunostained with BrdU and Sox_2 primary antibodies (Figure 13a) and unpaired t test analysis showed that the number of proliferating $BrdU^+$ cells in the SGZ was significantly decreased in p75ko mice compared to wild type. The number of neural stem cells, $SOX2^+$ cells in the SGZ was also significantly decreased in p75 ko mice. Furthermore, both positive cells were also significantly decreased in p75ko mice (Figure 13b). Thus, these results indicate that the expression of p75NTR, plays a crucial role in the proliferation of NSCs in adult mice.

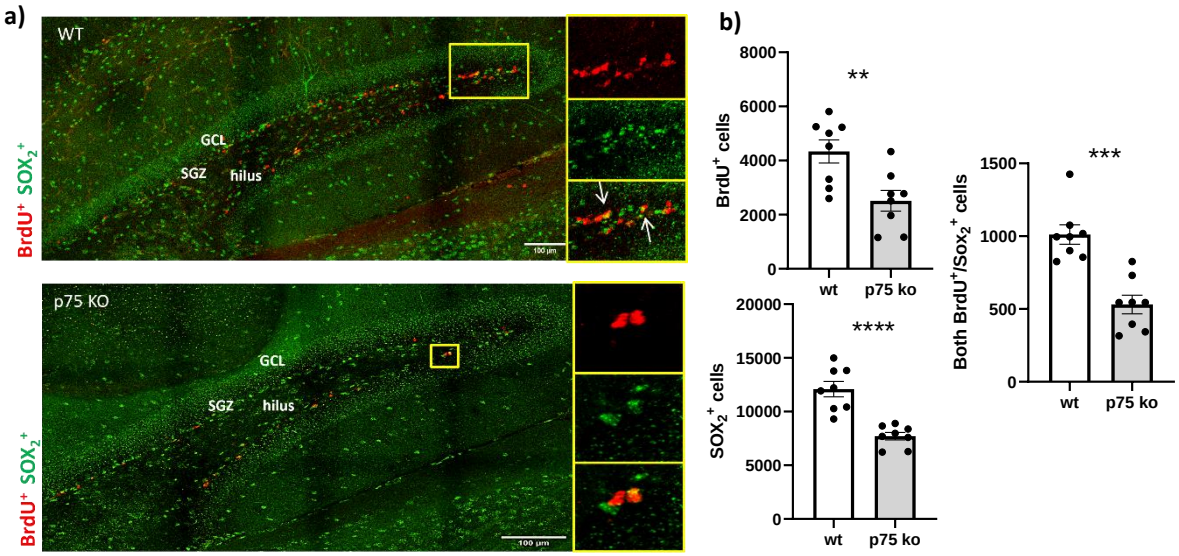

Figure 13. a) Coronal sections of DG from 2-months-old wild type and p75 knock out mice injected with BrdU for 5 days. Sections were co-immunostained for BrdU (red) and Sox_2 (green). Scale bar, 100 µm b) Quantification of $BrdU^+$ cells in DG, Sox_2^+ cells and $BrdU^+/Sox_2^+$ cells in injected mice. (N=8 WT, N=8 KO, unpaired t-test, ** p 0,0067, *** p 0,0001, **** p < 0,0001). BrdU, 5-bromo-2'-deoxyuridine; Sox_2, SRY-Box Transcription Factor 2; GCL, granule cell layer; SGZ, subgranular zone (Leica SP8, inverted confocal).

3.1.4. Increased proliferation rates of NSCs in 5xFAD mice of 2 months old.

In order to study the proliferation of NSCs under neurodegenerative conditions such as Alzheimer's disease, we performed immunofluorescent staining of the coronal sections of hippocampal DG of 2 months old 5xFAD mouse model (Figure 14a). As it is shown in Figure 14b, there is a statistically significant increase in the proliferation rates of NSCs in 5xFAD 2 months old mice when compared to WT. We assume that the

increased number of NSCs that we observed in our 5xFAD background is maybe due to the fact that there is an attempt for the creation of a compensatory, homeostatic mechanism against the neurodegenerative effects of AD-like pathology that could possibly reverse the neuronal loss that is a result of neurodegeneration generally seen at the onset of the disease - which is typically observed at around 4 months old. Furthermore, we also tested 5xFAD homozygous mice to choose which model is better suited to our experiments. Luckily there were no significant differences between 5xFAD heterozygous and homozygous mice, with the proliferation rates again being elevated when compared to WT mice (Figure 14b). So, we have decided to continue our studies with 5xFAD heterozygous mice which are also more commonly used in the literature.

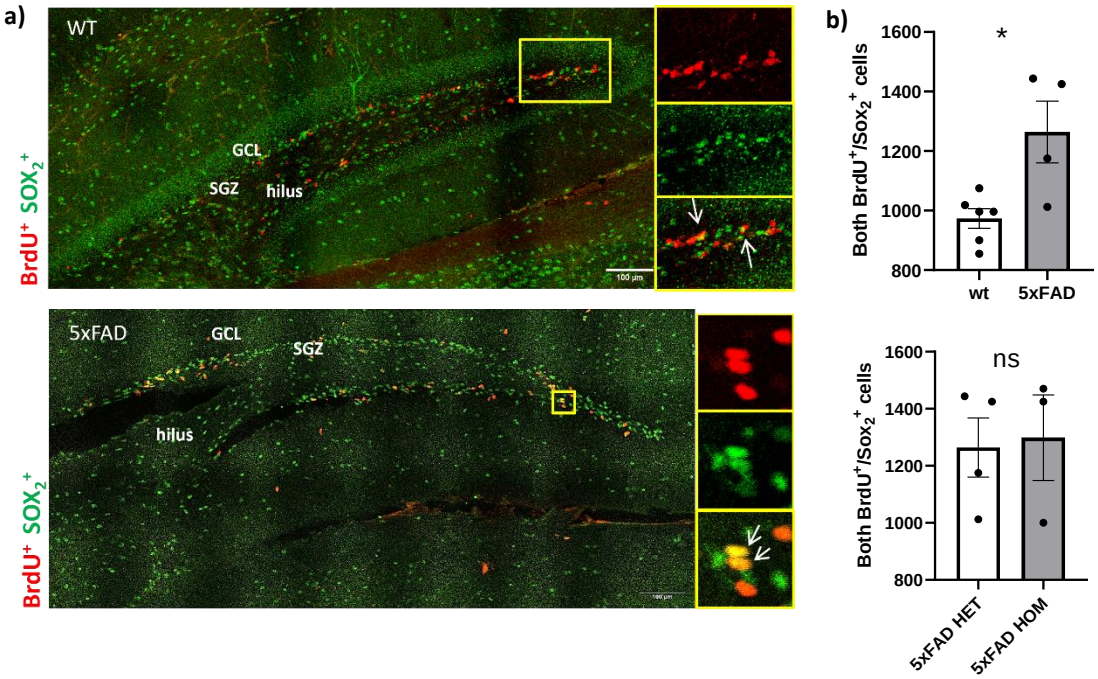

Figure 14. a) Coronal sections of DG from 2-months-old wild type and 5xFAD mice injected with BrdU for 5 days. Sections were co-immunostained for BrdU (red) and Sox2 (green). Scale bar, 100 µm b) Quantification of BrdU+/Sox2+ cells in injected mice. (N=6 WT, 4 5xFAD heterozygous & 3 5xFAD homozygous, unpaired t-test *p < 0,05, ns; no significant). BrdU, 5-bromo-2'-deoxyuridine; Sox2, SRY-Box Transcription Factor 2; GCL, granule cell layer; SGZ, subgranular zone (Leica SP8, inverted confocal).

3.1.5. Deficiency of p75NTR in 5xFAD 2 months old mice reversed the increased proliferation of NSCs.

Considering the above results and having tested our control animals, wild type, p75ko and 5xFAD, we tested the outcome of p75NTR's deletion in 5xFAD 2mo mice. We have generated double transgenic mice which carry 5xFAD mutations as well as a deficiency of p75NTR. So, through numerous crossings we created the mouse model of 5xFAD/p75NTR KO. We followed again the same experimental protocols with five consequent BrdU injections and immunofluorescent stainings of the coronal sections

of the hippocampal DG of these mice with BrdU and Sox_2 primary antibodies. The number of both $BrdU^+Sox_2^+$ cells revealed that in 5xFAD/p75NTR KO mice there was a significant decrease in the proliferation rates of NSCs when compared to WT mice, almost at the same levels as in p75NTR KO mice (Figure 15). This result confirms the crucial role of p75NTR for the proliferation of NSCs, even in the 5xFAD background where we observed an elevated proliferation. Thus, it seems that p75NTR's deletion is strong enough to abolish the high proliferation of 5xFAD model and drop the level of proliferation, lower than this of WT mice.

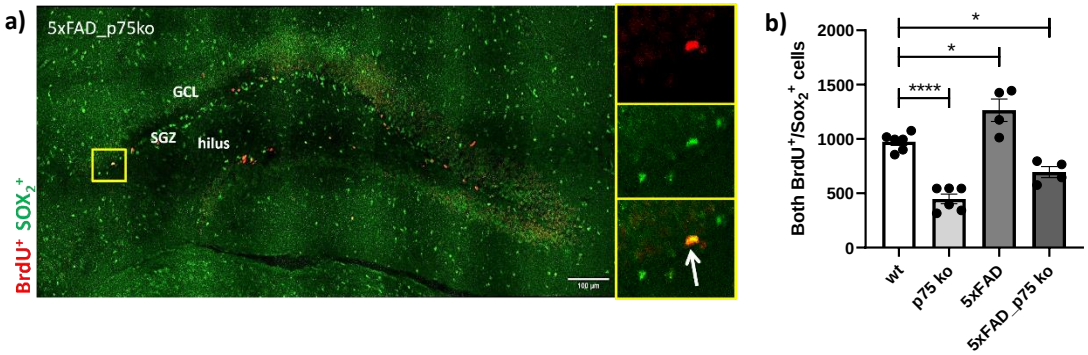

Figure 15. a) Coronal section of DG from 2-months-old 5xFAD/p75NTR KO mouse injected with BrdU for 5 days. Sections were co-immunostained for BrdU (red) and Sox_2 (green). Scale bar, 100 µm b) Quantification of $BrdU^+/Sox_2^+$ cells in injected mice. (N=6 WT, 6 p75 KO, 4 5xFAD & 4 5xFAD/p75NTR KO, one way ANOVA; p**** < 0,0001, p*< 0,05). BrdU, 5-bromo-2'-deoxyuridine; Sox_2, SRY-Box Transcription Factor 2; GCL, granule cell layer; SGZ, subgranular zone (Leica SP8, inverted confocal).

3.1.6. p75NTR is affecting adult neurogenesis in a cell non autonomous mechanism.

In order to validate if p75NTR expression derived by NSCs is the one controlling the adult neurogenesis in DG or if there are other cellular mechanisms derived from p75NTR expression in other cell types (like astrocytes or microglia) we also used another animal model with a specific deletion of p75NTR exonII, only in Nestin positive cells. Our aim was again to count $BrdU^+$, Sox_2^+ and both $BrdU^+/Sox_2^+$ cells, at the DG of the hippocampus. Coronal sections of dorsal DG were co-immunostained with BrdU and Sox_2 primary antibodies (Figure 16a) and unpaired t test analysis showed that the number of proliferating NSCs in the DG remained unchanged in p75fl/fl NestinCre mice compared to wild type (Figure 16b). Thus, it is shown that p75NTR is affecting adult neurogenesis in a cell non autonomous mechanism, driven by the expression of p75NTR in other cell types rather than NSCs.

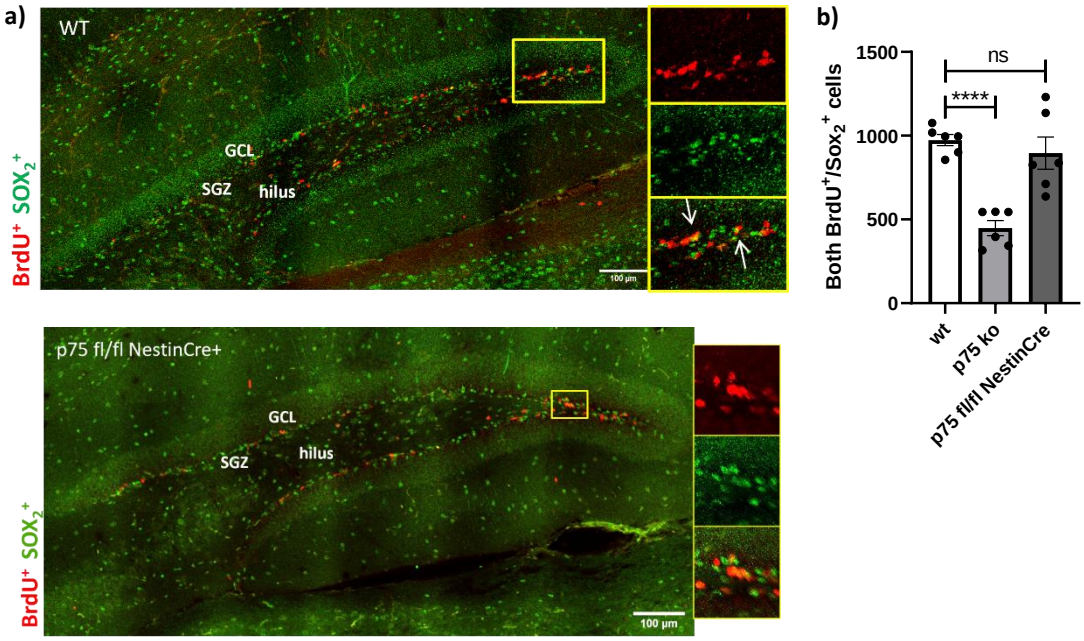

Figure 16. a) Coronal sections of DG from 2-months-old WT and p75 fl/fl NestinCre mice injected with BrdU for 5 days. Sections were co-immunostained for BrdU (red) and Sox_2 (green). Scale bar, 100 µm b) Quantification of $BrdU^+/Sox_2^+$ cells in injected mice. (n = 6 WT, 6 p75NTR KO & 6 p75fl/fl NestinCre, one way ANOVA; p**** < 0,0001, ns; no significant). BrdU, 5-bromo-2'-deoxyuridine; Sox2, SRY-Box Transcription Factor 2; GCL, granule cell layer; SGZ, subgranular zone (Leica SP8, inverted confocal).

3.1.7. p75NTR deficiency and AD background do not affect proliferation in 6 months old mice.

Finally, we studied the effect of p75NTR's deletion in NSC's proliferation in 6 months old mice. The immunofluorescent staining of DG hippocampus with BrdU and Sox_2 primary antibodies revealed no significant differences between all groups (Figure 17a). However, the number of both $BrdU^+Sox_2^+$ cells was importantly lower than this of 2 months old mice of the same groups (Figure 17b). This outcome suggests that the proliferation of NSCs diminishes to such an extent with aging that the reduction in proliferation caused by the p75NTR's deletion cannot be detected.

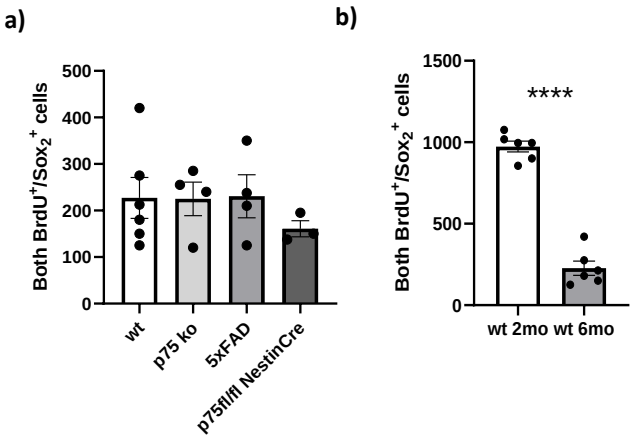

Figure 17. a) Quantification of $BrdU^+/Sox_2^+$ cells in injected 6 months old WT, p75NTR KO, 5xFAD & p75fl/fl NestinCre mice (n= 6 WT, 4 p75NTR KO, 4 5xFAD & 3 p75fl/fl NestinCre, ordinary one-way ANOVA). b) Quantification of $BrdU^+/Sox_2^+$ cells in injected 6 months old WT comparing to 2months old mice. (n= 6 WT, unpaired t-test **** p < 0,0001).

3.1.8. Immature neurons cannot proceed and differentiate into neurons in p75NTR deficient mice.

To test the effect of p75NTR deficiency on the differentiation of NSCs of the DG and the production of immature neurons at the age group of 2 months old, we first studied the number of Doublecortin positive cells, a protein marker expressed by immature neurons. Our aim was to count Dcx^+ cells at the DG of the hippocampus on coronal sections, which were immunostained with Dcx primary antibody (Figure 18a) and unpaired t test analysis showed that the number of immature neurons in the DG was significantly increased in p75 ko mice compared to wild type (Figure 18b). The area of Dcx cells' processes was also significantly increased in p75ko mice (Figure 18a,b). These results are showing that probably, immature neurons cannot proceed and differentiate into neurons when p75NTR expression is absent. Thus, it seems that these immature neurons remain in this state and they accumulate with the disability to proceed and differentiate into mature neurons and that is why the number of these cells is being increased.

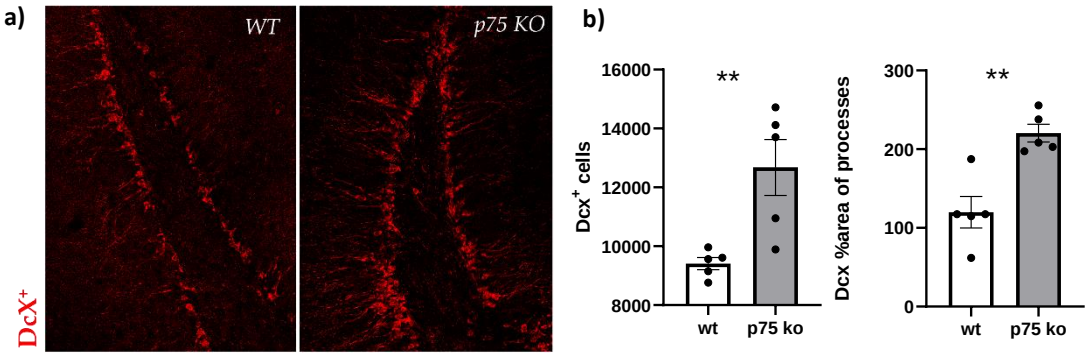

Figure 18. Coronal section of DG from 2-month-old wild type (left) & p75knock out (right) mice. a) Image depicts DcX (red) immunostained immature neurons. b) Quantification of DcX+ cells & cells' processes in p75 knock out & wild type mice of 2 months old (n=5 ko, n=5 wt, unpaired t-test, **p<0,005). DcX, doublecortin.

3.1.9. Reduction of NeuN expression is promoted in p75NTR ko mice.

To furthermore test the above hypothesis of immature neurons remaining at this state, we also studied what is happening with the production of mature neurons at the age group of 2 months old. For that reason, we pulsed mice with BrdU for the first 5 days of a 3-week period and after 21 days overall we sacrificed the mice and study the number of $BrdU^+$ neurons that survived. Our aim was to count $BrdU^+/NeuN^+$ (Neuronal nuclear marker) cells at the DG of the hippocampus to study the survival rates of NSCs. Coronal sections of DG were co-immunostained with BrdU and NeuN primary antibodies (Figure 19a). Unpaired t test analysis showed that the number of both positive cells in the DG, was significantly decreased in p75 ko mice compared to

wild type (Figure 19b). Thus, p75NTR is important for the immature neurons to procced and differentiate into neurons.

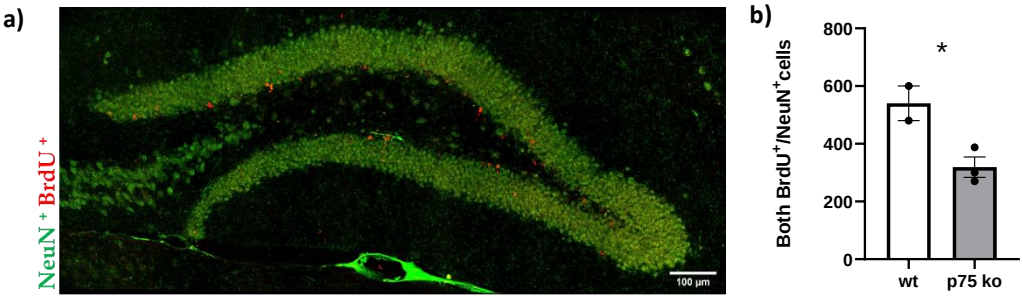

Figure 19. a) Image depicts BrdU (red) & NeuN (green) immunostained mature neurons from wt mouse. b) Quantification of both positive cells in p75 knock out & wild type mice of 2 months old (n=3 ko, n=2 wt, unpaired t-test, *p<0.05). NeuN, Neuronal Nuclear marker; BrdU, 5-bromo-2'-deoxyuridine.

3.1.10. Increased number 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 (Figure 20a) we concluded that although there is an increase, with a significant difference between the two groups (Figure 20b). So, it seems that AD background affects the proliferation of NSCs by increasing it and also has a major impact in the production of new immature neurons, since their number is higher than those of WT animals. Thus, we could suppose again that there is an attempt for the creation of a compensatory, homeostatic mechanism against the neurodegenerative effects of AD-like pathology.

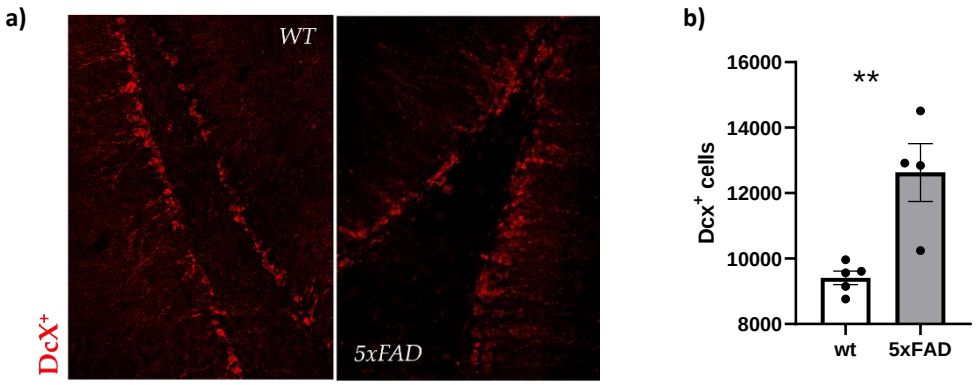

Figure 20. Coronal section of DG from 2-month-old wild type (left) & 5xFAD (right) mice. a) Image depicts DcX (red) immunostained immature neurons. b) Quantification of DcX+ cells in wild type and 5xFAD mice of 2 months old (n=5 wt, n=4 5xFAD, unpaired t-test, **p < 0,05). DcX, doublecortin.

3.1.11. p75NTR deficiency 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 number 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 21a,b). So, p75NTR's deletion is enough to abolish the increase in NSCs proliferation rates of 5xFAD and also to affect the production of immature neurons and decrease the number of Dcx$^+$ cells, compared to p75 ko and 5xFAD mice. These results indicated that p75NTR has significant effects in differentiation and not only in proliferation of NSCs, in an AD background.

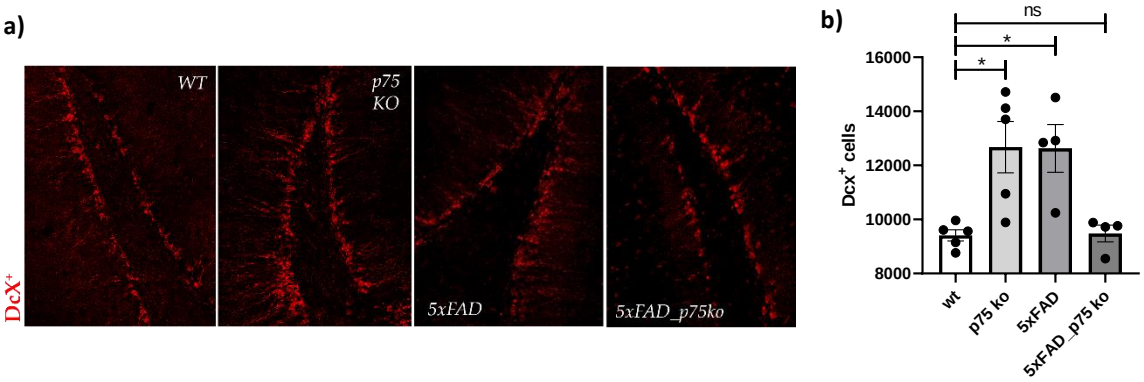

Figure 21. Coronal sections of DG from 2-months-old wt, p75 ko, 5xFAD and 5xFAD/p75NTR KO mice. a) Image depicts DcX (red) immunostained immature neurons. b) Quantification of DcX+ cells in wild type, p75NTR ko, 5xFAD, and 5xFAD/p75NTR KO of 2 months old (n=5 wt, n=5 p75 ko, n=4 5xFAD, n=4 5xFAD/p75NTR ko, one way ANOVA, *p < 0,05, ns; no significant). DcX, doublecortin.

3.1.12. p75NTR expressed by Nestin positive cells does not affect the production of immature neurons.

Furthermore, 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 p75fl/fl NestinCre mice which carry a conditional deletion of p75NTR only cells expressing Nestin marker (Figure 22a). Our results showed that there were no significant differences between the production of immature neurons in WT and p75fl/fl NestinCre mice (Figure 22b). Following up with the above data about the proliferation of NSCs, this suggests that p75NTR expressed by NSCs does not influence the proliferation of NSCs neither the production of immature neurons. Thus, it seems that there is a non-autonomous mechanism that controls the role of p75NTR in the proliferation and differentiation of the cells, driven by other cell types rather than NSCs.

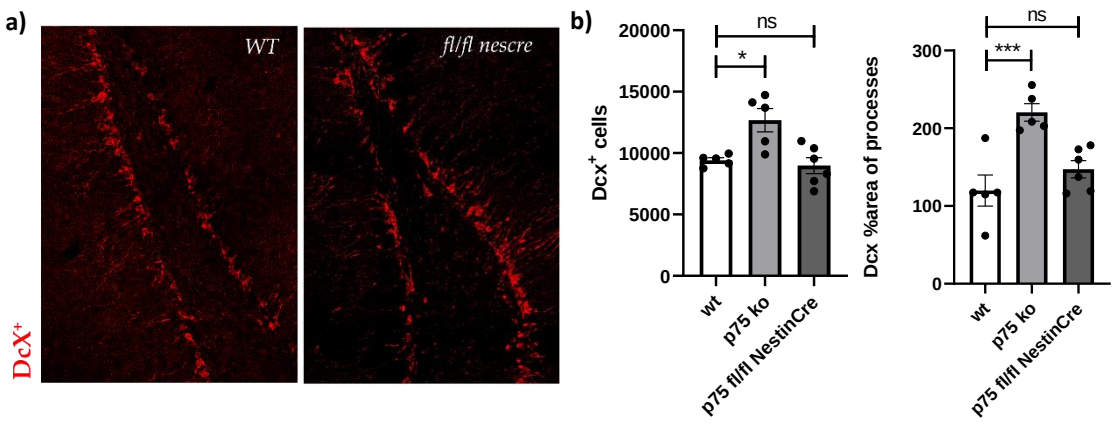

Figure 22. Coronal sections of DG from 2-month-old wild type (left) & p75fl/fl NestinCre (right) mice. a) Image depicts DcX (red) immunostained immature neurons. b) Quantification of DcX+ cells & cells' processes in p75 wild type and p75 fl/fl NestinCre mice of 2 months old (n=5 wt, n=5 p75 ko, n=6 fl/fl NestinCre, one way ANOVA, *p < 0,05, ***p < 0,005, ns; no significant). DcX, doublecortin.

3.1.13. No effect of p75NTR's deficiency and AD background in the production of immature neurons in 6 months old mice.

Finally, we studied the impact of p75NTR's deletion in the production of immature neurons in adult 6 months old mice. The immunofluorescent staining of coronal sections of the hippocampal DG from 6 months old mice DCX primary antibody revealed no significant differences between all groups (Figure 23a). However, the level of Dcx+ cells in 6 months old animals was significantly lower than those of 2 months old mice of the same groups (Figure 23b). 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.

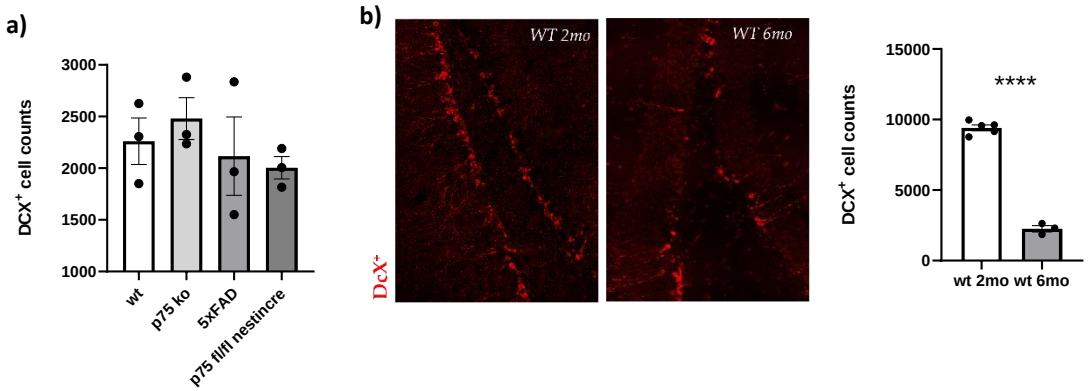

Figure 23. a) Quantification of Dcx+ cells in 6 months old WT, p75NTR KO, 5xFAD & p75fl/fl NestinCre mice (n=3 wt, n=3 p75NTR KO, n=3 5xFAD, n=3 fl/fl NestinCre, one way ANOVA, ns; no difference). b) Coronal sections of DG from 2-month-old wild type (left) & 6-month-old wild type (right) mice. Image depicts DcX (red) immunostained immature neurons. Quantification of Dcx+ cells in 2months old comparing 6 months old mice (n=5 wt 2mo, n=3 wt 6mo, unpaired t-test ****p <0,0001).

3.1.14. p75NTR expression and function on human induced pluripotent stem cells (hiPSCs)-derived NPCs

We have recently started exploring the p75NTR-dependent effects on human induced pluripotent stem cells (hiPSCs)-derived NPCs (in collaboration with Dr Chanoumidou), in order to validate the results of the existing animal models, but also to offer a novel platform for drug screening of new compounds with neuroprotective and/or neurogenic effects against AD. Of note, the role of p75NTR has not been studied before in human NPCs underlying the novelty of our work. So far, we have generated NPCs from three different iPSCs lines, two derived from healthy individuals (named as 841, 856) and one from an AD patient having the ApoE mutation. The identity of human NPCs was validated with immunostaining for key NPCs markers such as NESTIN (Figure 24). The generated NPCs lines can be expanded and cryopreserved enabling long term studies.

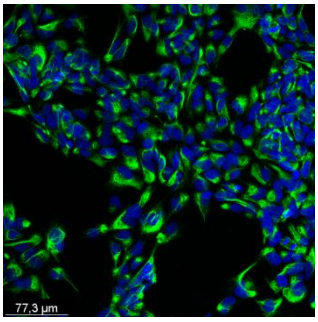

Figure 24. Immunostaining of human iPSCs-derived NPCs cultured on Matrigel for NESTIN (green) and HOECHST (blue). Scale Bar: 77µm.

The study of p75NTR involvement in human neurogenesis includes various research questions. Firstly, the expression and activity of p75NTR as well as other downstream mediators of the p75NTR signaling pathway need to be determined in human NPCs. Stable NPCs lines were generated from iPSCs provided by Professor Z. Cader. The stem cell identity of the cells was confirmed by immunostaining for NESTIN (Figure 24). To study the role of p75NTR in NPCs we firstly detected its expression in NPCs lysates with Western Blot analysis (Figure 25a). Apart from p75NTR, NPCs were found to express the p75NTR-interactors RIP2 and TRAF6 (Figure 25a) indicating an active state of p75NTR signaling in these cells. Furthermore, the actual interaction of p75NTR with the aforementioned TRAF6 protein also confirmed with co-IP (Figure 25b).

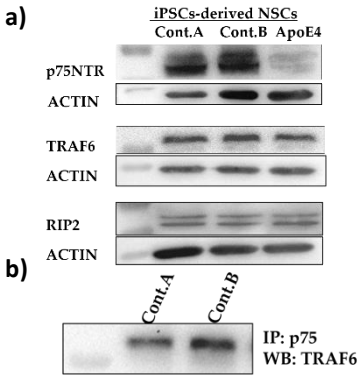

Figure 25. a) Western Blot showing the expression of p75, RIP2 and TRAF6 proteins in lysates of three iPSCs-derived NPCs lines (841,856, ApoE). b) Co-IP showing the interaction of p75NTR with TRAF6 protein.

The interesting thing here is also the declined expression of p75NTR in ApoE4 cell line, derived from AD patient having this specific mutation. Unfortunately, more experiments have to be done in order to specify these results, taking also in mind that when AD is present, we know by literature that p75NTR expression is elevated. Something that we do not observe here.

3.1.15. Inhibition of p75NTR rescues Aβ induced toxicity in human iPSCs – derived NPCs

Next, we were interested in investigating the effect of p75NTR inhibition on NPCs survival under healthy and AD relevant conditions. The activity of p75NTR was prohibited by using a p75NTR specific neutralizing antibody. AD pathology was mimicked by treating the cells with αβ-amyloid peptides (aβ1-42 oligomers) that are known to have a toxic effect on cells. The results of the Celltox assay provide evidence that p75NTR negatively influences human NPCs survival after treatment with aβ peptides (Figure 26 a,b) indicating a regulatory role of p75NTR in NPCs pathology of AD.

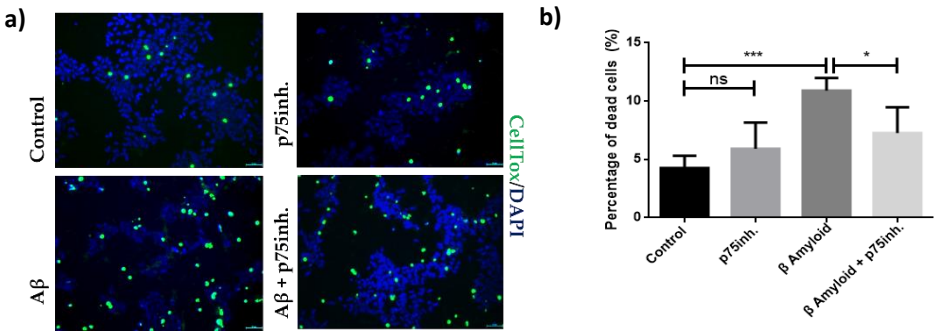

Figure 26: a) Dead cells (green) upon inhibition of p75NTR and/or treatment with aβ oligomers. b) Graph showing the percentage of dead cells (Celltox labeled cells/total number of HOECHST^{+} cells) n=3 from different NPCs lines (841,856) (unpaired t-test ***p < 0,0001, *p < 0,05, ns, no significant).

3.1.16. Inhibition of p75NTR has no effect in proliferation of human NPCs

Next, we were interested in investigating the effect of p75NTR inhibition on NPCs proliferation under healthy conditions. BrdU assay in NPCs lines derived from healthy individuals, revealed no alteration in the proliferation rate of the cells when p75NTR is inhibited (Figure 27) suggesting that p75NTR is not actively involved at least in human NPCs proliferation. This result is totally different from the results derived from our mouse models, but there is a probability that p75NTR inhibitor is not working properly. More trials should be done but the best solution would be to use shRNA for silencing p75NTR gene instead of using an antibody for blocking.

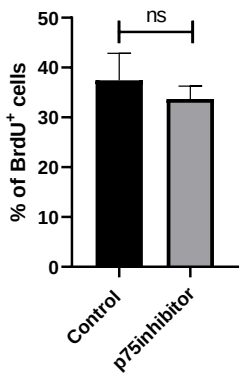

Figure 27: Graph showing the percentage of proliferating cells (BrdU⁺ cells/total number of HOECHST⁺ cells) upon inhibition of p75NTR (n=3 different NSC lines (841,856), unpaired t-test, no significant).

3.1.17. p75NTR is important for differentiation and maturation of human NPCs

Last but not least, it was also important to study what is happening with the role of p75NTR in the differentiation procedure of the human NPCs and to further confirm our results gained from our *in vivo* experiments on p75 knock out mice. To do so, we used human iPSCs-derived NPCs and we differentiated them to neurons. After 13d and 21d of differentiation, cells were used for immunofluorescence stainings. Here it seems, that p75NTR plays also a significant role in the differentiation processes of human NPCs. In 13 days, DCX⁺ cells are increasing in number when p75NTR inhibitor is used. Moreover, the processes of DCX⁺ cells seem longer, exactly like the results we have gained from our mouse models (Figure 28). After 8 more days, Tuj1⁺ cells seem less, when p75NTR inhibitor is used, which means that p75NTR is also important for NPCs to differentiate to mature neurons (Figure 28). Even if these results derived from one trial, it is really promising to gain exactly the same results with our *in vivo* experiments. Of course, more trials are going to be executed.

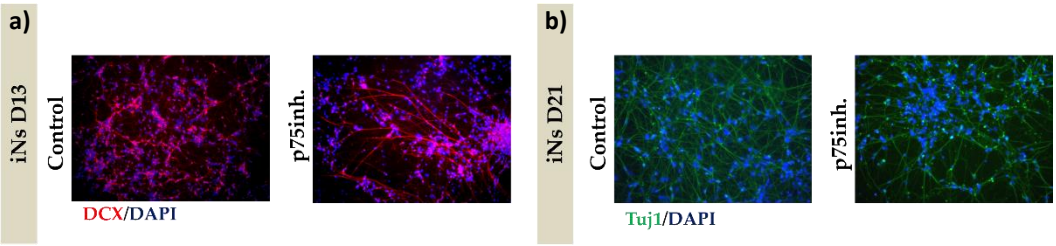

Figure 28. a) Neurons derived from healthy human iPSCs and cultured for 13 days, with or without treatment with p75NTR inhibitor. Cells were co-immunostained for Dcx (red) and DAPI (blue). b) Neurons derived from healthy human iPSCs and cultured for 21 days, with or without treatment with p75NTR inhibitor. Cells were co-immunostained for Tuj1 (red) and DAPI (blue) (n=1).

3.2 PART 2 - Activation of the p75 Neurotrophin Receptor by the Neurotrophin Analog ENT-A044

3.2.1. ENT-A044 activates TrkB receptor, but not TrkA

Our studies upon neurotrophin analog ENT-A044, started with assays on Trk receptors to study their activation. In collaboration with Dr. Rogdakis and PhD candidate Despoina Charou, we first tested ENT-A044 in activation of TrkB Receptor and its downstream signaling kinase Akt. For this reason, we used primary astrocytes isolated from the cortex of post-natal day 2 (P2) mouse pups, which endogenously express TrkB. Cells where starved of serum for 4 h, and treatment with BDNF (500 ng/mL) or ENT-A044 (500 nM) occurred for 20 min. Western blot analysis revealed that ENT-A044 induces the phosphorylation of TrkB receptor and the kinase Akt (Figure 29a), suggesting that ENT-A044 can activate TrkB receptor and its downstream signaling. Furtermore, we used NIH-3T3 cells that were stably transfected with the TrkB receptor and treated for 24h with BDNF or ENT-A044 in the absence of serum and cells were then stained with CellTox reagent and Hoechst to identify apoptotic cells. The inhibition of TrkB receptor totally abolishes the neuroprotective effect of ENT-A044 and BDNF (Figure 29b). In order to confirm the involvement of TrkB in the protective effect of ENT-A044, we treated cells with either BDNF or the compound in the presence of one selective TrkB inhibitor, ANA-12.

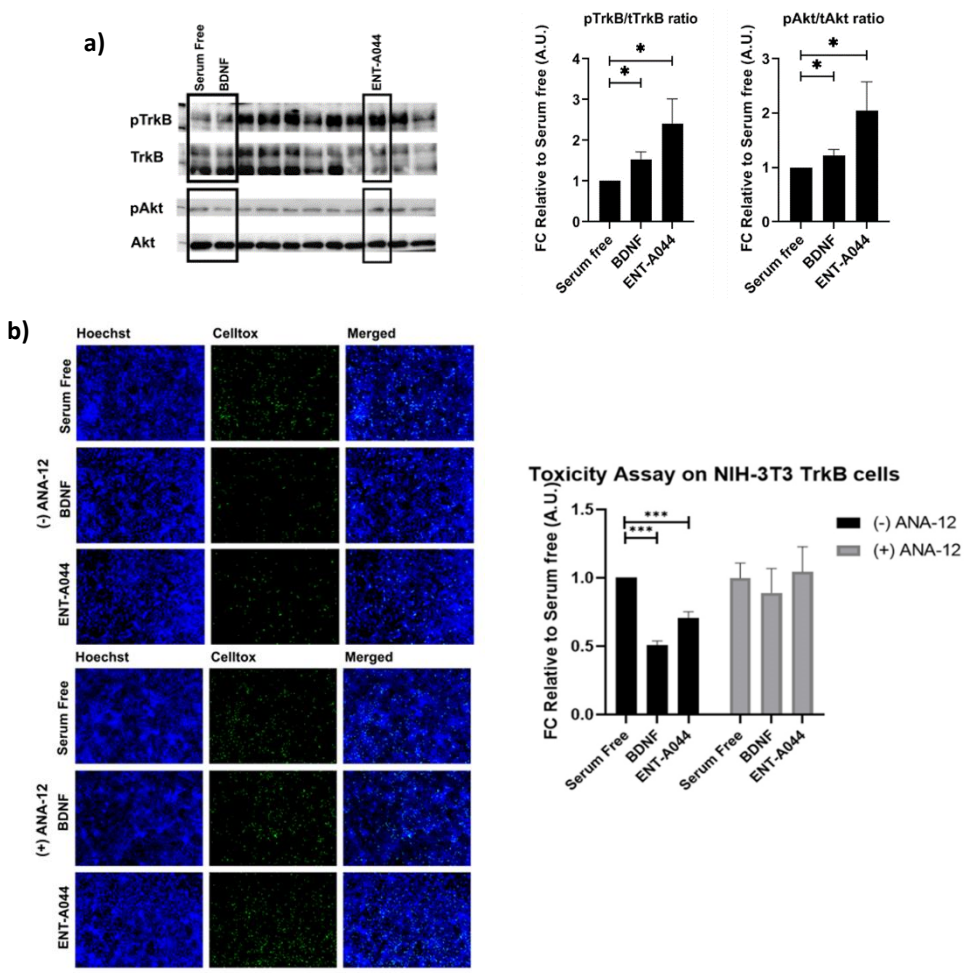

Figure 29 (previous page). (a) Representative blots determined via Western blot analysis of lysates belonging to primary mouse cultures of astrocytes after 20 min of treatment with BDNF (500 ng/mL) and the tested compound ENT-A044 (500 nM) & quantification analysis of pTrkB and pAkt expression (t-test against control serum free, * $p < 0.05$, mean ± SEM of five measurements). b) Cell tox assay on stable transfected NIH-3T3-TrkB cells after 24 h and treatment with the tested compound ENT-A044 (500 nM). ANA12, i.e., the TrkB inhibitor, was also used & quantification of cell tox+ cells (green)/Hoechst+ cells (blue), t-test against control serum free, *** $p < 0.001$, mean ± SEM of triplicate measurements.

Later on, we tested ENT-A044 in PC12 cells - expressing both TrkA receptor and p75NTR - under serum starvation conditions in order to induce cell death. PC12 cells were treated for 24h with NGF or ENT-A044 in the absence of serum and the CellTox cytotoxicity assay was performed to identify apoptotic cells. Our results showed that, ENT-A044 significantly augmented cell death (Figure 30a). Given that ENT-A044 promotes cell death in PC12 cells, we assumed that the observed cell death was probably mediated by the actions of the pro-apoptotic p75 death receptor.

To further investigate our hypothesis, we first explored the possibility that ENT-A044 interacts with the TrkA neurotrophin receptor and its downstream signaling kinases Akt and Erk1/2. Thus, PC12 cells were starved of serum for 4 h and then treated with NGF (100 ng/mL) or ENT-A044 (500 nM) for 20 min. In parallel, other analogs of the same chemical library were also tested. Western blot analysis revealed that ENT-A044 cannot induce the phosphorylation of kinases Akt and Erk1/2, which are known to be activated upon TrkA activation and have been associated with NGF promotion of cell survival (Figure 30b). Thus, considering the inability of ENT-A044 to activate the TrkA receptor, the observed cell death in PC12 is probably mediated by p75NTR.

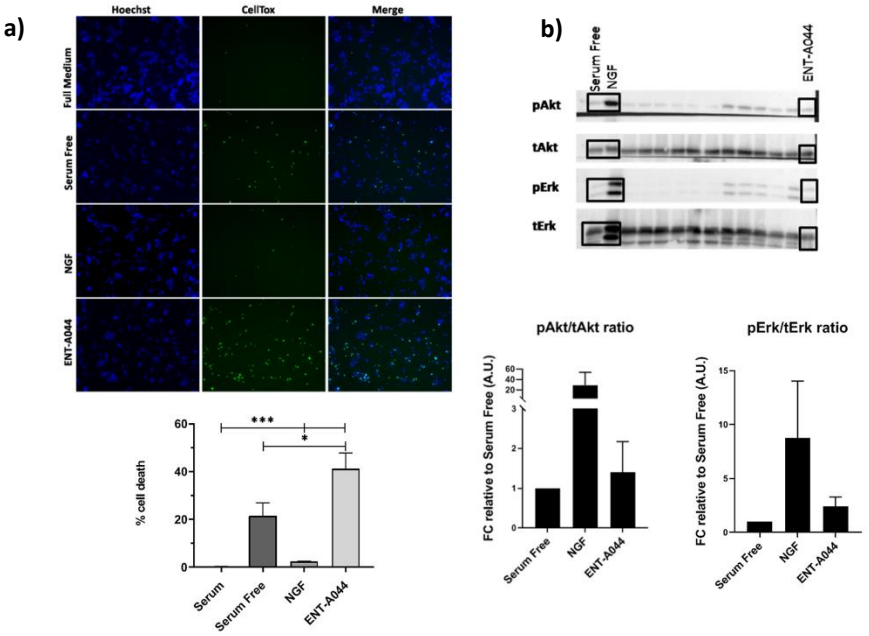

Figure 30. a) Cell tox assay performed on PC12 cells after 24 h and treatment with the tested compound ENT-A044 (500 nM). Quantification of cell tox+ cells (green)/Hoechst+ cells (blue), one-way ANOVA, * $p < 0.05$, *** $p < 0.001$ mean ± SEM of triplicate measurements. b) Representative blots determined

via Western blot analysis of lysates from PC12 cells after 20 min of treatment with NGF (100 ng/mL), synthetic analogs and the compound ENT-A044 (500 nM). Quantification analysis of pAkt and pErk expression (one-way ANOVA against control serum free, mean ± SEM of triplicate measurements).

3.2.2. ENT-A044 Promotes Cell Death in Transiently Transfected HEK293T Cells, by activating p75NTR

Following the observed cell death in PC12 cells and TrkB activation by ENT-A044, we proceeded to study our compound's implementation in p75NTR activation. HEK293T cells were transiently transfected with the plasmid for the expression of human p75NTR. Treatment of these cells with ENT-A044 revealed an increase in observed death following serum deprivation-induced cell death (Figure 31a,b). HEK293T cells that were not transfected with p75NTR plasmid (wild type HEK293T cells) were also treated with ENT-A044, having no differences from the serum-free condition (Figure 32). Phosphorylation of JNK protein was also measured in order to define the exact signaling pathways that are involved in the induction of cell death. After 30 min of treatment with NGF or our compound, phosphorylation of JNK was also increased (Figure 31c). Thus, ENT-A044 can activate JNK-mediated death signaling pathways via p75NTR.

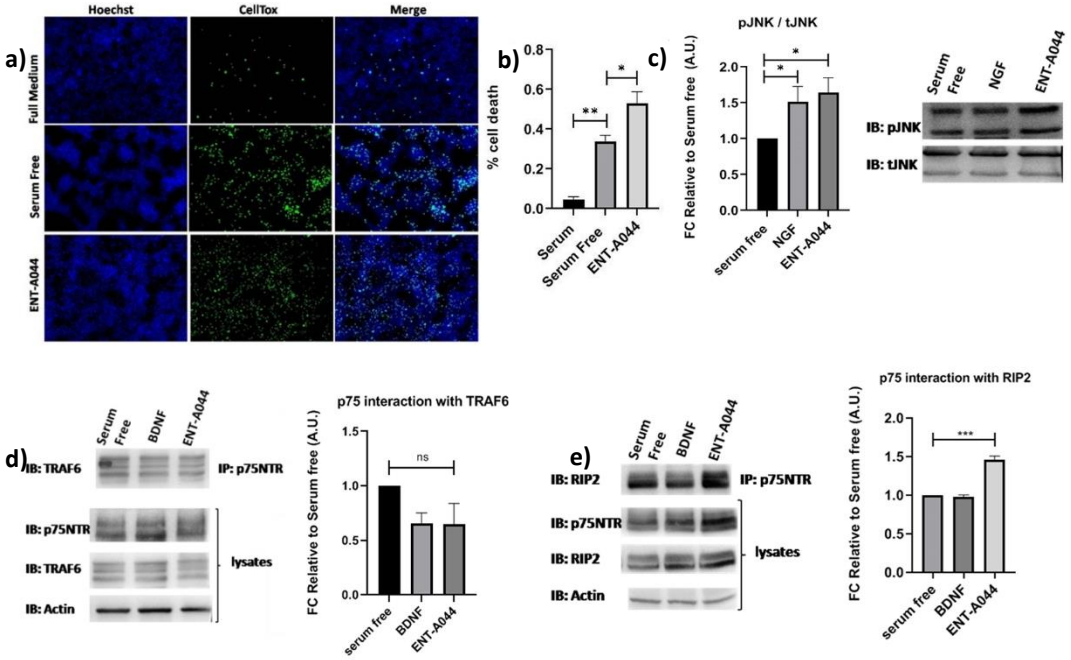

Figure 31: HEK293T cells were transiently co-transfected with the plasmid cDNAs of p75NTR and/or TRAF6 and/or RIP2. a) Cell tox assay performed on transfected HEK293T cells after 48 h and treatment with the tested compound ENT-A044 (500 nM). b) Quantification of cell tox+ cells (green)/Hoechst+ cells (blue), one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, mean ± SEM of triplicate measurements. c) Western blot analysis performed on transfected HEK293T cells after 30 min of treatment with the tested compound (500 nM) and quantification of pJNK expression (unpaired t-test against control serum free, * $p < 0.05$, mean ± SEM of triplicate measurements). d) Transfectants were exposed for 20

min to BDNF (500 ng/mL), and the tested compound and lysates were immunoprecipitated with p75NTR-specific antibody and immunoblotted with antibodies against TRAF6. Total lysates were analyzed for p75NTR or actin expression via immunoblotting (unpaired t-test against control serum free; ns, no significant; mean ± SEM of triplicate measurements). e) Transfectants were exposed for 20 min to BDNF (500 ng/mL), and the tested compound and 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, *** $p < 0.005$, mean ± SEM of triplicate measurements).

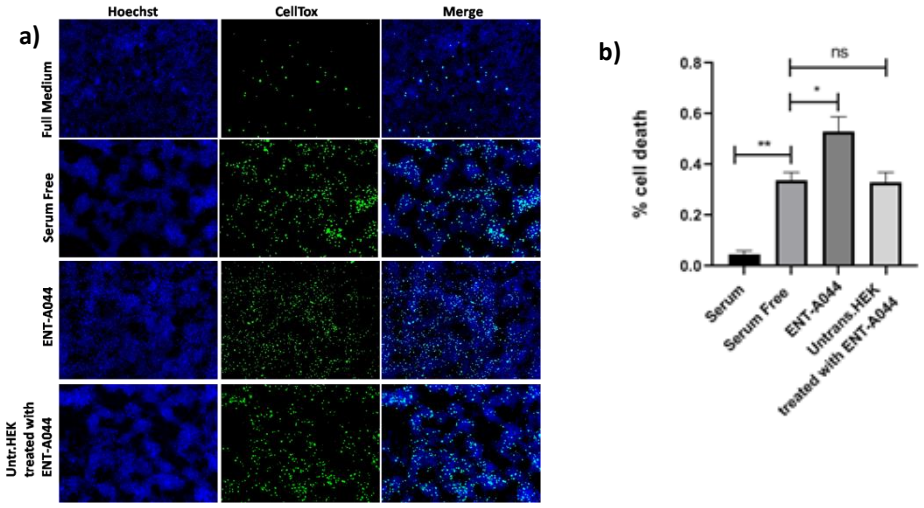

Figure 32. a) Cell tox assay on transiently transfected and non-transfected HEK293T cells with p75NTR plasmid, after 48 hrs and treatments with the tested compound ENT-A044 (500nM). b) Quantification of cell tox+ cells (green)/Hoechst+ cells (blue), one-way ANOVA, * $p < 0.05$, ** $p < 0.01$, mean ± SEM of triplicate measurements.

To better characterize the p75NTR signaling pathways that are activated by ENT-A044, we performed an immunoprecipitation assay. p75NTR lacks intracellular enzymatic activity and its signaling is dependent on interactions between proteins, like TRAF6 and RIP2, and the receptor's death domain. By performing co-immunoprecipitation experiments for p75NTR and its interactors TRAF6 and RIP2, we observed that there was no significant recruitment of TRAF6 upon p75 receptor activation using our compound (Figure 31d), while the RIP2 protein exhibits a significant interaction with the p75NTR in treated with ENT-A044 samples (Figure 31e).

As HEK293T cells only express p75NTR, it seems that ENT-A044 can induce cell death when only p75NTR is being expressed, while it has opposite effects when the TrkB receptor is also co-expressed, as in the case of astrocytes. Due to those results, we examined the effects of ENT-A044 in a system in which both TrkB and p75NTR are expressed. Firstly, we used co-transfected HEK293T cells with both TrkB and p75NTR plasmids. Cell tox assay then showed cell survival or death based on which receptor was present. More specifically, when both receptors were present, ENT-A044

predominantly led to survival (Figure 33). This result is a clear indication that ENT-A044-dependent signaling of the TrkB receptor can overcome the death signaling pathway that is triggered by p75NTR in a cell-specific manner.

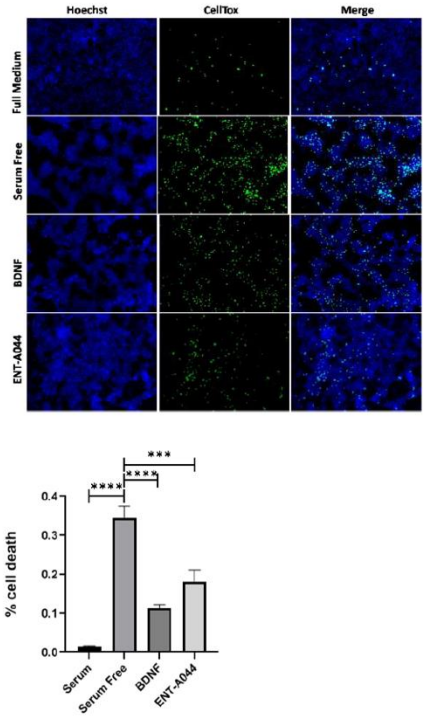

Figure 33. Cell tox assay on transiently transfected HEK293T cells with TrkB and p75NTR plasmids, after 48 hrs and treatments with the tested compound ENT-A044 (500nM) & quantification of cell tox+ cells (green)/Hoechst+ cells (blue), one-way ANOVA, ** $p < 0.01$,*** $p < 0.005$, **** $p < 0.0001$, mean ± SEM of triplicate measurements.

3.2.3. ENT-A044 Promotes Cell Survival in P7 Mouse Hippocampal NSCs

To further support this hypothesis, we used primary cultures from P7 mouse hippocampal NSCs that endogenously express both TrkB and p75NTR (Figure 34), showing that, when both of the receptors are present, ENT-A044 treatment for 48 h can lead to survival signaling (Figure 35).

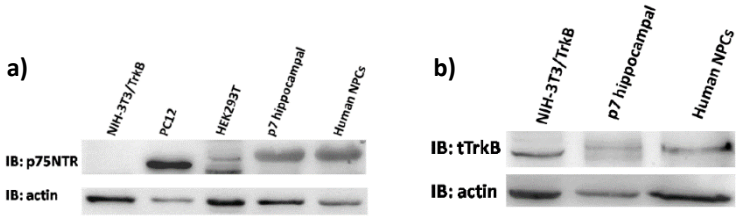

Figure 34. a) Western blot analysis on lysates from p7 mouse hippocampal NSCs and NPCs that were generated by human induced pluripotent stem cells (hiPSCs), for the detection of p75NTR expression. b) Western blot analysis on lysates from p7 mouse hippocampal NSCs and NPCs that were generated by human induced pluripotent stem cells (hiPSCs), for the detection of total TrkB expression. PC12 cells, stable transfected NIH-3T3 cells and transiently transfected HEK293T were used like controls for p75NTR and TrkB expression.

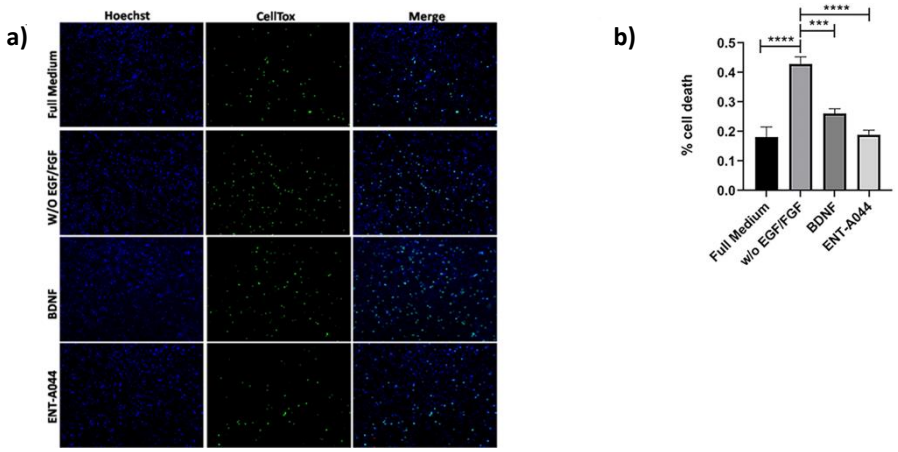

Figure 35: a) Cell tox assay on P7 mouse hippocampal NSCs after 48 h of treatment with the tested compound ENT-A044 (500 nM). b) Quantification of cell tox+ cells (green)/Hoechst+ cells (blue), one-way ANOVA, *** $p < 0.005$, **** $p < 0.0001$, mean ± SEM of triplicate measurements.

3.2.4. ENT-A044 Shows a Strong Pro-Apoptotic Effect on Human NPCs

Lastly, we focused on human cells of neural origin and tested the effect of ENT-A044 on Human Neural Progenitor cells (NPCs). NPCs were generated via human-induced Pluripotent Stem Cells (hiPSCs) and express both TrkB and p75NTR receptors (Figure 34). NPCs treatment with ENT-A044 for 48 h had a detrimental effect on cell survival, as revealed via cell tox cytotoxicity assay (Figure 36a,b). The apoptotic effect of ENT-A044 is in line with the increased levels of pJNK found in NPCs upon treatment with the compound for 24 h. Increased pJNK was also observed upon cells' treatment with NGF, suggesting the activation of an apoptotic signaling pathway totally mediated by p75NTR in these cells (Figure 36d). On the other hand, we observed that treatment with BDNF for 48 h could not induce cell death mediated by p75NTR, which occurs independently of its expression in human NPCs (Figure 36a,c). Thus, ENT-A044 leads to cell death mediated by p75NTR in human NPCs, although these cells express both p75NTR and the TrkB receptor, indicating significant differences between human and mouse neuronal cell functions that are mediated by NTRs.

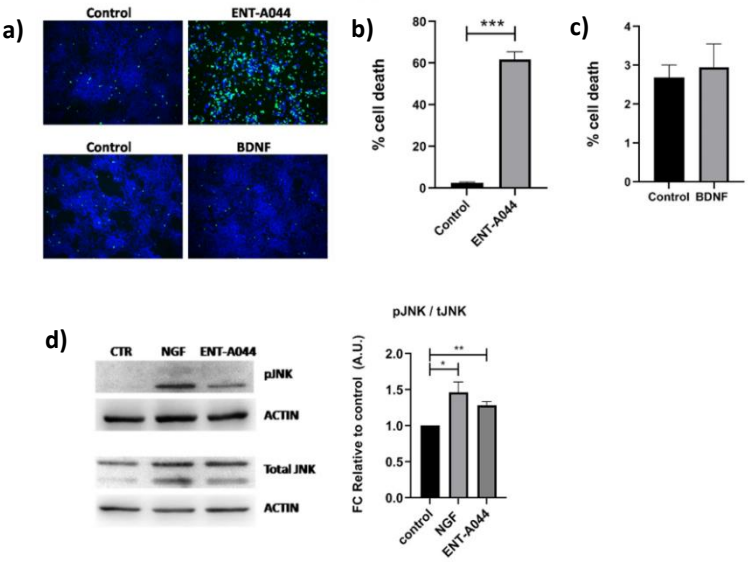

Figure 36 (previous page). a) Representative images of cell tox assay performed on human NPCs after 48 h of treatment with ENT-A044 (1 µM) and BDNF (500 ng/mL). b,c) Quantification of dead cells (green)/Hoechst+ cells (blue), *t*-test, ns no significant, *** $p < 0.001$, mean ± SEM of triplicate measurements d) Images and quantification of Western blot analysis of p-JNK and total JNK performed on NPCs treated with ENT-A044 (1 µM) or NGF (100 ng/mL) for 24 h (unpaired *t*-test against control, * $p < 0.05$, ** $p < 0.005$, mean ± SEM of triplicate measurements).

4. DISCUSSION

p75NTR in adult neurogenesis of the Dentate Gyrus of rodent hippocampus, under physiological and neurodegenerative conditions.

p75 Neurotrophin Receptor is a major regulatory receptor in many developmental but also pathophysiological processes and its signaling properties are continuously enriched and paired with specific diseases. As a neurotrophin receptor, its primary role is dedicated to neuronal effects, while its structural categorization to death receptors' family, assigns the pro-apoptotic mechanism as its most generalized effect. Thus, p75NTR has been implicated in the development, propagation and execution of neuronal and glial death during development or under neurodegenerative conditions. However, the very recent years, p75NTR has two sharply contrasting aspects or characteristics: not only induces cell death, but, in a cell-specific manner, it can also rescue neuronal cells from apoptosis or even induce the proliferation of their precursors.

The scientific community has spent the last thirty years trying to understand how the complicated function of p75NTR affects the neuronal cells. Its pleiotropic expression in a variety of tissues and its paradoxical survival/apoptotic function, have made its study a fascinating task in a variety of developmental and pathological processes. Furthermore, recent research has identified p75NTR as a novel regulator of adult neurogenesis and a target for preventing neurodegeneration in AD.

Pioneering work by Altman and Das (1965) first identified adult neurogenesis in the subgranular zone of the DG in rodents, and the following studies have confirmed that the DG supports adult hippocampal neurogenesis in humans (Altman & Das, 1965; Eriksson et al., 1998). The hippocampus, a brain area critical for learning and memory, is especially vulnerable to damage at early stages of AD. Emerging evidence has indicated that altered neurogenesis in the adult hippocampus represents an early critical event in the course of AD (Mu & Gage, 2011). Although, recent contradictory studies question even the existence of adult neurogenesis and thus its significance (Boldrini et al., 2018; Sorrells et al., 2018), very recent pioneering work by Choi and colleagues has shown the importance of adult neurogenesis in combination with exercise to prevent AD phenotype, using the 5xFAD mouse model that we also use (Choi et al., 2018).

The "end-point" of many neurological diseases, including AD, is neuronal cell death. Cognitive dysfunctions in dementia are caused by the apoptotic death of hippocampal and cortical neurons, while neuronal loss is sometimes seen as a natural part of aging. The most typical cause of dementia is AD. Globally, a new diagnosis is made every seven seconds, turning the disease into a worldwide pandemic (Ferri et al., 2005). According to Anand et al. (2014), there is no effective treatment for AD at the moment and according to Kodamullil et al. (2017), numerous clinical trials of potential

medications that target tau or amyloid dysfunctions have been unsuccessful (Anand et al., 2014; Kodamullil et al., 2017). It seems that the available treatments so far (cholinesterase inhibitors, memantine and recently licensed anti-amyloid antibodies), offer a solution only for the symptoms and partially reduce the disease's progression. Slowing or stopping disease progression and ultimately increasing neurogenesis to restore neuronal loss would be an effective strategy for treating neurodegenerative diseases (Magavi et al., 2000; Mu & Gage, 2011).

Thus, there is a growing interest in using neuroprotective and neuroregenerative techniques that can stop or even reverse the progressive loss of neuronal function as a result of a neurodegenerative disease. The detailed study of adult neurogenic potential and the pharmacological targeting of neural stem cells in the adult brain could pave new therapeutic avenues for treating neurological diseases, particularly through the exploitation of endogenous regenerative capacity. The detailed mapping of single cell properties and functions, based on the tempo-spatial expression of multifunctional regulatory p75NTR, could provide an advantage for controlling neurogenesis and the related cognitive functions. In order to bypass this limitation and enhance the endogenous neurogenesis, we propose to genetically characterize and decipher the multiple and extremely variable p75NTR signal transduction pathways in NSCs of the adult brain. Moreover, recent research findings have also introduced p75NTR as a novel and significant target for understanding major pathophysiological components of AD. However, no detailed mechanism on the aforementioned results has been proposed, while contradictory results have emerged (Dokter et al., 2015).

The present study deciphers the p75NTR's role in NSCs to confirm its therapeutic potential and suggest it as a tool to enhance neurogenesis. We investigate its role by studying various animal models *in vivo* and mouse cells *in vitro*. We also use human neural progenitor cells to verify the activity and functional implications of p75NTR signaling. In this line, human iPSCs-derived NPCs could provide access to a relevant biological system in which p75NTR could be targeted and further studied. We explore p75-dependent effects on human iPSCs-derived NPCs, in order to validate the existing animal models but also to offer a novel platform for drug screening of new compounds with neuroprotective and neurogenic effects against human AD. One of the major limitations in drug development is the discrepancy that is observed between animal models and human responses to a new compound. Thus, the validation of animal models and its correlation with human studies could importantly minimize the risk of failure, while the preclinical testing of new compounds in human tissue could significantly enhance drug selection and accelerate drug development.

Based on the existing knowledge, our aim in this PhD thesis was firstly to investigate the role of p75NTR in adult neurogenesis, and secondly to study this interplay under neurodegenerative conditions. Our study is deciphering the p75NTR role in adult neurogenesis using *in vivo* mouse models such as the p75 knock out under control (wild type background) or AD (crossing p75KO with the 5xFAD mouse model for AD). To determine whether p75NTR plays a role in dentate gyrus progenitor proliferation and/or differentiation, p75knockout and wild type mice underwent the 5 days BrdU injection protocol and proliferation and differentiation markers were assessed. Quantification assessment revealed a decreased number of both $BrdU^+$ / Sox_2^+ cells in p75knockout mice (Figure 13), pointing out the necessity of the p75NTR on adult neurogenesis. Most studies that were done in p75NTR deficient mouse model on exonIII, revealed also this decrease in knock out mice (Bernabeu & Longo, 2010; Catts et al., 2008). However, if we take into consideration how the deletion of p75NTR gene is achieved, we can observe a variant of contradictory results. The specific knock out model that we have used, has a deletion on exon III of p75NTR gene and still express the short isoform of the receptor (Nykjaer et al., 2005).

To determine whether the decrease in progenitor proliferation in p75 ko dentate gyrus was associated with altered differentiation of progenitors into neurons, sections from the above mice were assessed for Dcx expression and we observed an increase not only at the number of Dcx^+ cells in knock out animals but also in the area of processes from Dcx^+ cells (figure 18). Furthermore, in p75 ko mice, stereological estimates of $BrdU^+$ / $NeuN^+$ cells are showing a decrease compared to wild type mice (Figure 19), suggesting that the immature neurons are getting trapped at the state of Dcx^+ cells and cannot exit cell cycle, proceed and differentiate into neurons. Indeed, it has been shown that p75NTR can regulate the cell cycle and facilitate the cell cycle exit of neuronal cells (Vilar et al., 2006; Zanin et al., 2016; Zhang et al., 2009). Thus, our study further supports that p75NTR is really important in differentiation processes. We show that p75 receptor is promoting survival and/or differentiation of cells undergoing neuronal differentiation and it is necessary for this action. In order to evaluate the p75NTR-dependent potential for glial differentiation too, the study of the expression of a glial marker could also be examined. An increasing number of studies have shown that p75 can regulate differentiation of neural, non-neural cells and subventricular zone neuroblasts (Frade, 2000). Therefore, the mechanism which p75 regulates hippocampal neurogenesis is perhaps due to the failure of the cells to differentiate or mature appropriately. Moreover, one could expect that p75NTR deficient mice should display reduced rates of cell death. Nevertheless, Catts et al. showed that p75NTR knock out mice have an increased number of cell death (Catts et al., 2008). This fact, is maybe associated with our findings on decreased number of $BrdU^+$ / $NeuN^+$ cells in knock out mice. Thus, it seems that loss of p75NTR is promoting cell death and not neuronal survival as expected. Furthermore, it is known that this

mouse line that was used in the present study (deletion of exonIII) can still express the short p75NTR isoform (Paul, 2004; von Schack et al., 2001). Because of that, these animals do not express a p75NTR protein capable of ligand binding, but the truncated proteins mimic naturally occurring proteolytic fragments of p75NTR and are capable of proapoptotic signaling (Murray, 2004; Paul, 2004).

p75NTR was shown to be expressed in the DG (Barrett et al., 2016) and recent fate-mapping experiments revealed its expression by progenitor cells located in the SGZ and by cells of the neuronal lineage (Bernabeu & Longo, 2010). In our study, we haven't observed the expression of p75NTR at the DG of hippocampal formation, at least at wild type mice of 2 months old. This difficulty to exactly locate its expression in DG with immunofluorescence assays is probably due to the disability of available primary antibodies to detect p75NTR expression, or maybe because the detection threshold of immunofluorescence stainings of p75NTR expression in DG is really low (Figure 11). On the other hand, it seems that in wild type mice of 6 months old, we can detect the expression of the receptor specifically in the hilus of hippocampal formation (Figure 12), due to the fact that p75NTR expression is elevated according to cell death observed in 6 months old (Figure 17 & 23). According to literature, we assumed that p75NTR$^+$ cells here, are probably NSCs and glial cells like astrocytes.

Based on the aforementioned results about the expression of p75NTR in NSCs, we assume that the effects of p75NTR on the population of BrdU$^+$ / Sox$_2^+$ cells, Dcx$^+$ and BrdU$^+$ / NeuN$^+$ cells, might not be solely related to directly cell autonomous functions of the receptor in newborn cells, but also related to a cholinergic input or the association with striatum or hypothalamus as well. Related to this thought, our results are showing the expression of the receptor in Basal Forebrain, hypothalamus and striatum of 2 months old mice (Figures 10 & 11), as well, implicating a role for p75NTR being expressed by other cell types in other regions, that have an input in the hippocampus.

To be sure about the non-autonomous mechanisms that p75NTR is taking advantage of to regulate the proliferation and differentiation of NSCs, we study another mouse model with a specific deletion of exonII of p75NTR gene, only in Nestin$^+$ cells using a Cre recombinase system. Interestingly, we observed no differences in the number of proliferating NSCs and also in the number of immature neurons (Figures 16 & 22). Thus, it seems like p75NTR being expressed by NSCs has no role in adult neurogenesis and differentiation of these cells, and we assume that there is a non-autonomous mechanism that is being activated.

Cholinergic input in hippocampal DG – How it affects Adult Neurogenesis

According to the cholinergic input mentioned above, it seems like the hippocampus receives several inputs from the basal forebrain cholinergic system. The cholinergic system also seems likely to regulate hippocampal neurogenesis in the adult, positively promoting proliferation, differentiation, integration and potentially survival of newborn neurons. Given the crucial role of the cholinergic system for normal cognitive functioning and age-related dementia disorders, there is an influence of ACh on the formation of new hippocampal neurons and its relation to learning and memory. (Bruel-Jungerman et al., 2011). The evidence that ACh does regulate adult hippocampal neurogenesis comes from studies examining the consequences of experimental cholinergic forebrain lesions on proliferation, survival and apoptosis of newborn granule neurons in rodents (Mohapel et al., 2005). Poser et al., showed that cholinergic innervation is also increased in the DG of p75NTR deficient mice, which is likely to be a consequence of the increased numbers of cholinergic forebrain neurons (Poser et al., 2015). Thus, it seems that because of p75NTR deficiency, cholinergic input in the DG is being increased and the increased ACh levels are at least partially responsible for an increased neurogenesis. Interestingly p75NTR knock out mice used here would be expected, to have increased rather than decreased neurogenesis. However, it seems that alterations of intrinsic signaling mechanisms in progenitors are likely to account for that decrease (Bernabeu & Longo, 2010). However, if we take into consideration, that the increase in DCX cells that we observe in p75NTR knock out mice, is related to neurogenesis, we can say that indeed p75NTR deficiency, is increasing the cholinergic input in the DG and the increased ACh levels are responsible for an increased neurogenesis.

Furthermore, we can observe the expression of p75NTR in hypothalamus and striatum (Figure 11c, d). p75NTR is expressed in AgRP neurons and is necessary for the arcuate hypothalamus response to fasting (Podyma et al., 2020). On the other hand, the striatal cholinergic interneurons (CINs) are the main source of acetylcholine in the striatum and it is believed to play an important role in basal ganglia physiology and pathophysiology (Mallet et al., 2019). However, the role of p75NTR expression in these two brain regions, seems that has no relation with neurogenesis observed in the DG of hippocampus.

Neurogenesis levels in 5xFAD mouse model

We have also evaluated the impact of adult neurogenesis in an AD mouse model, the 5xFAD mouse of AD. Even though, we were waiting to observe a decrease in the neurogenesis levels of 2 months old mice, compared to wild type mice, we observed a significant increase, not only in both $BrdU^+$ / SOX_2^+ cells, but also in DCX^+ cells (Figures 14 and 20). Searching for other studies with the same results, we took into account the one contacted by Ziegler-Waldkirch et al. in 2018, where they determined

proliferation and neurogenesis in 5xFAD transgenic mice at different ages (6 weeks, 4 and 8 months) and compared them to their WT littermates. The number of DCX positive neuroblasts and immature neurons was significantly increased in 4-month-old 5xFAD mice when compared to WT controls at the same age. Likewise, the number of Ki67-positive proliferating cells in 5xFAD mice was significantly increased (Ziegler-Waldkirch et al., 2018). We propose that the increase observed in our results, is due to the fact there is a compensatory, homeostatic mechanism against the neurodegenerative effects of AD-like pathology that could possibly reverse the neuronal loss, generally seen at the onset of the disease, which is typically observed at around 4 months old.

Generally, there is a big variety of different aspects on how β-amyloid is affecting the proliferation and differentiation of NSCs. Even though Aβ peptides are considered neurotoxic, they can mediate many biological processes, both in adult brains and throughout brain development, and it seems that there are no many studies that do not agree with this statement. However, the problem is when we refer to the isoforms of amyloid, meaning Aβ40 and Aβ42, which are both present in amyloid plaques or when we refer to the concentrations of Aβ and the forms that are being created, meaning monomers, oligomers and fibrils. Moreover, the toxic effects of oligomeric Aβ depends on whether the peptide is found in a soluble or insoluble form (Amemori et al., 2015). Some studies have shown that soluble forms of Aβ oligomers exhibit strong neurotoxic effects and an increase in soluble oligomeric Aβ levels could be a potential cause of AD (Kayed & Lasagna-Reeves, 2012; J. Y. Kim et al., 2003) suggesting that soluble oligomeric Aβ peptides are the most toxic species (particularly to neurons) and is believed to be involved in problems of memory, dementia and synaptic depletion (Cleary et al., 2005; Dahlgren et al., 2002; Heo et al., 2007; Walsh et al., 2002).

Aβ42 peptide is known to be more abundant than Aβ40 within the plaques due to its greater capacity for self-aggregation. In addition, a few studies have shown that Aβ42 is more toxic than Aβ40 both *in vivo* and *in vitro*. On the other hand, the Aβ40 peptide is the major Aβ species generated by neurons in physiological conditions. In different pathologies like AD, the Aβ40 levels are diminished, which provokes a rise in the Aβ42/Aβ40 ratio and the following increase of Aβ42 levels (J. Y. Kim et al., 2003). When Bernabeu-Zornosa et al. used Aβ40, demonstrated that at high concentrations provokes apoptotic cellular death and the damage of DNA in human NSCs, while also increasing the proliferation and favors neurogenesis by raising the percentage of proliferating neuronal precursors (Bernabeu-Zornoza et al., 2019). While on the other hand, Lopez-Toledano showed that αβ peptide does not impair the neurogenic rate in NSCs progeny, but it increases the total number of neurons *in vitro* in a dose-dependent manner and neurogenesis is induced by Abeta42 and not Abeta40 (Lopez-Toledano, 2004). Furthermore, secreted amyloid precursor protein (sAPP) increased

the differentiation of human neural stem cells (hNSCs) *in vitro*, and hNSCs were differentiated into astrocytes rather than neurons (Kwak et al., 2006). As mentioned above, Aβ peptide in its monomeric form and at low concentrations may be neuroprotective, and some studies have shown that monomeric Aβ peptide enhances the survival of hippocampal neurons *in vitro* (J. Kim et al., 2007; Whitson et al., 1990).

According to that point of view, in 5xFAD of 2 months old that we are using in this PhD thesis, it is still early enough for the fibrils to be created, so maybe the concentration and the forms of Aβ amyloid can enhance, at least at this stage the proliferation and differentiation of NSCs.

Neurogenesis levels in 5xFAD_p75KO mouse model

In our next step, we analyzed the deletion of p75NTR in an AD background, using the 5xFAD_p75ko mouse model, derived from crosses between 5xFAD and p75 knock out mice (with the deletion of exon III). Deficiency of p75NTR in 5xFAD 2 months old mice reversed the increased proliferation of NSCs, that we observed in 5xFAD mouse model (Figure 15). Thus, it seems that p75NTR's deletion is strong enough to abolish the high proliferation from 5xFAD model and drop the level of proliferation lower than this of wild type mice. However, p75NTR deficiency in 5xFAD 2 months old mice does not affect the production of immature neurons (Figure 21). The levels of DCX^+ cells in these mice are the same with wild type mice, but if we compare these mice with their control groups, meaning 5xFAD model and p75 ko, we observe a significant reduction of immature neurons.

If we take into consideration all of the above information discussed here, we could say that in 5xFAD mice, where p75NTR expression is present, αβ amyloid could probably interact with the receptor, acting by leading to neuroprotection in general. However, in 5xFAD_p75ko mice, because of the deletion of the receptor, αβ amyloid cannot act on neuroprotection. Maybe this is why, we have a decreased number of proliferative NSCs and DCX^+ cells. The result of DCX^+ cells having the same levels with the wild type, may be derived by the fact that other factors are also playing a significant role in neurogenesis (for example the increased levels of Ach, especially in p75ko mice) and the p75NTR expression (WT mice) or not (p75ko), might not be so crucial after all.

Our *in vivo* experiments in mouse models are generally relying on studies on 2 months old. Having in mind that we want to expand our knowledge in different age groups, we also tested mice of 6 months old. Interestingly, when we compared 6 months old mice derived from different experimental control groups, such as wild type, p75 knock out and 5xFAD mice, we had no significant changes, neither in both $BrdU^+$ / SOX_2^+ cells, nor in DCX^+ cells (Figure 17, 23). We hypothesized here, that these mice of 6 months old are old enough to have decreased number of cells in general, making it difficult to observe any differences at all, between the tested group. Comparing wild type mice

of 6 months old and 2 months old age, we can see that there is a really significant difference between the numbers of both $BrdU^+$ / SOX_2^+ and DCX^+ cells (approximately around 75% difference) (Figure 17, 23). According to Yang et al., the rates of NSCs proliferation were largely reduced by about 85% during the time frame of 3 months old mice to 9 months old (Yang et al., 2015). Furthermore, using a fluorescence-activated cell sorting technique that allows for the prospective purification of the main neurogenic populations from the subventricular zone (SVZ), Daynac et al., demonstrated an early decline in adult neurogenesis with a dramatic loss of progenitor cells in 4 months old young adult mice. Whereas the activated and quiescent NSC pools remained stable up to 12 months, the proliferative status of activated NSCs was already altered by 6 months (Daynac et al., 2016). Generally, it seems that physical function progressively declines, starting as early as 6 months of age in mice and importantly, functional aging of male C57BL/6J mouse starts at younger relative ages compared to when it starts in humans (Yanai & Endo, 2021).

Having no differences in our six months old mice of all ages, we haven't proceeded with our experimental protocols to 5xFAD_p75KO mice. Our thought here is to study what is happening in 4 months old mice, where the differences would be observable. Furthermore, 5xFAD mice that we have used like a mouse model of AD, rapidly develops severe amyloid pathology, accumulating high levels of intraneuronal Aβ42, beginning around 1.5 months of age and extracellular amyloid deposition begins around 2 months (Oakley et al., 2006). However, spontaneous alternation in the Y-maze, a measure of spatial working memory, is impaired beginning at approximately 4 to 5 months of age (Devi & Ohno, 2010; Oakley et al., 2006) and in the hippocampus and cortex, the most pronounced differences to have emerged thus far involve genes related to neuroinflammation and their differential expression is apparent by 4 months (Landel et al., 2014) and persists until at least 18 months (Siwek et al., 2015). Furthermore at 4 months old, 5xFAD mice just start to develop plaques, whereas plenty of Ab deposits could be detected by 6E10 immunoreactivity in 8-months old 5xFAD mice (Ziegler-Waldkirch et al., 2018). Moreover, basal synaptic transmission and LTP in hippocampal area CA1 begin to deteriorate between 4 and 6 months (Crouzin et al., 2013; Kimura & Ohno, 2009). Thus, it is really important to study and observe any differences in neurogenesis, when significant characteristics totally implicated in AD, start arising in this mouse model, meaning at the age of 4 months old. All the results from our *in vivo* experiments are summarized in Figure 37.

Mice used		p75 KO vs wild type	5xFAD vs wild type	5xFAD/p75 ko vs 5xFAD	p75 floxed vs wild type
2 months old mice	BrdU/Sox2	⇩	⇧	⇩	--
	Dcx	⇧	⇧	⇩	--
	NeuN	⇩			
4 months old mice		To be studied…			

Mice used		p75 KO vs wild type	5xFAD vs wild type	p75 floxed vs wild type	2mo wt vs 6mo wt
6 months old mice	BrdU/Sox2	--			⇧
	Dcx	--			⇧

Figure 37. Summary of the results gained by our *in vivo* experiments in different genetic backgrounds of mice.

p75NTR and its role in human iPSCs – derived NPCs

Last but not least, we demonstrated that human NPCs produced by hiPSCs (derived from healthy individuals) express p75NTR for the first time to our knowledge (Figure 25). Furthermore, we evaluated the downstream mediators of the p75NTR signaling pathways, RIP2 and TRAF6, in human NPCs, indicating an active state of p75NTR signaling in these cells. The actual interaction of p75NTR with the aforementioned TRAF6 protein also confirmed with co-IP also confirming that p75 NTR is active in these cells (Figure 25).

Interestingly, in human NPCs derived from an ApoE4 mutation carrier we can observe that there is a really decreased expression of p75NTR. Having in mind, that p75NTR and its role in human NPCs is not yet clarified, we do not know exactly why this is happening, especially in an ApoE background and having only one sample derived from an AD patient with an ApoE4 mutation. However, we could hypothesize that there is an interaction between p75NTR and APOE4 in patients with AD in general. It is known that the apolipoprotein E (APOE) gene is one well-known gene that influences Alzheimer's risk. It is involved in making a protein that helps carry cholesterol and other types of fat in the bloodstream. Problems in this process are thought to contribute to the development of Alzheimer's. APOE comes in several alleles and APOE4 increases risk for Alzheimer's and is associated with an earlier age of disease onset in certain populations (J. Kim et al., 2009). As we have already discussed, it seems that the extracellular domain of p75NTR, plays an important role in the solubility of Aβ and might be one of the endogenous mechanisms in the regulation of Aβ plaque formation (Y.-J. Wang et al., 2011). According to Zhou & Wang, they found that p75NTR knockout increases the insoluble Aβ as reflected by the increased amyloid plaques and formic acid-extracted Aβ levels (Zhou & Wang, 2011). On the other hand, it is likely that ApoE protein plays a critical role in the solubility of Aβ, as well (Kim et al. 2009). The reduction in the Aβ binding ability of ApoE4 may

reduce the solubility of Aβ and it seems that ApoE4 variant may reduce Aβ solubility and increase aggregation in the brain (Zhou & Wang, 2011). Thus, it seems that in a healthy brain, the extracellular domain of p75NTR can act by increasing the solubility of αβ and preventing the creation of amyloid plagues. However, AD patients who are carriers of an APOE4 mutation, have a decreased solubility of αβ and an increased creation of αβ aggregates, which is exactly the same with what is happening in p75ko mice. Having all of these in mind, we could hypothesize that according to our results, there is a mechanism that may affect the expression of p75NTR by decreasing it (so the extracellular domain, cannot act on the solubility of αβ), allowing APOE4 to act by decreasing the solubility of αβ. More trials are needed here, to be sure about those speculations.

In order to validate our results from our *in vivo* experiments on mouse models and to observe whether there are any differences according to the origin of the cells that we are using, we evaluated the effect of p75NTR blocking on proliferation and differentiation processes. More specifically BrdU assay in NPCs lines derived from healthy individuals, revealed no alteration in the proliferation rate of the cells when p75NTR is inhibited (Figure 27) suggesting that p75NTR is not actively involved in human NPCs proliferation. Due to the fact that this result is totally different from the results derived from our mouse models, we took into consideration the possibility that p75NTR inhibitor is not working properly. More specifically, p75NTR blocking antibody, named MC192, has been used for blocking the activation of p75NTR in many published works (Eleftheriadou et al., 2014; Kimpinski et al., 1999; Rogdakis et al., 2022) although other studies support that MC192 is not at all suitable as a blocking agent, due to the fact that it was generated specifically by screening for monoclonals that had the ability to enhance the binding of NGF, the natural ligand for p75. Therefore, this antibody is particularly unusual (Chandler et al., 1984). However, the effect of MC192 on p75NTR depends on the concentration that is going to be used in each experiment. The best solution to avoid a problem like that, would be to use shRNA for silencing p75NTR gene instead of using an antibody for blocking, experiments that are going to follow the next months.

Interestingly, when we used human iPSCs-derived NPCs and we differentiated them to neurons, after 13 days DCX^+ cells are increasing in number when p75NTR inhibitor is used. Moreover, the processes of DCX^+ cells seem longer, exactly like the results we have gained from our mouse models (Figure 28). After 8 more days (day 21), $Tuj1^+$ cells seem less, when p75NTR inhibitor is used, which means that p75NTR is also important for NPCs to differentiate to mature neurons (Figure 28). Even if these results derived from one trial, it is really promising to gain exactly the same results with our *in vivo* experiments. Thus, it seems that p75NTR plays a crucial role in differentiation of human NPCs.

In a second step, we wanted to see the interaction between p75NTR and AD. According to previous studies it seems that p75NTR is an important player in regulating Aβ deposition in AD patients (Y.-J. Wang et al., 2011b; Yao et al., 2015). On the one hand, αβ amyloid can act like ligand and by activating cell death signaling pathways (Knowles et al., 2009; Saadipour et al., 2013; Yaar et al., 1997). On the other hand, p75NTR expression is protective against Ab-induced toxicity (Bengoechea et al., 2009; Sotthibundhu et al., 2009; Yao et al., 2015). To see the interaction of p75NTR and αβ amyloid in human NPCs, we treated cells with or without p75NTR blocking antibody and aβ1-42 oligomers. The results of Celltox assay provided evidence that p75NTR negatively influences human NPCs survival after treatment with aβ peptides (Figure 26) indicating a regulatory role of p75NTR in NPCs pathology of AD. Thus, it seems that αβ amyloid is acting like ligand for p75NTR activating cell death. Additionally, inhibiting p75NTR expression might overcome the cell death observed because of toxic αβ oligomers and maybe it could also lead to elevated levels of Ach, which would affect inadequate neurogenesis, according to previous thoughts analyzed above.

Thus, it is an imperative need in the p75 neurotrophin receptors' field to decipher the specific signaling properties of the receptor in a cell-specific manner (since the receptor is expressed in almost all cell types of the nervous system) in relation to the final cellular phenotype, under physiological and neurodegenerative conditions. In this way, and only if you take into consideration the exact contribution of each cell (NSC, mature neuron, astrocytes, oligodendrocytes and microglia) involved in this complex phenomenon, the different p75NTR-signaling on these cells, and by resembling the physiological architecture of the stem cell niche, we could efficiently also develop and test new drugs. Moreover, the use of human cells could improve the translational outcome and pave the way for precision pharmacology for AD.

Conclusively, the aforementioned scientific breakthrough that is developing in this Doctoral Thesis, represents an ambitious and concrete step towards the long-term vision of our studies. We strongly believe that the knowledge of the p75NTR's role in adult neurogenesis, rodent and human origin, will provide an important benefit for developing novel therapeutic applications against AD pathology.

Activation of p75 Neurotrophin Receptor by Neurotrophin Analog ENT-A044

Dehydroepiandrosterone (DHEA), an endogenous neurosteroid which is a small sized, lipophilic molecule, has been demonstrated by our research team to have the ability to act on TrkA and p75NTR and inhibit neuronal death (Charalampopoulos et al., 2004; Lazaridis et al., 2011). The long-term use of DHEA as a potential treatment for neurodegeneration, however, is problematic, especially in patients with genetic predisposition to hormone-dependent tumors (breast, endometrium, ovaries, prostate, etc.) because it is metabolized *in vivo* to estrogens and androgens, which affect the endocrine system (Calogeropoulou et al., 2009a; Compagnone & Mellon, 2000).

Calogeropoulou et al. created a chemical library of synthetic C17-derivatives of DHEA (Calogeropoulou et al., 2009) with anti-apoptotic properties but no androgenic/estrogenic effects that accompanies the use of DHEA. These new compounds are BBB-permeable and, more critically, they mimic the neuroprotective and neurogenic actions of endogenous neurotrophins by acting like them. It has been demonstrated that the C17-spiroepoxy steroid derivative BNN27 interacts and activates the TrkA receptor in a manner that selectively causes the phosphorylation of TrkA tyrosine residues and downstream neuronal survival-related kinase signaling (Pediaditakis, Efstathopoulos, et al., 2016). Additionally, it can interact with p75NTR, leading to the recruitment of the effector proteins RIP2 and TRAF6 to the receptor and the release of RhoGDI in the cerebellum's main granular cells in addition to causing neuroprotective effects (Pediaditakis, Kourgiantaki, et al., 2016). Recently, Rogdakis et al. demonstrated that ENT-A013 specifically activates TrkA receptor and exerts neuroprotective and anti-amyloid activities (Rogdakis et al., 2022). It is a chemically stable and more powerful molecule than BNN27.

In the current study, one of these synthesized molecules is being tested. Although it activates both TrkB and p75NTR neurotrophin receptors, the effects on the cells varies depending on which receptor is expressed and, interestingly, whether the neuronal cell derives from a mouse or a human species. Notably, ENT-A044 is a molecule that shares structural similarities with ENT-A013, a well-studied TrkA activator that was created using the same synthetic techniques (Rogdakis et al., 2022). However, it is clear from the complete lack of phosphorylation of the most important downstream signals of the TrkA receptor in TrkA-expressing PC12 cells, that ENT-A044 cannot activate TrkA. On the other hand, we noticed TrkB phosphorylation and Akt phosphorylation in cells expressing TrkB, demonstrating that ENT-A044 treatment activates signaling pathways mediated by TrkB. We looked more closely at our initial finding that ENT-A044 causes cell death in PC12 cells, which also express TrkA and p75NTR after serum deprivation-induced apoptosis and asked whether p75NTR specifically mediated this impact.

According to this theory, ENT-A044 had no effect on cell death in naive HEK293T cells but our chemical compound raised the levels of cell death in transiently transfected HEK293T cells that solely express p75NTR. As a result, it is clear that p75NTR activation is what causes cell death in response to therapy with ENT-A044. More specifically, the JNK protein, which is known to be involved in cell death signaling pathways that arise from the activation of p75NTR (Charalampopoulos et al., 2012), is activated in order to predominantly carry out this death signal.

The majority of the signaling functions of p75NTR, a member of the TNFR superfamily, are provided by the Death Domain located at its intracellular domain (Charalampopoulos et al., 2012). Its effects can range from cell survival to cell death depending on the ligand that activates the receptor and the kind of the cell, and the co-expression of Trk receptors or other co-receptors, like sortilin or Nogo, can also distinguish the final cellular fate (Lu et al., 2005; Meeker & Williams, 2015). The activation of NFkB following RIP2 recruitment has been linked to the promotion of neuronal survival by p75NTR (Hamanoue et al., 1999). More specifically, the recruitment of RIP2 protein to p75NTR activates the transcription factor NFkappaB, which in turn triggers survival signals on particular populations of neuronal cells (Carter et al., 1996; Vicario et al., 2015). In transiently transfected HEK293T-p75 cells, ENT-A044 effectively promoted the association and interaction between p75NTR and its effector protein RIP2, however it showed no appreciable effects on the relationship with TRAF6 protein, which causes cell apoptosis. A significant concern has arisen regarding the potential triangular connection between RIP2, p75NTR, and the TrkB receptor, which when combined leads towards survival signaling pathways. This is because ENT-A044 stimulates both TrkB and p75NTR, resulting in cell survival.

In primary cultures of mouse P7 hippocampal NSCs and transiently transfected HEK293T-TrkB/p75 cells, ENT-A044 can promote the survival signaling pathway initiated by the TrkB receptor and suppress cell death, which is induced by p75NTR in the absence of TrkB. The protective RIP2 signaling is overcome by the p75NTR-dependent stimulation of cell death, which most likely happens as a result of a large induction of the pro-apoptotic JNK signal. All of ENT-A044's signaling pathways are listed together with their biological effects in Figure 38.

Finally, we demonstrated that human NSCs produced by hiPSCs express p75NTR for the first time to our knowledge. The TrkB receptor is also expressed by these cells. The effects of ENT-044 on human neural precursor cells are entirely different from those observed in mice cells or cell lines, where both receptors were co-expressed, and result in a considerable induction of cell death. This apoptotic action is solely mediated by p75NTR as TrkA receptor is not expressed, and NGF treatment enhanced the levels of pJNK as well. These cellular differences between mouse and human cells may be attributable to endogenous neurotrophins and ENT-A044 having different affinities for

the responsive receptors as well as having distinct signaling capacities as a result of these differences.

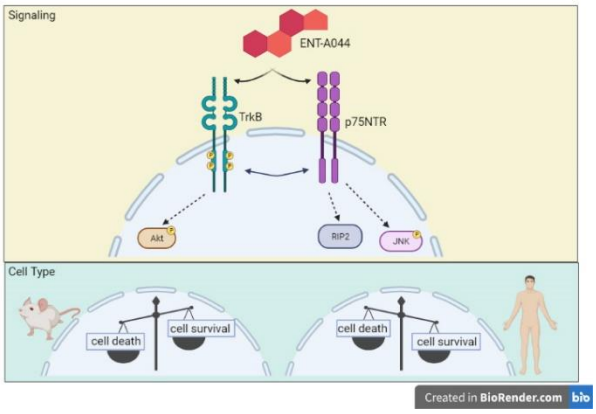

Figure 38. ENT-A044 is shown to result in cell survival in mouse P7 hippocampal NSCs, as it co-expresses both TrkB receptor and p75NTR. In contrast, in iPSCs-derived neuronal precursors, expressing both p75NTR and TrkB receptor, ENT-A044, results in cell death. Thus, ENT-A044 is able to activate both receptors, resulting in differential signaling in a cell type-specific manner, depending on the species of origin—mouse or human—of the neuronal cells ("Figure created with BioRender.com").

Furthermore, based on earlier research, it's probable that ENT-A044 is modifying the receptor's structure as well as how the p75NTR interacts with its co-receptors, such as Trks or sortilin (Nykjaer et al., 2005). We have demonstrated that the first compound we tried, BNN27, has different binding sites in TrkA and p75NTR than NGF, which supports this theory (Pediaditakis, Efstathopoulos, et al., 2016; Pediaditakis, Kourgiantaki, et al., 2016).

Last but not least, by utilizing ENT-A044's role in inducing cell death through p75NTR, we may be able to use it therapeutically to treat diseases like cancer by focusing on the eradication of malignant cells. The function of p75NTR in cancer is debatable. It has been demonstrated to function as a tumor suppressor and a prognostic factor in cancers, but it has also been found to contribute to tumor aggressiveness (Blondy et al., 2019; S.-D. Huang et al., 2009; Jin et al., 2007; Okumura et al., 2003, 2006; Yuanlong et al., 2008). Because many cancers, including neural crest-derived melanoma, display high levels of p75NTR, Goh et al. demonstrated that NSC49652, a small chemical that targets the p75NTR transmembrane domain, can induce apoptosis in melanoma cells by acting through p75NTR (Goh et al., 2018).

The controversial nature of the p75NTR is highlighted by all of the aforementioned facts, and more research on the signaling pathways and processes that this receptor affects is required. The pleiotropic signaling cascades and several different cellular effects that result from p75NTR activation are one of its drawbacks. Therefore, it would be quite fascinating to integrate data from -omics analysis at the level of a single cell when there are endogenous ligands and synthetic agonists in our future studies. Furthermore, *in vivo* studies of diseased animal models associated with the receptor's

effects, such as Alzheimer's Disease, may provide more precise evidence of the receptor's contribution to neurodegeneration. A useful experimental tool for analyzing the pleiotropic actions of these receptors in many cell types and species can be provided by molecules like ENT-A044. Small molecules with advantageous pharmacological characteristics, such as ENT-A044, may also provide new therapeutic options for the treatment of tumor and cancer growth as well as neurological diseases. Thus, cutting edge research is geared toward discovering and developing novel drugs that target specific p75 receptor-mediated adult neurogenesis in a specific manner. Neurogenic drugs might reverse or compensate deficits and impairments associated with neurological diseases and disorders in a more effective and less invasive way than the exogenous transplantation of neural precursors, since they can be pharmaco-dynamically controlled. Their potential for regenerative medicine is immense.

The findings of the present study will provide the pharmacological basis for proposing a brand-new therapeutic approach of neurodegenerative diseases via pharmacological activation of residual adult neural stem cell proliferation and migration towards the neuropenic, suffering brain areas of Alzheimeric brain.

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