"In vitro study of the effects of endogenous and synthetic neurotrophins in the survivability of neuronal cells."

by

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“In vitro μελέτη της επίδρασης ενδογενών και συνθετικών νευροτροφινών στην επιβίωση νευρικών κυττάρων”

του

Τσιμπόλη Αλέξανδρου

Διπλωματική Διατριβή

που υποβάλλεται στα πλαίσια εκπλήρωσης

των απαιτήσεων απόκτησης του μεταπτυχιακού τίτλου

στην

Μοριακή Βάση Νοσημάτων του Ανθρώπου – Τμήμα Ιατρικής

του

Πανεπιστημίου Κρήτης

Σεπτέμβριος 2020

Summary

Neurotrophins are necessary for the normal development and functioning of the central nervous system (CNS). Acting primarily through their tyrosine receptor kinase (Trk) receptors, they protect the neurons and ensure their survival and well-being. Dehydroepiandrosterone (DHEA) steroid hormone and its novel synthesized derivatives, microneurotrophins (BNNs), also exhibit significant neuroprotective properties, which they too exert through Trk signaling. In vivo experiments in 5xFAD mice, revealed decreased Aβ plaque formation in the hippocampus and increased proliferation and neuronal generation in the dentate gyrus, thus indicating a positive implication in adult neurogenesis. In glial cell populations, like astrocytes, neurotrophins can act through TrkB receptor and affect their reactive state and the subsequent cytokine expression profile. In the present thesis, we investigated the neuroprotective effects of BNN27 and BNN237 initially in P7 hippocampal NSCs. We provide evidence of increased proliferation in these cell populations under extreme stress conditions. We were also able to identify important receptors and downstream signaling pathways implicated in this process. Simultaneously, we begin examining their effects in reactive astrocytes and how these effects can alter the levels of pro- and anti-inflammatory cytokines in the surrounding microenvironment.

Περίληψη

Οι νευροτροφίνες είναι απαραίτητες για την φυσιολογική ανάπτυξη και λειτουργία του κεντρικού νευρικού συστήματος (CNS). Δρώντας κυρίως μέσω των υποδοχέων με δράση τυροσινικής κινάσης (Trk), προστατεύουν τους νευρώνες και εξασφαλίζουν την επιβίωση και ευημερία τους. Η στεροειδής ορμόνη δεϋδροεπιανδροστερόνη (DHEA) καθώς και τα καινοτόμα συνθετικά παράγωγά της, οι μικρονευροτροφίνες (BNNs), εμφανίζουν επίσης σημαντικές νευροπροστατευτικές ιδιότητες μέσω Trk σηματοδότησης. In vivo πειράματα που πραγματοποιήθηκαν σε 5xFAD ποντίκια, έδειξαν μείωση στον σχηματισμό αμυλοειδικών πλακών στον ιππόκαμπο ενώ ταυτόχρονα αύξηση του πολλαπλασιασμού και της νευρογένεσης στην οδοντωτή έλικα του ιππόκαμπου, υποδηλώνοντας έτσι την πιθανή ευεργετική τους επίδραση στην ενήλικη νευρογένεση. Σε πληθυσμούς γλοιακών κυττάρων, όπως τα αστροκύτταρα, οι νευροτροφίνες αλληλεπιδρούν με τον TrkB υποδοχέα και επηρεάζουν μέσω αυτού την ενεργοποίηση των αστροκυττάρων και ακολούθως το προφίλ έκφρασης των διαφόρων κυτοκινών. Στην παρούσα διατριβή, μελετήσαμε τις νευροπροστατευτικές ιδιότητες των BNN27 και BNN237 αρχικά σε P7 νευρικά βλαστοκύτταρα του ιππόκαμπου. Τα αποτελέσματά μας δείχνουν σημαντική αύξηση στον πολλαπλασιασμό των συγκεκριμένων κυτταρικών πληθυσμών ακόμα και σε συνθήκες ιδιαίτερα τοξικές για αυτούς. Καταφέραμε επίσης να αναγνωρίσουμε υποδοχείς αλλά και σηματοδοτικά μονοπάτια που εμπλέκονται σε αυτήν την διαδικασία. Ταυτόχρονα, ξεκινήσαμε να μελετάμε τις επιδράσεις των μικρονευροτροφινών στην ενεργοποίηση των αστροκυττάρων και πως αυτές επηρεάζουν τα επίπεδα των προ- και αντι-φλεγμονωδών κυτοκινών στο μικροπεριβάλλον τους.

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Introduction

Neurotrophins

Neurotrophins are growth factors expressed both in the brain and peripheral tissues. They play an important role in the proper development of the vertebrate nervous system by regulating neuronal survival and regeneration (Allen, Watson et al. 2011). They have also been implicated to many vital neuronal functions including synapse formation and axonal and dendritic growth (McAllister, Lo et al. 1995, Vicario-Abejon, Owens et al. 2002, Lykissas, Batistatou et al. 2007). There are four neurotrophic factors circulating the human body: Nerve Growth Factor (NGF), Brain-Derived Neurotrophic Factor (BDNF), Neurotrophin 3 (NT3) and 4 (NT4).

Neurotrophins are able to regulate all these neurogenic and neuroprotective actions via signaling through tyrosine receptor kinase (Trk) receptors (Huang and Reichardt 2003). There are three Trk receptors: TrkA, TrkB and TrkC. Not all Trk receptors interact with all of the neurotrophins. BDNF for example acts through TrkB receptor while NGF is recognized only by TrkA (Figure 1).

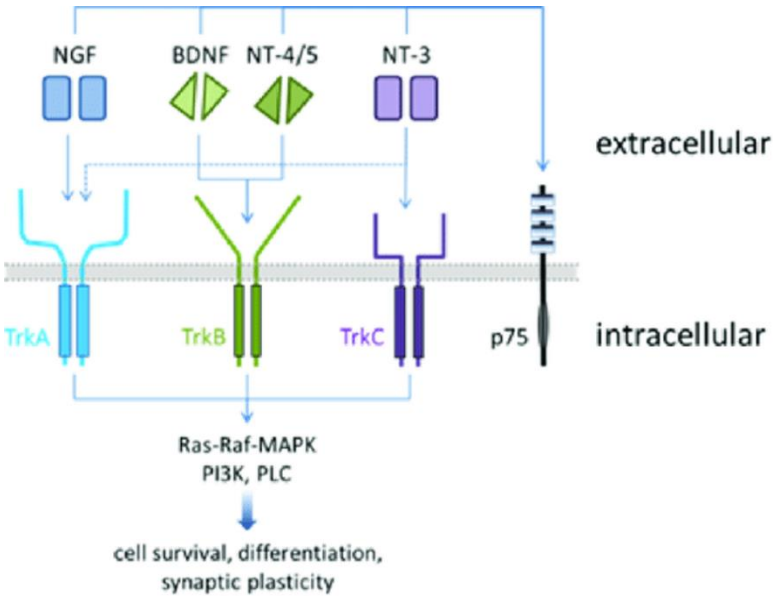


Figure 1: Diagrammatic representation of the different interactions between neurotrophins and their Trk and p75^{NTR} receptors (Skerratt, Andrews et al. 2016).

Sometimes though, neurotrophins can exert some apoptotic effects besides the neuroprotective ones. These actions are mediated by death receptor p75^{NTR} (Figure 1), a receptor with the unique ability to bind all neurotrophins (Dechant and Barde 1997). Although p75^{NTR} receptor was considered to be a principally proapoptotic receptor, this notion has started to change lately, as many evidences emerge implicating the receptor with neuronal survivability. Today, we know that p75^{NTR} receptor's functions are pleiotropic and their outcome highly depends on its complex interaction with other receptors (Becker, Cana et al. 2018).

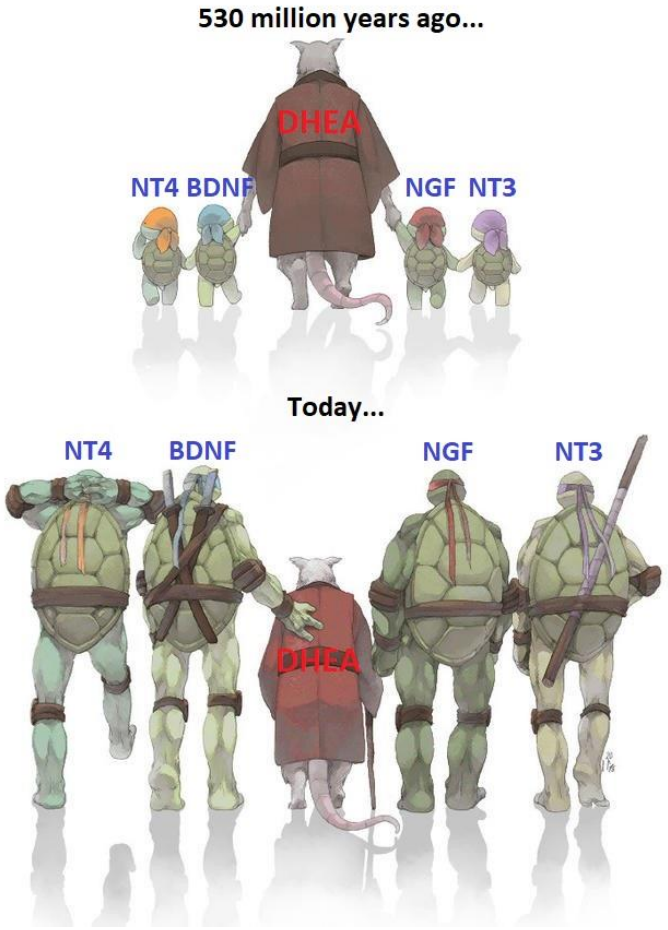
In the aged and degenerating brain, the levels of many neurotrophic factors have been found to be significantly diminished. This observation, along with the significant neuroprotective abilities that neurotrophins bare, turned the focus of many groups on how to utilize them for designing therapeutic approaches for neurodegenerative diseases. But it soon became clear that two characteristics of neurotrophins would render this task impossible. Their big molecular weight in combination with their non-lipophilic nature meant that they cannot freely cross the Blood-Brain Barrier (BBB) while at the same time denaturate quickly in the plasma. Thus, the need for the creation of a small lipophilic molecule with the ability to bind to Trk receptors and/or $p75^{NTR}$ receptor emerged.

Neurotrophin synthetic analogues: the "birth" of MicroNeurotrophins

Our group, for the past ten years is working with the most abundant neurosteroid in the human body, dehydroepiandrosterone (DHEA). DHEA is able to bind to and activate all three of the Trk receptors, as well as the $p75^{NTR}$ receptor, indicating a possible role as a neurotrophic factor as well.

DHEA mainly functions as a precursor for the metabolic biosynthesis of sex hormones, estrogen and androgen. It is also able to bind to many neurotransmitters (e.g. NMDA and $GABA_A$) and steroid hormone receptors (e.g. ERα and ERβ). DHEA's effects in the brain have been characterized as neuroprotective, a concept that comes in agreement with the observation that DHEA levels, just like neurotrophins, were found to be significantly reduced in patients with neurodegenerative disorders, as well as in the healthy aged population (Yanase, Fukahori et al. 1996, Bernardi, Lanzone et al. 2000).

DHEA's ability to bind to neurotrophin receptors, along with the fact that DHEA has preexisted in less complicated neural system, such as in the invertebrate amphioxus, in contrast to the other neurotrophins that only emerged after the need for more detailed regulation in organisms, such as vertebrates, with advanced neural system, lead us to the safe conclusion that DHEA may have been the first and only neurotrophic factor in the ancient less complex organisms, paving the way of neuronal survival for the neurotrophins to come (Meme 1).



Meme 1: Humorous representation of the evolution of neurotrophins, based on the existing hypothesis, that DHEA (represented as sensei Splinter In the Meme), acting as a neurotrophic factor in ancient organisms with simple neural systems, "conveyed" its neuroprotective abilities to the more evolved neurotrophins (represented as Leonardo, Michelangelo, Donatello and Raphael), to better serve and cope with the increased needs of the more complex and advanced neural systems observed in vertebrates today.

Previous work in our lab has unraveled the mechanism through which DHEA exerts its neuroprotective actions (Charalampopoulos, Tsatsanis et al. 2004). The neuroprotective mechanism of DHEA acts through the activation of TrkA and $p75^{NTR}$ receptor and their subsequent signaling pathways, the same way NGF does (Lazaridis, Charalampopoulos et al. 2011). Thus, its potential to fill in the gap for the therapeutic approaches in many neurodegenerative diseases that neurotrophins couldn't, immediately becomes obvious. But first, there was one barrier that we had to overcome. As we mentioned, DHEA is a steroid precursor for estrogens and androgens with many secondary effects via its binding to neurotransmitter and steroid hormone receptors, and its long-term use as a potential treatment would impair the sensitive hormonal balance inside the patient's brain.

For this reason, our group has recently tried to synthesize DHEA analogs that bear modifications at C3 and C17 positions with the expectation to retain the neuroprotective properties of DHEA and abolish its steroidal effects. After screening many such compounds, we concluded that the most potent compounds involved spiro-epoxy derivatives with modifications at C17 position. Through further screenings in compounds with these features, we were able to synthesize many DHEA derivatives with significant neuroprotective effects as well as the ability to bind to neurotrophin receptors (Figure 2). These small molecules are also highly lipophilic and thus able to permeate the BBB. We named these molecules "synthetic neurosteroidal microneurotrophins" or BNNs.

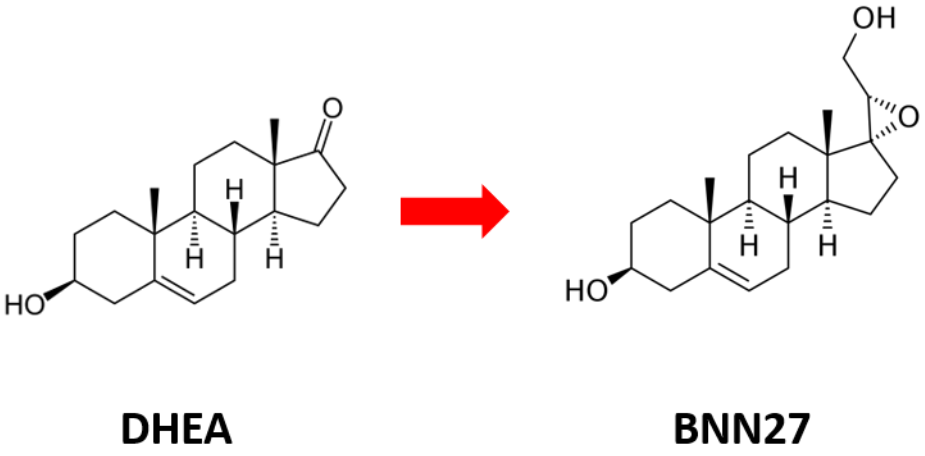


Figure 2: Chemical representation depicting the structural differences at C17 between DHEA and microneurotrophin BNN27.

Signaling through microneurotrophins

Studies performed all these years from our group in different microneurotrophin derivatives has led to the clarification of some of the receptors that interact with them and their downstream pathways. BNN27, is one of the most prominent synthetic neurotrophic analogues that our group has tested, with significant neuroprotective properties. We found that BNN27 interacts and activates both TrkA and $p75^{NTR}$ receptors, promoting this way neuronal survival. More specifically, when BNN27 interacts with NGF receptor TrkA (Figure 3A), downstream signaling through AKT and ERK pathways is activated leading to neuronal survival and differentiation (Pediaditakis, Efstathopoulos et al. 2016), while interaction with the $p75^{NTR}$ receptor (Figure 3B) blocks the activation of the pro-apoptotic factors JNK and Caspase-3 and promotes the activation of pro-survival factors Rho-GDI and RIP-2 (Pediaditakis, Kourgiantaki et al. 2016). BNN27 can also exert a synergistic effect promoting axonal outgrowth, when interacting with NGF directly (Figure 3A).

BNN20 is another well-studied microneurotrophin that our group synthesized. Extensive work using the BNN20 in the "weaver" mouse model, which is a Parkinson's Disease (PD) model with progressive dopaminergic neurodegeneration, illustrated the direct interaction of BNN20 with the TrkB receptor (Botsakis, Mourtzi et al. 2017). This interaction leads to the downstream activation of both the PI3K-Akt and the MEK-ERK pathways and subsequent regulation of the NF-kB expression (Figure 4). Due to the increased NF-kB activation, BDNF levels in the sick mice were restored to normal thus protecting dopaminergic neurons from apoptosis and promoting overall cell survival. BNN20 administration, also altered the microglia phenotype in the "weaver" mice to the neuroprotective, anti-inflammatory M2 phenotype (Panagiotakopoulou, Botsakis et al. 2020).

Results like the ones we just mentioned, reveal the extensive capabilities of the use of microneurotrophins as therapeutic interventions in many neurodegenerative diseases. Its unique neuroprotective effects, along with its ability to freely cross the BBB, create a formula for a potential drug that will be ideal for administration in a wide variety of neurodegenerative disorders, from the most common dementia

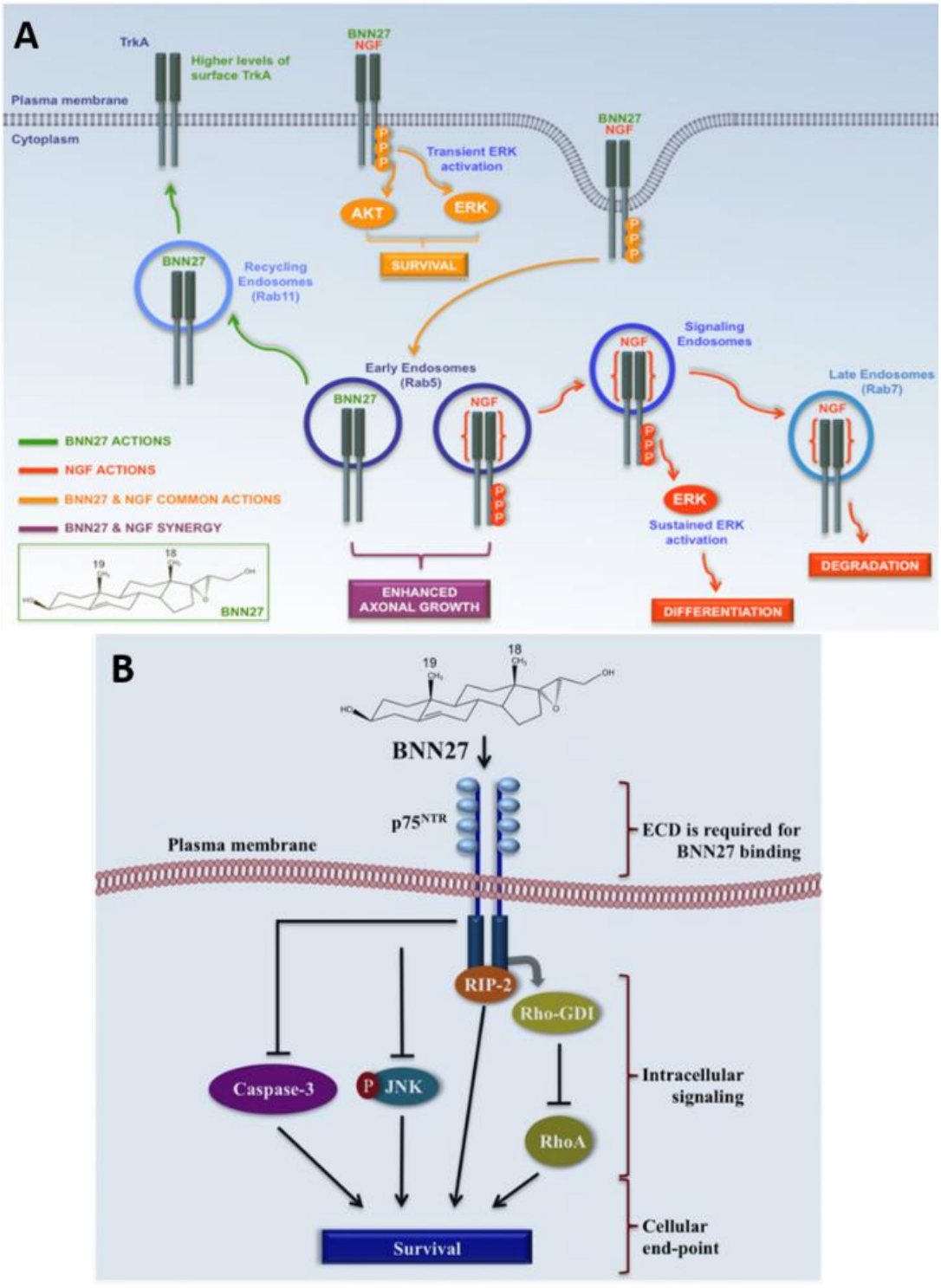


Figure 3: Schematic representation of the interactions of BNN27 with TrkA (A) and p75^{NTR} (B) receptors and the downstream signaling pathways activated (Pediaditakis, Efstathopoulos et al. 2016, Pediaditakis, Kourgiantaki et al. 2016).

diseases, AD and PD, to even the treatment of diabetic retinopathy (DR) (Iban-Arias, Lisa et al. 2018, Iban-Arias, Lisa et al. 2019).

Screenings of synthetic neurotrophic analogues is a perpetual process in our lab these days. Every now and then, a new promising microneurotrophin is synthesized and subsequent studies are taking place to evaluate its neuroprotective properties and unravel its mechanism of action. In the present study, we will work with two of the most prominent BNNs so far: BNN27 and BNN237.

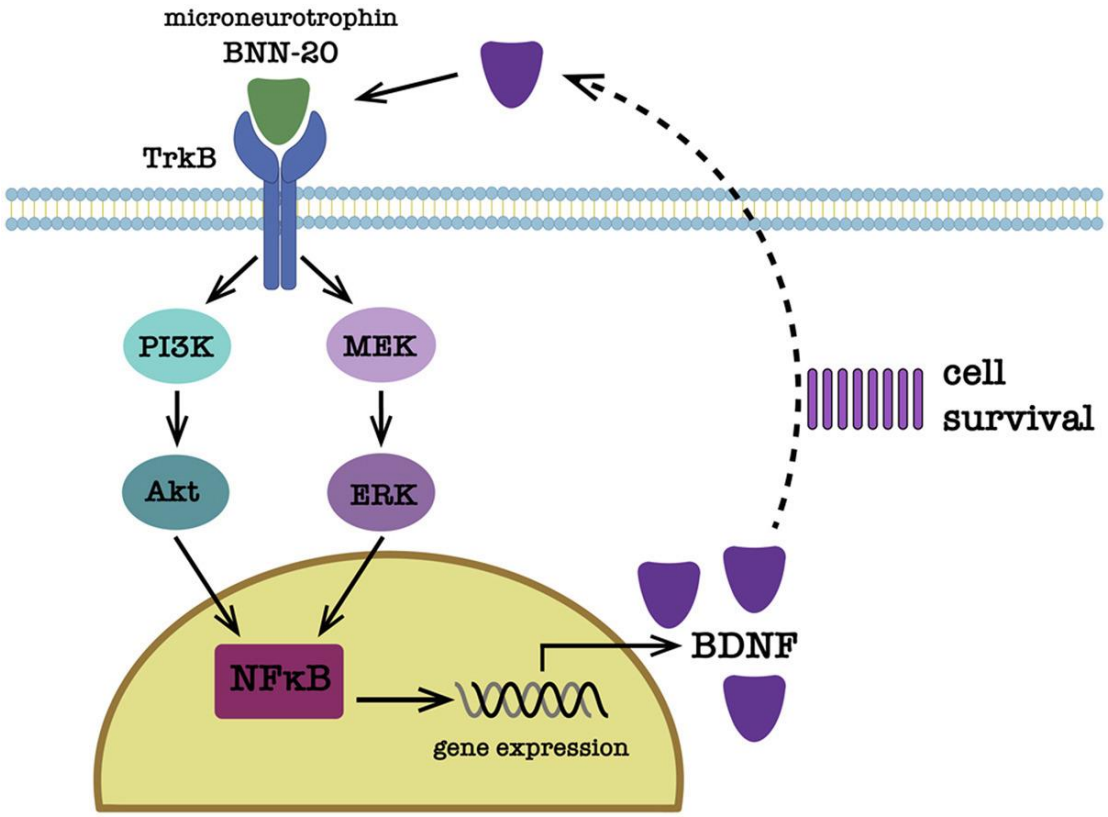


Figure 4: Schematic representation of the interaction of BNN20 with TrkB receptor and the downstream signaling pathways activated that lead to BDNF expression and dopaminergic neuronal survival (Panagiotakopoulou, Botsakis et al. 2020).

Adult neurogenesis: an alternate therapeutic target for microneurotrophins

Discovery of neurogenesis in the adult brain is one of the most important findings in the neuroanatomical research in the last 25 years, as it describes the formation of new neurons in the adult CNS, a process that was not considered possible since the early 90s (Kuhn, Toda et al. 2018). Despite earlier indications and observations by many groups, lack of technological knowledge and equipment forbid this, nowadays, textbook knowledge from being established at the time (Altman and Das 1965, Kaplan and Hinds 1977, Bayer 1983).

Hippocampal formation, and more specifically dentate gyrus, is now considered the niche of adult neurogenesis in many mammalian species. Although hippocampus is not the only neurogenic zone in the adult brain, it is the most important one, since it is involved in higher cognitive functions, including learning and memory. Interestingly, dentate gyrus, is also one of the three areas in the adult brain where Neural Stem Cells (NSCs) have been identified, and which is also known to be severely damaged in AD patients, possibly due to defective neuronal differentiation (Hollands, Bartolotti et al. 2016).

Unpublished data from our lab using BNN27 in vivo, unearthed a possible neuroprotective role for BNN27 in adult neurogenesis. More specifically, experiments were performed in the AD mouse model, 5xFAD (bares 5 human mutations characterized in AD patients, 3 in the APP gene and 2 in the PSEN1 gene), using a BNN27-pellet implantation (pellet was dissolvable in such a rate that it would release a certain amount of the microneurotrophin BNN27 every day) subcutaneously at 1.5 months of age where Amyloid-β (Aβ) plaques are already observed intra-neuronally but cognitive dysfunction has not yet developed in the young mice. After 2-months, the mice were subjected to spontaneous alternation test at a T-maze and after evaluation of the results, they were sacrificed and perfused. The isolated brains were sliced and immunostained for detailed molecular analysis. The results from the spontaneous alternation test indicated significant improvement in the working memory of BNN27-treated mice. This improved cognitive performance of the 5xFAD-BNN27-treated mice was further justified by the immunostained with anti-Aβ, brain slices, that clearly showed decreased Aβ plaque formation in the hippocampus compared to the placebo-treated mice, as well as by the immunostained for proliferating (BrdU) cells, slices, where our team detected significantly increased proliferation and neuronal generation in the dentate gyrus compared again to the control group.

These results paved the way for the in vitro experiments presented in this study, highlighting the brain areas and the cell populations where our work could potentially focus.

Reactive Astrocytes as Mediators of Neuroinflammation

Today, we know that chronic neuroinflammation in the brain is a phenomenon observed in many neurodegenerative diseases (Ransohoff 2016). Although, inflammation exists in the brain, just like in the rest of the body, as a protective physiological response to possibly damaging stimulators, such as microorganisms or cellular debris, alterations in the molecular components of this response could lead to severe damages in the brain. The innate inflammatory response in the CNS, bares some characteristic differences to its corresponding peripheral immune system. Due to the presence of the BBB, white blood cells are forbidden from entering the brain parenchyma and for this reason the role of the immune cells in the brain burdens the glia cells, microglia and astrocytes (Ransohoff, Schafer et al. 2015).

Astrocytes have been associated to neuroinflammation by many in vivo and in vitro studies over the past few years in such a way that today, increased levels of glial fibrillary acidic protein (GFAP), which is an astrocytic marker, are considered a clear sign of neuroinflammation in many neurodegenerative diseases (Millington, Sonego et al. 2014, Colombo and Farina 2016). Microglia and astrocytes are known to be able to react to many pro- and anti-inflammatory cytokines, changing their state between activated and deactivated, depending on the nature of the cytokines they interact with (Sofroniew 2014). Previous studies have also revealed that reactive astrocytes tend to allocate around senile plaques in mouse models of AD (Olabarria, Noristani et al. 2010).

In a recently published study, researchers looked further into the role of reactive astrocytes in AD brains and were able to characterize two distinguished populations of reactive astrocytes, which they term A1 and A2 (Liddelow, Guttenplan et al. 2017). A1 reactive astrocytes developed a purely neurotoxic phenotype promoting neuroinflammation, while A2 reactive astrocytes exerted neuroprotective and anti-inflammatory properties (Figure 5). In the same study, they also stained brain tissue samples from AD patients and found that the predominant phenotype of reactive astrocytes was A1, an observation that is in accordance with the chronic inflammation in these patients as we mentioned before.

Interestingly, some studies have clearly demonstrated the ability of neurotrophin BDNF to interact with astrocytes through TrkB signaling (Colombo and Farina 2016, Ponath, Park et al. 2018), while others directly associated BDNF to astrocytic activation (Bali, Banik et al. 2019, Ding, Chen et al. 2020). Recent reviews, have even attempted to associate, through literature, DHEA to the neuroprotective properties of astrocytes

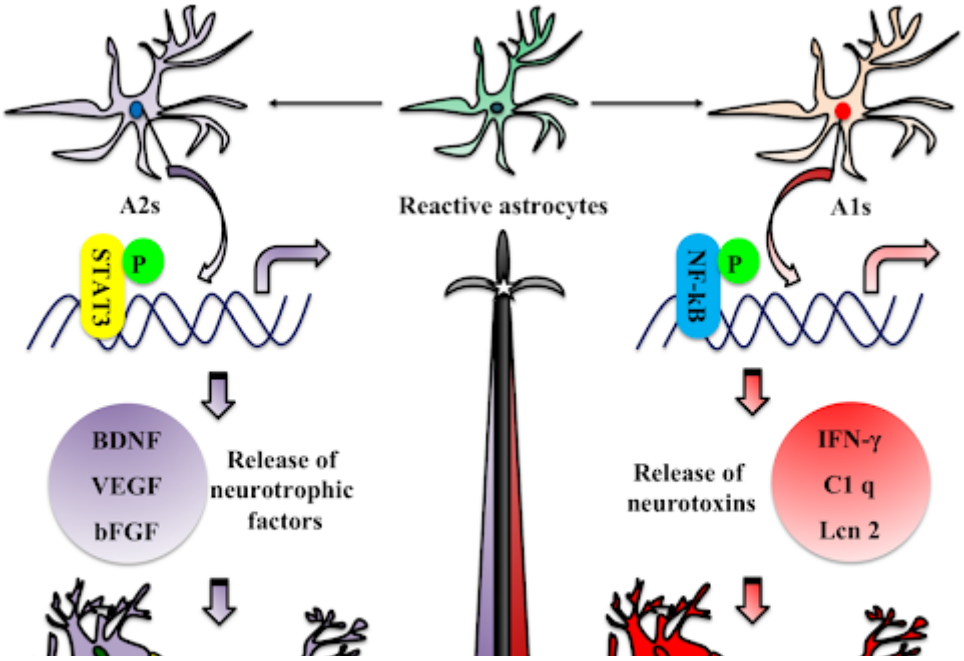


Figure 5: Schematic representation of the two different types of reactive astrocytes (A1 and A2) and their different effects on neuronal survival. It is of interest the choice of the illustrator to display the dual nature of reactive astrocytes as a double-edged sword (Li, Li et al. 2019).

in many brain traumas, including neurodegenerative diseases (Arbo, Bennetti et al. 2016, Arbo, Ribeiro et al. 2018). Although it is yet debatable whether neurotrophin effects on astrocytes are strictly beneficial for the brain, it is evident that they are promising therapeutic targets for many neurodegenerative diseases and worthy of further investigation (Poyhonen, Er et al. 2019).

All these data create a valid hypothesis, that needs to be tested, on whether our microneurotrophins could exert some of their neuroprotective properties observed in the in vivo experiments indirectly through alterations in the astrocytic phenotype and subsequently in the neuroinflammatory environment in the brain, the same way that BNN20 altered the microglia phenotype and reduced the neuroinflammatory load in the "weaver" mouse model as we mentioned previously (Panagiotakopoulou, Botsakis et al. 2020).

Aim of this study

Microneurotrophins are a novel class of DHEA-derived, neurotrophin synthetic analogues with the ability to bind to the neurotrophic receptors Trk and $p75^{NTR}$, while at the same time having abolished all of the steroid effects of DHEA. Their well-studied, neuroprotective properties demonstrate their unique therapeutic potential in many neurodegenerative diseases. The aim of this study will be twofold: a) to investigate the neuroprotective effects of BNN27 and BNN237 in P7 hippocampal NSCs and the downstream signaling pathways implicated in this process, and b) to examine the possible involvement of BNN27 and BNN237 in the activated astrocytic phenotype and their subsequent effects in neuroinflammation.

Materials and Methods

P7 hippocampal NSCs cell culture: Hippocampi were isolated from P7 mice and incubated in Dissection medium (contains 10x HBSS, sterile PBS and 5% Pen-Strep), before washed 5 times with fresh Dissection medium. Then the hippocampi were incubated in 1,5 ml accutase for 30' at 37^{o}C (every 5' gentle pipetting). After 30', we pipet extensively to achieve single cell cultures and then add 9 ml DMEM/F12 and centrifuge at 800 rpm for 5'. We then remove the supernatant and resuspend in 1 ml Complete medium for NSCs. The Complete medium contains B-27 Supplement 50X minus vitamin A (Gibco, 12587010), D-glucose solution 45% (Sigma, G8769-100ML), 2mM L-Glutamine (Gibco, A2916801), 100 µg/ml Primocin (Invivogen, ant-pm-1), 20 ng/ml FGF (R&D Systems, 233-FB-025), 20 ng/ml EGF (R&D Systems, 236-EG-200), 200 µg/ml Heparin (Sigma-Aldrich, H3149) and DMEM/F12. After resuspension in Complete medium, the cells are seeded in flasks coated with PDL (Sigma-Aldrich,

P6407) and Laminin and incubate overnight (37^{o}C, 5% CO_2). The next day, we add 1 ml of Complete medium. We supplement with fresh complete medium every 2-3 days. When the floating neurospheres start overpopulating the flask, we perform the first passage. It takes 3 passages for the cell culture to clear and contain strictly floating neurospheres suitable for experiments. For the treatments with either partially or totally deprived EGF, we used a medium with the exact same formula as the Complete medium, except the reduction of EGF concentration to 5 ng/ml or complete lack of it, respectively. For the treatments containing Aβ40 or Aβ42 toxic medium, after the synthesis of Aβ oligomers, as described in the Preparation of Aβ Oligomers protocol, we supplemented the Aβ-toxic DMEM/F12 with all of the remaining components as described in the Complete medium formula. The treatments with microneurotrophins BNN27 and BNN237, were taken place immediately after the addition of each medium in the culture. For the treatments with ketamine or bicuculline, the selected volume was diluted to the appropriate for each condition medium before added as a treatment to our cultures.

Astrocyte cell cultures: Cortices were isolated from P2 mice and incubated in 1X HBSS with Trypsin at 37^{o}C for 10'. 1X HBSS + Trypsin was then removed and the cortices were resuspended in 1X HBSS + sterile PBS. The cortices were then centrifuge at 1000 rpm for 5' and the supernatant was removed. Pellet was then resuspended in 1ml of Cortex medium (DMEM High-Glucose with 10% FBS and 2% Pen-Strep) with extensive pipetting for achieving single cell cultures. Then cells were seeded in flasks and incubated (37^{o}C, 5% CO_2) for 14 days. Fresh Cortex medium was added to the cultures twice a week. After 14 days, cultures were placed in shaker (200 rpm) for 1 hour at 37^{o}C to remove microglia. Old Cortex medium containing the microglia was discarded from the cultures and replaced with fresh and then the cultures were again placed in shaker (250 rpm) overnight at 37^{o}C for the oligodendrocytes to be removed. The next day, the Cortex medium containing the oligodendrocytes was discarded and replaced with fresh medium containing Ara-C 10μM (Sigma, C1768-100MG) for 4 days. Ara-C-containing medium was then removed and replaced with fresh Cortex medium for 24 hours. All plates and flasks used from this point on, were pre-treated with Laminin (Sigma, L2020-1mg) at a working concentration of 15μg/ml. For the treatments containing Aβ40 or Aβ42 toxic medium, after the synthesis of Aβ oligomers, as described in the *Preparation of Aβ Oligomers* protocol, we supplemented the Aβ-toxic DMEM with 2% Pen-Strep and 10% DCC-stripped FBS (Dextran-coated charcoal stripping of FBS protocol) in order to remove all of the steroids inside the FBS and thus avoid masking some of the effects from our microneurotrophins.

Dextran-coated charcoal (DCC) stripping of FBS: Activated charcoal (Sigma-Aldrich,C-5385) and Dextran (Sigma-Aldrich, 31390) were diluted to a final concentration of 0,3% and 0,03% respectively in dH_2O into final volume similar to the volume of the FBS that we plan to strip. The mix was left to gently shake overnight at 4^{o}C. The next day,

the mix was centrifuged at 4500g for 12' at 4°C and then the supernatant was removed. A washing step with dH_2O was then performed and similar centrifugation to remove the supernatant. The pellet was resuspended in the FBS to be stripped and incubated overnight at 4°C with gentle stirring. The next day the FBS was centrifuged at 4500g for 20' at 20°C for the charcoal-dextran-steroids complex to be removed. The supernatant was isolated and filtered using a 0.45 μm filter to sterilize it.

qRT-PCR: Total RNA was extracted from P7 hippocampal NSC cultures and astrocytic cultures using the TRIzol Reagent (Invitrogen, 15596026) and cDNA synthesis was performed using the High-capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific, 4368814) to the manufacturer protocols. 1 μl of RNA isolation product was loaded in 8% agarose gel and after electrophoresis (110V for 25') was checked for confirmation of RNA isolation and purification. 1 μl of synthesized cDNA was used for amplification of GAPDH housekeeping gene in a PCR machine using a standard cycling program of 95°C for 5' followed by 35 cycles of 95°C for 1', 58°C for 1' and 72°C for 1' and a hold step at 72°C for 10'. The amplified product was loaded in 8% agarose gel and after electrophoresis (110V for 30') was checked for successful cDNA synthesis. Primers were chosen from distinguished published papers and crosschecked using NCBI primer BLAST software (http://www.ncbi.nlm.nih.gov/tools/primer-blast/) to ensure low to zero probability of amplifying non-specific products (Table 1). All primers were tested for their efficiency compared to housekeeping gene Actin and all had 95-105% efficiency. For the qRT-PCR, 1 μl of synthesized cDNA was used along with KAPA SYBR FAST qPCR (Merck, KK4601) according to the supplier's protocol and a cycling program of 95°C for 20'', followed by 40 cycles of 95°C for 3'' and 60°C for 30'' on a StepOnePlus Real-Time PCR System (Applied Biosystems, 4376600). After qPCR completion, melting curve of amplified products was created according to instrument guidelines.

Western Blot: Protein samples were collected at 4°C in Lysis Mix containing Phosphatase Inhibitor Cocktail Set V (Millipore, 524629) and Protease Inhibitor Cocktail Set I (Millipore, 539131). After sonication, the total protein concentration was determined using Pierce BCA Protein Assay Kit (Thermo Fisher Scientific, 23227). Equal amounts of protein were loaded to 12% Tris-HCL gel, and after electrophoresis (110V for 90'), proteins were transferred to nitrocellulose membranes (Amersham Protran Wester, GE10600002). Blots were probed overnight at 4°C with anti-TrkA (1:1000, Millipore, 06-574), anti-TrkB (1:1000, Abcam, ab33655), anti-TrkC (1:1000, Cell Signaling Technology, C44H5), anti-$p75^{NTR}$ (1:1000, Promega, G323A), anti-GAPDH (1:3000, Sigma-Aldrich, G8795) and anti-Actin (1:3000, Sigma- Aldrich, MAB1501). Blots were then incubated with HRP-conjugated secondary antibodies for 1 hour at room temperature in 1:5000. For visualization, blots were treated with SuperSignal West Pico Chemiluminescent Substrate (Thermo Fisher Scientific, 34580).

Fluorescent Immunocytochemistry: Cultures were first washed with PBS for 1' at room temperature, before fixation with 4% PFA for 10'. After 2 washes with PBS and one more with PBSTx 0,1%, fixed cultures were blocked (PBSTx 0,3% with 10% Serum specific to the species the 2nd antibodies were raised in and 0,1% BSA) for 1 hour at room temperature. Then they were probed overnight at 4^{o}C with primary antibodies diluted in PBSTx 0,1% with 1% Serum (the same that was used for blocking). As primary antibodies for staining we used anti-Nestin (1:1000, Novus Biologicals, NB100-1604), anti-GFAP (1:1000, Merck-Millipore, AB5541), anti-TrkA (1:1000, Millipore, 06-574), anti-TrkB (1:1000, Abcam, ab33655), anti-TrkC (1:1000, Cell Signaling Technology, C44H5) and anti-p75^{NTR} (1:1000, Promega, G323A). The next day, the fixed cultures were washed twice with PBSTx 0,1% and secondary antibodies (1:1000) were added to them for 1 hour at room temperature. Stained cultures were then washed twice with PBSTx 0,1% and one more with PBS before nucleus stain Hoechst 33342 (Invitrogen, H3570) was used at 1:10000 diluted in PBS for 10'. After two more washes with PBS, the immunostained cultures were then analyzed via fluorescence microscope.

BrdU Fluorescent Immunocytochemistry: Based on the fluorescent immunocytochemistry protocol described earlier, it only differs at the steps right after fixation, where some extra handling needs to take place in order for the integrated BrdU (BD Biosciences, 562253) to be revealed for the anti-BrdU (1:200, Invitrogen, B35128) to bind. After fixation with 4% PFA, cells are being washed with PBS twice before incubated with 2N HCL at 37^{o}C for 30', a process necessary for the DNA to be unwinded. Then, 2N HCL is being removed and neutralized by 0,1 M sodium tetraborate (pH 8,5) for 10' at room temperature. After 2 washes with PBS and one more with PBSTx 0,1%, the protocol continues from the step of blocking as described in the fluorescent immunocytochemistry protocol.

Preparation of Aβ oligomers: For the preparation of Aβ oligomers, the necessary volume of Aβ1-42 (AnaSpec, AS-20276) and Aβ1-40 (AnaSpec, AS-24236) for each concentration was diluted in DMEM/F12, for experiments on P7 hippocampal NSCs, or DMEM, for experiments on astrocytes, and incubated overnight at 37^{o}C. The next day, the diluted amyloids were centrifuged at 14000 rpm and the supernatant was isolated. The Aβ-toxic DMEM/F12 or DMEM were then supplemented with the necessary components for each treatment, and added to the cell cultures.

Table 1: Table containing all of the primers used in qRT-PCR

Gene	Forward (5'->3')	Reverse (5'->3')
GAPDH	ATTGTCAGCAATGCATCCTG	ATGGACTGTGGTCATGAGCC
Actin	GGAGATTACTGCTCTGGCTC	GGACTCATCGTACTCCTGCT
TrkA	AGAGTGGCCTCCGCTTTGT	CGCATTGGAGGACAGATTCA
TrkB	TGGACCACGCCAACTGACATT	GAATGTCTCGCCAACTTGAG
TrkC	TGCAGTCCATCAACACTCACCAGA	TGTAGTGGGTGGGCTTGTTGAAGA
$p75^{NTR}$	GACTAACCTAGGCCACCCAA	CAGACGTCGTTTCCAGATGT
Amigo2	GAGGCGACCATAATGTCGTT	GCATCCAACAGTCCGATTCT
Srgn	GCAAGGTTATCCTGCTCGGA	TGGGAGGGCCGATGTTATTG
Serping1	ACAGCCCCCTCTGAATTCTT	GGATGCTCTCCAAGTTGCTC
S100a10	CCTCTGGCTGTGGACAAAAT	CTGCTCACAAGAAGCAGTGG
Emp1	GAGACACTGGCCAGAAAAGC	TAAAAGGCAAGGGAATGCAC
Ptx3	AACAAGCTCTGTTGCCCATT	TCCCAAATGGAACATTGGAT
Serpina3n	CCTGGAGGATGTCCTTTCAA	TTATCAGGAAAGGCCGATTG
Steap4	CCCGAATCGTGTCTTTCCTA	GGCCTGAGTAATGGTTGCAT
GFAP	AGAAAGGTTGAATCGCTGGA	CGGCGATAGTCGTTAGCTTC

Results

Unravelling the Expression Pattern of Neurotrophin Receptors in the P7 hippocampal NSCs

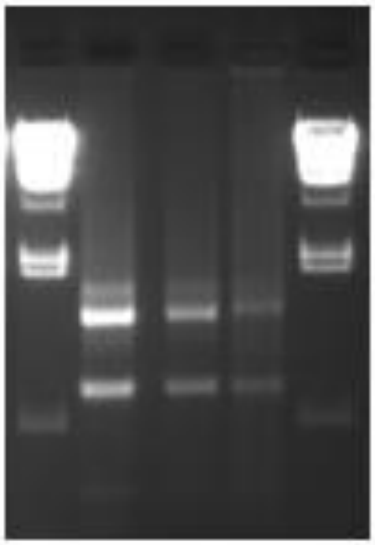
In order to proceed with our in vitro experiments in the P7 hippocampal NSCs we first had to check its expression patterns of neurotrophin receptors to ensure that microneurotrophins would be able to have direct effects on them and possibly eliminate some signaling pathways. For this reason, we searched for possible expression of all Trk and $p75^{NTR}$ receptors, first in transcriptional (qPCR) and then in translational level (Western Blot). The qPCR expression data were evaluated compared to our reference gene, the "housekeeping" GAPDH.

The qPCR results, as shown in Figure 6, reviled some first indications on the Trk and $p75^{NTR}$ receptors expression patterns of P7 hippocampal NSCs, with TrkB and TrkC receptors being present while TrkA and $p75^{NTR}$ receptor levels were not even detectable. These results were further confirmed translationally using Western Blot analysis (Figure 7).

A

ng	ΔCT TrkA-GAPDH	ΔCT TrkB-GAPDH	ΔCT TrkC-GAPDH	ΔCT p75-GAPDH
120	17.18424129	3.152795792	1.545186043	9.075639725
30	16.21406746	3.216535568	1.552399635	9.055954933
7.5	14.11979961	3.030491829	1.300670624	8.935656548
1.88	12.32657051	3.256639481	1.663111687	9.046094894
0.47	10.09077549	3.162248611	1.543512344	8.439278603

B



C

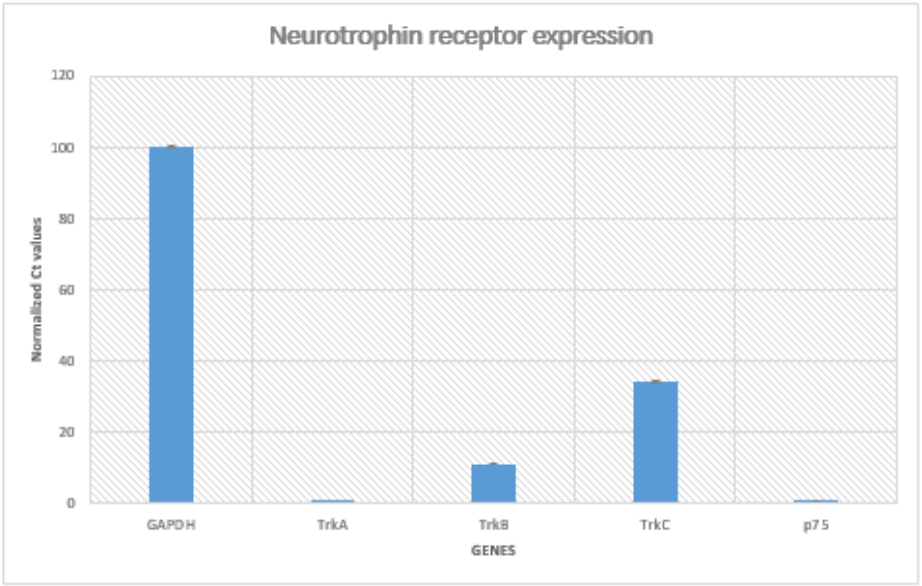


Figure 6: A. ΔCT values of the selected primers for TrkA, TrkB, TrkC and p75 compared to our reference gene GAPDH. B. Electrophoresis gel of the synthesized cDNAs, depicting the purity of the RNA extraction in 3 different samples of P7 hippocampal NSCs (the 3 samples between two ladders). C. The qPCR results of the 4 examined receptors.

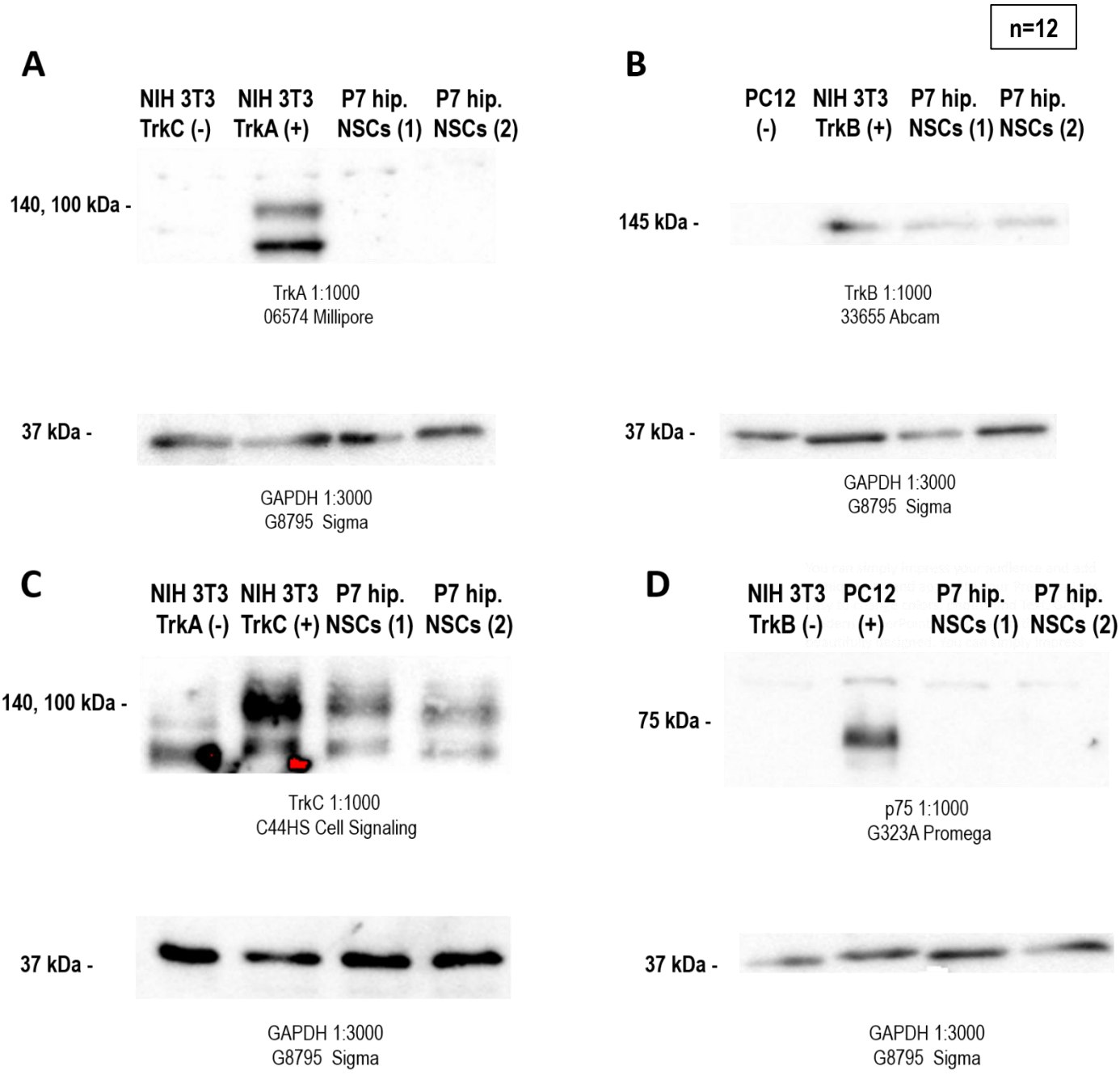


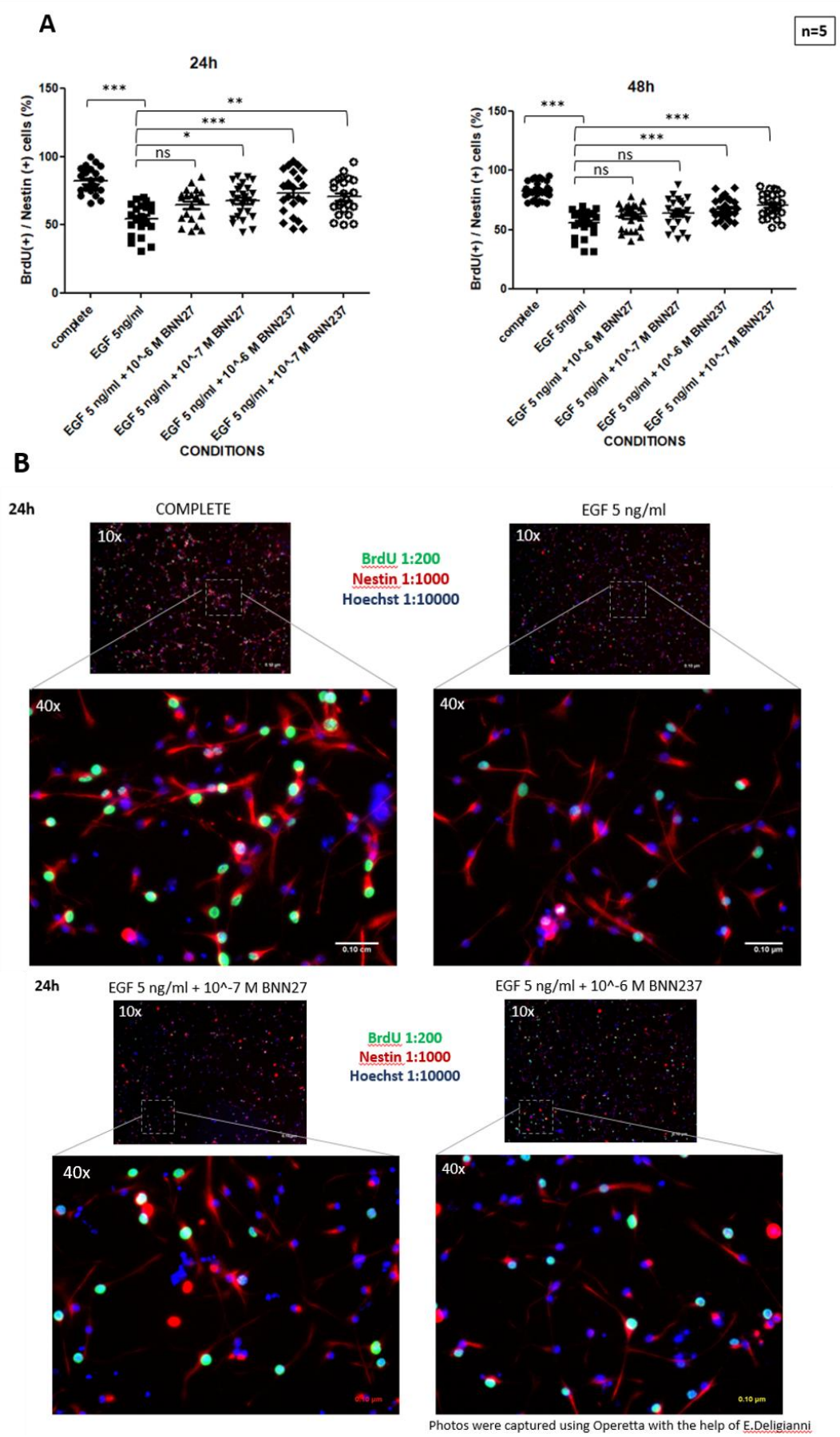
Figure 7: Western Blot analysis of the expression levels of TrkA (A), TrkB (B), TrkC (C) and p75 (D) receptors in two different samples of P7 hippocampal NSCs lysates. Negative (-) and positive (+) controls were used in each membrane to verify our results.

Positive effects on P7 hippocampal NSCs proliferation in vitro under partial deprivation of the Epidermal Growth Factor (EGF) by microneurotrophins BNN27 and BNN237

Having established the expression of TrkB and TrkC in our P7 hippocampal NSCs cell line, and thus, the possibility for BNNs to act on them, we then moved on to search for possible neuroprotective effects on this cell line by our microneurotrophins. For this reason, we chose to check the BNNs effects on P7 hippocampal NSCs ability to proliferate under stress conditions due to EGF deprivation. EGF is an important mitogen for the proper development of the nervous system, and has been associated with the induction of the proliferation and migration of NSCs (Threadgill, Dlugosz et al. 1995). Low levels of plasma EGF are also considered a potent biomarker of cognitive decline in AD, while its receptor (EGFR) is a promising target for therapeutic approaches on Aβ-induced memory loss (Wang, Chiang et al. 2012, Lim, Swanson et al. 2016).

After isolation, P7 hippocampal NSCs were cultivated in complete medium for 17h, and then were partially deprived of EGF (EGF 5ng/ml instead of 20ng/ml) for 24h or 48h with and without the presence of BNN27 (two different concentrations: 10^{-6}M and 10^{-7}M) or BNN237 (two different concentrations: 10^{-6}M and 10^{-7}M). BrdU was added to the cultures 4h prior to the end of the treatments at concentrations of 10nM. The cultures were immunostained using the immunocytochemistry protocol and the results were obtained and analyzed using the Operetta High-Content Imaging System.

In EGF deprived conditions the BrdU positive cells detected are significantly lower compared to the complete condition in both 24h and 48h (Figure 8A). BNN27 was able to significantly rescue proliferation of NSCs only in the 24h treatments in 10^{-7}M concentrations, while BNN237 exerted its neuroprotective properties on proliferation both at 24h and 48h treatments with slightly better results observed at 10^{-6}M concentrations (Figure 8A).



Photos were captured using Operetta with the help of E.Deligianni

*Figure 8: A. Quantification of the BrdU(+)/ Nestin(+) cells ratio under 24h and 48h partial deprivation of EGF (5ng/ml instead of 20ng/ml) with and without BNN27 or BNN237 treatments (1-way ANOVA, Bonferroni's multiple comparison test, p<0,0001,***). B. Representative images of conditions: Complete, EGF 5ng/ml, EGF 5ng/ml + BNN27 10^{-7}M, EGF 5ng/ml + BNN237 10^{-6}M, obtained using Operetta (Nestin was used as a marker for NSCs and Hoechst as a nucleus marker).*

Positive effects on P7 hippocampal NSCs proliferation in vitro under complete deprivation of the EGF by microneurotrophins BNN27 and BNN237

After that, we decided to step up the stress conditions in P7 hippocampal NSCs and search if our microneurotrophins could perform as well as they previously did. For this reason, we repeated the exact same experiments as before, only this time we completely deprived our P7 hippocampal NSC cultures from EGF.

In conditions lacking EGF, the number of BrdU positive cells was almost knocked out (Figure 9A). BNN27 at 24h treatment, was able to rescue proliferation significantly at concentrations of 10^{-7}M. BNN237 also exhibited significantly elevated neuroprotection of NSCs proliferation at 24h treatment, again with better results at concentrations of 10^{-6}M. None of the two microneurotrophins was able to show any significant rescue in proliferation on P7 hippocampal NSCs after 48h complete EGF deprivation (Figure 9A).

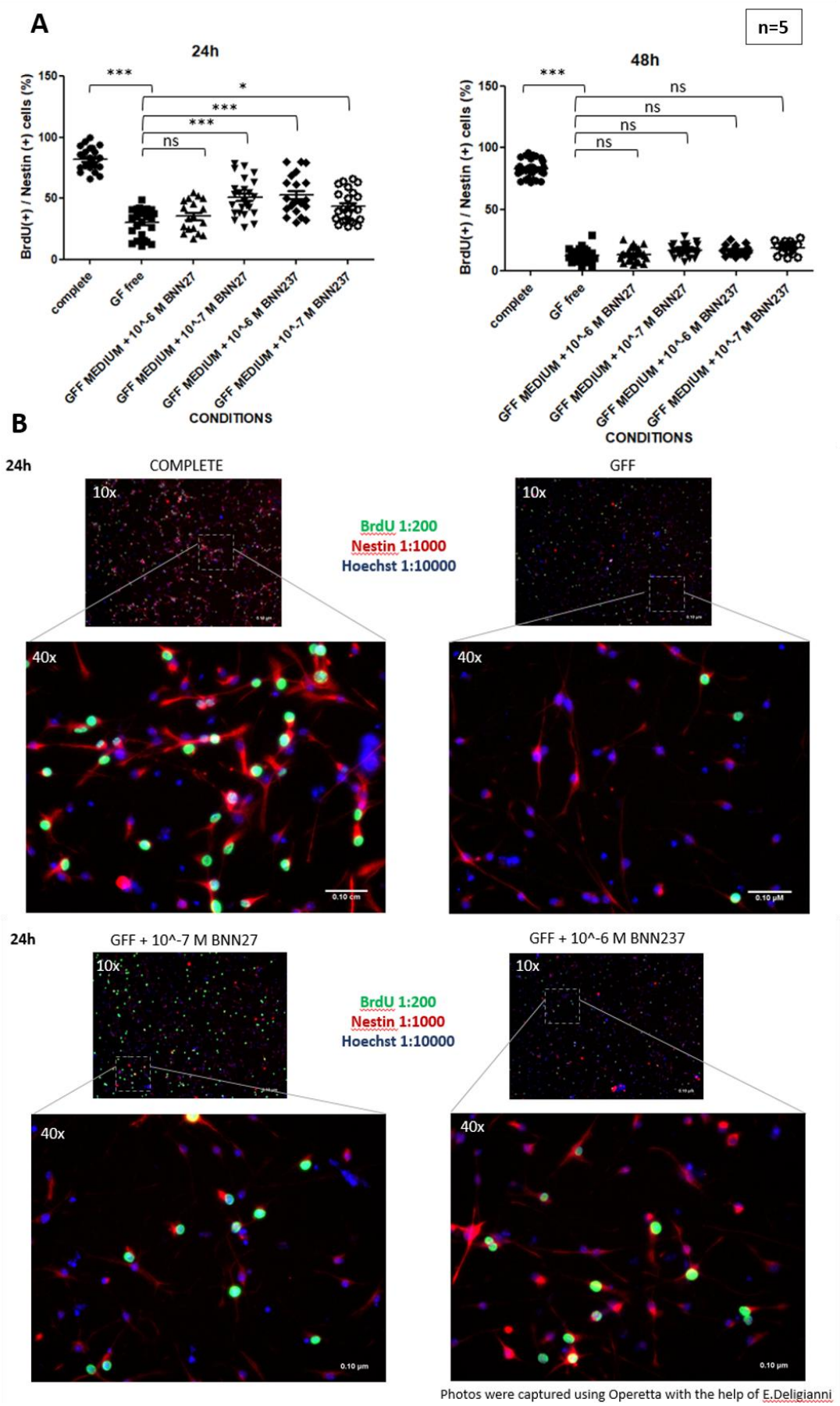


*Figure 9: A. Quantification of the BrdU(+)/ Nestin(+) cells ratio under 24h and 48h complete deprivation of EGF (instead of 20ng/ml) with and without BNN27 or BNN237 treatments (1-way ANOVA, Bonferroni's multiple comparison test, p<0,0001,***). B. Representative images of conditions: Complete, GFF (Growth Factor Free), GFF + BNN27 10^{-7}M, GFF + BNN237 10^{-6}M, obtained using Operetta (Nestin was used as a marker for NSCs and Hoechst as a nucleus marker).*

Quantification of Nestin (+) cell intensity in partial and complete EGF deprived conditions

Nestin (neuroepithelial stem cell protein) is a type VI intermediate filament protein of the cytoskeleton expressed mainly in neural stem cells. Nestin plays an important role in proliferation, differentiation and migration, functions that are characteristic of stem cells, thus making it an important marker of NSCs and their stemness ability (Bernal and Arranz 2018).

Based on our previous results showing reduced proliferation at EGF deprived conditions, we wanted to examine whether our cultures would lose their stemness ability and start differentiating after EGF deprivation, an effect already observed in human neural precursor cells (Schwindt, Motta et al. 2009). For this reason, we cultured P7 hippocampal NSCs for 17h in complete medium and then in medium either partially (EGF 5ng/ml) or completely deprived of EGF for 24h.

The results showed no statistical significance compared to our control cultures (complete medium) in the nestin intensity and subsequently in the stemness capabilities of culture (Figure 10).

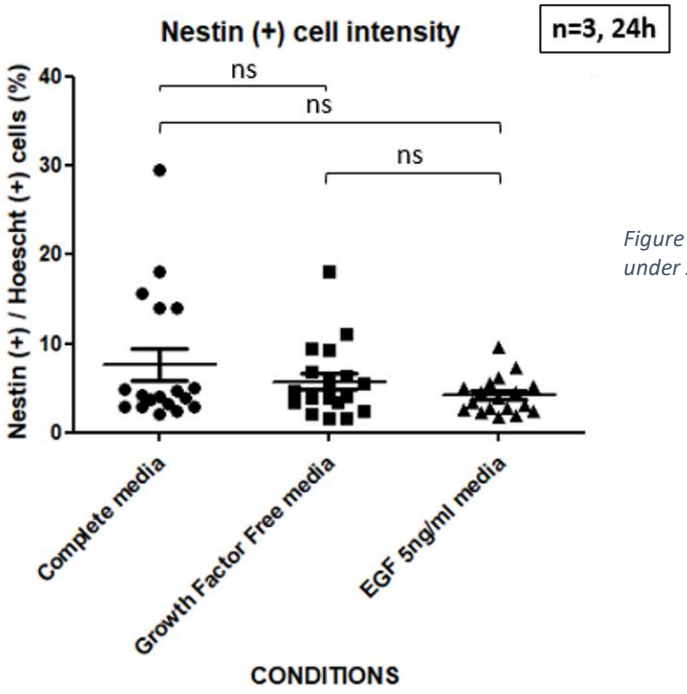


Figure 10: Quantification of the Nestin(+)/ Hoechst(+) cell ratio under 24h of complete or partial (EGF 5ng/ml) EGF deprivation

Investigation of the Aβ oligomer toxicity on proliferative P7 hippocampal NSCs

In order to simulate the AD pathology in our P7 hippocampal NSCs, we introduced Aβ oligomers in our cultures and investigated its effects on the proliferation. As we mentioned before, there are two toxic isoforms of Aβ oligomers found in amyloid plaques, Aβ40 and Ab42, with the latter existing in higher concentrations in the Aβ deposits and considered significantly more toxic.

For this reason, we cultured isolated P7 hippocampal NSCs for 24h under partially (5ng/ml) EGF-deprived conditions with the addition of either Aβ40 or Aβ42 (2μM concentration) and microneurotrophins BNN27 and BNN237. 4 hours prior to the end of our experiment we introduced BrdU in the culture and then proceed with the ICC protocol staining with anti-BrdU for proliferating cells, nestin for NSCs and hoechst as a nucleus marker. Our results suggested that, although BNN27 and BNN237 rescued proliferation significantly when applied, the lack of difference between EGF-deprived conditions and EGF-deprived in conjunction with Aβ oligomers conditions forbid us from making safe conclusions on whether the microneurotrophins acted in a neuroprotective way against the AD pathology or just the EGF deprivation (Figure 11). In fact, in the case of Aβ40-oligomer-treated cultures, we even observed a significantly higher percentage of BrdU (+) cells. This result is backed up by previous studies indicating that Aβ40 is implicated in neuronal progenitor cell (NPC) self-renewal by promoting their entry into the S-phase (Chen and Dong 2009).

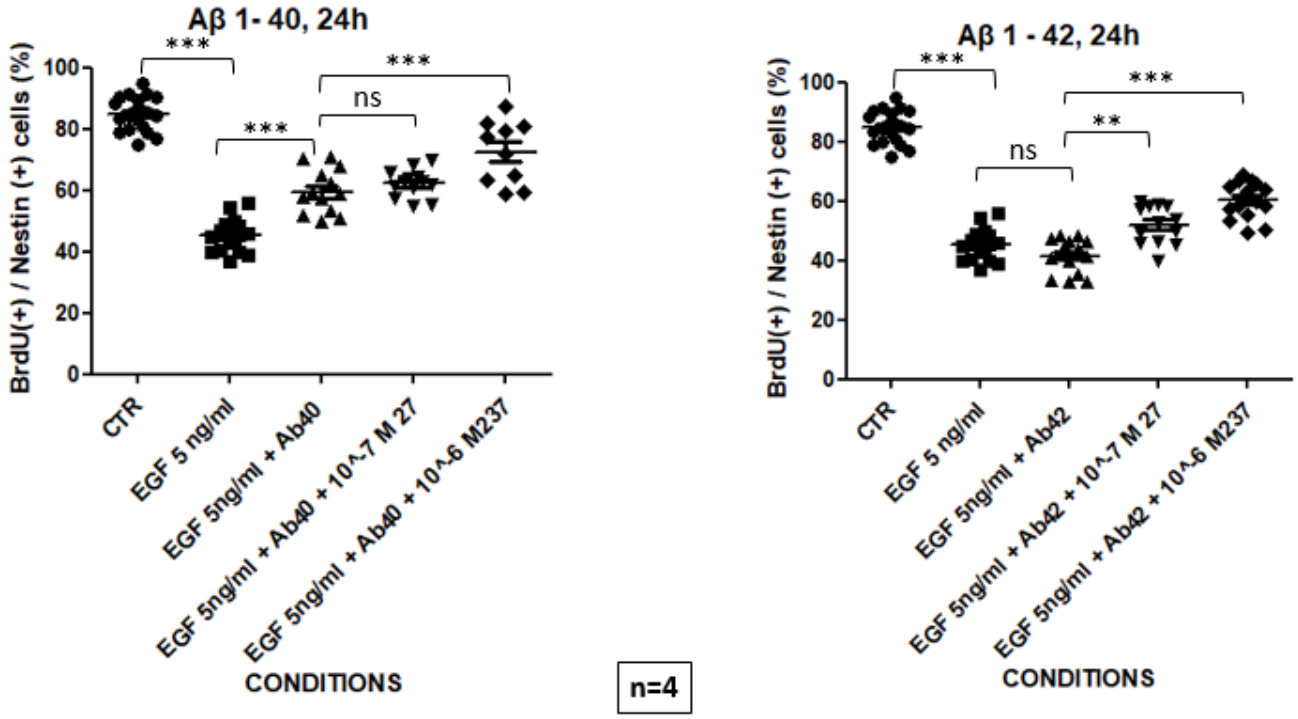


Figure 11: Quantification of BrdU (+)/ Nestin (+) cell ratio in partially EGF-deprived cultures with the addition of either Aβ40 or Aβ42 oligomer-toxicity (2μM concentration) and microneurotrophins BNN27 and BNN237 after 24h.

In order to achieve a clear distinction between partially EGF-deprived conditions and partially EGF-deprived + Aβ-oligomer-treated conditions we tried to increase the Aβ-related stress in our P7 hippocampal NSC cultures through two changes: increase the Aβ load in our cell cultures and prolong the treatments to 48h. In addition, we will repeat the experiments in complete conditions too, to eliminate the effects of EGF deprivation.

Indeed, as indicated by our results in Figure 12, the experiments should be repeated in complete conditions and not in partially EGF-deprived ones. Also, the concentration of Aβ oligomers both for Aβ40 and Aβ42 should be increased to 5μM and our cultures should be treated with the different conditions for at least 48h.

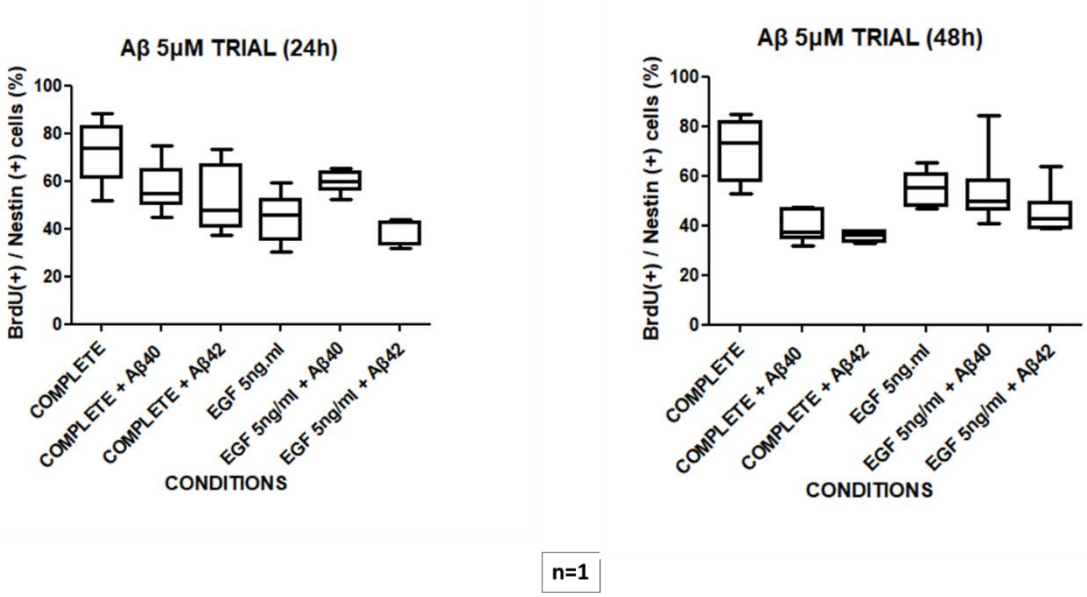


Figure 12: Quantification of BrdU (+)/ Nestin (+) cell ratio in partially EGF-deprived cultures with the addition of either Aβ40 or Aβ42 oligomer-toxicity (5μM concentration) after 24h and 48h.

Investigation for potential implication of other receptors on BNNs effects

Neurotrophins like BDNF, have already been associated with synaptic plasticity in the Central Nervous System (CNS) and Long-Term Potentiation (LTP) in the CA3-CA1 hippocampal region (Minichiello, Korte et al. 1999). In AD patients, abnormal neuronal anatomy and neurodegeneration leads to synaptic dysfunction and subsequent progressive decline in cognition and memory. Activation of glutamate receptor N-methyl-D-aspartate (NMDA), has recently implicated in synaptic dysfunction through excess Ca^{2+} influx leading to excitotoxicity (Paoletti, Bellone et al. 2013, Kodis, Choi et al. 2018). Taken together, these results raise the question of possible involvement of the NMDA receptor in the actions of our microneurotrophins.

BDNF is also an important mediator of LTP at glutamatergic and GABAergic synapses in the CNS (Villette and Dutar 2017). In AD patients, glutamatergic neurotransmission is significantly impaired due to APP-mediated alterations in the GABAergic

interneurons that disrupt the balance between excitatory and inhibitory neurotransmission leading to neurodegeneration (Limon, Reyes-Ruiz et al. 2012). $GABA_A$ receptor is therefore another potent receptor that may be implicated in the neuroprotective effects of the microneurotrophins that we already observed.

To address these questions, we repeated our EGF-deprived experiments with BNNs but this time we added different concentration of either ketamine, which is an NMDA receptor inhibitor, or bicuculline, a $GABA_A$ receptor antagonist.

Our results from 2 replicates so far, indicate that NMDA receptor is possibly not involved, as no differences in the proliferation rescue capabilities of both BNN27 and BNN237 are observed when the NMDA inhibitor is added to the culture, in contrast to $GABA_A$ receptor, where in the presence of its inhibitor, both of our microneurotrophins tend to lose their neuroprotective effects (Figure 13). Of course, due to the small number of replicates, statistically significant differences have not yet been observed and more replicates need to be added.

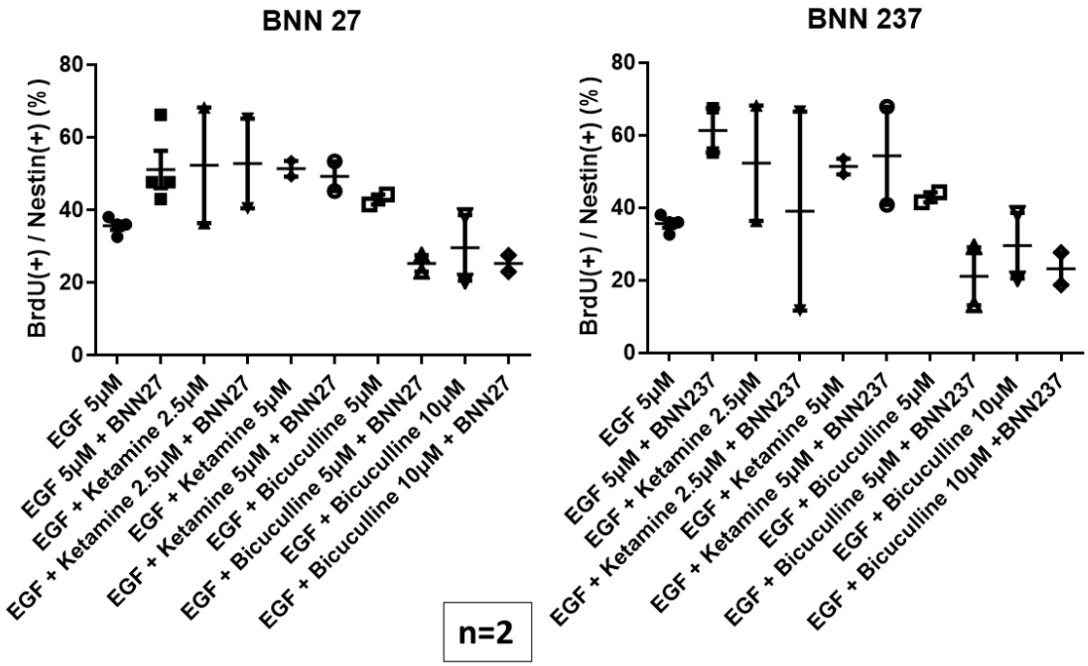


Figure 13: Quantification of BrdU (+)/ Nestin (+) cell ratio in partially EGF-deprived cultures after treatment with either BNN27 (left) or BNN237 (right) in the presence of different concentrations of NMDA inhibitor, ketamine, and $GABA_A$ inhibitor, bicuculline.

Developing a protocol for high purity-astrocytic culture

The protocol used for isolation of astrocytes from mice cortex, as described in Materials and Methods section, yields cultures of quite good purity which soon degrades to very low levels due to rapidly proliferating microglia remnants during the isolation protocol. In order to proceed with our experiments, we first had to develop

a protocol that would increase our astrocytic culture yield both right after isolation and after a long-period treatment.

For this reason, we first wanted to determine the best substrate that would facilitate the adhesion of astrocytes while at the same time it would impede microglia adhesion. We used two different substrates, Poly-D-Lysine (PDL) and Laminin, as well as their combination and measured through immunostaining the number of GFAP (+) cells in each condition. The results in Figure 14A, demonstrate that the use of laminin-coated plates and flasks yields significantly more purified astrocytic cultures compared to the PDL-coated and PDL-Laminin-coated ones. These results are backed up by literature suggesting that PDL facilitates the adhesion of microglia and oligodendrocytes, while laminin facilitates the adhesion only for astrocytes (Solà, Cristòfol et al. 2011).

But still, the culture purity wasn't high enough, so we decided to try adding Cytosine β-D-arabinofuranoside (Ara-C) at constantly increasing concentrations for 4 days. Ara-C is a cytosine base analog that is able to get incorporated in the DNA of proliferating cells during DNA replication and arrest the cell cycle in the S-phase while at the same time it inhibits the action of DNA and RNA polymerases. Ara-C is quickly converged to cytosine arabinoside triphosphate which can also have detrimental effects on the DNA. Thus, Ara-C mainly affects rapidly dividing cells. In our astrocytic cultures, after the removal of oligodendrocytes through shaking, what is left in the flask is a super-confluent monolayer of non-proliferating, due to lack of space, astrocytes along with a few constantly proliferating microglia cells. In Figure 14B, we can clearly see the beneficial effects for our astrocytic culture purity after 4 days of treatment with Ara-C at 10 μM concentration (purity above 92%).

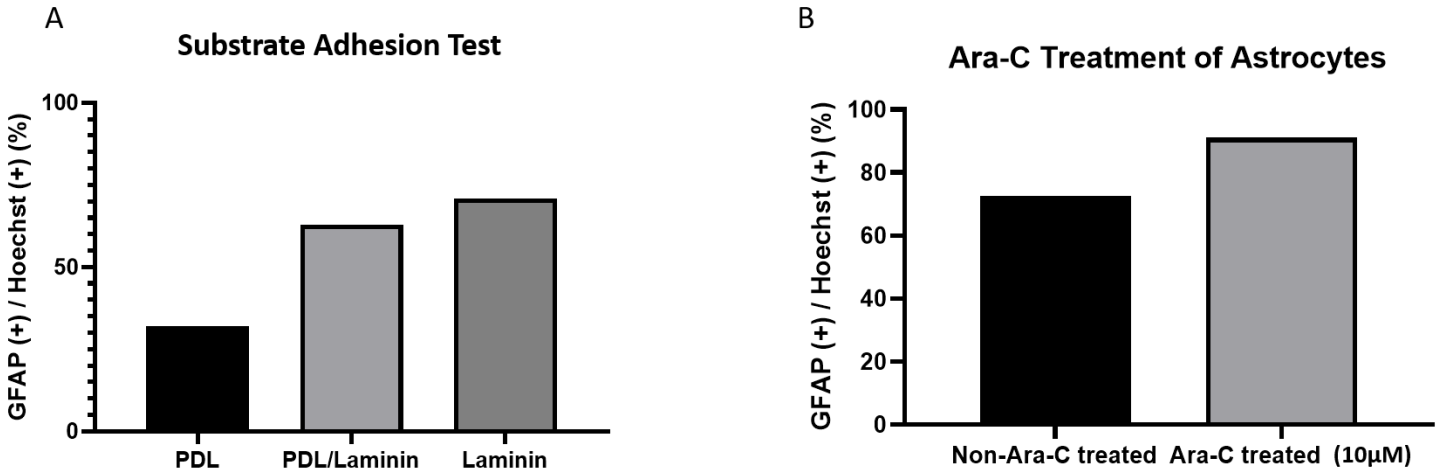
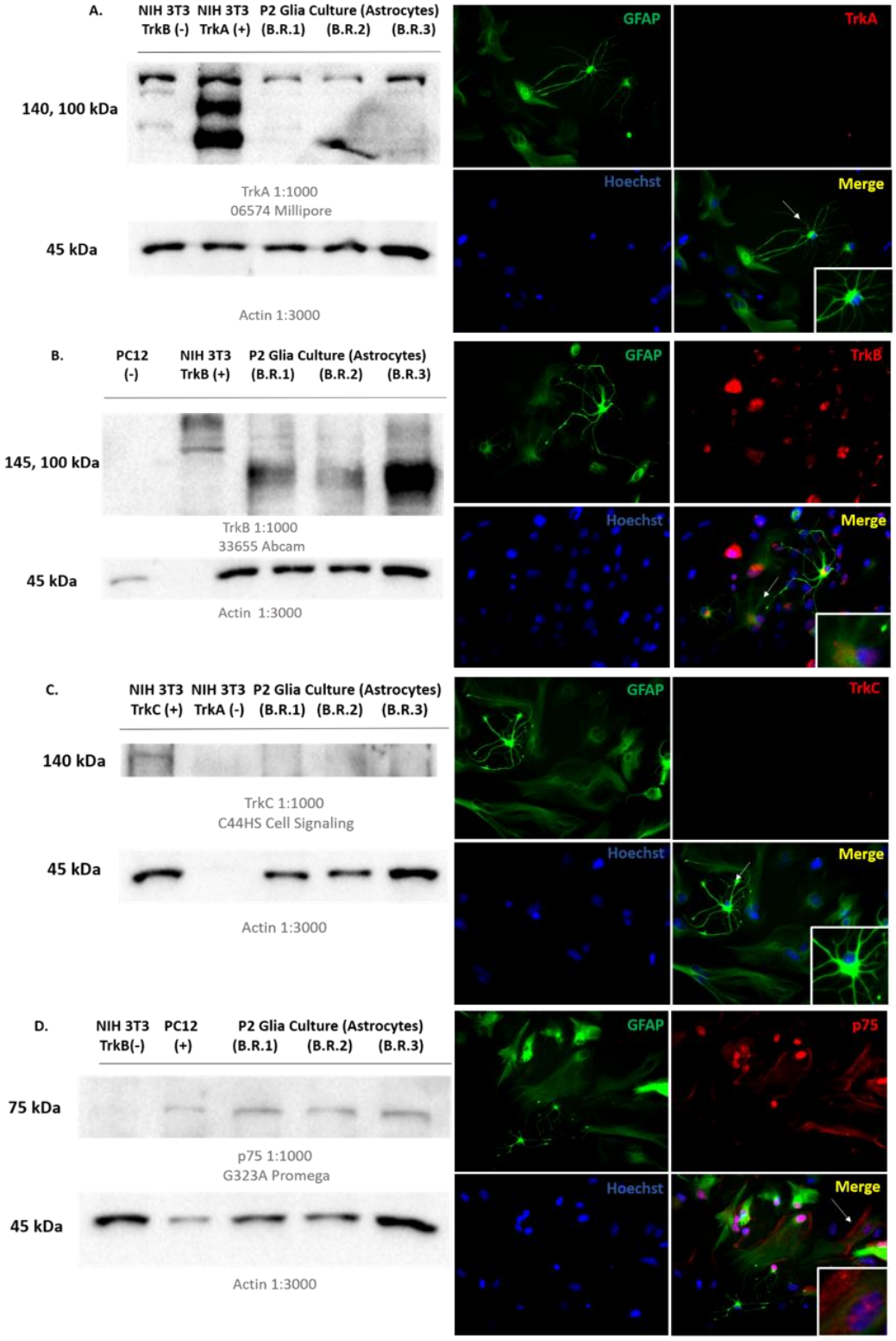


Figure 14: A) Quantification of GFAP (+) /Hoechst (+) cell ratio in plates coated with PDL, PDL + Laminin and Laminin substrate. B) Quantification of GFAP (+) /Hoechst (+) cell ratio in cultures treated with and without 10 μM Ara-C for 4 days.

Unravelling the Expression Pattern of Neurotrophin Receptors in Astrocytes

Just like we did with P7 hippocampal NSCs, before proceeding to test the effects of microneurotrophins, we should first examine if and which of the Trk or $p75^{NTR}$

receptors do astrocytes express. We performed both immunostainings as well as Western Blot analysis for all 4 receptors. The results, as depicted in Figure 15, indicate that astrocytes express both TrkB and $p75^{NTR}$ receptors but none of the TrkA or TrkC.



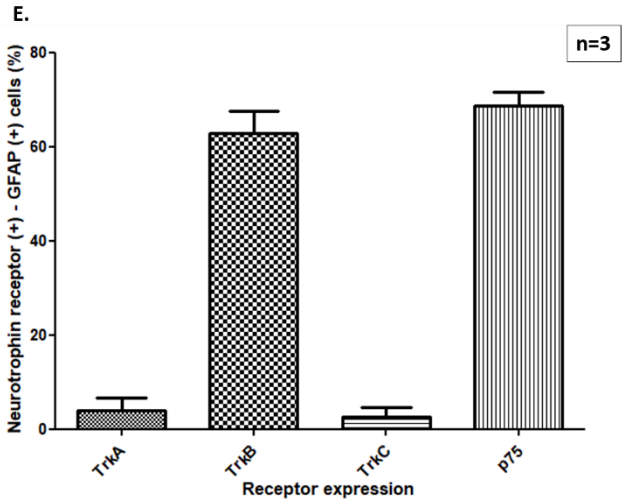


Figure 15: A) Western Blot for TrkA receptor in 3 biological replicates of astrocytic cultures and representative fluorescent photos at 32x magnification. B) Western Blot for TrkB receptor in 3 biological replicates of astrocytic cultures and representative fluorescent photos at 32x magnification. C) Western Blot for TrkC receptor in 3 biological replicates of astrocytic cultures and representative fluorescent photos at 32x magnification. D) Western Blot for p75 receptor in 3 biological replicates of astrocytic cultures and representative fluorescent photos at 32x magnification. E) Quantification of Neurotrophin receptor (+) / GFAP (+) cell ratio for all the Trk and p75 receptors in fixed, immunostained astrocytic cell cultures.

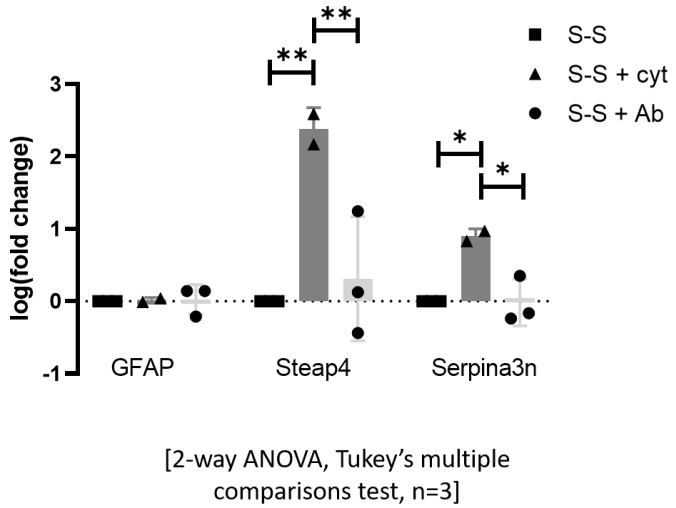
Amyloid-beta cannot directly activate astrocytes

After clarifying that the astrocytic cultures do express the necessary receptors to interact with our microneurotrophins, it was time to establish a protocol for astrocytic activation mediated by Aβ upon which we would test our hypothesis that microneurotrophins can alter the reactive astrocytes profile in 5xFAD mice. Our first attempt was to try and activate the astrocytic population directly through treatment with Aβ42 oligomers (10μM) for 24h. To properly measure astrocyte activation through qRT-PCR, we will use 3 pan-reactive markers (GFAP, Steap4 and Serpina3n) whose overexpression has been associated to astrogliosis (Liddelow, Guttenplan et al. 2017). As a positive control of astrocytic activation, we will use a combination of the cytokines IL-1β (10ng/ml) and TNFa (30ng/ml), which have been found to be able to induce an inflammatory reactive astrocyte phenotype (Hyvarinen, Hagman et al. 2019). For all of the treatments we used a serum-stripped medium that lacks steroids.

Our results showed that Aβ42 failed to activate astrocytes directly, even at a so high concentration, as we cannot observe any differences in the expression levels between the untreated cultures and the Aβ42-treated ones (Figure 16). These results have also been highlighted by another group that too failed to activate astrocytes through direct administration of Aβ42 (Xu, Zhang et al. 2018). The reason for this, could be the fact that Aβ42-mediated neuroinflammation response in microglia is conveyed through the activation of TLR-4 receptor and the downstream MYD88 pathway, which astrocytes lack (Walter, Letiembre et al. 2007, Zhang, Sloan et al. 2016).

This result also confirms that reactive astrocytic activation in neurodegenerative diseases, is indeed induced as a downstream effect of microglia activation (Liddelow, Guttenplan et al. 2017). For this reason, our next approach to successfully activate astrocytes through Aβ treatment would be an indirect method that will involve the

Aβ-mediated activation of microglia culture for 24h and the use of the isolated conditioned medium from these cultures to activate the astrocytic population.



[2-way ANOVA, Tukey's multiple comparisons test, n=3]

Figure 16: Graphic representation of the logarithmic fold change of the 3 pan-reactive markers (GFAP, Steap4, Serpina3n) of astrogliosis in cultures treated with 10μM Aβ42 compared to the untreated cultures.

Discussion

Neurotrophic factors are of extreme importance in the regulation of the proper development and maintenance of the neural network in the CNS. Their neuroprotective capabilities in the degenerating brain are well-characterized but their big non-lipophilic structure in combination with their quick denaturation in the plasma acted as a deterrent to their potential therapeutic exploitation.

In our lab, we are currently working with an innovative drug category named "microneurotrophins" or simply BNNs. These are small, lipophilic analogues of DHEA, a neurosteroid with well characterized neuroprotective effects, which it exerts via signaling through neurotrophin receptors Trk and $p75^{NTR}$. Microneurotrophins bear DHEA's ability to bind to all Trk and $p75^{NTR}$ receptors, while at the same time lacking its steroidal properties, rendering them ideal for therapeutic interventions. In vivo work in our lab has revealed that microneurotrophins also act in a neuroprotective way in the CNS in many mouse models including the "weaver" mouse (PD), the streptozotocin-induced diabetic rat (DR) and the 5xFAD mouse model (AD). In the case of 5xFAD mice, treatment at 1.5 months of age, before cognitive dysfunction emerges, resulted in significantly improved working memory, decreased Aβ plaque formation in the hippocampus and increased proliferation and neuronal generation in the dentate gyrus.

Through this work, we aim to replicate these neuroprotective effects in vitro in order to find out which brain cells interact with the BNNs, and at the same time, unravel the pathways implicated in this process.

Initially, we worked with primary cells derived from the hippocampus of newborn mice (P7 hippocampal NSCs), in order to establish the positive effects of microneurotrophins in adult neurogenesis. Indeed, using BrdU immunostainings to measure the proliferating capabilities of our cultures, we were able to observe significant BNN-mediated rescue of the proliferation when our cultures were partially or completely-deprived of the necessary mitogen EGF. These results reveal the direct interaction between microneurotrophins and P7 hippocampal NSCs and highlights the importance of this interaction in the ameliorated in vivo phenotype observed by our team.

The next step was to simulate the AD pathology in vitro and test the neuroprotective properties of microneurotrophins under Aβ-induced toxicity. Both BNN27 and BNN237 significantly increased the proliferation of the Aβ-toxic cultures under partial EGF deprivation, compared to the Aβ-toxic cultures under partial EGF deprivation without the BNN treatment. But lack of significant proliferative difference between the partially EGF-deprived cultures and the partially EGF-deprived, Aβ-toxic cultures forbid us from strongly suggesting that BNNs protect our cultures from Aβ-toxicity.

For this reason, we tried different treatments for our cultures, including higher Aβ concentrations and longer exposure, in order to effectively distinguish the Aβ-toxic effects in our P7 hippocampal NSC cultures and the possible neuroprotective effects of microneurotrophins to them. The results indicated that our experiments should be performed in complete medium with increased Aβ concentration (5μM) and longer exposure to the treatments (48h). These experiments are already on-going and will soon be incorporated to this study, further enriching our data regarding the neuroprotective capabilities of microneurotrophins on adult neurogenesis.

At the same time, we sought out to unravel the receptors involved in the P7 hippocampal NSCs increased BNN-mediated proliferation. Results in transcriptional and translational level shown in the present work, reveal the Trk's and $p75^{NTR}$ receptor expression patterns of our cultures, with TrkB and TrkC being the only two receptors present. Future work involves the use of Trk inhibitors in Aβ-toxic cultures treated with BNNs in an effort to clarify if and which Trk receptor is involved.

Besides Trk and $p75^{NTR}$ receptors, we wanted to test the possible involvement of other receptors known through literature to interact with DHEA, like $GABA_A$ and NMDA receptors. To investigate this, we used ketamine and bicuculline as inhibitors of NMDA and $GABA_A$, respectively, in partially EGF-deprived cultures where we have observed before rescue in the proliferation of P7 hippocampal NSCs in the presence of microneurotrophins. The results show a trend for reduced proliferation when bicuculline is added to the culture, thus indicating a possible implication of the $GABA_A$ receptor in the neuroprotective effects of the microneurotrophins. Ketamine addition to the cultures, on the other hand, does not seem to have any effect in the actions of microneurotrophins. Due to lack of statistical significance, at least two more replicates of this experiment are underway in order to intensify the observed differences.

Another brain cell population that emerged through the literature as a possible target of microneurotrophins was astrocytes. Astrocyte activation has clearly been implicated in AD and is considered one of the key regulators of chronic neuroinflammation, a hallmark of many neurodegenerative diseases. Astrocytes nowadays are known to have two distinctive reactive phenotypes, A1 and A2. A1 reactive astrocytes are mainly neurotoxic, releasing pro-inflammatory cytokines and leading to excess neuroinflammation and neurodegeneration. A2 reactive astrocytes on the other hand, are considered neuroprotective with anti-inflammatory properties. In AD patients, the rates of A1 reactive astrocytes are significantly elevated and they were found to be located mainly around senile plaques. Astrocytes express the TrkB receptor, as we also demonstrated in this study, and it has been proposed to interact through this receptor with BDNF, an interaction that may even lead to their activation.

Microneurotrophins, could theoretically exploit this interaction and through TrkB signaling, alter the final outcome of astrocytic activation back to the resting state or even to A2 reactive state, and thus ameliorating neuroinflammation.

To test this hypothesis, we first wanted to establish an Aβ-mediated astrocytic activation protocol to simulate the AD pathology effects in astrocytes in vitro. Our first attempt involved the direct treatment of our astrocytic cultures with Aβ42 (10μM). Using qRT-PCR, we were unable to detect any significant astrocytic activation between the control group and the Aβ42-treated one. This result indicate that astrocytes probably lack the receptors needed to recognize the Aβ molecules and subsequently activate to initiate the neuroinflammatory response. Also, it further highlights the importance of the microglia population in the induction of astrogliosis as many studies already have demonstrated. For this reason, there are on-going experiments that involve the Aβ-mediated activation of microglia cultures for 24h and subsequent transfusion of the conditioned medium to astrocytic cultures for another 24h, in order to achieve in vitro the desired Aβ-mediated astrogliosis.

The next step, would be to measure transcriptionally (qRT-PCR) and translationally (immunocytochemistry) the levels of A1 and A2 reactive astrocytes in these cultures using specific markers from the literature and then compare those results to Aβ42-toxic cultures treated with BNN27 and BNN237. If indeed we observe a significant difference in the levels of A1 and A2 reactive astrocytes amongst the differently treated cultures, it would be of value to check for differences in the levels of pro-inflammatory and anti-inflammatory cytokines after BNN treatment.

Our approaches to investigate the scientific questions set in the aim section of this study, although being the appropriate ones based on the existing literature, do bare the limitation of not being able to perfectly simulate temporally, the way neurosteroids, like DHEA and glucocorticoids, affect neurogenesis and neuroinflammation by having certain ultradian expression rhythms. In other words, ideally, we would like to imitate the ultradian rhythm of DHEA, which has barely been studied and taken into account as a possible contributing factor, for our BNN administration to achieve maximum performance of the drug, as its stimulative

efficacy could be related to this rhythm. Thus, the ability to conduct chronopharmacological experiments to test this hypothesis became necessary.

The first step towards the successful creation of a platform that would allow the conduction of such experiments was concluded within this study as a side project. This step included the designing of all the necessary features and the creation of a 3D model to better illustrate the platform and work on it.

The platform would rely on a peristaltic pump attached to a flow controller that would allow for the administration of up to 10 different medium/treatments to the culture. The medium to be administered to the culture at each time would travel through the microfluidic tubing system to the Feeding Tank where it would be released at the exact time the user chooses to and at the desired flow. The microfluidic tubing system will remain air-tight at all times to ensure constant flow and pressure to move the medium around. Initially, the cell cultures will be seeded in detachable microfluidic channel slides, but as the platform would get upgraded, we plan to replace those slides with brain-on-a-chip slides that would be able to host and connect different cell culture strains at the same time. As fresh medium would be added to the cell culture, the conditioned one will travel through the microfluidic tubing system all the way to the Isolation Tank where it would be isolated in order to be used in molecular in vitro experiments. Peristaltic pump will receive control commands regarding flow speed and flow start/stop through wi-fi, via a portable desktop computer attached to the pump (Raspberry Pi 4). The whole platform will be placed inside a CO_2 incubator to ensure ideal conditions for the cell culture, while the peristaltic pump along with the portable desktop computer will be properly sealed in order to protect them from humidity.

A platform like this will become a valuable instrument in our hands as its capabilities can only be limited by the user's imagination. It will allow for the conduction of chronopharmacological experiments for any combination of drugs, substances or endogenous molecules that we will wish to examine at any in vitro disease model. Its integration to our daily in vitro experiments will revolutionize our approach to study the different diseases.

References

Allen, S. J., J. J. Watson and D. Dawbarn (2011). "The neurotrophins and their role in Alzheimer's disease." Curr Neuropharmacol **9**(4): 559-573.

Altman, J. and G. D. Das (1965). "Autoradiographic and histological evidence of postnatal hippocampal neurogenesis in rats." J Comp Neurol **124**(3): 319-335.

Arbo, B. D., F. Bennetti and M. F. Ribeiro (2016). "Astrocytes as a target for neuroprotection: Modulation by progesterone and dehydroepiandrosterone." Prog Neurobiol **144**: 27-47.

Arbo, B. D., F. S. Ribeiro and M. F. Ribeiro (2018). "Astrocyte Neuroprotection and Dehydroepiandrosterone." Vitam Horm **108**: 175-203.

Bali, P., A. Banik, B. Nehru and A. Anand (2019). "Neurotrophic Factors Mediated Activation of Astrocytes Ameliorate Memory Loss by Amyloid Clearance after Transplantation of Lineage Negative Stem Cells." Mol Neurobiol **56**(12): 8420-8434.
Bayer, S. A. (1983). "3H-thymidine-radiographic studies of neurogenesis in the rat olfactory bulb." Exp Brain Res **50**(2-3): 329-340.
Becker, K., A. Cana, W. Baumgartner and I. Spitzbarth (2018). "p75 Neurotrophin Receptor: A Double-Edged Sword in Pathology and Regeneration of the Central Nervous System." Vet Pathol **55**(6): 786-801.
Bernal, A. and L. Arranz (2018). "Nestin-expressing progenitor cells: function, identity and therapeutic implications." Cell Mol Life Sci **75**(12): 2177-2195.
Bernardi, F., A. Lanzone, R. M. Cento, R. S. Spada, I. Pezzani, A. D. Genazzani, S. Luisi, M. Luisi, F. Petraglia and A. R. Genazzani (2000). "Allopregnanolone and dehydroepiandrosterone response to corticotropin-releasing factor in patients suffering from Alzheimer's disease and vascular dementia." Eur J Endocrinol **142**(5): 466-471.
Botsakis, K., T. Mourtzi, V. Panagiotakopoulou, M. Vreka, G. T. Stathopoulos, I. Pediaditakis, I. Charalampopoulos, A. Gravanis, F. Delis, K. Antoniou, D. Zisimopoulos, C. D. Georgiou, N. T. Panagopoulos, N. Matsokis and F. Angelatou (2017). "BNN-20, a synthetic microneurotrophin, strongly protects dopaminergic neurons in the "weaver" mouse, a genetic model of dopamine-denervation, acting through the TrkB neurotrophin receptor." Neuropharmacology **121**: 140-157.
Charalampopoulos, I., C. Tsatsanis, E. Dermitzaki, V. I. Alexaki, E. Castanas, A. N. Margioris and A. Gravanis (2004). "Dehydroepiandrosterone and allopregnanolone protect sympathoadrenal medulla cells against apoptosis via antiapoptotic Bcl-2 proteins." Proc Natl Acad Sci U S A **101**(21): 8209-8214.
Chen, Y. and C. Dong (2009). "Abeta40 promotes neuronal cell fate in neural progenitor cells." Cell Death Differ **16**(3): 386-394.
Colombo, E. and C. Farina (2016). "Astrocytes: Key Regulators of Neuroinflammation." Trends Immunol **37**(9): 608-620.
Dechant, G. and Y. A. Barde (1997). "Signalling through the neurotrophin receptor p75NTR." Curr Opin Neurobiol **7**(3): 413-418.
Ding, H., J. Chen, M. Su, Z. Lin, H. Zhan, F. Yang, W. Li, J. Xie, Y. Huang, X. Liu, B. Liu and X. Zhou (2020). "BDNF promotes activation of astrocytes and microglia contributing to neuroinflammation and mechanical allodynia in cyclophosphamide-induced cystitis." J Neuroinflammation **17**(1): 19.
Hollands, C., N. Bartolotti and O. Lazarov (2016). "Alzheimer's Disease and Hippocampal Adult Neurogenesis; Exploring Shared Mechanisms." Front Neurosci **10**: 178.
Huang, E. J. and L. F. Reichardt (2003). "Trk receptors: roles in neuronal signal transduction." Annu Rev Biochem **72**: 609-642.
Hyvarinen, T., S. Hagman, M. Ristola, L. Sukki, K. Veijula, J. Kreutzer, P. Kallio and S. Narkilahti (2019). "Co-stimulation with IL-1beta and TNF-alpha induces an inflammatory reactive astrocyte phenotype with neurosupportive characteristics in a human pluripotent stem cell model system." Sci Rep **9**(1): 16944.
Iban-Arias, R., S. Lisa, N. Mastrodimou, D. Kokona, E. Koulakis, P. Iordanidou, A. Kouvarakis, M. Fothiadaki, S. Papadogkonaki, A. Sotiriou, H. E. Katerinopoulos, A. Gravanis, I. Charalampopoulos and K. Thermos (2018). "The Synthetic Microneurotrophin BNN27 Affects Retinal Function in Rats With Streptozotocin-Induced Diabetes." Diabetes **67**(2): 321-333.
Iban-Arias, R., S. Lisa, S. Poulaki, N. Mastrodimou, I. Charalampopoulos, A. Gravanis and K. Thermos (2019). "Effect of topical administration of the microneurotrophin BNN27 in the diabetic rat retina." Graefes Arch Clin Exp Ophthalmol **257**(11): 2429-2436.
Kaplan, M. S. and J. W. Hinds (1977). "Neurogenesis in the adult rat: electron microscopic analysis of light radioautographs." Science **197**(4308): 1092-1094.

Kodis, E. J., S. Choi, E. Swanson, G. Ferreira and G. S. Bloom (2018). "N-methyl-D-aspartate receptor-mediated calcium influx connects amyloid-beta oligomers to ectopic neuronal cell cycle reentry in Alzheimer's disease." Alzheimers Dement **14**(10): 1302-1312.
Kuhn, H. G., T. Toda and F. H. Gage (2018). "Adult Hippocampal Neurogenesis: A Coming-of-Age Story." J Neurosci **38**(49): 10401-10410.
Lazaridis, I., I. Charalampopoulos, V. I. Alexaki, N. Avlonitis, I. Pediaditakis, P. Efstathopoulos, T. Calogeropoulou, E. Castanas and A. Gravanis (2011). "Neurosteroid dehydroepiandrosterone interacts with nerve growth factor (NGF) receptors, preventing neuronal apoptosis." PLoS Biol **9**(4): e1001051.
Li, K., J. Li, J. Zheng and S. Qin (2019). "Reactive Astrocytes in Neurodegenerative Diseases." Aging Dis **10**(3): 664-675.
Liddelow, S. A., K. A. Guttenplan, L. E. Clarke, F. C. Bennett, C. J. Bohlen, L. Schirmer, M. L. Bennett, A. E. Munch, W. S. Chung, T. C. Peterson, D. K. Wilton, A. Frouin, B. A. Napier, N. Panicker, M. Kumar, M. S. Buckwalter, D. H. Rowitch, V. L. Dawson, T. M. Dawson, B. Stevens and B. A. Barres (2017). "Neurotoxic reactive astrocytes are induced by activated microglia." Nature **541**(7638): 481-487.
Lim, N. S., C. R. Swanson, H. R. Cherng, T. L. Unger, S. X. Xie, D. Weintraub, K. Marek, M. B. Stern, A. Siderowf, P. Investigators, I. Alzheimer's Disease Neuroimaging, J. Q. Trojanowski and A. S. Chen-Plotkin (2016). "Plasma EGF and cognitive decline in Parkinson's disease and Alzheimer's disease." Ann Clin Transl Neurol **3**(5): 346-355.
Limon, A., J. M. Reyes-Ruiz and R. Miledi (2012). "Loss of functional GABA(A) receptors in the Alzheimer diseased brain." Proc Natl Acad Sci U S A **109**(25): 10071-10076.
Lykissas, M. G., A. K. Batistatou, K. A. Charalabopoulos and A. E. Beris (2007). "The role of neurotrophins in axonal growth, guidance, and regeneration." Curr Neurovasc Res **4**(2): 143-151.
McAllister, A. K., D. C. Lo and L. C. Katz (1995). "Neurotrophins regulate dendritic growth in developing visual cortex." Neuron **15**(4): 791-803.
Millington, C., S. Sonego, N. Karunaweera, A. Rangel, J. R. Aldrich-Wright, I. L. Campbell, E. Gyengesi and G. Munch (2014). "Chronic neuroinflammation in Alzheimer's disease: new perspectives on animal models and promising candidate drugs." Biomed Res Int **2014**: 309129.
Minichiello, L., M. Korte, D. Wolfer, R. Kuhn, K. Unsicker, V. Cestari, C. Rossi-Arnaud, H. P. Lipp, T. Bonhoeffer and R. Klein (1999). "Essential role for TrkB receptors in hippocampus-mediated learning." Neuron **24**(2): 401-414.
Olabarria, M., H. N. Noristani, A. Verkhratsky and J. J. Rodriguez (2010). "Concomitant astroglial atrophy and astrogliosis in a triple transgenic animal model of Alzheimer's disease." Glia **58**(7): 831-838.
Panagiotakopoulou, V., K. Botsakis, F. Delis, T. Mourtzi, M. N. Tzatzarakis, A. Dimopoulou, N. Poulia, K. Antoniou, G. T. Stathopoulos, N. Matsokis, I. Charalampopoulos, A. Gravanis and F. Angelatou (2020). "Anti-neuroinflammatory, protective effects of the synthetic microneurotrophin BNN-20 in the advanced dopaminergic neurodegeneration of "weaver" mice." Neuropharmacology **165**: 107919.
Paoletti, P., C. Bellone and Q. Zhou (2013). "NMDA receptor subunit diversity: impact on receptor properties, synaptic plasticity and disease." Nat Rev Neurosci **14**(6): 383-400.
Pediaditakis, I., P. Efstathopoulos, K. C. Prousis, M. Zervou, J. C. Arevalo, V. I. Alexaki, V. Nikoletopoulou, E. Karagianni, C. Potamitis, N. Tavernarakis, T. Chavakis, A. N. Margioris, M. Venihaki, T. Calogeropoulou, I. Charalampopoulos and A. Gravanis (2016). "Selective and differential interactions of BNN27, a novel C17-spiroepoxy steroid derivative, with TrkA receptors, regulating neuronal survival and differentiation." Neuropharmacology **111**: 266-282.

Pediaditakis, I., A. Kourgiantaki, K. C. Prousis, C. Potamitis, K. P. Xanthopoulos, M. Zervou, T. Calogeropoulou, I. Charalampopoulos and A. Gravanis (2016). "BNN27, a 17-Spiroepoxy Steroid Derivative, Interacts With and Activates p75 Neurotrophin Receptor, Rescuing Cerebellar Granule Neurons from Apoptosis." Front Pharmacol **7**: 512.
Ponath, G., C. Park and D. Pitt (2018). "The Role of Astrocytes in Multiple Sclerosis." Front Immunol **9**: 217.
Poyhonen, S., S. Er, A. Domanskyi and M. Airavaara (2019). "Effects of Neurotrophic Factors in Glial Cells in the Central Nervous System: Expression and Properties in Neurodegeneration and Injury." Front Physiol **10**: 486.
Ransohoff, R. M. (2016). "How neuroinflammation contributes to neurodegeneration." Science **353**(6301): 777-783.
Ransohoff, R. M., D. Schafer, A. Vincent, N. E. Blachere and A. Bar-Or (2015). "Neuroinflammation: Ways in Which the Immune System Affects the Brain." Neurotherapeutics **12**(4): 896-909.
Schwindt, T. T., F. L. Motta, G. F. Barnabe, C. G. Massant, O. Guimaraes Ade, M. E. Calcagnotto, F. S. Conceicao, J. B. Pesquero, S. Rehen and L. E. Mello (2009). "Short-term withdrawal of mitogens prior to plating increases neuronal differentiation of human neural precursor cells." PLoS One **4**(2): e4642.
Skerratt, S. E., M. Andrews, S. K. Bagal, J. Bilsland, D. Brown, P. J. Bungay, S. Cole, K. R. Gibson, R. Jones, I. Morao, A. Nedderman, K. Omoto, C. Robinson, T. Ryckmans, K. Skinner, P. Stupple and G. Waldron (2016). "The Discovery of a Potent, Selective, and Peripherally Restricted Pan-Trk Inhibitor (PF-06273340) for the Treatment of Pain." J Med Chem **59**(22): 10084-10099.
Sofroniew, M. V. (2014). "Multiple roles for astrocytes as effectors of cytokines and inflammatory mediators." Neuroscientist **20**(2): 160-172.
Solà, C., R. Cristòfol, C. Suñol and C. Sanfeliu (2011). Primary Cultures for Neurotoxicity Testing. **56:** 87-103.
Threadgill, D. W., A. A. Dlugosz, L. A. Hansen, T. Tennenbaum, U. Lichti, D. Yee, C. LaMantia, T. Mourton, K. Herrup, R. C. Harris and et al. (1995). "Targeted disruption of mouse EGF receptor: effect of genetic background on mutant phenotype." Science **269**(5221): 230-234.
Vicario-Abejon, C., D. Owens, R. McKay and M. Segal (2002). "Role of neurotrophins in central synapse formation and stabilization." Nat Rev Neurosci **3**(12): 965-974.
Villette, V. and P. Dutar (2017). "GABAergic Microcircuits in Alzheimer's Disease Models." Curr Alzheimer Res **14**(1): 30-39.
Walter, S., M. Letiembre, Y. Liu, H. Heine, B. Penke, W. Hao, B. Bode, N. Manietta, J. Walter, W. Schulz-Schuffer and K. Fassbender (2007). "Role of the toll-like receptor 4 in neuroinflammation in Alzheimer's disease." Cell Physiol Biochem **20**(6): 947-956.
Wang, L., H. C. Chiang, W. Wu, B. Liang, Z. Xie, X. Yao, W. Ma, S. Du and Y. Zhong (2012). "Epidermal growth factor receptor is a preferred target for treating amyloid-beta-induced memory loss." Proc Natl Acad Sci U S A **109**(41): 16743-16748.
Xu, X., A. Zhang, Y. Zhu, W. He, W. Di, Y. Fang and X. Shi (2018). "MFG-E8 reverses microglial-induced neurotoxic astrocyte (A1) via NF-kappaB and PI3K-Akt pathways." J Cell Physiol **234**(1): 904-914.
Yanase, T., M. Fukahori, S. Taniguchi, Y. Nishi, Y. Sakai, R. Takayanagi, M. Haji and H. Nawata (1996). "Serum dehydroepiandrosterone (DHEA) and DHEA-sulfate (DHEA-S) in Alzheimer's disease and in cerebrovascular dementia." Endocr J **43**(1): 119-123.
Zhang, Y., S. A. Sloan, L. E. Clarke, C. Caneda, C. A. Plaza, P. D. Blumenthal, H. Vogel, G. K. Steinberg, M. S. Edwards, G. Li, J. A. Duncan, 3rd, S. H. Cheshier, L. M. Shuer, E. F. Chang, G. A. Grant, M. G. Gephart and B. A. Barres (2016). "Purification and Characterization of Progenitor and Mature Human Astrocytes Reveals Transcriptional and Functional Differences with Mouse." Neuron **89**(1): 37-53.