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PRODUCTION, PURIFICATION AND CHARACTERIZATION OF THE EXTRACELLULAR DOMAIN OF p75

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Title of the TFG: *Production, purification and characterization of the extracellular domain of p75*

Summary: The objective of this TFG is to develop a protocol for the purification of the extracellular domain of the neurotrophin receptor p75, as well as to purify this same receptor and depending on the results, characterize its function and structure.

To that end, two different constructs of the receptor will be transfected into HEK 293 cells:

-One construct with the extracellular domain of p75 linked to a histidine tail and to an HA tag.

-One construct with the same domain, with a mutation in the 228th residue, so a threonine is converted to a cisteine residue so that constitutive dimerization happens. It also has a histidine tail and a HA tag.

The purification will be performed mainly by the use of an affinity column for histidine tails, as well as other complementary methods such as dialysis. The aim is to obtain the highest possible quantity of purified protein for further assays.

After production and purification of both constructs, if results are favorable, several tests and assays will be carried out in order to characterize the structure and interaction of the receptor with its ligand, neurotrophin NGF.

Resumen: El objetivo del TFG es el de desarrollar un protocolo de purificación del dominio extracelular del receptor de neurotrofinas p75, así como purificar este mismo receptor y en función de los resultados, caracterizar su función y estructura.

Para ello se realizará la trasfección de dos construcciones distintas de dicho receptor en células HEK 293:

-Una construcción con el dominio extracelular de p75, junto a una cola de histidinas y una región HA, que abarca un total de 244 aminoácidos.

-Una construcción con el mismo dominio, con una mutación en el aminoácido 228, que pasa de ser una treonina a presentar una cisteína, lo que permite la dimerización constitutiva del receptor. También presenta cola de histidinas y región HA.

La purificación se realizará principalmente mediante columnas de afinidad para las colas de histidina, así como con otros métodos complementarios como la diálisis. Se trata de obtener la mayor cantidad de proteína pura posible, para realizar a continuación diversos ensayos.

Tras la producción y purificación de ambas construcciones, si los resultados son favorables, se realizarán también diversas pruebas y ensayos para caracterizar la estructura e interacciones del receptor al unirse su ligando, la neurotrofina NGF.

Key words: p75, NGF, neurotrophin, purification, Western Blot

Palabras clave: p75, NGF, neurotrofina, purificación, Western Blot

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Y a mi familia, por estar ahí todo el camino.

INDEX

FIGURE INDEX

1. INTRODUCTION

1.1. Overview

The p75 neurotrophin receptor ($p75^{NTR}$) has been an object of study in neurobiology for years, as it participates in different pathways that lead either to neuron survival or cell death. A member of both the tumor necrosis factor family of receptors (TNFSR) and a neurotrophin receptor, the disparities in function of $p75^{NTR}$ arise from the great number of ligands this protein can bind to.

Several efforts have been made to discover how this receptor carries out its function, as well as its structure and how it affects signal transmission inside the cell. However, we still don't fully understand how this receptor works in the signaling pathways of the neurons, and many studies seem to contradict each other, usually raising more questions than answers. The main aim of this short study is to try to understand how this pathway might be working, by producing and purifying the extracellular domain of the $p75^{NTR}$ from the wild type and from a constitutively active mutant, and characterizing its interaction with the neurotrophin NGF.

1.2. Neurotrophins

Neurotrophins are a family of small dimeric proteins that participate in a great number of physiological processes in vertebrates. Neurotrophins such as the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT3) and neurotrophin-4/5 (NT4/5) have been studied in mammals (see Figure 1), and all share similarities in both structure and function (Dechant & Barde, 2002; Dechant & Neumann, 2000-2013). Moreover, neurotrophin-6/7 have been identified in fish, although there doesn't seem to be orthologues in mammals or birds (Gotz et al., 1994; Nilsson et al., 1998).

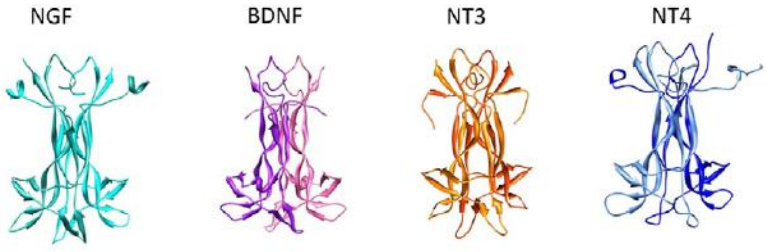

Figure 1. Structure of mammalian neurotrophins (Allen et al., 2013).

Neurotrophins are synthesized by sympathetic and sensory organs (Korsching, 1993). These growth factors are then taken in by the nerves and transported through the axon to the neural body, where they promote cell growth and differenciation. For

the neurotrophins to be biologically active, they must form a homodimer by a non-covalent bond between two identical peptide chains. Although the basic structure of all four neurotrophins is similar, there are crucial differences that are unique to each one, allowing their wide range of behaviors in the organism (Dechant G, Neumann H, 2000-2013).

Of all neurotrophins that have been characterized, NGF is perhaps the most well known. It was discovered in 1950 as a molecule that enhanced neuronal survival and development in the peripheral nervous system (Levi-Montalcini & Hamburger, 1951). Since then, numerous studies have increased our knowledge on the role of this molecule in the development of the nervous system, such as its participation in the maintenance of the structural integrity of cholinergic neurons in the central nervous system (Aloe et al., 1997).

NGF is synthesized at first as a precursor called proNGF, and is then cleaved by a serin protease to produce the mature and biologically active form of NGF. Generally, neurotrophic precursors are inactive; however, proNGF has been found to participate as a ligand in apoptosis processes (Bradshaw et al., 2015; Li et al., 2001).

As to how this neurotrophin sends signals to the cell, it's thanks to its two well known receptors, TrkA and $p75^{NTR}$. Both of these receptors are transmembrane proteins that bind to NGF and send a signal towards the cell to induce different changes. TrkA (short for Tropomyosin rearranged kinase receptor A) is a member of the Trk protein family of receptors, which is comprised by the receptors A, B and C (See Figure 2). These receptors dimerize upon binding to NGF, and their intracellular tyrosine-kinase domain is phosphorilated and converted into a docking site for intracellular enzymes (Reichardt, 2006).

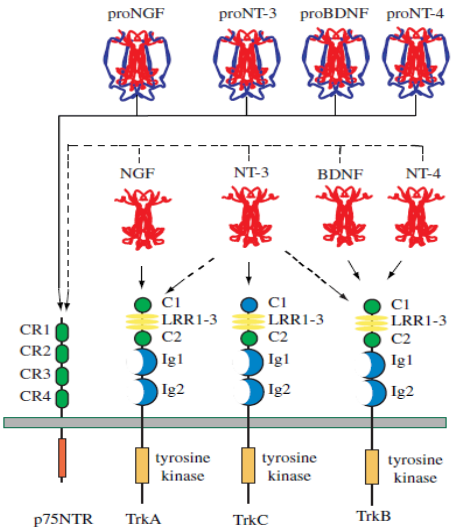

Figure 2. Interaction of $p75^{NTR}$ and the Trk family with neurotrophins (Reichardt, 2006).

$p75^{NTR}$ was the first NGF receptor to be discovered, and also the founding member of the tumor necrosis factor family of receptors (TNFR) (Vilar, 2017). However, following studies have made this receptor a rather peculiar case of study, isolating it from other similar receptors.

1.3. p75 neurotrophin receptor

The p75 neurotrophin receptor ($p75^{NTR}$) is a membrane protein mainly present in neurons in mammalian brain, though it can also be found in many other organs and tissues. It belongs to the family of tumor necrosis factor superfamily of receptors (TNFSR), composed by 29 different receptors that have been studied to date as well as 18 ligands. It is of no surprise, then, that this family is related to a wide variety of physiological processes such as cell death or survival (Cabal-Hierro & Lazo, 2012).

$p75^{NTR}$ participates in a wide variety of physiological processes involving neuron development and survival. The role of this neurotrophin receptor is complex and contradictory, as it both promotes cell survival and cell death (See Figure 3). Other processes in which $p75^{NTR}$ has been studied to have an effect are axon elongation or synaptic transmission.

Cell survival

It's common occurrence that neurotrophic receptors p75 and Trk are coexpressed in neurons. In cases where p75 is coupled with TrkA, it has been studied that p75 produces a positive modulatory effect of TrkA, thus improving cell differentiation and survival (Mamidipudi & Wooten, 2002). It is believed that this is due to $p75^{NTR}$ acting as an affinity enhancer between TrkA and NGF, which also increases cell survival if the ratio p75:TrkA is big (Casaccia-Bonnefil et al., 1998). Although the binding affinity of both p75 and TrkA to neurotrophins are similar (10^{-9} M), when in presence of p75, TrkA can bind to neurotrophins with an affinity almost 100 times greater (10^{-11} M) (Esposito et al., 2001).

Moreover, knockout models for p75NTR show a massive decrease in peripheral sensory neurons (von Schack, 2001), which confirms the important role of this receptor in neuronal survival and differentiation.

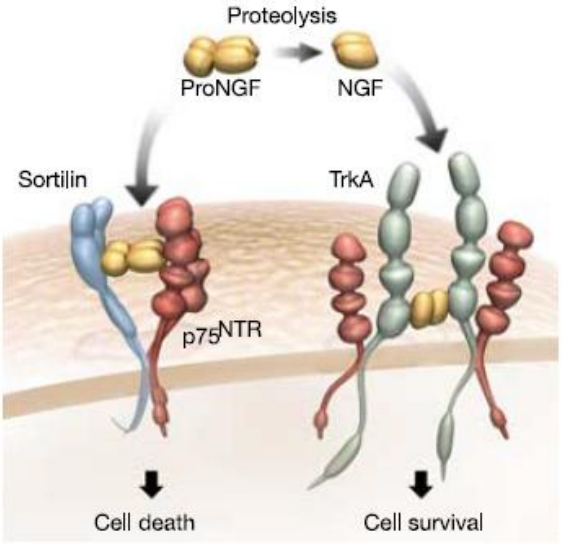

Figure 3. Comparison between the mechanisms involving p75NTR of cell death and survival (Nykjaer et al., 2004).

Cell death

As opposed to the previously explained effect, neurotrophin receptor p75 can transmit a death signal to the cell when it binds to the pro-NGF. Since this immature neurotrophin is unable to bind to the Trk receptors, it cannot promote cell survival (Dechant, 2002). Another factor that enhances this apoptotic effect is the interaction between p75NTR and sortilin, a membrane receptor located in the central and peripheral nervous system. In presence of proNGF, the sortilin/p75 complex has been found to be a critical factor in neuronal death during the development and aging of the nervous system, as well as a big player in several neurodegenerative diseases (Nykjaer & Willnow, 2012).

However, this is not the only way it has been studied $p75^{NTR}$ can send a death signal. In *in vitro* experiments, overexpression of the receptor alone carries an apoptotic meaning (Rabizadeh et al., 1993).

Axon elongation

The role of p75NTR in axonal growth has been studied in embryonic rat hippocampal neurons and chick ciliary neurons, where this receptor stimulates neurite outgrowth. This is due to the interaction of p75DD with the Rho protein.

RhoA is a GTPase that regulates actin polymerization, turning the cytoskeleton rigid and stopping neurite growth. p75 has been found to activate Rho by dissociating it from the RhoGDI complex, a downregulator of the Rho family (Yamashita & Tohyama,

2003), thus decreasing axonal elongation. However, if p75 binds with a neurotrophin. Rho activity is decreased and there's a neurite outgrowth (Yamashita et al., 1999).

Synaptic transmission

Recent studies have discovered that p75 has a role in synaptic transmission. In cocultures of myocites and neurons, p75 has been observed to alter the neurotransmitter release between the two types of cells in presence of neurotrophin BDNF. It seems that the primary role of this receptor is to release cholinergic vesicles to decrease contraction frequency by favoring the release of the inhibitory neurotransmitter acetylcholine (Yang et al., 2002).

It's also been detected that both Trk and $p75^{NTR}$ participate in synaptic plasticity in the central nervous system. Although p75 isn't directly related to the long term potentiation (LTP; a persistent strengthening of the synapses) in the hippocampus, there's evidence that supports that animals lacking this receptor have an impaired long term depression (LTD; the opposite process to LTP, it balances the strength of the synapses to encode new information) (Rösch et al., 2005).

1.4. p75 structure and how it relates to cell signaling

A common point in the members of this family is that none have enzymatic activity by themselves, and instead must recruit different signaling proteins to their intracellular regions (Li et al., 2013). In the case of $p75^{NTR}$, this receptor it's a death domain (DD) that needs to recruit intracellular factors and adaptors to be able to send the signal to the cell.

The main focus of study for $p75^{NTR}$ has been focused mainly in either its extracellular, transmembrane or intracellular domain individually (See Figure 4). That is, a structure of the whole receptor has not yet been achieved.

First of all, we have the extracellular domain, which consists of cysteine-rich domains and the stalk domain. $p75^{NTR}$ has 4 cysteine-rich motifs in this region, which can each bind to the NGF and form homodimers of the receptor. This gives rise to a stoichiometry of 2:2 for p75ECD:NT3 (Feng et al., 2010), though it has also been seen that for p75ECD:NGF the stoichiometry is actually 2:1 (He and Garcia, 2004). This domain can bind different neurotrophins and proneurotrophins, such as NGF, proNGF or NT-3, as several groups have demonstrated by crystallization of these complexes (Aurikko et al., 2005; Feng et al., 2010; Gong et al., 2008). However, p75ECD not only can bind to neurotrophins. It has been reported to bind to the neurotoxic prion protein fragment PrP, the Aβ-peptide of the amyloid precursor protein or APP, and the

envelope of the rabies virus (Della-Bianca et al., 2001; Yaar et al., 1997; Tuffereau et al., 1998).

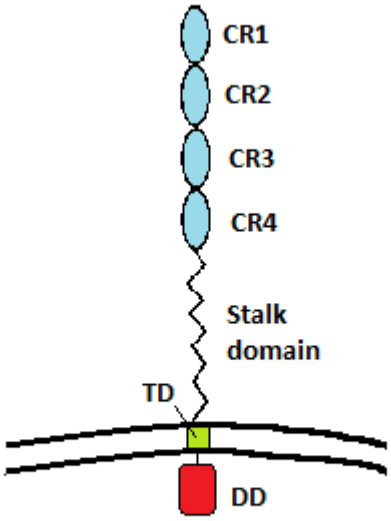

Figure 4. Domains of p75NTR. CR1-4 (cysteine-rich domains); stalk domain; TD (transmembrane domain); DD (death domain).

As for the stalk domain, there is not a lot of information about it yet. It's been studied that this domain is target of posttranslational modifications such as O-glycosilation, and that it seems to have a role in the sorting of proteins to the apical or basolateral surface of polarized epithelial cells, a critical process for the correct cell function and architecture (Youker et al., 2013).

Next is the intracellular region of $p75^{NTR}$, which is constituted by the juxtamembrane domain and a death domain. It has a disordered and flexible structure, and several studies have discovered protein adaptors such as TRAF4 and TRAF6 binding to this region (Ye et al., 1999; Khursigara et al., 1999).

As for the death domain, this region doesn't have any catalytic activity by itself, but instead recruits factors to transmit the signal to the inside of the cell. Such factors can be either other death domains from a different p75 dimer or membrane receptor p45, or it can form complexes with intracellular factors like RhoGDI or RIP2-CARD (Vilar, 2017). Depending on the factor recruited, the effect on the cell will be different; the complex p75DD/RhoGDI, for example, favors axonal elongation (Yamashita et al., 1999); and the p75DD/RIP2-CARD works as a modulator of the NF-κB pathway and the innate immunity (McCarthy et al., 1998; Kobayashi et al., 2002).

Lastly, the transmembrane domain presents a cysteine in position 257, which allows for constitutive dimerization of the p75 receptor without presence of neurotrophins (Vilar et al., 2009a). This has given rise to the question as to how can the signal be transmitted inside the cell if the receptor is already in dimeric form.

The current hypothesis is that the dimeric receptor acts in a way similar to a scissor model, where the binding of NGF to the extracellular domain induces an opening of the intracellular region, thus allowing the entrance of adaptors inside the cell (Vilar et al., 2009a). This model, however, might not be explaining completely p75-mediated signaling if the rigidity of the domain isn't taken into account; since the constitutive dimer is formed by covalent binding, it should not be able to freely move to induce this scissor-like movement.

The present work will focus on another hypothesis, which is that the conformational dimer of $p75^{NTR}$ binds to NGF and recruits another dimer of the same type to send a signal to the cell (See Figure 5). This theory is backed by the knowledge that the transmembrane domain is too rigid to suffer any kind of conformational change upon NGF binding to send a signal, so a possible option could be that p75 is forming clusters that would trigger the signaling pathways, very much like other members of the TNFSR family.

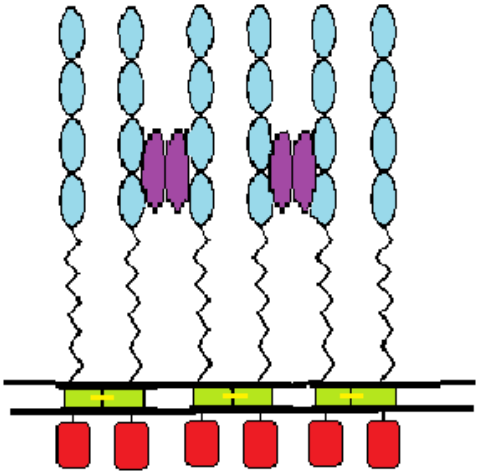

Figure 5. Representation of our proposed model of p75 clustering. In purple, NGF dimmers between constitutively active dimmers of p75.

To study this model, we will use two different constructs: a wild-type construct with the four cysteine-rich domains and the stalk domain, and a constitutively active variant with a mutation at T248C (Vilar et al., 2009b).

2. OBJECTIVES

The aim of this study is to produce, purify and characterize the whole extracellular domain, not only the cysteine-rich domains, which are the constructs used for the current studies of stoichiometry.

As for the objectives we want to achieve, they are the following:

1. To set up a production and purification protocol of p75-ECD from culture in HEK 293.

2. To optimize the purification protocol for the constructs with the wild-type p75 and a constitutively active mutant.

3. To characterize the wild-type and mutant constructs' interaction with NGF.

3. MATERIALS AND METHODS

3.1. Production of p75-ECD in cell lines

The production of p75-ECD was carried out in cell cultures that where transfected with a plasmid containing the DNA sequence of the protein.

Culture and maintenance of the cell lines

The chosen cell line to carry out the production was HEK 293 (human embryonic kidney cells) due to the ease with which they can be transfected and grown, as well as their high transfection efficiency (See Figure 6). The media culture used to maintain these cells was a complete DMEM media, prepared in the laboratory using commercial DMEM, fetal bovine serum (FBS) at 10%, L-Glutamine and antibiotics (penicillin and streptomycin).

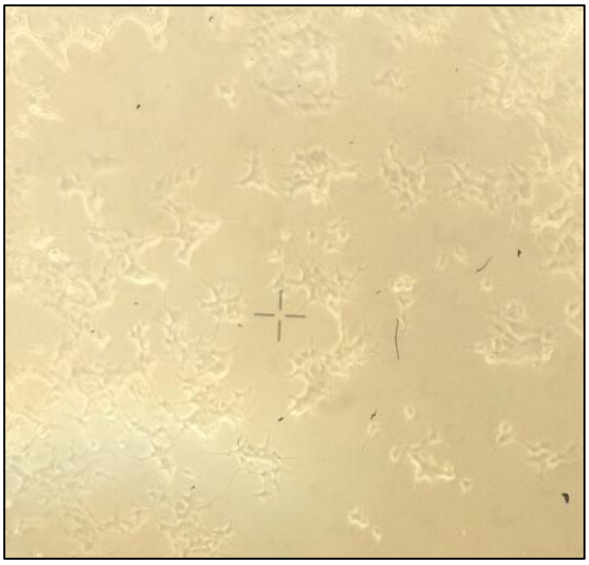

Figure 6. Plate with HEK 293 culture.

HEK cells were stored at -80ºC and kept in DMSO (dimetilsulfoxide), a cryoprotector. To unfreeze the cells and start the culture, we used the following procedure:

1 mL of frozen stock from HEK 293 is taken and seeded in 8 mL of complete media, and left to incubate at 37ºC for 24 hours. After a day has passed, the media is changed to avoid the toxicity of the DMSO to harm the cells.

- Alternatively, the frozen stock can be added to ~7 mL of complete media and centrifuged immediately after for 4 minutes at 1000 rpm. Then the media is removed and the pellet is resuspended in 1 mL of complete DMEM, and seeded in a new plate with 8 mL of media.

- This step greatly dilutes the DMSO and allows for the cells to grow faster.

Once the culture plates are fully covered in cells, that is, once they are convergent, the media is eliminated and the cells are washed with ~8mL of PBS to remove any traces of media left.

Afterwards ~3 mL of warm tripsin is added to the plate to separate the cells from the plastic surface. In the case of HEK, they could be separated only with PBS due to their low adherence, but they would become clumps.

After a few minutes the tripsin and the cells are put in a 15 mL Falcon tube with 2 mL of new complete media to stop the action of the tripsin. The cells are then centrifuged for 3 minutes at 1000 rpm to completely stop the tripsin.

During centrifugation, new plates are prepared with ~8 mL of new complete media. Once centrifugation is over, the supernatant is discarded and replaced with ~7 mL of complete DMEM. The cells are resuspended by gently aspiring them with the pipette.

Lastly, 1 mL of the resuspended cells is taken for each plate that has been prepared and mixed with the complete media by gently moving the plate. The plates are left to incubate at 37ºC.

Transfection of p75 in the cell culture

Before transfection, the cell media is changed, the optimal time being from 1 to 2 hours before starting the transfection. While in wait, we prepare the constructions to be trasnfected:

- A construct of wild type p75-ECD (wt) with an HA tag and a histidine tail. Construct #148.
- A construct of a conformational active p75-ECD (mut) with the point mutation T248C, with a HA tag and a histidine tail. Construct #343.

The transfection is carried out by a special mixture of media , the desired DNA and PEI reagent. The media used is called Opti-MEM, a minimal essential media (MEM) that reduces the need for serum complementation in cell culture, and is recommended for trasnfection procedures. For each plate of cells to be transfected, we use 100µL of Opti-MEM, 10µL of PEI and 10µg of DNA.

- With a simple calculation we determine the volume needed to get the 10 µg needed for the transfection. Since DNA is obtained from a purified extract from *E. coli*, the concentration of DNA in the case of the #148 and #343 plasmid is different. Plasmid #148 is at a concentration of 2.7 µg/ µL, and #343 at 1.4 µg/ µL. For #148 we need a total volume of 3.7 µL per plate and 7.1 µL for #343.
- By the end of the experiment, a DNA extraction was performed to produce more plasmid #148, this time with a concentration of 2.34 µg/ µL, so the volume needed in this case is 4.2 µL.

The process of transfection is as it follows:

The cell media is changed 1-2 hours before transfection with complete DMEM. This allows the cells to be in optimal conditions to resist the stress of the transfection process.

While in wait for the cells to adapt to the new media, the transfection media is prepared in a sterile eppendorf. The order of addition of the reagents is critical for the transfection to happen: first, we put the OPTIMEM, then the DNA, and finally, the PEI reactive.

- Order is critical because PEI is a reagent that forms micelles in solutions such as the media. The objective is for these micelles to take in the DNA plasmid with the construct and become a vector to insert the plasmid into the cell culture to synthesize the desired protein. After the mixture has been made, we wait for 15 minutes for the micelles to get created around the DNA. We can see how the media becomes turbid as time passes; it's a sign that PEI has started to form micelles.

After the hour has passed and the transfection mixture has been incubating for 15 minutes, we take the mixture with our DNA plasmid and gently put it in the cell plate, drop by drop to avoid damage to the cells, since the mixture is rather toxic.

The cells are left in the incubator at 37ºC for 24 hours. Next day, the cells are passed to another plate or a flask (to scale up protein production), and left again to incubate.

Retrieval of media culture with p75-ECD constructs

After transfection, we wait for 5-6 days to retrieve the culture media where the cells have been ejecting the p75-ECD. Since our construct doesn't have the transmembrane region, it doesn't stay in the membrane of the cells and is thrown into the media culture.

The retrieved media is put into falcon tubes and centrifuged at 1000 rpm for 5 minutes to eliminate any cell waste, and transferred to clean tubes once again. The media can be frozen at -20ºC if the purification cannot be carried out immediately after recollection.

3.2. Purification of p75-ECD constructs

Purification with an affinity column

Once we have our media with the produced protein in it, we need to purify the extract and remove all other protein extracts in the media that could, such as BSA (bovine serum albumin).

Although there are several methods for protein purification (such as precipitation or filtration), the one chosen for this experiment is the affinity column, a chromatographic method for purification. In it, we use a column made from resin coated in metal particles that bind to the histidine tail of the constructs we want to purify, capturing only our protein. Afterwards, the column is washed with a solution with imidazole, a compound that competes with the metallic beads, binds to the histidine tail, and releases the protein.

Before building the column, we prepared the needed solutions for the protocol (for the composition of each one, see ANNEX 1):

- NaCl 5 M.
- Phosphate buffer (pH 7,5), to equilibrate the pH and wash the column.
- Imidazol 0,5 M in phosphate buffer (pH 7,5), to elute the protein once it's captured by the column.
- $NiCl_2$ 0,1 M, a metalic solution to capture the protein in the column.
- WE (wash estringent), a mixture of phosphate buffer and imidazol to perform a stronger wash.

To avoid the degradation of the protein, we add protease inhibitors to the sample media we want to purify: PMSF (phenylmethylsulfonyl fluoride) and 5X inhibition cocktail, that contains several protease inhibitors.

Next step is to prepare an affinity column. A flowchart of the whole process can be seen in Figure 7.

For the preparation of the column we add 0.3 mL of resin (Chelating Agarose Beads Bulk Resin, ABT or similars) to a support. The resin is then washed to remove the preservative (ethanol) with 10 column volumes of distilled water (3 mL). Next, we add 10 volumes of a metallic salt solution, in this case $NiCl_2$, and wash with distilled water (10 volumes) twice to remove any non retained metal particle.

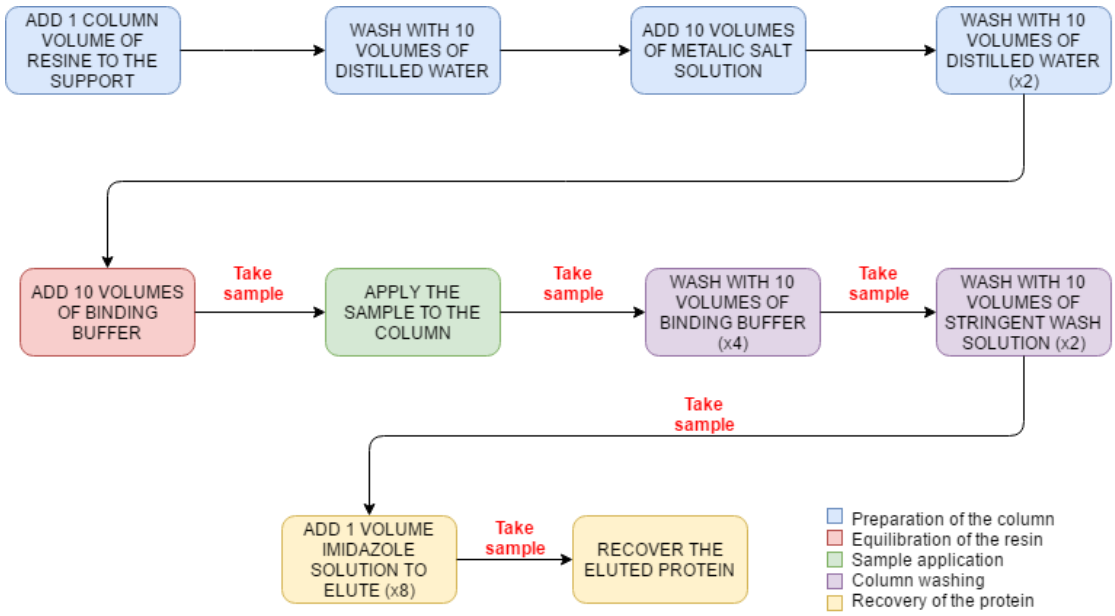

Figure 7. Schematic of the purification process.

Following this is the stabilization of the column with binding buffer (phosphate buffer pH 7.5), by adding 10 volumes of the solution.

Next step is the application of the sample to the column, adding the culture media to the column slowly, until all the liquid has passed through. A sample of the media is taken before and after it passes through the column for a polyacrilamide gel, and another to measure the absorbance (see Results for the values).

Afterwards, the column is washed with 10 volumes of binding buffer, four times. For each washing step, we take a sample for the gel and to measure the absorbance. After we finish the washes, we apply two stringent washes of 10 volumes each to further remove anything that is not our protein in the column. Samples as with the previous steps are taken.

Lastly, we elute the protein in 1.5 mL eppendorf. We add 1 volume of 0.5 M imidazole solution to the column for each eppendorf, with a total number of 8 elutions. From the eppendorf we take samples for the gel and the absorbance.

Protein dialysis

Before the process of dialysis it is necessary to make sure with a polyacrylamide gel that we have our protein (for the explanation on protein gel electrophoresis, see next point, Protein Gel Electrophoresis and Western Blot). Once we are sure our protein is correctly purified, we will dialyze it to remove the imidazole.

For this process, we use a cellulose tubular membrane. This membrane has pores that allow a washing buffer to remove small molecules such as imidazole from the protein, while our sample remains inside the membrane. The process of dialysis is as follows:

First of all, we prepare 2 L of PBS buffer for the dialysis. It can be done by diluting 200 mL of commercial 10X PBS into 1800 mL of distilled water.

Depending on the volume of sample we are going to dialyze, we will cut a length equivalent to 1 cm of membrane per 1 ml of sample. However, it's convenient to cut in excess, since we need to clip both extremes of the membrane to avoid losing the protein sample into the buffer. Generally speaking, we only take as protein samples the two first elutions from the purification step, since there's no signal of protein being in further elutions.

When we have the membrane cut, we put it into the dialysis buffer to rehydrate it. We also need to gently open it by a corner, taking care not to touch the sides of the membrane, and pour buffer into the membrane to complete the regeneration.

Once the membrane has been completely rehydrated, we close one of the sides with a clip, and carefully pipette the protein sample into the membrane. After all the sample has been put inside, the membrane is closed at the other side, and left into the dialysis buffer.

The buffer and protein will be left overnight spinning into the cold chamber (at 10ºC), and recovered the next day into an eppendorf.

We will measure again the absorbance of this recovered protein, and we will calculate it's concentration by means of the Lambeert-Beer equation:

$A = \varepsilon \times l \times c$; where A is the absorbance of the sample, ε is the extinction coefficient, l is the length that light travels in the sample, and c is the concentration of the protein.

To obtain the extinction coefficient we made use of the online resource ExPasy ProtParam, a tool that given an amino acid sequence, it returns parameters such as its molecular weight, theoretical pI and the extinction coefficients. The obtained extinction coefficient for both constructs was ε = 25910 M^{-1} cm^{-1}.

Storage of the purified protein

After dialysis, we can preserve the eluted protein in the fridge at 4 ºC for a couple of days while we perform other tests, but to correctly keep the protein we must freeze it with liquid N_2 at -186ºC and store it at -80ºC.

To improve the quality of the protein, the sample is divided in aliquots of smaller volume it doesn't suffer from consecutive cycles of freezing and unfreezing. For both constructs, we aliquot into tubes with 40 µl of sample.

3.3. Protein Gel Electrophoresis and Western Blot

Polyacrylamide gel

During the experiments, two different types of gels were used: the most common one is the denaturalizing gel, and the other is the native gel. Though the preparations for both are similar, the native gel will need a special preparation and running buffer.

The denaturing gels are those that have sodium dodecyl sulphate (SDS) in their composition, as well as in the sample buffer. SDS is a compound that denaturalizes and gives the protein a negative charge. Due to this negative charging, when connected to the current, the protein can migrate in a gel, from the negative pole to the positive one.

On the other hand, native gels don't have any SDS, thus the proteins maintain their 3D conformation and migrate according to their shape, instead of their charge.

Gel preparation

We make a polyacrylamide gel at 10-12% in the resolving gel and at 5% at the stacking gel (see Annex. Recipes). For the native gel, we won't use the 10% SDS.

Sample preparation

For denaturing conditions, we take a volume of the protein sample (usually 30 µl) and mix it with a fifth of its volume of denaturing sample buffer (SDS PAGE sample buffer with Coomassie Blue; for 30 µl of sample, it would be 6 µl).

- For native conditions, we take a volume of protein sample and mix it with a fifth of it of native sample buffer (Poinceau + Glycerol).

Running buffer preparation

Running buffer was prepared following the protocol of the laboratory (see Annex. Recipes).

- A special buffer was made for native gel electrophoresis, without SDS.

Gel run

We used the BioRad platform and protocol to perform our protein gel electrophoresis. The conditions in which we performed were a current of 120 V for about 1.5 hours.

Samples were put into the lanes with a Hamilton syringe, and a molecular marker was added to identify the weights of our protein. After that, the samples were added in the lanes, and sample buffer was added to avoid a wrong migration at the ends of the gel.

Western Blot

After electrophoresis, we transferred the protein from the gel to a nitrocellulose membrane. This was done by pressing the gel and the membrane together between filter paper, in a special plastic support. Once correctly pressed, the membrane and gel are connected again to electric current, this time at 100 V for 2 hours, to mobilize the proteins.

- A critical point for the transference to work is the correct collocation of the gel and the membrane in respect to the anode and the cathode. The membrane has to be put at the anode and the gel towards the cathode so that once electrical current flows, the protein migrates towards the membrane and not the filter paper.

Afterwards, we can dye the membrane with a solution of Poinceau to corroborate the presence of protein in the membrane, at the height we expect it to be due to its weight.

Once we have this step done, we wash the membrane with TTBS buffer to remove any remaining Poinceau and prepare milk for the membrane blocking. Membrane blocking is needed to avoid any unspecific binding of the antibodies. The milk solution is prepared by diluting powder milk and TTBS, with a 5% milk being enough to block the membrane. The membrane is left to block in milk for about an hour and with continuous agitation.

Next is a washing step with TTBS. This must be done for an hour with frequent changes of TTBS to remove any remaining milk protein that hasn't bound to the membrane. After washing, the membrane is incubated in the primary antibody, which is specific to the HA tag of the protein construct and comes from mouse.

- This incubation is mainly done overnight, but if need be it can also be performed by leaving the antibody 1 hours in agitation and then washing.

After the incubation with the primary antibody, a new round of washes is needed, once again to remove any unspecific binding of the antibody.

Then, the secondary antibody is put on the membrane, and incubated for an hour. The secondary antibody is an anti-mouse with specificity for the primary antibody. It also has a fluorophore group to detect the hybridized protein in the membrane with a fluorescence scanner. In our case, the fluorophore is red.

The last step is a final washing cycle, and then we can obtain the image of our protein in the membrane with the fluorescence scanner.

3.4. Membrane stripping

Membrane stripping consists of removing the blocking proteins as well as the antibodies on a Western Blot. This is done to repeat the incubation process if the protein has somehow become dirty or there has been a mistake during the process of incubation and the results cannot be extracted from it.

The process of stripping is as follows:

For a membrane, 20 ml of stripping solution is taken and mixed with 0.16 ml of β-mercaptoethanol (BME) inside a bottle. The mix is then heated in the microwave to the ebullition point, taking care in not letting it boil so the BME doesn't evaporate. This is done a couple of times, until the solution is well heated.

The membrane is washed in TTBS, which is later discarded, and the stripping solution is poured hot on top of the membrane. This must be done inside a _ due to the BME being a toxic volatile.

The membrane is then covered and left in agitation for 30 minutes. After the time has passed, the stripping solution is heated again and the process is repeated once more. Afterwards, the membrane is washed and it can be blocked again, and later incubated in the antibodies.

3.5. Reducing and non-reducing conditions assay

This assay is performed by adding BME to the sample buffer of the samples in a gel assay. The BME breaks the bonds of the cysteines, thus turning the dimers of the mut construct into monomers. For a sample of 30 µl of protein, 2µl of BME is added.

- Alternatively, a mix of SDS and BME can be made to avoid adding the reducing reagent to the sample each time. In that case, we take a volume of SDS buffer and add 10% of that volume of BME. If a long time passes, it is convenient to refill the BME as it is an extremely volatile compound and could be evaporating with time.

3.6. Cross-linking assay

A cross-linking assay is a type of experiment that allows the study of interactions between proteins close in space. A chemical molecule is used as a spacer arm, which has reactive ends that are able to join different molecules that are close enough to each other, recreating *in vitro* the process of bioconjugation that happens *in vivo*.

The cross-linker in our assay is BS3, which can join proteins that are apart from each other about 11.4 Å (See Figure 8).

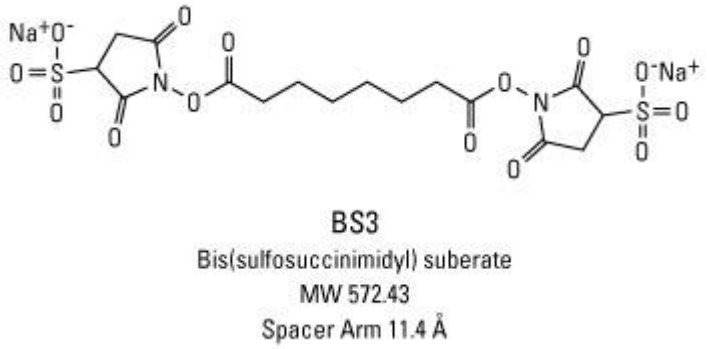

Figure 8. Chemical structure of BS3 cross-linking reagent (ThermoFisher).

Our assay consisted in a control sample, without cross-linker; a sample with only cross-linker; a sample with only NGF; and a sample with both NGF and cross-linker (for illustrative volumes, see Table 1). The process to carry out the assay is the following:

First of all, BS3 is weighted to reach a concentration of 25mM; 1 µg in 70 µl of PBS buffer. However, at this point we only need to weight the BS3.

Next, 4 aliquots of sample are prepared (40 x 4) and marked as 1 to 4. In tubes 3 and 4, we add 5 µl of NGF (100 ng/µl) and incubate the mix for 5 minutes at room temperature. During this time, we dilute the BS3 in the buffer solution.

- The BS3 is prepared immediately before using it to prevent it from linking to other molecules of BS3 instead of our protein sample.

In tubes 2 and 4, we add 5 µl of BS3 (25mM) and incubate the mixture for 30 minutes, at room temperature and in the dark.

After 30 minutes have past, we add a quencher to stop the BS3 from joining molecules. This quencher is Tris buffer at 1.5M, and we add 3 µl to each tube and wait for another 15 minutes.

After waiting, the samples are prepared to be used as usual for a polyacrylamide gel. To keep a uniform volume, we add to the tubes enough dialysis buffer to reach 50 µl.

Table 1. Cross-linking assay volumes (in µl). Final volume per tube: 50µl

Tube	Dialysis buffer	Protein sample	BS3	NGF	Quencher
1	10	40	-	-	3
2	5	40	5	-	3
3	5	40	-	5	3
4	-	40	5	5	3

It is important to note that these volumes are illustrative; depending on the experiment, some reduction of the protein sample might be needed, as well as the equivalent with the other reagents.

3.7. Plasmid DNA purification

Once the plasmid used for transfections was used, we performed a plasmid DNA extraction with the Qiagen commercial kit or similars. The process is as instructed by the kit, with all the reagents being already prepared.

4. RESULTS AND DISCUSSION

4.1. Production of p75

The production protocol of p75-ECD for the wt and mut constructs was optimized for transfection in p100 plates, and growth and production of the protein extract in flasks of 175 cm^2. Each flask produced ~35 ml of media that was later purified.

Troubleshooting

The main problems we found while setting up the production protocol for the p75-ECD was the contamination of the cell culture. These contaminations were due to several factors:

- **Manipulation**. Mistakes during manipulation of the cell plates such as opening them outside the cabin, not cleaning the material with ethanol 70%, touch the tips and using them on the culture, not wearing the lab coat or wear a wrong one, and other similar actions. The only way to avoid them is to focus on the experiment and be mindful of everything that is done at the cell culture room.
- **Wrong preparation of DMEM media**. One of the requirements of this complete media is the use of antibiotics; forgetting to put them in the media might be a source of contamination. The contaminated DMEM must be put away and any contaminated plate must be washed with bleach and thrown away.
- **Contaminated serum (FBS**). In one occasion, there was a contamination caused by the use of a contaminated serum in the complete DMEM used for cell culture. The result was that all plates that used that media were contaminated, and so they had to be discarded, as well as the contaminated media.
- **Use of serum (FBS) past its expiration date.** Although not a problem in itself, it was detected that the cells were growing at a slower rate than usual. Besides this, they didn't show abnormalities in morphology or transfection ability.
- **Contaminated washing buffer**. Contamination of PBS, which was used for washing the plates, was another source of contamination. The cause might have been an incorrect sterilization of the PBS in the autoclave, or a wrong storage one sterilized. Contaminated plates and PBS were removed.

Each time a contamination was detected, the cells were disposed of by rinsing them with bleach before throwing them away.

4.2. Purification of p75

The process of purification was repeated multiple times, and optimized by taking into account the results seen in the Western Blots.

First of all, it was confirmed that four steps were enough to remove any unspecific binding in the column, as can be seen from the absorbance data of the washing steps (see Figure 9). By the fourth washing step, the absorbance was reduced to a value of almost 0, meaning the column had been cleaned from every molecule not bound to it.

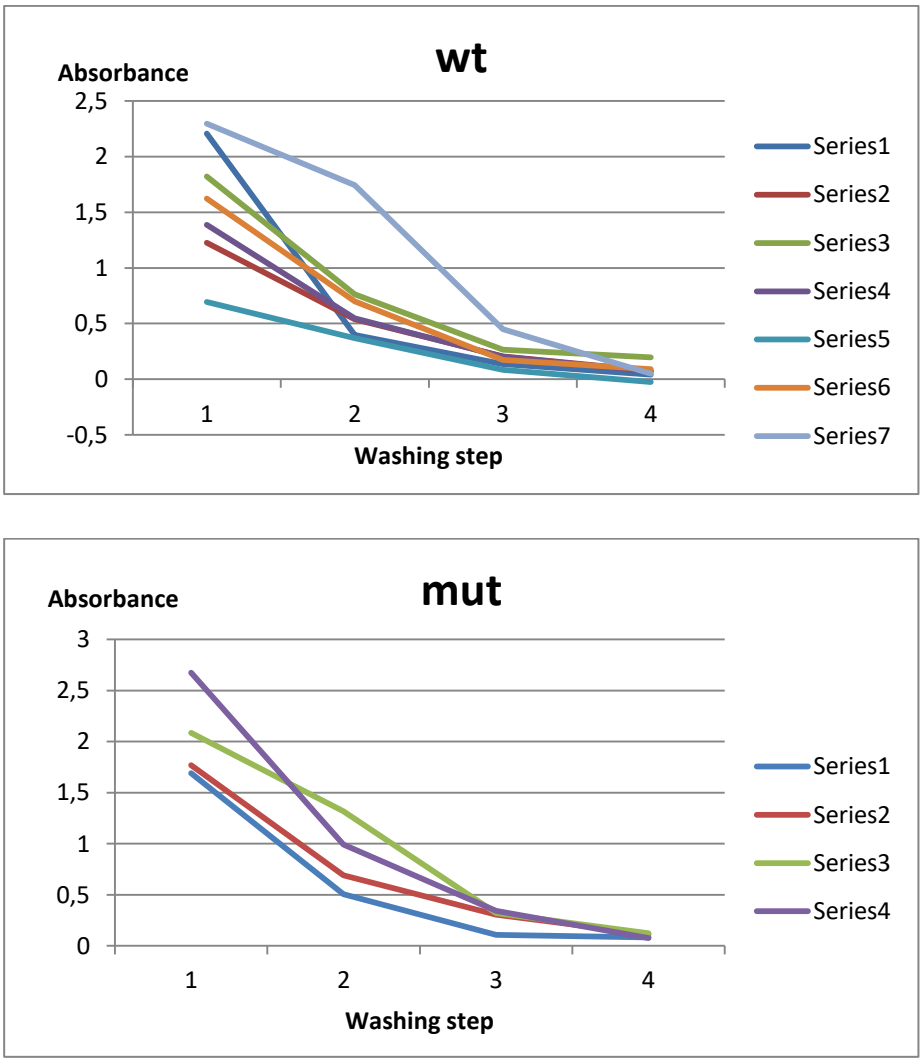

Figure 9. Top: absorbances for the washing steps of the wt construct. Bottom: absorbances for the washing steps of the mut construct. Each serie represents a different purification process.

From the protocol explained in MATERIALS AND METHODS, we decided to remove the stringent washes, due to the loss of the mutant protein during the first purification (see Figure 10). Our constructs of p75-ECD weight 26 kDa, but at this region is heavily glicosylated, we take into account an extra weight in sugars of around 25 kDa. Thus, the total weight of our constructs is of ~50 kDa.

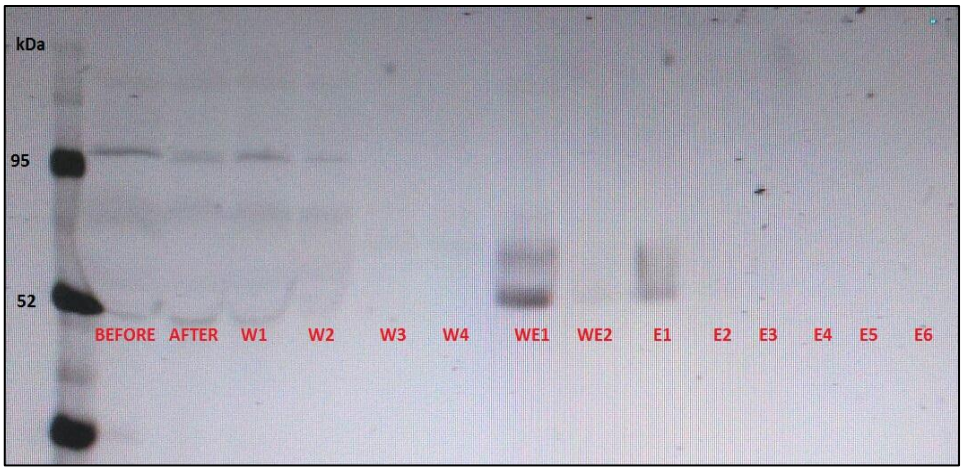

Figure 10. Western Blot with samples from each step of the purification process of the mut construct. Most of the protein can be seen in step WE1, which was discarded and therefore, lost during the washing steps.

After purification, we made sure we had protein by performing a Western Blot with a sample of each elution, and using a Poinceau staining solution before incubation with the antibodies (see Figure 11 for an example of both).

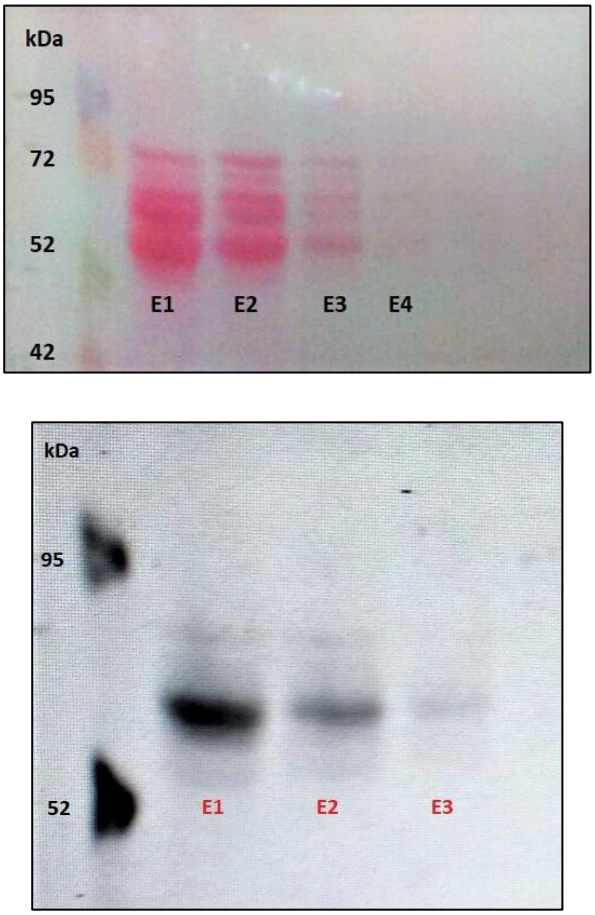

Figure 11. Top: Poinceau staining of a Western Blot with elutions from the purification of wt protein. Most of the staining is, in fact, BSA. Bottom: for the same Western, fluorescent signal of the tagged protein.

Once we were sure the elutions had our protein, we dialyzed them and measured the absorbances of the retrieved protein. These values are compiled in Table 2, along with the concentrations obtained from applying the Lambert-Beer equation (see MATERIALS AND METHODS for details).

Table 2. Values for the absorbance and concentration of the dialyzed sample of wt and mut.

	Abs	C
Wt	0,059	**6 µg/ml**
	0,231	**230 µg/ml**
	0,580	**568 µg/ml**
mut	0,479	**469 µg/ml**

The dialysis of the mut construct was carried out only one time, since it yielded good results on the first try. For the wt protein, each new purification yielded better results Once we obtained the concentrations of protein, we carried on with the experiments to characterize p75-ECD.

Troubleshooting

Some of the problems that arose during the purification of the protein were the following:

- **Loss of the protein during a stringent wash.** As previously stated, we lost our protein in a stringent wash during one of the first purifications. We decided to remove those steps to avoid losing the sample.
- **Wrong freezing method for the purified protein.** Instead of using a fast-freezing method like N_2 at -186 ºC and then storing the protein at -80 ºC, the first weeks the protein had been wrongly stored at -20 ºC and without previously using liquid nitrogen. This protein was deemed useless and was disposed of.
- **Problems with the protein gel electrophoresis.** The glass is not correctly put in the support so the gel flows down, the gel doesn't polymerize correctly, not enough running buffer in the support, sample flowing out of the lane, the sample diffuses if it's left too much time in the gel without connecting it to the current.
- **Problems with the Western Blot.** The membrane and the gel are put backwards so the sample migrates to the filter paper, insufficient time for the transference to happen, incomplete blocking, insufficient washing of the membrane, old antibodies.

4.3. Characterization of p75

The characterization of p75-ECD was carried out performing several assays and visualizing the results with protein gel electrophoresis and Western Blot.

Our objective was to have two types of both native and denaturing gel experiments: for the native gels, a simple migration assay of both constructs and a cross-linking assay; and for the denaturing gels one with the constructs treated with reducing and non-reducing conditions, and another cross-linking assay.

4.3.1. Native gel

A simple native gel was performed, without any prior treatment of the samples, to determine if the constructs migrated differently depending on their ability to form dimers or not (see Figure 12).

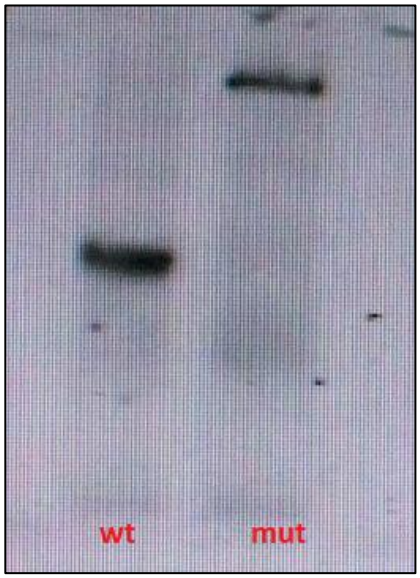

Figure 12. wt and mut protein in a native gel.

As it can be seen, the constructs have indeed differences in the migration; wt has migrated farther down than mut, which means that the mut construct weights more than the wt. This result is consistent with what we expected: since wt is not able to dimerize, all of it is in monomeric form so it migrates more than the mut construct. This one, as it has a constitutive cysteine bridge, forms dimmers and migrates much less than the other construct.

4.3.2. Denaturing gel in reducing and non-reducing conditions

The aim of this assay was to determine whether the mut construct was forming the constitutive cysteine bridge. By comparing each of the protein variants in two different conditions it would be clear if the mutated protein was indeed being dimerized.

What we were expecting was that in the reducing conditions both proteins migrate the same, since the BME breaks the cysteine bridges, and even the mut protein should be in monomeric form. For the non-reducing conditions, we expected the mut construct to show a band higher up than the wt, since it should be forming dimers and weight more than the monomers of the wt.

When we obtained the image of the membrane, we saw several fluorescent lines which impeded the correct reading of our experiment (see Figure 13). Although the protein can be seen in the membrane, we decided to perform a stripping protocol to remove the antibodies, wash the membrane and start again, to make sure a mistake wasn't made during the process of blocking and incubation.

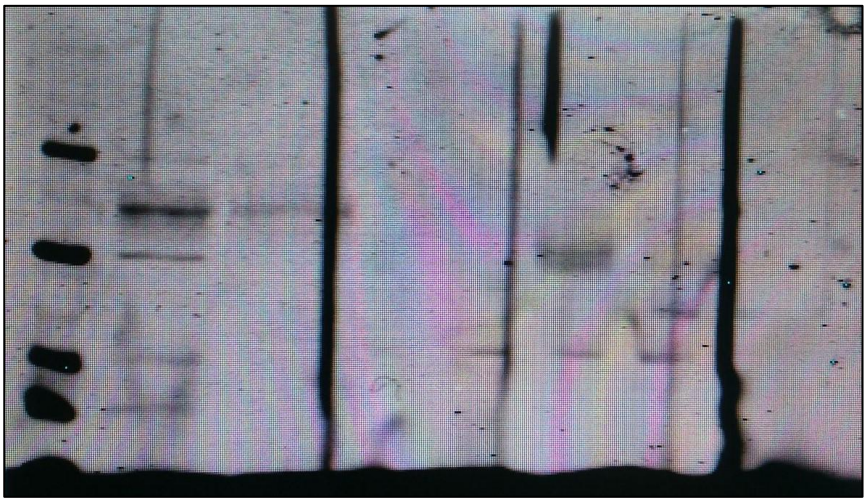

Figure 13. Denaturing gel with reducing and non-reducing conditions. Although the bands for the marker and the reduced protein can be seen, there are several vertical lines which show a very strong fluorescence that make impossible to see the non-reducing conditions.

The results for the stripping process were a slight increase in the clarity of the image, but the overall result didn't change even after the stripping. This points to a mistake during the gel electrophoresis, as well as the fact that the florescent lines only appear in the zone of the membrane where the gel was put during transference.

It was then decided that the experiment would be repeated (see Figure 14). This time, both constructs can be clearly seen in both treatments.

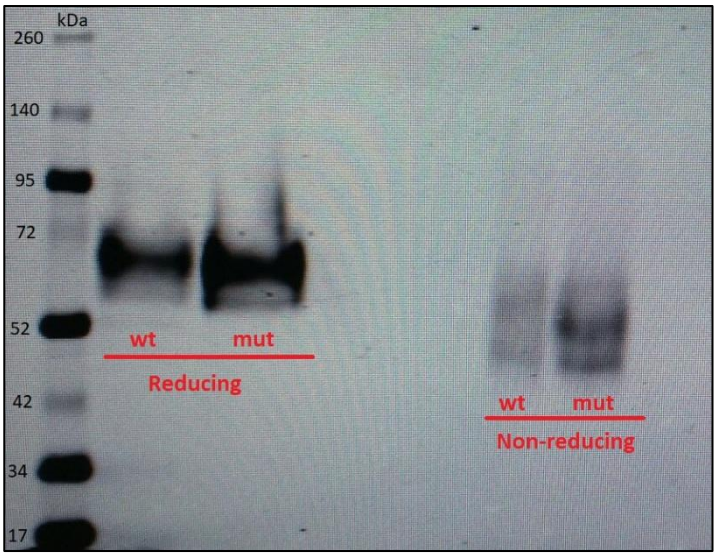

Figure 14. Definitive reducing and non-reducing gel assay.

We find that there seems to be no differences between the wt and mut constructs in any case. The samples treated with non-reducing conditions migrate further than the others because the structure of the protein is more compact, so they cross the pores of the gel easily.

If everything had been as we expected, we should have been able to see a higher band in the mut lane around ~100 kDa for the non-reducing conditions, as the dimer between cysteines should still be there. However, the band is actually at the same height that the monomeric wt construct. This might be due to the fact that the mut construct isn't actually forming dimers between cysteines, so the addition of BME is irrelevant. This contradicts with the native gel previously seen in Figure 12, though, which seemed to show us the dimer of p75-ECD in the mut construct.

4.3.3. Denaturing cross-linking assay

The denaturing cross-linking assay was performed with only the wt construct, as we were in the process of producing mut protein (see Figure 15). The protocol for the cross-linking assay is explained in its corresponding point of MATERIALS AND METHODS.

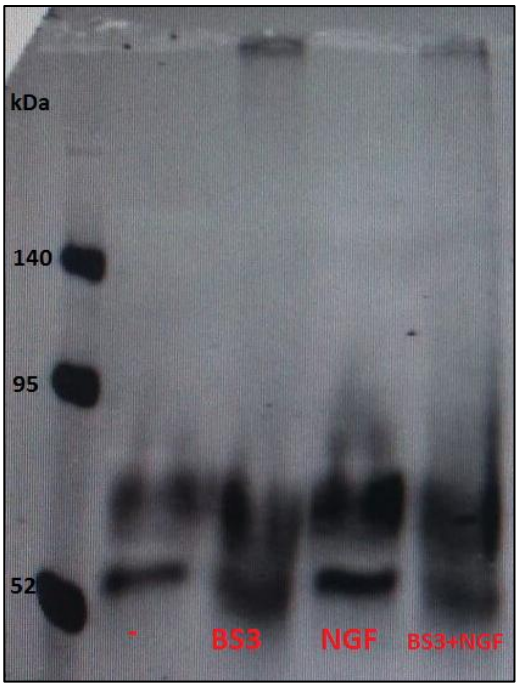

Figure 15. Cross-linking assay for the wt construct in denaturing conditions. The volume injected in each lane is of 40µl. From left to right, each lane has: a control with only protein; cross-linker and protein; NGF and protein; and cross-linker, NGF and protein.

In the lanes with BS3, it can be seen there is protein on the top of the gel. This could mean there are aggregates so big that haven't been able to enter through the pore size of the gel.

This might be due to a high concentration of cross-linker, which might have aggregated with itself as well as the protein. However, another reason as to why these aggregates are forming is the stalk domain of the protein constructs. Since they don't have a fixed structure, they might be facilitating an aggregation between molecules, which is then increased by the presence of the cross-linker.

It also appears that there is a double band in each lane. Judging by the weight, the bottom bands should be our protein, while it is possible that the upper bands are BSA that hasn't been correctly purified, thus cross-linking as well and appearing on top of the p75-ECD since BSA weights around ~66 kDa.

4.3.4. Native cross-linking assay

As opposed to the denaturing cross-linking assay, this second study was performed to visualize the behavior of both protein constructs under native conditions, without a denatured structure. Besides, the tube with only NGF was also removed since it had showed to have no effect whatsoever in the migration of the protein.

The aim was to see the differences between the wt construct, which doesn't form dimmers, and the mut construct which does so.

If our hypothesis was right, we should have been able to see how the mut construct migrated less than the wt, as the BS3 would have joined together dimers of the protein and NGF together, forming clusters. Meanwhile, the wt construct should have only been able to form dimers. The result of this assay were not quite satisfactory (see Figure 16).

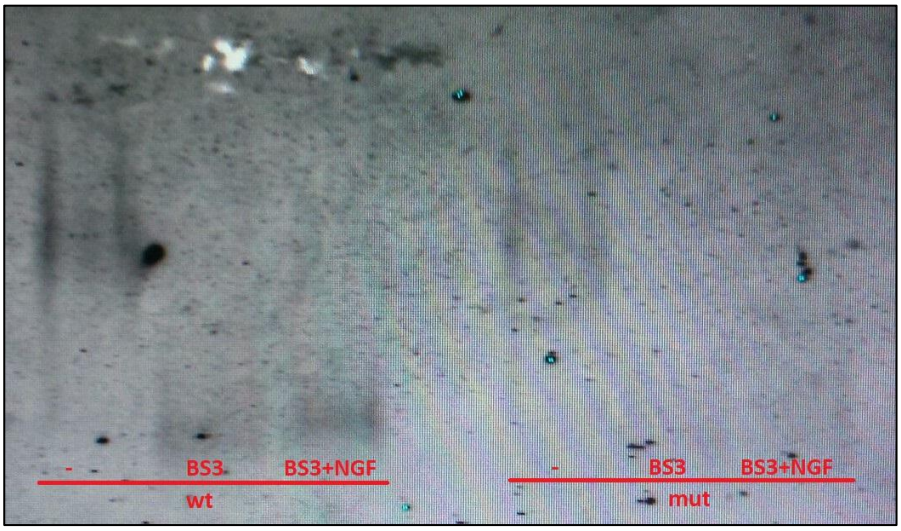

Figure 16. Native crosslinking assay with the wt and mut constructs. Each lane has a volume of sample of 15 µl. While the wt lanes can be seen, the samples from mut are barely seen.

As it can be seen, there is very little protein in the membrane, even less in the mut construct lanes than in the wt ones. This is most probably due to the lower concentrations of purified mut construct, since we used the same volumes from each sample, not the same concentration. Besides, due to shortage of purified protein, we had to use a smaller volume of protein (15 µl) to carry out the experiment, as opposed to other cross-linking assays (40 µl).

As for the migration of the protein, we see that in the lanes where the cross-linker has been added, we have lower bands, as if the protein had reduced its size in comparison to the control lane. We theorize this might happen because the BS3 is in fact compacting the whole structure, thus making it easier for the protein to move through the pores of the gel with more ease. In an experiment performed with only the mut construct, we found similar results (see Figure 17).

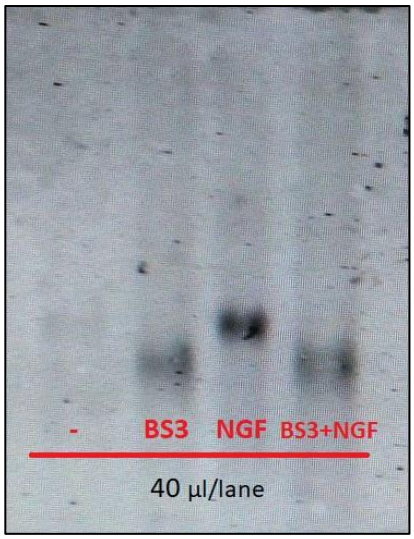

Figure 17. Native cross-linking assay of the mut construct. The volume of injected sample is 40 µl.

As with the first cross-linking assay, we can see that it seems that the BS3 is indeed compacting the structure of p75, increasing its mobility. On the other hand, both figures show that addition of NGF alone doesn't seem to have an effect on either protein construct, as the band is at the same height than the control.

Troubleshooting

In contrast with the production and purification steps, the troubleshooting of the characterization step is much smaller, since there was no time to carry out a deep study on the mistakes and their cause, and repeat the experiment with the adequate corrections of protocol. However, some of the problems that we noticed during the characterization of the p75-ECD where the following:

- **Mistakes during the cross-linking assay**. Bad timing during the addition of the cross-linker and quencher, use of excessive/insufficient cross-linker for the sample of protein, use of a buffer with the wrong pH.
- **Problems with the protein gel electrophoresis and Western Blot.** This set of problems is the same as the ones in the purification point.

5. DISCUSSION

From our primary objectives, we can say that two of them were met with satisfactory results. The development of the production and purification protocol as well as the optimization of the purification process, were a success in terms of protein construct obtained.

Up until this date, there hadn't been any previous study on whether HEK were a good cell line for the production of the p75-ECD, and our results show that although the obtained concentrations of protein were small, the cells were competent enough to be a producer for this construct.

As for the purification protocol, the use of an affinity column and subsequent dialysis yielded interesting concentrations of purified protein in both of the constructs. Moreover, the storage of protein aliquots at -80º C after a quick freezing with liquid N_2 was seen to keep the constructs in optimal conditions for later assays.

Then, for the characterization of the p75-ECD interaction with NGF, we must say that the obtained results were not quite satisfactory, as there was not enough time to conduct the number of assays needed to obtain reproducible data.

One of the most conflicting points of the characterization step was our inability to determine whether the mut construct was forming covalent dimers or not, due to contradicting results in the native and denaturing gels (Figures 12 and 13). There are several explanations as to why this contradiction might be happening; the simplest being that the results shown in Figure 12 might not have been representative of the real situation. Since there was no opportunity to repeat the experiment, they cannot be fully trusted to be an accurate representation.

Another possibility would be that the denaturing assays were not correctly performed. Usually, assays in reducing and non-reducing conditions are usually performed in separate gels. This is due to the fact that the used molecular weight marker has BME in its composition, and might reduce the samples from the non-reducing treatment.

Initially we assumed that by separating the reducing and non-reducing conditions with sample buffer without BME we would be able to get good results. However, after seeing them, we might have underestimated the BME ability to diffuse in a gel and break the mut dimers. Also, the fact that in non-reducing conditions the protein migrates faster suggests that it hasn't actually been reduced by the BME. Further

investigation should be done, this time performing separate gels for reducing and non-reducing conditions.

As for the cross-linking assays, it is regrettable that we weren't able to extract any conclusive results. There was no time to perform repetitions of the assays, and we were also unable to purify enough protein to perform as many experiments as we had hoped. However, from the results we obtained, it seems that NGF doesn't have a role in creating dimers of protein, as seen with the bands treated with NGF being at the same height as the controls. This could mean that the constructs are forming dimers without the need for NGF, so the addition of the neurotrophin wouldn't have an effect.

Other important point which needs to be studied further is the real ability of the mut construct to form dimers. Although the wt construct has shown itself in monomeric form all the time, the mut construct presents contradictory results that need to be solved with further studies.

Another important point to optimize is the concentration of cross-linker used in the assays. Although we used a reasonable amount since too little would yield no results and too much would only form aggregates, we didn't take into account the differences in concentration of protein in each sample. This should be optimized, though we didn't have the time to do so for this work.

In conclusion, it is regretful to say that we have not been able to make our results reproducible, as we have been unable to repeat most of the experiments due to lack of time and protein samples. However, it should be noted that there are also very preliminary experiments and should be understood as a setting for a deeper study on the matter now that a production and purification protocol has been established and optimized.

6. BIBLIOGRAPHY

Allen, S. J., Watson, J. J., Shoemark, D. K., Barua, N. U., & Patel, N. K. (2013). GDNF, NGF and BDNF as therapeutic options for neurodegeneration. *Pharmacology & therapeutics*, *138*(2), 155-175.

Aloe, L., Bracci-Laudiero, L., Bonini, S., Manni, L., & Aloe, L. (1997). The expanding role of nerve growth factor: from neurotrophic activity to immunologic diseases. *Allergy*, *52*(9), 883-994.

Arakawa, T., Haniu, M., Narhi, L. O., Miller, J. A., Talvenheimo, J., Philo, J. S., ... & Louis, J. C. (1994). Formation of heterodimers from three neurotrophins, nerve growth factor, neurotrophin-3, and brain-derived neurotrophic factor. *Journal of Biological Chemistry*, *269*(45), 27833-27839.

Aurikko, J. P., Ruotolo, B. T., Grossmann, J. G., Moncrieffe, M. C., Stephens, E., Leppänen, V. M., ... & Blundell, T. L. (2005). Characterization of symmetric complexes of nerve growth factor and the ectodomain of the pan-neurotrophin receptor, p75NTR. *Journal of Biological Chemistry*, *280*(39), 33453-33460.

Bradshaw, R. A., Pundavela, J., Biarc, J., Chalkley, R. J., Burlingame, A. L., & Hondermarck, H. (2015). NGF and ProNGF: regulation of neuronal and neoplastic responses through receptor signaling. *Advances in biological regulation*, *58*, 16-27.

Cabal-Hierro, L., & Lazo, P. S. (2012). Signal transduction by tumor necrosis factor receptors. *Cellular signalling*, *24*(6), 1297-1305.

Casaccia-Bonnefil, P., Kong, H., & Chao, M. V. (1998). Neurotrophins: the biological paradox of survival factors eliciting apoptosis. *Cell death and differentiation*, *5*(5), 357-364.

Dechant G, Neumann H. Neurotrophins. In: Madame Curie Bioscience Database [Internet]. Austin (TX): Landes Bioscience; 2000-2013. Available from: https://www.ncbi.nlm.nih.gov/books/NBK6480/?report=classic

Dechant, G., & Barde, Y. A. (2002). The neurotrophin receptor p75NTR: novel functions and implications for diseases of the nervous system. *Nature neuroscience*, *5*(11), 1131.

Della-Bianca, V., Rossi, F., Armato, U., Dal-Pra, I., Costantini, C., Perini, G., ... & Della Valle, G. (2001). Neurotrophin p75 receptor is involved in neuronal damage by prion peptide-(106–126). *Journal of Biological Chemistry*, *276*(42), 38929-38933.

Esposito, D., Patel, P., Stephens, R. M., Perez, P., Chao, M. V., Kaplan, D. R., & Hempstead, B. L. (2001). The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor. *Journal of Biological Chemistry*, *276*(35), 32687-32695.

Feng, D., Kim, T., Özkan, E., Light, M., Torkin, R., Teng, K. K., ... & Garcia, K. C. (2010). Molecular and structural insight into proNGF engagement of p75NTR and sortilin. *Journal of molecular biology*, *396*(4), 967-984.

Gong, Y., Cao, P., Yu, H. J., & Jiang, T. (2008). Crystal structure of the neurotrophin-3 and p75NTR symmetrical complex. *Nature*, *454*(7205), 789-793.

Götz, R., Köster, R., Winkler, C., Raulf, F., Lottspeich, F., Schartl, M., & Thoenen, H. (1994). Neurotrophin-6 is a new member of the nerve growth factor family. *Nature*, *372*(6503), 266-269.

Hall, A., & Lalli, G. (2010). Rho and Ras GTPases in axon growth, guidance, and branching. *Cold Spring Harbor perspectives in biology*, *2*(2), a001818.

He, X. L., & Garcia, K. C. (2004). Structure of nerve growth factor complexed with the shared neurotrophin receptor p75. *Science*, *304*(5672), 870-875.

Khursigara, G., Orlinick, J. R., & Chao, M. V. (1999). Association of the p75 neurotrophin receptor with TRAF6. *Journal of Biological Chemistry*, *274*(5), 2597-2600.

Kobayashi, K., Inohara, N., Hernandez, L. D., Galán, J. E., Núñez, G., Janeway, C. A., ... & Flavell, R. A. (2002). RICK/Rip2/CARDIAK mediates signalling for receptors of the innate and adaptive immune systems. *Nature*, *416*(6877), 194-199.

Korsching, S. (1993). The neurotrophic factor concept: a reexamination. *Journal of Neuroscience*, *13*(7), 2739-2748.

Lee, R., Kermani, P., Teng, K. K., & Hempstead, B. L. (2001). Regulation of cell survival by secreted proneurotrophins. *Science*, *294*(5548), 1945-1948.

Levi-Montalcini, R., & Hamburger, V. (1951). Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo. *Journal of Experimental Zoology Part A: Ecological Genetics and Physiology*, *116*(2), 321-361.

Li, J., Yin, Q., & Wu, H. (2013). Structural basis of signal transduction in the TNF receptor superfamily. *Advances in immunology*, *119*, 135.

Mamidipudi, V., & Wooten, M. W. (2002). Dual role for p75NTR signaling in survival and cell death: can intracellular mediators provide an explanation?. *Journal of neuroscience research*, *68*(4), 373-384.

McCarthy, J. V., Ni, J., & Dixit, V. M. (1998). RIP2 is a novel NF-κB-activating and cell death-inducing kinase. *Journal of Biological Chemistry*, *273*(27), 16968-16975.

Nilsson, A. S., Fainzilber, M., Falck, P., & Ibáñez, C. F. (1998). Neurotrophin-7: a novel member of the neurotrophin family from the zebrafish. *FEBS letters*, *424*(3), 285-290.

Nykjaer, A., & Willnow, T. E. (2012). Sortilin: a receptor to regulate neuronal viability and function. *Trends in neurosciences*, *35*(4), 261-270.

Nykjaer, A., Lee, R., Teng, K. K., Jansen, P., Madsen, P., Nielsen, M. S., ... & Hempstead, B. L. (2004). Sortilin is essential for proNGF-induced neuronal cell death. *Nature*, *427*(6977), 843-848.

Rabizadeh, S., Oh, J., Zhong, L. T., Yang, J., Bitler, C. M., Butcher, L. L., & Bredesen, D. E. (1993). Induction of apoptosis by the low-affinity NGF receptor. *SCIENCE-NEW YORK THEN WASHINGTON-*, *261*, 345-345.

Radziejewski, C., & Robinson, R. C. (1993). Heterodimers of the neurotrophic factors: formation, isolation, and differential stability. *Biochemistry*, *32*(48), 13350-13356.

Reichardt, L. F. (2006). Neurotrophin-regulated signalling pathways. *Philosophical Transactions of the Royal Society B: Biological Sciences*, *361*(1473), 1545-1564.

Rösch, H., Schweigreiter, R., Bonhoeffer, T., Barde, Y. A., & Korte, M. (2005). The neurotrophin receptor p75NTR modulates long-term depression and regulates the expression of AMPA receptor subunits in the hippocampus. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(20), 7362-7367.

Tuffereau, C., Bénéjean, J., Blondel, D., Kieffer, B., & Flamand, A. (1998). Low-affinity nerve-growth factor receptor (P75NTR) can serve as a receptor for rabies virus. *The EMBO journal*, *17*(24), 7250-7259.

Vilar, M. (2017). Chapter Four-Structural Characterization of the p75 Neurotrophin Receptor: A Stranger in the TNFR Superfamily. *Vitamins and Hormones*, *104*, 57-87.

Vilar, M., Charalampopoulos, I., Kenchappa, R. S., Reversi, A., Klos-Applequist, J. M., Karaca, E., ... & Ericson, J. (2009b). Ligand-independent signaling by disulfide-crosslinked dimers of the p75 neurotrophin receptor. *Journal of cell science*, *122*(18), 3351-3357.

Vilar, M., Charalampopoulos, I., Kenchappa, R. S., Simi, A., Karaca, E., Reversi, A., ... & Schiavo, G. (2009a). Activation of the p75 neurotrophin receptor through conformational rearrangement of disulphide-linked receptor dimers. Neuron, 62(1), 72-83.

von Schack, D., Casademunt, E., Schweigreiter, R., Meyer, M., Bibel, M., & Dechant, G. (2001). Complete ablation of the neurotrophin receptor p75NTR causes defects both in the nervous and the vascular system. *Nature neuroscience*, *4*(10), 977-978.

Yaar, M., Zhai, S., Pilch, P. F., Doyle, S. M., Eisenhauer, P. B., Fine, R. E., & Gilchrest, B. A. (1997). Binding of beta-amyloid to the p75 neurotrophin receptor induces apoptosis. A possible mechanism for Alzheimer's disease. *Journal of Clinical Investigation*, *100*(9), 2333.

Yamashita, T., & Tohyama, M. (2003). The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI. *Nature neuroscience*, *6*(5), 461-467.

Yamashita, T., Tucker, K. L., & Barde, Y. A. (1999). Neurotrophin binding to the p75 receptor modulates Rho activity and axonal outgrowth. *Neuron*, *24*(3), 585-593.

Yang, B., Slonimsky, J. D., & Birren, S. J. (2002). A rapid switch in sympathetic neurotransmitter release properties mediated by the p75 receptor. *Nature neuroscience*, *5*(6), 539-545.

Ye, X., Mehlen, P., Rabizadeh, S., VanArsdale, T., Zhang, H., Shin, H., ... & Reed, J. C. (1999). TRAF family proteins interact with the common neurotrophin receptor and modulate apoptosis induction. *Journal of Biological Chemistry*, *274*(42), 30202-30208.

Youker, R. T., Bruns, J. R., Costa, S. A., Rbaibi, Y., Lanni, F., Kashlan, O. B., ... & Weisz, O. A. (2013). Multiple motifs regulate apical sorting of p75 via a mechanism that involves dimerization and higher-order oligomerization. *Molecular biology of the cell*, *24*(12), 1996-2007.

7. ANNEXES

1. Recipes. Electrophoresis and Western Blot.

Polyacrylamide stacking gel (5%)

- H_2O 3.22 mL
- Tris HCl 0.5 M pH 6.8 1.25 mL
- Acrylamide (37.5%) 0.53 mL
- SDS (10%) 50 µL
- APS 50 µL
- TEMED 5 µL

Polyacrylamide separating gel (10%)

- H_2O 4.83 mL
- Tris HCl 1.5 M pH 7.5 2.50 mL
- Acrylamide (37.5%) 2.67 mL
- SDS (10%) 100 µL
- APS 100 µL
- TEMED 10 µL

Running buffer

- Glycine 144 g
- Tris base 30 g
- SDS 10 g
- H_2O 1 L

Native running buffer

- Glycine 28 g
- Tris base 6 g
- H_2O 2 L

Adjust to pH 7.5

SDS-PAGE sample loading buffer

- Tris HCl 0.5 M pH 6.8 — 4 mL
- SDS — 0.8 g
- Glycerol 100% — 4 mL
- 0.5 M EDTA — 1 mL
- Bromophenol Blue — 8 mg
- H_2O — 5 mL

Native sample loading buffer

- Tris HCl 0.5 M pH 6.8 — 2 mL
- Glycerol 100% — 4 mL
- Poinceau — 8 mg
- H_2O — 4 mL

Transfer buffer

- Glycine — 14.41 g
- Tris base — 3.03 g
- Methanol/ethanol — 200 mL
- H_2O — to 800 mL

Tris HCl 0.5 M pH 6.8

- Tris base — 30.29 g
- Water — to 500 mL

Adjust pH to 6.8

Tris HCl 1.5 M pH 8.8

- Tris base — 90.8 g
- Water — to 500 mL

Adjust pH to 7.5

TTBS

- 10x TBS — 100 mL
- Tween 20 — 1 mL
- H2O — to 900 mL

Poinceau

- Poinceau 100 mg
- Acetic acid 5 mL
- H_2O mQ up to 100 mL

Blocking milk

- Powder milk 1.25 g
- TTBS 25 mL

2. Recipes. Purification.

NaCl 5 M

- NaCl 58.44 g
- H_2O 200 mL

Phosphate buffer pH 7.5

- Na_2HPO_4 3.5 g
- NaCl 2.9 g
- H_2O 500 mL

Imidazole 0,5 M (in phosphate buffer pH 7.5)

- Na_2HPO_4 3.5 g
- NaCl 2.9 g
- Imidazole 17 g
- H_2O 500 mL

Eluting solution (imidazole)

- NaCl 5 M 2.5 mL
- Imidazol 5 mL
- Phosphate buffer 42.5 mL

$NiCl_2$ 0,1 M

- $NiCl_2$ 1.3 g
- H_2O 100 mL