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ΔΕΛΗΒΑΝΟΓΛΟΥ ΝΙΚΟΛΕΤΑ

ΕΠΙΒΛΕΠΩΝ ΚΑΘΗΓΗΤΗΣ: ΙΩΑΝΝΗΣ ΧΑΡΑΛΑΜΠΟΠΟΥΛΟΣ

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MASTER THESIS

MOLECULAR AND GENETIC STUDY OF THE MULTIPLE SIGNALING PATHWAYS OF THE P75 NEUROTROPHIN RECEPTOR IN SCHWANN CELLS

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HERAKLION
2014

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PROLOGUE

This master thesis started on March 2014 and was concluded on November 2014 at the Department of Pharmacology of Medical School, University of Crete. The issue of this thesis "Molecular and Genetic Study of the Multiple Signaling Pathways of the p75 neurotrophin receptor in Schwann cells", aims at the understanding of role, function and mechanisms mediated by this receptor. More specifically, it was studied p75 receptor interaction with other proteins after ligand stimulation and their possible role in protecting Schwann cells from cell death.

I would like to express my sincere gratitude to my teacher and supervisor, I. Charalampopoulos, Assistant Professor of Pharmacology, for his guidance, encouragement and continuous support through the course of this work. The extensive knowledge, vision and creative thinking of Prof. Charalampopoulos have been the source of inspiration for me throughout this work.

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Finally, this work is dedicated to the beloved memory of my grandfather, Nickolaos Trikyllidis.

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ΠΕΡΙΛΗΨΗ

Ο υποδοχέας θανάτου p75 είναι ένας παν-νευροτροφικός υποδοχέας, ανήκει στην οικογένεια υποδοχέων TNFR και εκφράζεται, τόσο σε νευρώνες όσο και στη γλοία, κατά την ανάπτυξη και την ενήλικο ζωή και μπορεί να επάγεται η έκφρασή του ύστερα από τραύμα ή/και ασθένεια. Έχει περιγραφεί εκτενώς, ότι ο υποδοχέας p75 μπορεί να διαμεσολαβεί μια πληθώρα λειτουργιών όπως ο κυτταρικός θάνατος, η επιβίωση, η αξονική αύξηση, ο πολλαπλασιασμός και η μυελίνωση. Επιπλέον, ο υποδοχέας p75 συνδέει όλες τις νευροτροφίνες και είναι ο υψηλής συγγένειας σύνδεσης υποδοχέας για τις ανώριμες ισομορφές των νευροτροφινών (προ-νευροτροφίνες). Μπορεί, επίσης, να συνδέει άλλους μη νευροτροφικούς συνδέτες όπως το Αβ-αμυλοειδές, οι πρωτεΐνες prion (PrP) ή πρωτεΐνες μυελίνωσης όπως η γλυκοπρωτεΐνη που σχετίζεται με τη μυελίνη (MAG). Λόγω της έλλειψης εγγενούς ενζυμικής ενεργότητας, αλληλεπιδρά με μια πληθώρα ενδοκυτταρικών πρωτεϊνών για να ενεργοποιεί διάφορους σηματοδοτικούς καταρράκτες. Έχει εδραιωθεί σε μελέτες δομής-λειτουργίας ότι οι διαφορετικοί συνδέτες προκαλούν διαφορετικές αλλαγές διαμόρφωσης του μορίου του υποδοχέα που ρυθμίζουν διαφορικά την κυτταρική απόκριση. Η κατανόηση της φυσιολογίας, γενετικής και μοριακής βιολογίας του υποδοχέα μπορεί να συμβάλλει στην ανάπτυξη καινοτόμων θεραπευτικών προσεγγίσεων της δυσλειτουργίας του νευρικού συστήματος σε παθολογικές καταστάσεις. Σε αυτή τη μελέτη, διερευνήσαμε την εξαρτώμενη από συνδέτη αλληλεπίδραση μεταξύ του υποδοχέα p75 και της πρωτεΐνης RIP-2, μιας πρωτεΐνης προσαρμογής, που σχετίζεται με την κυτταρική επιβίωση των κυττάρων Schwann και παρουσιάζουμε τον εν δυνάμει προστατευτικό ρόλο δύο καινοτόμων συνδετών του p75, της δεϋδροεπιανδροστερόνης (DHEA) και του συνθετικού της αναλόγου BNN27, στην απόπτωση.

Λέξεις κλειδιά: νευροτροφίνη, p75 υποδοχέας, απόπτωση, RIP-2, κύτταρα Schwann, δεϋδροεπιανδροστερόνη (DHEA), BNN27.

ABSTRACT

The p75 death receptor is a pan-neurotrophin receptor, belongs to TNFR family and is expressed, both on neurons and glia, during development and adulthood and it can be induced after nerve injury or/and disease. It has been extensively described that p75NTR can mediate a variety of functions such as cell death, survival, axonal growth, proliferation and myelination. In addition, p75NTR binds to all neurotrophins and is the high-affinity receptor for the immature isoforms of neurotrophins (pro-neurotrophins). It can also bind other non neurotrophic ligands such as Aβ-amyloid, prion proteins (PrP) or myelin derived proteins such as myelin-associated glycoprotein (MAG). Due to lack of intrinsic enzymatic activity, it interacts with a plethora of intracellular proteins in order to activate different signaling cascades. In structure-function studies has been established that different ligands evoke different conformational changes of the receptor molecule that regulate differentially the cellular response. Understanding of its physiology, genetic and molecular biology may contribute to novel therapeutic approaches to malfunction of the nervous system in pathological conditions. In this study, we investigated the ligand-depended interaction between p75NTR and receptor-interacting protein 2 (RIP-2), an adaptor protein involved in cell survival in Schwann cells and we demonstrate the potential protective role of two novel p75NTR ligands, dehydroepiandrosterone (DHEA) and its synthetic analogue BNN27, in apoptosis.

Key words: neurotrophin; p75 receptor, apoptosis; receptor-interacting protein; Schwann cells; dehydroepiandrosterone; BNN27.

INTRODUCTION

The proper development and function of the nervous system requires the production and secretion of specific biological molecules that affect/guide different types of cells within it. These molecules act as trophic/supporting factors and include the nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3) and neurotrophin-4 (NT-4), known as neurotrophins (Purves et al., 1988; Lewin and Barde, 1996). Neurotrophins are responsible for the generation, proliferation, differentiation, survival, shape, axonal growth, synaptic connectivity and response to trauma/disease of neurons (Bibel and Barde, 2000). Neurotrophins exert their role by binding as dimmers to a second set of transmembrane proteins, which are located on the surface of cells (Figure 1). These receptors proteins fall into two categories, the first is the tropomyosin-related kinase tyrosine kinase (Trk) receptor and the second one is a member of tumor necrosis factor (TNF) receptor family, the p75 pan-neurotrophin receptor (p75NTR) (Friedman and Greene, 1999; Kaplan and Miller, 2000; Lee et al., 2001a). Trk family includes TrkA, TrkB and TrkC which bind with specificity different neurotrophin, NFG, BDNF/NT-3 and NT-4, respectively. On the other hand, the p75NTR interacts with all neurotrophins with low affinity (Kd 10^{-9}M), as well as with their immature forms, the pro-neurotrophins with high affinity (Kd 10^{-11}M), carrying a pro-domain which is cleaved to produce the mature neurotrophins (Hempstead et al., 1991). In the last decades, many studies have been shown the important and numerous functions of Trk receptors within the nervous system, but little is known about p75NTR. It was believed that p75NTR only works together as complex with the Trks to bind neurotrophins with high affinity (Kd 10^{-11}) and regulate the specificity of the binding ligand to each Trk receptor. Also, it was under question if p75 could mediate signaling by itself. In the recent years, valuable information is coming to light, about p75NTR's involvement as a key-player in different signaling pathways.

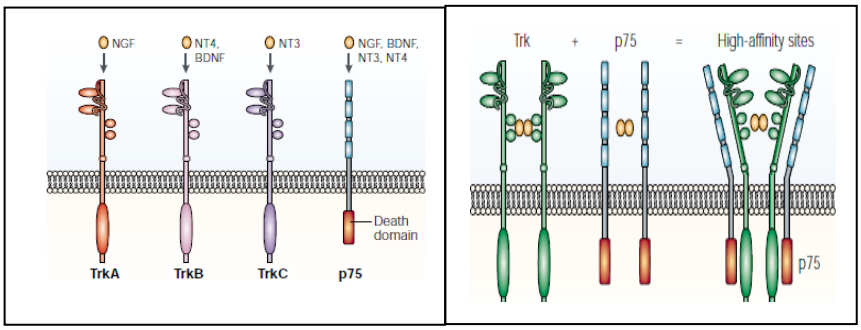

Figure 1: Left: Neurotrophins, receptors and binding selectivity. Right: High affinity receptor complex (Chao, 2003).

Gene, protein structure and isoforms of p75NTR

The p75NTR gene is located on 17th chromosome and its promoter lacks of the typical regulating sequences such as CAAT and TATA box, but has many GC rich sequences (Huebner et al., 1986; Sehgal et al., 1988b) and also, there are regions that can be regulated by retinoic acid, Vitamin D3 and testosterone (Metsis et al., 1992, Naveilhan et al., 1996).

This gene encodes for a 75kD type I transmembrane protein. P75NTR is the 16th member of the TNFR family and has no intrinsic enzymatic activity, so it can signal through recruitment of various adaptor proteins. Structurally, p75NTR consists of an extracellular domain (ECD), a juxtamembrane domain, a transmembrane domain (TM) and an intracellular domain (ICD) with an 80 amino acid formation called death domain (DD) (Figure 2). At the extracellular domain, there are 4 conservative cysteine rich regions, which is a typical characteristic for this receptor family (TNFR) and are responsible for the ligand's binding (Yan and Chao, 1991). There are sites of N-linked and O-linked glycosylation, as well. The juxtamembrane domain is the most highly conserved site of the receptor, is very flexible and involves in protein-protein interactions (Large et al., 1989). The transmembrane domain (TM) is of high importance due to the Cys257 residue that regulates the "snail-tong" mechanism of activation (Vilar et al., 2009) (Figure 3). As far as concerns the ICD, unlike the other members of TNFR, p75NTR has an ICD that is divided into sub-domains and regulate various functions. There is the Chopper domain with a Cys279 residue which can be palmitoylated and play role either in direction and localization of the p75NTR or in cell-death. At the C-terminal tail there is a PDZ consensus, which binds adaptor molecules and has a role in protein trafficking and receptor

complex association. At last, there is a type II death domain that consists of six α-helices and recruits various adaptor proteins to execute its functions, most prominent cell death (p75NTR is called death receptor, too).

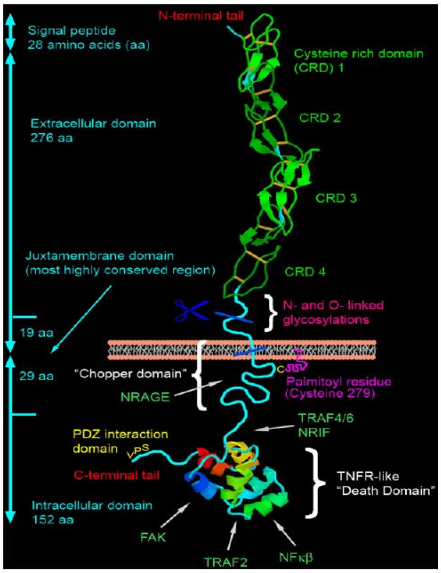

Figure 2: p75NTR structure. Here is shown the cysteine-rich domains responsible for ligand binding, Chopper domain implicates in cell death and can be pamlitylated, ICD that can interact with various adaptor proteins, PDZ domain and type II DD (Underwood & Coulson, 2007).

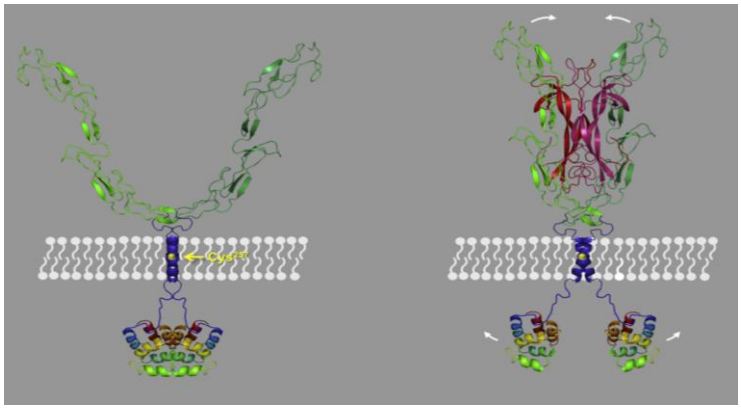

Figure 3: Activation of p75NTR by neurotrophin binding. Schematic representation of the proposed "Snail-Tong mechanism". Cys257 residue allows the movement of p75NTR subunits to close on the extracellular (stimulated by ligand-binding) and to open intracellularly (Vilar et al., 2009).

Alternative splicing and proteolytic processing yield truncated isoforms of p75. There has been identified a short isoform (s-p75) of 45kD that lacks the three out of four cysteine rich regions in the ECD and cannot bind neurotrophins (Dechant and Barde, 2002) (Figure 4). Also, p75NTR is cleaved by metalloproteinase and give a soluble fragment of ECD and another fragment of TM and ICD. These fragments are highly produced during development or after injury, but their functional role is still unknown (Di Stefano 1991).

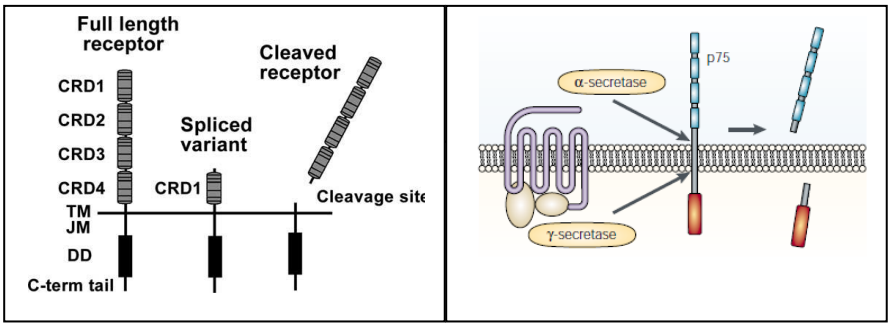

Figure 4: Left: p75NTR isoforms. Right: Cleavage sites by metalloproteinases like α-secretase and preselinin-dependent γ-secretase (Roux and Barker, 2002; Chao, 2003).

Co-receptors and ligands

As mentioned before, p75NTR works synergistically with Trks to form a high affinity complex for neurotrophins that promote survival, proliferation and differentiation of neurons. Previous studies demonstrate that for both receptors the association/disassociation constant (individually) for neurotrophin binding is low approximately Kd 10^{-9}M, but when they get together their complex binds neurotrophins with high constant such as Kd 10^{-11}M 9 (Barker, 2004).

On the other hand, p75NTR promotes cell death, growth inhibition and malfunction of synapses, by association with the co-receptor sortilin and binding to pro-neurotrophins (Figure 4) (Kd 10^{-11}M) (Nykjaer, 2004; 2005, Woo 2005, Yang 2009).

In addition, P75NTR associates with Nogo receptor (NogoR) and Lingo-1. This complex is activated by myelin derived ligands such as myelin-associated

protein (MAG) and Nogo and leads to growth cone collapse, axonal growth inhibition and myelination of axons (Figure 5).

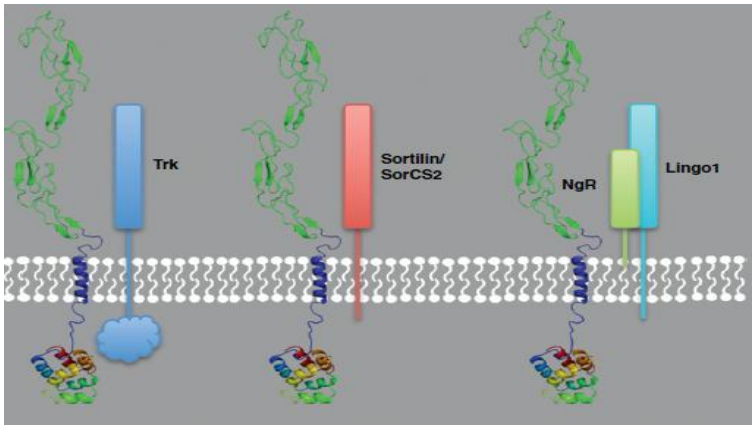

Figure 5: p75NTR co-receptors. (Ibanez and Simi, 2012).

p75NTR binds except of immature and mature neurotrophins also, to ligands without neurotrophic properties such as Aβ peptide and prion protein (PrP) and causes cell death (Figure 6). As well as, the receptor is a target for rabies virus. They interact through a glycoprotein on the virus's envelope and this is how the virus manages to enter the nervous system (Dechant and Barde, 2002).

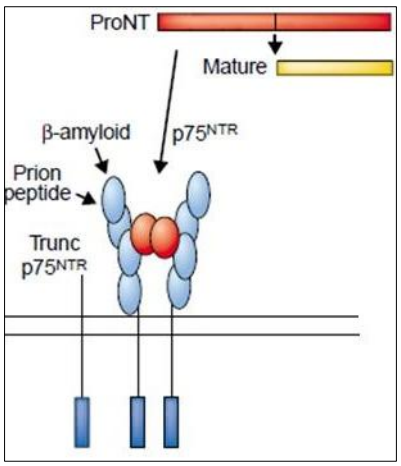

Figure 6: p75NTR neurotrophin and non-neurotrophin ligands (Hempstead, 2002).

p75NTR biological functions and interacting/adaptor factors

p75NTR's response to its ligands and environmental stimuli is mediated by a number of interacting proteins that compensate the receptor's lack of enzymatic function and lead to various physiological outcomes by activating the right signaling pathway each time. p75NTR signals for cell death or survival, neurite outgrowth or myelination and is implicated in synaptic connectivity and plasticity (Figure 7).

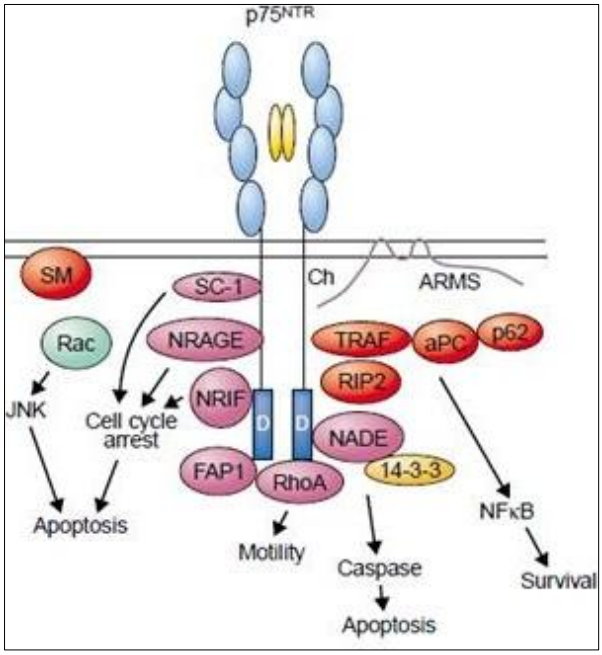

Figure 7: p75NTR implicates in various signaling pathways with different biological outcomes (Hempstead, 2002).

Cell death

Apoptosis through p75NTR takes place under physiological conditions during development for the proper connectivity, spatially and temporally, and refinement of the nervous system. This requires neurotrophin binding that are secreted at high levels at developmental procedures. So, when the innervation of a specific tissue target is improper, neurons undergone p75NTR-mediated apoptosis to ensure the physiological function of this system (Kohn et al., 1999). There are many studies that support p75NTR-mediated apoptosis both in vitro and in vivo. It has been shown that NGF binding to p75NTR leads to cell death binding in rat oligodendrocytes (Gu et al., 1999), sympathetic neurons (Salehi et al., 2000), retinal ganglion (Frade, 2000b),

Schwann cells (Soilu-Hanninen et al., 1999) and other cell types. Also, in in vivo studies that have been conducted in transgenic mice p75NTR-/- have found a great loss of sensory, sympathetic and cortical neurons (Majdan et al., 1997). Of high significance is its role after injury or in disease. In these conditions p75NTR-induced apoptosis happens in an attempt to maintain the integrity and function of the nervous system. In this case the cell death is a consequence of pro-neurotrophin binding that is known to accumulate under pathological states. In addition p75NTR can mediate cell death in the absence of neurotrophins or other ligands due to receptor's high levels (over-expression) and spontaneous multimerization of its monomers (Rabizadeh et al., 1993; Casha et al., 2001).

The mechanism by which p75NTR leads to cell death involve (pro-)neurotrophin binding (pro-neurotrophin binding is more effective), activation of caspases 1, 3 and 6 (correlation with mitochondria's integrity), activation of MAP kinase and-Jun N-terminal kinase (JNK) that in turn results in the transcription of c-Jun, ATF-2 and p53 genes (Yoon et al., 1998; Palmada et al., 2002). Although, apoptosis is independent to c-Jun activation and may be occurs through p53 (Aloyz et al., 1998).

Survival

Despite its "reputation" p75NTR can also have positive effects like cell survival, independently of Trks. Many studies suggest its protective role, neurotrophin binding promotes survival of neocortical neuron during development (De Freitas et al., 2001), of sensory neurons (Hamanoue et al., 1999), in developing retina (Hutson and Bothwell, 2001) and in cultured Schwann cells (Khursigara et al., 2001). The proposed mechanism involves the activation of the NF-κB transcription. This factor regulates the expression of many genes that promote cell survival (Karin and Lin, 2002). NF-κB forms a heterodimer of p65/p50 units which is located to cytoplasm to its inactivated form by binding to IκB, an inhibitory protein. Upon stimuli, IκB is phosphorylated by IKK complex and degradates, thus inducing the translocation of the heterodimer p65/p50 from cytoplasm to nucleus. There, pro-survival genes are transcribed such as IAP and Bcl-2 and other proteins are produced to inhibit apoptosis (Pahl, 1999). Another important survival pathway that is activated is the phosphatidyl inositol 3-kinase (PI3-K)/AKT. This pathway can be activated not only by interaction

with Trk but independently, as well (Roux et al., 2001). AKT inactivates caspase 9, Bad a pro-death protein and Forkhead transcription factors (Peso et al., 1997; Cardone et al., 1998; Brunet et al., 1999). Also, there is evidence that Akt phosphorylates IκB and activates NF-κB, as well (Beraud et al., 1999).

Neurite outgrowth and myelination

Apart from regulating cell death or survival, p75NTR involves in other functions like axonal elongation and myelin formation, during development and after injury. NGF binding to p75NTR has been shown to promote neurite outgrowth of hippocampal neurons (Bran et al., 1999), of ciliary neurons (Collins and Dawson, 1983) and of Schwann cells (Yamashita et al., 1999). As far as concerns, modulation of myelin formation Chan et al., have found that in Schwann co-cultures with dorsal root ganglia (DRGs), activation of p75NTR by BDNF binding promotes myelination. The proposed mechanism of these outcomes lies on the activation/inactivation of RhoA by RhoGDI. RhoA is a small GTPase protein regulating the actin cytoskeleton. RhoGDI is a guanine exchanging factor (GEF) and its role is to regulate RhoA's states (bound or unbound to GTP) and thus, its activation or inactivation. RhoA is sequestered by RhoGDI that prevents its association with downstream effectors (Cosgaya et al., 2002). In the absence of a ligand p75NTR associates with RhoGDI and RhoA is activated inhibiting axonal elongation and enhancing myelin formation. When NGF binds to p75NTR induces dissociation of RhoGDI and then RhoGDI binds and inhibits activation of RhoA. This results in growth cone collapse and neurite outgrowth (Wang et al., 2002; Wong et al., 2002). On the other hand, the myelin derived ligands such as myelin associated glycoprotein (MAG) and oligodendrocyte myelin glycoprotein (OMGP) are capable of binding to p75NTR through its indirect interaction with NogoR and Lingo-1 (Yang et al., 1996). This receptor complex activates RhoA which in turn inhibits axonal growth favoring, at the same time, myelin formation and myelination of the axons (Yamashita and Tohyama, 2002).

Synaptic modulation

The first evidence for the role of p75NTR on synaptic transmission comes from studies with neurons and cardiac myocytes. It has been shown that BDNF

binding to p75NTR induces acetylcholine release and subsequently decreases the twiching frequency of myocytes (Lockhart et al., 1997; Yang et al., 2002). Also, pro-BDNF binding to p75NTR modulates excitatory synapses in hippocampus leading to long-term depression (LTD) (Woo et al., 2005). It has been observed in p75NTR-/- mice greater spine density and dendritic circuit (Zagrebelsky et al., 2005). Finally, it is established that in neuromuscular synapse binding of pro-BDNF to p75NTR results in reduction in synaptic dynamics and deformation of presynaptic terminals by presynaptic p75NTR (Yang et al., 2009).

p75NTR interactors

p75NTR is involved in different pathways because of its ICD ability to recruit and interact with various proteins under different physiological contexts. A yeast two-hybrid screening with the p75NTR ICD identified a number of adaptor proteins indicated in table 1 (Figure 8).

Table 1: p75 interactor proteins.

Protein class	Example of p75-interacting proteins	Function
MAGE, MAGE-like	NRAGE, necdin	Cell cycle progression
GTPase	RhoA	Neurite extension
TRAF	TRAF4	Survival
	TRAF6	Survival
	DTRAF1	Survival
Trk	TrkA	Neurotrophic signaling
	TrkB	Neurotrophic signaling
	TrkC	Neurotrophic signaling
Other	NRIF	Cell death
	NADE	Cell death
	SC-1	Cell cycle progression
	FAP-1	Survival
	RIP-2	Survival
	Caveolin-1	Neurotrophic signaling

TRAF is a group of proteins that interact with TNFR and interleukin family receptors (Wajant et al., 2001). There are six members and all of them interact with p75NTR, but only TRAF-4 and TRAF-6 activate JNK and NF-κB pathways (Ye et al., 1999b; Krajewska et al., 1998; Khursigara et al., 1999). TRAF-6 is expressed in the nervous system and interacts transiently with p75NTR through its juxtamembrane domain (Khursigara et al., 1999; Wooten et al., 2001).

RIP-2 is a serine/threonine kinase. It has a caspase recruitment domain (CARD), by which binds to p75NTR (Khursigara et al., 1999) and activates caspase-1 or pro-survival molecules like inhibitor of apoptosis-1 (IAP-1) and CARD only protein (COP) (Thome et al., 1998; Lee et al., 2001b). Also, RIP-2 can associate with TRAF-6. The differential expression of RIP-2 regulates survival by activation of NF-κB or cell death under certain circumstances, through p75NTR (Mc Carthy et al., 1998).

RhoA is a small GTPase protein that affects actin polymerization. When it is activated (unbound to RhoGDI) stabilizes the cytoskeleton and inhibits axonal growth and promotes myelin formation. When it is inactivated (bound to RhoGDI) leads to growth cone collapse and induces neurite outgrowth. Generally, RhoA is involved in many neuronal procedures like migration, polarity and morphogenesis of the nervous system (Luo, 2000).

NRIF-1 and NRIF-2 are proteins containing Kruppel boxes (KRAB domains) and zinc fingers (Casademut et al., 1999). They heterodimerize and work as transcriptional repressors (Friedman et al., 1996). They interact with ICD and DD of p75 NTR. Usually, are located in the nucleus but when are associated with the p75NTR can be found in the cytosol, too (Benzel et al., 2001). These proteins are involved in apoptosis and promote the exit from the cell cycle (cell cycle arrest) (Spengler et al., 1997).

Neurotrophin receptor interacting MAGE homologue (NRAGE) is a member of MAGE protein family. Its cellular function is not fully elucidated yet, but it seems to induce apoptosis and cell cycle arrest by interacting with the p75NTR (Salehi et al., 2000b; Taniura et al., 1998).

NADE is characterized by the presence of a nuclear export sequence (NES) and is a p75NTR-associated death executor. When p75NTR binds NGF, NADE activates caspases -2 and -3 and results in apoptosis (Mukai et al., 2000; 2002).

SC-1 is another transcription factor containing a zinc finger domain, o positive regulatory (PR) domain and a potential amino-terminal PEST sequence (Chittka and Chao, 1999). Locates in the cytoplasm where interacts with the juxtamembrane of p75NTR and after NGF binding to p75NTR translocates to the nucleus. It has a role in cell cycle arrest (Ren et al., 1999).

FAP-1 is a PTPase with PDZ domains. FAP-1 binds to the C-terminus of p75NTR and it is believed that blocks p75NTR-incuduced survival by NF-κB activation, promoting this way cell death (Sato et al., 1995; Roux et al., 2001).

Ankyrin repeat-rich membrane spanning protein (ARMS) is a protein with unique characteristics. Consists of four transmembrane domains, many ankyrin repeats, an α motif and a PDZ motif. Its role is to link both physical and functional p75NTR-Trk complexes (Kong et al., 2001).

Ceramide is a product of sphingomyelin's hydrolysis which is mediated by neurotrophin binding to p75NTR (activates sphingolyelinase). Ceramide has opposing effects depending what cause its production. NFG-dependent ceramide production is responsible for JNK activation and apoptosis (Casaccia-Bonnefil et al., 1996) but p75NTR-dependent ceramide production promotes differentiation and survival (Brann et al., 1999; De Freitas et al., 2001).

Caveolin is a protein that forms the caveolae, formations on the plasma membrane that may facilitate endocytosis and receptor recycle and trafficking (Parton, 1996; Okamoto, 1998). Both p75NTR and Trk associate directly with caveolin-1.

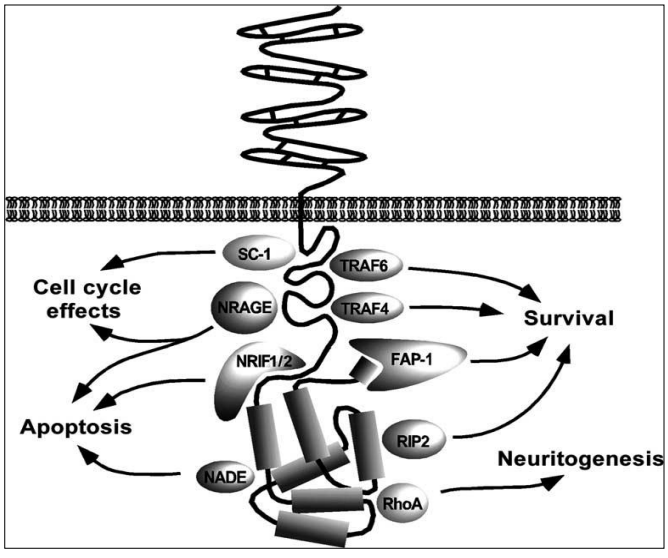

Figure 8: p75NTR mediates its functions by recruitment of various effectors proteins due to its lack of enzymatic activity (Roux and Barker, 2002).

p75NTR expression and association with diseases

During early stages of development p75NTR is widely expressed in the nervous system controlling the elimination of high number of immature neurons (Oppenheim, 1991). This guarantees the normal circuit, innervation and function of nervous system. p75NTR is expressed at some developmental stages at different cell populations and tissues both in the central (CNS) and peripheral (PNS) nervous system. In CNS mostly in neurons and glial cells in the cerebral cortex, cerebellum, hippocampus, basal forebrain, spinal cord and brainstem neurons (Wyatt & Henter 1998; Ernfors et al., 1989). In PNS is expressed in sensory and sympathetic ganglia, as well as, in parasympathetic and enteric neurons (Carroll et al., 1992; Schatteman et al., 1993). p75NTR is expressed in Muller cells of retina, kidney, pituitary and other developing organs and also, in glands, perivascular cells and meninges (Byers et al., 1990; Russo et al., 1994; Wheeler et al., 1998).

On the other hand, p75NTR is not widely expressed in the adult nervous system. More specific, its expression stops at most of the areas except for the cholinergic neurons of the basal forebrain, sensory neurons, spinal cord motor neurons and glial cells, where its levels decrease substantially (Ernfors et al., 1989).

It is of high interest that p75NTR expression is induced after injury or under pathological conditions at the areas that was expressed during development (Table 2). This re-expression is believed to work as a homeostatic mechanism to protect and maintain the proper function of the nervous system (Ibanez and Simi, 2012). Such paradigms are after peripheral crush nerve, spinal cord injury, axotomy, focal cerebral ischemia, seizures, in demyelinating lesions and diseases like multiple sclerosis, amyotrophic lateral sclerosis, Alzheimer's disease, diabetic neuropathy and various tumors (Dechant and Barde et al., 2002; Zhang et al., 2003; Copray et al., 2003; Ito et al., 2002; Bui et al., 2002).

Table 2: Types of trauma and diseases in which p75NTR re-expression is induced.

Injury/lesion/model
Nerve ligation or transection
Axotomy or demyelinating lesions
SOD1-G39A transgenic mouse model and ALS patients
Spinal cord injury, multiple sclerosis
Nerve crush, transection, oxidative stress
Optic nerve axotomy, ocular hypertension, ischemic injury, glaucoma
Axotomy
Axotomy
Seizures
Excitotoxic insults, seizures
Ischemic stroke
Hypo-osmolar stress
Alzheimer's disease

(Ibazez and Simi, 2012).

p75NTR in glia

As it was mentioned previously, p75NTR regulates cell death or survival and axonal growth or myelination. All these functions have been studied primarily in neurons of CNS and PNS. Since, p75NTR is also expressed in glia it's interesting to examine its role in migration, myelination and regulation of cell cycle. p75NTR is expressed in myelinating glia like oligodendrocytes, olfactory ensheathing cells (OECs) and Schwann cells, in non myelinating glia such as astrocytes and in microglia, too.

p75NTR in Schwann cells

Schwann cells (SCs) derive from neural crest and are directed in the peripheral nervous system, where are the most abundant cell type (LeDuarin and Kalcheim, 2009). These cells are differentiated in many phenotypes including SCs forming myelin (Scherer and Arroyo, 2002), non myelin forming SCs of PNS and autonomous nervous system (Yamazaki et al., 2011), perisynaptic SCs (Ko et al., 2007), perineuronal satellite cells (PSCs) of DRGs and the autonomic ganglia (Zhou et al., 1999) (Figure 9). SCs involve in maintenance of the normal nervous system function,

response in trauma, repair and pain, even modulation of immune system (Zhou et al., 2000). SCs are studied primarily, due to their role in myelination of PNS neurons. Their ratio Trk:p75NTR is low and it has been observed that SCs express highly p75NTR in development and after injury, so it is obvious that their biological responses are mediated through p75NTR (Cragnolini and Friedman, 2008). These reasons render Schwann cells the ideal cell type for studying the biology and physiology of p75NTR.

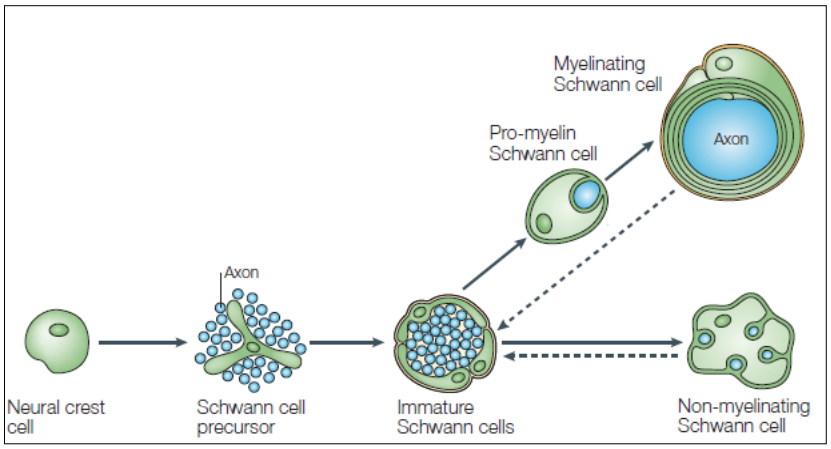

Figure 9: The main Schwann cell types. The origin and developmental stages of SCs (Jessen and Mirsky, 2005).

Before SCs be able to myelinate, they have to undergo though a restricted regulating program that consists of proliferation, migration along the axons and up-regulation of the myelinating proteins (Figure 10). It is believed that p75NTR not only affects myelination but regulates all the pro-myelination maturation procedures. As far as concerns proliferation, SC-1 is thought to be implicated in inhibition of proliferation. After NGF treatment, SC-1 translocates into the nucleus and represses transcription of cyclin E (Chittka and Chao, 1999). In addition, it has been observed in p75NTR-/- mice that SCs lack the ability to migrate. In other studies, is shown that TrkC/NT-3 promotes the migration and inhibits myelination while BDNF binding to p75NTR have the exact opposite function (Yamauchi et al., 2004). This supports the idea that different receptors and neurotrophins are required during different developmental stages of maturation.

When it comes to myelination, p75NTR's role is more elucidative. First, by the time SCs make contact with axons, a transcriptional "cascade" starts and activates NFκ-B, Oct-6 and Krox-20 which in turn results in transcription of myelinating genes and subsequent myelination. Second, BDNF plays a major role in regulating through p75NTR the myelination. In studies where SCs are in co-cultures with DRGs, BDNF (derived from DRGs) recruits a polarity protein PAR-3 that interacts with p75NTR and has as an outcome the induction of myelination (Nickols et al., 2003).

Also, in situations that re-myelination is needed such as after nerve crush or axotomy, there is up-regulation of both p75NTR and BDNF (Zhang et al., 2000). In p75NTR-/- mice there are fewer myelinated axons with thinner sheaths due to lower levels of myelin proteins compared to their wild type littermates (Song et al., 2006). Also, in a study where SCs of p75NTR-/- and wild type mice were transplanted in mice without endogenous p75NTR, those which have received SCs from p75NTR-/- mice displayed deficits in myelination, decreased number of axons and damage in motor neurons growth (Tomita et al., 2007). In addition, p75NTR is expressed also in peripheral neurons, so there is the possibility of a bidirectional relationship between SCs and axons for induction of re-myelination (Zhou and Li, 2007). Another aspect of p75NTR affecting re-myelination is through a pathway where p75NTR inhibits the tissue plasminogen activator (tPA). In this pathway p75NTR interacts with phosphodiesterase (PD34) that results in cAMP's degradation and down-regulation of tPA's expression. This pathway is believed to be a mechanism of structural remodeling of the extracellular matrix that contributes to re-myelination and regeneration (Sachs et al., 2007).

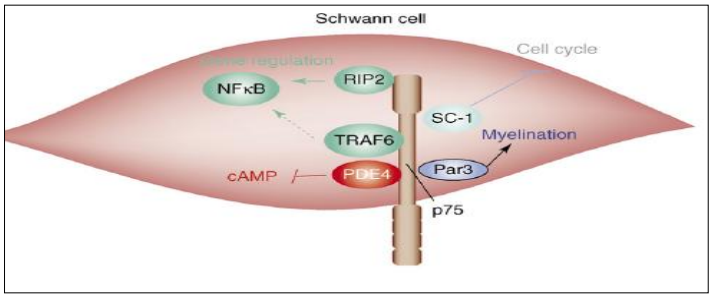

Figure 8: Function of p75NTR in the most studied cell type, Schwann cell (Cragnolini and Friedman, 2007).

Potent therapeutic strategies

p75NTR induce cell death, inhibit axonal growth and synaptic plasticity and for this reason constitutes a "hotspot" target of therapeutic target. Until today, all the efforts aimed at the ligand-binding properties of p75NTR and tried to develop molecules that despair this interaction and by this, abolishing the activation of the devastating pathways. In contrast to these approaches, there are efforts to design and synthesize compounds that can modulate its function towards the receptor's positive signaling pathways such as survival and regeneration. These molecules should mimic the neurotrophin properties both in signaling and in pharmacokinetic characteristics (Longo and Massa, 2013). Since, neurosteroids have been shown to have apart from their genomic but also non genomic effects and can interact with neurotrophins and neurotransmitters receptors and other proteins, they have been produced synthetic peptides that maintain the beneficial effects and lack the harmful properties. Neurosteroids are steroids that are synthesized de novo in the brain by the mevalonate pathway to cholesterol, independently from plasma levels (Baulieu and Robel, 1995). In previous studies, the neurosteroid dehydroepiandrosterone (DHEA), which is produced both in neurons and glia, has been shown to interact with p75NTR and induces the recruitment of adaptor proteins TRAF-6, RIP-2 and RhoGDI. Also, DHEA protects and rescues neurons from NGF-deficiency apoptosis (Lazaridis et al., 2011) (Figure 9). In this context, they have been designed synthetic neurosteroids (DHEA analogs) with neuroprotective but non-endocrine properties to contribute as therapeutic drugs with minimum side effects. Another, aspect of how important is DHEA presence in nervous system arises from evolutionary findings. More specifically, is has been found that the responsible enzyme for its biosynthesis existed in early evolutionary stages just before the appearance of vertebrate in Branchiostoma floridae also known as Amphioxus. This organism belongs to cephalochordata which are in the pro-stage of vertebrates. In addition DHEA has no specific receptor and exerts its role by binding, apart from TrkA as well, to γ-aminobutiric acid type A ($GABA_A$), N-methyl-D-aspartate (NMDA), sigma receptors or by converting to estrogens and androgens to ER receptors. For these reasons, it is was tempting to speculate that DHEA may acted like an early neurotrophic molecule and when the vertebrate nervous system had evolved, DHEA was replaced by more complicated molecules, the peptidic neurotrophins (Mizuta and Kubokawa, 2007; Miller, 2009).

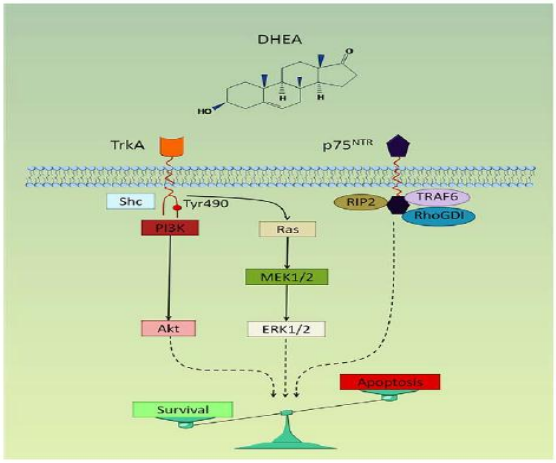

Figure 9: DHEA binds with high affinity to p75NTR and TrkA. DHEA binding to p75NTR affects the interaction between p75NTR and the adaptor proteins RIP-2, TRAF-6 and RhoGDI (Lazaridis et. al, 2011).

PURPOSE OF THE STUDY

The aim of this study is to decipher the functional role of the death receptor p75NTR in glial cells. More specific, it is investigated how this receptor is stimulated by different ligands. Also, it is studied what adaptor proteins are recruited after the ligand-dependent stimulation and most important which signaling pathways are activated as a response to receptor's structural conformation. The outline of the theoretical and experimental approaches is as follows:

- Isolation and expansion of high quality Schwann cells culture
- Adaptor protein detection and temporal determination of expression
- p75NTR/adaptor protein interaction
- Ligand-dependent p75NTR interaction
- Ligand-dependent p75NTR response in Schwann cells physiology

The potential answers to the before mentioned questions, would contribute to establish our hypothesis which is the ability to manipulate p75NTR pharmacologically by developing neurotrophin agonists. These substances should be specific at promoting the positive effects that p75NTR mediates (activation of survival pathways) while having minimum side effects in cellular and whole organism level.

MATERIALS AND METHODS

Isolation and expansion of Schwann cell culture

In this study Schwann cells are used as a source of highly expressed p75NTR cells. Schwann cells can be isolated and expanded in great numbers. They are in close contact with axons of PNS. A Schwann cell can myelinate an axon or a part of it but an axon can be myelinated by many Schwann cells. Here, the source of Schwann cells is the sciatic nerve because of its easy accessibility and large size. The sciatic nerves originate from rat pups and the following protocol concerns an entire litter.

The use of rats and protocols are approved by the Animal Care and Use of Committee of the University of Crete.

The followed protocol is based on the method of Brockes et al., (1979), as modified by Kleitman et al., (Honkanen, et al., 2007) for rat Schwann cells.

Note: All the following procedures are carried out to aseptic conditions as possible and all cultures are handled into a continuous laminar flow and are maintained in a 5% CO_2 humidified incubator at 37◦C.

Materials

- P0 to P4 rat pups
- A pair of forceps no: 5
- Dissecting scissors
- Scalpel handle with blade
- Stereoscope
- 15ml falcon (Sarstedt)
- PBS pH= 7.4 (Sigma)
- Penicillin/Streptomycin (Gibco)
- Trypsin (Gibco)
- 10mg/ml Collagenase type A (Sigma)
- Bright-Line Hemacytometer (Sigma)
- Culture medium: Dulbecco's modified essential medium (DMEM) (Gibco)
- Fetal bovine serum (FBS) (Gibco)
- 100μg/ml Primocin (Invivogen)
- Culture dishes (10mm and 20mm) (Sarstedt)
- 0.05% Trypsin/EDTA (Gibco)
- Recombinant Human NRG1-beta 1/HRG1-beta (R&D Systems)
- Forskolin (Sigma)
- 1000x Cytosine β-D-arabinofuranoside, HCl salt (Ara C) (Sigma)

Dissolve 27.9 mg Ara-C in 10 ml water to yield a 10 mM stock solution. Filter through a 20μm filter and store at −20°C, stable up to 1 year.

- Fluoro-deoxyuridine (FDU) (Sigma)
- Mouse anti-rat CD90 anti-Thy-1.1 (Serotec)
- Rabbit serum (Sigma)
- Ligands: pro-NGF (pro-NFG recombinant protein, My BioSource) NGF (Milipore), Dehydroepiandrosterone (DHEA) and BNN27.

  pro-NGF and NGF are reconstituted to 1000mg/ml concentration. 250μl of sterile water is added to the bottle and dissolve with thoroughly pipetting up and down. Aliquots are stored at -20◦C. DHEA and BNN27 are dissolved in DMSO to final concentration of 10^{-2}M and stored at -20◦C.

- Culture media:

The compositions of the media used in this study are as followed:

- ✓ Complete growth medium: DMEM, containing 10% inactivated fetal bovine serum (FBS), 1% penicillin/streptomycin, 100 μg/ml Primocin, 50 ng/ml recombinant human heregulin-β1 (rGGF) and 2μM forskolin.
- ✓ Cytolysis medium for complement-kill purification: complete DMEM, containing 4μg/ml mouse anti-rat CD90 anti-Thy-1.1.
- ✓ Medium for treatments: serum free DMEM (sf DMEM), containing no trophic factor.

Protocol

- Neonatal rat pups from postnatal day 0 (P0) to 4 (P4) are placed on ice to be anesthetized, one at a time. When it stops moving, it is decapitated and sprayed with 70% ethanol. Then the dorsal skin is removed from the legs until the lower torso (around the waist).
- The animal is pinned at the ventral side down with the palmal side up at the hind limbs.
- Under a stereoscope an incision is made along the leg, from sciatic notch to knee, to reveal the sciatic nerve (figure 10). The nerve is separated from connective tissue and perineurium and it is gently removed.
- All the nerves from the whole litter are collected in 15ml falcon with PBS+1%P/S.
- The nerves are spinned down to be pelleted.
- PBS is aspirated and 1 ml is left.
- 10% trypsin and 10% collagenase are added and nerves are incubated for 30 min to 37◦C.
- The enzymatic reaction is ended by addition of 3-fold volume of DMEM +10%FBS.

- The nerves are centrifuged for 2 minutes to be pelleted.
- The pellet is resuspended in 6ml DMEM+10% FBS (this volume concerns a whole litter) and then the tissue is dissociated mechanistically.
- Triturated cells are counted with a hematocytometer.
- The cells are placed in 100 x 20mm plate if the total Schwann cell number is $>10^6$ otherwise are placed in a 60 x 15mm plate, with complete DMEM.

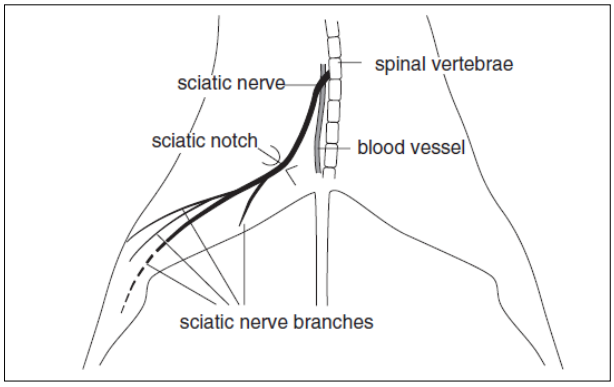

Figure 10: Schematic representation of the anatomy of sciatic nerve (Weinstein and Wu, 1999).

Purification of primary Schwann cells culture

At this point the primary culture contains axonal debris and fibroblasts. To eliminate the fibroblasts two different methods are used with different results in purifying the SCs culture. In both methods were used anti-mitotics because these substances stop DNA synthesis in all proliferating cells and are effective in fibroblasts because they proliferative in higher rate than SCs (Morrissey et al., 1991). In second method, complement cytolysis is performed because is a way to remove all the undesired cells from a culture. This method is based on the identification on the cell surface specific marker-antigen. It is known that fibroblast, but not SCs, express on their surface Thy-1.1 and by using a specific antibody and subsequently incubating with a different serum, they can be eliminated (Broockes et.al., 1979).

1)

- After 2 days in culture 2.5M of the anti-mitotic factor fluoro-deoxyuridine (FDU) is added (1/250).

- After a day the medium is changed to complete DMEM containing 50ng/ml NRG1-beta 1 and 2 μM forskolin.
- These steps are repeated every 2 days until the fibroblasts are reduced in number by microscopic (bright-field) observation.

2)

- After a day in culture the DMEM is removed and replaced with DMEM containing 10mM Ara C (1:1000 from stock) for 24 hours. Ara C as anti-mitotic factor affects mostly the fibroblasts because these are rapid dividing cells in contrast to SCs.
- On the second day in culture DMEM + Ara C is removed, cells are washed twice with sf DMEM and complete DMEM is added, containing 50ng/ml NRG1-beta 1 and 2 μM forskolin, for 24 hours.
- On the third day complement cytolysis is performed to remove the remaining fibroblasts.
- Cells are washed twice with sf DMEM and then are incubated at 37◦C for 15minutes in 2ml of cytolysis medium.
- 400μl of rabbit serum is added and incubation is continued for additional 35 minutes at 37◦C. The fibroblasts should die and detach.
- SCs are washed twice with sf DMEM and 10 ml of complete medium is added.

Expansion of culture

Schwann cells are kept in culture changing the medium every 2 days. When the culture is confluent, around 80%, the cells are passaged as following:

- Complete DMEM is removed
- Cells are washed once with PBS or twice with sf DMEM
- 0.05% trypsin/EDTA is added and cells are incubated for 3-5 minutes at 37◦C, while observing at microscope to see cells detaching from the plate. The volume of used trypsin/EDTA is based on the surface of the dish i.e. 1ml for a 100 x 20mm or 500μl for a 60 x 15mm plate.
- The enzymatic reaction is inactivated by the addition of with 3-fold volume of complete DMEM.
- Cells are collected in a falcon tube and centrifuged for 2 minutes to be pelleted.
- SCs are resuspended with the appropriate volume (6-10 ml), depending on the initial cell number, and are replated on culture dishes with complete DMEM containing 50ng/ml NRG1-beta 1 and 2μM forskolin.

Immunostaining for Schwann cell marker S100

Materials

- 48-well multidish (Nunc, Thermo Scientific)
- BrightLine Hemacytometer (Sigma)
- PBS pH=7.4 (Sigma)
- Fixation/Permeabilization Solution kit BD Cytofix/Cytoperm (BD Biosciences) (1ml of wash buffer is diluted in 9ml dH_2O).
- RabMab Anti- S100 beta antibody (abcam)
- Alexa Fluor 488 Goat Anti-Rabbit IgG
- Hoechst (Life Technologies)

Protocol

SCs of different days in culture (6 and 24) are plated on a 48-well plate in $3x10^4$ cells/well and after 2 days are immunostained as followed:

- SCs are washed with PBS.
- 150μl cytofix is added to each well and left for 20minutes.
- Cells are washed 2x 500μl wash buffer for 10minutes.
- 1^{st} antibody rabbit anti-S100 beta is added in 1/200 (dilute in wash buffer) overnight at 4◦C or for an hour in room temperature on rotator.
- 2x 500μl washes with wash buffer for 10 minutes are made.
- SCs are incubated with the 2^{nd} antibody Alexa Fluor anti-rabbit 488 1/500 for 1hour at room temperature on rotator.
- Cells are washed twice with 500μl wash buffer for 10 minutes.
- 150μl of 1/10000 Hoechst to each well and left for 10 minutes on rotating device.
- 2x 10 minutes PBS washes are made.
- S100 positive cells are observed with 10x lens on fluorescent microscope and the percentage results from the average number counting 5 different fields.

Immunoprecipitation (IP)

Materials

- 6-well multidish (Nunc, Thermo Scientific)
- BrightLine Hemacytometer (Sigma)
- sf DMEM (Gibco)
- Ligands: pro-NGF (pro-NFG recombinant protein, My BioSource) NGF (Milipore), Dehydroepiandrosterone (DHEA) and BNN27
- PBS pH=7.4 (Sigma)

- Pierce IP Lysis Buffer: 25mM Tris-HCl pH 7.4, 150mM NaCl, 1mM EDTA, 1% NP-40 and 5% glycerol (Thermo Scientific) (before use protease inhibitors are freshly added)
- Aprotinin from bovine lug saline solution: trypsin/ serine protease inhibitor(Sigma)
- Phenylmethanesulfonyl fluoride (PMSF) (Sigma)
- Mouse RICK (25) (RIP-2) (dilution 1:250, Santa-Cruz Biotechnology)
- Protein A/G PLUS-agarose (Santa-Cruz Biotechnology)
- 2x SDS-PAGE sample buffer (50ml) (pH=7.4)
- ✓ 5ml 1M Tris-HCl
- ✓ 20ml SDS 10%
- ✓ 10ml glycerol
- ✓ 0.1gr Bromophenol Blue (BPB)
- ✓ 15ml dH_2O
- ✓ 5% beta-mercaptoethanol

Protocol

- After 4 days in culture SCs are detached and replated in 6-well multidishes in $1.5x10^6$ cells/well.
- After 2 days SCs are washed 5 times with sf DMEM and are treated or left untreated for 10 or 20 minutes with ligands (pro-NGF, NGF, DHEA AND BNN27) at a concentration of 100ng/ml. There are 5 sample conditions: 1) serum free (sf) 2) pro-NGF 3) NGF 4) DHEA 5) BNN27.
- After the treatment, the plate is placed on ice and SCs are washed with 1ml ice-cold PBS to stop the reaction.
- Cells are lysed in 300μl lysis buffer, containing aprotinin and PMSF, and then divided into aliquots of different volumes, 200μl and 100μl.
- 30μl of SDS-PAGE sample buffer are added to 100μl lysates and the samples are kept to -20◦C for Western blot analysis.
- 1μg/ml anti-RIP-2 is added to 200μl lysates and the samples are incubated on rotating device overnight at 4◦C.
- 30μl of A/G agarose beads are added to each sample and left on rotating device for 4 hours at 4◦C
- Lysates are 2x washed with 500μl lysis buffer, containing aprotinin and PMSF, and centrifuged at 2500 g for 3 minutes.
- The supernatant is removed and 50μl of SDS-PAGE sample buffer is added to each sample.
- Samples are stored at -20◦C and analyzed by Western blot.

Western blot analysis

Materials

- Separating gel buffer (pH =8.8) (100ml) (store at 4^oC)
- ✓ 18.165 gr Tris
- ✓ 0.4gr SDS 0.4% w/v

- Stacking gel buffer (pH =6.8) (100ml) (store at 4^oC)
- ✓ 6.05gr Tris 0.5M
- ✓ 0.4gr SDS 0.4% w/v

- Buffer ηλεκτροφόρησης 10X (pH =8.3) (1lt) (store at 4^oC)
- ✓ 30.3gr Tris
- ✓ 144.2gr glycine
- ✓ 10gr SDS

- Buffer transfer 10X (pH =8.3) (1lt) (store at 4^oC)
- ✓ 30.3gr Tris
- ✓ 144.2 g Glycine

- 30% acrylamide stock (100ml) (store at 4^oC)
- ✓ 29.2gr Acrylamide
- ✓ 0.8gr Bis-acrylamide
- ✓ Filter solution through 0.45μm filter

- Ammonium Persulfate (APS) (10ml)
- ✓ 1gr APS
- ✓ Add d H_2O to 10ml

- 10% SDS-polyacrylamide gel (2 gels)
- ✓ 6ml acrylamide stock
- ✓ 3.843ml separate buffer
- ✓ 5ml dH2O
- ✓ 150 μl APS
- ✓ 7 μl TEMED

- Stacking gel
- ✓ 1.275ml acrylamide stock

- ✓ 0.937ml stacking buffer
- ✓ 5.1ml dH2O
- ✓ 75 µl APS
- ✓ 2.5 µl TEMED

- TBS 10X (pH =7.6) (1lt) (store at 4^{o}C)
- ✓ 24.2gr Tris
- ✓ 80gr NaCl
- ✓ TBST 1x
- ✓ 1% Tween-20 (1ml/lt)
    - Mini-Protean Tetra Cell (Bio-Rad)
    - Precision Plus Protein Kaleidoscope (Bio-Rad)
    - Nitrocellulose transfer membrane 0.45µm (Bio-Rad)
    - Bovine serum albumin (BSA) (Sigma)
    - Anti-Human p75 pAb (Promega)
    - Mouse RICK (25) (RIP-2) (Santa Cruz Biotechnology)
    - Rabbit GAPDH (Santa Cruz Biotechnology)
    - HRP-goat-anti-rabbit (Life Technologies)
    - Pierce ECL Western Blotting Substrate (Thermo Scientific)
    - ChemiDoc XRS+ System (Bio-Rad)
    - ImageLab Software (Bio-Rad)

Protocol

- Samples in SDS-PAGE sample buffer are resolved on a 10% SDS-polyacrylamide gel under reducing conditions for 2.5 hours.
- Proteins are transferred to nitrocellulose membrane for 1 hour and blocked with 5% BSA TBST blocking solution for 30 minutes at 4◦C
- The membrane is incubated with first antibody 1/1.000 anti-p75 in TBST for IP samples and 1/250 anti-RIP-2 in TBST for the lysates overnight at 4◦C.
- After 3x10 minutes washes with TBST the membrane is incubated with the secondary antibody anti-rabbit 1/5.000 and 1/2.000 anti-mouse for an hour at room temperature.
- 3x10 minutes washes with TBST.
- The membrane is developed using the ECL solution, the lighting signal is detected by Chemi-Doc XRS+ system and analyzed by ImageLab software.

Flow cytometry (FACS) analysis for apoptosis by detection of active Caspase-3

Materials

- 6-well multidish (Nunc, Thermo Scientific)
- BrightLine Hemacytometer (Sigma)
- sf DMEM (Gibco)
- Ligands: pro-NGF (pro-NFG recombinant protein, My BioSource) NGF (Milipore), Dehydroepiandrosterone (DHEA) and BNN27
- PBS pH=7.4 (Sigma)
- 2ml eppendorf tubes (Sarstedt)
- Fixation/Permeabilization Solution kit BD Cytofix/Cytoperm (BD Biosciences) (1ml of wash buffer is diluted in 9ml dH_2O).
- Rabbit Caspase-3 (abcam)
- Alexa Fluor 488 Goat Anti-Rabbit IgG (Life Technologies)
- PP Test tubes with Snap Cap (Corning)
- BD FACSCalibur Cell Analyzer (BD Biosciences)

Protocol

- Schwann cells after 2 or 7 days in culture are detached and replated in 6-well multidishes in $1.5x10^6$ cells/well.
- SCs are washed 5 times with sf DMEM and are treated or left untreated for 72 hours with ligands (pro-NGF, NGF, DHEA AND BNN27) at a concentration of 100ng/ml. There are 5 sample conditions: 1) serum free (sf) 2) pro-NGF 3) NGF 4) DHEA 5) BNN27.
- After this period of treatment, the plate is placed on ice and SCs are washed with 1ml ice-cold PBS to stop the reaction.
- SCs are detached mechanistically with pipetting up and down and collected into 2 ml eppendorf tubes.
- Cells are centrifuge at 6000 g for 2 minutes at 4◦C and then are resuspended in 400μl wash buffer.
- SCs are centrifuged as previously and resuspended in 80μl of cytofix solution 5 times (pipetting up and down) and left for 20 minutes on ice.
- 500μl of wash buffer are added and SCs are centrifuged as previously.
- Cells are incubated with primary rabbit anti-Caspase-3 (1/500 diluted in wash buffer) for 30 minutes on ice.
- 2x 500μl wash buffer are added and cells are centrifuged as previously.
- SCs are incubated with secondary Alexa Fluor anti-rabbit 488 (1/500) for 30 minutes on ice.
- 2x 500μl wash buffer are added and cells are centrifuged as previously.
- SCs are resuspended in 1ml PBS and transferred into tubes for FACS analysis.

- Cells are detected by BD FACSCalibur and analyzed by CellQuest Pro Software.

Flow cytometry (FACS) analysis for detection of p75NTR levels in Schwann cells

Schwann cells after 4 days in culture are detached and replated in 24-well multidish in $5x10^4$ cells/well. After 2 days SCs are processed for FACS as described previously. As negative control is used naive HEK293 cells (they do not express endogenously p75NTR). The antibodies used in this procedure are primary rabbit anti-human p75NTR at 1/50 dilution (50μl) and as secondary Alexa Fluor anti-rabbit 488 at 1/500 dilution (100μl).

Culture of HEK293 cells

Materials

- Complete DMEM (+10% FBS, +1% pen/strep) (Gibco)

HEK293 are plated in 24-well plate in $6x10^4$ cells/well.

After 2 days in culture DMEM can be used in experimental procedures as described previously.

MATERIALS AND METHODS FOR BINDING DISPLACEMENT ASSAYS

Transfection of HEK293 cells

Materials

- Culture media DMEM (Gibco)
- Fetal Bovine Serum (Gibco)
- Penicillin/Streptomycin
- 100 x 20mm culture dishes
- Plasmids: AmphiTrk, ApTrk, Ltrk
- TurboFect Transfection Reagent (Fermentas, Thermo Scientific)
- Complete medium composition: DMEM +10%FBS +1% pen/strep
- 15ml falcon tube (Sarstedt)

Protocol

- HEK293 cells are plated on 8x 100x20mm plates for each plasmid and untransfected HEK293.
- After 2 days in culture DMEM is replaced with 3ml of fresh complete medium.
- Transfection mix is prepared as following:
- For 100x20mm plate, in a 15ml falcon tube are added:
- 1ml sfDMEM/plate
- 10μg DNA/plate
- 160μl Turbofect Transfection Reagent/plate
- The transfection mix is left 15min for forming DNA/lipid complexes that will enter the cell.
- 1ml of transfection mix is added to each plate
- After 5hours of incubation the medium is replaced with 8-10 ml of fresh complete DMEM.
- HEK293 are used 2 days after transfection.

[3H]-DHEA Binding Assays

Materials

- PBS pH=7.4 (Sigma)
- 1mM PMSF (protease inhibitor) (Sigma)
- 1μg/ml aprotinin (protease inhibitor) (Sigma)
- 50ml falcon tubes (Sarstedt)
- 50mM glycine pH=3 (Sigma)
- DHEA-S (Sigma)
- [3H]-DHEA
- GF/B filters (GE Healthcare Life Sciences)
- Poly-ethyleneimine (PEI) solution (Sigma)
- Fluor High Performance LSC cocktail for aqueous samples (Scintillation fluid, Sigma)
- Scintillation counter (Perkin Elmer, Foster City, CA)

Protocol

Membrane preparation (The whole procedure is performed on ice)

- HEK293 cells transfected and untransfected were collected in 50ml falcon and centrifuged at 1.200 rpm to form a pellet.
- Cell pellets were washed with cold PBS.
- After centrifugation cells were homogenized in 3ml PBS, containing freshly added protease inhibitors (1 mM PMSF and 1 mg/ml aprotinin, referred as PBS mix).
- Crude membrane fractions were isolated by 2x differential centrifugation at 5000rpm (5 minutes at 4◦C, to remove unbroken cells and nuclei) and 2x 27.000rpm (1 h, at 4◦C). Between centrifugations membranes were washed once with ice-cold PBS mix and re-suspended in the same buffer.
- Membranes were then briefly acidified with 50 mM glycine pH 3 for 3 min on ice to elute membrane adsorbed proteins
- They were washed once, resuspended in PBS mix (at a concentration of 2 mg/ml) and used immediately for binding displacement experiments.

Binding conditions

- 2 mg/ml of membrane suspension in triplicate were incubated with a constant concentration of [3H]-DHEA (1 nM) in the presence of increasing concentrations of DHEA-S (10^{-14}–10^{-6} M) in PBS mix in a final volume of 100 ml.
- Membranes were incubated overnight at 4◦C, on a rotating plate.
- Then they were collected on GF/B filters, prewetted in 0.5% PEI solution at 4◦C. Filters were washed five times with ice-cold PBS, dried, and counted in scintillation fluid in a scintillation counter with 60% efficiency for Tritium.

Statistical Analysis

Statistical analysis of our data was performed using Analysis of Variance, post hoc comparison of means, followed by the Fisher's least significance difference test. For data expressed as percent changes we have used the nonparametric Kruskal-Wallis test for several independent samples.

RESULTS

Part 1

Schwann cells culture purification

To ensure the quality, validity and reproducity of all the experimental approaches, SCs culture have to be as pure as possible. In this study, different purified methods are tested to yield higly enriched in SCs number cultures. First, it is tested the efficacy of the anti-mitotic factor FDU which is prefered for purifying DRGs culture. Then, the alternative approach combines two methods, the use of the anti-mitotic factor Ara C and cytolysis by complement kill based on the presence or absence of Thy-1.1 antigen on cell surface. Here, it is shown that use of anti-mitotic FDU results in 69.4% SCs purification in contrast to the combined use of the anti-mitotic Ara C and complement cytolysis which yields approximately 99% purified SCs cultures (figure 11). Finally, it is of high importance to investigate whether long term (>24 days) SCs culture are enriched and if SCs maintain their morphology and physiology. After staining for S-100 positive cells, it is observed that SCs either in short term (6 days) and long term (>24 days) cultures maintain all of their unique features, at the same percentage 99% (figure 12).

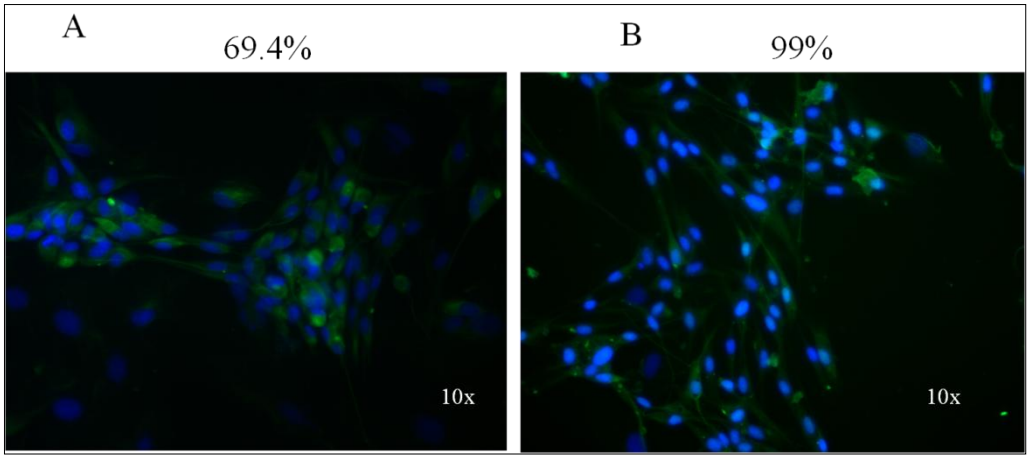

Figure 11: Purification of SCs culture. A) 69.4% SCs (S-100 positive cells) using 2.5M FDU. B) 99% SCs (S-100 positive cells) using 10μM Ara C and complement cytolysis.

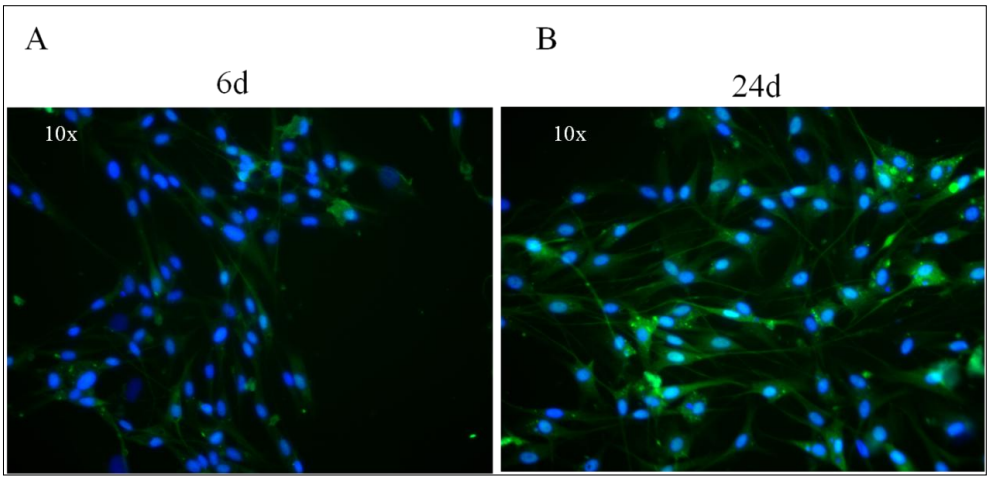

Figure 12: Physiology and number of early and late SCs culture. Both cultures are purified by Ara C and complement cytolysis combined method. A) SCs in 6 days culture. B) SCs in 24 days culture.

Expression of p75NTR

In this study is studied the endogenous expression of p75NTR in Schwann cells which express highly p75NTR. Here, we detected the p75NTR expression firstly by cell immunostaining and secondly by flow cytometry FACS analysis, using a specific antibody for the receptor's intracellular domain. (figure 13).

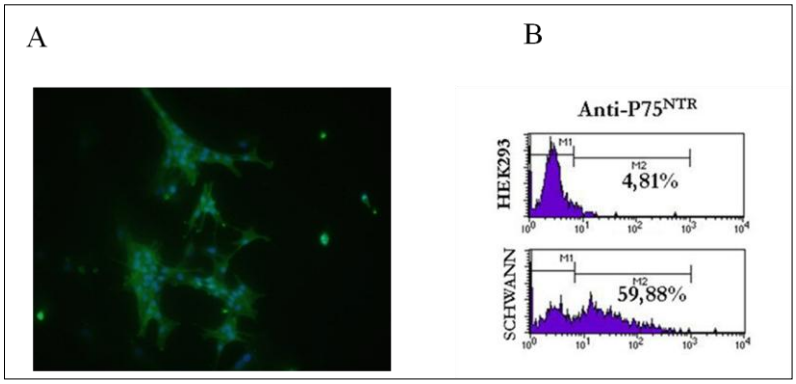

Figure 13: Endogenous expression of p75NTR in SCs. A) Immunostaining: SCs express p75NTR. B) Flow cytometry (FACS analysis) for the detection of p75NTR expression levels in Schwann cells and HEK293 (negative control). Schwann cells are from culture purified with 2.5M FDU. Cells were cultured for 6 days before any experimental processing.

Expression of RIP-2

RIP-2 is one of the numerous p75NTR adaptor proteins and is considered as the key protein for transducing survival signals. Thus, it is obvious that its presence within SCs have to be determined under our established culture conditions in time-depented context. To investigate this, Schwann cells were maintened in culture for different timepoints of 6, 12, 18, 24 and 30days. Then, lysates were collected from each timepoint and analyzed by Western blot with antibody against RIP-2 (figure 14). The immunoblot shows that RIP-2 is stable expressed in primary Schwann cell cultures for a long period of time. Unexpectedly, it is found that RIP-2 expression is detectable even in 30 day. p75NTR expression was evaluated as well, in the same culture conditions and timepoints. Generally, RIP-2 and p75NTR are stable expressed in early points of culture (6 and 12 days), in intermediate points (18 and 24 days) and in late cultures (30 days).

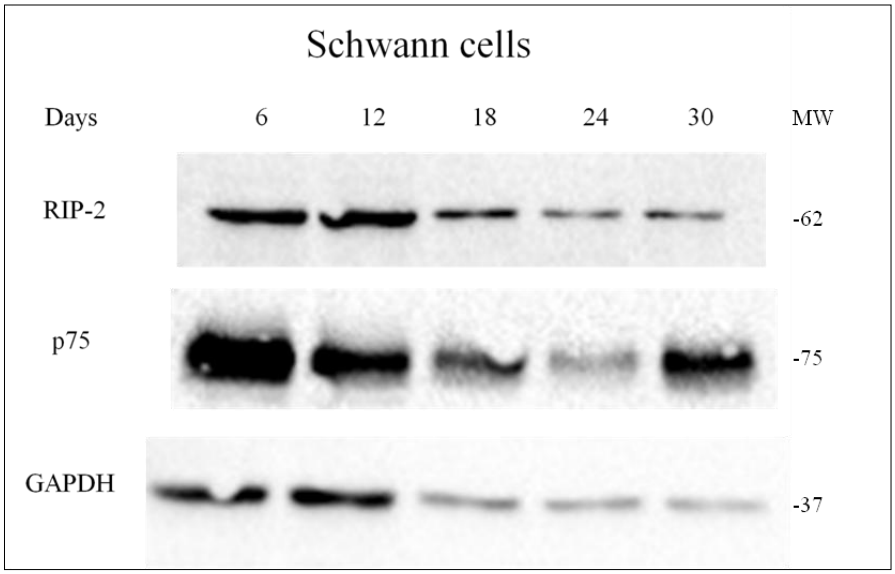

Figure 14: Expression of RIP-2 and p75 in Schwann cells for 6, 12, 18, 24 and 30 days in culture. Lysates were collected and the expression of RIP-2 and p75 was assessed by Western blot analysis. The blot was reprobed with GAPDH as a loading control.

RIP-2 binds and interacts with p75NTR

Since, RIP-2 is determined in terms of time all experiments were conducted in early cultures (6 days). As it is established, RIP-2 by p75NTR binding activates NF-κB which in turn regulates the expression of genes important for survival. To verify the interaction between RIP-2 and p75NTR, in first place, and secondly to test whether our ligands induce RIP-2 interaction with endogenous p75NTR, Schwann cells in early culture (6 days) were treated or left untreated with 100ng/ml (10^{-7}M) of pro-NGF, NGF and 100nM of DHEA and BNN27 for either 20 and 10 minutes. Then lysates were collected, immunoprecipitated with anti-RIP-2 and immunobloted for p75 and RIP-2 (figures 15 and 16). These results indicate an endogenous interaction between RIP-2 and p75 that is ligand- and time- dependent. Their interaction after different ligand-stimulation is transient and it can be observed after 10 minutes for all four ligands but not for longer time (20 minutes) of treatment with DHEA.

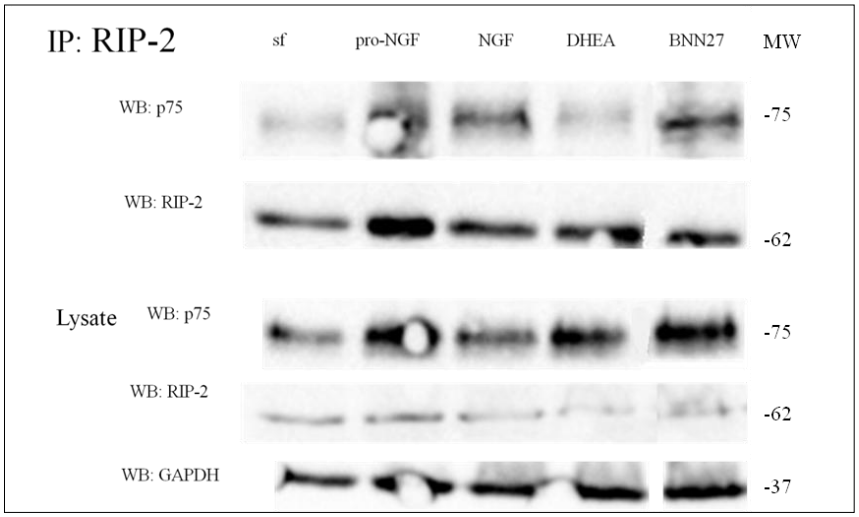

Figure 15: Schwann cells cultured for 6 days and then treated for 20min with 100ng/ml of pro-NFG, NGF and 100nM DHEA and BNN27. It is observed an endogenous ligand-dependent association of RIP-2 with p75NTR, except for DHEA. The lysates were collected, immunoprecipitated with anti-RIP-2 and subsequently immunobloted for p75 and RIP-2. Lysates were assessed for p75 and RIP-2 levels. The blot was reprobed with GAPDH as loading control.

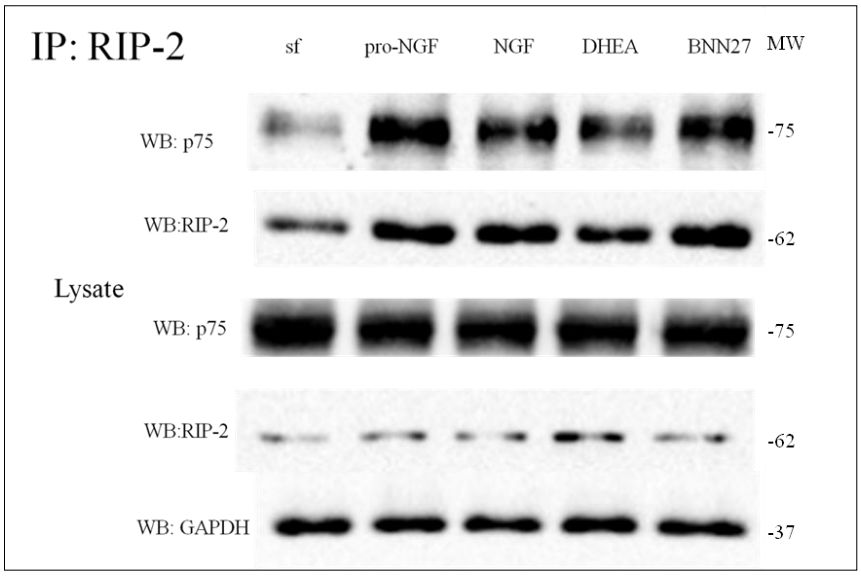

Figure 16: Schwann cells cultured for 6 days and then treated for 10min with 100ng/ml of pro-NFG, NGF and 100nM DHEA and BNN27. An endogenous ligand-dependent association of RIP-2 with p75NTR is shown. The lysates were collected, immunoprecipitated with anti-RIP-2 and subsequently immunobloted for p75 and RIP-2. Lysates were assessed for p75 and RIP-2 levels. The blot was reprobed with GAPDH as loading control.

The ligand-dependent cell-death of primary Schwann cells

Depending on previous experiments of our laboratory on the ability of these ligands to affect cell viability, Schwann cells of 2 and 6 days in culture were subjected to serum starvation over a period of 72 hours and incubation with or without 100ng/ml of pro-NGF, NGF and 100nM of DHEA and BNN27 (figure 17). Our findings suggest that in 2 days of culture the percentage of apoptotic cells is higher for NGF-dependent and lower for pro-NGF-dependent cell death. In contrast to this, in 6-day culture more cells die as a response to pro-NGF, while killing by NGF was decreased. Of high importance is that both DHEA and BNN27 exhibit an anti-apoptotic function, in both experimental approaches.

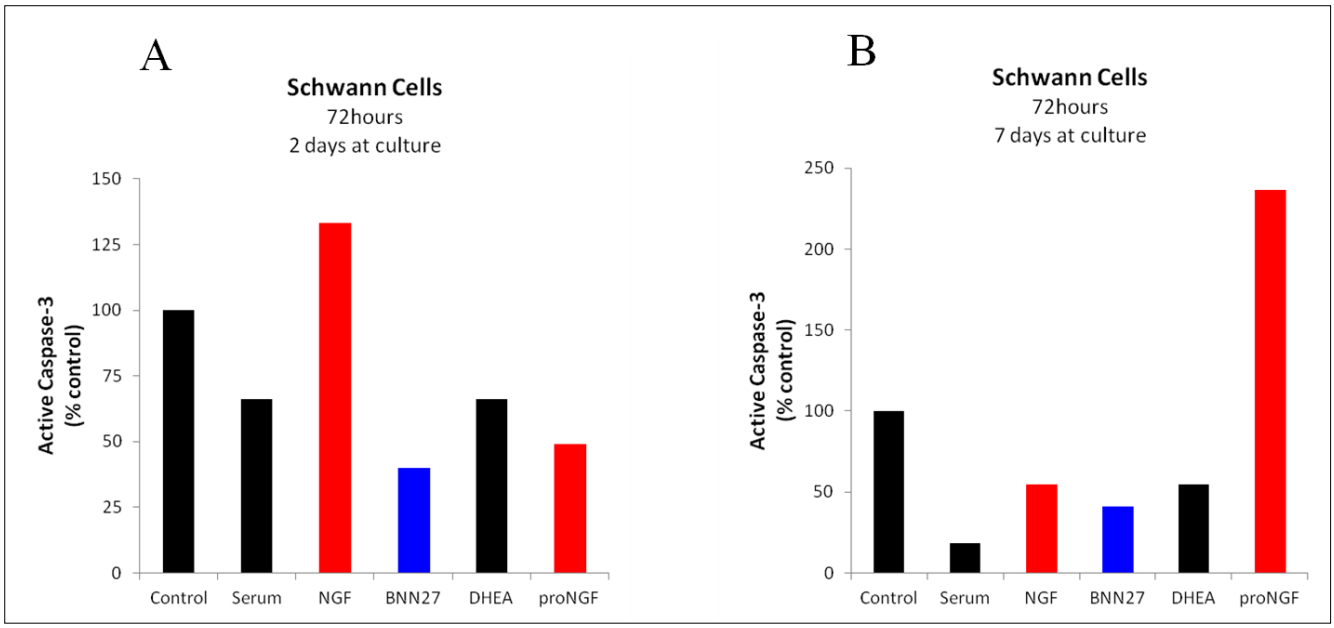

Figure 17: Evaluation of apoptotic Schwann cells by assessment of Caspase-3 activity over a period of 72 hours of serum starvation and incubation with or without 100ng/ml of pro-NGF, NFG and 100nM DHEA and BNN27. Schwann cells were in culture for 2 (A) and 7 (B) days.

Part 2

DHEA-S interacts with Trk-homologue receptors of invertebrate organisms

In parallel with the before mentioned work, there was another experiment executed as a continuity to an already existing research project. The goal of this work was to investigate DHEA sulfate ester and its ability to bind to specific Trk-homologous receptors of invertebrate organisms such as the mollusk Lymnaea stagnalis (Ltrk), the marine mollusk Aplysia californica (ApTrk) and the cephalochordate amphioxus Branhiostoma floridae (AmphiTrk). The prior work on this project concluded that DHEA interacts with all Trk receptors and Ltrk, ApTrk and AmphiTrk (Figure 18) (Pediaditakis et al., 2014). It is already known that DHEA-S interacts with TrkA (Lazaridis et al., 2011) and now in this part is shown, for the first time, that DHEA-S also interacts at nanomolar concentrations with invertebrate Trk-homologous receptors Ltrk (Ki: 1.16± 0.11nM), ApTrk (Ki: 1.13±0.35nM) and AmphiTrk (Ki: 6.58±0.27nM) (Figure 19).

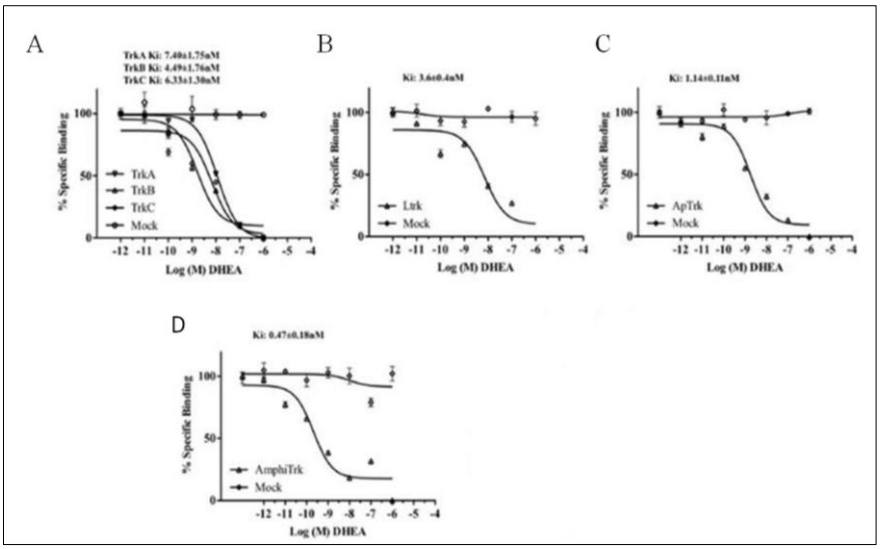

Figure 18: [3H]-DHEA binding to membranes from HEK293 cells transfected with mammalian and invertebrate Trk receptors cDNAs. Competition binding assays of tritiated [3H]-DHEA in the presence of increasing concentrations of nonlabeled DHEA. These assays were performed using membranes isolated from HEK293 cells transfected with the cDNAs of mammalian TrkA, TrkB, TrkC receptors (A) or invertebrate Ltrk(B), ApTrk (C), AmphiTrk receptors (D).

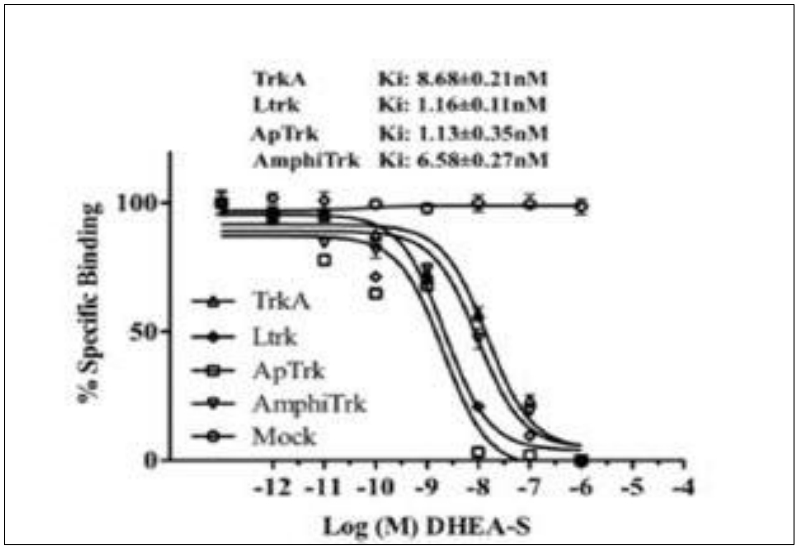

Figure 19: [3H]-DHEA binding to membranes from HEK293 cells transfected with invertebrate Trk receptors cDNAs. Competition binding assay of tritiated [3H]-DHEA in the presence of increasing concentrations of nonlabeled DHEA-S. This assay was performed using membranes isolated from HEK293 cells transfected with the cDNAs of invertebrate Ltrk, ApTrk and AmphiTrk receptors.

DISCUSSION

Schwann cells express in high levels the NGF low-affinity receptor p75NTR, which its biology is investigated in this study. The first aim was to isolate and expand highly purified SCs culture from rat neonatal sciatic nerve. The ideal method had to be fast, inexpensive and efficient. For this reason, two already existing purifying methods were evaluated in eliminating other cell types such as fibroblasts, which are present in great number. SCs cultures were treated with fluoro-deoxyuridine (FDU) and Cytosine arabinofuranoside (AraC) followed by complement depletion. It is found, that the combined method of treatment with AraC and complement depletion was more efficient in yielding enriched SCs cultures, approximately 99%, compared to treatment only with FDU which yields only 69.4%. These results were verified by immunostaining with S100 which is a glial marker specific for Schwann cells. Initially, it is observed that SCs are not "healthy" after treatment with FDU and in addition they cannot be maintained in culture for many days in contrast to the alternative method. These facts raise the question in what way using FDU would affect the cells physiology and biology and moreover the experimental results concerning functional elucidation of p75NTR. In addition, it is known that 3-day treatment FDU in neurons-glia co-cultures eliminates most of the glial cell population (Morrissey et al, 1991). Moreover, in our test it is observed that in FDU treating cultures the fibroblasts exceeded the number of SCs at some point and took over the culture (as mentioned before SCs could not be maintained for long period in culture), but in AraC/complement depletion cultures SCs maintained not only their number but also their physiology and phenotype. Finally, it has come to a conclusion that treatment with AraC and complement cytolysis, is enough to obtain purified SCs culture and this method is preferable because it is fast, inexpensive, gentle on cells and effective.

The next step was to see p75NTR expression level both directly by immunostaining and quantitively by FACS analysis. Cell immunostaining is a direct and fast way to detect p75NTR but FACS analysis is a high quality and precise method with trustable and quantitive results.

Another goal of this study was to determine the temporal expression of one of the most important adaptor protein RIP-2. It is found that RIP-2 is constantly present within 30 days of culture. These findings are partially consistent with the study of Khursigara et al., in which levels of RIP-2 are high at 6 days but RIP-2 cannot be

detect after 30days in culture or more. It can be assumed that SCs are subjected to stress, indicating from the slight elevated expression, when are maintained for such a long period in culture conditions and this is the reason that RIP-2 is still expressed, trying to play a protective role and contribute to their survival. Another explanation to this observation could be, due to an environmental or trophic factor, SCs could lose RIP-2 expression in even longer culture period for example 40 days. In Khursigara et al., study SCs were maintained in absence of neuregulin-β. Finally, as it was expected the expression of p75NTR is maintained in all days of culture.

Since, RIP-2 expression is determined, next it was investigated its ligand-depended binding and interaction with p75NTR. The main p75NTR ligands are pro-NGF and NGF and were used as control ligands and also two other ligands DHEA and BNN27 were used as novel ligands of endogenous p75NTR. In previous studies, DHEA has been shown to bind and interact with TrkA and p75NTR and exert neuroprotective and anti-apoptotic role in neurons (Lazarides et al., 2011). In order to utilize DHEA's positive functions leaving out its side effects, they have been designed and synthesized analogues without its endocrine effects the so-called "spiro-analogues", BNN27 is one of them. First, it was tried to be demonstrated that RIP-2 immunoprecipitates with p75NTR after 20 minutes of ligand-treatment for all 4 ligands something that haven't been observed for DHEA, although it is known that NGF treatment lead to CARD domain of RIP-2 binding to DD of p75NTR and precipitation (Khursigara et al., 2001). In that study NGF addition was only for 5 minutes, so based on it, it can be speculated that there cannot be detected any interaction in 20 minutes treatment because RIP-2 interacts with p75NTR transiently and this is lost within 20 minutes. This suggests that RIP-2 may dissociate following its activation. To confirm this hypothesis, the same experimental procedure was repeated but this time ligands were added for 10 minutes. It is found that proteins can be coimmunoprecipitated from SCs cells within 10 minutes of ligand-stimulation including DHEA, confirming the previous hypothesis.

The final aim of this study was to assess SCs ligand-depended cell death by evaluation of Caspase-3 activation. SCs in 2- and 7-days culture subjected to serum and trophic factor starvation for 72 hours in presence of 100ng/ml pro-NGF, NGF and 100nM DHEA and BNN27. It was shown that in SCs of 2 days culture pro-NFG unexpectedly didn't induced cell death, in contrast, acted in a protective way, while cells were more vulnerable to NFG and died in great number, which is a surprising

fact as well. On the other hand, in SCs of 7 days culture is observed a higher percentage of cell death induced by pro-NGF while NGF seems to act in a promote survival, as was expected. Pro-NFG binds with high affinity to p75NTR and induces apoptosis while NGF has been reported to promote survival in early SCs culture due to high RIP-2 expression (Khursigara et al., 2001). Finally, both DHEA and BNN27 in 2 and 7 days culture had a neuroprotective role by preventing cell death of SCs. DHEA as mentioned before interacts with p75NTR and prevents apoptosis in sensory and sympathetic neurons (Lazarides et., 2011).

The role of DHEA and its sulfate ester (DHEA-S) is a research subject in our laboratory and is included in many projects. In this context, there is a phylogenetic study concerning the evolutionary process of Trk receptors and DHEA ability to bind to invertebrate and mammalian Trk receptors. It has been shown that indeed DHEA binds to all mammalian Trks. Also, DHEA interacts with invertebrate Trk-homologous receptors and more specifically with the mollusk Lymnaea stagnalis (Ltrk), the marine mollusk Aplysia californica (ApTrk) and the cephalochordate amphioxus Branhiostoma floridae (AmphiTrk). Since this was found and as it was previously shown that DHEA-S also interacts with TrkA, came as a consequence the need to investigate whether DEHA-S binds and interacts with the Ltrk, ApLtrk and AmphiTrk, as well. Here, it is proved that DHEA-S can interact at nanomolar concentrations with all of the three receptors. Also, DHEA-S binds with lower affinity to AmphiTrk in contrast to DHEA due to small but significant structural differences. These results suggest that DHEA may played the role of neurotrophin in the less complicated nervous system of organisms at early evolutionary stages in absence of the dimeric peptidic neurotrophins, which they have evolved because of the development of a more complex nervous system.

PERSPECTIVES

p75NTR is a unique receptor as far as concerns its physiology and function. It was believed to be a non-signaling receptor and now has come to light that mediates a number of very interesting and important functions. It is implicated in apoptosis, survival, myelination, synaptic function and regeneration. It co-operates with many receptors, binds with various ligands and interacts with numerous adaptors proteins. All these accumulating evidence make p5NTR capable of responding differently under various conditions depending of the environmental context every time. p75NTR signaling is implicated not only during development but in a number of human diseases such as Alzheimer's disease, Multiple Sclerosis (MS) and Motor neuron Disease (MND).

It is of high importance that research focuses on elucidating how this receptor responds to its ligands, how recruits adaptor proteins depending on the cellular context or state and how mediates its signal. In my opinion, first should be determined which segment of the receptor molecule is required for a particular function and this requires the development of genetic tools for correlating the ligand binding with structural conformation and finally the functional outcome. Second, there should be strategies that would aim at determining what happens in vivo taking into consideration the whole organism something that requires the development of effective animal model of diseases. This will provide information for the human disease state by identifying in which processes p75NTR involves and contributes physiologically (for example during development) or pathologically. In addition, the pharmaceutical approaches have to be upgraded not only aiming at the level of silencing the receptor but manipulating it in a way that p75NTR activates/promotes its positive functions with the minimum side effects. Last but not least, should be taken advantage of studies of stem cells that express p75NTR and are able to proliferate and differentiate and could be utilized in treating degenerative diseases or trauma like spinal cord injury.

More specifically, our intention is, first of all, to elucidate the interaction with other adaptor proteins, such as TRAF-6 and SC-1 and p75NTR and the signaling pathways that are activated. Furthermore, there is need to test not only the ligands that were mentioned here but even more DHEA analogues that our laboratory has already screen for their actions in other experimental systems. As a next step, it has to be development a detailed map of how different segments of the receptors molecule

contribute to its function. In that context, Charalampopoulos et al., after having screen a number of point mutations of the p75NTR DD managed to construct various mutants of this receptor carrying different mutation or combination of mutations. These mutants are valuable genetic tools to decipher the physiology, structural conformation and respond of p75NTR in the presence or absence of ligand and its functional outcome under different ligand-stimulation. We have already in our hands a p75-/- mouse model to study in a bimodal way. First, our aim is to isolate and expand p75NTR-/- SCs cultures for testing specific mutants of the receptor with our ligands and as a further step to breed these mice and treat them with DEHA analogues for in vivo investigation of their role (neuroprotective and regenerative or not). At the end, another future experimental approach is to manage to develop combined mouse models such as the knock-in p75NTR Cys^{275} model for evaluating the receptor activation mechanisms by different ligand-stimulation and the p75-/- and 5xFAD for in vitro and in vivo studying the role of p75NTR in Alzheimer's disease. Although, there are many aspects of p75NTR to elucidate and even more things to reveal, its pharmacological manipulation by small peptidic molecules acting as neurotrophin agonists is of high-priority significance.

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