Universitat de Lleida

TrkA COOH-terminal tail: its relevance on receptor
stability and signaling for cell differentiation and
survival

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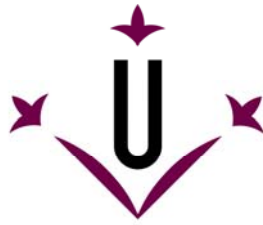
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Thesis supervisor:

Marta Llovera Tomas

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ABBREVIATIONS

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aa	Amino acid
AMSH	Associated Molecule with the SH3 domain of STAM
ARMS	(Kidins220) Ankyrin repeat-Rich Membrane Spanning protein
BDNF	Brain-Derived Neurotrophin Factor
CaM	Calmodulin
CBD	Calmodulin Binding Domain
CHK	Csk Homologous Kinase
CIPA	Congenital Insensitivity of Pain with Anhydrosis disease
CR	Cysteine-Repeat domains
CRD image	Colocalization-Rate Displaying image
CREB	cAMP Response Element-Binding
DAG	Diacylglycerol
DD	Death Domain
DRG	Dorsal Root Ganglion
DUB	De-Ubiquitinating enzyme
EGFR	Epidermal Growth Factor Receptor
ENaC	Epithelial Sodium Channel
ERK	Extracellular Regulated Kinase
ESCRT	Endosomal Sorting Complexes Required for Transport
FGF	Fibroblast Growth Factor
FKHRL1	Forkhead transcription factor
FRS2	Fibroblast Growth Factor Receptor Substrate 2
GDNF	Glial cell-Derived Neurotrophic Factor
GHR	Growth Hormone Receptor
GPCR	G-Protein-Coupled Receptor
GSH	Glutathione
GST	Glutathione S-Transferase
HGF	Hepatocyte Growth Factor
ICD	Intracellular Domain
Ig-C2	Immunoglobulin-like C2 type domain
IGF-1R	Insulin-like Growth Factor 1 Receptor
IKK	IκB kinase
IP3	Inositol trisphosphate
KD	Kinase Death
MAPK	Mitogen Activated Protein Kinase
MVB	Multivesicular Body

NGF	Nerve Growth Factor
NMDAR	N-Methyl-D-Aspartic acid Receptor
NRAGE	Neurotrophin Receptor-interacting MAGE homolog
NRIF	Neutrophin Receptor Interacting Factor
NT3	Neurotrophin 3
NT4/5	Neurotrophin 4/5
p75NTR	p75 Neurotrophin Receptor
PC12	Pheochromocytoma Cell line
PC12nnr5	subclone of PC12 cell line non responsive to NGF
PDGFR	Platelet-Derived Growth Factor Receptor
PFA	Paraformaldehyde
PKC	Protein Kinase C
PLCγ	Phospholipase Cγ
PTB	Phosphotyrosine Binding Domain
PtdIns (4,5) P2	Phosphatidylinositol 4,5-bisphosphate
PVDF	Polyvinylidene Fluoride
Rac-1	Ras related C3 botolinum toxin substrate 1
RasGrf1	Ras Guanine-releasing factor 1
RhoA	Ras homologous member A
RISC	RNAi-Induced Silencing Complex
RSK	Ribosomal S6 Kinase
RT	Room Temperature
RTK	Receptor Tyrosine Kinase
SAP	SLAM-Associated Protein
SC1	Schwann Cell 1
SCG	Superior Cervical Ganglion
SH2	Src-Homology domain
SH2,3	Src Homology 2,3 domain
Shc	SH2 containing transforming protein C
shRNA	small hairpin RNA
siRNA	small interfering RNA
SOS	Son Of Sevenless
TK	Tyrosine Kinase
TNFR	Tumor Necrosis Factor Receptor
TRAF	TNF Receptor Associated Factor
Ub	Ubiquitin
UBD	Ubiquitin Binding Domain
UIM	Ubiquitin Interacting Motif
UPS	Ubiquitin–Proteasome System

INTRODUCTION

INTRODUCTION

1. Neurotrophins and their receptors

1.1. **Neurotrophic theory**

The complexity of processes governed by the nervous system is in a complete accordance with its high level of specialization and exceptional morphology, enabling the formation of synapses with the corresponding tissue, thus creating the neuronal network. Development, structure and maintenance of the vertebrate nervous system critically depend on strictly regulated survival and death signals. Trophic stimuli provided by neurotrophins, a family of growth factors, ensure survival of developing neurons, whereas apoptotic signals function to match the number of neurons to the target size and to refine target innervation. (Nykjaer, Willnow et al. 2005). Limiting quantities of neurotrophins secreted by the target organs and tissues during development control the number of surviving neurons and is a requirement for a suitable density of target innervation (Reichardt 2006). The presence of limited neurotrophin levels upon innervation results in considerable reduction of the number of neurons (Oppenheim 1991). This period of massive cell death, when more than 50% of neurons dye is named "critical phase" and is preceded by a phase of sprouting, when neurons do not express receptors for neurotrophins. Neurons dye mostly by apoptosis, which occurs while they already have high level of differentiation, when the axonal processes of the innervating neurons arrive at and invade their peripheral target tissues. The selective survival of only a fraction of the initial number of neurons is accomplished by the competition of the innervating processes for a limited amount of trophic factor elaborated by the target tissue. Neurons, such as those in the sympathetic and dorsal root ganglia, become neurotrophin dependent, once they reach their targets. This dependence is related to the concomitant expression of neurotrophin receptors (Davies, Bandtlow et al. 1987) and represents the basis for the neurotrophic theory (Oppenheim 1989). This mechanism can account in part for the adjusting of the number of neurons that connect with their targets in the periphery and the size of the target field, resulting

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in a stable neuronal population which size is essentially determined at birth. The competition of neurons for neurotrophins not only regulates neuronal survival, but also the axonal and dendrite growth and dendrite ramification, which is in accordance with the quantity of neurotrophic factor, captured by neurons.

Once the nervous system has reached its complete development, neurotrophins continue their action, since they are necessary for the proper function of several neuronal populations, like sympathetic neurons, for example. They are also required for the maintenance of the synaptic structure and the synaptic plasticity, which is one of the important neurochemical basics of higher-order processes such as memory and learning (McAllister, Katz et al. 1999). Neurotrophins are also important for neurotransmitter release (Yang, Slonimsky et al. 2002), axonal and dendritic growth and guidance or for controlling the ramifications of sensory neurons, assuring their recovering after injury.

1.2. Neurotrophin's family and their receptors

Although neurotrophins were initially identified as target-derived neuronal survival factors, now as mentioned above they are recognized to mediate a wide range of responses, including: proliferation, differentiation, myelination, axonal growth, synaptogenensis, long-term potentiation (Huang and Reichardt 2001; Chao 2003; Arevalo and Wu 2006) and in addition, some higher-order activities, such as learning, memory and behavior (Chao, Rajagopal et al. 2006). Cellular effects of neurotrophins depend on their levels of availability, affinity to their corresponding transmembrane receptors and the activation of the downstream signaling cascades stimulated after receptor activation. Alteration in neurotrophin levels has been implicated in neurodegenerative disorders, such as epilepsy, Alzheimer's, Huntington's and Parkinson's disease, as well as psychiatric disorders, including depression, aggression and substance abuse and cancer (Chao, Rajagopal et al. 2006).

The family of neurotrophins comprises four members in mammals: NGF (nerve growth factor), BDNF (brain-derived neurotrophic factor), NT-3 (neurotrophin-3) and NT-4/5 (neurotrophin-4/5). In the nervous system, all neurotrophins are secreted in limited amounts from the target tissue, where they exert an effect on the pre-synaptic neuron. After receptor binding and internalization, they are transported

retrogradely into endosomal vesicles towards the cell body in order to accomplish their specific actions.

Neurotrophins are initially synthesized as precursors or pro-neurotrophins that are proteolitically cleaved by furin or pro-convertases to release the mature biologically active proteins (Hempstead 2002; Chao 2003). Proneurotrophins, secreted from cells are biologically active and since their intracellular processing is not always efficient, substantial amounts of proneurotrophins are secreted in some tissues. Proneurotrophins bind with high affinity to the p75 neurotrophin receptor and activate signaling pathways controlled by this receptor, which in many cells results in promotion of apoptosis. Thus, the regulation of proneurotrophin maturation is an important post-transcriptional control point that limits and adds specificity to their actions (Lee, Kermani et al. 2001). Data suggest that secretion of proneurotrophins is increased following brain injury or degeneration, and binding of these proteins to p75NTR may increase neuronal loss in these injury and disease models (Fahnestock, Michalski et al. 2001; Harrington, Kim et al. 2002; Pedraza, Podlesniy et al. 2005).

Mature neurotrophins are glycosilated and form stable, non-covalent dimers. Nerve growth factor (NGF), the first member of the family to be discovered, was originally purified as a factor able to support survival of sympathetic and sensory neurons in culture (Levi-Montalcini 1966). It was initially purified from mouse submaxillary glands under acidic conditions as a 26,5 kDa protein, named 2.5S form. Subsequently, a higher molecular weight form, the NGF 7S was isolated at neutral pH. This NGF form is comprised of three subunits—α, β and γ, but only the β subunit is the biologically active, exerting effect on the proliferation and survival of neurons. The NGF 2.5S fraction contains homodimers of β subunits. NGF is present in the peripheral nervous system where it acts on sympathetic neurons and sensory neurons involved in nociception and temperature sensation. It is also present in the hippocampus, Schwann cells, fibroblast and mastocytes. (Levi-Montalcini, Skaper et al. 1996; Frossard, Freund et al. 2004). In the central nervous system, NGF promotes the survival and function of cholinergic neurons in the basal forebrain. These neurons, believed to be important for memory processes as they project to the hippocampus, are shown to be specifically affected in Alzheimer's disease (Reichardt 2006).

Brain-derived neurotrophic factor (BDNF) was the second purified member of the neurotrophin family (Barde, Edgar et al. 1982). BDNF is a homodimer, consisting of 12.3 kDa monomers. BDNF is required during development by midbrain

dopaminergic neurons, cerebellar granule cells, hippocampal neurons, and cortical neurons and supports the survival of trigeminal and vestibular ganglion neurons, as well as dorsal root and retinal ganglia (Hofer and Barde 1988; Snider 1994).

NT-3 is secreted by astrocytes to support the survival of oligodendrocyte precursors and also differentiated oligodendrocytes and neurons (Barres and Raff 1994). Neurotrophin-4/5 exists as a homodimer and its expression in skeletal muscle is controlled by neuronal stimulation. NT-4/5 supports the growth and functional maturation of neuromuscular functions in the post-natal developmental period (Funakoshi, Belluardo et al. 1995).

1.3. Structure of Trk receptors

Neurotrophins mediate their effects through binding to two different membrane receptors: the tropomyosin-related tyrosine kinase receptors (Trk) and the p75 neurotrophin receptor (p75NTR).

While the Trk receptors are responsible for the survival and growth properties of the neurotrophins, the actions of p75NTR could be divided in two categories. First, p75NTR is a Trk co-receptor that can enhance or suppress neurotrophin-mediated Trk receptor activity. Second, p75NTR independently, activates signaling cascades that result in the induction of apoptosis or in the promotion of survival.

The first member of the Trk family TrkA, was discovered as an oncogene, isolated from a colon carcinoma. The oncogene consisted of a non-muscle tropomyosin fused to the kinase domain of TrkA. Consequently, the corresponding proto-oncogene was found to be a transmembrane protein whose structure suggested that it was a receptor tyrosine kinase (Huang and Reichardt 2003). The other two members of the family, TrkB and TrkC were identified because of their high homology to TrkA. Trk receptors are type I membrane proteins, which share a conserved structure and possess a tyrosine kinase domain in its intracellular part.

Trk receptors contain an extracellular domain composed of three leucine-rich motifs flanked by two cysteine clusters and two immunoglobulin-like C2 type domains (Ig-C2), Neurotrophins interact with the membrane proximal immunoglobulin-like domain, while the first Ig-C2 domain prevents ligand independent activation of the receptor (Reichardt 2006). A single transmembrane domain spans the membrane to a cytoplasmic region that contains the tyrosine kinase domain, situated between a juxtamembrane region of 60 aa and a C-terminal domain of 15 aa (Figure 1).
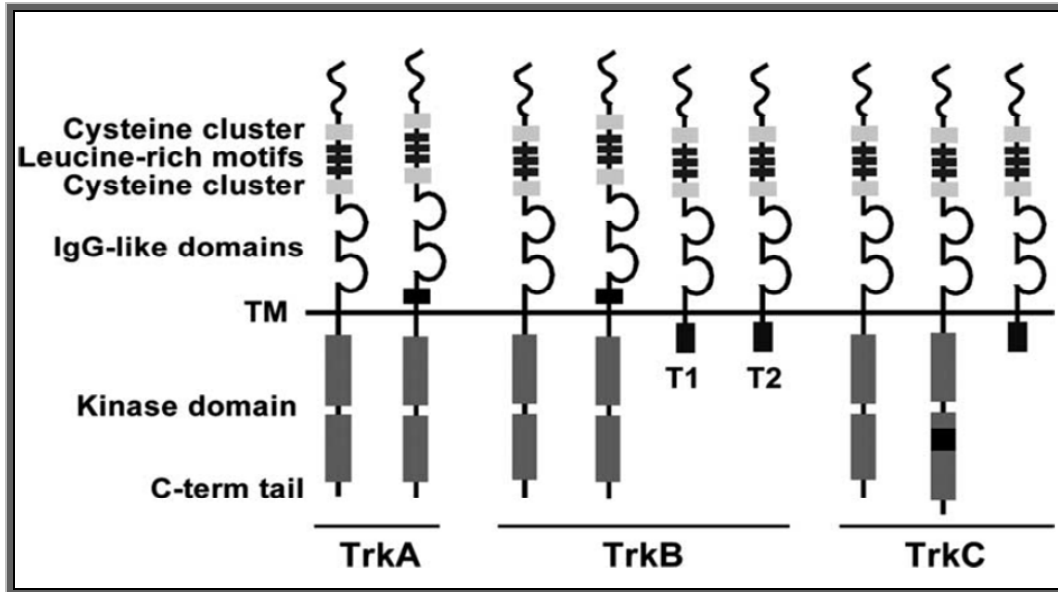
Figure 1. Schematic representation of the mammalian Trk tyrosine kinase isoforms. The three *trk* genes (*trkA*, *trkB*, and *trkC*) encode a full-length receptor, and multiple alternatively spliced isoforms. Splicing leads to TrkA and TrkB receptors that lack a short amino acid sequence in their extracellular domains, TrkB and TrkC receptors that lack the intracellular kinase domain, as well as TrkC receptor with an insert in the kinase domain. Taken from (Roux and Barker 2002)

Differential splicing of Trk receptor mRNAs leads to the generation of different transcriptional isoforms (Figure 1) (Clary and Reichardt 1994; Garner, Menegay et al. 1996; Strohmaier, Carter et al. 1996).

TrkA cognate ligand is NGF, but it could also bind NT-3 and NT-4/5. The neuronal form of the receptor includes exon 9, which codifies for six amino acids, located in the extracelular region. The presence of exon 9 favors NT-3 binding to the receptor (Clary and Reichardt 1994). TrkB is the preferred receptor for BDNF and NT-4/5. An increased affinity for NT-3 and NT-4/5, without affecting BDNF binding is observed when the optional exon in the juxtamembrane region is present. As it has been noticed, the insertion of the sequence into the extracellular juxtamembrane domain enhances the binding of the receptor to non-preferred ligands on both TrkA and TrkB receptors, thus TrkB is activated by NT-3 and NT-4/5 in addition to BDNF (Strohmaier, Carter et al. 1996). Trk C binds only NT-3. Isoforms containing insertions into the intracellular domain are inactive.

Splicing also generates isoforms of TrkB and TrkC that include comparatively short cytoplasmic motifs lacking the tyrosine kinase domain. Expression of these isoforms

has been shown to inhibit dimerization of kinase-containing Trk receptors, thereby inhibiting responses to neurotrophins (Eide, Vining et al. 1996). For many years, it was believed that these truncated receptors do not directly signal, but instead their function was to restrict the diffusion of neurotrophins. However, it has been demonstrated that BDNF-mediated activation of the truncated T1 isoform of TrkB controls the Ca^{2+}release from intracellular stores through a G protein and inositol trisphosphate (IP_3)-dependent pathway (Rose, Blum et al. 2003). In summary, neuronal responses to neurotrophins will depend on the type of receptor, on the splicing isoforms present and on the co-expression of p75NTR.
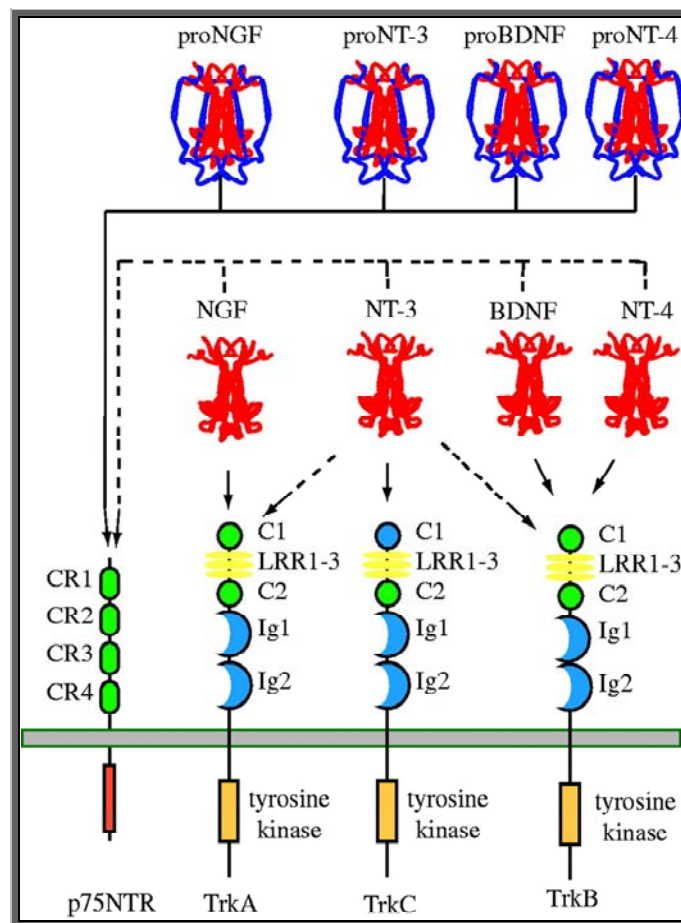
Figure 2. Neurotrophin–receptor interactions. The major interactions of each of the four mammalian neurotrophins are depicted. Each proneurotrophin binds p75NTR, but not the Trk receptors. Following maturation through proteolysis of the proneurotrophins, each mature neurotrophin is able to bind and activate p75NTR, but exhibits more specific interactions with the three Trk receptors. NGF binds specifically TrkA; BDNF and NT-4/5 recognize TrkB; NT-3 activates TrkC. In some cellular contexts, NT-3 is also able to activate TrkA and TrkB with less efficiency. Taken from (Reichardt 2006).

1.4. Structure of p75 NTR

p75NTR is named pan-neurotrophin receptor or common neutrophin receptor because of its ability to bind with similar affinity all of the known neurotrophins. p75NTR was the first identified member of the TNFR (tumor necrosis factor receptor) super family, although it is an unusual member of this family due to its ability to act as a tyrosine kinase co-receptor and because neurotrophins are structurally unrelated to the ligands which typically bind TNFR family members (Roux and Barker 2002). p75NTR is a Type I transmembrane protein with an extracellular domain that consists of four cysteine-repeat domains (CR). Both CR2 and CR3 have been implicated in neurotrophin-binding interactions. The cytoplasmic domain does not have any enzymatic activity, but contains different docking sites for interacting with other receptors or adaptor proteins. The p75NTR could regulate the responsiveness of Trk receptors to neurotrophins, since its coexpression with TrkA increases the rate of NGF association with the receptor by about 25 folds, resulting from the generation of high-affinity binding sites (Mahadeo, Kaplan et al. 1994; Esposito, Patel et al. 2001). The presence of p75NTR enhances the specificity of TrkA and TrkB for their primary ligands, NGF and BDNF, respectively (Clary and Reichardt 1994).

1.5. p75NTR isoforms and expression

Alternative splicing of *p75ntr* gene creates a variant that lacks exon 3, generating an isoform incapable of binding neurotrophins, but capable of triggering apoptosis (Dechant and Barde 1997). Truncated p75NTR isoforms are produced both by alternative splicing and by proteolysis. The soluble form-extracellular domain of p75NTR is produced at very high levels during development and following peripheral nerve injury. Full-length p75NTR is able to promote signaling cascades autonomously which result in the induction of apoptosis or in the promotion of survival, depending on the cellular context. p75NTR could also modulate axonal growth with different outcomes depending on the molecule that binds it. Pro-neurotrophins bind with high affinity to the p75NTR receptor and activate signaling pathways controlled by this receptor, which in many cells results in the promotion of apoptosis (Lee, Kermani et al. 2001).

p75NTR is widely expressed during development in the central nervous system (CNS): telencephalon, neocortex, hippocampus, etc. However, in the mature

CNS, p75NTR expression is limited to basal forebrain cholinergic neurons, motor neurons, and the cerebellar Purkinje neurons. In the developing peripheric nervous system (PNS), p75NTR is expressed in the sympathetic and dorsal root ganglia as well as in enteric and parasympathetic neurons. p75NTR is widely expressed outside the nervous system as well: kidneys, lungs, testes, inner ear, hair follicles, etc. (Rabizadeh and Bredesen 2003).

1.6. Specific functions of neurotrophins

The discovery of NGF occurred due to its capacity to induce dendritic outgrowth of sensory and sympathetic ganglion explants (Levi-Montalcini and Hamburger 1951). Its importance for the proper function of some neuronal populations was validated later, during the search for factors, that could help the survival of motor and sensory neurons, thus compensating the lethal effects produced by removal of their target tissues (Levi-Montalcini 1987). The first *in vivo* and *in vitro* experiments, using antibodies that specifically block NGF, showed the loss of specific neuronal populations. In addition, NGF treatment resulted in an increase of the number of neurons due to the inhibition of the programmed cell death (Burek and Oppenheim 1996).

A very useful approach to critically address the role of the neurotrophins was the generation of *knockout* (KO) mice of the neurotrophins and their receptors. Mice that completely lack neurotrophins die during the first few weeks following birth. Heterozygous mice in which neurotrophin levels are reduced by half are viable but they show some deficits. Decreasing the level of NGF leads to several deficits in memory acquisition and retention and when this happens with levels of BDNF, mice show enhanced aggressiveness, hyperactivity and hyperphagia (Chao 2003). The loss of proprioceptors in NT-3 mutants correlates with postural and movement abnormalities. Pain-sensitive (nociceptive) neurons, which express TrkA, are selectively lost in the dorsal root ganglion of knockout mice lacking NGF or TrkA, so that heterozygotes show a decreased sensitivity to pain consistent with a reduction in the number of nociceptive neurons. Moreover, sympathetic neurons are almost completely lost in these mice. In view of the fact that exist compensatory or redundant mechanisms, some neuronal populations survive, because of their dependence on more than one neurotrophin. Because of that KO mice only have some partial loss due to the activation of neurotrophin receptors by different neurotrophins. These compensatory mechanisms are also present in the central

nervous system (CNS), since no strong deleterious effects are observed in KO mice (Huang and Reichardt 2001). Two different KO mice have been obtained for p75NTR: Exon 3 KO mice, that express the short splicing isoform and exon 4 KO mice, which do not have any functional p75NTR fragment. These KO mice show increased number of neurons on birth, which correlates with the loss of function of p75NTR, leading to the inability to regulate neuronal cell death. Shortly, the number of neurons decrease comparing to the *wt* mice due to the lower capacity of Trk receptors to bind neurotrophins with high affinity (Roux and Barker 2002). Additionally to functions strictly related to the nervous system, neurotrophins, and especially NGF, exert other functions such as:

- Promote survival of some neuronal populations not only upon development, but also in the adulthood.
- Define the morphology of synaptic connections, as well as neurotransmitter mixture content released by neurons, this way helping the adjustment of neurons to the environment and contributing to the synaptic plasticity. NGF could also regulate the expression of neurotransmitters in a specific moment (Yang, Slonimsky et al. 2002).
- Exert some effects on the mediators of inflammation, promoting the degranulation of mastocytes and provoking leukocyte chemotaxis (Levi-Montalcini, Skaper et al. 1996).

The discovery of the molecular basis of the congenital insensitivity to pain with anhydrosis (CIPA) disease, which consists in a loss of function mutation of the gene encoding TrkA, provides a rare opportunity to explore the developmental and physiological function of the NGF-dependent neurons. CIPA is an autosomal recessive hereditary disorder characterized by repeated episodic fever, anhidrosis (inability to sweat), and absence of reaction to noxious stimuli, self-mutilating behavior, and mental retardation. Defects in NGF-TrkA signal transduction leads to failure of survival of sympathetic ganglion neurons, nociceptive sensory neurons of the dorsal root ganglion (DRG) and neurons that innervate sweat glands, therefore those neurons dye by apoptosis during development (Indo 2001). KO mice for TrkA die within the first month of life, so behavioral studies are hampered, but human patients with CIPA often survive to adulthood. Keeping this fact in mind, human patients could be very useful for behavioral studies of TrkA deficient individuals, which could help to reveal the diversity of functions of sympathetic and afferent neurons in the human physiology. A recent study suggests that NGF-TrkA system

plays an important role in establishing at least partially the neural networks involved in homeostasis, arousal and emotion. Since CIPA patients lack NGF dependent neurons responsible for interoception (sensitivity to stimuli originating inside of the body) and also lack autonomic sympathetic regulation, they are unable to express emotional responses to various interoceptive stimuli (Indo 2009). Potential links between NGF-dependent neurons and emotion may also provide further insights into the neural basis of human emotions and feelings.

2. Neurotrophin signaling mediated by Trk receptors

Neurotrophin binding to Trk receptors leads to receptor dimerization, auto or transphosphorylation and consequently, recruitment of different adaptor proteins and activation of several signaling pathways (Huang and Reichardt 2003). The unique combination of receptor docking sites, adaptor proteins and trafficking contributes to the specificity of neurotrophin action. Trk receptors are activated specifically by mature neurotrophins and not by proneurotrophins (Lee, Kermani et al. 2001). Trk receptors contain ten evolutionary conserved Tyr in their cytoplasmic domain, three of which, Y670, Y674 and Y675 on TrkA, are situated in the activation loop of the tyrosine kinase domain. Phosphorylation of these three tyrosines leads to an enhanced tyrosine kinase activity. Phosphorylation on other positions creates docking sites for signaling proteins containing phosphotyrosine binding (PTB) or Src-homology (SH2) domains. Two main phosphotyrosine docking sites have been described on Trk receptors, Y490 and Y785 in the human TrkA and their corresponding Tyr in TrkB and TrkC.
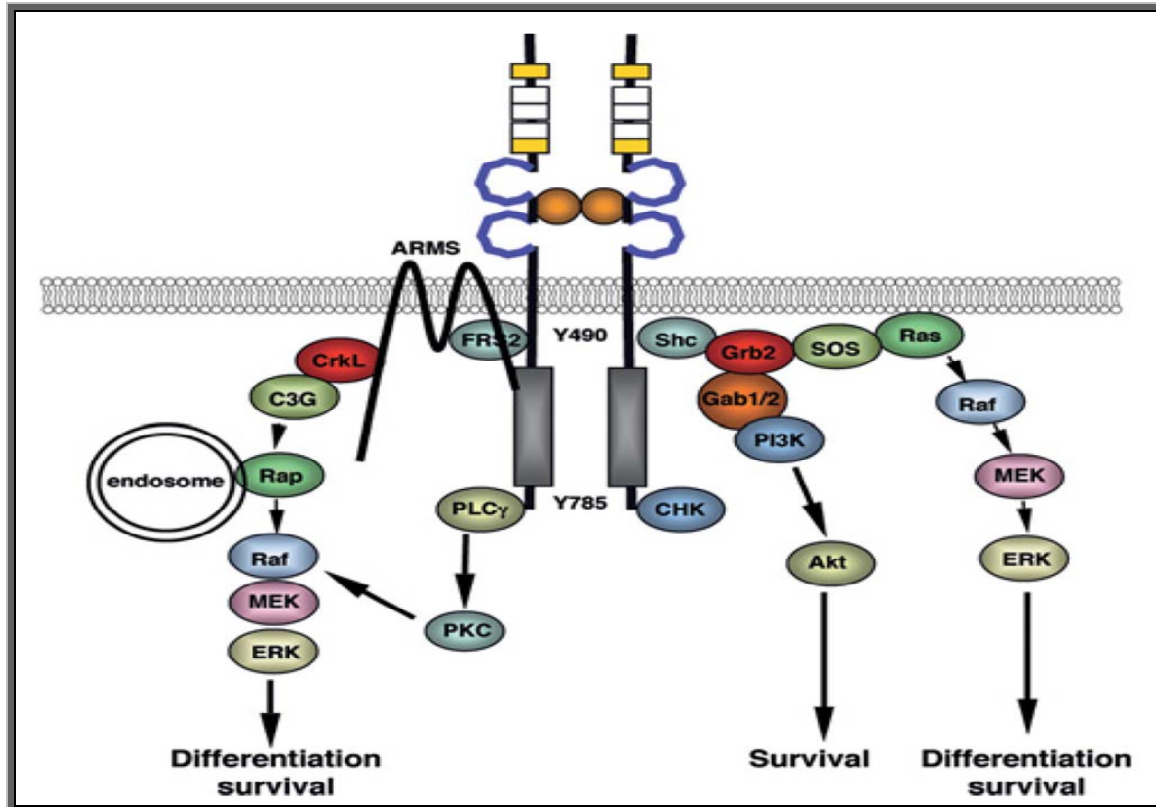
Figure 3. Trk receptor-mediated signaling pathways. Neurotrophin binding to Trk receptors leads to their dimerization and activation and to the recruitment of different proteins that associate with specific phosphotyrosine residues in the cytoplasmic domain of Trk receptors. These interactions trigger the activation of various signaling pathways, such as the Ras/Rap-MAPK, PI3K-Akt, and PLCγ pathways, which result in survival, neurite outgrowth, gene expression, and synaptic plasticity. Tyrosine residue nomenclature is based on the human sequence of TrkA non-neuronal form. Taken from (Arevalo and Wu 2006).

The major pathways activated by the Trk receptors are Shc-Ras-MAPK, PI3K-Akt and PLCγ- protein kinase C (PKC) and their downstream effectors (Kaplan and Miller 2000; Huang and Reichardt 2003; Arevalo and Wu 2006; Reichardt 2006). Phosphorylation on Y490 provides a recruitment site for both Shc and Frs2, which provide links to Ras, PI 3-kinase and Rap1-B-Raf pathways (Figure 3).

2.1. **MAPK signaling pathway**

Phosphorylated Y490 recruits the adaptor protein Shc, which consequently binds to the complex formed by Grb-2 and the Ras exchange factor-son of sevenless (SOS) (Figure 3). Grb-2 could also bind directly to the activation loop tyrosines Y674 and Y675 and Y785 of the C-terminal tail (MacDonald, Gryz et al. 2000), thus initiating a cascade of signaling events (Reichardt 2006). The small GTPase Ras is mainly involved in signaling for neuronal differentiation, but in many neurons it also promotes neuronal survival, through PI 3-kinase or through MAPK/ERK pathways. In PC12 cells, different adaptors appear to mediate transient versus prolonged activation of ERK signaling. To promote a transient activation, the recruitment of Shc and further engagement of the adaptor protein Grb2 that binds to the Ras exchange factor SOS is required. In this case, active Ras stimulates signaling through Raf, which phosphorylates MEK1/2 that in turn activate ERK1/2 kinases. Among the targets of ERK are the ribosomal S6 kinases (RSKs), MAPK-activating protein kinase 2 phosphorylate CRE-binding protein (CREB) and other transcription factors (Xing, Kornhauser et al. 1998). The prolonged activation of MAPK/ERK signaling in PC12 cells depends on a distinct signaling pathway initiated through binding to phosphorylated Y490 of the adaptor protein fibroblast growth factor receptor substrate FRS-2 (Meakin, MacDonald et al. 1999). Several phosphorylated tyrosines on FRS-2 function as binding sites for some additional proteins, including the adaptor proteins Grb-2 and Crk, the Src homology protein tyrosine phosphatase-2 (SH-PTP) and Src. In a cascade initiated by binding to FRS-2, Crk binds and activates the exchange factor C3G, which in turn activates the small G protein Rap-1. Rap-1 activates B-Raf and subsequently triggers the activation of ERK/MAPK signaling pathway, thus providing a sustained activation of MAPK signaling pathway (York, Yao et al. 1998; York, Molliver et al. 2000; Watson, Heerssen et al. 2001).

While transient activation involves Shc/Grb-2/SOS/Ras/Raf1/MAPK, instead the prolonged activation requires another set of adaptor proteins, such as CrkII/Crk, guanine nucleotide exchange factor C3G, the small GTPase Rap-1, the protein tyrosine phosphatase Shp2 and the serine threonine kinase B-Raf (Marshall 1995; Wu, Lai et al. 2001). Rap-1 activated by C3G, signals through B-Raf, which results in a sustained activation of MAPK. Receptor internalization is required for the activation of this pathway (York, Molliver et al. 2000; Wu, Lai et al. 2001), therefore a signaling platform is established linked to TrkA on endosome vesicles. FRS-2 binds TrkA

juxtamembrane domain on the KFG motif (441-443), acting as an adaptor protein anchored to the membrane (Kouhara, Hadari et al. 1997). *In vitro,* FRS-2 competes with Shc for binding to Y490 and interacts with the SH2 domain of CrkII. MAPK phosphorylation results in the activation of two downstream kinases Rsk and MSK1, which in turn control the expression of genes crucial for neuronal differentiation and survival (Ginty, Bonni et al. 1994). Furthermore, it has been shown, that BDNF activates ERK5 through Rap1 in cortical neurons (Wang, Su et al. 2006), whereas in PC12 cells NGF activates ERK5 via the small G-protein Ras (Kamakura, Moriguchi et al. 1999). MEKK2/3-MEK5-ERK5 signaling pathway, plays an important role in the regulation of cell proliferation and differentiation, together with the MAPK1/2-ERK1/2 cascade (Obara and Nakahata). Besides, its regulation by the neurite outgrowth multiadaptor, NOMA–GAP has been recently shown to be required for the NGF-stimulated neurite outgrowth and extension in PC12 cells and for the sustained ERK5 activation (Rosario, Franke et al. 2007). Moreover, the activation of ERK5 during retrograde signaling of endocytosed Trk receptors leads to nuclear translocation of ERK5 and phosphorylation of CREB which results in enhanced neuronal survival (Watson, Heerssen et al. 2001).

2.2. PI3K-Akt signaling pathway

Signaling through the PI3K-Akt pathway is essential for the survival of several neuronal populations during development. PI3K binds directly to the phosphorylated Y751 of TrkA (Obermeier, Halfter et al. 1993) and it is activated through an adaptor protein complex (Holgado-Madruga, Moscatello et al. 1997). Shc participates in this signaling pathway, but this time associates with Grb2 and Gab1 to activate PI3K (Figure 3). Disruption of PI3K/Gab1 association decreases the NGF-dependent survival of PC12 cells, which confirms that Gab1 is required for PI3K activation (Holgado-Madruga, Moscatello et al. 1997). Lipid products generated by PI3K recruit many proteins containing pleckstrin-homology domains to the plasma membrane, including the Akt kinase and 3-phosphoinoside-dependent kinases (PDKs). Akt is activated by phosphorylation on Thr308 by PDK1 and on S473 by the mammalian target of rapamycin *(*mTOR*),* after which it phosphorylates several proteins important for controlling the cell survival (Datta, Brunet et al. 1999; Yuan and Yankner 2000). These include Bad (Bcl-2/Bcl-x-associated death promoter), IκB, the forkhead transcription factor FKHRL1, glycogen synthase kinase 3-β (GSK3B), and human, but not mouse caspase-9 (Brunet, Bonni et al. 1999). Bad is a Bcl-2 family

member, which promotes apoptosis when unphosphorylated by binding to Bcl-xL. Phosphorylation of Bad by Akt, or by MAPKs results in its association with 14-3-3 protein, thus preventing binding to Bcl-xL. Akt phosphorylates FKHRL1 and prevents its transcriptional activity, which is involved in the regulation of the expression of several pro-apoptotic proteins. The phosphorylation by Akt of the inhibitor of the NF-κB pro-survival transcription factor, IκB, targets it for degradation (Wooten, Seibenhener et al. 2001). Elevated GSK3B activity has been shown to promote apoptosis in cultured neurons (Hetman, Cavanaugh et al. 2000). Neurotrophin withdrawal increases GSK3 activity, whereas phosphorylation by Akt decreases it. Apart from its pro-survival function, active Akt causes axonal growth and increases axon caliber and branching, when it is present at the growth cone of sensory neurons. (Atwal, Massie et al. 2000; Zhou, Zhou et al. 2004).

2.3. PLC γ signaling pathway

Upon TrkA activation, phosphorylation of Y785 leads to PLCγ recruitment and activation, which results in the hydrolysis of PtdIns (4,5) P_2 and generation of inositol trisphosphate (IP3) and diacylglycerol (DAG) (Obermeier, Halfter et al. 1993). The former provokes a transient increase in intracellular free Ca^{2+}, while the latter serves as a direct activator of protein kinase C. The liberation of Ca^{2+} from internal stores additionally stimulates Ca^{2+}/calmodulin dependent protein kinases (CaM kinases). PKC subsequently activate ERK signaling pathway via Raf-1 (Corbit, Foster et al. 1999).

2.4. TrkA interacting proteins

Besides the adaptor proteins described above, which are involved in the regulation of the signaling cascades activated by TrkA receptors, there exist a growing number of Trk interacting proteins. Their interaction with TrkA receptors could affect receptor activity, degradation and internalization rates, as well as receptor trafficking and function.

ARMS/Kidins220 (Ankyrin repeat-Rich Membrane Spanning protein) ARMS associates to TrkA and for this interaction transmembrane domains of both proteins are required. Ligand interaction with TrkA receptor results in tyrosine phosphorylation of ARMS. ARMS remains active for a long time, thus creating

docking sites for Crk, which consequently results in Rap1-dependent sustained ERK activation (Arevalo, Yano et al. 2004).

Grit binds to the C-terminal domain of the TrkA receptor. Grit regulates neurite outgrowth on PC12 cells by modulating the Rho family of small GTPases (Nakamura, Komiya et al. 2002).

Kalirin is a multi domain guanine nucleotide exchange factor (GEF), which induce cytoskeletal rearrangement in neurons by activating Rho proteins. The interaction with TrkA occurs through its N-terminal pleckstrin homology domain. Endogenous Kalirin is important for neurite outgrowth in PC12 cells (Chakrabarti, Lin et al. 2005).

Csk homologous kinase (CHK) binds directly to the phosphorylated Y785 of TrkA, through its SH2 domain, following NGF stimulation in PC12 cells. CHK participates in TrkA signaling by regulating NGF-induced neurite outgrowth on PC12 cells (Yamashita, Avraham et al. 1999).

RasGrf1 (Ras guanine-releasing factor 1) interacts with the intracellular domain of TrkA receptor through its N-terminal fragment and becomes phosphorylated. This interaction and phosphorylation is dependent on the HIKE domain of TrkA (a region that interact with pleckstrin homology domains), thus regulating neurite outgrowth in PC12 cells (Robinson, Manto et al. 2005)

The **c-Abl** non-receptor tyrosine kinase is involved in many cellular functions, one of which is the control of the axonal cytoskeleton. c-Abl binds to phosphotyrosine residue(s) in the kinase activation loop of TrkA (Koch, Mancini et al. 2000). This interaction does not require TrkA receptors to be autophosphorylated. c-Abl may be recruited to the NGF-TrkA receptor complex and is thought to be important for the neuritogenesis (Yano, Cong et al. 2000).

Calmodulin (CaM) binds directly to the C-terminal domain of TrkA in a calcium dependent manner. This interaction is important for the regulation of TrkA trafficking and cleavage. CaM seems to play a protective role in TrkA cleavage under basal conditions, what may be important for the maintenance of a pool of receptors at the plasma membrane ready for NGF binding and the induction of cellular response (Llovera, de Pablo et al. 2004).

Caveolin can interact with both p75NTR and TrkA and this interaction may regulate neurotrophin responses. This interaction promotes the transientness of NGF-induced TrkA phosphorylation. Furthermore, caveolin can directly block NGF-induced TrkA autophosphorylation *in vitro*, which negatively regulates NGF-induced

differentiation. Direct and inhibitory interaction of caveolin with TrkA modulate the duration of ligand-induced receptor autophosphorylation, thus regulating the responses to neurotrophins (Bilderback, Gazula et al. 1999).

SHP-1 is a phosphotyrosine phosphatase, which can function as a TrkA phosphatase in PC12 cells and in sympathetic neurons, controlling both the basal and NGF-regulated level of TrkA activity in neurons. SHP-1 directly regulates TrkA activity, thus controlling the number of neurons during the developmental cell death period (Marsh, Dubreuil et al. 2003).

SLAM-associated protein (SAP) interacts with all members of Trk receptor family *in vitro* and *in vivo*. Binding of SAP requires Trk receptor activation and phosphorylation of Y674 in the activation loop of the kinase domain. Overexpression of SAP in PC12 cells suppresses the NGF-dependent activation of ERK1/2 and PLC-γ, leading to the inhibition of neurite outgrowth. SAP may serve as a negative regulator of Trk receptor activation and downstream signaling (Lo, Chin et al. 2005).

Pincher mediates the internalization of activated TrkA and TrkB receptors in both sympathetic and hippocampal neurons. It also participates in the formation of newly identified clathrin-independent macroendosomes for Trk receptors in soma, axons, and dendrites. A unique characteristic of Pincher-Trk endosomes is their insusceptibility to lysosomal degradation, which ensures persistent signaling through Erk5. Block of Pincher function *in vitro*, prevents Trk macroendosome formation, thus invalidating neuronal survival by retrograde transport (Valdez, Akmentin et al. 2005).

Nedd4-2 is an E3 ubiquitin ligase that associates directly with TrkA through the PPXY domain located in the COOH-terminal domain of the receptor. Nedd4-2 is activated upon NGF binding and leads to ubiquitination and downregulation of TrkA, but not TrkB receptors. Increased expression of Nedd4-2 in dorsal root ganglion (DRG) neurons showed decreased levels of activated TrkA receptors without affecting TrkB receptors, which results in apoptosis of the NGF-dependent neurons. On the other hand, downregulation of Nedd4-2 by siRNA experiments in sensory neurons results in more active TrkA, but not TrkB, receptors. Therefore, Nedd4-2 regulates TrkA by ubiquitination and consequent downregulation, thus modulating the survival of NGF-dependent neurons (Arevalo, Waite et al. 2006).

p62 interacts with TrkA and the formation of TRAF6-p62 complex functions as a bridge connecting p75NTR with TrkA, thus serving as a common platform for communication of both p75NTR and TrkA receptor signaling. Moreover, p62 serves as

a scaffold for the activation of the NF-kB pathway, which mediates NGF survival and differentiation responses (Wooten, Seibenhener et al. 2001).

3. p75NTR-mediated signaling pathways

p75NTR is a unique receptor, whose extracellular domain consists of four cystein rich repeat domains (CR), three of which-CR2, CR3 and CR4 are implicated in neurotrophin binding. p75NTR has a single transmembrane domain.and in the cytoplasmic region it contains a death domain (DD), similar to those identified in TNF receptors (Figure 4). p75NTR could signal either independently or by modifying the binding and signaling capabilities of its co-receptors, Trk receptor tyrosine kinases. Firstly, crystal structure studies reported the binding of a NGF homodimer to one molecule of unglycosylated p75NTR (He and Garcia 2004). However, recently the crystal structure of NT-3 bound to the glycosylated ectodomain of p75NTR has been solved and NT-3 forms a central homodimer around which two glycosylated p75NTR molecules bind symmetrically (Gong et al 2008). Therefore, the 2:2 architecture of the complex establishes a NT-induced dimerization model for p75NTR activation.

Because of the lack of catalytic activity in the cytoplasmic domain of p75NTR, the interacting proteins carry out its signaling. These proteins are either constitutively associated to p75NTR or the receptor recruits them in response to neurotrophins. This way p75NTR receptor participates in several signaling platforms with different co-receptors, thus contributing to different cell effects.

p75NTR associates with ARMS which serves as a bridge binding it to Trk receptors. ARMS functions to connect both receptors physically and functionally and is important for the sustained MAPK activation (Arevalo, Yano et al. 2004). Additionally, neurotrophin binding promotes the association of TRAF6 to the cytoplasmic domain of p75NTR, leading to its phosphorylation and consequent activation of NFκB signaling pathway, thereby promoting NFκB dependent neuronal survival (Figure 4). As previously described, p62 binds TrkA and the complex of TRAF6/p62 connects it to p75NTR. p62 can modulate IκB kinase (IKK) activity, thus affecting NFκB dependent survival and differentiation (Wooten, Seibenhener et al. 2001).

Another molecule, the protein kinase RIP2, binds to the DD of p75NTR in a ligand dependent manner. The recruitment of RIP2 to p75NTR mediates anti-apoptotic signals (Khursigara, Bertin et al. 2001) (Figure 4).

RhoA is a member of the family small GTPases, which is involved in several aspects of neuronal morphogenesis. It has been shown that RhoA interacts with the intracellular domain (ICD) of p75NTR and this interaction could play an important role for p75NTR-dependent neurite outgrowth (Luo 2000).

NGF and proNGF bind with similar affinity to p75NTR. The authentic proNGF receptor is Sortilin, a 95 KDa member of the Vsp10-domain receptors family. The affinity of Sortilin to proNGF increases 20-fold in cells that coexpress p75NTR. proNGF simultaneously associates with Sortilin and p75NTR thereby acting as a crosslinker, creating a ternary complex (Qian, Riccio et al. 1998)(Figure 4). Sortilin acts as an essential coreceptor, which enables cells coexpressing TrkA and p75NTR to respond to proNGF by apoptosis induction rather than survival. In the absence of Sortilin, proNGF might be cleaved by extracellular proteases and the obtained NGF could stimulate TrkA instead.
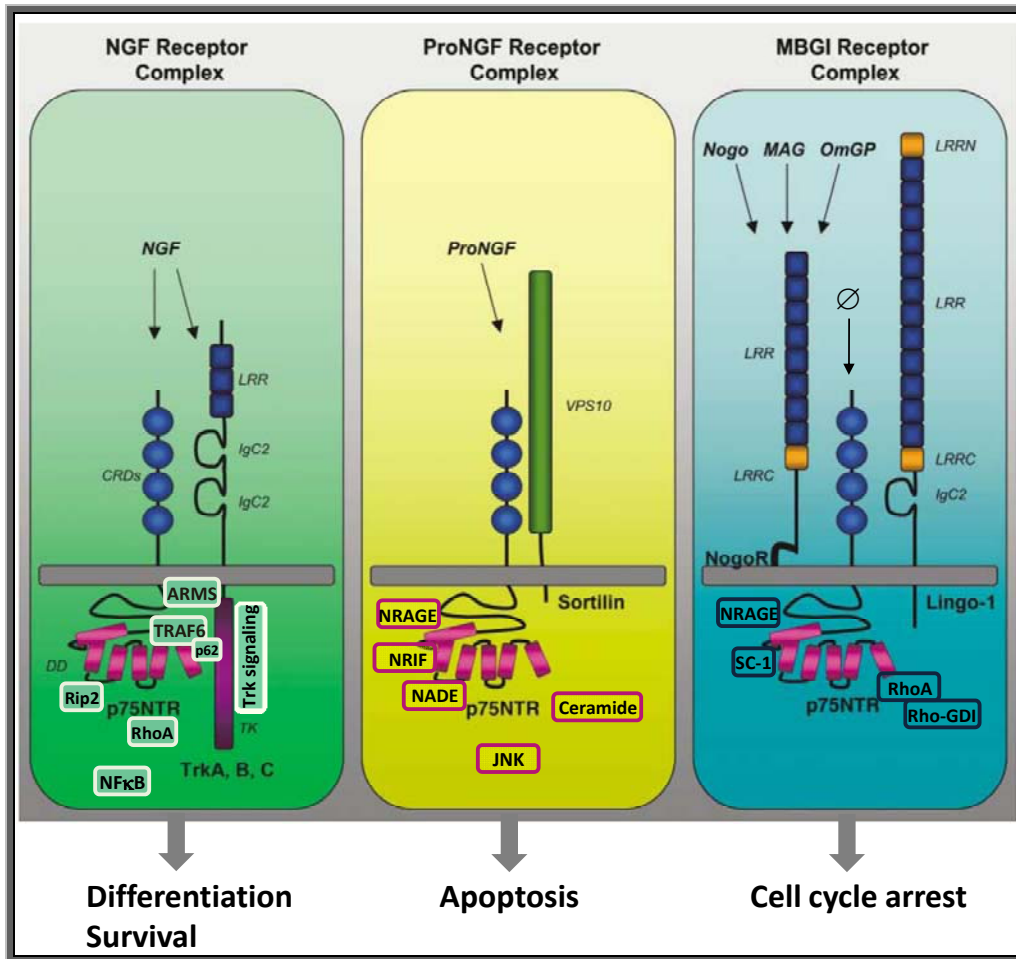
Figure 4. General mechanisms of p75NTR signaling. p75NTR interacts with the TrkA receptor and enhances the ability of TrkA to respond to NGF (in green). p75NTR and

Sortilin complex mediates proapoptotic signals in response to proNGF binding (in yellow). In absence of ligand p75NTR may form a tripartite complex with the NogoR and with Lingo-1 that results in growth inhibitory signals to be transduced in response to Nogo, MAG (myelin-associated glycoproteins), or OMgp (oligodendrocyte myelin glycoprotein)(in blue). Some of the proteins that participate in the p75 signaling are shown. Modified from (Barker 2004).

The apoptotic effect of p75NTR depends on its interaction with several downstream molecules. **NRIF** induced cell death through p75NTR requires p53 and NRIF nuclear translocation. Neurotrophin receptor-interacting MAGE homolog (**NRAGE**) also interacts with p75NTR through a mechanism that involves cell cycle arrest, JNK activation, cytochrome C accumulation, and activation of caspases 3, 7 and 9 (Salehi, Roux et al. 2000).

The p75NTR-associated cell death executor **(NADE protein)** may induce cell death following ligand binding. p75NTR/NADE-dependent apoptosis may be modulated by an interaction between NADE and 14-3-3 protein (Kimura, Irie et al. 2001). Ligand engagement of p75NTR activates acidic sphingomyelinase, which leads to the accumulation of **ceramide** (Dobrowsky, Werner et al. 1994). NGF mediated ceramide production correlates with JNK activation and apoptosis (Song and Posse de Chaves 2003) and it has also been shown to correlate with cell differentiation and survival (DeFreitas, McQuillen et al. 2001).

Another p75NTR coreceptor is the glycolipid-anchored Nogo receptor **(Nogo-R)**. Nogo-R binds myelin-based growth inhibitors, including Nogo-A, myelin-associated glycoprotein and oligodendrocyte myelin glycoprotein, and restricts axonal regeneration by promoting growth cone collapse of injured neurons. For this effect the coexpression with p75NTR and the transmembrane protein LINGO-1 are required (Kao, Jaiswal et al. 2001).

p75NTR has been shown to activate RhoA through a direct interaction, thereby inhibiting neurite outgrowth (Yamashita, Avraham et al. 1999). Neurotrophin binding to p75NTR eliminates p75NTR-dependent activation of RhoA, stimulating neurite outgrowth. p75NTR could function as a displacement factor, interacting directly with RhoGDI, thus facilitating the release of **RhoA** from **RhoGDI** (Yamashita and Tohyama 2003). The activators of the Nogo receptor-signaling complex increase this association, thereby increasing the activation of RhoA. The zinc finger protein Schwann cell factor **(SC1)** is normaly present in the cytoplasm, but NGF binding to p75NTR and not to TrkA, results in its nuclear translocation. Together with NRAGE, SC1 induces cell-cycle arrest when overexpressed (Roux and Barker 2002).

The p75NTR receptor undergoes an α-secretase-mediated release of the extracellular domain followed by a γ-secretase-mediated intra-membrane cleavage, which is accompanied by translocation of the p75NTR-ICD to the nucleus. Coexpression of the soluble ICD with TRAF6 leads to the activation of NFκB signaling pathway. Cleavage of p75 may represent a general mechanism for transmitting signals as an independent receptor and as a co-receptor for other signaling systems.

The outcome of p75NTR activation depends on the ligand type and the ability of the coreceptors to crosslink and coordinate, thereby facilitating the activation of specific signaling pathways. p75NTR is abundantly expressed during development, but is downregulated in the adult organism, where it is expressed in conditions of increased neuronal cell death including mechanical damage, ischemia, stroke and Alzheimer's disease (AD). These findings suggest a physiological role for p75NTR during neuronal degeneration (York, Yao et al. 1998).

3.1. p75NTR and Trk interactions

p75NTR and Trk receptors bind directly through their extracellular domains (Chang, Arevalo et al. 2004). It has also been demonstrated that both the transmembrane and the cytoplasmic domain are necessary for this interaction to occur (Esposito, Patel et al. 2001).
Trk receptor function is regulated in different ways by the presence of the p75NTR:

1. p75NTR inhibits activation of Trk receptors by non-preferred neurotrophins both *in vitro* and *in vivo* (Bibel, Hoppe et al. 1999).

2. *In vitro* studies indicate that the presence of p75TR increases the activation of TrkA by suboptimal concentrations of NGF (Mahadeo, Kaplan et al. 1994) and that the p75NTR collaborates with TrkA to form high-affinity binding sites for NGF (Esposito, Patel et al. 2001).

3. p75NTR is capable to promote the retrograde transport of several neurotrophins (Curtis, Adryan et al. 1995).

4. The presence of p75NTR prevents the ligand-independent constitutive Trk activation.

5. Degradation effects on Trk: p75NTR suppresses ubiquitination of TrkA and TrkB receptors, thereby delaying Trk receptor internalization and degradation (Makkerh, Ceni et al. 2005). Another study indicates that the presence of p75NTR promotes endocytosis of the Trk receptors through the recruitment of ubiquitin ligases and subsequent trafficking of the Trk receptors to endosomal

compartments, which results in enhanced cell differentiation (Geetha, Jiang et al. 2005).

6. Ligand binding of p75NTR can activate ceramide-involving signaling cascade that results in reduced TrkA activity through serine phosphorylation of its intracellular domain (MacPhee and Barker 1997).

7. The activation of Trk receptors has strong effects on p75NTR signaling. On the one hand, Trk signaling suppresses the p75NTR-mediated Jun kinase cascade activation and sphingomyelin hydrolysis, therefore preventing apoptosis. On the other hand, TrkA receptor activation does not affect the induction of the NF-κB cascade, known to promote survival.

In conclusion, p75NTR modulates Trk receptor function at several levels: by promoting ligand binding, by increasing accessibility to neurotrophins and by helping endocytosis and retrograde transport of neurotrophins. It also refines the ligand specificity of Trk receptors, regulates axon growth and helps the regulation of survival and apoptosis of different neuronal populations.

4.   Kinetics of TrkA neurotrophin receptor

TrkA is a transmembrane protein type I of 80kDa, while inserted into the membrane of endoplasmatic reticulum. Generally, the maturation takes place in the Golgi complex, where after glycosylation two forms are generated. One is the N-glycosylated 110 kDa precursor form, which after sialylation leads to the mature 140-kDa form, the one present on the plasma membrane. In PC12 cells, cell surface gp140 TrkA half-life is approximately 138 minutes and is reduced to approximately 86 min by NGF treatment (Jullien, Guili et al. 2002). The half-life of immature 110-kDa form decreases from 24 to 20 min after NGF stimulation.

4.1. Internalization

Internalization of cell surface receptors occurs by invagination of certain zones of the plasma membrane. This process usually follows ligand-induced activation of receptors, when they are taken into endocytic vesicles and are targeted for lysosomal or proteasomal degradation. Internalization process also occurs under basal conditions when it usually participates in the renewal of proteins on the membrane.

The mechanism of receptor internalization is not unique, three different mechanisms have been described for Trk receptors (Figure 5) (Zweifel, Kuruvilla et al. 2005).

1. **Clathrin-mediated endocytosis: this is** the best studied endocytic pathway for internalization of membrane receptors. Forming of specific clathrin-enriched zones on membrane surface, called clathrin-coated pits, forces the spherical membrane structure and consequently creates invaginations. Upon ligand binding, receptors undergo dimmerization, recruitment to clathrin-coated pits, invagination and scission from plasma membrane to the cytosol. Uncoating of vesicles to form endosomes is accompanied by clathrin liberation, which is returned for recycling. Dynamin, a small GTPase, which is involved in the cutting of clathrin-coated vesicles from the plasma membrane, is also important for this type internalization.

2. *Caveolae-mediated endocytosis*: Activated NGF-TrkA complexes were found in specific sphingomyelin and cholesterol enriched compartments of the plasma membrane called "lipid rafts". When caveolin is present in these compartments, invaginations called caveolae are formed. Calveolae are implicated in vesicular transport and signaling. Dynamin also participates in caveolin-dependent internalization.

3. **Pinocytosis and membrane "ruffling":** This mechanism involves the participation of Pincher, a pinocytic chaperone as a membrane trafficking protein that mediates endocytosis and trafficking of NGF-TrkA complexes. Pincher-enhanced internalization is initiated at the ruffling plasma membrane, where pinocytic structures are formed. The sorting and trafficking of TrkA-containing vesicles occurs latter in tubular structures associated with Pincher. Pinocytic endocytosis is an important mechanism for trafficking of NGF-TrkA containing vesicles in PC12 cells (Shao, Akmentin et al. 2002). Pincher-associated endosomes are associated with Erk5 and are not easily degraded by lysosomes.

The diversity of pathways by which receptors can be internalized indicates that the mechanism of ligand–receptor internalization might depend on the type of receptor, the cellular context in which the ligand is present, and/or the magnitude of the signal generated by ligand stimulation. It seems that the activation of Trk is required for internalization to occur, since ligand-binding to the receptor increases its internalization rate. Interestingly, the inhibition of tyrosine kinase activity of Trk is not capable of preventing internalization in PC12 cells, although it could affect the

direction of internalized receptor, since more recycling than degradation is observed (Saxena, Howe et al. 2005). Therefore, internalization is ligand-dependent but independent of the kinase activity of the receptor.
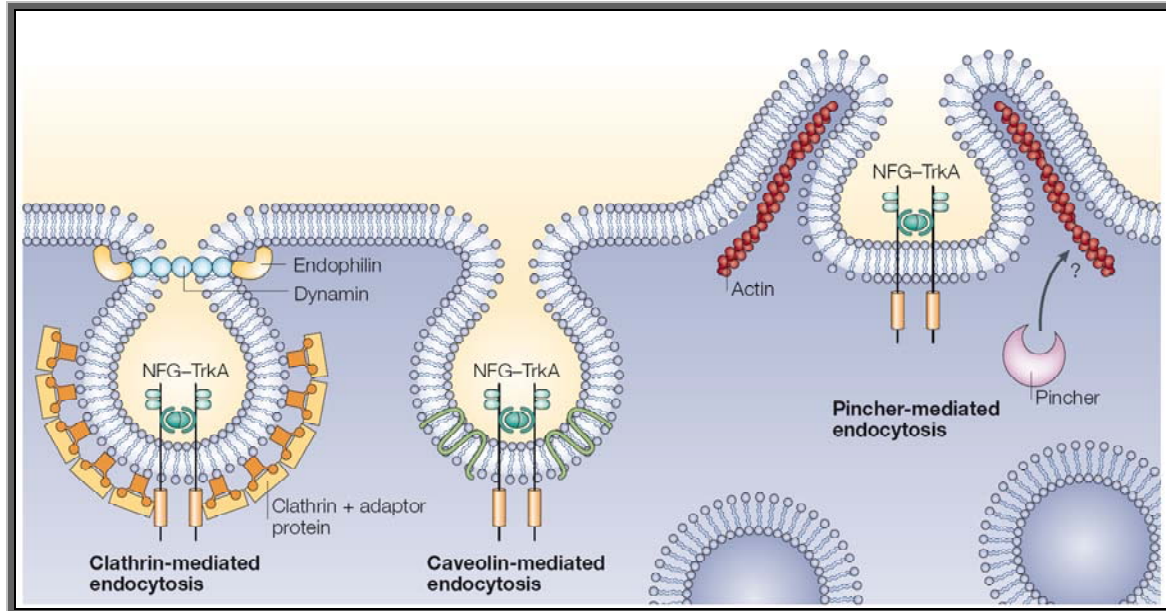
Figure 5. Different internalization mechanisms of neurotrophins and their receptors: Clathrin-mediated endocytosis, caveolae-mediated endocytosis and pinocytosis. Taken from (Zweifel, Kuruvilla et al. 2005)

After internalization, all proteins participating in the formation of endocytic vesicles such as, clathrin, caveolin, pincher, etc. are recycled back to the plasma membrane. Receptors are recruited to the clathrin-coated pits by direct interacting with the clathrin coat adaptor complex AP2 or by binding to other adaptor proteins, which in turn interacts with the clathrin heavy chain and/or AP2. Clathrin-coated pits invaginate inwards with the help of several accessory proteins, forming a clathrin-coated vesicle, a process that requires the GTPase dynamin. Several clathrin-independent pathways of endocytosis also exist, although the precise mechanism is not well understood. Endocytic vesicles derived from both clathrin-dependent and clathrin-independent endocytosis fuse with early endosomes, which are also referred to as sorting endosomes. Endosomal trafficking is controlled by several Rab proteins -small GTP-binding proteins of the Ras superfamily. Each GTP-bound Rab protein resides in a particular type of endosome and functions by recruiting specific effector proteins. Following their internalization into early Rab5-containing endosomes,

receptors can rapidly recycle back to the plasma membrane by a Rab4-dependent mechanism, traffic to the recycling compartment that contains Rab11A or remain in endosomes, which mature into multivesicular bodies (MVBs) and late endosomes. MVBs are defined by the presence of intraluminal vesicles (ILVs) that are formed in a process of inward membrane invagination involving endosomal sorting complex required for transport (ESCRT) complexes. Endosome maturation (early-to-late) involves the acquisition of Rab7 and the removal of endosomal components that are necessary for recycling. Cargo destined for degradation is incorporated into ILVs of MVBs. Fusion of late endosomes and MVBs with lysosomes carrying acid hydrolases results in cargo degradation (Sorkin and von Zastrow 2009).
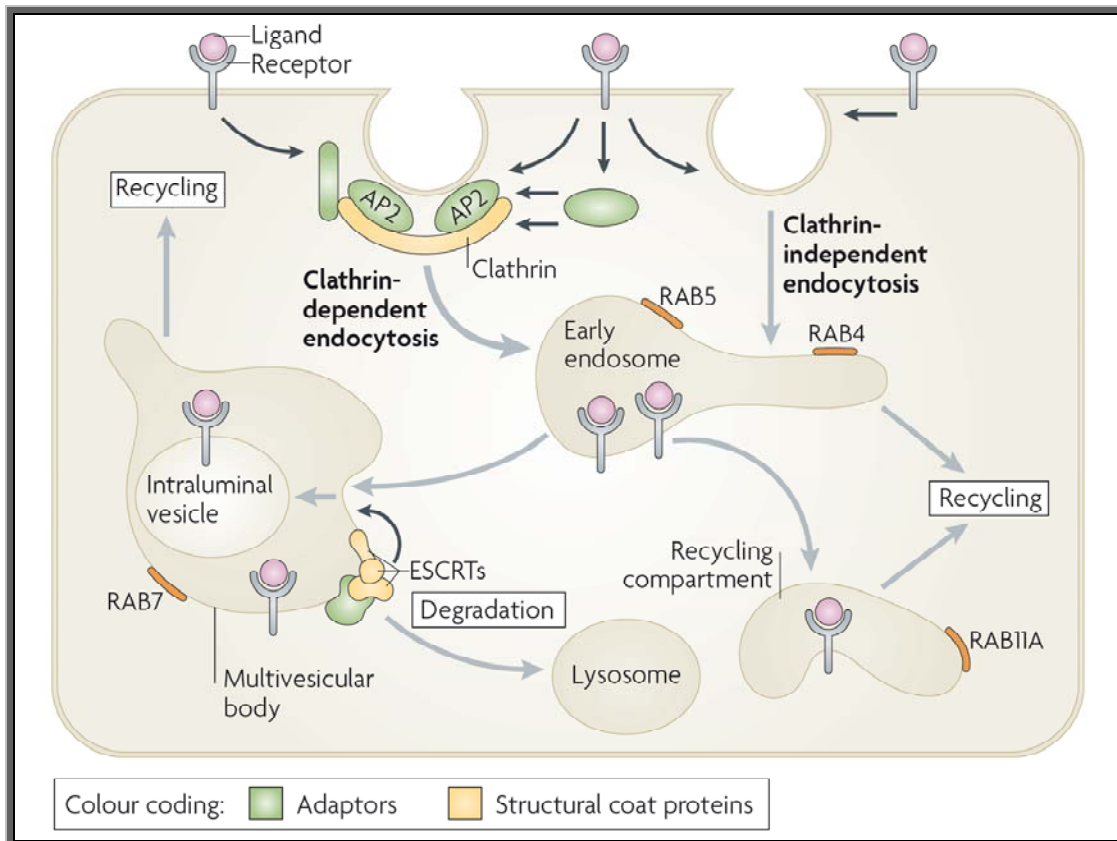
Figure 6. Mechanisms of receptor endocytosis. Schematic representation of consecutive events accompanying the receptor internalization following ligand-induced activation. Taken from (Sorkin and von Zastrow 2009).

After its internalization, NGF-TrkA receptor system continues to signal from the endosome vesicles, called "signaling endosome" until it is transported through the

axon to the neuronal cell body (Chao 2003; Howe and Mobley 2005). Inactivation of the endosomal complex is mainly mediated by lysosomal degradation.

Different motifs from Trk receptors play a role in the mediation of the internalization process. These motifs bind determinate adaptor proteins, thus defining the course of internalized receptors. One of such motifs is the YRKF701-704, whose implication in the internalization through the interaction with the clathtin endocytosis machinery has been shown (de Pablo, Perez-Garcia et al. 2008). Substitution of the Y701, either with Asp(D) or with Phe(F) lead to a strong reduction of the receptor co-precipitation and colocalization efficiency with CHC (clathrin heavy chain). Additionally, analysis of receptor degradation of both mutants showed that the targeting of the endocytic vesicles to the lysosomes was affected when Y701 was substituted. These results were in accordance with previous studies on other membrane proteins, demonstrating that the YxxΦ motif mediates the targeting of certain proteins to lysosomes (Williams and Fukuda 1990; Harter and Mellman 1992). This data provide evidence that the YRKF701-704 motif of TrkA is involved not only in the regulation of receptor internalization via a clathrin-dependent mechanism, but also in the targeting of the receptor to the lysosomes.

Ubiquitin (Ub), a protein containing 76-amino acids, has also been shown to be implicated in the regulation of receptor internalization, trafficking and degradation (Piper and Luzio 2007). The ubiquitination of membrane proteins has been revealed to act as a trafficking signal, involved in the regulation of receptor internalization and sorting upon ligand-binding (Bonifacino and Traub 2003).

4.2   Retrograde signaling

It is interesting to understand how the active ligand-receptor signaling complex formed on the cell surface can reach the neuronal cell body, where it might transmit neurotrophin signals to the soma and mediate survival, growth and gene expression. In human, the distance between neuronal terminations and neuron cell body could easily reach 1m of length. Neurotrophins could exert their effects on the axonal growth without the necessity to reach neuronal cell body. However, survival and some morphological responses require transcriptional activation mediated by CREB, which elicit the expression of the pro-survival molecule Bcl-2. Because of that reason, NGF-TrkA complexes recruit the cellular machinery responsible for endocytosis, trafficking and finally retrograde transport.

Retrograde endosomal signaling in neurons is a complex process including the internalization of ligand–receptor complexes in axon terminals, the sorting of complexes into active signaling vesicles, physical translocation of these endosomes along the axonal microtubule network to cell bodies, endosomal signaling and ultimately destroying of the retrograde endosomal signaling complex. Several mechanisms of retrograde transport have been proposed (Zweifel, Kuruvilla et al. 2005):

- "Signaling endosome model": It represents the endocytosis and transport of NGF and Trk (in a complex or separately) which travel the whole distance from the neuronal terminations to the soma in a special endocytic vesicle. The name of this vesicle-"signaling endosome" originates from the fact that it contains not only NGF-Trk complex, but also signaling molecules, activated by the receptor.
- Retrograde propagation of a phosphorylation signal without transport of signaling molecules: it is characterized by retrograde waves of Trk receptor activation along the plasma membrane. According to this model, transmitting of the activation does not require physical transport of the receptor along the axon.
- Retrograde transport of signaling molecules downstream of Trk: corresponds to calcium or cAMP waves deriving from activated Trk receptors.

The widely accepted theory is the "signaling-endosome model", according to which Trk is internalized and transported together with NGF, thus being maintained active during the whole process. Some studies also indicate that ligand internalization might not be crucial for retrograde neurotrophin receptor signaling (MacInnis and Campenot 2002). Therefore, obviously the signaling-endosome model is not the only possibility for retrograde transport of NGF, even though it is the most accepted.

NGF-Trk receptor complexes can be localized in endocytic vesicles, covered or not with clathrin and caveolin, and also in multivesicular bodies. It was demonstrated that these vesicular structures transported along the axon, contain "early endosome" markers such as RAb-5 and EEA1 (Delcroix, Valletta et al. 2003). For that reason, it is necessary for them to avoid the degradation on the synaptic terminal. They also serve as signaling platforms, since it was demonstrated that after NGF stimulation they are found to be associated with different signaling molecules, such as PI3K, MAPKs, PLCγ and Rap-1 (Howe, Valletta et al. 2001).

In sensory neurons, NGF–TrkA-containing vesicles have characteristics of early endosomes and are associated with signaling components of the MAPK, PI3K and phospholipase C-γ signaling pathways, which supports the signaling endosome hypothesis (Wu, Lai et al. 2001; Delcroix, Valletta et al. 2003).

It is still unclear how retrograde transport is regulated and only few implicated proteins have been identified so far. Evidences exist that retrograde transport of NGF–Trk receptor vesicles occurs in a dynein dependent manner along the microtubule network (Yano, Lee et al. 2001). Cytoplasmic dynein is a negative end-directed motor that transports organelles along microtubules in a retrograde direction. It was demonstrated *in vivo* (Hafezparast, Klocke et al. 2003) and *in vitro* (Heerssen, Pazyra et al. 2004) that dynein-mediated retrograde transport is crucial for neuronal survival. It is interesting how dynein/dynactin motor proteins interact with membrane cargoes. It was reported that an endosomal vesicle protein, named retrolinkin functions as a receptor, binding vesicles to dynein/dynactin and that the deletion of retrolinkin membrane associated domains disrupts retrograde vesicular transport in mice sensory neurons (Liu, Ding et al. 2007).

Another report show that retrograde transport pathway of p75NTR, TrkB and BDNF, is strictly dependent on the activities of two small GTPase, Rab5 and Rab7 and that precisely Rab7 plays an essential role in axonal retrograde transport by controlling a vesicular compartment implicated in neurotrophin traffic (Deinhardt, Salinas et al. 2006).

Growing evidence that abnormal retrograde neurotrophic factor signaling is involved in the etiology of neurological diseases such as Alzheimer's disease (Salehi, Delcroix et al. 2004) Huntington's disease and amyotrophic lateral sclerosis, confirms that it is essential to gain more knowledge of how it could be influenced, not only during development but also in adulthood.

5. Neurotrophin intersection with other signaling pathways

Trk receptors are generally activated upon binding of their cognate ligands, but they can also be transactivated, without involvement of neurotrophins in response to G protein-coupled receptor (GPCR) signaling (Arevalo and Wu 2006). Transactivation of Trk receptors triggers the PI3K-Akt signaling pathway, which

results in increased cell survival after NGF or BDNF withdrawal in PC12 cells and hippocampal neurons, respectively. It has also been shown that Trk receptors undergo activation in intracellular membranes and not at the cell surface (Rajagopal, Chen et al. 2004). Activation of tyrosine kinase receptors trough GPCR transactivation demonstrates an alternative way in which signals may be transmitted by intracellular receptors in the absence of ligand.

Just as Trk receptors are activated by GPCRs, other receptors and channels can be activated as a result of Trk receptor stimulation by neurotrophins. For example, members of the TRP (transient receptor potential) family of cation channels, like TRPC3 and TRPV1, are stimulated through Trk receptors (Chuang, Prescott et al. 2001). Likewise, the probability for N-methyl-D-aspartic acid (NMDA) receptor to open and consequent glutamate induction to occur are increased by BDNF-activated TrkB receptor, which phosphorylates the specific NMDA receptor subunit NR2B (Lin, Wu et al. 1998).

In addition, Trk receptors have the capability to activate the Ret51 receptor tyrosine kinase isoform in postnatal superior cervical ganglion (SCG) neurons (Tsui-Pierchala, Milbrandt et al. 2002).

All data mentioned above indicate that cross talk between neurotrophins and other signaling pathways may play an essential role in the modulation of different molecules necessary for proper functioning of the nervous system.

6. Regulation of neurotrophin receptors by ubiquitination

Neurotrophin signaling should be strictly regulated to elicit the correct cellular response. Downregulation of the neurotrophin signal can be achieved by degradation of the receptors, as occurs with other transmembrane proteins. Two studies demonstrated that Trk receptors undergo ligand-dependent ubiquitination which may be modulated by the presence of p75NTR (Geetha, Jiang et al. 2005; Makkerh, Ceni et al. 2005). However, significant disagreements were raised between the two reports, since Makkerh et al. suggest that co-expression of p75NTR negatively regulates the ubiquitination of Trk receptors, impairing their internalization and degradation, while Geetha et al. propose that p75NTR regulates positively the ubiquitination of TrkA. A third work by Arelavo et al. reported that Nedd4-2 binding

to TrkA receptor leads to its downregulation and degradation, following ubiquitination (Arevalo, Waite et al. 2006).

It is well known that ubiquitination of membrane proteins plays a critical role in many cellular processes, such as regulation of protein stability and endocytosis (Staub, Dho et al. 1996), regulation of receptor internalization, trafficking and degradation (Piper and Luzio 2001). Ubiquitination of membrane proteins acts as a trafficking signal, involved in the regulation of receptor internalization and sorting upon ligand-binding (Bonifacino and Traub 2003). For that reason it is important to address the role of this post-translational modification on the regulation of TrkA activity, trafficking and function, because the elucidation of the molecular mechanisms that modulate Trk receptor activity and receptor inactivation are important for understanding the particular features of neurotrophin receptors.

6.1. The ubiquitin-proteasome system

Ubiquitin (Ub) is a 76-amino-acid protein that is highly conserved throughout eukaryotes, with only three amino-acid changes from yeast to human. Its covalent conjugation to other proteins, denominated ubiquitination or ubiquitylation, is essential for the degradation of proteins whose levels are regulated either constitutively or in response to changes in the cellular environment.

The classical vision of ubiquitylation is that it targets proteins for degradation by the proteasome (Kloetzel 2001). In addition to its role in proteasomal degradation, ubiquitylation is also referred as a signal that targets plasma membrane proteins for destruction in lysosomes (Hicke 2001). Indeed, the best-characterized signal for entering membrane proteins into the degradative-MVBs pathway is their ubiquitination. ESCRTs play a central role in protein-protein and protein-lipid interactions that underline ubiquitin-mediated sorting to the lysosome. Four different ESCRTs, denominated as ESCRT-0, I,-II and –III are recruited to endosomes through both protein and lipid interactions. ESCRT-0,-I and –II, which participate in the early degradative MVB pathway, have ubiquitin-interacting modules, necessary for cargo sorting, whereas ESCRT-III, the final complex in the pathway, actively recruits de-ubiquitinating enzymes (DUBs) to remove Ub from the cargo before its incorporation into the MVBs (Williams and Urbe 2007).

Although, the traditional view of ubiquitylation focused on its role in targeting misfolded proteins for degradation by the proteasome, now it is clear that ubiquitylation may target proteins from distinct locations to two fundamentally

different proteolytic structures, proteasomes and lysosomes, which represent the two main degradation systems in mammalian cells.

The ubiquitin–proteasome system (UPS) (Figure 7) is critically important for protecting cells against the toxic effects of misfolded proteins and it is the principal mechanism for proteolysis in the mammalian cytosol and nucleus. The 26S proteasome consists of a central, barrel-shaped catalytic (20S) complex, carrying multiple active sites, which are sequestered in an interior chamber, only accessible through a narrow pore. Two peripherally positioned regulatory complexes (19S) unfold the substrate polypeptide chain and transfer it into the active-site chamber, using integral chaperone subunits. The requirement for unfolding means that proteins, which have reached the proteasome, but cannot be unfolded, could function as potential dominant inhibitors. Defective regulation of ubiquitin-proteasome system is manifested in diseases that range from developmental abnormalities and autoimmunity to neurodegenerative diseases and cancer (Dahlmann 2007).

Ubiquitin is essential for multitude of processes such as; cell cycle progression, organelle biogenesis, apoptosis, regulation of cell proliferation, cellular differentiation, quality control in the endoplasmic reticulum, protein transport, inflammation, antigen processing, DNA repair and stress responses (Weissman 2001). The universality of ubiquitin conjugation demonstrates a fundamental resemblance to phosphorylation. Ubiquitination occurs when the first ubiquitin is covalently joined to the substrate through an isopeptide bond between the C-terminus of ubiquitin and a lysine residue of the target protein. This conjugation typically involves three types of enzyme: E1 (ubiquitin-activating enzyme), which hydrolyses ATP and forms a thioester-linked conjugate between itself and ubiquitin; E2 (ubiquitin-conjugating enzyme), which receives ubiquitin from E1 and forms a similar thioester bond with ubiquitin and E3 (ubiquitin ligase), that binds both E2 and the substrate and transfers the ubiquitin to the substrate (Ross and Pickart 2004).

Like phosphorylation, ubiquitylation is a reversible process, with an activity analogous to that of phosphatases carried out by deubiquitylating enzymes (DUBs). Proteins may be phosphorylated or hyper-phosphorylated, which leads to functional differences. Similarly, ubiquitin may be conjugated to one Lys (monoubiquitination) or to a number of Lys on a target molecule (multi-monoubiquitination). On the other hand, the ubiquitylation machinery is distinct from the phosphorylation machinery, since ubiquitin itself contains seven lysine residues that can be potentially used as acceptors for the attachment of other ubiquitin molecules, allowing the formation of

different types of ubiquitin chains, named polyubiquitination (Figure 8). Phosphorylation can occur on either tyrosine, serine, or threonine residues leading to greater signaling diversity. Correspondingly, poly-ubiquitin chain formation can be linked by any of the internal ubiquitin lysines (e.g. Lys 48 or Lys 63). Polyubiquitin chains can be stabilized and further extended by the action of E4 enzymes. It has been shown that all seven lysine residues are used *in vivo* for chain formation (Xu and Peng 2006). Among the many emerging functions of ubiquitylation, exocytosis and endocytosis appear to be regulated at more than one step by protein ubiquitylation. Different ways of substrate ubiquitination contribute to the generation of a diversity in ubiquitin dependent cellular processes (Figure 7). At last, phosphorylation can increase or inhibit ubiquitylation, by modifying either the protein designed to be ubiquitylated or the enzymes that catalyse the ubiquitination process.
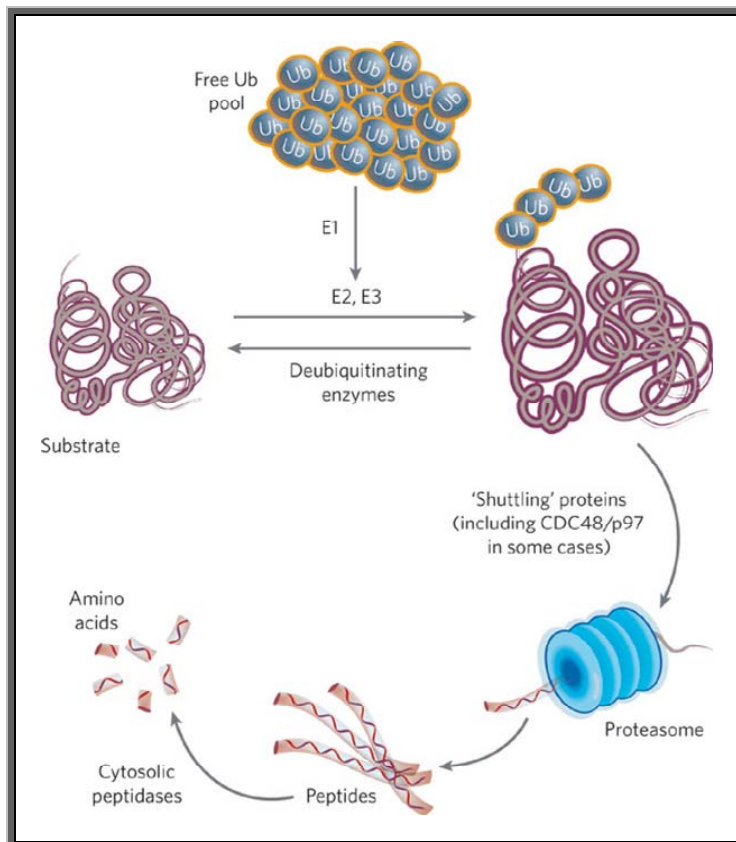
Figure 7. ***Schematic diagram of the ubiquitin–proteasome system.*** Before being targeted for proteasomal degradation, most proteins are covalently modified with ubiquitin (Ub). Usually, three enzyme types are involved in this process — ubiquitin-activating (E1), ubiquitin-conjugating (E2) and ubiquitin ligase (E3) enzymes. Proteins tagged with chains of four or more ubiquitins are shuttled to the proteasome by various proteins such as

CDC48/p97. In the proteasome, proteins are reduced to peptides, which are released into the cytosol and further destroyed by peptidases. Taken from (Rubinsztein 2006).

Monoubiquitination or multi-monoubiquitination has been demonstrated to be required for the entry of certain proteins into vesicles at different stages of the secretory/endocytic pathway (Hicke 2001). It is also involved in virus budding, DNA repair, gene expression and transcription, endocytosis and activation of protein kinases (Woelk, Sigismund et al. 2007). The best-studied examples for polyubiquitination are chains of four or more ubiquitin moieties linked through Lys 48. This type of chain targets proteins for degradation via the 26S proteasome (Pickart 2000). Nevertheless, analysis of Ub-chains formed through Lys63 show that similarly to monoubiquitination, such chains generate a non-proteolytic signal involved in DNA repair, transcriptional regulation, inflammatory response, endocytosis and activation of protein kinases (Huang and D'Andrea 2006). Nuclear Magnetic Resonance (NMR) data confirmed the existence of a conformational difference between Lys48 and Lys63-linked chains, which finally take an extended, linear conformation of Ub units arranged head to tail (Varadan, Assfalg et al. 2004). This type of structure suggests that Lys63 chains might be recognized as a signal topologically similar to monoubiquitin.

Much less is known about the precise function and topology of chains that are linked through Lys6, Lys11, Lys27, Lys29 and Lys33. Recent studies demonstrate that atypical, mixed polyUb chains can be formed, contributing to an additional level of complexity in the ubiquitin system. Several groups have reported the identification of branched chains containing different types of linkages, but their functions remain unclear (Peng, Schwartz et al. 2003; Ben-Saadon, Zaaroor et al. 2006).

Specific deubiquitinating enzymes (DUBs) are used for the removal of Ub-moieties from ubiquitinated substrates and its consequent recycling. DUBs serve to compensate ubiquitination reactions within the cell, thus dynamically contributing to the regulation of various cellular processes, such as endosomal sorting (Clague and Urbe 2006). Ubiquitin could provide an additional surface for protein–protein interactions, since several Ub binding domains (UBDs) have been identified and characterized (Hicke, Schubert et al. 2005; Hurley, Lee et al. 2006). These Ub binding domains, which do not display strict sequence conservation, interact with Ub via diverse three-dimensional folds and direct the flow of information to specific signaling pathways.
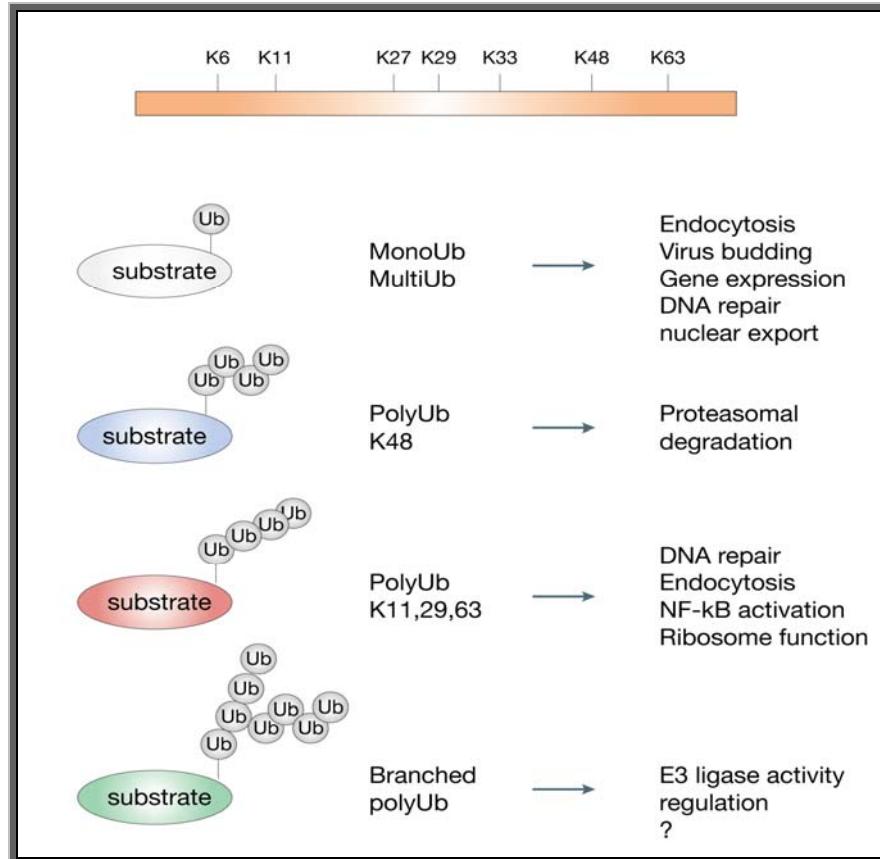
Figure 8. Schematic representation of the different Ub modifications with their functional roles. Taken from (Woelk, Sigismund et al. 2007)

6.2. Ubiquitination and endocytosis of membrane proteins

Ligand-initiated degradation of most receptor tyrosine kinases requires that the receptor is internalized and transported to lysosomes and/or proteasomes (Bonifacino and Traub 2003; Dikic and Giordano 2003). Receptor degradation, following receptor endocytosis is related to receptor down-regulation and its function is to terminate the signal elicited upon ligand binding. The ubiquitin system is of extreme importance for the physiological modulation of many cellular processes, including endocytosis of transmembrane receptors.

Studies performed in yeast, could be considered as one of the first evidence for ubiquitin non-proteasomal function. They have demonstrated that Ub is required for the first step in cargo internalization, as well as for targeting of cargos to vacuoles, which suggests that it is critical in determining intracellular protein trafficking (Hicke

and Riezman 1996). Indeed, monoubiquitination alone of several yeast transmembrane receptors (alpha-factor receptors, permeases and transporters) is sufficient to trigger their internalization, although modification with Lys63-linked Ub chains can speed up this process (Galan and Haguenauer-Tsapis 1997).

The situation is more complex in mammalian cells since not only the receptor but also the endocytic adaptors, are often ubiquitinated in response to extracellular stimuli. Moreover, more than one internalization pathway exists in the cell and not all of them appear to be regulated by ubiquitin. Early studies on the growth hormone receptor (GHR) have shown that an intact ubiquitination machinery is required, although ubiquitination of the receptor is not indispensable for its internalization (Govers, ten Broeke et al. 1999). This statement is also true for a class of G Protein-Coupled Receptors (GPCRs) that are ubiquitinated upon agonist stimulation (Shenoy, McDonald et al. 2001). Mutation of all the lysine residues in the cytoplasmic tail of the β2-adrenergic receptor and the chemokine receptor-CXCR4, does not affect the initial internalization event, but severely damage the downstream endocytic sorting step that targets receptors for degradation (Marchese and Benovic 2001; Shenoy, McDonald et al. 2001). This study propose that polyubiquitination of GPCR-associated β-arrestins is essential for the internalization of receptors in clathrin-coated pits (Shenoy, McDonald et al. 2001).

Platelet-derived growth factor receptor (PDGFR) and epidermal growth factor receptor (EGFR) were the first mammalian tyrosine kinase receptors found to be ubiquitinated (Mori, Heldin et al. 1992; Galcheva-Gargova, Theroux et al. 1995) and targeted for lysosomal degradation, as a mechanism of signaling attenuation (Haglund, Di Fiore et al. 2003; Haglund, Sigismund et al. 2003). Later on, several other receptor tyrosine kinases, such as Met and Ret have been shown to be ubiquitinated by the E3 ligase Cbl in a ligand-dependent manner. In the case of Met, its stimulation with hepatocyte growth factor (HGF) leads to detectable receptor ubiquitination and enhanced degradation (Peschard, Fournier et al. 2001; Abella, Peschard et al. 2005). The juxtamembrane region of Met contains a docking site for Cbl, the phosphorylated Tyr 1003, which is required for ligand-dependent ubiquitination and degradation of the receptor (Peschard, Fournier et al. 2001) and this Cbl docking site is absent in the Tpr-Met oncogene (Peschard and Park 2007). Met mutants, insensitive to Cbl-dependent ubiquitination are tumorigenic due to their enhanced stability and the sustained downstream signaling pathways activation (Peschard, Fournier et al. 2001; Abella, Peschard et al. 2005). Indirect recruitment of

Cbl to Met is also possible through the Grb2 adapter protein (Peschard, Fournier et al. 2001), thus proposing a mechanism whereby the direct and indirect binding sites cooperate to place Cbl correctly on the receptor. This modality of Cbl recruitment could also be seen for other receptor tyrosine kinases, such as EGFR and Ret (Scott, Eketjall et al. 2005). A constitutive complex of Grb2 and Cbl could be recruited to both Ret receptor isoforms-Ret51 and Ret9 via docking of Shc to phosphorylated Tyr1062. Phosphorylation of Tyr1062, which is present in Ret51, but absent in Ret9, provide the longer isoform with a second way to recruit the Grb2-Cbl complex. This leads to a stronger association of Cbl ubiquitin ligase to the activated Ret51 receptor than to Ret9 isoform, resulting in increased ubiquitination and faster turnover of Ret51 (Scott, Eketjall et al. 2005).

Ret could become poly or monoubiquitinated depending on the growth factor stimulation. An accumulation of monoubiquitinated, but not polyubiquitinated Ret occurs in NGF-treated neurons, in contrast to GDNF that promotes a strong polyubiquitination of the receptor. Ret monoubiquitination is preceded by NGF-induced accumulation of autophosphorylated receptor, predominantly on the plasma membrane. In contrast, GDNF promotes the internalization of activated Ret. Thus, NGF stimulates Ret activity in mature sympathetic neurons by inhibiting the ongoing ubiquitin-mediated degradation of Ret, before its polyubiquitination and internalization (Pierchala, Tsui et al. 2007). However, the role of ubiquitination on receptor internalization is not a general rule. Mutation of the major ubiquitination sites of Fibroblast Growth Factor receptor (FGFR) does not affect its internalization, thus questioning the involvement of ubiquitin in FGFR internalization (Haugsten, Malecki et al. 2008).

Ligand-induced EGFR internalization and sorting to the lysosome is one of the best-characterized mechanisms of how the regulation of receptor turnover is delicately modulated by the ubiquitin signal. Once activated by its cognate ligand, EGFR undergoes dimerization and trans-phosphorylation at different tyrosine residues, which serve as docking sites for several signalling/adaptor molecules, one of which is Cbl Ub ligase, which mediates receptor ubiquitination (Levkowitz, Waterman et al. 1998; Levkowitz, Waterman et al. 1999; Woelk, Sigismund et al. 2007). EGFR was initially shown to be modified by multiple monoubiquitination *in vivo* (Mosesson, Shtiegman et al. 2003). The utilization of a chimeric protein composed of EGFR and an ubiquitin mutant that could not be extended by polyubiquitination, showed that a single ubiquitin was sufficient to drive internalization, even though at a lower rate

compared to the wild-type receptor (Haglund, Sigismund et al. 2003). Recent analysis based on quantitative mass-spectrometry approach, has revealed that, in addition to multiple monoubiquitination the EGFR is modified by short Lys63-linked chains within the kinase domain (Huang, Kirkpatrick et al. 2006). This data suggest that although monoubiquitination is sufficient for internalization, as observed in yeast, polyubiquitination through Lys63 generates a more efficient internalization signal, possibly by increasing the binding affinity to UBD-containing proteins.

Nevertheless, EGFR mutants displaying negligible ubiquitination were still internalized at a rate comparable to that of the wild-type receptor, showing that EGFR ubiquitination is not essential for internalization through clathrin-coated pits (Huang, Goh et al. 2007). This apparent discrepancy between ubiquitination being sufficient, but not required, for EGFR internalization can be explained by the existence of alternative internalization pathways possibly regulated by different signals (Sorkin 2004).

The best-characterized role for receptor ubiquitination is the negative regulation of signaling by targeting receptors for lysosomal degradation (Raiborg, Rusten et al. 2003; Clague and Urbe 2006). The controversy on the requirement for ubiquitin at the initial step of EGFR internalization (Jiang and Sorkin 2003; Grovdal, Stang et al. 2004), was solved by conducting studies under physiological conditions (Sigismund, Woelk et al. 2005). The EGFR uses different pathways depending on its ubiquitination state, which is in a direct correlation with the amount of EGF used to stimulate the receptor. At low EGF doses, receptor ubiquitination is not detected and the EGFR is internalized only through clathrin-mediated endocytosis, while at high EGF doses, both clathrin-dependent and independent internalization routes are employed as the receptor becomes ubiquitinated (Figure 9). Indeed, ubiquitination appears to be required for clathrin-independent endocytosis while it is dispensable for clathrin-dependent internalization (Sigismund, Woelk et al. 2005).
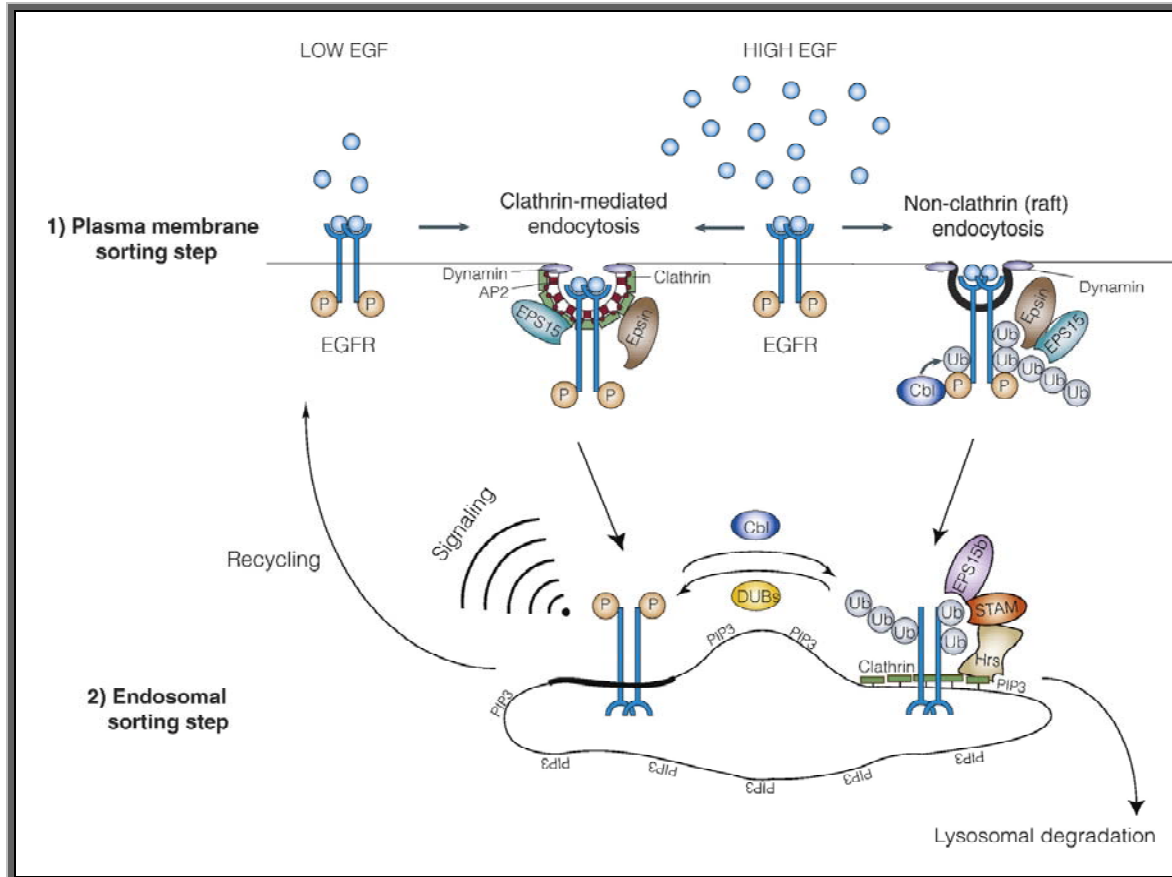
Figure 9. EGFR can be internalized through different endocytic pathways as a function of EGF dose. In the clathrin route, receptors are mainly directed to recycling and signaling, while in the non-clathrin route they are preferentially targeted for lysosomal degradation. A second sorting step is present at the level of the endosomes, where the two internalization pathways seem to converge. A flat clathrin pattern on the endosomal membrane stabilizes the interaction between the ESCRT complex (Hrs, STAM and Eps15b) and ubiquitinated EGFR, which is then targeted for degradation. Taken from (Acconcia, Sigismund et al. 2009)

Mutations targeting the tyrosine docking-site for Cbl or the ubiquitinated lysines, which result in Ub-defective receptor, abolish receptor down-regulation but not its internalization via the clathrin pathway (Grovdal, Stang et al. 2004; Huang, Kirkpatrick et al. 2006). At higher, but still physiologically relevant, EGF concentration (20 ng/ml), an additional clathrin-independent, raft-dependent pathway is employed, along with ubiquitination of the receptor. Three adaptor molecules, eps15, eps15R and epsin, are recruited to the EGFR at the plasma membrane upon ligand activation. These proteins have been traditionally associated to clathrin-dependent endocytosis (Coda, Salcini et al. 1998; Hawryluk, Keyel et al.

2006), but recent evidence, indicates the requirement for these adaptors in targeting ubiquitin-conjugated proteins to clathrin-independent endocytosis (Chen and De Camilli 2005; Sigismund, Woelk et al. 2005). Both eps15/R and epsin present single or multiple ubiquitin interacting motifs, through which they recognize the ubiquitinated receptor and mediate its clathrin-independent internalization (Hofmann and Falquet 2001; Polo, Sigismund et al. 2002). Interestingly, these proteins are themselves modified by monoubiquitination in a ligand-dependent manner (Hicke, Schubert et al. 2005). Monoubiquitination of adaptors could contribute to signal amplification by creating additional surfaces of interaction (Hicke, Schubert et al. 2005; Mukhopadhyay and Riezman 2007), thus being responsible for sorting of receptors along the endocytic pathway. An alternative model proposed that monoubiquitination of the adaptor leads to an intramolecular interaction mediated by the UBD that folds back on the ubiquitin attached in *cis* and allows the detachment of the adaptor from the ubiquitinated cargos (Hoeller, Crosetto et al. 2006).

Interestingly, the majority of EGFRs internalized via clathrin-mediated endocytosis are not targeted for degradation, but rather are recycled to the cell surface (Figure 8). On the contrary, clathrin-independent internalization preferentially leads to receptor degradation. This has important implications for signaling, as by shifting EGFR fate towards recycling rather than degradation, clathrin-mediated endocytosis prolongs the duration of the signal (Sigismund, Argenzio et al. 2008). Thus, Ub might play a critical role during the internalization step, when depending on the ubiquitination type it could conduce to receptor signaling amplification or to receptor downregulation, resulting in the termination of the signal. These findings suggests that cells are able to sense growth factor concentration and to interpret the information stored in the stimulus strength, converting it to different outputs such as distinct ubiquitination patterns, distinct endocytic routes and finally, distinct biological responses. A similar mechanism has been recently described for IGF-1R (Sehat, Andersson et al. 2008) and PDGFR. In the second case, cells switch from a migrating to a proliferating phenotype in response to an increasing PDGF gradient, which suggests that the decision to proliferate or migrate depends on the different endocytic route followed by the receptor in response to ligand concentration (De Donatis, Comito et al. 2008). The idea that cells can exert distinct biological responses depending on ligand concentration is not novel. It was established that during development gradients of morphogens could induce distinct signaling events in cells positioned differently along the gradient, revealing an essential role of

receptor trafficking also in the regulation of this kind of response (Fischer, Eun et al. 2006).

6.3. Ubiquitination of Trk neurotrophin receptors

Ubiquitin plays an important role on TrkA signaling and down-regulation. Ubiquitination has been reported to play a role in both internalization and sorting of the receptor (Schnell and Hicke 2003). As previously described TrkA internalization in distal axonal processes is required for retrograde signaling to neuronal soma, which finally controls neuronal differentiation (Zweifel, Kuruvilla et al. 2005). Three reports have described the ubiquitination of TrkA receptor upon NGF stimulation (Geetha, Jiang et al. 2005; Makkerh, Ceni et al. 2005; Arevalo, Waite et al. 2006). Geetha and colleagues reported that in PC12 cells, TrkA becomes polyubiquitinated in a Lys63-dependent manner. This process was mediated by the formation of a complex with the p75NTR and its associated E3 ligase-TRAF6, which ubiquitinates TrkA at Lys485. Previous study of this group demonstrated that TRAF6 interacts and form a complex with p75NTR, p62 adaptor protein and TrkA (Wooten, Seibenhener et al. 2001). They propose that TrkA-Lys63 polyubiquitination regulates receptor internalization and signaling. However, they suggest that TrkA may also be polyubiquitinated independently of p62/TRAF6 and (Geetha, Jiang et al. 2005). Another recent study of the same group propose that both proteasome and lysosomes play a coordinated role in the degradation of internalized TrkA receptors, emphasizing on a new and important step for proteasome in regulating deubiquitination of the receptor, prior to its degradation into the lysosomes (Geetha and Wooten 2008).

Simultaneously, Arevalo and colleagues demonstrated that in DRG neurons the E3 ubiquitin ligase Nedd4-2 is responsible for NGF-dependent multi-monoubiquitination of TrkA receptor (Arevalo, Waite et al. 2006). They demonstrated that Nedd4-2 binds directly to the PPXY motif of TrkA receptor, a sequence surrounding the residue Y785 in TrkA, which is a putative binding site for WW domains of Nedd-4 E3 ubiquitin ligase family (Ingham, Gish et al. 2004). This binding was specific for TrkA, since PPXY motif is not conserved in the closely related members of Trk family, TrkB and TrkC. Nedd4-2 was bound constitutively to inactive TrkA receptor and caused its down-regulation by producing a negative effect on the steady state levels of TrkA receptors (Arevalo, Waite et al. 2006).

Other published results, such as Kuruvilla and colleagues study, were contrary to some Geetha and colleagues observations. They showed that TrkA receptors undergo efficient internalization in the absence of p75NTR in cultured sympathetic neurons (Kuruvilla, Zweifel et al. 2004). Additionally, another group demonstrated that the removal of TrkA juxtamembrane sequences containing the Lys485 residue did not show any effect on NGF-dependent TrkA recycling (Chen, Ieraci et al. 2005) or degradation (Sommerfeld, Schweigreiter et al. 2000). Another report, described that TrkA and TrkB receptors underent robust ligand-dependent ubiquitination, but that the co-expression of p75NTR attenuated the their ubiquitination and delayed NGF-induced TrkA internalization and degradation. These results, obtained in HEK293 cells expressing TrkA, revealed that p75NTR had more likely a negative effect on TrkA ubiquitination and delayed TrkA internalization in PC12 cells. These data indicate that p75NTR may prolong cell-surface Trk-dependent signaling events by negatively regulating receptor ubiquitination (Makkerh, Ceni et al. 2005).

In conclusion, more work is required to understand how these findings can be integrated into a comprehensive picture. Certainly, the ubiquitination either monoubiquitination or Lys63-linked polyubiquitination regulates the internalization and trafficking of membrane proteins in different ways, depending on the receptor system. The different positioning of the ubiquitin-based signals may be important for the fine modulation of the receptor endocytosis, which is finally critical for the balance between receptor induced signal transduction and receptor down-regulation.

6.4 Regulation of the Nedd4-2 ubiquitin ligase activity

In mammals, there is a growing number of proteins that can be regulated by Nedd4-2 and its best-characterized target is the epithelial sodium channel (ENaC). Nedd4-2 interacts with ENaC at the cell surface through multiple WW domains. The specificity of this interaction is dictated by the PPXY motif of ENaC. After binding, the Nedd4-2 active domain catalyses the ubiquitination of Lys residues in the N-terminus of ENaC which functions as a signal to remove ENaC from the cell surface and target it to the lysosomes for degradation. Therefore, the major function of Nedd4-2 is to decrease the number of ENaC channels at the cell surface, thus reducing the rate of epithelial Na^+ absorption. In accordance, the disruption of the interaction between Nedd4-2 and ENaC causes the Liddle's syndrome, an autosomal dominant disorder with severe sodium retention and hypertension, caused by the excessive Na^+ intake (Staub, Dho et al. 1996). To increase the number of surface ENaC channels, Nedd4-2

must be downregulated, and this is achieved by its phosphorylation (Snyder 2009). Phosphorylation modulates Nedd4-2 activity by altering its ability to bind to ENaC, thus the same interaction that controls the specificity of Nedd4-2 binding is used to regulate its activity. Under basal state Nedd4-2 binds to ENaC and catalyses its ubiquitination whereas, upon phosphorylation it is unable to bind ENaC, so the channel remains at the cell surface and thus the Na^+ absorption is enhanced. However, Hallows and collaborators showed that phosphorylation deficient Nedd4-2 mutants are unable to either ubiquitinate ENaC or efficiently inhibit ENaC currents. They demonstrate the importance of novel phosphorylation sites, such as Thr-899 situated within the catalytic domain of Nedd4-2, whose mutation is sufficient to disrupt the Ub ligase activity of Nedd4-2 (Hallows, Bhalla et al.).

6.5 Regulation of endocytosis by protein monoubiquitination

Ubiquitin-dependent internalization of receptors requires the specific molecular recognition of the ubiquitinated cargo by UBD-containing proteins. A single ubiquitin-UBD interaction would not be sufficient for the efficient internalization of the ubiquitinated receptor however; there exist several mechanisms to transform low-affinity contacts into physiologically relevant interactions. Endocytic adaptors, such as eps15 or epsin, contain multiple UBDs as well as additional protein modules that recognize other endocytic proteins. The existence of multiple UBDs allows the ubiquitinated receptor to increase its binding affinity by either binding several monoubiquitinated molecules in multi-monoubiquitinated cargos (Hurley, Lee et al. 2006) or engaging a single monoubiquitin through different UBDs (Lee, Tsai et al. 2006; Penengo, Mapelli et al. 2006). Alternatively, ubiquitinated receptors may be preferentially targeted to their ubiquitinated cargos by simultaneous binding to ubiquitin (in the cargo) and to the plasma membrane (Mukhopadhyay and Riezman 2007). An increase in the binding affinity between ubiquitinated receptors and ubiquitinated proteins can also be achieved by a linear topology of Lys63-linked polyubiquitination. Ubiquitin receptors themselves are frequently monoubiquitinated by a process that requires the presence of a UBD and is referred to as coupled monoubiquitination. This process strictly depends on the ability of the ubiquitin interacting motif (UIM) to bind to monoubiquitin, since the underlying molecular mechanism is based on interaction between the UIM of the ubiquitinated receptor

and a HECT-type E3 Ub ligase, such as Nedd4-2 which itself has been modified by ubiquitination (Hicke, Schubert et al. 2005; Woelk, Oldrini et al. 2006). In some cases, the ubiquitinated receptor can skip the E3 Ub ligase, through direct binding to the E2 conjugating enzyme, which process is called E3 independent ubiquitination (Hoeller, Hecker et al. 2007). Both types of ubiquitination, E3-independent and E3-dependent can coexist, thus E3-independent ubiquitination may control the level of constitutive protein monoubiquitination, whereas E3-dependent ubiquitination may be responsible for monoubiquitination in physiologically relevant, signaling-mediated processes.

Two hypotheses concerning the functional role of the monoubiquitination of endocytic proteins could be proposed. One is that coupled monoubiquitination would extend the range of intermolecular interactions of the ubiquitinated receptor, creating a network of ubiquitin-mediated interactions, leading to signal amplification and progression of ubiquitinated cargos along the endocytic pathway (Woelk, Sigismund et al. 2007). The other is that intramolecular interactions between monoubiquitination and UBDs within the endocytic adaptor may lead to an autoinhibitory mechanism, which causes the dissociation of the ubiquitinated receptor from the ubiquitinated cargo (Hoeller, Crosetto et al. 2006; Mukhopadhyay and Riezman 2007). These two possibilities are not mutually exclusive and both mechanisms could be involved in the regulation of endocytic processes, possibly by acting at distinct trafficking steps and/or regulating different endocytic adaptors.

6.6   Functional role for deubiquitination in receptor trafficking

Deubiquitination of proteins of the endocytic machinery critically influences the regulation of intracellular trafficking. The first role discovered for deubiquitination enzymes (DUBs) in trafficking was the removal of ubiquitin from cargos prior to their translocation into the lumen of MVBs (Swaminathan, Amerik et al. 1999). This is an essential step in sorting, which ensures recycling of ubiquitin in order to preserve its intracellular concentration, which in yeast is carried out by the DUB hydrolase, Doa4. In mammals, this deubiquitination step is performed by several DUBs. For the EGFR two DUBs have been described USP8 (Ubiquitin Specific Protease 8, also called UBPY) and AMSH (Associated Molecule with the SH3 domain of STAM). Recent studies have

established crucial, but distinct roles for these two DUBs in the fine regulation of EGFR degradation. AMSH rescues EGFR from sorting to MVBs and from its subsequent degradation, by removing ubiquitin from the receptor at early stages of endosomal sorting. This way AMSH promotes EGFR recycling (McCullough, Clague et al. 2004; Bowers, Piper et al. 2006; McCullough, Row et al. 2006). In contrast, UBPY activity seems to be required for the final delivering of the EGFR to MVBs and degradation. For that purpose, UBPY acts on multiple substrates along the sorting pathway, targeting the receptor itself, as well as endocytic adaptors and components of the ESCRT machinery for degradation (Bowers, Piper et al. 2006; Row, Prior et al. 2006). Functioning at different steps along the EGFR route to lysosomes, these DUBs balance the fate of EGFR between downregulation and recycling (Millard and Wood 2006). It was demonstrated that AMSH is involved in lysosomal but not proteasomal-dependent pathways, since it can process Lys63-linked polyubiquitin, but not Lys48-linked one (McCullough, Clague et al. 2004). On the contrary, UBPY shows dual specificity for both Lys63 and Lys48-linked chains, suggesting a more extensive role for this DUB in both lysosomal and proteasomal degradation (Mizuno, Iura et al. 2005; Row, Prior et al. 2006). Another DUB, the UCH37, has been implicated in the regulation of the type-I TGFβ receptor degradation (Wicks, Haros et al. 2005). A relatively unexplored field regards the identification of DUBs that act on monoubiquitinated endocytic proteins. Initial studies in *Drosophila melanogaster* showed that DUB fat facets (faf) is important for the correct development of the Drosophila eye. Faf exerts its regulatory role on the stability of liquid facet (lqf), a homologue of the mammalian endocytic protein epsin (Cadavid, Ginzel et al. 2000). Subsequent studies in mammalian cells confirmed the functional interaction between epsin and the mammalian homologue of Faf, named FAM/USP9X (Chen, Polo et al. 2003), although the role of FAM/USP9x in receptor endocytosis is still unclear.

Many questions, especially those concerning the regulation of DUBs and how substrate specificity is achieved and also their subcellular localization and substrate-binding partners, should be addressed to better understand the function of these crucial enzymes in receptor trafficking and other cellular pathways. The variety of structurally distinct ubiquitin modifications, the dynamism and reversibility of the ubiquitination cascade and the capability of UBDs to translate, transduce and amplify the Ub-based signal, make Ub a highly versatile intracellular messenger (Woelk, Sigismund et al. 2007).

7.   TrkA receptor implication in human diseases

TrkA was originally isolated from a human colon carcinoma as a transforming oncogene activated by a somatic rearrangement that fused a non-muscle tropomyosin gene to the kinase domain of a novel tyrosine kinase receptor, thus rendering a constitutive tyrosine kinase activity (Martin-Zanca, Oskam et al. 1989). A systematic analysis of TrkA ectodomain performed by Arévalo et al.(Arevalo, Conde et al. 2000) demonstrated that the immunoglobulin-like NGF binding region of the extracellular domain prevents the spontaneous dimerization and activation of the receptor in the absence of NGF. Thus, the TrkA onocogene was one of the first transforming genes identified in human cancers. Similar oncogenic rearrangement of the TrkA gene, causing ligand independent receptor activation was found in a consistent fraction of human thyroid papillary carcinomas as well as in acute myeloid leukemia (Nakagawara 2001). In addition, an autocrine loop, involving NGF and TrkA was shown to be responsible for tumor progression in prostatic carcinoma (Djakiew, Delsite et al. 1991) and was also demonstrated in breast cancer, medullary thyroid carcinomas, pediatric sarcomas, etc. (Brodeur, Minturn et al. 2009). The expression of TrkA in human neuroblastoma is a good prognostic marker, most likely because its signaling is important for growth arrest and/or differentiation of the neural crest derived cells from which these tumors originate (Brodeur, Maris et al. 1997). TrkA expression is low or absent in most advanced stage tumors, and they do not undergo terminal differentiation in response to NGF, which shows that the NGF/TrkA pathway is responsible for differentiation and regression of favorable neuroblastomas.

Thus, rearrangement or aberrant expression of Trk genes clearly plays an important role in a variety of cancers, therefore a better understanding of the expression, function and regulation of Trk proteins may facilitate targeting these receptors with therapeutic agents.

On the other hand, mutations affecting different TrkA domains and causing loss of activity are associated with congenital insensitivity to pain with anhidrosis (CIPA), a rare recessive genetic disease characterized by loss of pain and temperature sensation, defects in thermal regulation and occasionally mental retardation (Indo, Tsuruta et al. 1996). CIPA mutations may cause the inactivation of TrkA receptor through at least three different mechanisms: complete inactivation, protein processing alteration, and reduction of receptor activity (Pierotti and Greco 2006).

7.1. Impaired receptor downregulation is related to cancer

It is worth noted that from the 58 genes that encode RTKs, the deregulation of more than 30 has been associated with cancer development (Blume-Jensen and Hunter 2001). The increased RTK signaling is often caused by gene amplifications, chromosomal translocations or mutations that promote ligand-independent autophosphorylation, as described for TrkA receptor above. However, in addition to these positive mechanisms, there is growing evidence that escape from negative regulatory mechanisms is an important event in RTK deregulation, causing similarly neoplastic growth (Dikic and Giordano 2003).

The downregulation of tyrosine kinase activity and hence signaling can be modulated reversibly through the action of tyrosine phosphatases as well as irreversibly through their ligand-induced internalization by means of endocytosis, followed by degradation in lysosomes. As previously described, this process involves the covalent binding of ubiquitin and retention of the receptor from the recycling pathway by ubiquitin-binding proteins localized on sorting endosomes, such as the hepatocyte growth factor-regulated tyrosine kinase substrate-Hrs (Hicke and Dunn 2003). Non-ubiquitinated RTKs can recycle back to the cell surface and escape lysosomal degradation (Katzmann, Odorizzi et al. 2002).

Several lines of evidence exist which support the role for c-Cbl E3 Ub ligase for the internalization of RTKs, in addition to their targeting for lysosomal degradation following ubiquitination (Levkowitz, Waterman et al. 1999; Soubeyran, Kowanetz et al. 2002). Alterations that uncouple RTKs from c-Cbl-mediated ubiquitination and thereby downregulation are tightly associated with the pathogenesis of cancer. Examples include the Met receptor, colony-stimulating factor-1 receptor (CSF-1R), PDGFR and EGFR. These receptors have all been identified as substrates for ubiquitination, dependent on the interaction with c-Cbl for proper degradation.

It was described for Met receptor, that the c-Cbl tyrosine-kinase binding (TKB) domain binds to a juxtamembrane tyrosine (1003) residue of the receptor, an interaction which is essential for ubiquitination and degradation of the Met receptor (Peschard, Fournier et al. 2001). An oncogenic form of the Met receptor, Tpr-Met, is generated following a carcinogen-induced chromosomal rearrangement that results in the deletion of the juxtamembrane tyrosine binding site for c-Cbl (Y1003). The

Tpr-Met RTK oncoprotein is constitutively activated and non-ubiquitinated, because it fails to bind c-Cbl. This suggested that loss of Cbl recruitment and ubiquitination contribute to the oncogenic deregulation of Tpr-Met receptor, which has a prolonged half-life and is oncogenic in cell culture and in tumorigenesis assays. This result demonstrates that c-Cbl and ubiquitination are important negative regulators for this receptor (Peschard, Fournier et al. 2001).

Mutations of the Cbl binding site are frequently observed in CSF-1R in human myelodysplasia and acute myeloblastic leukemia, further implicating loss of c-Cbl binding in oncogenic deregulation of CSF-1R in human cancer (Ridge, Worwood et al. 1990). Additionally, EGFR mutant lacking only the direct c-Cbl-binding site elicits stronger mitogenic signals than the wild-type receptor (Waterman, Katz et al. 2002). A truncated EGFR receptor where an intact kinase domain is present, but the c-Cbl TKB direct binding site (Y1045) and the internalization signals are missing has been identified in human glioblastoma (Frederick, Wang et al. 2000). Furthermore, another member of the EGF receptor family, HER2/ErbB2, is overexpressed in many human tumors such as breast, ovary, prostate, and brain tumors. Overexpression of HER2 shifts the formation of EGFR homodimers toward the formation of EGFR/HER2 heterodimers. While ligand-stimulated EGFR homodimers undergo rapid ubiquitination, internalization, and degradation, EGFR/HER2 heterodimers recruit c-Cbl to a reduced extent and are slowly internalized and rapidly recycled to the cell surface (Muthuswamy, Gilman et al. 1999). Hence, the overexpression of HER2 constitutes a mechanism through which EGFR escapes Cbl mediated downregulation. Indeed, overexpression or amplification of Cbl binding RTKs, frequently observed in human cancers, could act as a mechanism to sequester Cbl proteins, enhancing the stability of other RTKs that are Cbl substrates.

Together, all described examples represent how a variety of oncoproteins avoid lysosomal downregulation by loss of the c-Cbl-binding site, inefficient c-Cbl recruitment or through the formation of fusion proteins that escape endocytosis and the degradative lysosomal pathway (Peschard and Park 2003). As ubiquitination regulates a large variety of cellular processes (Hicke and Dunn 2003) it is not surprising that it is actually a suitable target for anticancer therapy.

Examples mentioned above reveal a role for impaired endocytosis in cancer. Due to the malignant potential of RTKs of the EGFR family, these have been rational targets for novel therapeutics. One strategy involves preventing ligand interaction by blocking the extracellular ligand-binding domain using monoclonal antibodies specific

to this site. Another method is to disturb receptor activation by introducing small molecules that bind to the kinase pocket and prevent post-receptor signalling. The advantage with this approach is that it would also include receptors that are constitutively active, independently of ligand binding. A third strategy, which takes advantage of our knowledge of receptor downregulation, is to induce RTK internalization and degradation in tumours. All the three methods have been proven promising in clinical trials (Rowinsky 2004).

7.2 Growth factor receptor degradation as a cancer therapy

Understanding of the mechanisms that negatively regulate growth factor signaling and characterization of the function of such negative regulators in both normal and transformed cells can lead to identification of therapeutic targets for treatment of human cancers. Indeed, there are data to suggest that some existing therapies may act, at least in part, to enhance such negative regulatory mechanisms, although they were not developed specifically to enhance receptor degradation. Therapies that enhance growth factor receptor downregulation have been tested for activity in human cancers. Much of the information regarding downregulation of growth factor receptors has been generated by studies focusing on the EGFR. However, the basic mechanisms described for the EGFR are likely to regulate signaling elicited by many other growth factor receptors. Monoclonal antibodies that target cell surface receptors have been developed to inhibit receptor function, either by blocking activity or by inducing an immune response to the cells bearing those receptors. *In vitro* studies have shown that a variety of anti-ErbB-2 antibodies can inhibit cell proliferation. Recent work has shown that the downregulation induced by these antibodies involves recruitment of Cbl proteins and subsequent ubiquitination of ErbB-2 (Klapper, Waterman et al. 2000). In contrast, the normal mechanism of activation of ErbB-2 by heterodimerization with one of the other family members results in masking of the Cbl-binding sites (Muthuswamy, Gilman et al. 1999). Moreover, it is likely that the synergistic interaction between Trastuzumab (monoclonal antibody that interferes with ErbB-2 receptor, used for breast cancer treatment) and chemotherapy in patients is due in part to enhanced receptor downregulation. This class of drugs was initially identified as inhibitors of the Src tyrosine kinase, but subsequent studies revealed that they had no direct

effect on tyrosine kinase activity but instead enhanced degradation of a wide range of proteins, including EGFR and ErbB-2. These drugs target the molecular chaperones Hsp90 and Grp94, involved in the stabilization, folding, trafficking, and degradation of proteins. By binding to these chaperone proteins they force the chaperone complex to adopt a protein destabilizing conformation, which results in the ubiquitination and degradation of the proteins bound to the chaperones (Lipkowitz 2003).

Additional studies demonstrated that an irreversible tyrosine kinase inhibitor (TKI)-CI-1033 enhanced endocytosis, ubiquitination, and proteasomal degradation of mature ErbB-2 molecules. In addition, the TKI enhanced degradation of nascent ErbB-2 molecules. Mechanistically, it appears that TKIs perturb the structure of the chaperone protein complex associated with ErbB-2. Thus, the ligand-independent ubiquitination and degradation of membrane receptors may be relevant to the clinical efficacy of TKIs.

Altogether, these data show that ubiquitylation plays a pivotal role in desensitization of oncogenic kinases, thus being conceivable that future therapeutic strategies would employ the ubiquitylation system. The development of antibodies and/or drugs that bind to the extracellular domains of growth factor receptors and induce ligand dependent downregulation can be envisioned for a variety of receptors that are known to be important in the pathogenesis of cancer. Additionally, as more is learned about the downregulation pathway, additional targets should become apparent. Finally, agents that alter downregulation of growth factor receptors have shown to be promising as a cancer therapy. Thus, novel agents designed specifically to enhance growth factor receptor downregulation may provide a useful class of agents in the treatment of cancer.

OBJECTIVES

OBJECTIVES

A previous study performed by our group showed that CaM binds directly to the most distal part of TrkA COOH-terminus in a calcium dependent manner. Therefore, our initial objective was:

➢ Identify the amino acids on the C-terminal domain of TrkA, which conform to the calmodulin binding site.

After discovering that the candidate CaM binding domain sequence was not implicated in CaM interaction, we observed that L784A and L784A/V790A TrkA mutants were expressed at a lower amount than wild-type receptor. Therefore, we focused on the following objectives:

➢ Characterize the effect of L784A and L784A/V790A TrkA mutations on receptor stability and endosomal trafficking.

➢ Analyze the effect of TrkA C-terminal mutations on post-translational modifications, important for receptor activity and function, such as phosphorylation and ubiquitination.

➢ Identify the ubiquitin ligase involved in the overubiquitination of TrkA-L784A and TrkA-L784A/V790A mutants.

➢ Assess the influence of C-terminal mutations on TrkA signal transduction and activation of downstream signaling cascades.

➢ Analyze the capability of mutant TrkA receptors to promote cell differentiation in PC12nnr5 cells.

➢ Analyze the effect of C-terminal tail alterations on the morphology and survival of sympathetic neurons.

MATERIALS AND METHODS

MATERIALS AND METHODS

1. Cell cultures

Cells were incubated in a CO_2 incubator under a humid atmosphere at 37°C, supplied with 5% CO_2. Cell culture media, supplements, and sera were from **GIBCO®**-Invitrogen: Dulbecco's modified Eagle's medium (DMEM) with L-glutamine, 4500mg/L D-glucose, without sodium piruvate (#41965-039); Serums: FBS-fetal bovine serum (#10270-106), heat inactivated at 56°C for 30 minutes and HS-horse serum heat-inactivated (#26050-088). L-Glutamine 200mM (#25030-024), NEAA-non essential amino acids (#11140-035), sodium pyruvate 100 mM (#11360-039), HEPES 1M (#15630-056) and penicillin-streptomycin antibiotics (#15140-122) were bought as commercial sterile solutions 100x to Invitrogen. NGF Grade II (2.5S)(#N-100) was purchased from Alomone Labs (Jerusalem, Israel).

1.1. Cell culture dishes coating:

Poli-DL-ornitine: (Sigma, #P8638) Poli-DL-Ornitine stock solution was 10 mg/ml, diluted in sodium boric borate buffer (150mM sodium tetraborate, 150mM boric acid pH 8.3 approx.) We use it at a final concentration of 30µg/ml diluted in MiliQ sterile water. Cell culture dishes were covered and incubated at room temperature (RT) with this solution for one hour.

Poli-D-lysine: (Sigma, #P7280), this reagent was prepared in water at 0,01mg/ml final concentration. Cell culture dishes were covered and incubated at 37 °C for one hour. After removing the solution, cell culture dishes could be stored at 4 °C for up to one month.

Collagen: type I collagen from rat-tail (BD,#354236) at a final concentration of 100µg/ml diluted in 0.02N acetic acid. Cell culture dishes were covered with a minimum volume of the solution and left in the laminar flow hood/cabinet to be evaporated. We add medium and aspirate several times in case that the solution is not completely evaporated.

Gelatin: (Type A gelatin from swine skin, Sigma, #G9539) used at 0.1% final concentration in water.

1.2 Culture of PC12wt, PC12^{nnr5} and PC12 ^{615} cell lines

The PC12wt cell line derived from a rat pheochromocytoma has the characteristic to express TrkA and to acquire sympathetic neuron phenotype upon differentiation in presence of NGF. The PC12 $^{6/15}$ cell line is a clone of PC12 cells, stably expressing the non-neuronal form of human TrkA (donated by D.Kaplan, Montreal, Canada). On the contrary, the PC12 ^{nnr5} cell line (donated by Y.A. Barde, Basel, Switzerland) was obtained by chemical mutagenesis and clone selection of non-responsive cells to NGF, therefore it does not express detectable amounts of rTrkA (Green, Rydel et al. 1986).

Medium used for growing all PC12 cell lines was Dulbecco's modified Eagle's medium (DMEM), supplemented with 6% FBS and 6% HS; 10 mM/L HEPES, 20 U/ml of penicillin and 20 µg/ml of streptomycin. We added G418 at 250 µg/ml to PC12 $^{6/15}$ to maintain the selective stable expression of human TrkA.

While splitting the cells, the medium was renewed and 1/3 or 1/4 dilution of the cells was carried out every 3-4 days. No trypsinization was required to detach the cells, but only a mechanical method was used consisting of pipetting few times on top of the cell monolayer.

For the application of some kind of treatment, which could be aggressive for cells, it is necessary to do a coating of the culture vessels, for example poli-DL-ornitine or poli-D-lysine/collagen in case we want to perform a differentiation experiment. Coating is not required for cell maintenance.

1.3. Maintaining of HEK 293T cells

This is a cell line with an epithelial morphology,obtained from human embryonic kidney cells. We used it because of its capacity to over-express exogenous proteins and its ability to become easily transfected, in addition it is the cell line of choice for the packing and production of lentiviral particles. The growing medium is DMEM, supplemented with 20 U/ml of penicillin, 20 µg/ml of streptomycin, 10 mM sodium pyruvate and 10% heat-inactivated FBS.

To perform the subculture of this cell line we do a washing with PBS and we add trypsin at 37°C, which we leave to act for 1-2 minutes at room temperature until

cells detach. Then, we add 5 ml of DMEM-10% that will inactivate the trypsin and transfer the cell suspension to a sterile tube, Then we centrifuge for 5 minutes at 800g, remove the medium, and suspend the cell pellet in fresh complete medium. We seed the cells in new vessels at a 1/10 dilution.

1.4. **Superior cervical ganglion dissection and sympathetic neurons maintenance**

Superior cervical ganglions (SCGs) were dissected from postnatal rat pups (P1-P3) under dissecting scope, using dissection scissors and two pair of forceps. During dissection SCGs were maintained on ice in a Falcon tube containing 12 ml Leibovitz's L15 medium (Gibco,#11415-064) supplied with L-Glutamine. Medium was carefully discarded and SCGs were incubated with 300µl of 1mg/ml final concentration collagenase (Worthington Biochemical, CLS4 #4188) for 30 min at 37°C. After the time elapsed, SCGs were washed with L15 medium, which was totally removed and then ganglions were trypsinized with 500 µl of 0.25% final concentration of Trypsin (2,5mg/ml)(Worthington Biochemical TRLVMF #4454) for 30 min at 37°C, gently mixing every 10 minutes. Trypsin was inactivated by adding 10 ml of C-Medium, containing Minimum Essential Medium (MEM) with Earle's Salt and L-Glutamine (Gibco #11095-080), 10% FBShi and additional L-Glutamine (Gibco #25030-081, 200mM) at 2mM final concentration, 20 U/ml of penicillin and 20 µg/ml of streptomycin. Cells were washed several times and then ganglions were re-suspended in 1ml C-Medium and homogenized using syringe with a 21G needle, 10 times and then another 10 times through a 23G needle until the cellular suspension becomes homogenous. After careful rinsing of both needles in 1ml of C-Medium, in order to collect as many cells as possible, 10µl of the cell suspension were mixed with 1µl Trypan Blue and right after cells were counted. Cells were seeded on 4-well plates, previously coated with rat-tail collagen type I, at an approximate cell density of 5x10^4 cells/well and were maintained in the presence of 50ng/ml NGF. C-Medium was replaced on the next day with one containing anti-mitotic agents: 50µM 5-Fluoro-2'-Desoxyuridine (Sigma #F-0503) and 50µM Uridine (Sigma U-3003) to obtain 5-Flourouracil and 3.33µg/ml final concentration of Aphidicolin (A.G. Scientific A-1026). Sympathetic neurons (each 4-well plate) were transduced with 2µl of concentrated lentiviruses codifying for the empty vector, TrkAwt receptor and both mutant receptors. NGF withdrawal was initiated 5 days later to assure the expression

of exogenous receptor. Neurons were maintained in culture up to 2 weeks before being processed. Following 24h, 48h or 72h of NGF withdrawal nuclei of live neurons were stained with Hoechst 33342 and 5-7 pictures were taken on a fluorescent microscope. Same samples were lyzed and processed for western blot analysis of the activated caspase 3. Fragmented or condensed apoptotic nuclei as well as normal nuclei were counted using the MBF Image J application "Cell counter". The apoptosis was calculated as a percentage of all Hoechst positive sympathetic neurons nuclei.

1.5. Transitory transfection

Lipofectamine ™ 2000 (LF2000) transfection: we used 5µl of LF2000 and 4µg of DNA (1,25:1 ratio) for each 50% confluent, 35mm cell culture dish (p35)(approx. 750.000 cells). We pre-dilute the LF2000 and the DNA in 125µl of medium w/o antibiotics and then we mix both pre-dilutions and incubate for 20 min at room temperature, leaving the complexes DNA/LF2000 to form. Before adding to cells, we aspirate the medium and add the mixture drop by drop, covering the whole cell culture dish surface. We let for another 5 min of incubation and then we add small amount (up to 2 ml) of complete PC12 medium (with sera and antibiotics). We change the medium after 6h of incubation or on the next day.

Polyethilenimine (PEI) transfection: One hour before transfection, replace the medium with one w/o sera and antibiotics. For a p35 50% confluent cell culture dish we use 10µg of DNA diluted in 250µl of 150 mM NaCl and 50µl of PEI 1x (10µM) in 200µl of 150mM NaCl. We add the PEI solution to the DNA solution and mix immediately for 1 min (vortex). After 10 min of incubation at room temperature, we add the mixture drop by drop to the cells and swirl gently. We leave in the incubator for 3-4h and then replace the medium by complete medium.

1.6. Cell transduction

For shRNA experiments and for over-expression during the long time course of stimulation, we used lentiviral transduction of the cells. For that purpose, approx. 500.000 cells were seeded in p35 cell culture dishes, previously coated with poli-D-lysine and collagen. Immediately after seeding 5µl of the concentrated virus was added to the culture medium (for details on lentivirus production see page 80). The medium was replaced on the next day and the efficiency of the transduction was

continuously monitored under the fluorescence microscope. The percentage of GFP-positive cells was detected to be between 90-99%.

2. Molecular biology

2.1. Materials:

Enzymes: Enzymes from Roche: DpnI, NheI, XbaI, EcoRI, EcoRV SalI, KpnI, HindIII and Alcaline phosphatase, shrimp. Enzymes from New England Biolabs: BglII, FseI, SacII, AflIII and Klenow fragment DNA polimerase I. Enzymes from TaKaRa: BamHI, XhoI, SfiI, ClaI, BstXI, SphI and T4 DNA ligase. SupraTherm™ DNA Polymerase was from GeneCraft, TaqMan® Reverse Transcriptase from Applied Biosystems and *PfuUltra™* High-Fidelity DNA Polymerase from Stratagene.

Plasmids: The plasmids used in this work are: pBluescript (BSK) and pcDNA3 vector (Invitrogen), pEYFP-N1 (Clontech), pSUPER.retro.puro (OligoEngine), pCRII-SK, pEIGW and pLVTHM viral vectors were kindly donated by Dr. Trono (Trono 2000). The over-expression lentiviral vector pEIGW has a polylinker region with few restriction sites. Because of that reason, we had to sub-clone the gene of interest (hTrkA, neuronal form) into pCRII-SK vector first, and then using the restriction sites for SfiI, present in both plasmids. Another transferring strategy we applied was the following: 1) Cutting the vector sequence using the EcoRI restriction enzyme.2) Filling in the 5' overhang left after the restriction using the $3' \rightarrow 5'$ polymerase activity of the Klenow fragment, which strategy permitted us to create blunt ends. 3) Transferring the TrkA sequence to the pEIGW over-expression vector, using ClaI and NdeI restriction enzymes.

We used the cDNA for human neuronal TrkA form kindly given by Dr Dionisio Martín-Zanca (Instituto de Microbiología Bioquímica, Salamanca). TrkAi deletion mutants, sub-cloned in pGEX vector, were kindly donated by Dr Moses Chao (Skirball Institute of Biomolecular Medicine, New York).

Bacteria: We used two different *E.coli* strains DH5α for plasmid constructs and *E.coli* One shot ® Stbl3™ (Invitrogen) for lentiviral constructs. Competent bacteria were transformed using the heat-shock protocol or following provider's instructions. Frozen aliquots of transformed bacteria were stored at -80ºC in 20% glycerol.

2.2 Site-directed mutagenesis

Generation of COOH terminal mutants: We performed the mutagenesis by PCR, to obtain TrkA L784A and TrkA L784A/V790A mutants over pBSK vector, using primers which introduce the desired amino acid changes with a new restriction site, so that the selection of the transformants with mutant plasmids would be easier (Figure M1). Each pair of primers overlaps completely and has a minimum of 10 completely specific bases from the last mutation until the end. We used *PfuUltra™* High-Fidelity DNA Polymerase under the conditions recommended by the provider. After checking onto an agarose gel that the amplification was completed correctly, we digested the parental DNA template with DpnI enzyme. DpnI needs methylation of adenine residues for activity and thus digests only GmATC sequences containing N6-methyladenine. We use this characteristic to digest specifically the template DNA, which has been methylated, while produced by bacteria. On the contrary, the PCR product, which contains introduced mutations, has not been methylated and remains intact. After the digestion, we transform the PCR product in *E.Coli* DH5α and then we identify which colony contains the mutation by a restriction analysis with the appropriate enzymes. Next, we additionally verified the chosen clones by sequencing, to assure that there are no other mutations introduced on the hTrkA cDNA. Finally, we subcloned TrkA mutants into the pcDNA3 mammalian expression vector.

L784A   SacII
Forward 5'-GCCCGGCTGCAAG CCGCGG CCCAGGCACCTCC-3'
Reverse 3'-CGGGCCGACGTTC GGCGCC GGGTCCGTGGAGG-5'

V790A   XbaI
Forward 5'-CCCAGGCACCTCCTGCCTA TCTAGA TGTCCTGGGC-3'
Reverse 3'-GGGTCCGTGGAGGACGGAT AGATCT ACAGGACCCG-5'

Figure M1. Primers used for the introduction of mutations into the TrkA sequence by side deirected mutagenesis. Restriction sites used for the selection of the mutant-positive clones are highlighted in boxes.

2.3. General technique for gene cloning

Plasmid DNA from *E.coli* was extracted using Qiagen plasmid kit (Qiagen, Hilden, Germany) or Nucleobond ® AX (Macherey-Nagel) Kit following manufacturer's instructions. The concentration of DNA was determined in a Micro-Volume UV-visible Recording Spectrophotometer (NanoDrop, Thermo Scientific). Plasmid and insert were both digested with correspondent enzymes, except for the cloning of shRNAi into pSUPER plasmid (described in page 79), where the insert (annealed primers) already contain the compatible cohesive ends. At the end of the digestion, we dephosphorylated the vector with shrimp alkaline phosphatase (Roche), to avoid the religation of empty vector. The insert and the vector should be cleaned-up of salts, restriction enzymes and/or undesirable DNA fragments, so we used the QIAquick Gel extraction kit (QIAGEN). Then purified insert and vector were quantified, and the ligation reaction was set-up with a molar ratio 1:3 vector/insert respectively. We added the T4 ligase (TaKaRa) the reaction was incubated overnight at 16°C. Ligations were transformed into the *E.Coli* DH5α, using the heat-shock protocol. We checked for correct insertion by PCR, using primers annealing close to the insert region, thus the size of the amplicons obtained allowed us to distinguish between empty vector and a vector containing the insert.

2.4. Principle of RNAi action:

RNA interference (RNAi) methodology was popularized by groups working with *Caenorhabditis elegans*, when double-stranded RNAs, injected into a worm's gonad, blocked the expression of endogenous genes in a sequence specific manner. Introduced RNA fragments induce the degradation of a specific messenger RNA by binding to a particular homologue RNA sequence (Fire, Xu et al. 1998). RNAi can regulate endogenous gene expression. It also has a role in antiviral defense, in which viral double-strand RNAs are targeted for destruction by the RNAi machinery. When long dsRNAs enter cells, they are recognized and cleaved by Dicer, which is a member of the RNase III family of dsRNA-specific nucleases. This cleavage creates short dsRNAs, characterized by long 3' overhangs, called small interfering (si) RNAs. siRNAs can form a ribonucleoprotein complex called RISC (RNAi silencing complex). RISC mediates the unwinding of the siRNA duplex, so that a single stranded siRNA, coupled to RISC, then binds to a target mRNA in a sequence-specific manner, which mediates target mRNA cleavage by the "Slicer" Argonaute proteins.
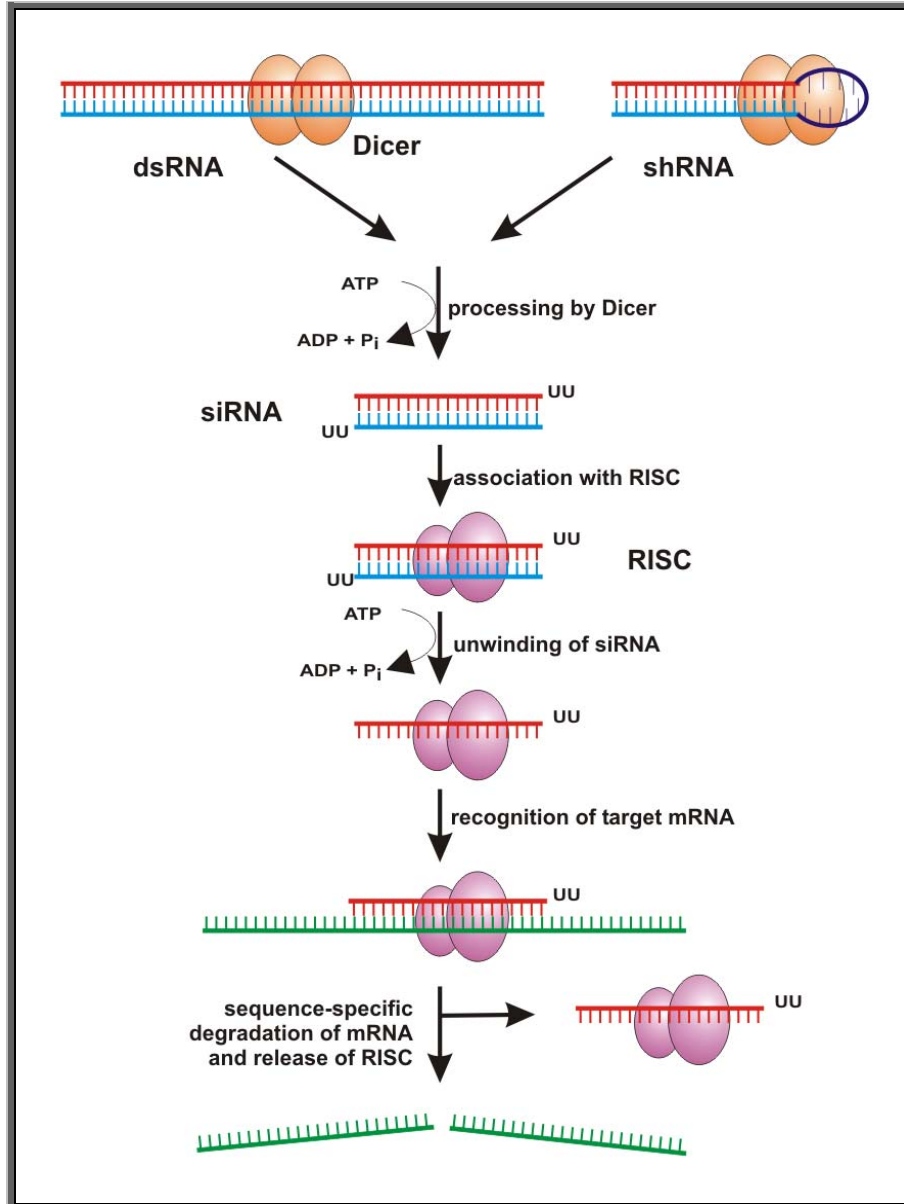
Figure M2. shRNA and siRNA mediated gene silencing*.* Different delivery strategies and processing of shRNAs in the cell are shown on the scheme above. Previously annealed shRNA primers introduced to the cell by viral transduction are processed by the enzyme Dicer and then bind to the RISC complex. RISC unwinds the double-strand siRNA and the antisense siRNA strand from the activated complex targets the homologous mRNA transcript for cleavage and subsequent degradation. The reduction in transcript level, denominated as gene silencing results in a diminished levels of the target protein. Taken from Rutz and Scheffold (2004).

The cleaved mRNA can be recognized by the cell as being abnormal and then it is destroyed by nucleases, preventing its translation. This mechanism of specific mRNA destruction results in silencing of the gene expression.

The RNA interference technology serves as a revolutionary tool for studying gene function, biological pathways, and the physiology of disease. In the present work we have used the strategy to introduce shRNA (small hairpin RNA) by lentiviral vectors into PC12 cells to silence the expression of specific endogenous genes.

2.5. Primer design for Nedd4-2 shRNAi and subcloning procedures

The pSUPER retro.puro vector (Oligo Engine) leads to efficient and specific reduction of the expression levels of the target gene. It is a mammalian expression vector, which directs the synthesis of shRNA transcripts. The subcloning strategy is based on choosing a sequence into the target gene, of 19-21 nucleotide long. For that purpose, we used specific bioinformatics programs accessible online:

Invitrogen: https://rnaidesigner.invitrogen.com/rnaiexpress/

Promega: http://www.promega.com/siRNADesigner/program/)

Thus, we designed several primer pairs against the target gene (Nedd4-2 ubiquitin ligase). Selected sequences are presented on the table below.

shRNAi for Nedd4-2 gene (5'→3')

Fwd1	gatccccAACCACAACACAAAGTCACAGttcaagagaCTGTGACTTTGTGTTGTGGTTtttttta
Rev1	agcttaaaaaAACCACAACACAAAGTCACAGtctcttgaaCTGTGACTTTGTGTTGTGGTTggg
Fwd2	gatccccAAGTGGACAATTTAGGCCGAAttcaagagaTTCGGCCTAAATTGTCCACTTtttttta
Rev2	agcttaaaaaAAGTGGACAATTTAGGCCGAAtctcttgaaTTCGGCCTAAATTGTCCACTTggg

Table 1. shRNA primer sequences chosen for the down-regulation of rat Nedd4-2 gene.

Primers were ordered to Invitrogen and upon arrival, lyophilized primers were dissolved in sterile milli-Q water at a final concentration of 3mg/ml.

Primer annealing was done in 50 mM Hepes pH 7.4, 100mM NaCl buffer using 1 µL of each primer stock (3mg/ml) to a final volume of 50 µL (primer final concentration 60 µg/ml). The protocol for primer annealing consists of progressively reducing the temperature from 90°C to 10 °C for increasing time intervals (90 °C, 4min- 70 °C,

10min- 60 ℃, 20min- 50 ℃, 30min- 37 ℃, 45min- 10 ℃, 60min). Annealed primers have protuberant ends compatible with restriction sites for BglII and HindIII, ready to be introduced directly into digested pSUPER vector.
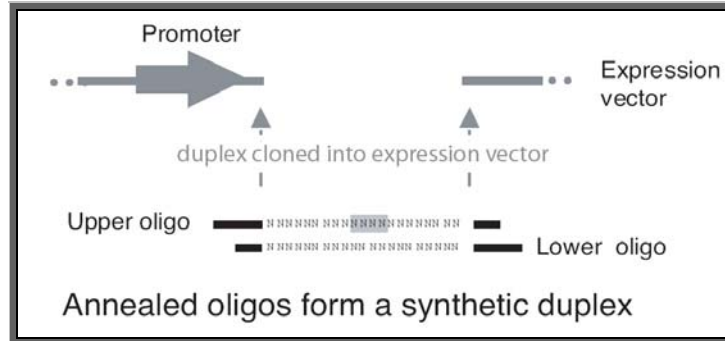
Figure M3. Design strategy for creating shRNA template inserts. Annealed complementary oligos can be used to create a synthetic DNA duplex

2.6. Subcloning of shRNAi into pLVTHM vector

The pLVTHM plasmid is the lentiviral vector we are going to use for the transduction of specific shRNAi into mammalian cells. Therefore, the pSUPER plasmid is used as a shuttle vector for our shRNA constructs. The promoter fragment H1 of pLVTHM is replaced by the"H1-shRNA"pSUPER fragment, using the restriction sites for EcoRI and ClaI. This way, we obtained shRNA constructs (Table 1) into the lentiviral vector and proceeded with the employment of the RNA interference technique to silence the expression of Nedd4-2 ubiquitin ligase in PC12nnr5 cells.

2.7. Lentivirus production

Dr Trono and colleagues have developed three generations of vectors for lentiviral production (Trono 2000). Naldini and colleagues described lentivirus production protocols in 1996 (Naldini, Blomer et al. 1996; Naldini, Blomer et al. 1996). Plasmids used for lentivirus production that belong to the second generation are the following:

1) Vectors pEIGW and pLVTHM were used for over-expression or inhibition of the gene expression, respectively. The vector itself is the only genetic material transferred to the target cells, as they have lost the transcriptional capacity of the viral long terminal repeat (LTR). The vector sequence includes the

transgene cassette flanked by cis-acting elements, required for its encapsidation, reverse transcription and integration into the genome.

2) pSPAX2 vector codifies for viral packaging proteins. psPAX2 contains a very efficient promoter (CAG), which allows the expression of viral packaging compounds, such as: TAT protein, DNA polymerase and Reverse Transcriptase. The CAG promoter includes the CMV enhancer, the chicken β-actin promoter and an intron.

3) pM2G vector codifies for a viral envelope protein from Vesicular Stomatitis Virus (VSV) and triggers the transduction of a wide range of tissues and cell lines.

We use HEK293T cells as packaging cell line for lentivirus production. Cells were seeded at a density $2,5x10^6$ in 100 mm cell culture dishes, coated with 0,1% of gelatin. Each cell culture dish was transfected with:

pLVTHM or pEIGW   20µg

pSPAX2   13µg

pM2G   7 µg

The transfection was performed according to the PEI transfection method (see page 74 for details). The efficiency of transfection was checked after 24 h on the fluorescence microscope. Cells were let to produce virus for 48h, medium was collected and centrifuged on 4000xg for 5 min to eliminate dead cells and subsequently filtered through a 45µm filter. Lentiviruses were concentrated by centrifugation at 50.000xg for 3h; and medium was removed, thereby preventing that component of cell culture medium could affect experiments. To obtain a high transduction efficiency, lentiviruses were finally suspended in 1% BSA in PBS and stored at -80 $^{\circ}$C, to preserve their transduction capacity. The biological titer of viruses was expressed as the number of transducing units per mL (TU/mL) and was determined by transducing HEK293T cells at two dilutions-1µl and 0.1µl of concentrated virus. After 48h of incubation, the percentage of GFP-positive cells was counted and viruses with $5x10^8$-$1x10^9$ TU/ml were used for the experiments.

3. Biochemistry

General reagents for preparing biochemical solutions: salts, detergents, sepharose beads were from SIGMA. The cocktail of protease inhibitors (EDTA free) was from Roche. Proteasome and lysosome inhibitors-MG132 and Lactacystin were purchased from Calbiochem.

Antibody	Dilution	Manufacturer	Catalog number
anti-pan Trk α-203	1:1500	*	*
anti-Rat TrkA	1:1000	Upstate Biotechnology	#06-574
anti-Phospho-Tyrosine Clone 4G10	1:10 000	Upstate Biotechnology	#05-321
anti-Phospho-TrkA (Y490)	1:1000	Cell Signaling	#9141
anti-Phospholipase Cγ1	1:5000	BD Biosciences	#620027
anti-Phospho PLCγ1 (Y783)	1:1000	Cell Signaling	#2821
anti-Phospho-p44/42 MAP kinase (Thr202/Tyr204)	1:5000	Cell Signaling	#9101
anti-Phospho-Akt (Ser473)	1:1000	Cell Signaling	#9271
anti-α-tubulin clone B-5-1-2	1:40 000	SIGMA	#T5168
anti-β actin	1:2000	SIGMA	#A5441
anti-FLAG® clone M2	1:5000	SIGMA	#F3165
anti-FLAG epitop (DYKDDDDK tag)	1:1000	Affinity BioReagents	#PA1-984B
anti-Ubiquitin clone P4D1	1:1000	Santa Cruz Biotechnology	#sc-8017
anti-polyubiquitin clone FK1	1:1000	BioMol International	#PW-8805
anti-Nedd4-2	1:2000	**	**

cleaved caspase-3 (Asp 175)	1:1000	Cell Signaling	#9661
anti-mouse IgG-HRP	1:5000	SIGMA	#A9917
anti-rabbit IgG-HRP	1:10 000	SIGMA	#A0545

Table 2. Table of antibodies applied in the experiments

Peptide α-203 donatedkindly provided by D. Martín-Zanca and antiserum obtained by Roser Pané in our Laboratory

**Kindly provided by Juan Carlos Arévalo, Universidad de Salamanca*

3.1 Western-blot (WB)

Cell lysis

After 32 h of transfection, cells were serum deprived for 16 h and treated with NGF (100 ng/mL) for several time intervals, as indicated. Cell monolayer was washed twice with ice-cold phosphate-buffered saline (PBS) and lysed in sodium dodecyl sulfate (SDS) lysis buffer (2% SDS and 125 mmol/L Tris–HCl pH 6.8) for western blot analysis. When immunoprecipitation experiment was performed, cells were lysed in Nonidet P-40 or RIPA lysis buffer (see pages 87-88 for details).

Sample preparation: We quantified the protein concentration of cell lysates according to the BioRad protein assay kit (BioRad, #500-0113,#500-0114,#500-0115). We prepared the samples, so that they had the same final volume and protein concentration. We added loading buffer 5x (10%SDS, 250mM Tris pH 6.8, 50% glycerol, 720 mM β-mercaptoethanol and bromophenol blue). For the samples, containing sepharose beads, we aspirate the residues of lysis buffer with an insulin syringe and then we added loading buffer 2x concentrated. We boiled the samples for 5 min and spined down at 13.000g for 1 min to collect the whole sample volume. SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE): SDS-polyacrylamide gels were prepared according to the size of the target proteins. For proteins of 100 kDa to 200kDa such as TrkA (110-140 kDa), PLCγ (148 kDa) we used 8% polyacrylamide gel and for proteins with molecular weight ranging from 20 to 100 kDa such as ERK1/2 (42-44kDa), Akt (60kDa), etc. we used 10% polyacrylamide gels. Resolving gel was prepared in 375µM Tris-HCl buffer pH 8.8, 0.1 % SDS and finally we added 0.08% ammonium persulfate (PSA) and 1µl/ml TEMED. Stacking gels were of 4 or 5% polyacrylamide in 125 µM Tris-HCl buffer pH 6.8, 0.1%SDS, 0.067%PSA and 1.7

μl/ml TEMED. Electrophoresis running buffer contained 1,44% glycine, 0,1% SDS and 25mM Tris. We loaded the samples, attempting that all wells have the same volume, adding 1x loading buffer into empty wells. Electrophoresis was run at constant current, 20 mA per gel of 1mm thickness and 8cm wide, during the time required until the front leaked out of the gel.

Transfer: We used a Hoefer semi-dry system to transfer the resolved proteins from the gel to a PVDF membrane.We cut Blotting Paper sheets (GE, #80-6211-29) and the PVDF membrane (Immobilon-P, Millipore) at the exact size of the gel. We hydrated the PVDF membrane with methanol for 1 min and then we washed it with water. Then we submerge the membrane, blotting paper and gel (separately) in transfer buffer (48mM Tris, 0.0375% SDS, 39mM glycine, 20% methanol). We placed 2 pieces of wet blotting paper on the anode (+), then the membrane and the gel and then another 2 pieces of blotting paper. To remove all the air bubbles that could have formed between the layers we rolled a plastic tube on top of the transfer sandwich carefully. Next, we placed the cathode (-) over the "sandwich" and run at constant current 0.8 mA /cm^2 of membrane surface for 1 hour.

Blocking: Once the transfer was finished we washed the membrane in TBS-T (20mM Tris pH 7.6, 150 mM NaCl, 0,1% Tween 20) and consequently we blocked in 5% non-fat powdered milk, diluted in TBS-T, for 1h at RT. For some special antibodies, such as anti-Phospho-Tyr 4G10, we blocked with 5% Bovine Serum Albumin (BSA) in TBS-T, because the milk has a significant number of proteins, phosphorylated in Tyr, which could augment the background.

Incubation with primary antibody: We diluted the primary antibody in TBS-T, according to table 2. We usually performed the incubation overnight at 4°C with constant shaking. Some antibodies, such as pan-Trk (α-203), α-tubulin or β-actin could be incubated for 1h at RT. We could re-utilize the primary antibody, by adding 0,02% of sodium azide to preserve from contamination and storing the diluted antibody at 4°C.

Incubation with secondary antibody: We wash three times with TBS-T and incubate with the appropriate secondary antibody conjugated to horse radish peroxidase (HRP), diluted in blocking solution for one hour at RT.

Development: The secondary antibody is conjugated to peroxidase, therefore to detect its presence we use a commercial reagent, the EZ-ECL kit (Biological Industries) that associate the catalysis of hydrogen peroxide with the luminol oxidation, which leads to the emission of light (chemiluminescence). With

antibodies that produce a faint signal with this method, we develop with Super Signal® West Dura (Pierce), which has a stronger and prolonged signal. Right after the ECL reaction, we expose membranes on photosensitive films Fuji super RX, at several time intervals and we use the Kodak HC 110 developer and Kodak Tmax fixation solution for film development.

Removing of antibody complexes from the membrane (membrane stripping): If we want to incubate the membrane with a differnet primary antibody, and the first iimmunodetection can interfere with the result, we must eliminate the antibody complexes by "membrane stripping". We have used two different stripping solutions: the first was 62.5 mM Tris, pH 6.8, 2% SDS and 100mM β-mercaptoethanol for 30 min at 60°C with constant shaking. Alternatively, we used a special commercial stripping solution-Restore ™ Western Blot Stripping Buffer (Pierce) and we incubated the membrane at 37°C for 15-30 minutes. This stripping, in a difference to the previous one is softer and does not require re-blocking of the membrane. After stripping, membrane was washed well and we initiate the second immunodetection process with or without blocking, depending on the type of stripping solution we used.

3.2 Recombinant protein purification

We generated TrkAi recombinant proteins, fused to the glutathione (GSH)-binding domain of the glutathione S-transferase (GST), which allowed us to purify proteins by using GSH-Sepharose beads.

Bacteria culture: *E.coli* DH5α transformed with the pGEX vector containing de gene of interest were inoculated and grown overnight at 37°C, under constant shaking in 10 ml of LB medium and 100 µg/ml of Ampicillin. Next day, the pre-culture was diluted in 200 ml of LB medium with Ampicillin and were incubated at 37°C until the OD$_{600}$ ≈0.8.

Expression: We induced the expression of the recombinant protein by adding 1mM IPTG, and bacteria were incubated overnight with constant shaking at RT. After induction, cells were centrifuged at 2600g for 20 min at 4°C. We washed the pellet with PBS and centrifuged again. The procedure could be stopped at this point by storing the pellet at -80°C, or proceed with the lysis.

Lysis: We suspended the bacterial pellet in 15ml of STE buffer (40mM Tris pH 8.0, 25mM EDTA, 150mM NaCl) with 0,1 mg/ml lysozyme and protease inhibitors, such as PMSF aprotinine, leupeptine, benzamidine. The buffer should be replenished

with PMSF during the process. Cell lysate was incubated on ice for 20 min, mixing with the pipette from time to time. Then we added 6mM Dithiothreitol (DTT) and 1.2%Sarcosyl and the lysate was sonicated during 5min for 5sec intervals at highest potential and 1min pause, while maintaining the sample on ice. At the end of the procedure, the sample became translucent. If the lysis was not complete it stayed turbid and most of the protein will be lost. Further, we added 2%Triton X-100 and we brought the lysate to a final volume of 30 ml with STE and incubate for 30 min at RT mixing periodically with the pipette. Finally we centrifuged at 16 000 rpm for 30 min at 4°C and kept the supernatant.

Purification: To purify the protein we incubated the supernatant for 3h at 4°C on the orbital shaker, with 1ml of glutathione-sepharose beads, previously washed with STE buffer. At the end of the incubation, three washes were performed in 40mM Tris pH 8, 150 mM NaCl, 2% Triton X-100, 1mM DTT For the elution we used: two volumes of 10 mM reduced Glutathione, in 50 mM Tris pH 8.0, 150mM NaCl, 0,1% Triton X-100 for 10 min at RT on the orbital shaker and we repeated the elution procedure three times. We quantified the eluted protein by SDS-PAGE and Coomassie Brilliant Blue R-250 staining, comparing to a known BSA amount range.

Concentration: We collected all the protein fractions in a concentration filter with a exclussion cut-off membrane of 10 kDa (Amicon), previously washed with water. We centrifuged at 3000g at 4°C until remaining around 500 µl. Further, we changed the buffer by adding 20mM Tris pH 8, 50mM NaCl, 1mM DTT. Then we concentrate again up to the desirable volume and we added 5% glycerol for its proper conservation. Protein concentration was quantified by SDS-PAGE and coomassie blue staining and we stored protein aliquots at -80°C.

Note: RT induction slows down the expression, which increase the proportion of correctly folded protein. We could induce the expression of soluble proteins at 30-37°C and it is not necessary to use Sarcosyl for the lysis. Another critical step is the sonication, because if the lysis is incomplete we lose a big part of the protein with the pellet. We could collect aliquots at each step of the procedure: before and after the induction, from the lyisis pellet and before the GST-Sepharose binding. If we have a small yield, we could run all aliquots in a SDS-PAGE and do a Coomassie staining. We always lose greater part of the protein in the lysate pellet, usually the denatured fraction of the recombinant protein. If the proportion of protein in the supernatant is smaller than the one we have in the pellet, we have a problem in the protein solubility. If we do not see it total lysate we have a problem in the induction.

We should avoid warming the samples and take them out of the ice only when the protocol requires it, thus avoiding degradation.

3.3 Calmodulin Pull down Assay

We treated cells according to the experiment requirements, then we washed them with ice cold PBS and lysed in Triton X-100 lysis buffer: 20mM Tris pH 7.4, 150mM NaCl, 1% Triton X-100, 1mM $Na_3 VO_4$, 25 mM NaF, 50mM β- glycerophosphate and cocktail of protease inhibitors.. We used 1 mg of protein from total cell lysates or 0,25-1µg of purified recombinant protein. We added 20µl of CaM-Sepharose beads (40µl of a 50% slurry), previously blocked with 1% BSA and then we added $CaCl_2$ or EGTA up to the required final concentration. Then, we incubated for 2h at 4^oC on the orbital shaker. We washed the beads three times with $CaCl_2$ or EGTA buffers, taking care not to mix them and spinning down at 6000g for 3min. Finally, we added the loading buffer and detected the presence of the protein of interest bound to the Sepharose beads, by western blot (WB).

3.4 Immunoprecipitation of TrkA/Co-IP Nedd4-2/TrkA

There are specific antibodies, which detect phosphorylated Tyr in TrkA, but undoubtedly a more sensitive approach is to detect phosphorylated Tyrosines with the anti-Phospho-Tyr 4G10 antibody on TrkA immunoprecipitates.

We use NP-40 lysis buffer for cell lysis and TrkA immunoprecipitation: 20mM Tris 7.4, 140mM NaCl, 10mM EDTA, 10% glycerol, 1% Nonidet P-40, 1mM $Na_3 VO_4$, 25 mM NaF, 40mM β- glycerophosphate and cocktail of protease inhibitors.

Once cell treatment finished, we washed the cell monolayer with ice cold PBS and placed the dishes on ice. If we wanted to stop the procedure here, we removed completely the PBS and froze the cells at -80^oC. The whole immunoprecipitation protocol was performed on ice. For cell lysis we added some buffer (150µl/p60 cell culture dish), we recovered all the cells debris with a plastic scraper and collected them in a 1.5ml tube. Lysates were incubated on the orbital shaker for 20 min to complete the lysis and then were centrifuged 15 min at 13 000rpm to eliminate the insoluble cell debris and nuclei.

We performed the immunoprecipitation with a fixed amount of total protein (300µg-1mg) adjusting to the same concentration for each sample and adding NP-40 lysis buffer up to 500µl. Then 1µl of α-203 antibody or 20 µl of FLAG-Sepharose

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beads/sample were incubated for 1-2h on the orbital shaker at 4 C°. Afterwards, 20µl protein A-Sepharose beads suspension (40µl suspension 50%) were added to the samples and incubated for additional 2h or left overnight. The incubation could be performed for 2h or overnight on the orbital shaker at 4°C. Then, we washed three times with the lysis buffer w/o protease inhibitors, and to pellet the beads we centrifuged at 6000rpm for 3 min. Finally, we added the loading buffer and analyzed samples by SDS-PAGE and WB.

3.5 Biotin labeling of membrane proteins

Assessment of TrkA stability by biotin labeling:

We use PC12nnr5 cells transiently transfected with TrkA constructs, following the protocol of LF2000 transfection. First, we washed cells twice with PBS at RT to remove any medium component that could affect the procedure. The biotinylation was performed by adding EZ-Link®Sulfo-NHS-Biotin, 1mg/ml (Pierce), a non-permeable and non-cleavable biotin, which reacts with primary amines present on proteins. It will just label the extracellular domain of membrane proteins. Cells were incubated in presence of the biotin at RT for 15 min and at the end, cells were washed twice with TBS to quench non-conjugated biotin. Then cell treatment was started by adding medium with or w/o NGF (100ng/ml) at 37ºC and placing the dishes into the CO_2 incubator. At 2h time intervals, dishes of each condition were removed and cells were washed with ice-cold PBS and freezed at -80°C.

 Cells were lysed in RIPA buffer: 50 mM Tris pH7.4, 150mM NaCl, 1% NP-40, 0,1% sodium deoxycholate, 0,1% SDS, 5mM EDTA, 40mM NaF, 1mM Na_3VO_4 and 1x EDTA-free protease inhibitors cocktail. We measured sample protein concentration with a BioRad Protein assay kit. The same amount of total protein (0.3-0.5mg) was transferred to a 1.5ml eppendorf tube and brought to a final volume of 500 µl with RIPA buffer. Then 20µl Streptavidin-Sepharose beads were added to each sample and a Streptavidin pull-down of biotinylated proteins was perfomed. Samples were incubated for 2-4h on the orbital shaker at 4°C. After washing three times in RIPA buffer, loading buffer was added and samples were analysed by WB. Biotinylated membrane proteins were immunodetected with anti-pan-Trk α-203 antibody, which identifies only the non degraded, after time intervals have elapsed, biotinylated receptor, pulled down with the streptavidin-sepharose beads.

The estimation of receptor degradation was performed by quantifying the amount of biotinylated TrkA on each lane, using MBF Image J software. Note that for the

L784A/V790A mutant a longer film exposure was used on the measurement, due to the lower amount of membranal TrkA receptor.

Receptor internalization experiment using biotin labeling

PC12nnr5 transfected cells following the LF2000 protocol were serum deprived overnight and used for the biotinylation of membrane proteins. Cells were washed twice with PBS and biotinylated with EZ-Link®Sulfo-NHS-SS-Biotin (Pierce) for 5 min at 25C^o. Non-conjugated biotin was removed by two washes with TBS, and cells were incubated in medium with or without NGF for several time intervals. At the end of each incubation period, cells were placed on ice, membrane biotin was cleaved with glutathione cleavage buffer (50 mmol/L glutathione, 75 mmol/L NaCl, 10 mmol/L EDTA, 1% bovine serum albumin, and 75 mmol/L NaOH) and cells were washed twice with 5mg/mL iodoacetamide in PBS and once with TBS. Cells were lysed in RIPA buffer: 50 mM Tris pH7.4, 150mM NaCl, 1% NP-40, 0,1% sodium deoxycholate, 0,1% SDS, 5mM EDTA, 40mM NaF, 1mM Na_3VO_4. 1x EDTA-free complete protease inhibitor cocktail and equal amounts of protein were submitted to a streptavidin–agarose pulldown for 2 h. After three washes in lysis buffer, Laemmli loading buffer was added to the samples and a western blot was performed using anti-pan-Trk antibodyand anti-TfR antibodies.

4. Cell biology

4.1 Immunofluorescence (IF)

Immunofluorescence was performed on cells growing on coverslips, for the acquisition of confocal images on the Olympus FluoViewTM FV500 confocal laser scanning microscope. Coverslips were sterilized in 70% ethanol and placed in 4-well cell culture plates (Nunc). Before seeding, coverslips were coated with poli-D-lysine and collagen.

Cell culture: Cells were seeded at low density, since it was important to acquire images of isolated cells. Therefore 10 000 cells/well were seeded for confocal microscope image acquisitionm and 25 000cells/well for differentiation experiments.

Fixation: Once cell treatment was finished, cells were fixed for 15 min in 4% paraformaldehyde (PFA)-PBS. Then cells were washed three times with PBS (at this step fixed cells could be kept at 4°C in PBS).

Permeabilization: 0.5% Triton X-100 in PBS for 1 min.

Blocking: 0.5% BSA, 1% FBS in PBS for 30 min.

Antibodies: Antibodies were diluted in PBS, supplied with 1/5 of blocking solution: anti- TrkA α-203 was used at 1:1500 dilution, anti-FLAG was added at a 1:2000 dilution. Primary antibody was incubated for 1h at RT or overnight at 4°C. When we performed an overnight incubation or when we had limiting volume of the antibody, we incubated with a small volume of primary antibody applied as a drop (50 µl) on each cover slip, performing the incubation in a humid chamber to avoid drying. After the primary antibody, coverslips were washed three times with PBS and incubation with the appropriate secondary antibodyat a 1:500 dilution: anti-Rabbit IgG Alexa Fluor-488 (Molecular probes, #A-11008) or anti- Mouse Alexa Fluor-594 (Molecular probes, #A-11005).

Mounting: Before mounting a final washing step with milliQ water was performed, to avoid salt crystals formation. Coverslips were mounted on slides with Mowiol or Vectashield, taking care that no air bubbles remain captured. For confocal microscopy only Mowiol was used.

Notes:

*When nuclear staining with Hoechst 33342 was performed, it was added together with the secondary antibody.

*Diluted antibodies with Hoechst were centrifuged 5 min at 13 000rpm to eliminate aggregates which could have formed and could contaminate the preparation.

*Samples were protected from light after adding the secondary antibody.

4.2 Differentiation assay of PC12nnr5 cells

PC12nnr5 cells were seeded at a low density (25 000 cells/well) into 4-well plates, previously coated with poly-D-lysine/collagen. Cells were transfected with LF2000 and after 24h of expression cells were treated with 100ng/ml of NGF in DMEM medium supplied with 0,5% HS. Medium was renewed with NGF on the third day of the treatment, changing just the half of the medium to avoid cell detachment. After 5 days of treatment, cells were fixed with 4% PFA/PBS and processed with the immunofluorescence procedure previously described. The following parameters were used: permeabilization: 1min in 0,5% Triton X-100in PBS; primary antibody: anti-

Trk α-203, 1h at RT; secondaryantibody: anti-Rabbit IgG-Alexa Fluor-488 at a 1:1000 dilution, 30 min at RT.

Cell counting was performed in a fluorescence microscope Olympus IX-70 inverted microscope (*Olympus* Corporation, Tokyo, Japan) equipped with a U-RFL-T module. Cells with very faint fluorescence and those very bright were excluded, since the overexpression of transfected TrkA was out of the physiological range. Total number of TrkA expressing cells and differentiated cells were counted. Only cells with prolongations twice longer than the body size were considered as differentiated. With the obtained data the percentage of cell differentiation was calculated.

4.3 Anti-Rat TrkA induced TrkA internalization

PC12nnr5 cells were transfected with TrkAwt and mutant receptors, following LF2000 method. After 48h of expression cells were treated with anti-Rat TrkA antibody, which can activate TrkA in the absence of NGF, as it was described by Clary and colleagues (Clary and Reichardt 1994). A dilution 1:10 in a complete DMEM medium was used, and cells were incubated for two time intervals: 2h and 4h. At the end of the treatment cells were fixed and processed following the general immunofluorescence procedurel using as blocking and permeabilization solution 5% HS,5% FBS, 3mg/ml Glycine, 0,1% Triton X-100 in PBS. Then immunodetection of the Lysosomal marker LAMP1 was performed with an antibody from Abcam (#ab13523) at a dilution 1:500 in PBS for 1h at RT.After washing, cells were incubated with the secondary antibodies: anti-mouse IgG Alexa Fluor-594 and anti-rabbit IgG Alexa Fluor-488, both at a 1:500 dilution, 1h at RT. Coverslips were mounted on slides and pictures were obtained on the Olympus confocal microscope with the immersion objective 60x. Image J software with the Intensity Correlation Analysis plugin was used to obtain the overlapping coefficients of both channels and to perform the colocalization analysis for each condition.

5. Bioinformatics tools

5.1 ClustalW:

We use this program to perform the alignments of Trk sequences (Thompson, Higgins et al. 1994). ClustalW WWW Service at the European Bioinformatics

Institute. Rodrigo Lopez, Services Programme and Andrew Lloyd. The ClustalWWW server at the EBIembnet.news volume 4.2 1997

http://www.ebi.ac.uk/Tools/clustalw2/index.html

5.2 Calmodulin Target Database:

This is a database of proteins that bind CaM. It provides searching tools for putative CaM binding domains and description of their characteristics.

http://calcium.uhnres.utoronto.ca/ctdb/flash.htm

5.3 pDRAW32

pDRAW32 is a free program for plotting plasmid sequences, manipulate DNA constructions and to design cloning strategies. pDRAW32 by Kjeld Olessen. Acaclone software: http://www.acaclone.com/

5.4 MBF Image J

This application permits to visualize and manage images obtained with any microscope. It permits to limit select regions of interest, quantify the intensity, change the visualisation colors of the image, to perform a colocalization analysis and many other different operations with images. We used the Intensity Correlation Analysis plugin, which generates Mander's coefficient (R), an overlap coefficient that represents the percentage of red dye molecules that share their location with green dye molecules. We use +ve PDM images to show the colocalization rate for each conditions. Generated positive (+ve) PDM (Product of the Differences from the Mean) value images are two slice images, where the first slice represents values resulting from both pixels above the mean (i.e. (red intensity-mean red intensity) and (green intensity-mean green intensity) are both positive). The second slice represents pixels that have pixel values in each channel, which are both below the mean (i.e (red intensity-mean red intensity) and (green intensity-mean green intensity) are both negative). For facility, we have proposed the name of "colocalization-rate displaying images"- (CRD images), which mode we will refer to them further.

MBF Image J ver 1.36b by Wayne Rasband. NIH, USA.

http://rsbweb.nih.g

RESULTS

RESULTS

1.   Searching for the TrkA calmodulin binding domain

A previous study from our group demonstrated that CaM binds directly in a calcium dependent manner to TrkA. To define the exact region where this interaction takes place, an *in vitro* assessment was performed by using several recombinant TrkA proteins. Two of these proteins were: the entire intracellular fragment of TrkA (433-796), which was previously demonstrated to bind CaM in a Ca^{2+} dependent manner (Llovera, de Pablo et al. 2004) and a truncated form containing only the COOH terminal tail (720-796). Both proteins were produced as fusion proteins with a GST tag, used for their purification (Figure R1). CaM pull-down assay with both recombinant proteins confirmed previous results, showing that CaM binds to TrkAi (433-796) cytoplasmic domain and additionally demonstrated that the most distal TrkAi fragment (720-796) has a greater CaM binding capacity when Ca^{2+} was present in the medium, comparing to the entire TrkAi fragment. This result evidenced the presence of the CaM binding motif on the region 720-796 of TrkA, the COOH-terminus of the receptor.
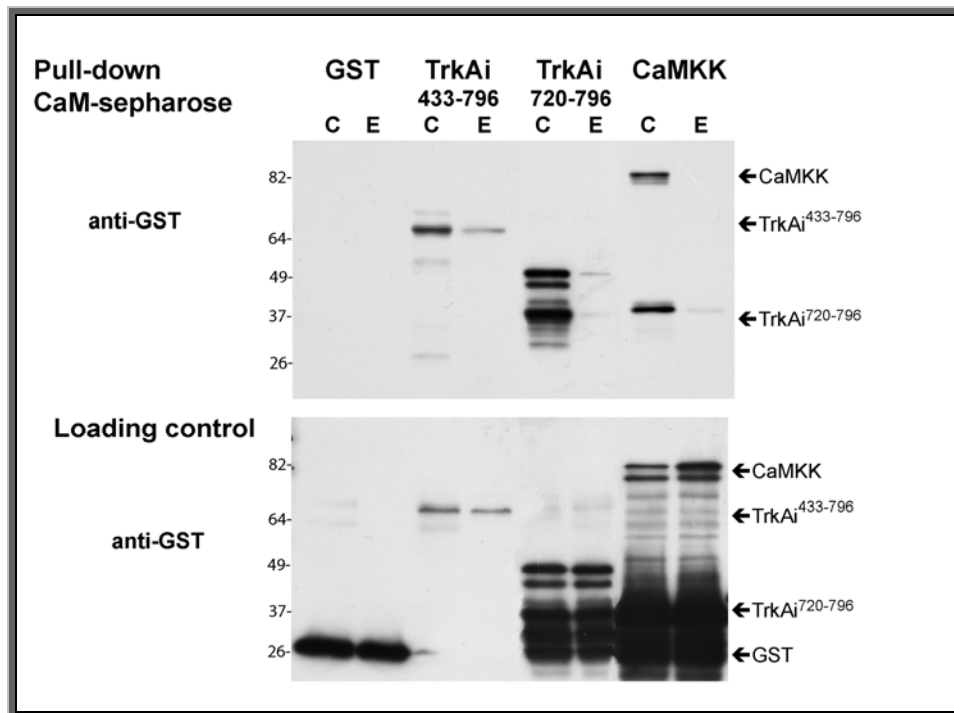

Figure R1. CaM binds directly to the TrkA COOH terminal domain (720-796) of TrkA in the presence of Ca^{2+} CaM sepharose interaction assay with the complete TrkA

intracellular domain (TrkAi$^{433-796}$) and a C-terminal fragment (TrkAi$^{720-796}$) fused to GST in the presence of Ca^{2+} (100µM) or EGTA (2mM). GST-fusion proteins were detected by western blot using an anti-GST antibody. GST alone and GST-CaMKK recombinant protein were used as negative and positive controls of the assay, respectively. The upper panel shows the pull-down result, i.e. the fraction bound to CaM sepharose beads, and the lower panel shows a loading control of the assay (input protein).

Calmodulin Binding Domain (CBD) prediction programs do not detect any candidate sequence in this region (Figure R2). Therefore, we consulted Dr Neil McDonald for his expertise in protein structure (Structural Biology Laboratory, Cancer Research UK). He proposed that the COOH terminal, situated out of the kinase domain could be implicated in the CaM interaction with TrkA. Analyzing the sequence in this region, we found a motif, which contains hydrophobic amino acids in positions 1-5-8-14 that could conform to a CaM binding site (Figure R4). We hypothesized that this sequence could contain a 1-14 type CaM binding motif ($\boxed{\text{V}}$HAR$\boxed{\text{L}}$QA$\boxed{\text{L}}$AQAPP$\boxed{\text{V}}^{777-790}$), because of the disposition of hydrophobic residues in positions 1-5-8-14 and the prediction for its structure, one α-helix part and another unfolded. Leaded by this hypothesis, we generated two TrkA mutants of the COOH-terminal tail, by substituting the hydrophobic amino acids on positions 8 and 14 by Ala: thus generating the TrkA-L784A mutant and the double mutant TrkAL784A-V790A. Subsequent pull-down assays were performed, either with recombinant proteins of the TrkA intracellular domain fused to GST, or on total cell lysates of PC12nnr5 cells, a subclone of PC12 cells that do not express detectable amounts of endogenous TrkA receptor and are nonresponsive to NGF. CaM pull-down assays, carried out on transiently transfected cells with the wild-type and mutant receptors showed that generated mutations in TrkA C-terminal tail did not result in a loss of CaM interaction, despite the different levels of protein expression (Fig R3, upper panel). In addition, pull-down assays performed with the same amount of recombinant protein demonstrated that TrkA C-terminal mutants had a similar binding affinity for Ca^{2+}/CaM (Figure R3, lower panel).
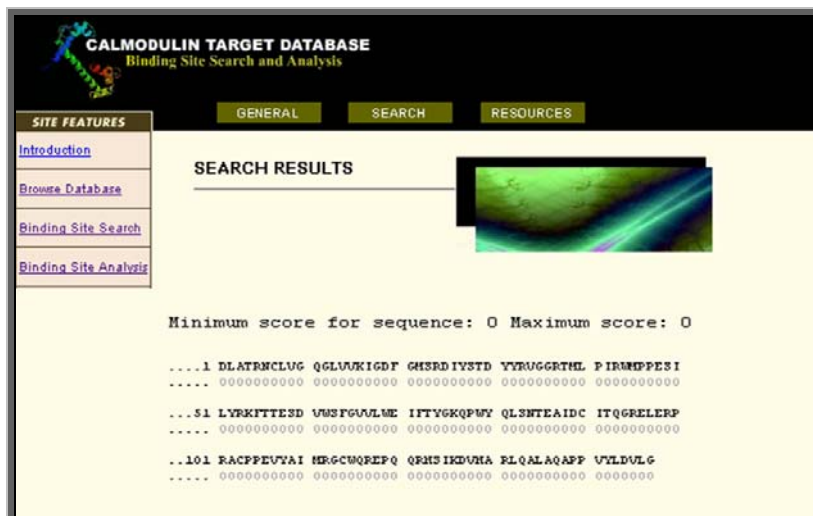
Figure R2. Pediction for a putative CBD in the C-terminal tail of the receptor, when the TrkAi (720-796) sequence was explored in the Calmodulin Target Database. *http://calcium.uhnres.utoronto.ca/ctdb/ctdb/home.html*

The prediction program showed that there is no candidate sequence in the C-terminal region. None was predicted in the complete TrkA intracellular sequence either.
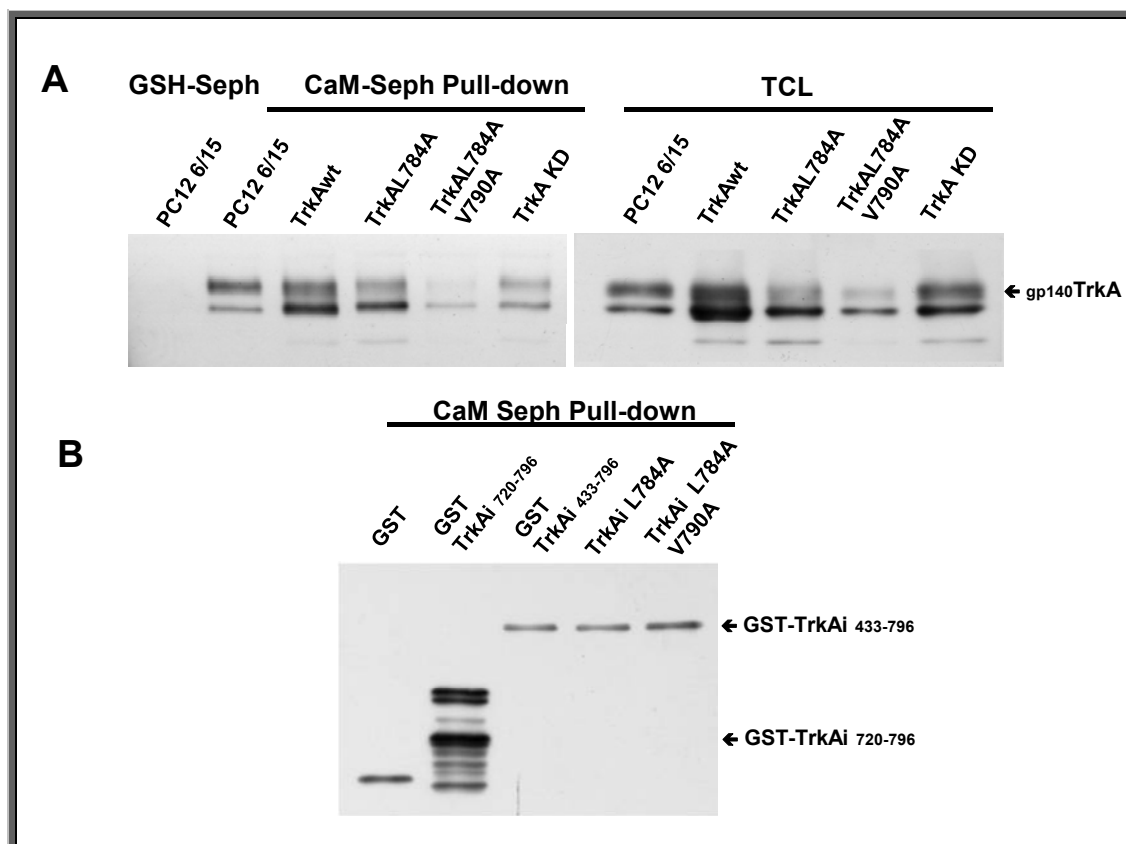
Figure R3. C-terminal mutants do not lose their capabiliity to interact with CaM.

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(A) CaM pull-down assay performed on total cell lysates of PC12nnr5 cells transiently transfected with TrkAwt, C-terminal mutants and the kinase death receptor (TrkA-KD; K544N). PC12 $^{6/15}$ cells, over-expressing hTrkA were used as a positive control for CaM interaction and as a negative control with Glutathion-Sepharose beads, proving the specificity of CaM-TrkA interaction. Note that a longer exposure was needed to be able to detect the precipitated TrkAL784A-V790A mutant. Total cell lysate samples, run in parallel to the pull-down samples showed a strong reduction in L784A-V790A mutant expression levels,.The pull down assay was carried out in the presence of 100μM Ca^{2+}. (B) In vitro CaM pull-down was performed with recombinant truncated form of TrkA (TrkAi$^{720-796}$) and the entire intracellular fragment of the wt receptor and L784A and L784A-V790A mutants fused to GST. TrkAi$^{433-796}$ wild type and TrkAi mutant receptor fragments show equal ability to bind CaM in presence of Ca^{2+} (100μM CaCl$_2$). CaM-bound proteins were analyzed by SDS–PAGE and western blot, using specific antibodies against GST.

Even though these mutant receptors did not lose their interaction with CaM, they showed lower expression levels, which encouraged us to continue working on the characterization of the effect of these mutations on the receptor activity and regulation.

2.   Characterization of the C-terminal TrkA mutants

The comparison of the C-terminal tail of Trk family members with several other RTKs present in neuronal cells (Figure R4) reveals that neurotrophin receptors, together with RYK and MusK, have a very short C-terminal tail, comparing to other members such as Ret, Met, InsR and EGFR that bear a long C-terminal tail with multiple functions. In addition, this region has a high content of hydrophobic amino acids in conserved positions, which points out to a role on the overall conformation of this region.
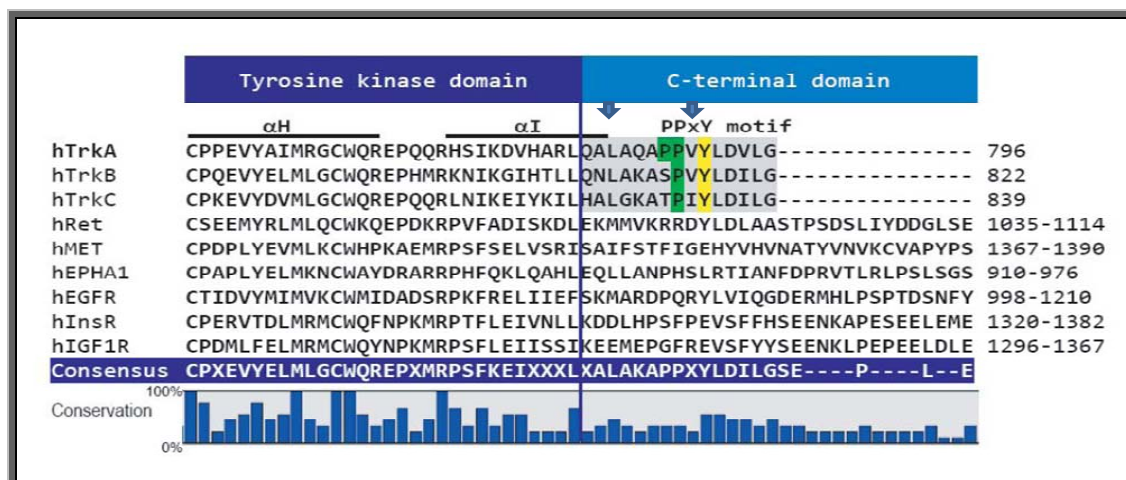
Figure R4. C-terminal domain alignment of human Trk family members with other tyrosine kinase receptors present in nervous system. Position of alpha-helices is indicated on the top (αH and αI). The position of the Nedd4-2 binding motif (PPXY) of TrkA is labeled and highlighted in green (P) and yellow (Y), which is absent in TrkB and TrkC receptors. Arrows show the hydrophobic amino acids that have been chosen for site-directed mutagenesis. The bottom histogram represents the amino acids conservation among the different TK receptors included in the alignment. Note that Trk family members have a very short C-terminus (shaded in grey).

3. Effect of TrkA C-terminal mutations on receptor stability

Expression of our TrkA constructs in PC12nnr5 cells showed a reduction on the amount of receptor present when L784 was mutated, and this effect was even more evident for the L784A-V790A mutant (Figure R3, upper panel). In order to verify whether this effect was due to a reduction on the antibody binding affinity, we verified TrkA levels with two anti-TrkA antibodies: first, the antibody used on Figure R3, anti-Trk α-203 raised against the last 14 amino acids sequence of hTrkA and second, the anti-Rat TrkA antibody raised against the extracellular domain of rTrkA, that cross-reacts with hTrkA. Western blot analysis with the anti-Rat TrkA antibody evidenced a reduction on the amount of L784A TrkA mutant that was even more apparent for the L784A-V790 TrkA mutant (Figure R5 A first panel). Similar results were obtained with the anti-Trk α-203 antibody (Figure R5 A second panel). Total cell lysates of PC12wt and PC12nnr5 were loaded as control samples. Endogenous rat TrkA on PC12 wt cell lysate was only detected with the anti-Rat TrkA antibody, because anti Trk α-203 has a higher affinity for human TrkA than for rat TrkA under

denaturing conditions. PC12nnr5 cells did not express detectable amounts of rat TrkA.
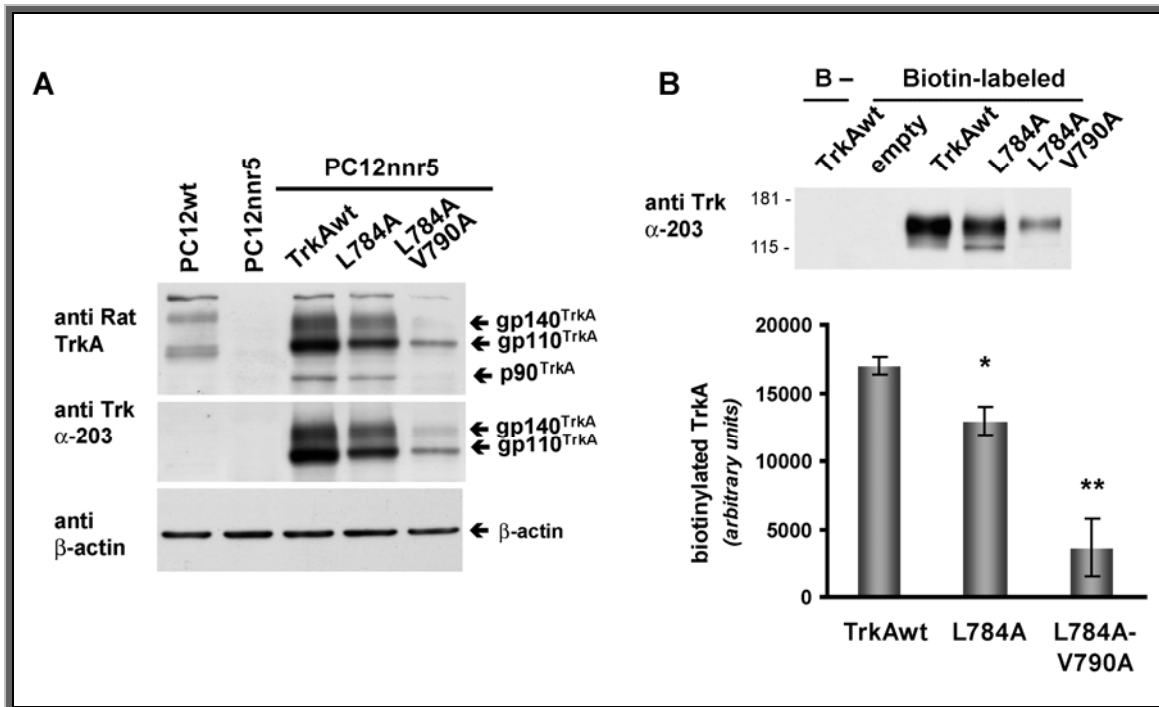
Figure R5. C-terminal TrkA mutant receptors show lower expression levels (A) Reduction of basal expression levels of the receptor observed in TrkA C-terminal mutants compared to the wild-type receptor, while overexpressed in PC12nnr5. Two different antibodies were used, the anti-Rat TrkA against the extracellular domain of Rat TrkA (upper panel) and the anti-Trk α-203 raised against the C-terminus of the hTrkA. Total cell lysates of PC12wt and PC12nnr5 cells were loaded as control samples. β-actin was used as a loading control. B) C-terminal mutants show a decreased level of plasma membrane receptor. The amount of receptor exposed to the surface was measured by biotinylation assay, streptavidin pull-down and then immunodetection by western blot. Bands on films were quantified by MBF Image J software, and results from three independent experiments were plotted in the graph below. Statistical significance was measured by the Student-T test (* $p<0.05$ ** $p< 0.01$), showing a reduction of the surface protein on both mutant receptors, being more significant for the TrkA L784A-V790A receptor.

After the observation that the amount of the mature isoform gp140^{TrkA} of the receptor is reduced when the C-teminus is mutated, we wanted to assess whether this alteration correlates with a reduction on the amount of plasma membrane receptor. To answer this question, we performed a cell surface protein biotinylation assay, using a non-cleavable biotin. Results showed a decrease in the amount of

biotinylated receptor, i.e. TrkA present at the plasma membrane (Figure R5 B). Receptor expression levels were quantified on the blots using MBF Image J software and are results are represented on the graph as arbitrary units.

As the vector and the promoter sequence are the same for all the TrkA constructs, we would not expect to find differences in the protein synthesis rate. In fact, we detected a proportional decrease in all the TrkA specific bands: gp140, gp110 and p90, without accumulation of the immature forms (gp110 and/or 90 kDa bands), indicating that the maturation step was not altered. Considering these observations, we have concluded that the strong decrease in the level of the C-terminal mutants was probably related to an increased degradation rate of the receptor.

4.   C-terminal TrkA mutants have an increased degradation rate and a faster lysosomal targeting

With the aim to verify whether the reduction of receptor's level, evident for TrkA C-terminal mutants, is due to an alteration of the receptor stability, we measured TrkA degradation rate by a biotinylation assay. Membrane proteins of PC12nnr5 transiently transfected cells were labeled and then cells were stimulated with NGF (100 ng/ml) or left untreated. Samples were obtained at 2 hours time intervals up to 8 hours and right after cells were lysed. The amount of biotinylated TrkA receptor was analyzed by streptavidin pull-down assay and western blot with anti-Trk α-203 antibody. Results obtained for the biotinylated TrkA in the absence of neurotrophin (Figure R6-left graph) or in the presence of NGF (Figure R6-right graph) show that total biotin labeling for non-stimulated TrkA dropped from 2 hours onwards and only around 30% of the labeling remained after 8 hours. We observed that the degradation of the TrkA-L784A and TrkA-L784A-V790A mutant receptors was significantly faster, since only 9,4% and 6,7% respectively of the labeling remained after 8h. A similar result was obtained on NGF stimulated cells. Only at the time point of 2 hours, a delay on TrkA receptor degradation was observed in the presence of the ligand (paired student T-test p= 0.033), but this effect was not maintained at longer time intervals (Figure R6). An estimation of TrkA degradation rate was performed by plotting the percentage of TrkA biotinylation on a logarithmic scale versus time. The slope value of the linear regression provides a measure of the degradation rate. Analysis of TrkA biotinylation data, confirmed that the degradation

rate of TrkA-L784A receptor was 1.9 times higher and the TrkA-L784A-V790A receptor one was 2.2 times higher than TrkAwt slope value (Figure R6 table).
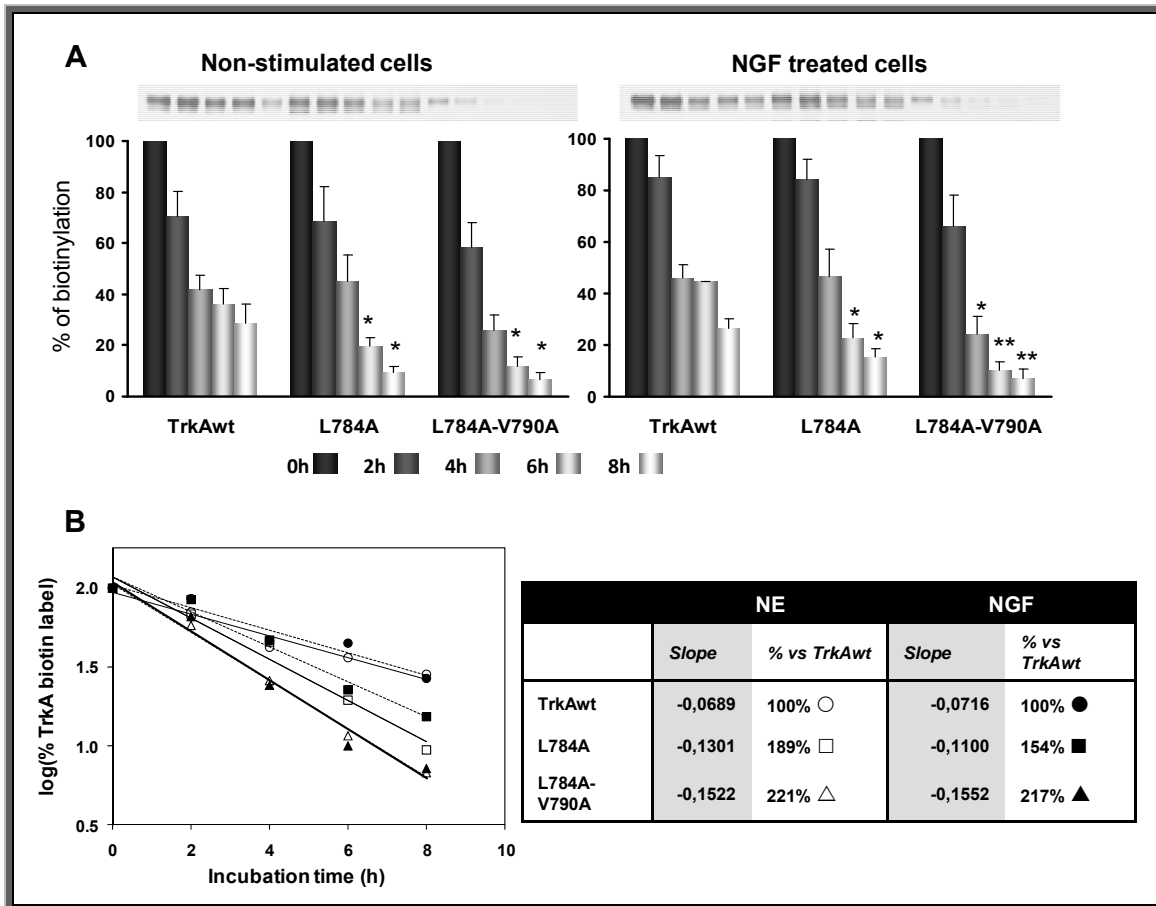
Figure R6. C-terminal TrkA mutants show a higher degradation rate. A) PC12nnr5 cells transiently transfected with mutant and wild-type TrkA receptors were submitted to membrane protein biotinylation and cells were incubated for the time intervals indicated in the presence or absence of NGF. Biotin-labeled TrkA was purified by streptavidin-agarose pull-down and detected by western blot (upper panel). One representative blot of the experiment is shown. Quantification of the reduction of TrkA biotin label was performed using values from four independent experiments and normalized versus the total biotin label at time 0. Results are represented on the bar-graphs for different time intervals as the means ± SEM. Comparison of the data obtained for the mutant receptors versus TrkAwt was carried out by the Student's t-test, $*p \leq 0.05$; $**p \leq 0.01$. B) The graph on the bottom represents an estimation of the degradation rate, obtained by plotting the data on a logarithmic scale and performing a linear regression. Symbols, ○● correspond for the TrkAwt; □■ for the TrkAL784A and Δ▲ for the TrkAL784A-V790A, open symbols correspond to non-stimulated cells (NE) and closed symbols to NGF treated cells. Values present in the table are the slopes and the relative

augment of receptor degradation rate (in %) versus wild-type receptor, whose value is taken as a reference (100%).

Our results show that both mutations alter the rate of receptor degradation, leading to a significant reduction of receptor stability. The addition of NGF slightly influenced the rate of receptor degradation, by transiently delaying the degradation rate of all three forms of the receptor, since the effect can be only observed at the 2 hours time point.

It has been reported that the degradation of TrkA receptors similarly to that of other RTKs such as the EGFR, involves the lysosomal pathway. We hypothesized that the increased degradation rate observed for the C-terminal mutants was probably due to the preferential targeting of endocytosed receptors towards the lysosomal compartment, which would result in a faster receptor turnover. Thus, to check our theory, we analyzed the colocalization of TrkA receptor with the late endosomal/lysosomal marker, LAMP1, in PC12nnr5 transiently transfected cells. In this experiment, we used the anti-Rat TrkA antibody, which binds to the extracellular domain of TrkA, thus specifically promoting the internalization of the receptor (Clary, Weskamp et al. 1994). Taking advantage of the agonist characteristics of the antibody, we labeled exclusively the receptors that were exposed to the media, thus being able to follow up the endocytosis of TrkA receptors within the cells. The colocalization analysis of TrkA and LAMP1 by confocal microscopy showed that as early as 2 hours after incubation, L784A and L784A-V790A TrkA mutants showed a higher Manders' colocalization coefficient than the wild-type receptor (Figure. R7, upper bar graph, marked with asterisk). However, at the later time point of 4 hours the colocalization coefficient of TrkAwt rose up to that observed for the mutant receptors (Figure R7 upper bar graph, marked with hush mark). This data suggests that mutant receptors reach the late endosomes and are targeted faster to the lysosomes than the wild-type receptor, which leads to a quicker receptor degradation.

Recent reports have demonstrated that lysosomal degradation of ligands, which remain bound to their receptors within the endocytic pathway (such as NGF-TrkA endosomal complex), is blocked in the presence of specific proteasome inhibitors (van Kerkhof, Alves dos Santos et al. 2001). Kerkhof and colleagues showed that the degradation of NGF, internalized via TrkA, was inhibited in the presence of proteasome inhibitors, such as Lactacystin and MG-132. It was also shown that the degradation of the EGFR receptor, which occurs in lysosomes,

depends on proteasomal activity, since the treatment of EGFR with proteasome inhibitors impedes its degradation (Longva, Blystad et al. 2002). These data suggest that the ubiquitin-proteasome system is involved in the endosomal sorting step of selected membrane proteins to lysosomes, thereby providing a mechanism for regulated degradation. Considering this finding, we decided to verify whether this statement was true for TrkA in our cell model. For that purpose, we performed a 4 hours treatment with Lactacystin and MG-132 of transiently transfected cells with the wild-type receptor and then we analyzed the colocalization of TrkA with the lysosomal marker LAMP-1. Colocalization analysis of TrkA with LAMP1 after 4h of antibody addition showed that proteasome inhibitors blocked the lysosomal targeting of TrkA receptors. This result corroborates published results, confirming the importance of proteasomal activity in the lysosomal trafficking of TrkA receptors. Taken together, these data suggest that the accelerated degradation of TrkA C-terminal mutants is mediated by the lysosome-degradation pathway.
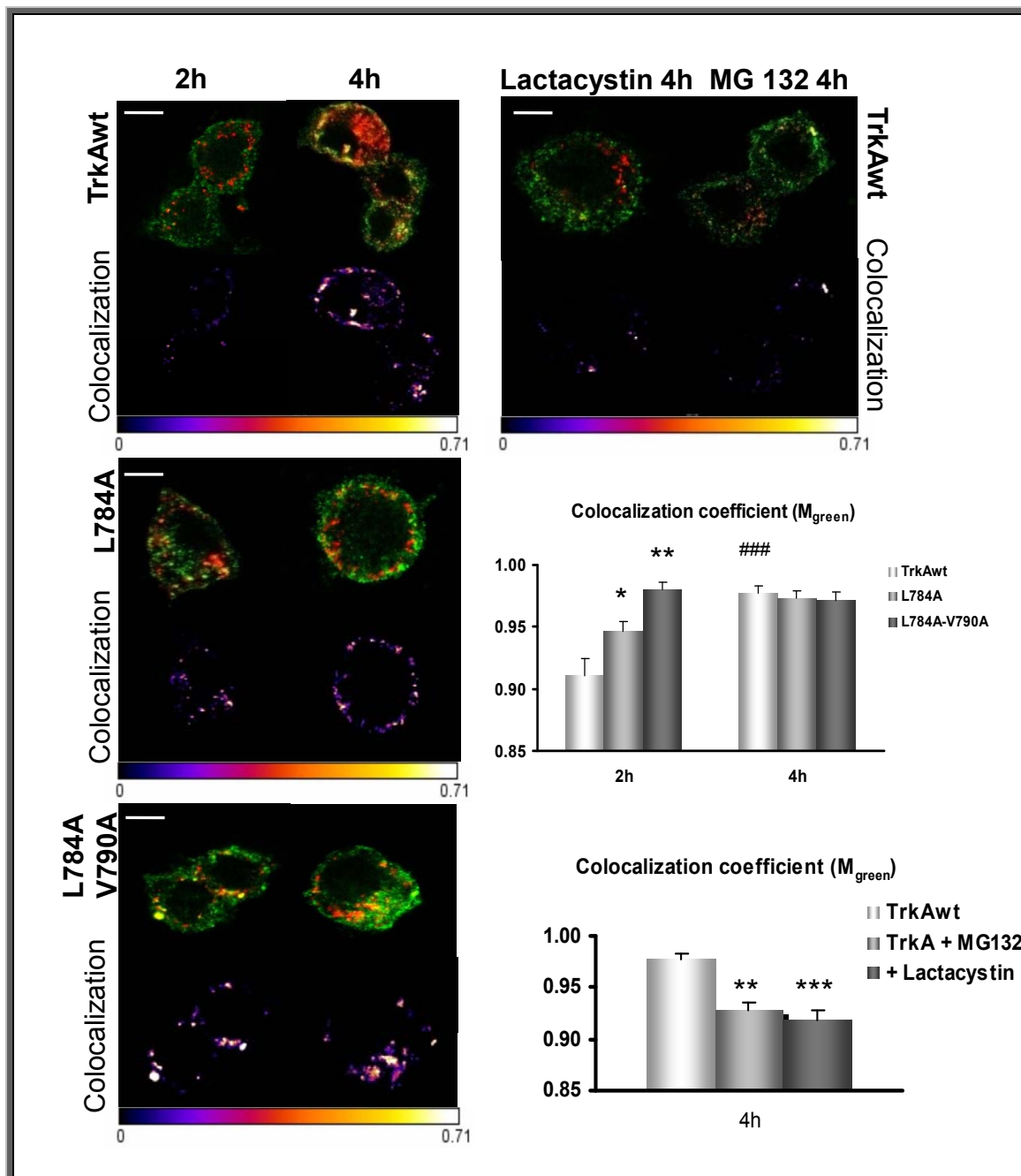
Figure R7. Colocalization analysis of TrkA receptors with the late endosome/lysosome marker LAMP1. PC12nnr5 transfected cells were incubated in the presence of the anti-Rat TrkA antibody to induce receptor internalization. After 2h and 4h cells were fixed and TrkA (green) and LAMP1 (red) were immunodetected with the appropriate antibodies. Two samples of TrkAwt expressing cells were pre-incubated for 30 min with the proteasome inhibitors lactacystin and MG-132, prior to the addition of the anti-Rat TrkA antibody. Mander's overlap coefficient was obtained by processing the acquired confocal images with the "Intensity correlation analysis" tool of the

MBF Image J software. Representative confocal images of the overlay are shown on the upper part of the composition. Images, resulting from the overlapping of pixels from both channels, called colocalization-rate displaying image (CRD image) are shown beneath. The histogram next to each CRD image, serves as a measuring scale that demonstrates the colocalization efficiency for each condition. being the lighter color the higher colocalization rate. Scale bar is equal to 10μm. Results of the Manders' colocalization coefficient for the green channel (Mgreen) are represented in the graph. Statistical significance was analysed by the Student-T test.* $p<0.05$ ** $p< 0.01$ *** $p< 0.001$ compared to TrkAwt, ### $p<0.001$ compared to 2h time-point. Scale bar = 5 μm.

5. TrkA C-terminal mutants have an increased ubiquitination rate

Our study demonstrated a lower amount of membrane and total receptor present in cells when TrkA C-terminus was altered (Figure R5), which was due to a higher degradation rate (Figure R6). Recent publications point out a crucial role of membrane protein ubiquitination on the regulation of receptor trafficking to degradation (Piper and Luzio 2001). Therefore, we decided to analyze whether the higher degradation rate observed for the TrkA mutants was due to an alteration of TrkA ubiquitination mechanism. For that purpose, we performed an immunodetection of ubiquitin on TrkA immunoprecipitates from PC12nnr5 transiently transfected cells. Western blot analysis showed a marked increase in the amount of ubiquitin molecules conjugated to the receptor in both C-terminal mutants compared to wild-type receptor. Higher ubiquitination level was only detected with the P4D1 anti-ubiquitin monoclonal antibody (that detects both mono and poly-ubiquitination), but not with de FK1 anti polyubiquitin specific monoclonal antibody (Figure R8 A, B). NGF addition for 15 min to cells promoted an increase in the ubiquitination state of the wild-type receptor, as it has been previously described (Arevalo, Waite et al. 2006), whereas mutant receptors did not display a further increase over basal ubiquitination levels. Taken together, these results suggest that in our cell model NGF activation of TrkA promotes mainly the monoubiquitination of wild type receptors, probably at multiple sites, while the C-terminal mutants display an increased basal monoubiquitination independently of NGF addition. Additionally, we observed that mutant receptors were able to become activated upon NGF binding, undergoing tyrosine phosphorylation but to a lower extent than TrkAwt (Figure R8 A, B second panel).

The involvement of two different ubiquitin ligases has been reported for the ubiquitination of TrkA upon NGF stimulation: TRAF6 and Nedd4-2. The site of Nedd4-2 binding to TrkA has been identified as the PPXY motif on the C-terminal tail of TrkA. Actually, Val-790 is the "X" residue of this motif. Therefore, it is expected that the alteration of TrkA C-terminal tail would mainly affect Nedd4-2 interaction.

In order to verify how the overexpression of these Ub-ligases affects the ubiquitination of TrkA receptor, we co-transfected TrkAwt with FLAG-Nedd4-2, FLAG-TRAF6 or both Ub-ligases together in PC12nnr5 cells. We detected a strong increase in TrkA ubiquitination when FLAG-Nedd4-2 was overexpressed, whereas the overexpression of FLAG-TRAF6 had a minor effect (Figure R8C, upper panel). When both ubiquitin ligases were overexpressed together, TrkA ubiquitination signal was stronger and a higher molecular weight smear appeared on the upper part of the membrane, indicating the appearance of larger ubiquitin chains conjugated to the receptor. Therefore, our results demonstrate that Nedd4-2 overexpression increases TrkA ubiquitination at a higher extent than TRAF6. Additionally, the result obtained from the cotransfection of both Ub-ligases suggests that monoubiquitination of TrkA by Nedd4-2 could prompt the subsequent K-63 polyubiquitin chain extension by TRAF6, indicating that mono and polyubiquitination of proteins could be sequential events carried out by different enzymes. However, additional experiments should be performed to demonstrate that possible mechanism. In accordance with this observation, a quantitative mass-spectrometry analysis revealed that EGFR receptors are both mono- and polyubiquitinated through K63-linked chains (Huang, Kirkpatrick et al. 2006). Additionally, PDGR and Ret have also been found to become ubiquitinated on both ways, therefore it may be a broader mechanism of membrane receptor regulation (Acconcia, Sigismund et al. 2009). The expression of both ubiquitin ligases was checked on the same samples with an anti-FLAG specific antibody showing that a better expression of exogenous FLAG-TRAF6 than FLAG-Nedd4-2 was attained (Figure R8C, lower panel).

In addition, overexpression of FLAG-tagged Nedd4-2 but not TRAF6 on PC12 cells produced a decrease in TrkA endogenous levels, providing additional evidence on the role of Nedd4-2 in the regulation of the TrkA receptor stability.
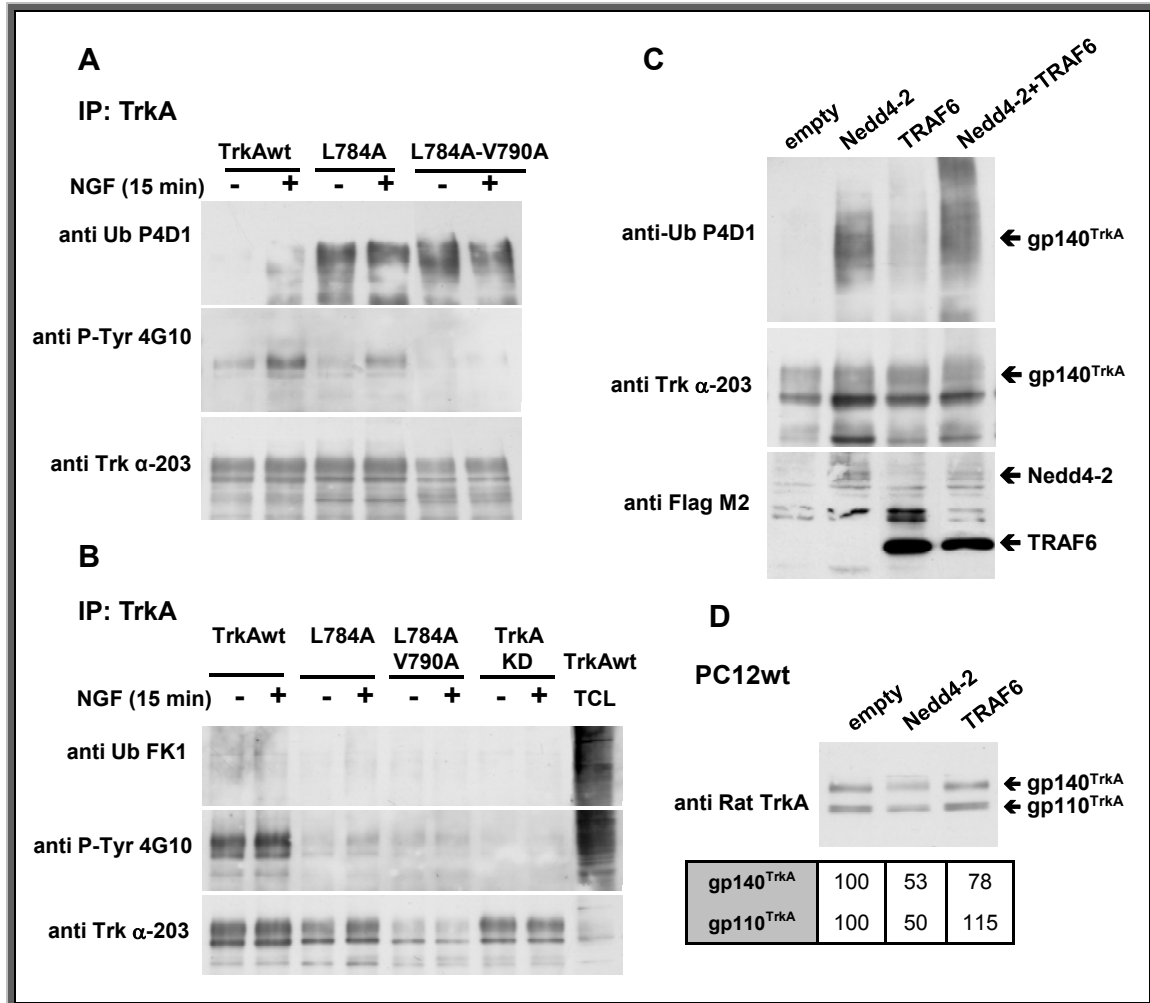
Figure R8. Alteration of the C-terminal tail of TrkA receptor results in an increase in the basal receptor ubiquitination. A) TrkA C-terminal mutants, expressed in PC12nnr5 cells, show an increased ubiquitination signal in comparison to TrkAwt. Cells were stimulated with 100 ng/ml of NGF for 15 min and then TrkA receptors were immunoprecipitated from total cell lysates and analyzed by western blot. Ubiquitination was assessed with the anti-ubiquitin P4D1 monoclonal antibody (upper panel), phosphorylation with the anti-phospho-tyrosine 4G10 antibody (middle panel) and the total amount of the immunoprecipitated receptor was checked by using anti-Trk α-203 antibody (lower panel). B) Samples, obtained as described above were immunodetected with anti-polyubiquitin FK1 monoclonal antibody (upper panel). A total cell lysate sample of MG-132 pre-treated cells (4h) was used as a control for the immunodetection. The same membrane was stripped and re-probed with anti-phospho-Tyr 4G10 antibody (second panel) and anti-Trk α-203 antibody (third panel). C) Overexpression of Nedd4-2 causes a stronger increase in TrkA ubiquitination than the overexpression of TRAF6. PC12nnr5 cells were co-transfected with TrkA and FLAG-Nedd4-2, FLAG-TRAF6 or both E3 ubiquitin ligases together. After 48 h of protein expression, the receptor was immunoprecipitated from total cell lysates, and its ubiquitination was analyzed

by western blot with anti ubiquitin P4D1 monoclonal antibody. Same membrane was stripped and re-probed for TrkA presence with anti-Trk α-203 antibody. Expression of Nedd4-2 and TRAF6 was checked on total cell lysates by western blot and immunodetection with anti-FLAG antibodies. D) Nedd4-2 overexpression reduces the stability of endogenous TrkA receptor in PC12 cells. PC12 wild-type cells were transfected with FLAG-Nedd4-2 or FLAG-TRAF6 expression plasmids. Forty-eight hours later, a western blot was performed with total cell lysates, using the anti-Rat TrkA specific antibodies. Quantification of the bands was carried out using MBF Image J software and normalized referring to the empty vector transfection condition.

Our experiments demonstrated that, in our cell model, TrkA receptor is mainly monoubiquitinated, since a stronger signal for the P4D1 antibody is detected and a very faint one while using the FK1 antibody. In order to confirm the fidelity of the FK1 antibody, we analyzed the ubiquitination of β-catenin. It is known that β-catenin stability is regulated by polyubiquitination, therefore, we performed an immunoprecipitation of the endogenous β-catenin as a control for the FK1 antibody. Additionally, we immunoprecipitated Trk from total cell lysates of PC12nnr5 cells, transiently transfected with the wild type receptor. Both samples were previously treated with MG132 for 4h to reinforce the ubiquitination event of both proteins, endogenous β-catenin and overexpressed TrkA receptor. The FK1 antibody effectively detected polyubiquitinated β-catenin and with less intensity TrkA polyubiquitination, while P4D1 antibody used in parallel showed no signal on β-catenin precipitates, but efficiently detected the ubiquitination of TrkA receptor (Figure R9). Note that after polyubiquitination, β-catenin corresponding band appears shifted at around 120 KDa. This data showed that FK1 antibody detects correctly the polyubiquitin chain bound to β-catenin and reinforce the statement that TrkA receptor is mainly monoubiquitinated.
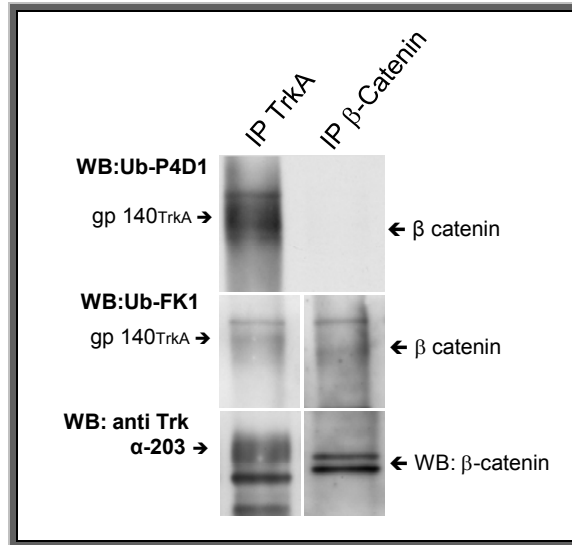
Figure R9. FK1 antibody detects polyubiquitination on β-catenin and on TrkA receptor after MG132 pre-treatment. β-catenin was immunoprecipitated from PC12nnr5 cells, transfected with TrkAwt receptor to prove the specificity of FK1 antibody for polyubiquitin chain detection. Same samples were blotted in parallel with P4D1 and FK1 antibodies. After stripping TrkA and β-catenin were detected. Note that MG132 treatment was necessary to detect the ubiquitination event under basal conditions.

In order to underline the role of Nedd4-2 on the regulation of TrkA we performed a colocalization analysis of TrkAwt and mutant receptors with both ubiquitin ligases, Nedd4-2 and TRAF6. For that purpose, we transfected PC12nnr5 cells with TrkAwt or mutant receptors with one of each FLAG-tagged ubiquitin ligases. After two days of expression, cells were serum deprived overnight and treated with NGF for 15 minutes. Afterwards, cells were fixed and immunostained with a red fluorochrom for the Ub ligases and with a green fluorochrom for TrkA receptors. Eight to ten confocal microscope images were acquired for each condition. Comparison of Mander's overlap coefficient values for the red channel (the channel for the ubiquitin ligases) showed that C-terminus mutations cause a significant increase in the colocalization coefficient of TrkA with FLAG-Nedd4-2 (Figure R10), whereas there was no effect on the FLAG-TRAF6 colocalization. Interestingly, a significant decrease ($p < 0.05$) of the Nedd4-2 colocalization efficiency with TrkAwt was observed after NGF stimulation of the receptor, detected only for TrkAwt receptor. This result is in accordance with previously published data showing that Nedd4-2 preferentially associates with the unphosphorylated TrkA receptor; therefore, Nedd4-2 does not compete with PLC-γ for binding to TrkA (Arevalo, Waite

et al. 2006). This observation reinforces the idea that the overubiquitination of both mutant receptors is mediated by Nedd4-2 ubiquitin ligase.

Searching for a further confirmation on the role of Nedd4-2 on TrkA function, we performed an immunoprecipitation assay of FLAG-tagged Nedd4-2 Ub ligase overexpressed in HEK293 cells together with TrkAwt and mutant receptors. Western blot analysis demonstrated a higher quantity of the C-terminus mutant receptors to be co-immunoprecipitated with Nedd4-2-FLAG, despite the lower amount of receptor present on total cell lysates (Figure R11). This result provide evidence for a stronger interaction occurring between Nedd4-2 ubiquitin ligase and TrkA C-terminal tail mutants, most likely due to the reduced hydrophobicity of the C-terminal tail.

Taken as a whole, these data indicate that the modification of the TrkA C-terminal domain results in an increase of Nedd4-2 binding to the receptor's C-terminal tail. Thus, the stronger binding promotes a higher level of receptor multimono-ubiquitination under resting conditions and leads to a raise in the receptor degradation. Therefore, overubiquitination detected on both mutant receptors could be due to their higher accessibility and/or binding affinity to the Nedd4-2 ubiquitin ligase.
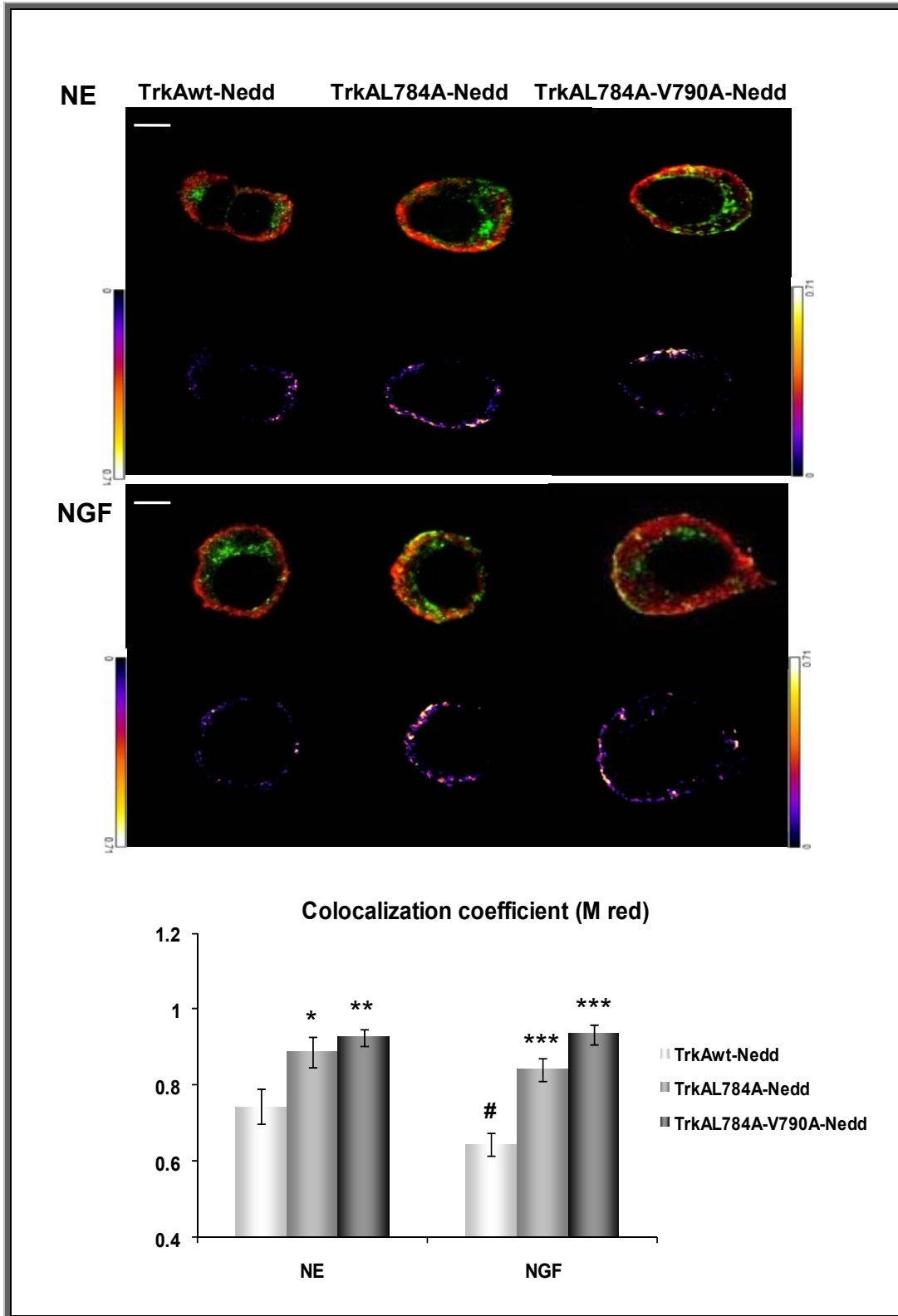
Figure R10. C-terminal tail TrkA mutants show a higher colocalization rate with Nedd4-2 than the TrkAwt. PC12nnr5 cells stably expressing TrkA variants were transfected with FLAG-Nedd4-2 construct and treated or not with NGF for 15 min. Cells were fixed and

processed for immunostaining of TrkA in green and Nedd4-2 in red. Confocal microscope images were acquired with a 60x oil immersion objective and an intensity correlation analysis was performed. Manders' colocalization coefficient referred to the red channel is represented on the bar graph for each experimental condition. Statistical significance was analyzed by the Student-T test.* $p<0.05$ ** $p< 0.01$ *** $p< 0.001$ compared to TrkAwt. Representative images of the overlays and the CRD images, resulting from the overlapping of pixels from both channels are shown for each condition. Comparison was made using values from eight different images (colocalization coefficient Mred). A decrease of the Nedd4-2 colocalization efficiency with TrkAwt was observed after NGF-mediated receptor activation. Scale bar = 5 μm.
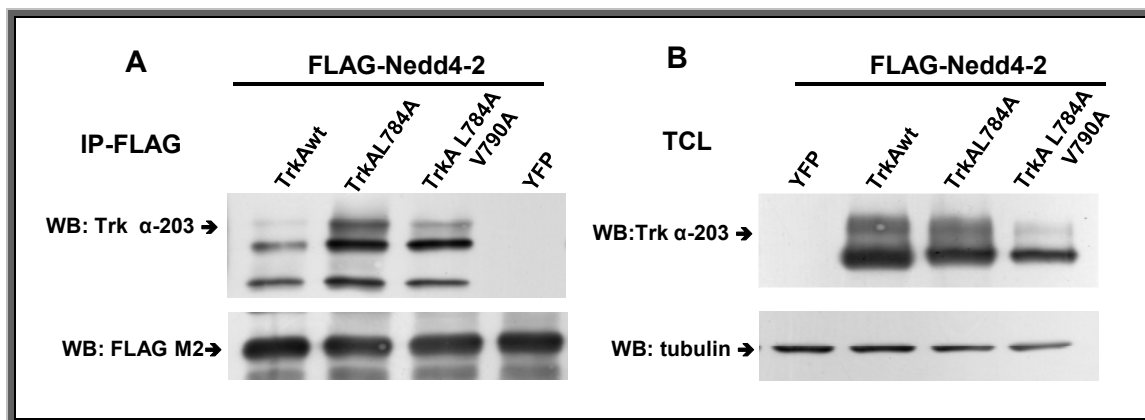
Figure R11. Nedd4-2 ubiquitin ligase binds more efficiently to the TrkA C-terminus mutant receptors. A) Total cell lysates from HEK293 cells co-transfected with Nedd4-2-FLAG and TrkAwt or mutant receptors were immunoprecipitated using anti FLAG-agarose beads. Precipitates were analyzed to western blot using the anti-Trk α-203 antibody, which revealed the quantity of receptor co-immunoprecipated with FLAG-Nedd4-2 (first panel). The total amount of immunoprecipitated FLAG-Nedd4-2 was evaluated using anti-FLAG M2 antibody (second panel). B) Total cell lysates from the same samples employed in the immunoprecipitation assay were run to check the expression levels of each receptor (first panel). Anti-tubulin was used as a loading control (second panel).

6.   Nedd4-2 mediates the multimonoubiquitination of TrkA C-terminal mutants

Two independent reports have described the ubiquitination of TrkA receptor upon NGF stimulation. Geetha and colleagues reported that in PC12 cells, TrkA becomes K63-polyubiquitinated through a process mediated by the p75 neurotrophin

receptor and its associated E3 ubiquitin ligase-TRAF6 (Geetha, Jiang et al. 2005). Simultaneously, Arevalo and colleagues demonstrated in dorsal root ganglion (DRG) neurons that the E3 ubiquitin ligase Nedd4-2 is responsible for NGF-dependent multi-monoubiquitination of endogenous TrkA receptor through its direct binding to the PPXY motif of the receptor (Arevalo, Waite et al. 2006). The alignment of the C-terminal domain of human Trk receptor family is shown on figure R12. The residues mutated in this work are indicated with arrows and the PPXY motif of TrkA is marked in green (P) and yellow (Y). As it can be observed on the sequence, the PPXY motif is only present in TrkA, which makes Nedd4-2 binding specific for this receptor, as it was described in Arevalo and colleague's study. The V790, one of the residues we have changed, is the X residue in the PPXY motif.
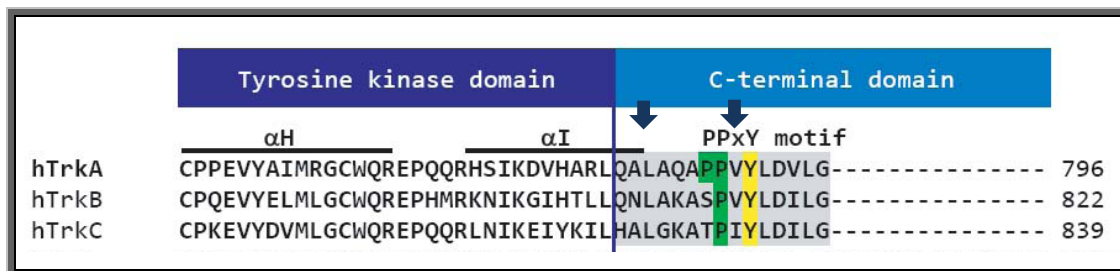
Figure R12. C-terminal domain alignment of human Trk family members. Position of alpha-helices are indicated on the top (αH and αI). The position of the PPXY motif of TrkA is indicated and highlighted in green (P) and yellow (Y), which is missing in TrkB and TrkC receptors.

In order to demonstrate that Nedd4-2 ubiquitin ligase is involved in the overubiquitination of TrkA C-terminal mutants, we next proposed to investigate the effect of knocking down the expression of the endogenous Nedd4-2 protein. Lentiviral transduction of specific rat Nedd4-2 shRNA sequences was used for that purpose. PC12nnr5 cells, transduced with two different shRNA sequences for Nedd4-2 were incubated for 5 days and then total RNA was extracted from cell lysates. The efficiency of the shRNA sequences was evaluated by semi-quantitative RT-PCR and by western blot in PC12nnr5 cells. The shRNA sequence, which caused a higher reduction of Nedd4-2 messenger RNA level, was chosen for employment in further experiments (Figure R13A left panel). Transduction of Nedd4-2 shRNA sequence reduced the amount of endogenous Nedd4-2 mRNA analyzed by semi-quantitative RT-PCR, and this reduction was confirmed at the protein level by western blot analysis (Figure R13A, right panel).

After checking the down regulation efficiency of shRNA sequence, we proceeded to the analysis of the effect of Nedd4-2 downregulation on TrkAwt and mutant receptors ubiquitination. TrkA receptors were immunoprecipitated from PC12nnr5 stable clones, transduced with the shRNA Nedd4-2 or a scrambled sequence. Results showed that the knockdown of Nedd4-2 protein expression strongly reduced the basal overubiquitination of TrkA mutants, which effect was less evident for the "wild-type" receptor, because of its lower level of basal ubiquitination. Nonetheless, the reduction on the ubiquitination signal for the TrkAwt receptor was as efficient as demonstrated for both mutants, according to the performed densitometric analysis, displayed beneath representative western blots (Figure R13 B). The ubiquitination event was detected again only with P4D1 antibody. Taken together these data provide evidence that TrkAwt and both mutant receptors become monoubiquitinated, and that Nedd4-2 is the ubiquitin ligase involved in this process. Moreover, our results demonstrate that mutations on the C-terminal tail of TrkA receptor, that affect its hydrophobicity, result in an increased basal monoubiquitination of the receptor, mediated by the Nedd4-2 ubiquitin ligase.
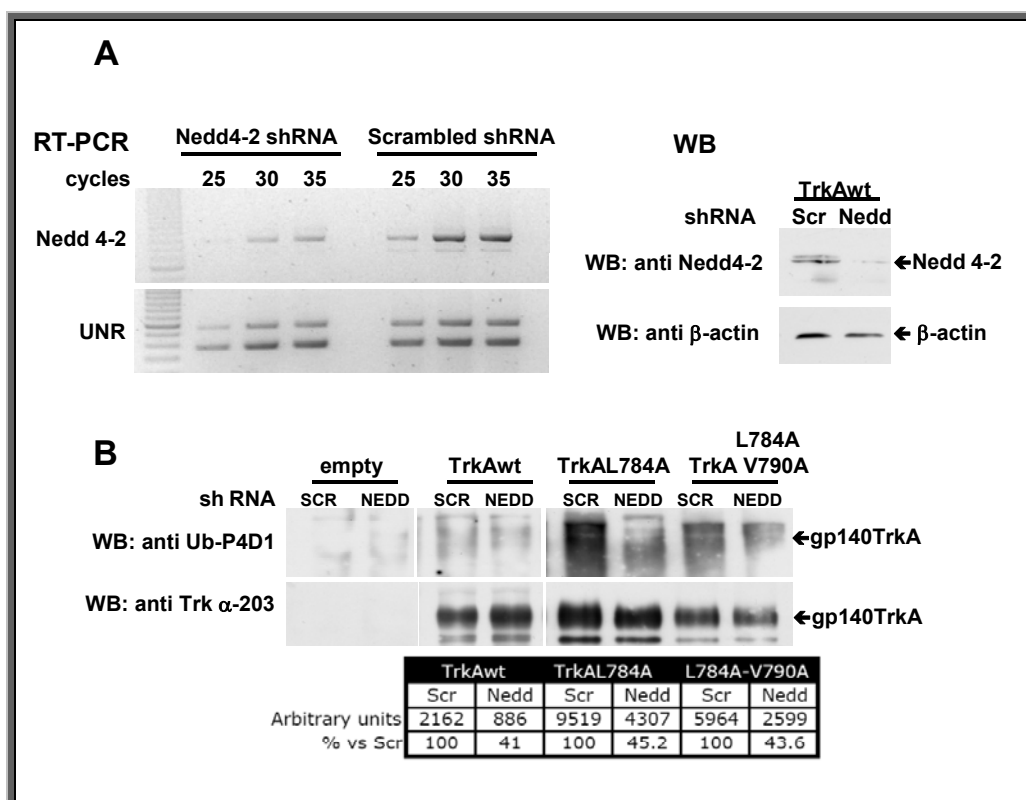
Figure R13. Nedd4-2 silencing leads to a decrease in TrkA basal ubiquitination. (A) Test of shRNA efficiency for the knockdown of the Nedd4-2 mRNA levels in PC12nnr5 cells by

semi-quantitative RT-PCR (left panel) and Nedd4-2 protein expression analyzed by western blot (right panel). PC12nnr5 cells were transduced with lentiviruses carrying the shRNA for rat Nedd4-2 or a scrambled sequence. After 5 days of transduction, RNA was extracted from cells, retro-transcribed and amplified by PCR with Nedd4-2 specific primers. Samples were taken at different cycles and ran in the same agarose gel. As a control for equivalent amount of cDNA used in the reaction the primers for Upstream of N-Ras (UNR) transcripts were used. The efficiency of the Nedd4-2 shRNA on protein expression level was checked by western blot with an anti-Nedd4-2 specific antibody. Samples used were total cell lysates of PC12nnr5 transduced with lentiviruses carrying the shRNA sequence for Nedd4-2 or a scrambled sequence. (B) The silencing of Nedd4- 2 protein expression resulted in a reduction of TrkA C-terminal mutant ubiquitination. PC12nnr5 cells stably expressing TrkAwt and the C-terminal mutants were transduced with lentiviruses carrying the Nedd4-2 shRNA or a scrambled sequence. After 5 days, cells were lysed and TrkA receptors were immunoprecipitated with anti Trk α-203 antibodies and analyzed by western blot with anti-ubiquitin P4D1 and anti-polyubiquitin FK1 antibodies (not shown) in parallel on top of the same samples splitted in two gels. The presence of TrkA in the precipitates was verified after membrane stripping and blotting with anti Trk α-203 antibody. Results from densitometric quantification of the anti-ubiquitin P4D1 blot are shown in a table below the blot images.

7. C-terminal TrkA mutants are not internalized in response to NGF

Ubiquitination of membrane proteins has been reported to be a signal for promoting receptor internalization. Therefore, we next checked whether NGF induced internalization of TrkA mutants was altered by Nedd4-2 mediated ubiquitination. TrkA internalization was assessed by biotinylation of membrane proteins and cleavage of the membrane label after two incubation intervals (5 min and 30 min) in the presence or absence of NGF. We used the 5 min time point as a reference before major internalization occurs. After 30 min of incubation, an NGF induced increase in receptor biotin label was observed only on TrkAwt samples (Figure R14, upper blot). Quantification of the pull-down blots showed that TrkAwt receptors were internalized after NGF-addition, whereas TrkA mutants did not undergo NGF-induced internalization. The amount of internalized receptor in the absence of neurotrophin was similar for the L784A mutant and lower for the L784A-V790A mutant. Therefore, the alteration of Nedd4-2 binding and the subsequently increased ubiquitination of TrkA results in a receptor which internalization cannot be induced by ligand binding,

and has a normal or reduced basal internalization rate (Figure R14 bar graph). These results provide evidence that Nedd4-2 mediated ubiquitination of TrkA does not play a role in the basal TrkA receptor internalization, since the overubiquitinated receptor is not internalized at a higher rate than TrkAwt. Nevertheless, ubiquitination may interfere with the NGF-induced receptor internalization, maybe by hiding specific internalization signals that are close to a ubiquitinated Lys residue.
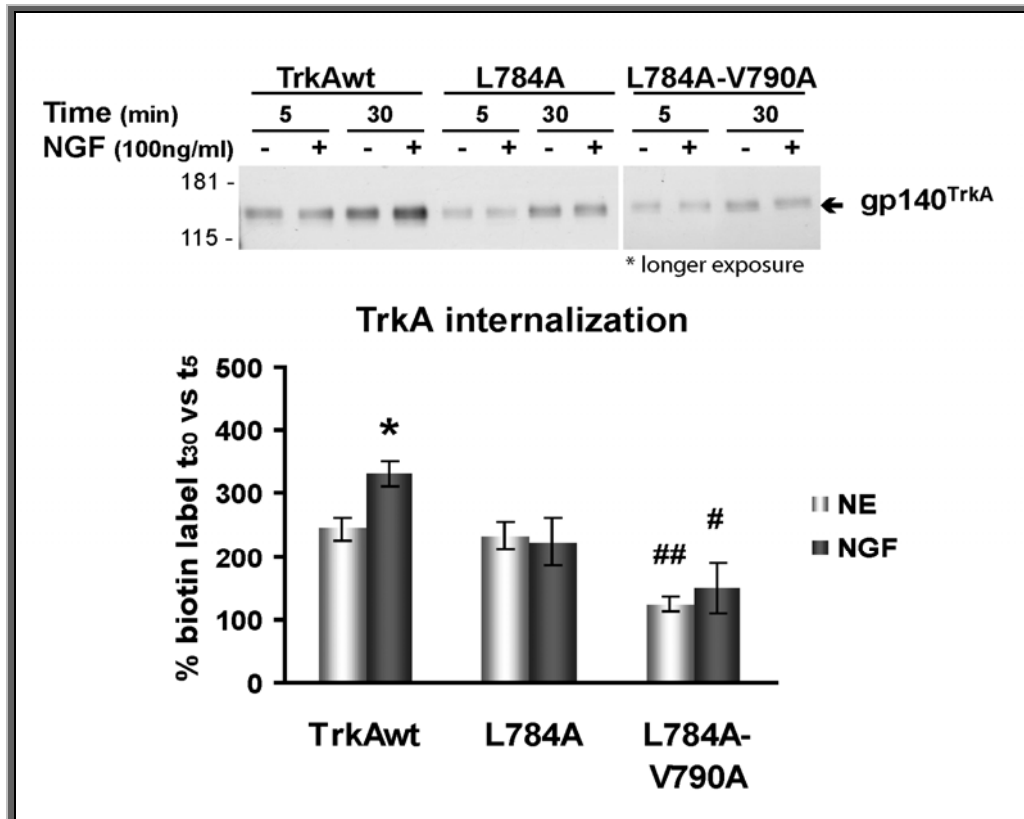
Figure R14. C-terminal mutant receptors do not undergo NGF induced receptor internalization TrkA internalization was analyzed by labeling membrane proteins with a cleavable biotin. PC12nnr5 cells transiently transfected with TrkAwt, L784A or L784A-V790A constructs were membrane protein labeled with biotin and incubated at 37ºC for 5 min and 30 min in the absence (-) or presence (+) of NGF (100 ng/mL). Then membrane biotin label was cleaved and only endocytosed receptors retained the biotin label. Cells were lysed and a streptavidin pull-down was performed. Precipitates were analyzed by SDS–polyacrylamide gel electrophoresis and western blot with anti Trk α-203 antibody. Blots from three independent biotinylation experiments were quantified with the MBF Image J software. A representative experiment is shown on the upper part. Note that a longer exposure was necessary to quantify the bands corresponding to the L784A-V790A mutant, due to the lower amount of receptor (right part of the blot). The obtained results are represented on the bar graph (mean ± SEM)

as the percentage of the amount of TrkA protected from cleavage at 30 min (t_{30}) versus 5 min (t_5) in NGF treated (NGF, dark grey bars) or untreated cells (NE, light grey bars). Statistical analysis of the results was performed by the Student's t-test: * $p < 0.05$ by comparing NGF against NE; # $p<0.05$, ## $p<0.01$ by comparing L784A-V790A mutant against TrkAwt.

8. TrkA C-terminal mutations do not affect the activation of signaling cascades

Alteration of the C-terminal tail of TrkA affects receptor turnover due to a higher degradation rate. An essential aspect of TrkA receptor is the transmission of the intracellular signal upon NGF binding. Therefore, we next analyzed the signaling capability of TrkA mutants in order to determine whether the ubiquitination of the receptor by Nedd4-2 could interfere with the activation of signaling cascades and how the faster degradation rate may attenuate the signal after long time periods of NGF exposure,

For that purpose, we transiently transfected PC12nnr5 cells with our TrkA constructs and we analysed receptor autophosphorylation after 5 minutes of NGF stimulation on TrkA immunoprecipitates. As expected, 5 min of NGF stimulation (100 ng/mL) induced a strong increase in the phospho-tyrosine reactivity of TrkAwt immunoprecipitates, (Figure R15A, upper panel, lane 1), whereas the amount of tyrosine phosphorylated receptor for both mutants was reduced accordingly to the lower amount of receptor present in the cells. Interestingly, the reduction in receptor autophosphorylation did not result in a decrease in the activity of downstream signaling molecules, such as ERK1/2 and Akt, whose activation levels remained comparable to those, observed for the wild-type receptor (Figure R15 A,B,C). We also added PC12wt cells as a control for the activation of ERK1/2 and Akt signaling molecules (Figure R15 C lane 1). This approach served us as a reference for the activation of signaling cascades by endogenous levels of TrkA receptor. The immunodetection of endogenous rat TrkA and over-expressed hTrkA was performed on the same membranes with anti-Rat TrkA antibody, in addition to the anti-Trk α-203 antibody. It was evident that even with less receptor expressed, C-terminal mutants were able to induce the activation of downstream signaling cascades, reaching similar levels of ERK1/2 and Akt activation to ones observed for the wild-type receptor. The response in PC12nnr5 transfected cells was lower than in PC12wt

cells, because only 30-40% of PC12nnr5 cells were transfected using Lipofectamine 2000 reagent.
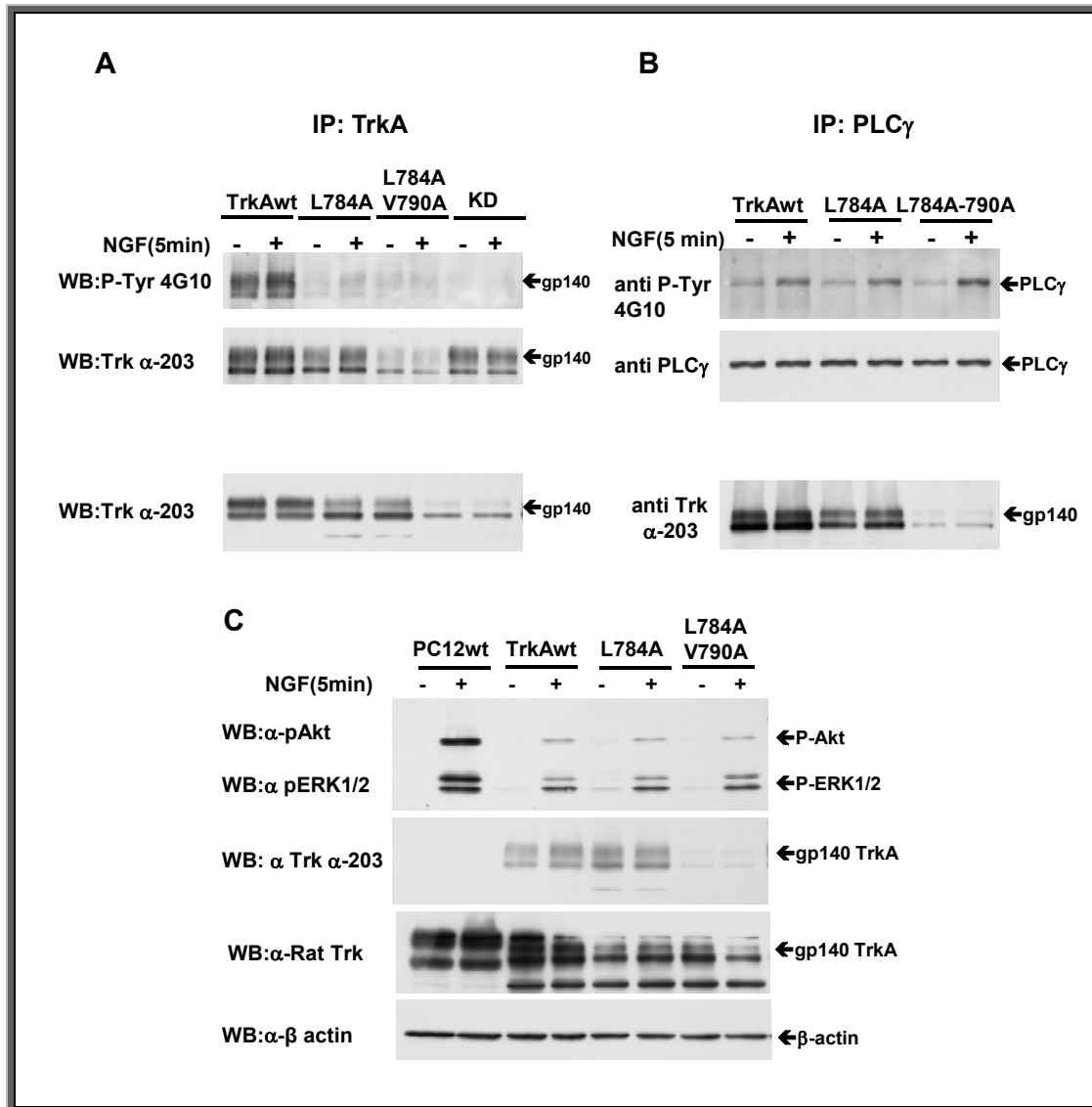
Figure R15. Lower levels of tyrosine phosphorylation observed on the mutant receptors do not result in an impairment of the activation of downstream signaling cascades. (A) Autophosphorylation of TrkA receptors in PC12nnr5 cells transiently transfected with TrkAwt and the mutant receptors (L784A, L784A-V790A) and a kinase death receptor K544N (KD) were serum deprived overnight and treated with NGF (100 ng/ml) for 5 min. TrkA was immunoprecipitated from total cell lysates and a western blot with anti-phospho-tyrosine 4G10 antibodies was performed (upper panel). Membrane was stripped and reprobed with anti Trk α-203 antibodies (second panel). The activation of downstream signaling pathways was detected using specific antibodies against the phosphorylated forms of ERK1/2 and Akt signaling molecules (fourth panel in A and B). Equal protein loading was assessed on the same membrane reblotting with anti-α-tubulin antibodies (fifth panel in A and B). (B) NGF induced

PLCγ phosphorylation on PC12nnr5 cells transiently transfected with TrkA constructs. Endogenous PLCγ was immunoprecipitated from total cell lysates and tyrosine phosphorylation was analyzed by western blot with anti-phospho-Tyrosine 4G10 antibodies (first panel). Immunoprecipitation efficiency was verified on the same membrane with anti-PLCγ antibodies (second panel). Total cell lysates (20 μg of protein) from the same experiment were ran and blotted for TrkA expression with anti Trk α-203 antibodies (third panel). (C) Activation of downstream signaling molecules by TrkA mutants is equivalent to that observed on TrkA wild-type receptor expressing PC12nnr5 cells. Total cell lysates from PC12wt expressing endogenous rat TrkA and PC12nnr5 over-expressing TrkA constructs were probed with Phospho(Ser473)-Akt and phospho(Thr202/Tyr204)-ERK1/2 antibodies (first panel). The expression of TrkA receptors was verified with anti Trk α-203 (second panel) and anti-Rat-TrkA antibodies (third panel). Finally, equivalent sample loading was checked with anti β-actin antibody (fourth panel).

To further investigate whether the generated mutations affected the recruitment of adaptor molecules implicated in the activation of downstream signaling pathways, we analyzed the phosphorylation of PLCγ, which is recruited to phospho-Y791, the Tyr residue of the PPXY motif, inside the binding site of Nedd4-2. For that purpose, we immunoprecipitated PLCγ from total cell lysates of PC12nnr5 cells transiently transfected with TrkA constructs and Tyr phosphorylation of PLCγ was analyzed. We were surprised to notice that the lower level of receptor activation not only had any effect on the activation of PLCγ, but even a slight increase in the phosphorylation of this signaling molecule was detected for the double mutant.

In addition, the analysis of Erk1, Erk2 and Akt phosphorylation in sites, which are relevant to their activation, showed that the response in the "wild-type", L784A and L784A-V790A TrkA receptors was equivalent at the usual neurotrophin concentration 100 ng/ml (Figure 15B-lower panel). At the submaximal concentration of 2.5 ng/ml of NGF only the L784A-V790A mutant showed a reduction in ERK1/2 and Akt phosphorylation (Figure R16). This result indicates that even with a lower level of TrkA receptor at the plasma membrane and less total TrkA phosphorylation, the activation of downstream signaling cascades are hardly affected by Nedd4-2 binding and receptor ubiquitination.

These data demonstrate that multimonoubiquitination of TrkA by Nedd4-2 does not substantially interfere with signal transduction, but on the contrary, it seems to amplify the intracellular signal. Cells transfected with the empty vector showed a response to NGF addition by activating ERK1 and ERK2 phosphorylation to

a lesser extent. This may be due to the NGF binding and activation of the endogenous p75NTR, thus leading to a low activation of the MAP kinase cascade.
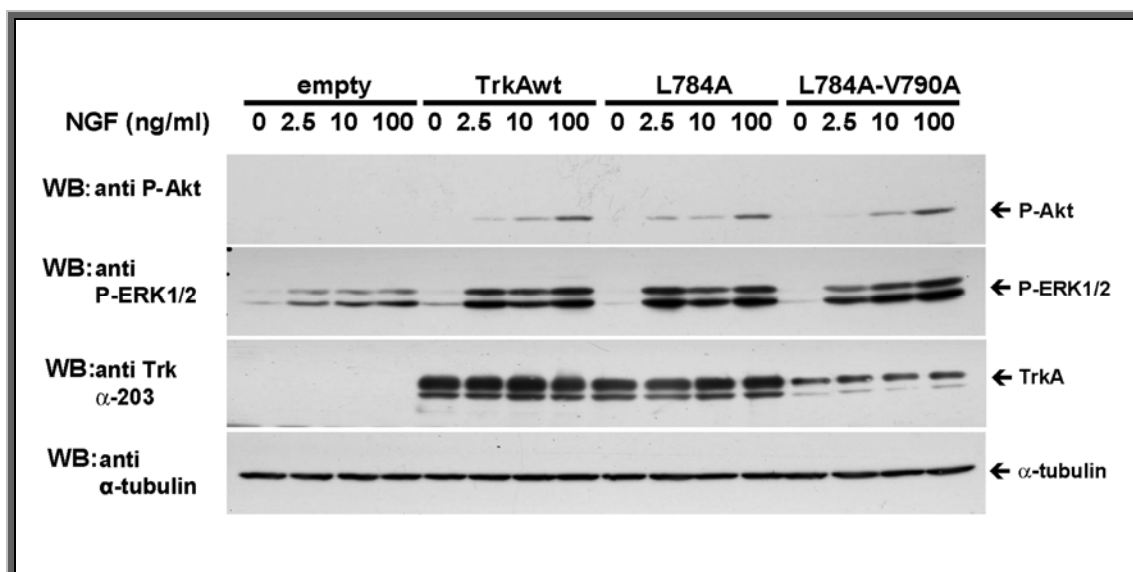
Figure R16. Activation of downstream signaling molecules by TrkA mutants on PC12nnr5 cells*. Total cell lysates from PC12nnr5 stably transduced with lentiviruses codifying for TrkAwt and mutant receptors (L784A, L784A-V790A) or with the empty vector (pEIGW) were serum starved overnight and stimulated with different NGF concentrations. Total cell lysates were loaded onto a SDS-polyacrylamide gel and Phospho(Ser473)-Akt (upper panel) and phospho(Thr202/Tyr204)-ERK1/2 (second panel) were detected by western blot. The expression of TrkA receptors was verified with anti Trk α-203 (third panel) and equal loading was assessed on the same membrane with anti-α-tubulin antibody (fourth panel).*

9.   L784A and L784A-V790A TrkA mutants are less competent in sustaining the activation of signaling cascades

One important aspect of TrkA receptor activation different from other RTK, such as the EGFR, is that in the presence of the ligand the activation of MAP kinase signaling cascade is sustained through the Rap1/B-Raf pathway. Considering this statement, we next analyzed the capability of the TrkA mutants to promote the sustained activation of two signaling effectors: PLCγ, and ERK1/2 MAP kinases. To obtain a more homogenous and stable expression of TrkA constructs, we transduced

PC12nnr5 cells with lentivirus, carrying an expression vector with the sequence codifying for the wild-type receptor and mutant receptors. We observed that both TrkA mutants were less competent to produce a sustained activation of Erk1/2 than the wild-type receptor (Figure R17), since at the 72h time point, the phosphorylation of PLCγ and ERK1/2 were back to the basal levels when C-terminus mutations were present. Therefore, alterations introduced into the TrkA C-terminal reduced the capability of the TrkA receptor to sustain intracellular signaling cascades for a long period.
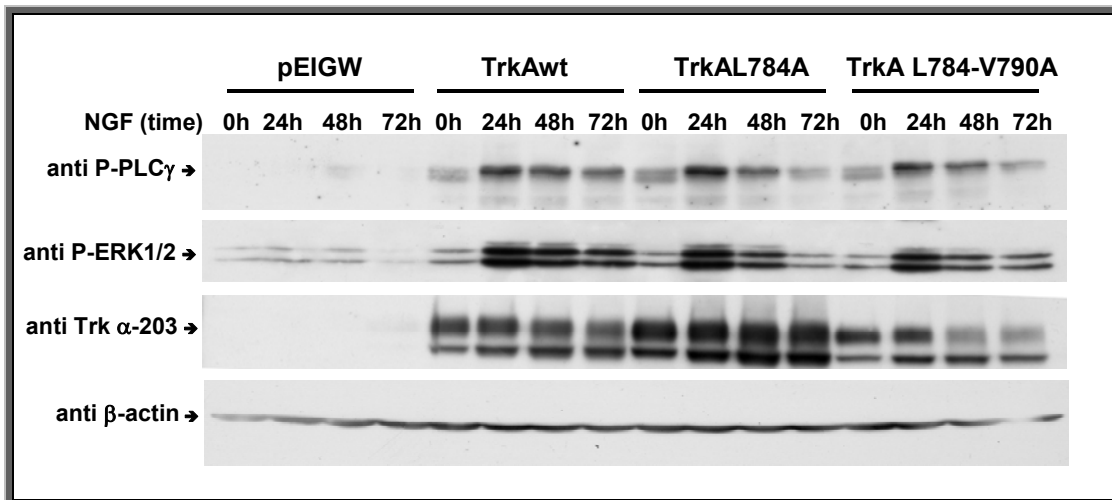
Figure R17**. **TrkA C-terminal mutations impair the sustained activation of signaling cascades. Long time-course of NGF activation of TrkAwt and C-terminal mutant receptors. PC12nnr5 cells stably transduced with lentiviruses codifying for TrkAwt, the mutant receptors (L784A, L784A-V790A) or the empty vector (pEIGW) were serum starved overnight, and treated with NGF (100 ng/ml) for different time intervals 24h, 48h and 72h. Phosphorylation of ERK1/2 (Thr202, Tyr204), Akt (Ser473) and PLCγ (Tyr783), was analyzed on total cell lysates (20 μg of protein). Expression of transduced TrkA receptors was checked by western blot and β-actin immunodetection was used to verify equal loading.

10. TrkA mutant receptors are able to promote PC12nnr5 cell differentiation to a higher extent that TrkAwt

Long-term activation of TrkA in PC12 cells causes changes on the cellular morphology, such as neurite extension and acquisition of a neuronal phenotype,

similar to the one characteristic for sympathetic neurons. Therefore, we were interested in analyzing how the point mutations introduced in the C-terminal tail of TrkA affect the neurite extension promoted by TrkA activation. For this experiment, the constructs were introduced by transient transfection into PC12nnr5 cells, and cells were treated with NGF. After 5 days of incubation in the presence of NGF (100 ng/mL), a counting of differentiated cells with neurites at least twice as long as the cell body was performed. Interestingly, cell differentiation was higher in L784A and L784-V790A transfected cells than in wild-type receptor expressing cells. Collected data allowed us to quantify the percentage of differentiation, demonstrating that mutant receptors are more efficient in promoting differentiation in comparison to the wild-type receptor (Figure R18, left graph). We used as a negative control for differentiation a kinase death TrkA receptor (TrkA-KD). This receptor does not respond to NGF stimulation, because the Lys (K544), acting as the ATP-binding site is substituted by an Asn (N), mutation that renders the receptor inactive.

Since the transfection strategy gives a very high level of exogenous gene expression, we tested another strategy of cellular gene transfer, the transduction of TrkA cDNAs with lentiviral vectors, which provides a moderate transgene expression level. Lentiviral transduction of TrkA receptors gave a higher percentage of cell differentiation in the presence of NGF compared to transient transfection under all tested conditions (Figure R18, right graph). Nonetheless, using this method we corroborated previously obtained results from transient transfection and confirmed that L784A and L784A-V790A mutants were more efficient in promoting cell differentiation than TrkAwt. Therefore, the changes introduced in the C-terminal tail of TrkA resulted in an increased capability to promote cell differentiation, in spite of the lower ability to sustain the activation of signaling cascades, such as Erk1/Erk2 MAP kinases.
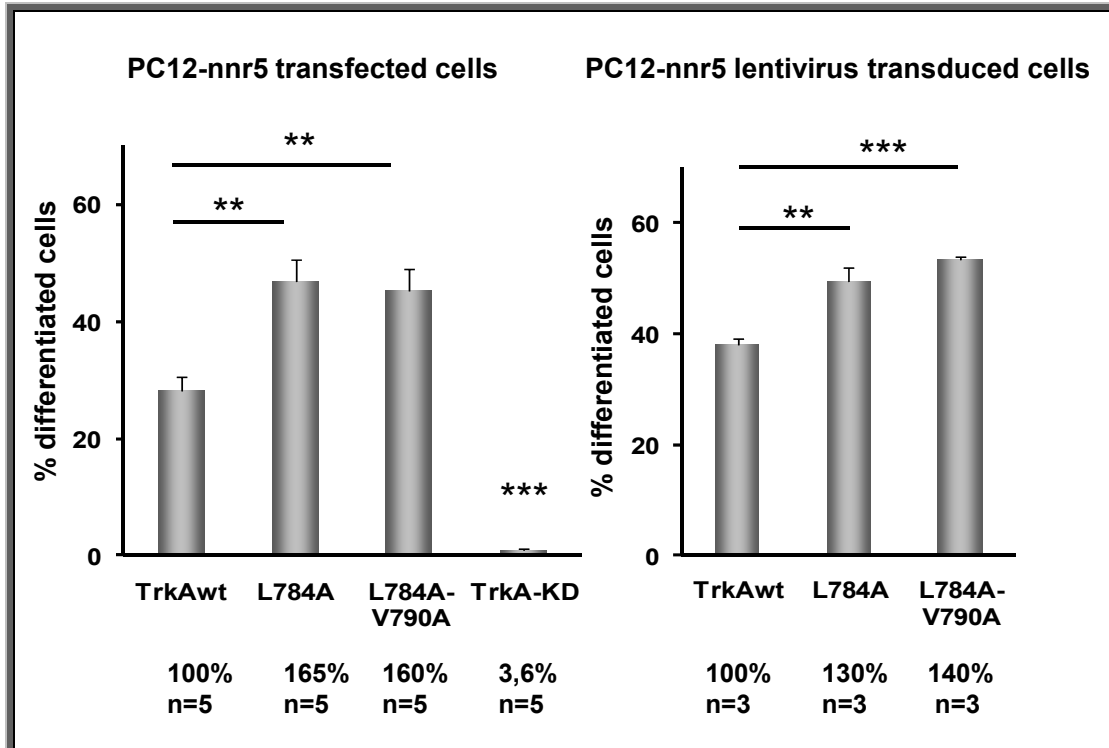
Figure R18. **TrkA C-terminal mutants are more efficient in promoting neurite outgrowth.** PC12nnr5 transiently transfected with TrkA constructs (left graph) or transduced with lentiviral vectors carrying TrkA cDNAs (right graph) were treated with NGF (100 ng/ml) for 5 days, fixed, and TrkA expression was immunodetected with anti Trk α-203 antibody and a secondary anti rabbit-Alexa Fluor 488 labeled antiserum. The percentage of differentiated cells was calculated by counting the number of stained cells with neurites at least twice as longer as the cell body and the total number of TrkA-positive cells. Data corresponds to the mean±SEM from 5 or 3 independent experiments, for transient transfection and lentiviral transduction respectively. Statistical analysis of the results was performed using Student's t-test: *p≤0.05;** p≤0.01;*** p≤0.001 compared to TrkAwt.

11. TrkA mutant receptors are less competent in promoting neuronal cell survival

It is well known that Trk expression is crucial for the correct development of the peripheral nervous system, nonetheless TrkA expression is the most important for the development of normal sympathetic neurons (Barbacid 1995). Indeed, NGF is necessary for the survival and differentiation of sympathetic neurons both *in vitro* and *in vivo* (Levi-Montalcini 1987). Sympathetic neurons in newborn animals treated

with NGF undergo hypertrophy, whereas addition of neutralizing antibody to NGF results in extensive neuronal cell death.

Therefore, on one hand we considered that an important aspect to be analyzed would be the capacity of the TrkA mutant receptors to promote the neuronal outgrowth and axonal sprouting in sympathetic neurons and on the other hand, to evaluate the effect of their overexpression on the neuronal survival. We performed these experiments in sympathetic neuron primary culture, isolated from superior cervical ganglions of postnatal rat pups (P1-3), where we analyzed the effect of the over expression of mutant TrkA receptors by competition with the endogenous receptors. For that purpose, sympathetic neurons, cultured at medium cell density (approximately 5×10^4 cells/well of a 4-well plate) were transduced by lentiviral vectors codifying for TrkAwt, both mutant receptors and empty vector. Neurons were maintained in a medium containing 50 ng/ml NGF. Following the confirmation of the lentiviral vector expression, performed by fluorescent microscopy, we compared the cell morphology and the neurite length and branching of sympathetic neurons expressing the empty vector to those neurons overexpressing either the TrkAwt receptor or both mutant receptors.

The comparison of the cell morphology showed no difference in all tested conditions. None alteration was either observed in the neurite length and branching capacity of neurons overexpressing mutant receptors, demonstrating that C-terminal alterations did not affect receptor signaling involved in neurite outgrowth. It was complicated to quantify the neurite length and density, because of the difficulty to maintain a single cell culture after plating, since sympathetic neurons normally tend to form clusters. Representative images are shown on figure R19.
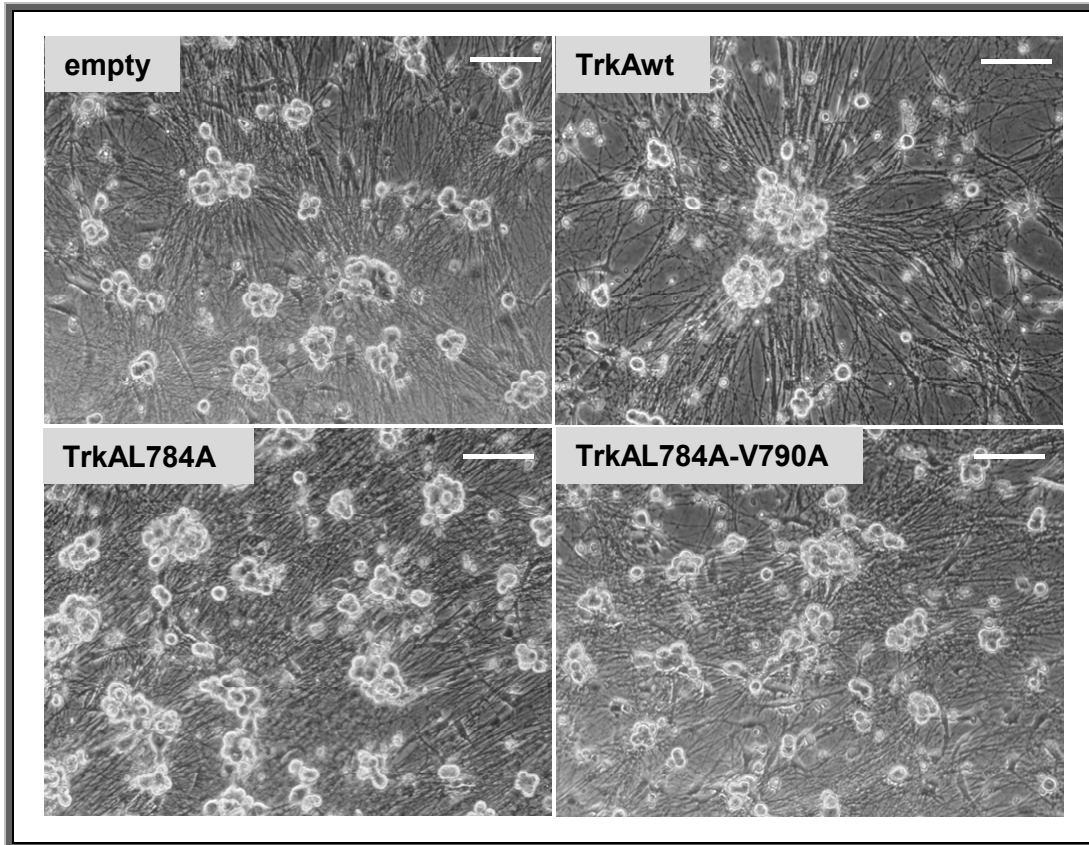
Figure R19. Sympathetic neurons transduced with lentiviruses codifying for TrkAwt, TrkAL784A, TrkAL784A-V790A or with the empty vector. Neurons were plated in 4-well plates previously coated with rat-tail type I collagen at medium cell density ($5x10^4$ cells/well approximately). They were maintained with 50ng/ml of NGF and antimitotic drugs. On the second day after plating they were transduced with lentiviral vectors containing the empty vector, TrkAwt and mutant receptors and were left for 5 days until green-positive cells were visible under fluorescent microscope. Scale bar = 50 µm.

Arevalo and collaborators (Arevalo, Waite et al. 2006) demonstrated that Nedd4-2 overexpression reduces TrkA stability and as a consequence promotes the death of NGF-dependent sensory neurons. Thus, they showed that the control of the steady-state level of TrkA receptor is critical in cells dependent on NGF for their survival. Moreover, the reduction in TrkA receptor levels resulted in a decreased TrkA signaling that cannot support cell survival.

Since our results showed that mutant receptors have a stronger interaction with Nedd4-2 Ub ligase, we decided to assess how the alteration of this interaction affects neuronal survival. For that purpose, sympathetic neurons previously transduced with lentivirus carrying the empty vector, wild type and mutant receptors were NGF

deprived for different time intervals- 24h, 48h and 72h. To reinforce the effect of the NGF withdrawal as sympathetic neurons produce small quantities of NGF, we additionally included anti-NGF antibodies in the medium. The susceptibility to apoptosis of these neurons was scored by two complementary approaches: 1) counting of the fragmented or condensed apoptotic nuclei by fluorescent microscopy, which allowed us to obtain a quantitative measurement of sympathetic neurons apoptosis and 2) detection of the active caspase 3 by western blot, which evidenced the triggering of the molecular mechanism implicated in the apoptotic event. NGF-deprived sympathetic neurons overexpressing empty vector or mutant receptors showed significantly higher percentage of apoptosis compared to neurons overexpressing the TrkAwt, and this was observed in all tested time points (Figure R20B). Moreover, the presence of the exogenous TrkAwt contributed to neuronal survival, since less apoptosis was observed for those neurons overexpressing TrkAwt together with the endogenous receptor compared to neurons expressing the empty vector, where only the endogenous receptor was present. Control neurons overexpressing each of the lentiviral vectors were maintained with NGF and were lysed together with the longest condition tested-72h, serving as a proper control only for this time point. We detected significantly higher number of apoptotic nuclei in sympathetic neurons subjected to NGF withdrawal for 72h compared to the control neurons (Figure 20B, hush mark). In accordance with the apoptotic nuclei counting, we detected an increase in active caspase 3 in sympathetic neurons overexpressing mutant receptors and empty vector compared to neurons transduced with lentivirus carrying the wild-type receptor (Figure R20A), thus reinforcing the results obtained.

Our results support previous observations by Arévalo and collaborators, demonstrating that mutant receptors that are less stable because of their faster targeting to late endosomal/ lysosomal compartment leading are less competent in promoting neuronal cell survival. In addition, our data demonstrate the relevance of Nedd4-2 on the regulation of TrkA protein levels, and emphasize the importance of the strength of these two molecules interaction. Moreover, we evidence that the conformation of the region surrounding the PPXY motif, implicated in Nedd4-2 interaction, is relevant for the binding affinity to TrkA and for Nedd4-2 activity. In conclusion, Nedd4-2 controls the amount of TrkA receptor affecting the fate of cells dependent on NGF for their survival.
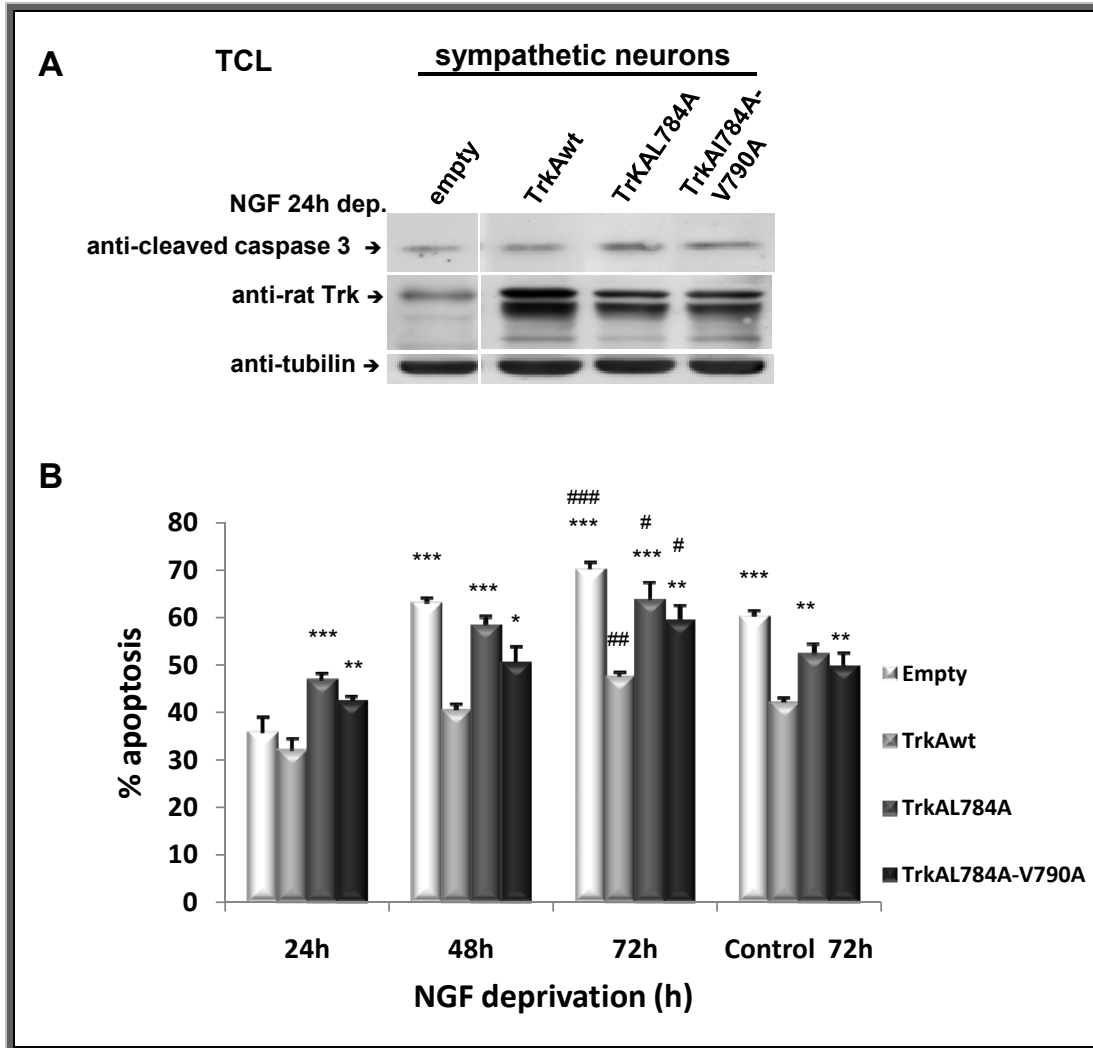
Figure R20. Sympathetic neurons overexpressing mutant receptors are more susceptible to NGF withdrawal induced apoptosis compared to those overexpressing TrkAwt and empty vector. A) Total cell lysates from sympathetic neurons overexpressing empty vector, TrkAwt and mutant receptors were collected after 24h of NGF withdrawal and subjected to Western blot analysis. The activation of caspase 3, one of the key executioners of apoptosis was detected by using a specific antibody, which recognizes only the cleaved-active form of caspase 3. Same membrane was further blotted with anti-rat Trk antibody to check the level of exogenous receptor expression in addition to the endogenous expression. Immunodetection with α-tubulin was used to verify equal loading. B) The percentage of apoptosis was calculated by staining with Hoechst 33342 and counting of fragmented or condensed nuclei of neurons transduced with the empty vector, TrkAwt and mutant receptors. Data corresponds to the mean±SEM from 5-6 independent counts performed for each of the tested conditions. Statistical analysis of the results was performed using Student's t-test: *$p \leq 0.05$;** $p \leq 0.01$;*** $p \leq 0.001$ compared to TrkAwt. # $p \leq 0.05$;## $p \leq 0.01$;### $p \leq 0.001$ condition 72h NGF withdrawal compared to Control 72h.

DISCUSSION

DISCUSSION

1. The C-terminal tail of TrkA as a putative CaM binding site

In a previously performed study, our group demonstrated that CaM binds directly to TrkA in a calcium-dependent manner. It was defined that this binding takes place in the C-terminal end of TrkA (Llovera, de Pablo et al. 2004). A wide CaM binding region was defined by using GST fusion proteins for two TrkA intracellular fragments- the membrane proximal fragment (GST-TrkAi$^{433-674}$) and the membrane distal fragment (GST-TrkAi$^{675-796}$). When recombinant proteins were employed in pull-down experiments in the presence of Ca^2, CaM-sepharose precipitates contained only the most distal C-terminal fragment of TrkA (GST-TrkAi$^{675-796}$), but not the membrane proximal fragment (GST-TrkAi$^{433-674}$), indicating that the CaM-binding domain is located inside the region between residues 669–790. The relevance of CaM interaction was studied by the use of pharmacological CaM antagonists, such as Trifluoperazine, W13 and W7, which induced TrkA cleavage and the formation of p41 and p38 TrkAi fragments. Therefore, a role for CaM on the maintenance of TrkA integrity at the plasma membrane was proposed.

Subsequently, we sought to find a more specific way to block calmodulin/TrkA interaction by the generation of TrkA variants with an impaired CaM binding site, thus focusing our research on finding the calmodulin binding domain of TrkA. Using a smaller fragment of TrkA intracellular domain (TrkAi$^{720-796}$) we restricted the region of TrkA where the calmodulin binding domain is contained to the last 76 amino acids on the C-terminus. For that purpose, we first assessed the bioinformatics CBD prediction programs, which unfortunately did not detect any candidate sequence in the region aa 720-796. Then we contacted Dr Neil McDonald for his expertise in protein structure (Structural Biology Laboratory, Cancer Research UK), who proposed that the C terminal tail of TrkA, situated out of the kinase domain could be implicated in the CaM interaction.

Analyzing in detail the sequence of this region, we found a motif which contains hydrophobic amino acids at positions 1-5-8-14 that fitted with a putative 1-

14 type CaM binding domain (☐HARL☐QAL☐AQAPP☐$^{777-790}$). Moreover, considering the 3D structure prediction of this region, which could conform to α-helix structure, we decided to mutate the two bulky hydrophobic amino acids at positions 8 and both 8+14, corresponding to amino acids Leu-784 and Val-790. We substituted them by the small hydrophobic amino acid, Ala, a mutation that does not change the net charge of the region but affects its hydrophobicity. We introduced the L784A change in the hTrkA cDNA sequence, and next V790 was mutated on top of the L790A mutant, thus generating TrkA-L784A and TrkA-L784A-V790A. Full length TrkA receptors were expressed on PC12nnr5 cells by transient transfection and GST-fused TrkAi domains were expressed and purified from *E. coli* DH5α. We performed a series of CaM-sepharose pull-down assays in the presence or absence of calcium using both types of recombinant proteins. Obtained results showed that amino acid changes introduced into TrkA C-terminal tail did not affect the binding of CaM. Therefore, we concluded that L784 and V790 were not conforming to the CaM binding domain of TrkA.

Interestingly, introduced mutations on TrkA sequence caused an important reduction of receptor's expression levels, thus suggesting a role of the C-terminal tail in the regulation of TrkA protein levels. This unexpected finding prompted us to characterize the effect of both mutations on TrkA receptor biology and activity.

2. Effect of C-terminal mutations on TrkA steady state levels

The comparison of the C-terminal tail of Trk family members with several others RTKs present in the nervous system and target tissues reveals that neurotrophin receptors, together with RYK and MusK, have a very short C-terminal tail, unlike other members such as Ret, Met, InsR and EGFR that bear a long C-terminal tail with multiple functions. In EGFR for example it plays an important role in receptor activation, since all of the identified autophosphorylation sites are situated in its long C-terminal tail (Hubbard and Till 2000). The C-terminal domain of the hepatocyte growth factor receptor Met is crucial not only for the regulation of the receptor activity, but also for the recruitment of signal transducers (Gual, Giordano et al. 2001). Similarly, the C-terminus of Ret receptor contains Tyr residues

important for its activation (Arighi, Borrello et al. 2005). In contrast, the C- terminal tail of TrkA is only 15 amino acids long and contains the phosphorylation site (Y791), which mediates PLCγ activation. Recently, Arévalo and collaborators discovered the presence of a Nedd4-2 ubiquitin ligase binding motif (PPXY) on this small region (Arevalo, Waite et al. 2006). They described that the binding of E3 ubiquitin ligase, Nedd4-2, to the C-terminus of the TrkA receptor induces receptor downregulation. Unlike other motifs in the Trk tyrosine kinase family, the site of Nedd4-2 association is not conserved in TrkB and TrkC receptors.

In addition to the PLCγ and Nedd4-2 Ub ligase binding sites, the C-terminal region of TrkA contains a high percentage of hydrophobic amino acids in conserved positions, including the two bulky hydrophobic amino acids Leu-784 and Val-790 that have been switched to Ala in the present study. Considering the relevance of the hydrophobicity for the maintenance of the 3D protein conformation it is likely that these mutations will alter the folding of this region, therefore affecting protein interactions. By overexpressing the TrkA mutants in PC12nnr5 for the CaM pull-down experiments, we observed that the introduction of one (L784) or two (L784A-V790A) amino acid changes into the C-terminal tail of TrkA caused a marked reduction of the amount of expressed receptor. Therefore, we wanted to elucidate whether the C-terminal tail was implicated in the regulation of TrkA turnover. We observed that a single point mutation on Leu-784 led to a reduction of TrkA protein levels, likewise the addition of V790A modification caused even a stronger effect. The immunodetection, performed with both the anti Trk α-203 and the anti-Rat TrkA antibody showed no accumulation of immature forms and a proportional reduction of all TrkA isoforms- p90, gp110 and gp140, indicating that the maturation step was not altered. Moreover, cell surface protein biotinylation assay confirmed that the amount of receptor present at the plasma membrane was accordingly reduced.

As a transmembrane protein Type I Trk receptor contains an endoplasmatic reticulum (ER) export signal. The receptor is initially synthesized and inserted into the ER membrane as an immature form with an apparent molecular weight of 90 kDa. The maturation process occurs mainly in the trans-Golgi network, where after undergoing N-glycosylation is converted to a glycoprotein of 110 kDa, the gp110 form. For its full maturation the receptor is submitted to sialylation of the sugar at the Golgi Network leading to the gp140 mature and plasma membrane form. Under basal conditions, the pool of the gp140 mature form is internalized and recycled back to the plasma membrane at a constant rate, independently of ligand stimulation,

thus being continuously shuttled between endocytic vesicles and plasma membrane. Two alternate endocytic pathways that Trk receptors can follow are: trafficking to lysosomes or recycling back to the plasma membrane. The degradation pathway to lysosomes leads to receptor desensitization and down-regulation, whereas the recycling pathway can lead to functional sensitization and prolongation of the cell surface specific signaling events. Different endocytic sorting routes have been described for both TrkA and TrkB receptors. Whereas activated TrkB receptors are mainly sorted to the degradation pathway, a portion of activated TrkA can be recycled back to the cell surface, a process that requires a specific recycling signal located in the cytoplasmic juxtamembrane region (aa 473-493) (Chen, Ieraci et al. 2005). Interestingly, this domain contains a Lys residue (K485) that has been described to become polyubiquitinated by TRAF6 (Geetha, Jiang et al. 2005; Jadhav, Geetha et al. 2008). Mutation of this residue results in the retention of the receptor on the plasma membrane and impairment of the activation of specific signaling pathways (Geetha, Jiang et al. 2005).

Different diseases are characterized by mutations which impair their receptor expression at the cell surface (Sanders and Myers 2004). One example of such alterations are loss-of-function mutations in the extracellular domain of the RTK Ret, associated with Hirsch sprung disease, where immature forms of mutant Ret protein accumulate intracellularly and are prevented from interaction with the co-receptor GFR1 and the ligand GDNF (Kjaer and Ibanez 2003). Similar observations have been reported for the insulin receptor in some rare cases of insulin-resistant diabetes (Rouard, Bass et al. 1999). As a common principle, these disease-causing mutations impair the folding efficiency of the kinases, leading to their entrapment by the chaperones, components of the ER quality control. Additionally, the mechanism of maturation arrest was observed in some constitutive active receptors, which could be due to a negative regulation of the components that participate in the maturation process, such as molecules of the quality control mechanism or molecules with importance for ER-Golgi vesicle transport (Schmidt-Arras, Bohmer et al. 2005). Considering all given examples one could assume that the reduction in the expression levels of both TrkA mutants could be due to a maturation problem. However, our observations demonstrate that the reduction of the protein levels of both TrkA mutants occur on all of the TrkA specific bands: p90, gp110 and gp140. Additionally to the absence of immature form accumulation we neither detected constitutive kinase activity of the mutants under basal conditions. On the contrary,

we observed reduced autophosphorylation of both mutant receptors, related to the lower amount of receptor present on the membrane and we further detected a proper activation of molecules mediating the downstream signal propagation. Therefore, we concluded that the alteration in the amount of receptor present on the membrane could not be explained by a problem in receptor maturation, but it could be rather related to an alteration of the rate of receptor degradation.

3. TrkA receptor degradation and lysosomal targeting

Further studies demonstrated that the amount of mutated receptors was affected as a consequence of their higher degradation rate, which correlated with an increased colocalization with LAMP1. To analyze receptor degradation we performed a biotinylation assay of membrane proteins and streptavidin pull-down at different incubation intervals in the presence or absence of NGF. Results showed that both mutant receptors had a higher degradation rate, leading to a significant reduction on the amount of total and surface receptor. The addition of NGF led to a transient delay in the degradation of all three forms of the receptor, effect that could only be observed at the 2h time point. Considering that the degradation of Trk receptors involves the lysosomal pathway, similarly to other RTKs such as EGFR, we analyzed the colocalization of TrkA receptor with the late endosome/lysosomal marker LAMP1 in PC12nnr5 overexpressing TrkA constructs. Results showed that the degree of colocalization for the mutant receptors with LAMP1 was higher at the earlier time point investigated (2h). Nonetheless, the colocalization of TrkAwt receptor reached similar levels to those observed for both mutants when analyzed at the 4h time point. These data suggest that mutant receptors reach the late endosome and lysosomal compartments faster than the wild-type receptor, which leads to a quicker receptor degradation and a reduction in the amount of total receptor.

4. Ubiquitination of TrkA receptor

The present study demonstrates a lower amount of membrane and total receptor present in cells when TrkA C-terminus is altered which is due to a faster targeting to late endosome and lysosomal compartments, leading to a higher

degradation rate. Considering these observations and taking into account that ubiquitination is known to be involved in the regulation of receptor trafficking and degradation, we decided to analyze whether the increased degradation rate of mutant receptors was due to a modification of the TrkA ubiquitination mechanism.

TrkA immunoprecipitates from PC12nnr5 overexpressing TrkAwt and mutant receptors were analyzed by western blot with two different antibodies: the P4D1 anti-ubiquitin antibody, that detects both mono and poly-ubiquitination and with the FK1 anti polyubiquitin specific antibody. We detected a higher ubiquitination signal on both C-terminal mutants with the P4D1 antibody, whereas we did not detect polyubiquitination with the FK1 antibody for none of the immunoprecipitated receptors. As it was previously described by Dr. Arévalo and colleagues (Arevalo, Waite et al. 2006), NGF addition for 15 min promoted an increased ubiquitination of TrkAwt, which was detected only with the anti-ubiquitin P4D1 antibody. This observation implies that TrkA is mostly monoubiquitinated in our cell system. Therefore, our results demonstrate that the alteration of the C-terminal tail of TrkA promotes an increased basal ubiquitination of the receptor which in turn leads to a higher lysosomal degradation of mutant receptors.

Lysosomal degradation and trafficking through LAMP-1 positive endosomes have been demonstrated for several chemokine receptors (CRs) after prolonged exposure to their ligands (Meiser, Mueller et al. 2008). Although the degradation process occurred by ubiquitin-independent mechanism for chemokine receptors 2(CXCR2) and 3 (CXCR3) (Meiser, Mueller et al. 2008), it was shown to be ubiquitin dependent for the chemokine receptor 4 (CXCR4) (Marchese, Raiborg et al. 2003). Interestingly, CXCR3 undergoes constitutive degradation in the absence of ligand (Meiser, Mueller et al. 2008) as we observe to occur with our TrkA mutant receptors.

Few years ago, two independent groups described the polyubiquitination of TrkA and TrkB upon activation and showed the importance of this event for the regulation of receptor internalization and degradation (Geetha, Jiang et al. 2005; Makkerh, Ceni et al. 2005). Dr Makkerh and colleagues observed that Trk receptors are polyubiquitinated in a ligand dependent manner. They detected a more robust ubiquitination of endogenous TrkB receptors of cortical neurons compared to TrkA receptors from PC12 cells. The ubiquitination of TrkA and TrkB receptors was checked with both anti-ubiquitin P4D1 and anti-polyubiquitin FK1 antibodies. Moreover, they showed that p75NTR co-expression attenuated the ubiquitination of TrkA and TrkB receptors overexpressed in HEK293 cells. Furthermore, they

concluded that p75 co-expression in PC12 cells reduced the rate of TrkA degradation and delayed NGF-induced TrkA internalization (Makkerh, Ceni et al. 2005). Few months later, Dr. Geetha and colleagues reported in PC12 cells the polyubiquitination of TrkA in a K63-dependent manner and showed that this process was mediated by p75 neurotrophin receptor (p75NTR) and its associated E3 ligase-TRAF6. The site of TRAF6 polyubiquitination was identified to be Lys485. A previous study of this group demonstrated that TRAF6 interacts and forms a complex with p75NTR, p62 adaptor protein and TrkA (Wooten, Seibenhener et al. 2001). Since they observed that p62 is recruited to TrkA in a kinase dependent manner (Geetha and Wooten 2003) and that the treatment with K252a did not completely diminish NGF-induced polyubiquitination of TrkA, they suggested that TrkA may also be polyubiquitinated independently of p62/TRAF6. Consequently, they assumed the possibility that K485 may be targeted by another Ub-ligase as well. Furthermore, they proposed that TrkA-Lys63 polyubiquitination regulates receptor internalization and signaling (Geetha, Jiang et al. 2005). From these reports, a disagreement regarding the relevance of p75NTR on the ubiquitination event was raised. Whereas Makkerh group showed evidence that p75NTR interfered with TrkA and TrkB ubiquitination and delayed receptor internalization, Geetha and collaborators demonstrated that p75NTR was necessary for TrkA polyubiquitination, acting as a link between the ubiquitin ligase TRAF6 and the Trk receptor, through p62. Further research will be necessary to solve this discrepancy.

Soon after, Dr. Arevalo and collaborators demonstrated that TrkA and TrkB receptors became multimonoubiquitinated in response to neurotrophins. Additionally, they identified Nedd4-2 to be the E3 ubiquitin ligase mediating this process in TrkA and mapped the motif of binding in the C-terminal tail of TrkA (PPXY). The PPXY motif is absent in TrkB or TrkC receptors, for that reason Nedd4-2 mediated multimonoubiquitination was only observed on TrkA receptors. Since the "X" residue of the PPXY motif, described to be involved in Nedd4-2 Ub ligase interaction is the Val-790 residue mutated in our study, we expected that the alteration of TrkA C-terminal tail would mainly affect Nedd4-2 interaction.

With the aim to analyze the involvement of Nedd4-2 and/or TRAF6 on TrkAwt ubiquitination we performed overexpression experiments with both E3 ubiquitin ligases. We co-transfected TrkAwt with FLAG-Nedd4-2, FLAG-TRAF6 or both Ub-ligases together in PC12nnr5 cells. We detected a strong increase in TrkA ubiquitination when FLAG-Nedd4-2 was overexpressed, whereas the overexpression

of FLAG-TRAF6 had a slighter effect. Results obtained from the simultaneous overexpression of both ubiquitin ligases showed a stronger ubiquitination signal and higher molecular weight bands, indicating the appearance of polyubiquitin chains covalently attached to the receptor. Hence, we could speculate that most probably two sequential ubiquitination events take place in TrkA receptors, initially, the monoubiquitination of TrkA at multiple sites catalyzed by Nedd4-2, and afterwards, the K63-polyubiquitin chain extension by TRAF6. However, further analysis is required to elucidate whether this sequential ubiquitination process on TrkA takes place under physiological conditions and to disclose the role of both ubiquitination types on TrkA regulation. Actually, the combination of multimonoubiquitination and K63- polyubiquitination was also identified for PDGR and Ret receptors (Acconcia, Sigismund et al. 2009) and it was additionally showed by quantitative mass spectrometry analysis that EGFRs are both mono and polyubiquitinated, being the K63 chain the most abundant type of ubiquitination (Miranda and Sorkin 2007).

In addition to its effect on the receptor ubiquitination the overexpression of the FLAG tagged Nedd4-2, but not TRAF6 produced a decrease in TrkA endogenous levels in PC12 cells, which provide further evidence on the role of Nedd4-2 in the regulation of TrkA receptor stability. Indeed, Dr Arévalo's study showed that the overexpression of Nedd4-2 in DRG neurons leads to the downregulation of TrkA receptor which subsequently affects neuronal survival by causing apoptosis of these neurons (Arevalo, Waite et al. 2006). On the contrary, down-regulation of Nedd4-2 protein led to an increase in phospho-TrkA levels consistent with decreased degradation of Trk receptor, showing that changing the endogenous levels of Nedd4-2 in sensory neurons can modulate the activity of Trk receptors. Similarly, Zhou and colleagues demonstrated that Nedd4-2 overexpression resulted in a higher epithelial sodium channel (ENaC) ubiquitination, which was sufficient to decrease the surface expression of ENaC. Nedd4-2 overexpression selectively reduced ENaC expression at the cell surface, without altering the quantity of immature ENaC in the biosynthetic pathway. On the opposite, Nedd4-2 silencing led to decreased ENaC ubiquitination (Zhou, Patel et al. 2007).

Our results demonstrate that in our working system TrkA receptor is mainly monoubiquitinated, since a stronger signal for the P4D1 antibody is detected compared to a very weak when using the FK1 antibody. Dr. Makkerh and colleagues have mentioned in their study that the level of TrkA ubiquitination in PC12 cells was very low and difficult to achieve using both P4D1 and FK1 antibodies, so they needed

to perform very long immunoblot exposures in order to detect it (Makkerh, Ceni et al. 2005). In our case the detection of the ubiquitination event for the TrkAwt receptor was only possible upon NGF stimulation, Ub ligase overexpression or after the treatment with proteasome inhibitors (MG132 or lactacystin). The situation was different for both mutant receptors whose ubiquitination was very robust under basal conditions and was not further enhanced after NGF stimulation. Unfortunately, Dr. Geetha did not specify the source of the anti-ubiquitin antibody used in his published work, neither the ubiquitination event was checked with different anti-Ub antibodies. They assumed that TrkA was polyubiquitinated by demonstrating that TRAF6 is the Ub ligase mediating this process. However, they proposed that TrkA could be also ubiquitinated by other Ub ligases, since NGF mediated ubiquitination of TrkA was only diminished, but not totally blocked upon inhibition of TrkA activity.

We made some efforts trying to reproduce Makkerh's results on PC12 cells with the anti-polyubiquitin FK1 monoclonal antibody but we did not obtain a consistent positive signal after NGF treatment, without the addition of proteasome inhibitors. It is possible that the low ubiquitination of TrkA receptors itself combined with the reduced sensitivity of the antibody could be a limiting factor for the detection of the polyubiquitination on Trk receptors in our working conditions.

Our results demonstrate that C-terminal TrkA mutants are less stable than TrkAwt due to receptor ubiquitination by Nedd4-2 and its subsequent lysosomal degradation, which was shown to occur faster compared to the TrkAwt receptor. In this sense, it has been described that during the endocytosis, cargo destined for degradation concentrates in multivesicular bodies (MVBs), whilst recycling proteins are removed (Woodman and Futter 2008). During this process, the MVB moves from the cell periphery to the cell centre and, when all the recycling proteins have been removed, interacts with the lysosome.

Therefore, one possible scenario could be that, under basal conditions, Nedd4-2 ubiquitinates TrkA mutants on K485 residue, the ubiquitination site described by Geetha et al (Geetha, Jiang et al. 2005). This event would hide the specific recycling signal present in the juxtamembrane region (Chen, Ieraci et al. 2005), thus shifting the sorting of TrkA receptors from recycling to degradative pathway, and therefore directing all the endocyted receptors towards the late endosome/lysosome pathway. It is likely that the hiding of the recycling signal could speed up the process of MVB's fusion with lysosomes, in this manner assisting

mutant receptors to reach faster late endosome/lysosome compartment. However, additional experiments would be necessary to demonstrate this hypothesis.

5.   Role of Nedd4-2 on TrkA ubiquitination

Since mutations introduced in the C-terminal tail of TrkA altered the hydrophobicity of the region surrounding the Nedd4-2 binding motif and this may cause a conformational change, we thought that the increased ubiquitination rate on TrkA mutants could be due to an increased binding affinity of Nedd4-2 to TrkA leading to an increased Ub ligase activity towards TrkA. Therefore, we analyzed Nedd4-2/TrkA interaction by two complementary approaches, a colocalization analysis of both proteins in intact cells and a co-precipitation assay of these proteins on total cell lysates.

Arévalo and collaborators showed that Nedd4-2 associates endogenously with TrkA since TrkA was co-immunoprecipitated with Nedd4-2 on sensory neurons isolated from DRGs. Moreover, colocalization analysis indicated a partial intracellular colocalization of TrkA and Nedd4-2 in the presence of NGF (Arevalo, Waite et al. 2006). In our study we performed a colocalization analysis of TrkAwt and mutant receptors with both ubiquitin ligases, Nedd4-2 and TRAF6. Results showed a significantly higher colocalization efficiency of Nedd4-2 with both mutants than with TrkAwt under basal conditions, whereas no changes were detected for TRAF6. Interestingly, a significant decrease of the Nedd4-2 colocalization efficiency was observed after NGF stimulation of TrkAwt receptor. This result corroborates previously published data showing that Nedd4-2 preferentially associates with the unphosphorylated TrkA receptor; thus not competing with PLC-γ for binding to TrkA (Arevalo, Waite et al. 2006). Intriguingly, the stimulation with NGF did not result in reduction of the colocalization coefficient of both mutants with Nedd4-2 as it was demonstrated for the TrkAwt receptor. In addition, by immunoprecipitation assays we revealed a higher quantity of the C-terminus mutant receptors bound to FLAG-Nedd4-2. Therefore, these results suggest that there is a stronger interaction between Nedd4-2 ubiquitin ligase and the C-terminal tail mutants due to the modification introduced on L784 and V790 which increases Nedd4-2 affinity for the receptor. This observation implies that not only the expression level of Nedd4-2 ubiquitin ligase is important for the mediation of the ubiquitination process as

described for the ENaC by Zhou and colleagues (Zhou, Patel et al. 2007), but also its capacity for interaction with the target molecule plays an essential role. Altogether, these data indicate that the modification of the TrkA C-terminal domain results in an increase of Nedd4-2 binding to the receptor. Thus, the stronger binding promotes a higher level of receptor multimonoubiquitination in resting conditions and leads to a raise in the receptor degradation rate, probably by interfering with the recycling pathway. Therefore, overubiquitination observed on both mutant receptors could be due to their higher accessibility and binding affinity for the Nedd4-2 ubiquitin ligase.

Considering previous data pointing to a role of Nedd4-2 on the ubiquitination of TrkA receptor we decided to down-regulate the expression of Nedd4-2 in PC12nnr5 cells overexpressing the "wild type" and mutant receptors. Importantly, Nedd4-2 silencing led to a reduction of the ubiquitination signal, observed for all tested conditions. A decrease in the basal ubiquitination of the TrkAwt receptor was also observed despite the lower signal detected. Consequently, our observations strongly suggest that Nedd4-2 regulates the steady state levels of TrkA receptor by ubiquitination and it is responsible of the over-ubiquitination of the C-terminal mutant receptors. Similar results were obtained by Zhou and collaborators on the characterization of ENaC ubiquitination by Nedd4-2. They showed that Nedd4-2 silencing decreased ENaC ubiquitination (Zhou, Patel et al. 2007). An alternative study of Sorkin's group, in which the mechanisms of Nedd4-2-mediated ubiquitination of the dopamine transporter (DAT) were investigated, showed that Nedd4-2 depletion by different siRNA sequences inhibited PKC-induced DAT ubiquitination. Furthermore, DAT ubiquitination was rescued by reconstitution of Nedd4-2 in siRNA-depleted cells of various cellular backgrounds (Vina-Vilaseca and Sorkin). Moreover, overexpression of Nedd4-2 increased the ubiquitination of DAT, supporting the role of Nedd4-2 in DAT ubiquitination. Both studies demonstrate the relevance of Nedd4-2 on the ubiquitination mechanism of two unrelated membrane proteins.

It is well known that in the process of receptor downregulation, sustained ubiquitination of RTKs marks them for degradation. In the present study we demonstrated that TrkA multimonoubiquitination observed on the C-terminal mutants is able to target inactive TrkA receptors to the lysosomal degradation pathway, in the absence of ligand. In addition to previous studies our results demonstrates that not only the quantity of Nedd4-2 Ub ligase is important for the ubiquitination of target proteins, but also the binding capability of Nedd4-2 with the

substrate is crucial. In this sense, we show that Nedd4-2 binding affinity is dependent on the conformation of the region where the association occurs. Therefore, our results are in agreement with previous studies and besides, they strongly suggest that Nedd4-2 can actively participate in the regulation of TrkA receptor stability, even under basal conditions.

6. Effect of ubiquitination on NGF induced receptor internalization

Ligand-induced endocytosis has been described as a mechanism of negative regulation of receptor signaling activities, reducing the number of membrane receptors available for ligand activation. However, it has been demonstrated that signaling can also occurs from endosomes, where receptors can interact with intracellular proteins through their exposed intracellular domains. Thus, internalized receptors can continue to transduce signal events initiated at the plasma membrane or they can assemble in newly formed signaling complexes generating specific signals (Sadowski, Pilecka et al. 2009).

Ubiquitination of membrane proteins has been reported to be a signal for promoting receptor internalization. Studies performed in yeast have demonstrated that Ub is required for the first step in cargo internalization, as well as for targeting of cargos to vacuoles, which suggests that it is critical in determining intracellular protein trafficking (Hicke and Riezman 1996). Indeed, monoubiquitination alone of several yeast transmembrane receptors (alpha-factor receptors, permeases and transporters) is sufficient to trigger their internalization, although modification with Lys63-linked Ub chains can speed up this process (Galan and Haguenauer-Tsapis 1997).

The situation is more complex in mammalian cells since not only the receptor but also the endocytic adaptors, are often ubiquitinated in response to extracellular stimuli.
Nonetheless, more than one internalization pathway exists in the cell and not all of them appear to be regulated by ubiquitin. Early studies on the growth hormone receptor (GHR) have shown that an intact ubiquitination machinery is required, although ubiquitination of the receptor is not indispensable for its internalization (Govers, ten Broeke et al. 1999). This statement has also been proved for a class of

G Protein-Coupled Receptors (GPCRs) that are ubiquitinated upon agonist stimulation (Shenoy, McDonald et al. 2001). Subsequent studies of various GPCRs and RTKs, such as EGFR suggested that ubiquitination is not essential for their clathrin-dependent endocytosis (Huang, Goh et al. 2007).

At least four mechanistically diverse and highly regulated endocytic routes exist for membrane receptors: macropinocytosis, clathrin-mediated endocytosis, caveolae-mediated endocytosis, and clathrin- and caveolae-independent endocytosis. (Zweifel, Kuruvilla et al. 2005). At present, there is some debate regarding which endocytic route is the dominant mechanism for the uptake of neurotrophins and Trk receptors, and there is evidence to support both clathrin-dependent (Grimes, Zhou et al. 1996; Beattie, Howe et al. 2000) and -independent mechanisms. TrkA has also been localized to caveolin-like domains in the PC12 cell line (Bilderback, Gazula et al. 1999; Peiro, Comella et al. 2000) and recently Pincher was identified as a membrane trafficking protein that mediates endocytosis and the trafficking of NGF–TrkA complexes by macropinocytosis (Shao, Akmentin et al. 2002).

Considering the fact that increased ubiquitination has been shown to be a signal that regulates receptor internalization for several membrane receptors, we next assessed the effect of the overubiquitination of both TrkA mutants on the internalization of the receptor. TrkA internalization was assessed by biotinylation of membrane proteins and cleavage of the membrane label after two incubation intervals (5 min and 30 min) in the presence or absence of NGF. An NGF-induced increase in receptor internalization was only observed on TrkAwt expressing cells, whereas TrkA mutant receptors did not undergo NGF-induced internalization. The amount of receptor protected from cleavage in the absence of neurotrophin was similar to TrkAwt for the L784A mutant and lower for the L784A-V790A mutant. Our results indicate that multimonoubiquitination by Nedd4-2 does not promote a higher rate of receptor internalization by its own, because overubiquitinated TrkA receptors internalize at a similar or even lower rate than TrkAwt, under basal conditions. However, after NGF addition, ubiquitination of TrkA seems to interfere with the NGF-induced receptor endocytosis mechanism. These results provide evidence that Nedd4-2 mediated ubiquitination of TrkA is not a signal, driving receptor internalization; on the contrary, it blocks TrkA activity-dependent endocytosis. One possibility could be that the covalent binding of ubiquitin on lysine residues would hide an internalization motif responsible of the recruitment of endocytic machinery. Our group described the existence of a clathrin dependent internalization motif inside

the TK domain of TrkA, the YRKF[701-704] sequence (de Pablo, Perez-Garcia et al. 2008), which participates in NGF-induced TrkA endocytosis. This motif contains a Lys residue (K703) that is a putative ubiquitination substrate for Nedd4-2. On the prediction of TrkA TK 3D conformation, K703 is located in an exposed region of the intracellular domain; therefore the covalent binding of an ubiquitin molecule to this residue could hide the AP-2/CHC complex binding site and disrupt the clathrin-dependent endocytosis. Considering these data, we could hypothesize that a major part of TrkA mutant receptors are endocyted in a clathrin-independent manner and are targeted for lysosomal degradation, since their enhanced ubiquitination rate may interfere with the recognition of the motifs essential for receptor recycling and for clathrin-dependent endocytosis. However, further research will be necessary to explore and demonstrate this theory.

Our results showed that TrkA multimonoubiquitination was able to target inactive TrkA receptors for lysosomal degradation in the absence of any stimulus, thus indicating that Nedd4-2 can actively participate in the regulation of the levels of TrkA receptors under basal conditions. Moreover, TrkA activation is not necessary for its sorting towards the lysosomal compartment. Therefore multimonoubiquitination of TrkA may favor the endosome-late endosome-lysosome route against the recycling pathway. Since it was demonstrated that TrkA ligand-dependent recycling depends on the juxtamembrane region (aa 473–493) of the receptor, which is absent in TrkB (Chen, Ieraci et al. 2005), and that this TrkA region contains K485, the residue identified as the TRAF6 ubiquitination site (Geetha, Jiang et al. 2005; Jadhav, Geetha et al. 2008), it can be inferred that ubiquitination on K485 may interfere with the recruitment of the recycling machinery on the juxtamembrane region.

We must have into account that protein ubiquitination depends on the activity of both, E3 Ub ligases and deubiquitination enzymes (DUBs), which are responsible for the removal of ubiquitin from the substrate, and that both groups of enzymes work together in a strictly coordinated manner. They assure the transferring of ubiquitin to substrates and maintain the pool of free Ub in the cell. Another possibility to explain our results could be that the increased number of ubiquitin molecules, covalently attached to both C-terminus mutants could not be completely removed by DUBs, while receptors are still in the early/recycling endosome, leading to predominant targeting of mutant receptors to late endosome/lysosome instead of recycling back to the membrane, as it was described for TrkA receptors under normal

conditions (Chen, Ieraci et al. 2005). However, further investigation is required to check whether any of these hypotheses is true.

7.   Regulation of the Nedd4-2 ubiquitin ligase activity

Two major mechanisms implicated in the regulation of Nedd4-2 ubiquitin ligase activity have been proposed. The first one, regarding the ENaC shows that Nedd4-2 Ub ligase activity depends on its binding affinity to the substrate, since disruption of this interaction causes the Liddle's syndrome, a disorder associated with severe sodium retention and hypertension, resulting from reduced ubiquitination and impossibility of ENaC to be targeted for lysosomal degradation (Staub, Dho et al. 1996). The second mechanism implies that the downregulation of Nedd4-2 is achieved by its catalytic site phosphorylation, which alters its ability to bind to ENaC (Snyder 2009). Thus, under basal conditions Nedd4-2 binds to ENaC and catalyses its ubiquitination whereas, upon phosphorylation it is unable to bind ENaC, so the channel remains at the cell surface resulting in enhanced Na^+ absorption. Nonetheless, another study demonstrates that phosphorylation deficient Nedd4-2 mutants are unable to either ubiquitinate ENaC or efficiently inhibit ENaC currents (Hallows, Bhalla et al.), therefore no conclusive data on the role of Nedd4-2 phosphorylation are available up to date.

Here we demonstrate that mutant receptors have a stronger affinity for Nedd4-2 Ub ligase, which is sufficient to promote an increased ubiquitination rate of the receptor in the absence of stimulus. Therefore, our results support the first model, where Nedd4-2 activity depends on its binding affinity. Furthermore, Arévalo and colleagues demonstrated that Nedd4-2 preferentially associates to inactive TrkA receptor, i.e. unphosphorylated on Y791, thus there is no competition with PLCγ for TrkA binding. Moreover, NGF addition led to a rapid Nedd4-2 phosphorylation which was dependent on TrkA activity. This observation together with the reduction in TrkA/Nedd4-2 colocalization upon NGF addition are in agreement with the second model, where Nedd4-2 phosphorylation decreases the binding affinity for the substrate and inactivates the enzyme.

In addition, our results demonstrate that the composition of the PPXY motif surrounding region is relevant for the regulation of Nedd4-2 activity and binding strength.

8. TrkA signal transduction and cell differentiation

In spite of the lower level of mutant receptors present at the plasma membrane, due to their higher ubiquitination and degradation rates, TrkA C-terminus altered receptors were able to transmit correctly the intracellular signal. Indeed, upon NGF addition an equivalent activation of the downstream signaling molecules, such as PLCγ, ERK1/2 and Akt was detected, despite the lower amount of Tyr phosphorylated receptor present on the membrane. A slight reduction in ERK1/2 and Akt phosphorylation was only detected for the L784A-V790A mutant at the submaximal concentration of 2.5 ng/ml of NGF. This result indicates that even with a lower level of TrkA receptor at the plasma membrane and a reduced total TrkA phosphorylation, the activation of downstream signaling cascades are hardly affected by Nedd4-2 binding and receptor ubiquitination.

Therefore, alterations introduced into the C-terminal tail, causing an increased Nedd4-2 binding did not unfavorably affect receptor activity. In addition, TrkA mutant receptors have shown to be able to transmit the differentiation signal in a higher percentage of cells than the wild-type receptor, even though with a shorter ERK1/2 activation sustainment. Therefore, surprisingly, TrkA receptors with a faster turnover and a shorter half-life are more efficient in transmitting the differentiation signal in PC12 cells in the presence of NGF. One possible explanation to these findings could be that the ubiquitin molecules serve as mediators for the recruitment of adaptor/substrate proteins to the TK domain, thus playing an additional important role in signal transduction. Another possibility could be that receptor ubiquitination previous to TK domain activation renders the activation of signaling cascades more efficient, providing docking sites for signaling molecules.

Our results provide evidence that Nedd4-2-mediated TrkA multimono-ubiquitination instead of interfering, may participate in the activation of signaling pathways, involved in the promotion of neurite outgrowth. In addition, it indicates that for the induction of neurite extension, the localized activation of signaling molecules may be sufficient, whereas the sustained ERK1/ERK2 or PLCγ activation is dispensable. In fact, it has been demonstrated that localized activation of phosphatidylinositol 3-kinase at the growth cone is essential for the rapid axon growth induced by NGF (Zhou, Zhou et al. 2004), and more recently, David and collaborators demonstrated that the localized phosphorylation of β-catenin by TrkB at the axon tip is implicated in axon growth in hippocampal neurons (David, Yeramian

et al. 2008). Altogether these results provide evidence that multimonoubiquitination of TrkA by Nedd4-2 participate in TrkA induced cell differentiation, probably by promoting the recruitment and activation of signaling molecules locally, or by the activation of other signaling cascades involved on cell differentiation, such as Erk5 or Rho/Rac GTPases. In this regard, a relevant role of ubiquitination on the regulation of signaling cascades has been demonstrated on death receptor and NFκB signaling pathways (Bianchi and Meier 2009; Wertz and Dixit 2010; Wertz and Dixit 2010) but no data are available for RTK signaling.

9. Effect of C-terminal alterations on neuronal survival

Complementary to the role on cell differentiation, Arevalo and collaborators (Arevalo, Waite et al. 2006) demonstrated that Nedd4-2 overexpression promotes the death of NGF-dependent sensory neurons. Furthermore, they showed that the downregulation of Nedd4-2 enhanced TrkA phosphorylation whilst the overexpression of Nedd4-2 caused a reduction in TrkA phosphorylation levels, related to the overall decrease in TrkA levels. Therefore, the control of the steady-state level of TrkA receptor was critical in cells dependent on NGF for their survival and the reduction in TrkA receptor stability in this cell context resulted in a decreased TrkA signaling that cannot support cell survival. This result indicates that the stability of the receptor is crucial for the survival signal.

Since PC12 cells are independent on TrkA signaling for survival they were a useful tool to elucidate the role of Nedd4-2 ubiquitin ligase on TrkA stability and signaling for differentiation. However, to clarify the importance of Nedd4-2 on TrkA activation leading to neuronal branching and cell survival we employed primary culture form superior cervical ganglion (SCG) neurons which express endogenous TrkA and are NGF-dependent for their survival. For that purpose we infected SCG neurons with lentiviruses codifying for the TrkAwt and mutant receptors and analyzed the effect of mutant receptor signaling on neuronal branching and survival in competition with the endogenous receptor. The overexpression of mutant receptors showed no alteration on the differentiation capacity of sympathetic neurons, since these neurons preserved the morphology and branching characteristic for the naïve sympathetic neurons and those transduced either with the empty or

with the TrkAwt receptor. However, NGF-deprived SCG neurons overexpressing mutant receptors showed a higher percentage of cell death compared to neurons overexpressing the TrkAwt or empty vector, analyzed by apoptotic nuclei counting and detection of active caspase 3. Moreover, the presence of the exogenous TrkAwt contributed to neuronal survival, since less apoptosis was observed for those neurons overexpressing TrkAwt together with the endogenous receptor.

Our results support Arévalo's group findings, demonstrating that mutant receptors with altered stability and their sorting directed to the lysosomes have reduced signaling capabilities for promoting cell survival. Thus, ubiquitin-promoted receptor downregulation of the C-terminal mutant receptors affects SCG neuron survival. Here we provide evidences that Nedd4-2 is one of the important regulators of TrkA protein levels, thus affecting the fate of cells dependent on NGF for their survival.

Interestingly, the alteration of RTKs downregulation has been tightly associated with the pathogenesis of cancer, including as examples the Met receptor, colony-stimulating factor-1 receptor, PDGFR and EGFR. These receptors have all been identified as ubiquitination substrates of the c-Cbl Ub ligase and for all of them receptor ubiquitination target proteins for degradation. In case that c-Cbl fails to bind and ubiquitinate Met, then the constitutively active-oncogenic form of the receptor Trp-Met is generated (Peschard and Park 2007). Furthermore, an EGFR mutant lacking only the c-Cbl-binding site elicits stronger mitogenic signals than the wild-type receptor (Waterman, Katz et al. 2002). Together, these receptors represent examples of how some oncoproteins avoid lysosomal downregulation by the loss of the c-Cbl-binding site or by inefficient c-Cbl recruitment. Taken all data together it is important to emphasize that RTK downregulation should be strictly regulated, since any alteration could have significant physiological effects such as those described above.

Research groups working on the characterization of the role of RTK ubiquitination have mainly introduced point mutations on substrate proteins, aiming at hampering the ubiquitination sites and disturb the ubiquitination process. On the contrary, in our study we have generated TrkA mutants that have an increased binding affinity to Nedd4-2 and consequently are highly ubiquitinated, which makes them an invaluable tool for analyzing the role of Nedd4-2 multimonoubiquitination on TrkA receptor biology. We obtained evidence for the relevance of multimonoubiquitination on the receptor lysosomal targeting in the absence of ligand

and interestingly, we demonstrated that mutant receptors with a reduced half life were able to transmit NGF signal and promote cell differentiation even at a higher extent than the wild type receptors. These findings suggest a new role of TrkA receptor ubiquitination on the promotion of signaling cascades that direct neurite outgrowth.

CONCLUSIONS

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CONCLUSIONS

- **First**: Hydrophobic amino acids L784 and V790 situated in TrkA C-terminal tail are not implicated in CaM binding to the receptor

- **Second**: TrkA C-terminal mutant receptors have a stronger affinity for Nedd4-2, demonstrating that residues surrounding the Nedd4-2 binding motif are relevant for Nedd4-2 recruitment and activity

- **Third**: Nedd4-2 binding to TrkA mutants promotes a higher multimonoubiquitination rate that targets inactive TrkA receptors for lysosomal degradation.

- **Fourth**: Nedd4-2 induced multimonoubiquitination of TrkA C-terminal mutants does not increase TrkA basal endocytosis, but blocks NGF-induced receptor internalization

- **Fifth**: TrkA mutant receptors are able to transmit normally the intracellular signal, although they are incapable of sustaining the activation of PLCγ and ERK1/ERK2 for more than 2 days

- **Sixth**: TrkA C-terminal mutants have an enhanced capability to promote neurite outgrowth in PC12nnr5 cells.

- **Seventh**: Sympathetic neurons overexpressing mutant receptors are more susceptible to NGF withdrawal-induced apoptosis

ABSTRACTS

ABSTRACT

The nerve growth factor receptor TrkA contributes to the survival and differentiation of several neuronal populations. Although main signaling pathways and adaptor molecules activated after ligand binding to the receptor are well studied, recently a growing number of TrkA interacting proteins have been described as novel mechanisms of receptor regulation. Receptor trans-autophosphorylation, additionally to activating signaling cascades relevant for cell differentiation and survival, such as MAPKs and PI3K-Akt, also induces a transient increase in intracellular calcium concentration. Therefore, the Ca^{2+} sensor calmodulin (CaM) also participates in TrkA signaling. Our group was interested in elucidating the relationship between CaM and TrkA, and discovered that CaM interacts directly with the C-terminal tail of TrkA in a Ca^{2+} dependent manner. Consequently, we sought to find the exact position of the calmodulin binding site on TrkA sequence. Since available prediction software was unable to find a putative CaM binding site in TrkA intracellular domain, we focused on the C-terminal tail, a domain rich in hydrophobic amino acids in conserved positions. We introduced point mutations on Leu784 and Val790 by switching them to Ala, located on our predicted CaM binding site. However, TrkA-L784A and TrkA-L784A/V790A mutants did not lose CaM binding, therefore they were not involved on CaM binding. Nonetheless, we demonstrated that these hydrophobic aminoacids are important for the binding and regulation of Nedd4-2 Ub ligase. We observed a stronger interaction of both C-terminal mutant receptors with Nedd4-2, corroborated with a higher colocalization of both proteins by immunofluorescence. Consequently, we observed an enhanced basal multimonoubiquitination of mutant receptors by Nedd4-2. These results correlated with a decrease in TrkA abundance due to a faster late endosome/lysosome targeting, leading to a higher degradation rate of mutant receptors. Despite the reduction in the amount of membrane receptor caused by the C-terminal changes, TrkA mutants were able to activate signaling cascades and were even more efficient in promoting neurite outgrowth than the wild-type receptor. Our results demonstrate that the C-terminal tail conformation of TrkA regulates Nedd4-2 binding and activity. Moreover, TrkA ubiquitination by Nedd4-2 promotes TrkA endosomal trafficking to late endosome/lysosome leading to receptor degradation. In addition, TrkA multimonoubiquitination does not interfere with the activation of signaling cascades, but rather potentiates receptor signaling leading to differentiation.

RESUMEN

El receptor del factor de crecimiento nervioso TrkA contribuye a la supervivencia y diferenciación de varias poblaciones neuronales. A pesar del buen conocimiento de las principales vías de señalización y las proteínas adaptadoras activadas después de la unión del ligando al receptor, últimamente se ha descrito un número creciente de proteínas que interaccionan con el receptor participando en su regulación. La trans-autofosforilación del receptor, adicionalmente a la activación de vías de señalización relevantes para la diferenciación y la supervivencia celular, como son las vías de las MAPKs y PI3K-Akt, también conlleva un aumento transitorio del calcio citoplasmático. En consecuencia la proteína sensora de calcio calmodulina (CaM) también participa en la señalización por TrkA. Nuestro grupo tenía interés en estudiar la relación entre CaM y TrkA y descubrió que la CaM interacciona de forma directa y calcio dependiente con el extremo COOH-terminal del receptor TrkA. A partir de estos resultados nos plantemaos buscar la posición exacta del sitio de unión a calmodulina dentro de la secuencia de TrkA. Puesto que los programas de predicción no encontraron ninguna secuencia candidata de unión a calmodulina en el dominio intracelular de TrkA, nos centramos en el extremo COOH-terminal, dominio rico en ácidos hidrofóbicos en posiciones conservadas. Introducimos mutaciones puntuales en el sitio candidato de unión a CaM cambiando la L784 y la V790 por Ala. Los ensayos de unión a CaM realizados mostraron que los mutantes TrkA-L784A y TrkAL784A-V790A no perdían la unión a CaM, por lo tanto no formaban parte del dominio de unión a CaM de TrkA. Sin embargo, hemos demostrado que estos aminoácidos son importantes para la unión y la regulación de la ligasa de ubiquitina Nedd4-2. . Observamos una mayor interacción de los dos mutantes del extremo COOH-terminal con Nedd4-2, resultado que era corroborado por una mayor colocalización de las dos proteínas detectada por immunofluorescencia. Como consecuencia observamos una intensa multimonoubiquitinización basal de los receptores mutados mediada por Nedd4-2. Estos resultados correlacionan con un importante descenso de la cantidad de receptor a causa de un direccionamiento de este hacia los endosomas tardíos/lisosomas. Todo ello resultaba en un incremento en la degradación de los receptors mutados. A pesar de la reducción en la cantidad de receptor en membrana, los mutantes de TrkA mostraron ser capaces de activar las vías de señalización e incluso ser más eficientes promoviendo el crecimiento de neuritas comparando con el receptor normal. Estos resultados demuestran que la composición del extremo COOH-terminal de TrkA regula la unión y la actividad de la ligasa de ubiquitina Nedd4-2. Además, la ubiquitinización de TrkA mediada por Nedd4-2 promueve el direccionamiento de los receptores mutados hacia los endosomas tardíos/lysosomas dando lugar a una mayor degradación del receptor. Por otro lado, se observa que la multimonoubiquitinización de TrkA no interfiere con la activación de las vías de señalización, sino más bien potencia la señalización del receptor que conduce a la diferenciación celular.

RESUM

El receptor del factor de creixement nerviós TrkA contribueix a la supervivència i a la diferenciació de diferents poblacions neuronals. Tot i que les principals vies de senyalització i molècules adaptadores activades després de la unió del lligant al receptor estan ben estudiades, recentment un creixent nombre de proteïnes que interaccionen amb TrkA han estat descrites com a nous mecanismes de regulació del receptor. La trans-autofosforilació del receptor, a més d'activar les cascades de senyalització rellevants per la diferenciació i supervivència cel·lular, com són les vies de les MAPKs i PI3K-Akt, també indueix un augment transitori de la concentració de calci citoplasmàtic. En conseqüència la proteïna sensora de Ca^{2+} calmodulina (CaM) també participa en la senyalització per TrkA. El nostre grup estava interessat en discernir la relació entre CaM i TrkA, i vàrem descobrir que CaM interacciona directament i de manera Ca^{2+}-dependent amb l'extrem carboxi-terminal de TrkA. A partir d'aquests resultats ens vàrem plantejar de buscar la posició exacta del domini d'unió a calmodulina en la seqüencia de TrkA. Donat que els programes de predicció no van trobar una seqüència candidata d'unió a CaM en el domini intracel·lular del TrkA, vàrem centrar-nos en el extrem COOH-terminal del receptor, un domini ric en aminoàcids hidrofòbics en posicions conservades. Vàrem introduir mutacions en dos aminoàcids del domini candidat, Leu784 i Val790 substituint-los per Ala. Els assajos d'unió van mostrar que els mutants TrkA-L784A i TrkA-L784A-V790A no perdien la unió a CaM, per tant no formaven part del domini d'unió a CaM de TrkA. Tanmateix, vàrem demostrar que aquests aminoàcids hidrofòbics són importants per a la unió i la regulació de la lligasa d'ubiquitina Nedd4-2. Vàrem observar una forta interacció dels dos receptors mutants amb Nedd4-2, que es corroborava amb una augmentada co-localització de les dues proteïnes detectada per immunofluorescència. Com a conseqüència s'observà una intensa multimonoubiquitinització basal dels receptors mutants mitjançada per Nedd4-2. Aquests resultats correlacionen amb un important descens en la quantitat de receptor a causa d'un direccionament d'aquest cap als endosomes tardans/lisosomes. Tot plegat resultava en un increment en la degradació dels receptors mutats. Malgrat la reducció en la quantitat de receptors de membrana, els receptors TrkA mutats van ser capaços d'activar vies de senyalització, i fins i tot eren més eficients en promoure l'extensió de neurites que els receptors TrkA normals. Els nostres resultats demostren que la composició de l'extrem COOH-terminal de TrkA participa en la regulació de la unió i activitat de Nedd4-2. Així mateix, s'evidencia que la ubiquitinització de TrkA per Nedd4-2 promou el tràfic endosomal de TrkA cap als endosomes tardans/lisosomes augmentant d'aquesta manera la degradació del receptor. Per altra banda, s'observa que la multimonoubiquitinització de TrkA no interfereix en l'activació de les vies de senyalització, tanmateix potencia la senyalització del receptor conduent a la diferenciació cel·lular.

BIBLIOGRAPHY

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Abella, J. V., P. Peschard, et al. (2005). "Met/Hepatocyte growth factor receptor ubiquitination suppresses transformation and is required for Hrs phosphorylation." Mol Cell Biol **25**(21): 9632-45.

Acconcia, F., S. Sigismund, et al. (2009). "Ubiquitin in trafficking: the network at work." Exp Cell Res **315**(9): 1610-8.

Arevalo, J. C., B. Conde, et al. (2000). "TrkA immunoglobulin-like ligand binding domains inhibit spontaneous activation of the receptor." Mol Cell Biol **20**(16): 5908-16.

Arevalo, J. C., J. Waite, et al. (2006). "Cell Survival through Trk Neurotrophin Receptors Is Differentially Regulated by Ubiquitination." Neuron **50**(4): 549-59.

Arevalo, J. C. and S. H. Wu (2006). "Neurotrophin signaling: many exciting surprises!" Cell Mol Life Sci **63**(13): 1523-37.

Arevalo, J. C., H. Yano, et al. (2004). "A unique pathway for sustained neurotrophin signaling through an ankyrin-rich membrane-spanning protein." EMBO J **23**(12): 2358-68.

Arighi, E., M. G. Borrello, et al. (2005). "RET tyrosine kinase signaling in development and cancer." Cytokine Growth Factor Rev **16**(4-5): 441-67.

Atwal, J. K., B. Massie, et al. (2000). "The TrkB-Shc site signals neuronal survival and local axon growth via MEK and P13-kinase." Neuron **27**(2): 265-77.

Barbacid, M. (1995). "Structural and functional properties of the TRK family of neurotrophin receptors." Ann N Y Acad Sci **766**: 442-58.

Barde, Y. A., D. Edgar, et al. (1982). "Purification of a new neurotrophic factor from mammalian brain." EMBO J **1**(5): 549-53.

Barker, P. A. (2004). "p75NTR is positively promiscuous: novel partners and new insights." Neuron **42**(4): 529-33.

Barres, B. A. and M. C. Raff (1994). "Control of oligodendrocyte number in the developing rat optic nerve." Neuron **12**(5): 935-42.

Beattie, E. C., C. L. Howe, et al. (2000). "NGF signals through TrkA to increase clathrin at the plasma membrane and enhance clathrin-mediated membrane trafficking." J Neurosci **20**(19): 7325-33.

Ben-Saadon, R., D. Zaaroor, et al. (2006). "The polycomb protein Ring1B generates self atypical mixed ubiquitin chains required for its in vitro histone H2A ligase activity." <u>Mol Cell</u> **24**(5): 701-11.

Bianchi, K. and P. Meier (2009). "A tangled web of ubiquitin chains: breaking news in TNF-R1 signaling." <u>Mol Cell</u> **36**(5): 736-42.

Bibel, M., E. Hoppe, et al. (1999). "Biochemical and functional interactions between the neurotrophin receptors trk and p75NTR." <u>EMBO J</u> **18**(3): 616-22.

Bilderback, T. R., V. R. Gazula, et al. (1999). "Caveolin interacts with Trk A and p75(NTR) and regulates neurotrophin signaling pathways." <u>J Biol Chem</u> **274**(1): 257-63.

Blume-Jensen, P. and T. Hunter (2001). "Oncogenic kinase signalling." <u>Nature</u> **411**(6835): 355-65.

Bonifacino, J. S. and L. M. Traub (2003). "Signals for sorting of transmembrane proteins to endosomes and lysosomes." <u>Annu Rev Biochem</u> **72**: 395-447.

Bowers, K., S. C. Piper, et al. (2006). "Degradation of endocytosed epidermal growth factor and virally ubiquitinated major histocompatibility complex class I is independent of mammalian ESCRTII." <u>J Biol Chem</u> **281**(8): 5094-105.

Brodeur, G. M., J. M. Maris, et al. (1997). "Biology and genetics of human neuroblastomas." <u>J Pediatr Hematol Oncol</u> **19**(2): 93-101.

Brodeur, G. M., J. E. Minturn, et al. (2009). "Trk receptor expression and inhibition in neuroblastomas." <u>Clin Cancer Res</u> **15**(10): 3244-50.

Brunet, A., A. Bonni, et al. (1999). "Akt promotes cell survival by phosphorylating and inhibiting a Forkhead transcription factor." <u>Cell</u> **96**(6): 857-68.

Burek, M. J. and R. W. Oppenheim (1996). "Programmed cell death in the developing nervous system." <u>Brain Pathol</u> **6**(4): 427-46.

Cadavid, A. L., A. Ginzel, et al. (2000). "The function of the Drosophila fat facets deubiquitinating enzyme in limiting photoreceptor cell number is intimately associated with endocytosis." <u>Development</u> **127**(8): 1727-36.

Chakrabarti, K., R. Lin, et al. (2005). "Critical role for Kalirin in nerve growth factor signaling through TrkA." <u>Mol Cell Biol</u> **25**(12): 5106-18.

Chang, M. S., J. C. Arevalo, et al. (2004). "Ternary complex with Trk, p75, and an ankyrin-rich membrane spanning protein." <u>J Neurosci Res</u> **78**(2): 186-92.

Chao, M. V. (2003). "Neurotrophins and their receptors: a convergence point for many signalling pathways." <u>Nat Rev Neurosci</u> **4**(4): 299-309.

Chao, M. V., R. Rajagopal, et al. (2006). "Neurotrophin signalling in health and disease." <u>Clin Sci (Lond)</u> **110**(2): 167-73.

Chen, H. and P. De Camilli (2005). "The association of epsin with ubiquitinated cargo along the endocytic pathway is negatively regulated by its interaction with clathrin." <u>Proc Natl Acad Sci U S A</u> **102**(8): 2766-71.

Chen, H., S. Polo, et al. (2003). "Rapid Ca2+-dependent decrease of protein ubiquitination at synapses." <u>Proc Natl Acad Sci U S A</u> **100**(25): 14908-13.

Chen, Z. Y., A. Ieraci, et al. (2005). "A novel endocytic recycling signal distinguishes biological responses of Trk neurotrophin receptors." <u>Mol Biol Cell</u> **16**(12): 5761-72.

Chuang, H. H., E. D. Prescott, et al. (2001). "Bradykinin and nerve growth factor release the capsaicin receptor from PtdIns(4,5)P2-mediated inhibition." <u>Nature</u> **411**(6840): 957-62.

Clague, M. J. and S. Urbe (2006). "Endocytosis: the DUB version." <u>Trends Cell Biol</u> **16**(11): 551-9.

Clary, D. O. and L. F. Reichardt (1994). "An alternatively spliced form of the nerve growth factor receptor TrkA confers an enhanced response to neurotrophin 3." <u>Proc Natl Acad Sci U S A</u> **91**(23): 11133-7.

Clary, D. O., G. Weskamp, et al. (1994). "TrkA cross-linking mimics neuronal responses to nerve growth factor." <u>Mol Biol Cell</u> **5**(5): 549-63.

Coda, L., A. E. Salcini, et al. (1998). "Eps15R is a tyrosine kinase substrate with characteristics of a docking protein possibly involved in coated pits-mediated internalization." <u>J Biol Chem</u> **273**(5): 3003-12.

Corbit, K. C., D. A. Foster, et al. (1999). "Protein kinase Cdelta mediates neurogenic but not mitogenic activation of mitogen-activated protein kinase in neuronal cells." <u>Mol Cell Biol</u> **19**(6): 4209-18.

Curtis, R., K. M. Adryan, et al. (1995). "Differential role of the low affinity neurotrophin receptor (p75) in retrograde axonal transport of the neurotrophins." <u>Neuron</u> **14**(6): 1201-11.

Dahlmann, B. (2007). "Role of proteasomes in disease." <u>BMC Biochem</u> **8 Suppl 1**: S3.

Datta, S. R., A. Brunet, et al. (1999). "Cellular survival: a play in three Akts." <u>Genes Dev</u> **13**(22): 2905-27.

David, M. D., A. Yeramian, et al. (2008). "Signalling by neurotrophins and hepatocyte growth factor regulates axon morphogenesis by differential beta-catenin phosphorylation." Journal of Cell Science **121**(16): 2718-2730.

Davies, A. M., C. Bandtlow, et al. (1987). "Timing and site of nerve growth factor synthesis in developing skin in relation to innervation and expression of the receptor." Nature **326**(6111): 353-8.

De Donatis, A., G. Comito, et al. (2008). "Proliferation versus migration in platelet-derived growth factor signaling: the key role of endocytosis." J Biol Chem **283**(29): 19948-56.

de Pablo, Y., M. J. Perez-Garcia, et al. (2008). "Tyr-701 is a new regulatory site for neurotrophin receptor TrkA trafficking and function." J Neurochem **104**(1): 124-39.

Dechant, G. and Y. A. Barde (1997). "Signalling through the neurotrophin receptor p75NTR." Curr Opin Neurobiol **7**(3): 413-8.

DeFreitas, M. F., P. S. McQuillen, et al. (2001). "A novel p75NTR signaling pathway promotes survival, not death, of immunopurified neocortical subplate neurons." J Neurosci **21**(14): 5121-9.

Deinhardt, K., S. Salinas, et al. (2006). "Rab5 and Rab7 control endocytic sorting along the axonal retrograde transport pathway." Neuron **52**(2): 293-305.

Delcroix, J. D., J. S. Valletta, et al. (2003). "NGF signaling in sensory neurons: evidence that early endosomes carry NGF retrograde signals." Neuron **39**(1): 69-84.

Dikic, I. and S. Giordano (2003). "Negative receptor signalling." Curr Opin Cell Biol **15**(2): 128-35.

Djakiew, D., R. Delsite, et al. (1991). "Regulation of growth by a nerve growth factor-like protein which modulates paracrine interactions between a neoplastic epithelial cell line and stromal cells of the human prostate." Cancer Res **51**(12): 3304-10.

Dobrowsky, R. T., M. H. Werner, et al. (1994). "Activation of the sphingomyelin cycle through the low-affinity neurotrophin receptor." Science **265**(5178): 1596-9.

Eide, F. F., E. R. Vining, et al. (1996). "Naturally occurring truncated trkB receptors have dominant inhibitory effects on brain-derived neurotrophic factor signaling." J Neurosci **16**(10): 3123-9.

Esposito, D., P. Patel, et al. (2001). "The cytoplasmic and transmembrane domains of the p75 and Trk A receptors regulate high affinity binding to nerve growth factor." J Biol Chem **276**(35): 32687-95.

Fahnestock, M., B. Michalski, et al. (2001). "The precursor pro-nerve growth factor is the predominant form of nerve growth factor in brain and is increased in Alzheimer's disease." Mol Cell Neurosci **18**(2): 210-20.

Fire, A., S. Xu, et al. (1998). "Potent and specific genetic interference by double-stranded RNA in Caenorhabditis elegans." Nature **391**(6669): 806-11.

Fischer, J. A., S. H. Eun, et al. (2006). "Endocytosis, endosome trafficking, and the regulation of Drosophila development." Annu Rev Cell Dev Biol **22**: 181-206.

Frederick, L., X. Y. Wang, et al. (2000). "Diversity and frequency of epidermal growth factor receptor mutations in human glioblastomas." Cancer Res **60**(5): 1383-7.

Frossard, N., V. Freund, et al. (2004). "Nerve growth factor and its receptors in asthma and inflammation." Eur J Pharmacol **500**(1-3): 453-65.

Funakoshi, H., N. Belluardo, et al. (1995). "Muscle-derived neurotrophin-4 as an activity-dependent trophic signal for adult motor neurons." Science **268**(5216): 1495-9.

Galan, J. M. and R. Haguenauer-Tsapis (1997). "Ubiquitin lys63 is involved in ubiquitination of a yeast plasma membrane protein." EMBO J **16**(19): 5847-54.

Galcheva-Gargova, Z., S. J. Theroux, et al. (1995). "The epidermal growth factor receptor is covalently linked to ubiquitin." Oncogene **11**(12): 2649-55.

Garner, A. S., H. J. Menegay, et al. (1996). "Expression of TrkB receptor isoforms in the developing avian visual system." J Neurosci **16**(5): 1740-52.

Geetha, T., J. Jiang, et al. (2005). "Lysine 63 polyubiquitination of the nerve growth factor receptor TrkA directs internalization and signaling." Mol Cell **20**(2): 301-12.

Geetha, T. and M. W. Wooten (2003). "Association of the atypical protein kinase C-interacting protein p62/ZIP with nerve growth factor receptor TrkA regulates receptor trafficking and Erk5 signaling." J Biol Chem **278**(7): 4730-9.

Geetha, T. and M. W. Wooten (2008). "TrkA receptor endolysosomal degradation is both ubiquitin and proteasome dependent." Traffic **9**(7): 1146-56.

Ginty, D. D., A. Bonni, et al. (1994). "Nerve growth factor activates a Ras-dependent protein kinase that stimulates c-fos transcription via phosphorylation of CREB." Cell **77**(5): 713-25.

Govers, R., T. ten Broeke, et al. (1999). "Identification of a novel ubiquitin conjugation motif, required for ligand-induced internalization of the growth hormone receptor." EMBO J **18**(1): 28-36.

Green, S. H., R. E. Rydel, et al. (1986). "PC12 cell mutants that possess low- but not high-affinity nerve growth factor receptors neither respond to nor internalize nerve growth factor." J Cell Biol **102**(3): 830-43.

Grimes, M. L., J. Zhou, et al. (1996). "Endocytosis of activated TrkA: evidence that nerve growth factor induces formation of signaling endosomes." J Neurosci **16**(24): 7950-64.

Grovdal, L. M., E. Stang, et al. (2004). "Direct interaction of Cbl with pTyr 1045 of the EGF receptor (EGFR) is required to sort the EGFR to lysosomes for degradation." Exp Cell Res **300**(2): 388-95.

Gual, P., S. Giordano, et al. (2001). "Differential requirement of the last C-terminal tail of Met receptor for cell transformation and invasiveness." Oncogene **20**(39): 5493-502.

Hafezparast, M., R. Klocke, et al. (2003). "Mutations in dynein link motor neuron degeneration to defects in retrograde transport." Science **300**(5620): 808-12.

Haglund, K., P. P. Di Fiore, et al. (2003). "Distinct monoubiquitin signals in receptor endocytosis." Trends Biochem Sci **28**(11): 598-603.

Haglund, K., S. Sigismund, et al. (2003). "Multiple monoubiquitination of RTKs is sufficient for their endocytosis and degradation." Nat Cell Biol **5**(5): 461-6.

Hallows, K. R., V. Bhalla, et al. "Phosphopeptide screen uncovers novel Nedd4-2 phosphorylation sites that potentiate its inhibition of the epithelial Na+ channel." J Biol Chem.

Harrington, A. W., J. Y. Kim, et al. (2002). "Activation of Rac GTPase by p75 is necessary for c-jun N-terminal kinase-mediated apoptosis." J Neurosci **22**(1): 156-66.

Harter, C. and I. Mellman (1992). "Transport of the lysosomal membrane glycoprotein lgp120 (lgp-A) to lysosomes does not require appearance on the plasma membrane." J Cell Biol **117**(2): 311-25.

Haugsten, E. M., J. Malecki, et al. (2008). "Ubiquitination of fibroblast growth factor receptor 1 is required for its intracellular sorting but not for its endocytosis." Mol Biol Cell **19**(8): 3390-403.

Hawryluk, M. J., P. A. Keyel, et al. (2006). "Epsin 1 is a polyubiquitin-selective clathrin-associated sorting protein." Traffic **7**(3): 262-81.

He, X. L. and K. C. Garcia (2004). "Structure of nerve growth factor complexed with the shared neurotrophin receptor p75." Science **304**(5672): 870-5.

Heerssen, H. M., M. F. Pazyra, et al. (2004). "Dynein motors transport activated Trks to promote survival of target-dependent neurons." Nat Neurosci **7**(6): 596-604.

Hempstead, B. L. (2002). "The many faces of p75NTR." Curr Opin Neurobiol **12**(3): 260-7.

Hetman, M., J. E. Cavanaugh, et al. (2000). "Role of glycogen synthase kinase-3beta in neuronal apoptosis induced by trophic withdrawal." J Neurosci **20**(7): 2567-74.

Hicke, L. (2001). "A new ticket for entry into budding vesicles-ubiquitin." Cell **106**(5): 527-30.

Hicke, L. (2001). "Protein regulation by monoubiquitin." Nat Rev Mol Cell Biol **2**(3): 195-201.

Hicke, L. and R. Dunn (2003). "Regulation of membrane protein transport by ubiquitin and ubiquitin-binding proteins." Annu Rev Cell Dev Biol **19**: 141-72.

Hicke, L. and H. Riezman (1996). "Ubiquitination of a yeast plasma membrane receptor signals its ligand-stimulated endocytosis." Cell **84**(2): 277-87.

Hicke, L., H. L. Schubert, et al. (2005). "Ubiquitin-binding domains." Nat Rev Mol Cell Biol **6**(8): 610-21.

Hoeller, D., N. Crosetto, et al. (2006). "Regulation of ubiquitin-binding proteins by monoubiquitination." Nat Cell Biol **8**(2): 163-9.

Hoeller, D., C. M. Hecker, et al. (2007). "E3-independent monoubiquitination of ubiquitin-binding proteins." Mol Cell **26**(6): 891-8.

Hofer, M. M. and Y. A. Barde (1988). "Brain-derived neurotrophic factor prevents neuronal death in vivo." Nature **331**(6153): 261-2.

Hofmann, K. and L. Falquet (2001). "A ubiquitin-interacting motif conserved in components of the proteasomal and lysosomal protein degradation systems." Trends Biochem Sci **26**(6): 347-50.

Holgado-Madruga, M., D. K. Moscatello, et al. (1997). "Grb2-associated binder-1 mediates phosphatidylinositol 3-kinase activation and the promotion of cell survival by nerve growth factor." Proc Natl Acad Sci U S A **94**(23): 12419-24.

Howe, C. L. and W. C. Mobley (2005). "Long-distance retrograde neurotrophic signaling." Curr Opin Neurobiol **15**(1): 40-8.

Howe, C. L., J. S. Valletta, et al. (2001). "NGF signaling from clathrin-coated vesicles: evidence that signaling endosomes serve as a platform for the Ras-MAPK pathway." Neuron **32**(5): 801-14.

Huang, E. J. and L. F. Reichardt (2001). "Neurotrophins: roles in neuronal development and function." Annu Rev Neurosci **24**: 677-736.

Huang, E. J. and L. F. Reichardt (2003). "Trk receptors: roles in neuronal signal transduction." Annu Rev Biochem **72**: 609-42.

Huang, F., L. K. Goh, et al. (2007). "EGF receptor ubiquitination is not necessary for its internalization." Proc Natl Acad Sci U S A **104**(43): 16904-9.

Huang, F., D. Kirkpatrick, et al. (2006). "Differential regulation of EGF receptor internalization and degradation by multiubiquitination within the kinase domain." Mol Cell **21**(6): 737-48.

Huang, T. T. and A. D. D'Andrea (2006). "Regulation of DNA repair by ubiquitylation." Nat Rev Mol Cell Biol **7**(5): 323-34.

Hubbard, S. R. and J. H. Till (2000). "Protein tyrosine kinase structure and function." Annu Rev Biochem **69**: 373-98.

Hurley, J. H., S. Lee, et al. (2006). "Ubiquitin-binding domains." Biochem J **399**(3): 361-72.

Indo, Y. (2001). "Molecular basis of congenital insensitivity to pain with anhidrosis (CIPA): mutations and polymorphisms in TRKA (NTRK1) gene encoding the receptor tyrosine kinase for nerve growth factor." Hum Mutat **18**(6): 462-71.

Indo, Y. (2009). "Nerve growth factor, interoception, and sympathetic neuron: lesson from congenital insensitivity to pain with anhidrosis." Auton Neurosci **147**(1-2): 3-8.

Indo, Y., M. Tsuruta, et al. (1996). "Mutations in the TRKA/NGF receptor gene in patients with congenital insensitivity to pain with anhidrosis." Nat Genet **13**(4): 485-8.

Ingham, R. J., G. Gish, et al. (2004). "The Nedd4 family of E3 ubiquitin ligases: functional diversity within a common modular architecture." Oncogene **23**(11): 1972-84.

Jadhav, T., T. Geetha, et al. (2008). "Identification of a consensus site for TRAF6/p62 polyubiquitination." <u>Biochem Biophys Res Commun</u> **371**(3): 521-4.

Jiang, X. and A. Sorkin (2003). "Epidermal growth factor receptor internalization through clathrin-coated pits requires Cbl RING finger and proline-rich domains but not receptor polyubiquitylation." <u>Traffic</u> **4**(8): 529-43.

Jullien, J., V. Guili, et al. (2002). "Molecular kinetics of nerve growth factor receptor trafficking and activation." <u>J Biol Chem</u> **277**(41): 38700-8.

Kamakura, S., T. Moriguchi, et al. (1999). "Activation of the protein kinase ERK5/BMK1 by receptor tyrosine kinases. Identification and characterization of a signaling pathway to the nucleus." <u>J Biol Chem</u> **274**(37): 26563-71.

Kao, S., R. K. Jaiswal, et al. (2001). "Identification of the mechanisms regulating the differential activation of the mapk cascade by epidermal growth factor and nerve growth factor in PC12 cells." <u>J Biol Chem</u> **276**(21): 18169-77.

Kaplan, D. R. and F. D. Miller (2000). "Neurotrophin signal transduction in the nervous system." <u>Curr Opin Neurobiol</u> **10**(3): 381-91.

Katzmann, D. J., G. Odorizzi, et al. (2002). "Receptor downregulation and multivesicular-body sorting." <u>Nat Rev Mol Cell Biol</u> **3**(12): 893-905.

Khursigara, G., J. Bertin, et al. (2001). "A prosurvival function for the p75 receptor death domain mediated via the caspase recruitment domain receptor-interacting protein 2." <u>J Neurosci</u> **21**(16): 5854-63.

Kimura, M. T., S. Irie, et al. (2001). "14-3-3 is involved in p75 neurotrophin receptor-mediated signal transduction." <u>J Biol Chem</u> **276**(20): 17291-300.

Kjaer, S. and C. F. Ibanez (2003). "Intrinsic susceptibility to misfolding of a hot-spot for Hirschsprung disease mutations in the ectodomain of RET." <u>Hum Mol Genet</u> **12**(17): 2133-44.

Klapper, L. N., H. Waterman, et al. (2000). "Tumor-inhibitory antibodies to HER-2/ErbB-2 may act by recruiting c-Cbl and enhancing ubiquitination of HER-2." <u>Cancer Res</u> **60**(13): 3384-8.

Kloetzel, P. M. (2001). "Antigen processing by the proteasome." <u>Nat Rev Mol Cell Biol</u> **2**(3): 179-87.

Koch, A., A. Mancini, et al. (2000). "Direct interaction of nerve growth factor receptor, TrkA, with non-receptor tyrosine kinase, c-Abl, through the activation loop." <u>FEBS Lett</u> **469**(1): 72-6.

Kouhara, H., Y. R. Hadari, et al. (1997). "A lipid-anchored Grb2-binding protein that links FGF-receptor activation to the Ras/MAPK signaling pathway." <u>Cell</u> **89**(5): 693-702.

Kuruvilla, R., L. S. Zweifel, et al. (2004). "A neurotrophin signaling cascade coordinates sympathetic neuron development through differential control of TrkA trafficking and retrograde signaling." <u>Cell</u> **118**(2): 243-55.

Lee, R., P. Kermani, et al. (2001). "Regulation of cell survival by secreted proneurotrophins." <u>Science</u> **294**(5548): 1945-8.

Lee, S., Y. C. Tsai, et al. (2006). "Structural basis for ubiquitin recognition and autoubiquitination by Rabex-5." <u>Nat Struct Mol Biol</u> **13**(3): 264-71.

Levi-Montalcini, R. (1966). "The nerve growth factor: its mode of action on sensory and sympathetic nerve cells." <u>Harvey Lect</u> **60**: 217-59.

Levi-Montalcini, R. (1987). "The nerve growth factor 35 years later." <u>Science</u> **237**(4819): 1154-62.

Levi-Montalcini, R. and V. Hamburger (1951). "Selective growth stimulating effects of mouse sarcoma on the sensory and sympathetic nervous system of the chick embryo." <u>J Exp Zool</u> **116**(2): 321-61.

Levi-Montalcini, R., S. D. Skaper, et al. (1996). "Nerve growth factor: from neurotrophin to neurokine." <u>Trends Neurosci</u> **19**(11): 514-20.

Levkowitz, G., H. Waterman, et al. (1999). "Ubiquitin ligase activity and tyrosine phosphorylation underlie suppression of growth factor signaling by c-Cbl/Sli-1." <u>Mol Cell</u> **4**(6): 1029-40.

Levkowitz, G., H. Waterman, et al. (1998). "c-Cbl/Sli-1 regulates endocytic sorting and ubiquitination of the epidermal growth factor receptor." <u>Genes Dev</u> **12**(23): 3663-74.

Lin, S. Y., K. Wu, et al. (1998). "BDNF acutely increases tyrosine phosphorylation of the NMDA receptor subunit 2B in cortical and hippocampal postsynaptic densities." <u>Brain Res Mol Brain Res</u> **55**(1): 20-7.

Lipkowitz, S. (2003). "The role of the ubiquitination-proteasome pathway in breast cancer: ubiquitin mediated degradation of growth factor receptors in the pathogenesis and treatment of cancer." <u>Breast Cancer Res</u> **5**(1): 8-15.

Liu, J. J., J. Ding, et al. (2007). "Retrolinkin, a membrane protein, plays an important role in retrograde axonal transport." <u>Proc Natl Acad Sci U S A</u> **104**(7): 2223-8.

Llovera, M., Y. de Pablo, et al. (2004). "Trk is a calmodulin-binding protein: implications for receptor processing." J Neurochem **88**(2): 422-33.

Lo, K. Y., W. H. Chin, et al. (2005). "SLAM-associated protein as a potential negative regulator in Trk signaling." J Biol Chem **280**(50): 41744-52.

Longva, K. E., F. D. Blystad, et al. (2002). "Ubiquitination and proteasomal activity is required for transport of the EGF receptor to inner membranes of multivesicular bodies." J Cell Biol **156**(5): 843-54.

Luo, L. (2000). "Rho GTPases in neuronal morphogenesis." Nat Rev Neurosci **1**(3): 173-80.

MacDonald, J. I., E. A. Gryz, et al. (2000). "Direct binding of the signaling adapter protein Grb2 to the activation loop tyrosines on the nerve growth factor receptor tyrosine kinase, TrkA." J Biol Chem **275**(24): 18225-33.

MacInnis, B. L. and R. B. Campenot (2002). "Retrograde support of neuronal survival without retrograde transport of nerve growth factor." Science **295**(5559): 1536-9.

MacPhee, I. J. and P. A. Barker (1997). "Brain-derived neurotrophic factor binding to the p75 neurotrophin receptor reduces TrkA signaling while increasing serine phosphorylation in the TrkA intracellular domain." J Biol Chem **272**(38): 23547-51.

Mahadeo, D., L. Kaplan, et al. (1994). "High affinity nerve growth factor binding displays a faster rate of association than p140trk binding. Implications for multi-subunit polypeptide receptors." J Biol Chem **269**(9): 6884-91.

Makkerh, J. P., C. Ceni, et al. (2005). "p75 neurotrophin receptor reduces ligand-induced Trk receptor ubiquitination and delays Trk receptor internalization and degradation." EMBO Rep **6**(10): 936-41.

Marchese, A. and J. L. Benovic (2001). "Agonist-promoted ubiquitination of the G protein-coupled receptor CXCR4 mediates lysosomal sorting." J Biol Chem **276**(49): 45509-12.

Marchese, A., C. Raiborg, et al. (2003). "The E3 ubiquitin ligase AIP4 mediates ubiquitination and sorting of the G protein-coupled receptor CXCR4." Dev Cell **5**(5): 709-22.

Marsh, H. N., C. I. Dubreuil, et al. (2003). "SHP-1 negatively regulates neuronal survival by functioning as a TrkA phosphatase." J Cell Biol **163**(5): 999-1010.

Marshall, C. J. (1995). "Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation." <u>Cell</u> **80**(2): 179-85.

Martin-Zanca, D., R. Oskam, et al. (1989). "Molecular and biochemical characterization of the human trk proto-oncogene." <u>Mol Cell Biol</u> **9**(1): 24-33.

McAllister, A. K., L. C. Katz, et al. (1999). "Neurotrophins and synaptic plasticity." <u>Annu Rev Neurosci</u> **22**: 295-318.

McCullough, J., M. J. Clague, et al. (2004). "AMSH is an endosome-associated ubiquitin isopeptidase." <u>J Cell Biol</u> **166**(4): 487-92.

McCullough, J., P. E. Row, et al. (2006). "Activation of the endosome-associated ubiquitin isopeptidase AMSH by STAM, a component of the multivesicular body-sorting machinery." <u>Curr Biol</u> **16**(2): 160-5.

Meakin, S. O., J. I. MacDonald, et al. (1999). "The signaling adapter FRS-2 competes with Shc for binding to the nerve growth factor receptor TrkA. A model for discriminating proliferation and differentiation." <u>J Biol Chem</u> **274**(14): 9861-70.

Meiser, A., A. Mueller, et al. (2008). "The chemokine receptor CXCR3 is degraded following internalization and is replenished at the cell surface by de novo synthesis of receptor." <u>J Immunol</u> **180**(10): 6713-24.

Millard, S. M. and S. A. Wood (2006). "Riding the DUBway: regulation of protein trafficking by deubiquitylating enzymes." <u>J Cell Biol</u> **173**(4): 463-8.

Miranda, M. and A. Sorkin (2007). "Regulation of receptors and transporters by ubiquitination: new insights into surprisingly similar mechanisms." <u>Mol Interv</u> **7**(3): 157-67.

Mizuno, E., T. Iura, et al. (2005). "Regulation of epidermal growth factor receptor down-regulation by UBPY-mediated deubiquitination at endosomes." <u>Mol Biol Cell</u> **16**(11): 5163-74.

Mori, S., C. H. Heldin, et al. (1992). "Ligand-induced polyubiquitination of the platelet-derived growth factor beta-receptor." <u>J Biol Chem</u> **267**(9): 6429-34.

Mosesson, Y., K. Shtiegman, et al. (2003). "Endocytosis of receptor tyrosine kinases is driven by monoubiquitylation, not polyubiquitylation." <u>J Biol Chem</u> **278**(24): 21323-6.

Mukhopadhyay, D. and H. Riezman (2007). "Proteasome-independent functions of ubiquitin in endocytosis and signaling." <u>Science</u> **315**(5809): 201-5.

Muthuswamy, S. K., M. Gilman, et al. (1999). "Controlled dimerization of ErbB receptors provides evidence for differential signaling by homo- and heterodimers." Mol Cell Biol **19**(10): 6845-57.

Nakagawara, A. (2001). "Trk receptor tyrosine kinases: a bridge between cancer and neural development." Cancer Lett **169**(2): 107-14.

Nakamura, T., M. Komiya, et al. (2002). "Grit, a GTPase-activating protein for the Rho family, regulates neurite extension through association with the TrkA receptor and N-Shc and CrkL/Crk adapter molecules." Mol Cell Biol **22**(24): 8721-34.

Naldini, L., U. Blomer, et al. (1996). "Efficient transfer, integration, and sustained long-term expression of the transgene in adult rat brains injected with a lentiviral vector." Proc Natl Acad Sci U S A **93**(21): 11382-8.

Naldini, L., U. Blomer, et al. (1996). "In vivo gene delivery and stable transduction of nondividing cells by a lentiviral vector." Science **272**(5259): 263-7.

Nykjaer, A., T. E. Willnow, et al. (2005). "p75NTR--live or let die." Curr Opin Neurobiol **15**(1): 49-57.

Obara, Y. and N. Nakahata "The signaling pathway leading to extracellular signal-regulated kinase 5 (ERK5) activation via G-proteins and ERK5-dependent neurotrophic effects." Mol Pharmacol **77**(1): 10-6.

Obermeier, A., H. Halfter, et al. (1993). "Tyrosine 785 is a major determinant of Trk--substrate interaction." EMBO J **12**(3): 933-41.

Oppenheim, R. W. (1989). "The neurotrophic theory and naturally occurring motoneuron death." Trends Neurosci **12**(7): 252-5.

Oppenheim, R. W. (1991). "Cell death during development of the nervous system." Annu Rev Neurosci **14**: 453-501.

Pedraza, C. E., P. Podlesniy, et al. (2005). "Pro-NGF isolated from the human brain affected by Alzheimer's disease induces neuronal apoptosis mediated by p75NTR." Am J Pathol **166**(2): 533-43.

Peiro, S., J. X. Comella, et al. (2000). "PC12 cells have caveolae that contain TrkA. Caveolae-disrupting drugs inhibit nerve growth factor-induced, but not epidermal growth factor-induced, MAPK phosphorylation." J Biol Chem **275**(48): 37846-52.

Penengo, L., M. Mapelli, et al. (2006). "Crystal structure of the ubiquitin binding domains of rabex-5 reveals two modes of interaction with ubiquitin." Cell **124**(6): 1183-95.

Peng, J., D. Schwartz, et al. (2003). "A proteomics approach to understanding protein ubiquitination." <u>Nat Biotechnol</u> **21**(8): 921-6.

Peschard, P., T. M. Fournier, et al. (2001). "Mutation of the c-Cbl TKB domain binding site on the Met receptor tyrosine kinase converts it into a transforming protein." <u>Mol Cell</u> **8**(5): 995-1004.

Peschard, P. and M. Park (2003). "Escape from Cbl-mediated downregulation: a recurrent theme for oncogenic deregulation of receptor tyrosine kinases." <u>Cancer Cell</u> **3**(6): 519-23.

Peschard, P. and M. Park (2007). "From Tpr-Met to Met, tumorigenesis and tubes." <u>Oncogene</u> **26**(9): 1276-85.

Pickart, C. M. (2000). "Ubiquitin in chains." <u>Trends Biochem Sci</u> **25**(11): 544-8.

Pierchala, B. A., C. C. Tsui, et al. (2007). "NGF augments the autophosphorylation of Ret via inhibition of ubiquitin-dependent degradation." <u>J Neurochem</u> **100**(5): 1169-76.

Pierotti, M. A. and A. Greco (2006). "Oncogenic rearrangements of the NTRK1/NGF receptor." <u>Cancer Lett</u> **232**(1): 90-8.

Piper, R. C. and J. P. Luzio (2001). "Late endosomes: sorting and partitioning in multivesicular bodies." <u>Traffic</u> **2**(9): 612-21.

Piper, R. C. and J. P. Luzio (2007). "Ubiquitin-dependent sorting of integral membrane proteins for degradation in lysosomes." <u>Curr Opin Cell Biol</u> **19**(4): 459-65.

Polo, S., S. Sigismund, et al. (2002). "A single motif responsible for ubiquitin recognition and monoubiquitination in endocytic proteins." <u>Nature</u> **416**(6879): 451-5.

Qian, X., A. Riccio, et al. (1998). "Identification and characterization of novel substrates of Trk receptors in developing neurons." <u>Neuron</u> **21**(5): 1017-29.

Rabizadeh, S. and D. E. Bredesen (2003). "Ten years on: mediation of cell death by the common neurotrophin receptor p75(NTR)." <u>Cytokine Growth Factor Rev</u> **14**(3-4): 225-39.

Raiborg, C., T. E. Rusten, et al. (2003). "Protein sorting into multivesicular endosomes." <u>Curr Opin Cell Biol</u> **15**(4): 446-55.

Rajagopal, R., Z. Y. Chen, et al. (2004). "Transactivation of Trk neurotrophin receptors by G-protein-coupled receptor ligands occurs on intracellular membranes." <u>J Neurosci</u> **24**(30): 6650-8.

Reichardt, L. F. (2006). "Neurotrophin-regulated signalling pathways." <u>Philos Trans R Soc Lond B Biol Sci</u> **361**(1473): 1545-64.

Ridge, S. A., M. Worwood, et al. (1990). "FMS mutations in myelodysplastic, leukemic, and normal subjects." <u>Proc Natl Acad Sci U S A</u> **87**(4): 1377-80.

Robinson, K. N., K. Manto, et al. (2005). "Neurotrophin-dependent tyrosine phosphorylation of Ras guanine-releasing factor 1 and associated neurite outgrowth is dependent on the HIKE domain of TrkA." <u>J Biol Chem</u> **280**(1): 225-35.

Rosario, M., R. Franke, et al. (2007). "The neurite outgrowth multiadaptor RhoGAP, NOMA-GAP, regulates neurite extension through SHP2 and Cdc42." <u>J Cell Biol</u> **178**(3): 503-16.

Rose, C. R., R. Blum, et al. (2003). "Truncated TrkB-T1 mediates neurotrophin-evoked calcium signalling in glia cells." <u>Nature</u> **426**(6962): 74-8.

Ross, C. A. and C. M. Pickart (2004). "The ubiquitin-proteasome pathway in Parkinson's disease and other neurodegenerative diseases." <u>Trends Cell Biol</u> **14**(12): 703-11.

Rouard, M., J. Bass, et al. (1999). "Congenital insulin resistance associated with a conformational alteration in a conserved beta-sheet in the insulin receptor L1 domain." <u>J Biol Chem</u> **274**(26): 18487-91.

Roux, P. P. and P. A. Barker (2002). "Neurotrophin signaling through the p75 neurotrophin receptor." <u>Prog Neurobiol</u> **67**(3): 203-33.

Row, P. E., I. A. Prior, et al. (2006). "The ubiquitin isopeptidase UBPY regulates endosomal ubiquitin dynamics and is essential for receptor down-regulation." <u>J Biol Chem</u> **281**(18): 12618-24.

Rowinsky, E. K. (2004). "The erbB family: targets for therapeutic development against cancer and therapeutic strategies using monoclonal antibodies and tyrosine kinase inhibitors." <u>Annu Rev Med</u> **55**: 433-57.

Rubinsztein, D. C. (2006). "The roles of intracellular protein-degradation pathways in neurodegeneration." <u>Nature</u> **443**(7113): 780-6.

Rutz, S. and A. Scheffold (2004). "Towards in vivo application of RNA interference - new toys, old problems." <u>Arthritis Res Ther</u> **6**(2): 78-85.

Sadowski, L., I. Pilecka, et al. (2009). "Signaling from endosomes: location makes a difference." <u>Exp Cell Res</u> **315**(9): 1601-9.

Salehi, A., J. D. Delcroix, et al. (2004). "Alzheimer's disease and NGF signaling." <u>J Neural Transm</u> **111**(3): 323-45.

Salehi, A. H., P. P. Roux, et al. (2000). "NRAGE, a novel MAGE protein, interacts with the p75 neurotrophin receptor and facilitates nerve growth factor-dependent apoptosis." Neuron **27**(2): 279-88.

Sanders, C. R. and J. K. Myers (2004). "Disease-related misassembly of membrane proteins." Annu Rev Biophys Biomol Struct **33**: 25-51.

Saxena, S., C. L. Howe, et al. (2005). "Differential endocytic sorting of p75NTR and TrkA in response to NGF: a role for late endosomes in TrkA trafficking." Mol Cell Neurosci **28**(3): 571-87.

Schmidt-Arras, D. E., A. Bohmer, et al. (2005). "Tyrosine phosphorylation regulates maturation of receptor tyrosine kinases." Mol Cell Biol **25**(9): 3690-703.

Schnell, J. D. and L. Hicke (2003). "Non-traditional functions of ubiquitin and ubiquitin-binding proteins." J Biol Chem **278**(38): 35857-60.

Scott, R. P., S. Eketjall, et al. (2005). "Distinct turnover of alternatively spliced isoforms of the RET kinase receptor mediated by differential recruitment of the Cbl ubiquitin ligase." J Biol Chem **280**(14): 13442-9.

Sehat, B., S. Andersson, et al. (2008). "Identification of c-Cbl as a new ligase for insulin-like growth factor-I receptor with distinct roles from Mdm2 in receptor ubiquitination and endocytosis." Cancer Res **68**(14): 5669-77.

Shao, Y., W. Akmentin, et al. (2002). "Pincher, a pinocytic chaperone for nerve growth factor/TrkA signaling endosomes." J Cell Biol **157**(4): 679-91.

Shenoy, S. K., P. H. McDonald, et al. (2001). "Regulation of receptor fate by ubiquitination of activated beta 2-adrenergic receptor and beta-arrestin." Science **294**(5545): 1307-13.

Sigismund, S., E. Argenzio, et al. (2008). "Clathrin-mediated internalization is essential for sustained EGFR signaling but dispensable for degradation." Dev Cell **15**(2): 209-19.

Sigismund, S., T. Woelk, et al. (2005). "Clathrin-independent endocytosis of ubiquitinated cargos." Proc Natl Acad Sci U S A **102**(8): 2760-5.

Snider, W. D. (1994). "Functions of the neurotrophins during nervous system development: what the knockouts are teaching us." Cell **77**(5): 627-38.

Snyder, P. M. (2009). "Down-regulating destruction: phosphorylation regulates the E3 ubiquitin ligase Nedd4-2." Sci Signal **2**(79): pe41.

Sommerfeld, M. T., R. Schweigreiter, et al. (2000). "Down-regulation of the neurotrophin receptor TrkB following ligand binding. Evidence for an

involvement of the proteasome and differential regulation of TrkA and TrkB." <u>J Biol Chem</u> **275**(12): 8982-90.

Song, M. S. and E. I. Posse de Chaves (2003). "Inhibition of rat sympathetic neuron apoptosis by ceramide. Role of p75NTR in ceramide generation." <u>Neuropharmacology</u> **45**(8): 1130-50.

Sorkin, A. (2004). "Cargo recognition during clathrin-mediated endocytosis: a team effort." <u>Curr Opin Cell Biol</u> **16**(4): 392-9.

Sorkin, A. and M. von Zastrow (2009). "Endocytosis and signalling: intertwining molecular networks." <u>Nat Rev Mol Cell Biol</u> **10**(9): 609-22.

Soubeyran, P., K. Kowanetz, et al. (2002). "Cbl-CIN85-endophilin complex mediates ligand-induced downregulation of EGF receptors." <u>Nature</u> **416**(6877): 183-7.

Staub, O., S. Dho, et al. (1996). "WW domains of Nedd4 bind to the proline-rich PY motifs in the epithelial Na+ channel deleted in Liddle's syndrome." <u>EMBO J</u> **15**(10): 2371-80.

Strohmaier, C., B. D. Carter, et al. (1996). "A splice variant of the neurotrophin receptor trkB with increased specificity for brain-derived neurotrophic factor." <u>EMBO J</u> **15**(13): 3332-7.

Swaminathan, S., A. Y. Amerik, et al. (1999). "The Doa4 deubiquitinating enzyme is required for ubiquitin homeostasis in yeast." <u>Mol Biol Cell</u> **10**(8): 2583-94.

Thompson, J. D., D. G. Higgins, et al. (1994). "CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice." <u>Nucleic Acids Res</u> **22**(22): 4673-80.

Trono, D. (2000). "HIV-based vectors: getting the best out of the worst." <u>J Gene Med</u> **2**(1): 61-3.

Tsui-Pierchala, B. A., J. Milbrandt, et al. (2002). "NGF utilizes c-Ret via a novel GFL-independent, inter-RTK signaling mechanism to maintain the trophic status of mature sympathetic neurons." <u>Neuron</u> **33**(2): 261-73.

Valdez, G., W. Akmentin, et al. (2005). "Pincher-mediated macroendocytosis underlies retrograde signaling by neurotrophin receptors." <u>J Neurosci</u> **25**(21): 5236-47.

van Kerkhof, P., C. M. Alves dos Santos, et al. (2001). "Proteasome inhibitors block a late step in lysosomal transport of selected membrane but not soluble proteins." <u>Mol Biol Cell</u> **12**(8): 2556-66.

Varadan, R., M. Assfalg, et al. (2004). "Solution conformation of Lys63-linked di-ubiquitin chain provides clues to functional diversity of polyubiquitin signaling." <u>J Biol Chem</u> **279**(8): 7055-63.

Vina-Vilaseca, A. and A. Sorkin "Lysine 63-linked polyubiquitination of the dopamine transporter requires WW3 and WW4 domains of Nedd4-2 and UBE2D ubiquitin-conjugating enzymes." <u>J Biol Chem</u> **285**(10): 7645-56.

Wang, Y., B. Su, et al. (2006). "Brain-derived neurotrophic factor activates ERK5 in cortical neurons via a Rap1-MEKK2 signaling cascade." <u>J Biol Chem</u> **281**(47): 35965-74.

Waterman, H., M. Katz, et al. (2002). "A mutant EGF-receptor defective in ubiquitylation and endocytosis unveils a role for Grb2 in negative signaling." <u>EMBO J</u> **21**(3): 303-13.

Watson, F. L., H. M. Heerssen, et al. (2001). "Neurotrophins use the Erk5 pathway to mediate a retrograde survival response." <u>Nat Neurosci</u> **4**(10): 981-8.

Weissman, A. M. (2001). "Themes and variations on ubiquitylation." <u>Nat Rev Mol Cell Biol</u> **2**(3): 169-78.

Wertz, I. and V. Dixit (2010). "Regulation of death receptor signaling by the ubiquitin system." <u>Cell Death Differ</u> **17**(1): 14-24.

Wertz, I. and V. Dixit (2010). "Signaling to NF-kappaB: Regulation by Ubiquitination." <u>Cold Spring Harb Perspect Biol</u> **2**(3): a003350.

Wicks, S. J., K. Haros, et al. (2005). "The deubiquitinating enzyme UCH37 interacts with Smads and regulates TGF-beta signalling." <u>Oncogene</u> **24**(54): 8080-4.

Williams, M. A. and M. Fukuda (1990). "Accumulation of membrane glycoproteins in lysosomes requires a tyrosine residue at a particular position in the cytoplasmic tail." <u>J Cell Biol</u> **111**(3): 955-66.

Williams, R. L. and S. Urbe (2007). "The emerging shape of the ESCRT machinery." <u>Nat Rev Mol Cell Biol</u> **8**(5): 355-68.

Woelk, T., B. Oldrini, et al. (2006). "Molecular mechanisms of coupled monoubiquitination." <u>Nat Cell Biol</u> **8**(11): 1246-54.

Woelk, T., S. Sigismund, et al. (2007). "The ubiquitination code: a signalling problem." <u>Cell Div</u> **2**: 11.

Woodman, P. G. and C. E. Futter (2008). "Multivesicular bodies: co-ordinated progression to maturity." <u>Curr Opin Cell Biol</u> **20**(4): 408-14.

Wooten, M. W., M. L. Seibenhener, et al. (2001). "The atypical protein kinase C-interacting protein p62 is a scaffold for NF-kappaB activation by nerve growth factor." J Biol Chem **276**(11): 7709-12.

Wu, C., C. F. Lai, et al. (2001). "Nerve growth factor activates persistent Rap1 signaling in endosomes." J Neurosci **21**(15): 5406-16.

Xing, J., J. M. Kornhauser, et al. (1998). "Nerve growth factor activates extracellular signal-regulated kinase and p38 mitogen-activated protein kinase pathways to stimulate CREB serine 133 phosphorylation." Mol Cell Biol **18**(4): 1946-55.

Xu, P. and J. Peng (2006). "Dissecting the ubiquitin pathway by mass spectrometry." Biochim Biophys Acta **1764**(12): 1940-7.

Yamashita, H., S. Avraham, et al. (1999). "The Csk homologous kinase associates with TrkA receptors and is involved in neurite outgrowth of PC12 cells." J Biol Chem **274**(21): 15059-65.

Yamashita, T. and M. Tohyama (2003). "The p75 receptor acts as a displacement factor that releases Rho from Rho-GDI." Nat Neurosci **6**(5): 461-7.

Yang, B., J. D. Slonimsky, et al. (2002). "A rapid switch in sympathetic neurotransmitter release properties mediated by the p75 receptor." Nat Neurosci **5**(6): 539-45.

Yano, H., F. Cong, et al. (2000). "Association of the Abl tyrosine kinase with the Trk nerve growth factor receptor." J Neurosci Res **59**(3): 356-64.

Yano, H., F. S. Lee, et al. (2001). "Association of Trk neurotrophin receptors with components of the cytoplasmic dynein motor." J Neurosci **21**(3): RC125.

York, R. D., D. C. Molliver, et al. (2000). "Role of phosphoinositide 3-kinase and endocytosis in nerve growth factor-induced extracellular signal-regulated kinase activation via Ras and Rap1." Mol Cell Biol **20**(21): 8069-83.

York, R. D., H. Yao, et al. (1998). "Rap1 mediates sustained MAP kinase activation induced by nerve growth factor." Nature **392**(6676): 622-6.

Yuan, J. and B. A. Yankner (2000). "Apoptosis in the nervous system." Nature **407**(6805): 802-9.

Zhou, F. Q., J. Zhou, et al. (2004). "NGF-induced axon growth is mediated by localized inactivation of GSK-3beta and functions of the microtubule plus end binding protein APC." Neuron **42**(6): 897-912.

Zhou, R., S. V. Patel, et al. (2007). "Nedd4-2 catalyzes ubiquitination and degradation of cell surface ENaC." J Biol Chem **282**(28): 20207-12.

Zweifel, L. S., R. Kuruvilla, et al. (2005). "Functions and mechanisms of retrograde neurotrophin signalling." <u>Nat Rev Neurosci</u> **6**(8): 615-25.