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# Regulation of TrkA signaling and trafficking by Nedd4-2


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## ABSTRACT

Ubiquitination of receptor tyrosine kinases has been implicated in the endocytosis, sorting and degradation of the receptors. Our group and others have shown that Trk receptors are ubiquitinated in response to neurotrophins. We have previously identified that Nedd4-2 ubiquitin-E3 ligase is responsible for ubiquitination of TrkA receptor. However, the role of ubiquitination in TrkA trafficking is not completely understood. Here we report that Nedd4-2 modulates the signaling and trafficking of TrkA receptor upon NGF treatment. Reduction of Nedd4-2 protein in DRG-cultured neurons increases TrkA levels and NGF-mediated signaling as measured by the levels of phosphorylated TrkA and MAPK. Although endocytosis of activated TrkA receptors in response to NGF is not affected after reducing Nedd4-2 expression, degradation and recycling of TrkA receptors are modified. A reduction in the degradation of TrkA receptors was observed, whereas recycling to the plasma membrane of internalized TrkA receptors is enhanced. As a result of both processes there is an accumulation of activated receptors in specific compartments in response to NGF. Phophorylated TrkA receptors are accumulated in early-endosome, late-endosome and recycling-endosome in neurons depleted of Nedd4-2. These effects are specific for TrkA receptor as p75 neurotrophin receptor levels are not affected after depletion of Nedd4-2 protein. Our data suggest that Nedd4-2 regulates TrkA receptor levels, trafficking and, therefore, NGF-mediated signaling.

## INTRODUCTION

### Neurotrophins and their receptors

Neurotrophins are a family of growth factors that include nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophins 3 (NT3) and neurotrophins 4/5 (NT4/5). Neurotrohpins are implicated in several different functions in the nervous system, including survival, proliferation, differentiation, myelination, apoptosis, axonal growth and synaptic plasticity (Chao, 2003). All the neurothophins exert their functions through binding to two different receptors: tropomyosin-related kinase (Trk) receptors and the p75 neurotrophin receptor ($p75^{NTR}$) **(Figure 1)** (Huang and Reichardt, 2001).

Trk receptors are a family of tyrosine kinases that consists of TrkA, TrkB and TrkC. They contain an extracellular domain composed of three leucine-rich motifs flanked by two cysteine clusters, two immunoglobulin-like C2 type domains (Ig-C2), a single transmembrane domain, and a cytoplasmic region with a kinase domain **(Figure 1)** (Arévalo and Wu, 2006b). Although the sequence of these family members is highly conserved, these family members are activated by different neurotrophins; TrkA by NGF, TrkB by BDNF and NT4/5, and TrkC by NT3 (Huang and Reichardt, 2001). Binding of neurotrophins to Trk receptors occurs mainly through the Ig-C2 domains, as described using chimeras between different Trk receptors, deletions, and point mutation analyses, with the domain closer to the transmembrane region playing a more prominent role (Arévalo et al., 2000). The leucine-rich motifs and the cysteine clusters may also be involved (Mcdonald and Meakin, 1996).

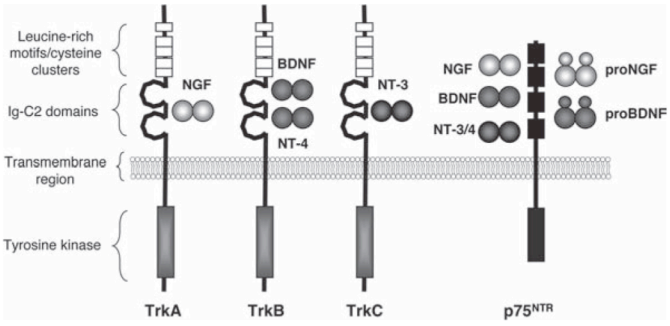


**Figure 1. Neurotrophins and their receptors (Arévalo and Wu, 2006b)** Neurotrophins are dimers that bind to the Trk and p75 NTR receptors. Neurotrophins are synthesized as pro-proteins that bind exclusively to p75NTR which belongs to the TNF receptor superfamily.

### Trk receptor-mediated signaling pathways

NGF binding induces the dimerization of TrkA receptors and the

*trans*-autophosphorylation of tyrosine residues in the cytoplasmic domain of TrkA receptors, which form docking sites for Src-homology-2 (SH2)-or phosphotyrosine-binding-domain-containing downstream effector proteins. The latter proteins trigger different signaling cascades that regulate the biological response to the ligand (Marmor and Yarden, 2004). Three signaling pathways initiated by NGF binding have been well characterized in regulating biological functions: Mitogen-Activated Protein Kinase (MAPK) pathway，the phosphoinositide 3-kinases (PI3K)-AKT pathway, and Phospholipase C gamma (PLC γ)-protein kinase C (PKC) pathway **(Figure 2)** (Arévalo and Wu, 2006b).

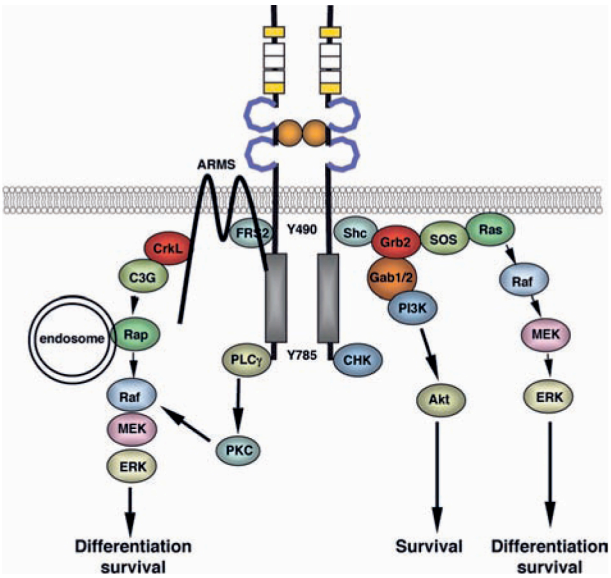


**Figure 2. Trk receptor-mediated signaling pathways. (Arévalo and Wu, 2006b)**

Neurotrophin binding to Trk receptors leads to their 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, PI3K and PLC̣ pathways, which result in survival, neurite outgrowth, gene expression, and synaptic plasticity.

Upon binding of NGF to Trk receptors, two different phases of MAPK are activated: Shc-Ras-MAPK and Rap-MAPK. Unlike Shc-Ras-MAPK pathway which is transient activated, the Rap-MAPK pathway provides a prolonged activation through the TrkA receptor internalization to the endosomal compartment (Wu et al, 2001). Phosphorylation of MAPK could modulate the activity of cyclic adenosine monophosphate response element-binding protein (CREB) to control the gene expression of the proteins necessary for the differentiation and survival of the neurons (Deak et al., 1998).

Binding of NGF to TrkA also activate PI3K (independent of Ras activity) by binding to adaptor proteins linked to TrkA. Active Trk receptors engage Shc, which associates Grb2 and Gab1 to activate PI3K and subsequently, Akt **(Figure 2)**. The role of PI3K-Akt signaling has been shown in primary neurons (Atwal et al, 2000). Several

different pro-apoptotic and pro-survival effectors downstream of Akt can mediate the survival actions of neurotrophins. For example, Bad phosphorylation by Akt leads to additional phosphorylation of Bad by other kinases, which prevents the pro-apoptotic effects of the protein (Datta et al, 1997). Similarly, the forkhead transcription factor, FKHRL1, which regulates the expression of several pro proapoptotic proteins, is phosphorylated by Akt in response to neurotrophins, preventing its transcriptional activity (Zheng et al, 2002). Moreover, the NF-κB pro-survival pathway is activated via Akt phosphorylation of the inhibitor IκB, targeting it for degradation (Foehr et al, 2000). PI3K-Akt can also exert a positive effect on the retrograde signaling that modulatesneuronal survival (Kuruvilla et al, 2000).

The activation of PLC $\gamma$ is induced by the phosphorylation at Y785 residue and consequently activate PKC and ERK1 signaling pathway through the Raf. This pathway plays an important role for TrkB signaling in response to BDNF that is involved in synaptic plasticity in the hippocampus (Patterson et al., 1996).

## Trafficking of Trk receptors

It has been established that upon binding to neurotrophins, Trk receptors are rapidly endocytosed in a clathrin-dependent manner (Grimes et al., 1997). Postendocytic sorting of Trk receptors to diverse pathways after ligand binding has significant impact on the physiological responses to neurotrophins because they also determine the strength and duration of the intracellular signaling cascades initiated by activated Trk receptors (Heerssen and Segal, 2002).

Three alternative endocytic pathways that Trk receptors can follow are trafficking to lysosomes, recycling to plasma membrane or retrograde transport. The degradative pathway to lysosomes is characterized by down-regulation of the total number of receptors at the cell surface and decreased responsiveness to ligand, while Recycling of receptors back to the plasma membrane can lead to functional resensitization and prolongation of cell surface specific signaling events (Zhe et al, 2005).

## Degradation of Trk receptors

In many cases, proteins destined for degradation are tagged by ubiquitin. The process of adding a ubiquitin, a 76 amino acid polypeptide, or a multiubiquitin chain, is called ubiquitination. The ubiquitinated proteins are directed to proteasome or lysosome, the two major degradative systems in eukaryotic cells, for protein degradation. The lysosome degrades most membrane and endocytosed proteins, while the ubiquitin-proteasome system (UPS) is responsible for degradation most intracellular, soluble proteins (Ciechanover, 2005).

Three enzymes were used to attach the ubiquitin to the proteins: E1, E2 and E3. The E1 (ubiquitin-activity) enzyme first activates ubiquitin in an ATP-dependent reaction by forming a thioester bound at its active-site cysteine with the COOH-terminus of ubiquitin. Ubiquitin is then transferred to the active site cysteine of a ubiquitin conjugating (carrying) enzyme (E2), and then sometimes to the active site cysteine of ubiquitin protein ligase (E3).

Given that the mammalian genomes encode just a few E1s, dozens of E2s and more than 500 E3s (Semple, 2003), the E3 enzymes therefore carry out the important task to recognize and target ubiquitin to the substrate proteins. Also, the functions of E3 are complicated, because of the fact that one E3 can have multiple substrates and that one substrate can be targeted by multiple E3s (Moore, 2006; Brooks and Gu, 2006).

There are two main types of E3 ligases: the RING (Really Interesting New Gene) type, that act as a large complex containing several proteins, like Cbl; and the HECT (Homologous to E6AP Carboxy Terminus) type, that acts as single chain enzymes that first accept the ubiquitin and then transfer it to the substrate protein, like Nedd4 (neural precursor cell expressed, developmentally down regulated 4). The Cbl family of RING-type ubiquitin ligases was well studies to facilitate ubiquitination of activated EGFRs and thereby promote their degradation in the lysosome (Haglund et al., 2003). The functions of Nedd4 were found in the regulation of epithelial sodium channels (ENaC). ENaC controls the salt and fluid reabsorption in the distal nephron, distal colon and lung epithelia. Abnormal elevation of its activity leads to the hypertension. It was found that Nedd4-2, not Nedd4-1, was a suppressor of ENaC by binding to the PY motif in the ENaC through its WW domains and thereby down regulated the ENaC level on the membrane and its activities (Kamynina et al., 2001) **(Figure 3)**.

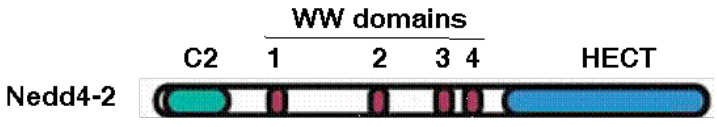


**Figure 3. Schematic diagram showing the structural organization of the nine mammalian Nedd4-like E3 ubiquitin ligases.** The C2 domain translocates the protein to the membrane upon calcium binding. The two to four WW domains are known to bind substrate proteins containing PY motifs. The catalytic cysteine within the HECT domain is responsible for ubiquitin transfer

Recently, it has been shown that Nedd4-2 associates with the TrkA receptor and is phosphorylated upon NGF binding. The binding of Nedd4-2 to TrkA through a PPXY motif leads to the ubiquitination and downregulation of TrkA (Arévalo et al., 2006a). However, the closely related TrkB receptor does not undergo ubiquitination though Nedd4-2, due to the lack of PPXY motif (Arévalo et al., 2006a). Other roles of Nedd4 family were found in TGF-b signaling, viral budding and axonal guidance (Daniela and Sharad, 2009).

More evidence has shown that the role of ubiquitination is not only for protein degradation, they also participate in protein sorting (Huang et al, 2007). However, the role of Nedd4-2 ubiquitination in TrkA trafficking is unknown. Here we hypothesized that Nedd4-2 may regulate TrkA receptor trafficking and therefore, affect the level of activated TrkA receptors in response to NGF.

In this study, TrkA expressing neurons were obtained by culturing the cells isolated from rat dorsal root ganglion (DRGs) in presence of NGF. In order to test the roles of Nedd4-2 in NGF induced TrkA signaling and trafficking, we first modulated the level of Nedd4-2 in the DRGs using lentivirus expressing shRNA Nedd4-2. Then, the level of activated TrkA receptors upon treatment with NGF was tested. We observed more activated TrkA in the DRGs expressing less Nedd4-2 and this is caused by the increased level of TrkA compared with the control DRGs. To analyze whether the increased level of TrkA was resulted from any defect of internalization or degradation of TrkA, biotinylation assays were performed. Data showed that Nedd4-2 could regulate the degradation rate of TrkA but not the internalization rate. By measuring the level of activated ERKs and AKT, we found that reduction of Nedd4-2 protein could influence NGF-mediated signaling.

To sum up, we have determined that depletion of Nedd4-2 results in an increase of TrkA receptors' stability and their activation time through decreasing TrkA degradation and enhanced recycling.

## MATERIALS AND METHODS

### Antibodies

The following antibodies were used in this study: antibodies to TrkC-14 and AKT1 (B1) were from Santa Cruz; antibodies to PTrk (Y490), MAPK and pMAPK were from Cell Signaling; antibodies to Rab5 was from BD; anti-pAKT (Ser473) was from Upstate; anti-Rab7 was from Sigma; antibodies to P75, PTrk (Y794) and Nedd4-2 (WW1) were described previously (Arevalo, 2006a).

### DRG Neuron Culture

DRGs were obtained from E15 rat embryos and dissected using 0.25% trypsin (Invitrogen) in L-15 (Invitrogen) media for 45min at 37℃. Cells were centrifuged to eliminate the trypsin and suspended with plating media, MEM (Invitrogen) supplemented with 10% FBS (Invitrogen), 0.4% Glucose (Sigma), 2mM glutamine (Invitrogen) and 100U/ml Pen/Strep (Invitrogen). Then cells were mixed using the pipette up and down for 50 times to get the single cell suspension. Cells were counted and plated on the plastic plate coated with 0.1mg/ml Poly-D-Lysine (Sigma) and Growth Factor Reduced Matrigel (BD Biosciences) in Plating Media with NGF (Harlan, 25ng/ml). On the next day, media was changed to NB media: Neurobasal (Invitrogen), B-27 (Invitrogen), 0.4% glucose (Sigma), 2mM glutamine (Invitrogen) and 100U/ml Pen/Strep (Invitrogen) with NGF (Harlan, 25ng/ml) and fluorodeoxyuridine (Sigma, 2.44μg/ml) and uridine (Sigma, 2.44μg/ml).

### Lentivirus Generation and Infection

Lentivirus used in this study was generated by co-transfection using Calcium phosphate in 293FT cells line. $2.5X10^6$ 293FT cells were plated one day before the transfection in a 10cm plate coated with 0.1mg/ml PDL. While bubbling 500μl 2X HBS pH 7.05 (140mM NaCl, 1.5mM $Na_2HPO4$ and 50mM HEPES), add 500μl $CaCl_2$/DNA solution (150μl 1M $CaCl_2$ and 9μg of PLVTHM-GFP or PLVTHM-GFP-shRNA nedd4-2 together with 6μg psPAX2 and 5μg pMD2.G) drop by drop. Cells were cultured for 16 hours before changing to fresh media without any antibiotics. Two days later, virus was collected by spinning down the media at 3000rpm for 15 minutes, filtering through a 0.45μM filter and kept at -80℃ in aliquot. To infect 100,000 DRG cells 300ul virus were added to the 1ml culturing media.

### Biochemical Assay for Signaling Transduction

DRGs at DIV3 were infected with lentivirus expressing control or Nedd4-2 shRNA for 5 days. After NGF-starved overnight with the presence of 20μM Z-VAD-FMK (R&D),

DRGs were stimulated with NGF (50ng/ml) for 0min, 5min, 15min, 30min, 45min and 60min at 37℃ and chilled on ice immediately. Cells were washed and lysed in 2XSDS loading buffer (100 mM Tris, 25% glycerol, 2% SDS, 0.01% bromphenol blue and Adjust to pH 6.8). Lysate then was denatured by boiling for 7 minute and was used for SDS-PAGE and immunoblotted for phospho-Trk Tyr490 (Cell signaling), TrkC-14 (Santa Cruz Biotechnology), phospho AKT Ser473 (Cell signaling), Akt1 B1 (Santa Cruz Technology), phospho-p44/p42 Thr202/Tyr204 MAP Kinase (Cell Signaling), p44/p42 MAP Kinase (Cell Signaling), Nedd4-2 and anti-βTubulinIII (Sigma)

## Surface Biotinalytion Assay

DRGs at DIV3 were infected with lentivirus expressing control or Nedd4-2 shRNA for 5 days. Subsequently, cells were washed using cold PBS twice, chilled on ice and incubated in 0.5ng/ml Sulfo-NHS-SS-biotin (Pierce) dissolved in Biotinylation Buffer (PBS, 1mM $CaCl_2$, 0.5mM $MgCl_2$) for 20min at 4℃ to label the membrane protein. Unreacted biotin was quenched and removed with 0.1M Glycine for 15min at 4℃. Cells were then washed with cold PBS, lysed with lysis buffer (20mM Tris PH 8.0, 137mN Nacl, 1% NP-40, 1mM PMSF, 1μg/ml Aprotinin, 2μg/ml Leueptine, 1mM Vanadate, 10mM NaF and 20mM β-glycerophosphate) for 40min at 4℃ with gently shaking and centrifuged at 12,000 rpm for 15min to eliminate the debris. Biotinylated protein were isolated from the total cell lysate by immobilization on NeutrAvidin-beads (Pierce) for at least 3 hours at 4℃, beads were washed three times and eluted with SDS sample buffer containing DTT, and eluted proteins were subjected to SDS-PAGE and immunoblotted using antibodies against TrkC-14 and p75.

## Surface Biotinylation based Internalization Assay

Unlike the Surface Biotinylation Assay, which tests the surface protein after the NGF induction, internalization Assay test the internalized surface protein. DRGs at DIV3 were infected and NGF-starved the same as the Surface Biotinylation Assay. Instead of adding NGF, the cells were washed with PBS and biotinylated with 0.5ng/ml Sulfo-NHS-SS-biotin (Pierce) dissolved in Biotinylation Buffer (PBS, 1mM $CaCl_2$, 0.5mM $MgCl_2$) for 20min at 4℃. Cells were then washed twice to eliminate the unreacted biotin and incubated in pre-warmed media at 37℃ with or without NGF for different time points (0min, 15min and 60min) to induce the internalization of biotinylated surface protein. Afterwards, the cells were chilled on ice immediately and the surface protein tagged biotin were striped using 50mM DTT for 15min at 4℃ to get the internalized surface protein only. Cells were then washed with cold PBS, lysed

with lysis buffer and biotinylated internalized surface protein were got in the same way as the Surface Biotinylation Assay and subjected to SDS-PAGE and immunoblotted using antibodies against TrkC-14 and p75.

### Surface Biotinylation based Degradation Assay

DRGs at DIV3 were infected with lentivirus expressing control or Nedd4-2 shRNA the same as described in the surface Biotinylation assay. After NGF-starved overnight with the presence of 20μM Z-VAD-FMK (R&D), DRGs were washed with PBS chilled on ice and biotinylated using 0.5ng/ml Sulfo-NHS-SS-biotin for 20min at 4℃. Then Cells were washed twice to eliminate the unreacted biotin and incubated in pre-warmed media at 37℃ with or without NGF for different time points (0, 15 and 60 min) to let the biotinylated receptors internalized and degraded. Subsequently Cells were washed and lysed using lysis buffer and the biotinylated proteins were precipitated by NertroAvidin-beads, washed and subjected to SDS-PAGE (see surface Biotinylation assay) and immunoblotted using antibodies against TrkC-14 and p75.

### Immunofluorescence and Confocal Microscopy

DRGs to do immunofluorescence were cultured on the coverslips which were coated with 1mg/ml PDL in 24-well-plate and infected with 10ul lentivirus expressing control or Nedd4-2 shRNA each well for 5 days. After the NGF starvation described as above, 50ng/ml NGF was added to induce the signaling transduction. Then, Cells were fixed with 4% paraformaldehyde (PFA) in PBS for 5min, washed with PBS three times and quenched with 50mM $NH_4Cl$ 10min followed by washing in PBS. Cells were blocked with blocking buffer (10% FBS, 5% BSA, 0.1% Tween 20 in PBS) for 30min at room temperature and were permeabilized using 0.1% Triton X-100 diluted in blocking buffer. Specimens were incubated with primary antibody overnight at 4℃, washed with PBS three times, followed by being incubated with the fluorescenated secondary antibodies for 40min at room temperature from light. Stained DRGs were examined by Leica confocal microscopy and Images were taken and analysed using software Image J.

## RESULTS

### Reduction of Nedd4-2 protein increases both total TrkA and surface TrkA in DRG neurons

Nedd4-2 was the first E3 ligase identified for TrkA receptor to bind to TrkA receptors through the PPXY motif. Moreover, it has been reported that increased expression of Nedd4-2 produced a negative effect upon the steady state level of TrkA receptors in HEK 293 cells (Arévalo, 2006a). To test whether Nedd4-2 affects the level of TrkA in the DRG neurons, we isolated the neurons and infected them with lentivirus containing control and Nedd4-2 shRNA. After five days' infection, lysate from the infected DRGs were subjected to Western blot with anti-Nedd4-2 and anti-Trk antibodies. Down regulation of the Nedd4-2 protein was observed clearly in the shRNA Nedd4-2 infected DRGs, but not in the control shRNA infected ones. Comparing with the TrkA level in the DRGs infected by the control lentivirus, the level of TrkA in the DRGs infected by shRNA Nedd4-2 is much higher **(Figure 4A, C)**. Interestingly, there is no difference of another membrane receptor p75, which suggests the specific effect of Nedd4-2 on TrkA. Therefore, these data indicated that less Nedd4-2 resulted in more total TrkA in the DRG neurons.

However, only TrkA receptors which are localized on the cell surface respond to NGF and cause the downstream cascades and finally regulate the cell functions. So, further study was emphasized on whether the TrkA receptors on the cellular membrane surface were affected by Nedd4-2 levels too. To address it, surface biotinylation assay was applied. DRGs were cultured and infected as mentioned above and the membrane proteins were labeled using NHS-SS-biotin which does not permeabilize the cells. Biotin labeled membrane proteins were pulled down using Neutro-Avidin agarose and subjected to SDS-PAGE and subsequently immunoblotted by anti-Trk and anti-p75 antibodies. It was observed that in the neurons whose Nedd4-2 level is downregulated by lentivirus infection containing shRNA Nedd4-2, the level of TrkA receptor on the surface is higher while the level of p75 is not affected by Nedd4-2 level **(Figure 4B-C)**. The results showed that both the total level of TrkA and the surface TrkA were increased followed by the shRNA Nedd4-2 lentivirus infection in the DRG neurons.

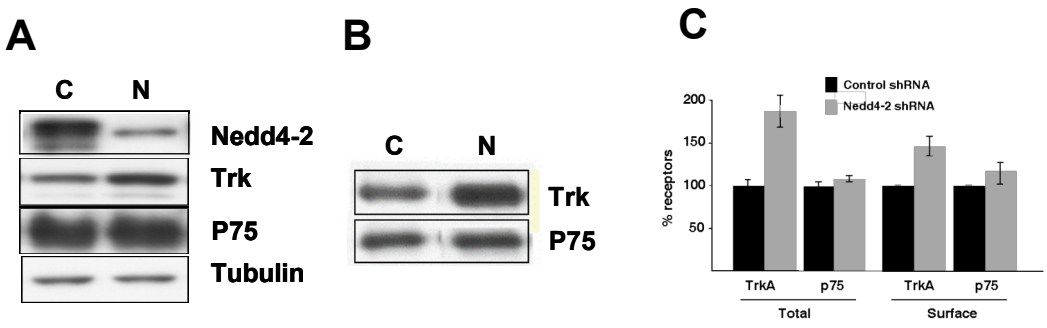


**Figure 4**. **Nedd4-2 regulates total and surface TrkA expression.** DRGs were grown in presence of NGF, infected with control and Nedd4-2 shRNA lentivirus. (A) Total lysates were subjected to Western blot of Nedd4-2, TrkA, p75 and tubulin. (B) Membrane proteins of DRGs were labeled by biotin, pulled down and subjected to Western blot of TrkA, p75 and tubulin. (C) The quantity of total and surface TrkA and p75 was normalized to its corresponding Tubulin and expressed as mean ± SD.

## Nedd4-2 depleted DRG neurons present enhanced TrkA activation

In order to find whether the activated TrkA level was also affected by Nedd4-2, sensory neurons isolated from DRGs were cultured on coverslips coated with poly D-lysine (PDL) in presence of NGF. After five days infection of lentivirus expressing control and Nedd4-2 shRNA, cells were starved from NGF overnight followed by NGF treatment for 30min; then immunofluorescence technique was applied using anti-phospho TrkA antibody. An increased level of phosphorylated TrkA receptor was observed in the neurons infected by the shRNA Nedd4-2 lentivirus **(Figure 5)**.

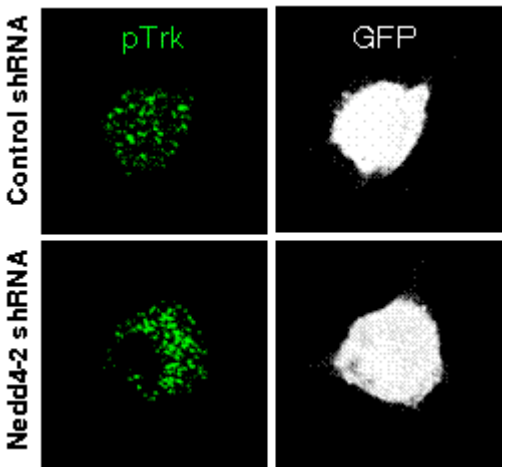


**Figure 5. Down regulation of Nedd4-2 leads to more activated TrkA in response to NGF in DRG neurons.**

Sensory neurons dissected from DRGs were cultured in coverslips for 4 days and infected using Lentivirus containing control and Nedd4-2 shRNA for 5 days. Cells were starved from NGF overnight and were treated with NGF for 30 minutes. Then, Coverslips were collected and stained with anti- pTrkA. GFP showed that the cells were well infected. Pictures were taken using Leica Confocal. Microscopy.

## Nedd4-2 does not influence the internalization rate of TrkA receptors

We have shown that less expression of Nedd4-2 in DRG neurons using lentivirus containing shRNA Nedd4-2 resulted in more TrkA receptors on the cell membrane. To explain this, we turned to the possible trafficking routes of the activated TrkA receptors. Like others receptor tyrosine kinases (RTKs), binding of NGF to TrkA receptor induce the dimerization and autophosphorylation of the TrkA receptors, then the activated receptors are endocytosed and trafficked to early endosome and then they either recycle back to the membrane or go to late endosome and then lysosome for degradation. Any change of these routes, such as the defect of internalization or/and degradation, more efficient recycling of the receptors, may cause more TrkA receptors in the Nedd4-2 downregulated cells.

To address this, we first tried to determine whether the level of Nedd4-2 affects the internalization of TrkA receptors after NGF treatment in the DRG neurons. To obtain the internalized proteins, we labeled the membrane protein of DRG using biotin as we did for the surface TrkA and then treated the labeled DRG neurons with NGF at 37℃ to let TrkA receptor internalize for 0min, 15min and 60min, followed by striping the membrane binding biotin using DTT to get only the internalized proteins labeled with biotin **(Figure 6A)**. Cells were lysed and biotin labeled proteins were pulled down and subjected to Western blot for Trk and p75.

Here we used cells without NGF treatment as the control which gave us the basal internalization rate of the inactive TrkA receptors. More TrkA receptors were found endocytosed in the DRGs infected with lentivirus containing shRNA nedd4-2 than that in the cells infected with control lentivirus in both 15min and 60min **(Figure 6B)**. However because the basal level of surface TrkA is also higher in the DRGs infected with lentivirus containing shRNA Nedd4-2, when we calculated the rate of TrkA internalization, we found there was not significance difference of the internalization rate between the DRGs infected with Lentiviurs contain shRNA nedd4-2 and control lentivirus as that of p75 **(Figure 6C-D)**.

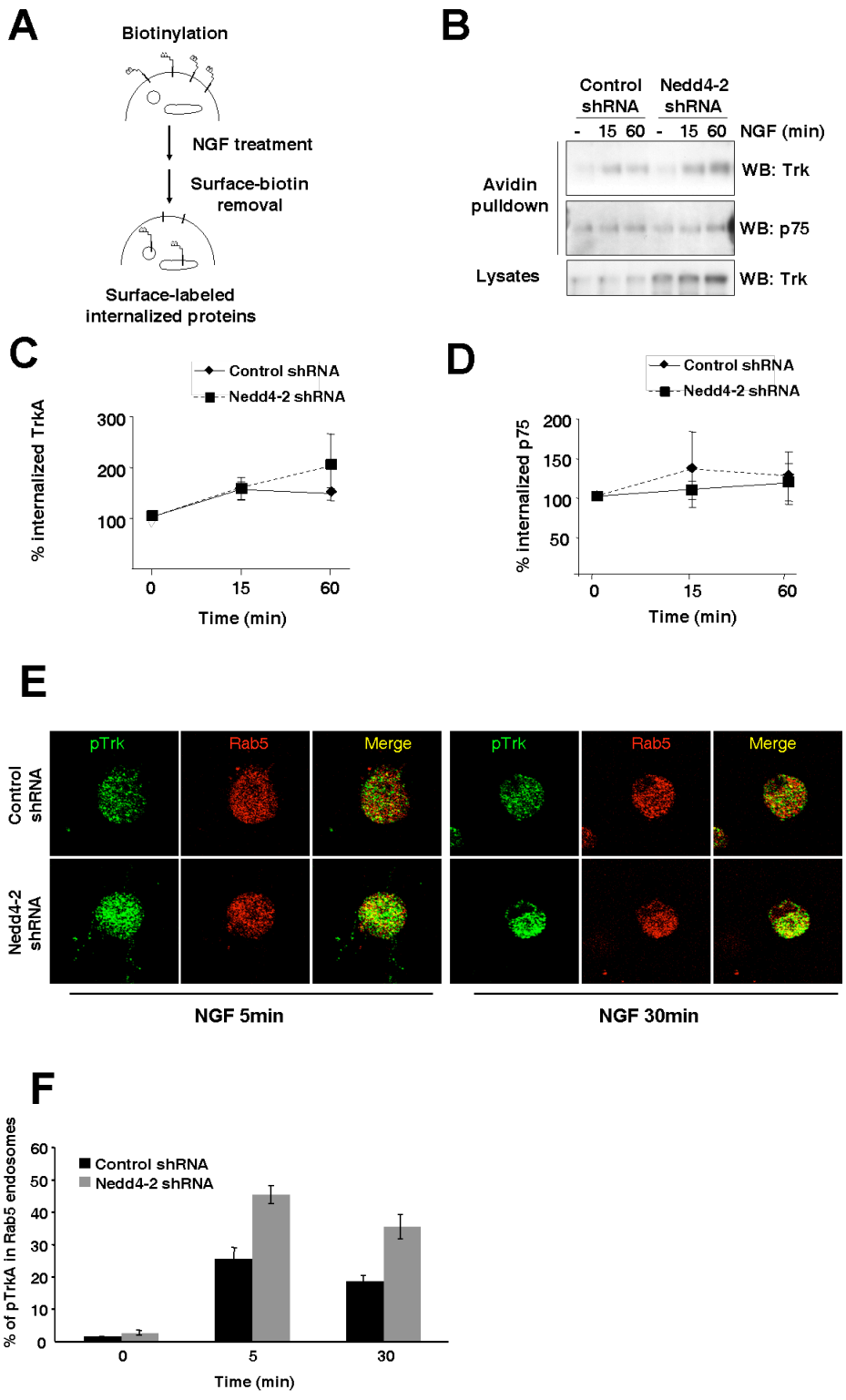


**Figure 6. TrkA receptor internalization in response to NGF is not affected by Nedd4-2 depletion.** (A) Experimental procedure. (B) WB showing surface labeled receptors that have been internalized in response to NGF in control and Nedd4-2 depleted DRG neurons. (C) Quantification of internalized TrkA receptors (*n*=3). (D) Quantification of internalized p75 receptors (*n*=3). (E) Stainings of Nedd4-2-depleted DRGs with pTrk and Rab5. (F) Quantification of pTrkA and Rab5 co-localization in response to NGF.

As we mentioned above, NGF binding to TrkA induces its activation followed by its internalization and trafficking. To further investigate if the level of Nedd4-2 influenced on the internalization of activated TrkA receptors to early endosome, the first step of the trafficking of activated TrkA receptor, we compared the activated TrkA receptors of control and knockdown groups localized in the early endosome by immunofluorescence using anti-phospho-TrkA antibody together with anti-Rab5 antibody, which is a marker of early endosome. The results showed that more activated TrkA colocalized with Rab5 with NGF treatment for 5 minutes than for 30 minutes in both the DRGs infected with control lentivirus and shRNA Nedd4-2 lentivirus, but when we compared the colocalization of activated TrkA with early endosome between the two groups of DRGs, it is shown that the more activated TrkA were found in the early endosome in the DRGs infected with shRNA Nedd4-2 lentivirus **(Figure 6E-F),** which is consistent with the results we got from Western blot. All of these data suggested that the internalization of TrkA receptors in response to NGF treatment was not affected by Nedd4-2.

## Nedd4-2 affects TrkA level through modulating its degradation rate

As we have identified that the Nedd4-2 did not affect the internalization rate of TrkA receptors, we hypothesized that Nedd4-2 might regulate TrkA receptors' degradation. To address that we first labeled the cells with biotin and stimulated with NGF to let TrkA receptor be internalized and degraded; then we pulled down the biotin labeled proteins and immunoblotted for TrkA and p75 **(Figure 7A)**.

The results showed that without NGF treatment, there are more TrkA receptors in the DRGs expressing less Nedd4-2 by infecting lentivirus containing shRNA Nedd4-2, which is consistent with what we found before **(Figure 4B)**. In the neurons infected with control lentivirus, TrkA receptors level decreased with the time of NGF treatment suggesting the degradation of TrkA. However, in the neurons infected with lentivirus containing shRNA Nedd4-2, the decreasing of TrkA receptors is much slower especially in the cells treated with NGF for 1h **(Figure 7B-C)**. The level of p75 is similar in all of the samples, since it is reported that p75, instead of going to degrade, it recycle back to the membrane at a very high speed **(Figure 7B, D)** (Saxena et al 2004).

After internalized to the early endosome, one of the trafficking routes is to go to late endosome and then lysosome for degradation. So we performed immunofluoresece using antibodies for Rab7, a late endosome marker and for pTrk to see their colocalization, therefore tracing the activated TrkA. Different from the results we

obtained from the colocolazition of pTrkA and Rab5, after treatment with NGF for 10min, there is less colocalization for pTrk and Rab7 in the DRGs infected with shRNA Nedd4-2 lentivirus, which mean although more pTrk was found in the early endosome they did not enter the late endosome as efficiently as the pTrk in the DRGs infected with Control lentivirus **(Figure 7E)**.

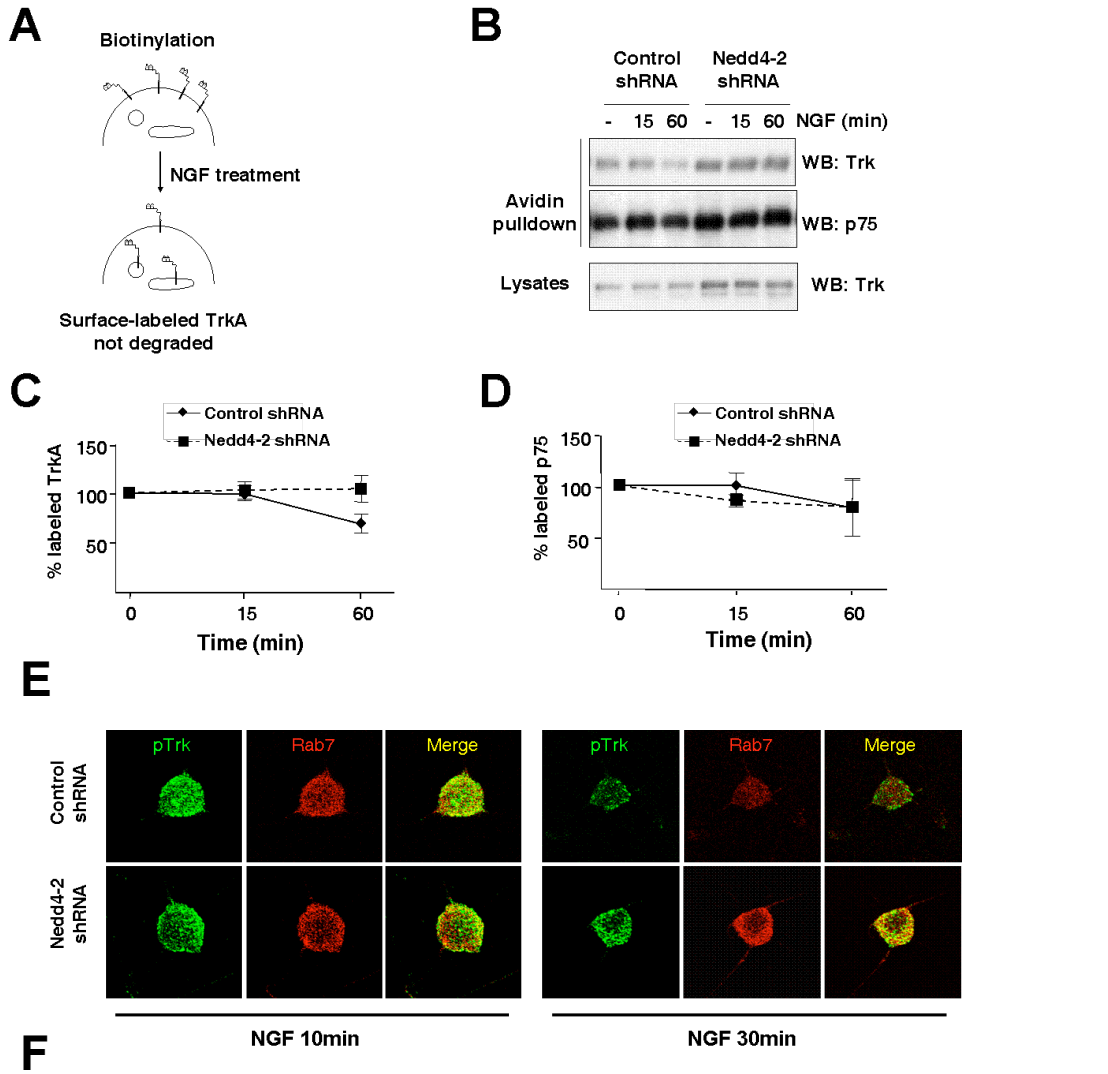


**Figure 7. Reduced TrkA degradation after Nedd4-2 depletion upon acute NGF treatment.** (A) Experimental procedure. (B) WB showing surface-labeled receptors in response to NGF in control and Nedd4-2 depleted DRG neurons. (C) Quantification of the degradation of surface-labeled TrkA receptors (*n*=3). (D) Quantification of the degradation of surface-labeled p75 receptors (*n*=3). (E) Stainings of Nedd4-2-depleted DRGs with pTrk and Rab7. (F) Quantification of pTrkA and Rab7 co-localization in response to NGF.

What's more, in the DRGs infected with Control lentivirus, we could see that pTrk entered the late endosome, accumulate there within 10 minutes' NGF treatment and then left the late endosome, as we have observed that there are less pTrk colocolized with Rab7 with NGF treatment for 30 minutes; However, in the DRGs with downregulated Nedd4-2 expression, pTrk continuously entered late endosome and accumulated there until NGF treatment for 30 minutes **(Figure 7F)**. The delay of entering pTrk into late endosome and the accumulation of pTrk there may explain what we have observed in the biotinylation based degradation experiment.

### Nedd4-2 depletion leads to an enhanced TrkA recycling

Upon binding of ligands, activated RTKs, which are endocytosed to the early endosome, also entered the recycling pathway in addition to going to the late endosome and lysosome for degradation. So we also took interest in whether more activated TrkA recycled back to the cell surface from the early endosome when the expression of Nedd4-2 is down regulated.

To address it, DRG neurons were infected with lentivirus containing control or Nedd4-2 shRNA. After NGF starving overnight, NGF was added for 0, 15 and 60 minutes to the media. Then surface protein was labeled by biotin **(Figure 8A)**, pulled down using NeutroAvidin agarose, subjected to SDS-PAGE, and immunobloted for Trk and p75.

As is shown, in the DRGs infected with control or Nedd4-2 shRNA virus, the level of surface TrkA receptors decreased with the time of NGF treatment **(Figure 8B-C)**; however, there is more surface TrkA receptors in the Nedd4-2 knockdown DRGs with NGF treatment for both 15 minutes and 60 minutes **(Figure 8B-C)**, while the surface level of p75 is similar to that in the DRGs infected with control virus **(Figure 8D)**. Since Nedd4-2 does not affect internalization rate of TrkA receptors in response to NGF, this increased TrkA receptors, compared to control group, should result from the enhanced recycling of the TrkA receptors when we down regulated the expression of Nedd4-2.

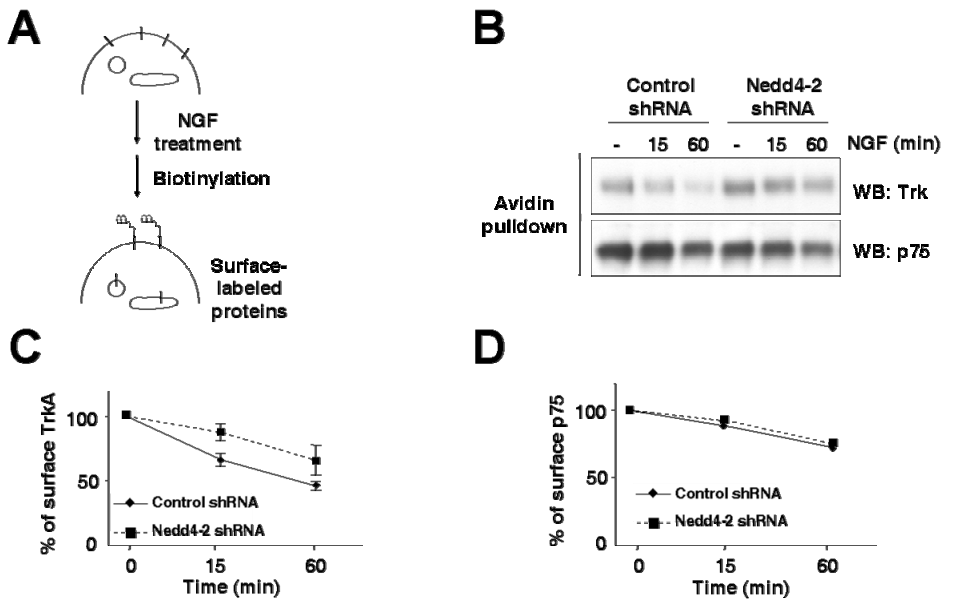


**Figure 8**. **Increased surface TrkA expression after Nedd4-2 depletion upon acute NGF treatment.** (A) Experimental procedure. (B) WB showing surface receptors in response to NGF in control and Nedd4-2 depleted DRG neurons. (C) Quantification of cell-surface TrkA receptors (*n*=3). (D) Quantification of cell-surface p75 receptors (*n*=3).

## Normal signaling pathways are affected by more TrkA receptors

Sensory neurons from the dorsal root ganglion critically depend on neurotrophin for their survival during development. We have already determined that downregulation of Nedd4-2 led to an increase of acitivated TrkA through slowing down its degradation rate. Here we were interested in if these increased activated TrkA receptor could affect their downstream cascades. Western Blot was performed using the cell extract from NGF-dependent sensory neurons infected by shRNA Nedd4-2 lentivirus or its control virus. By using antibodies to phospho-TrkA, phospho-MAPK, and phospho-AKT we addressed that less expression of Nedd4-2 led to more activated TrkA and consequently more activated MAPK **(Figure 9)**. So，Nedd4-2 not only affects the level of TrkA receptors but also influences its down stream signaling pathway.

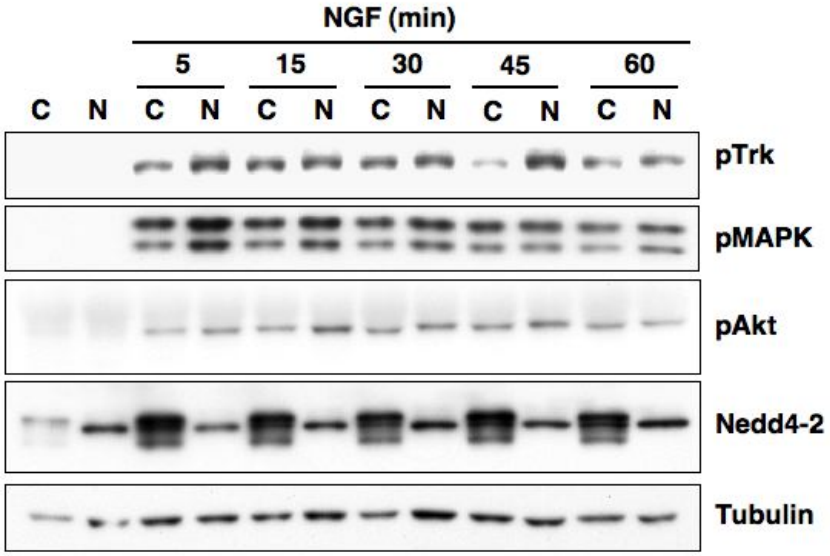


**Figure 9**. **Nedd4-2 regulates NGF-mediated signaling.** DRG neurons were grown in presence of NGF (25 ng/ml), infected with lentivirus expressing control or Nedd4-2 shRNA and NGF-starved O/N with Z-VAD-FMK. NGF (50 ng/ml) was added for the corresponding time and WB were performed. C: Control shRNA lentivirus; N: Nedd4-2 shRNA lentivirus.

## DISCUSSION

Nedd4-2 was firstly found to bind and ubiquitinate TrkA receptors but not TrkB receptors, because TrkA contains a PPXY motif which could be recognized by Nedd4-2 while TrkB does not have (Arévalo, 2006a). However, the detail of how Nedd4-2 regulates TrkA receptors is not clear. Through downregulating the expression of Nedd4-2 by RNA interference, we showed that knocking down Nedd4-2 led to an increase of both the total TrkA and surface TrkA in sensory neurons and consequently resulted in more activated TrkA. To further explain the effect of Nedd4-2 on TrkA receptors, we focused on the trafficking of activated TrkA receptors in response to NGF.

Endocytic trafficking of signaling receptors to alternate intracellular pathways has been shown to lead to diverse biological consequences. In mammalian cells, several ion channels and signal-transducing receptors that undergo regulated internalization are ubiquitylated in response to an extracellular signal, and ubiquitylation regulate their endocytic transport. Previous trafficking studies of receptor tyrosine kinases, namely, the EGF receptor, have shown that c-Cbl, and RING E3 ligase, promotes the removal of activated EGFR from the plasma membrane by directing the EGFR into an endocytic pathway that involves Eps15 (Annemieke et al 2004). So here the first experiments we performed were aimed to see whether Nedd4-2 affects the internalization of TrkA receptors in DRG neurons. Using WB analysis we have seen that downregulation of Nedd4-2 resulted in more TrkA receptor in the DRG neurons. In addition, there is more activated TrkA localized in early endosome; however, the internalization rate of activated TrkA after modifying Nedd4-2 protein level was not affected. So we concluded that Nedd4-2 did not function on the internalization of surface TrkA to early endosome in response to NGF treatment.

As is known that after internalization, the endocytosed activated TrkA could be sorted either to late endosome, lysosome for degradation or to recycling endosome to come back to the membrane, we were interested in whether Nedd4-2 had an effect on the degradation and recycling of TrkA. Using the biotinylation and immunofluorescence experiments, we observed that, in the Nedd4-2 knockdown DRGs, there is more activated TrkA accumulated in late endosome compared with that in the control DRGs. On one hand, this told us that the function of Nedd4-2 was mainly to regulate the activated TrkA to go to late endosome after they were sorted from the membrane to the early endosome; on the other hand, we could predict that the recycling of activated TrkA might also be modulated by the Nedd4-2. More experiments should be

done to confirm this point.

Increasing evidence shows that protein degradation has important roles in both neuronal development and long-term synaptic plasticity (Tai and Schuman, 2008). Moreover, many neurodegenerative disease, such as Alzheimer's (AD), Huntington's (HD) and Parkinson's (PD) diseases, are associated with abnormal protein aggregates, implicating degradative dysfunction. To determine the consequence of increased TrkA receptors in the DRG neurons with downregulated Nedd4-2, we firstly measured NGF-mediated signaling cascades. It is shown that there is more phosphorylated/activated MAPK, probably due to increased activation of TrkA after knockdown of Nedd4-2. It was found that NGF also plays an important role in the pain transduction. Investigation showed that: 1) NGF is up-regulated after inflammation injury of the skin (Weskamp and Otten, 1987); 2) Direct administration of NGF into the sciatic nerve produces hyperalgesia (Lewin et al, 1993); 3) Inflammation-related pain can be significantly reduced by neutralizing NGF bioactivity in animal models (Ugolini et al, 2006). So, we are also interested in whether the increased activation of TrkA receptors and the downstream cascades affect the pain transduction in DRG neurons with less Nedd4-2 expression.

To sum up, our data indicates that the level of Nedd4-2 directly modulate the degradation rate of TrkA receptors by functioning on the process from early endosome entering into late endosome but not affect the internalization of surface TrkA receptors to early endosome. Besides, Nedd4-2 affects NGF-mediated signaling by regulating TrkA receptor level. Further work will focus on the relationship between altered NGF-mediated signaling by downregulation of Nedd4-2 and the abnormal function of DRG neurons.

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