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Cell Survival through Trk Neurotrophin Receptors Is Differentially Regulated by Ubiquitination

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Summary

Specificity of neurotrophin factor signaling is dictated through the action of Trk receptor tyrosine kinases. Once activated, Trk receptors are internalized and targeted for degradation. However, the mechanisms implicated in this process are incompletely understood. Here we report that the Trk receptors are multimonoubiquitinated in response to neurotrophins. We have identified an E3 ubiquitin ligase, Nedd4-2, that 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. Activated TrkA receptor levels and the survival of NGF-dependent sensory neurons, but not BDNF-dependent sensory neurons, are directly influenced by Nedd4-2 expression. Unexpectedly, Nedd4-2 does not bind or ubiquitinate related TrkB receptors, due to the lack of a consensus PPXY motif. Our results indicate that Trk neurotrophin receptors are differentially regulated by ubiquitination to modulate the survival of neurons.

Introduction

Neurotrophin signaling is responsible for a wide variety of neuronal functions, including survival, differentiation, axonal and dendritic growth, cell death, neurotransmitter secretion, and neuronal activity (Chao, 2003; Huang and Reichardt, 2003; Poo, 2001). These actions depend upon two different transmembrane receptors, the Trk receptor tyrosine kinase and the p75 neurotrophin receptor (Huang and Reichardt, 2003). Nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and NT-4 all recognize the p75 receptor, but more specific interactions take place between NGF and TrkA; BDNF and NT-4 with TrkB; and NT-3 to TrkC receptors. The intracellular pathways stimulated by Trk receptors share many common protein substrates that are used by other receptor tyrosine kinases, such as Shc, Grb2, APS, FRS2, and phospholipase Cγ (PLC-γ).

While the signal transduction pathways used by the Trk receptors are well understood, little is known about the events that regulate Trk receptor internalization and turnover. Other receptor tyrosine kinases, such as the EGF and PDGF receptors, are rapidly degraded upon ligand activation. This process is controlled by ubiquitination of activated EGF and PDGF receptors by c-Cbl, an E3 ubiquitin ligase (Thien and Langdon, 2001). c-Cbl binds to the EGF receptor and promotes its ubiquitination at the cell membrane. After monoubiquitination at multiple sites, the receptors undergo degradation in lysosomes (Haglund et al., 2003b).

In contrast to many receptor tyrosine kinases, Trk receptors also undergo axonal transport after internalization (Ginty and Segal, 2002). Previous studies have indicated that the NGF-TrkA complex could be found in clathrin-coated vesicles and endosomes associated with tyrosine kinase substrates, such as PLC-γ. Several tyrosine phosphorylated proteins are associated with the TrkA receptor during transport, indicating that signaling by neurotrophins persists following internalization of their receptors (Delcroix et al., 2003; Ye et al., 2003). Stimulation of Erk/MAP kinase family members and activation of phosphatidylinositide-3 kinasE (PI3-K) and Akt kinases (Kuruvilla et al., 2000) have been observed in the cell body of dorsal root ganglia (DRG) neurons after retrograde signaling by neurotrophins (Watson et al., 2001). Turnover of Trk receptors is an ongoing process and regulates the extent of signal transduction in neurons. How degradation of Trk receptors is controlled following internalization is not understood.

Here we report that Trk receptors are differentially ubiquitinated and degraded after binding to neurotrophins. We have identified a ubiquitin E3 ligase, Nedd4-2, that binds specifically to the C-terminus of the TrkA receptor and induces the downregulation of the receptor. Unlike other motifs in the Trk tyrosine kinase structure, the site of Nedd4-2 association is not conserved in TrkB and TrkC receptors. As a result, expression of Nedd4-2 affects the survival of NGF-dependent, but not BDNF-dependent neurons. Our data provide a specific ubiquitination mechanism for modulating the level of Trk receptors at the cell surface.

Results

Trk Receptors Are Ubiquitinated in Response to Neurotrophins

Receptor tyrosine kinases, such as the EGF and PDGF receptors, frequently undergo attenuation of their signaling pathways via endocytosis and lysosomal degradation, processes in part regulated by ligand-induced receptor ubiquitination (Haglund et al., 2003a, 2003b). However, it is not known how Trk receptors are similarly regulated in response to neurotrophins.

To investigate if Trk receptors are ubiquitinated, we stimulated PC12-615 cells and primary cortical neurons

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with NGF and BDNF, respectively, for 10 min. Cell extracts were subjected to immunoprecipitation with pan-Trk antibodies and blotted in parallel with antibodies that recognize both multimonoubiquitination and polyubiquitination (P4D1) or only polyubiquitination (FK1) as it has been previously described (Haglund et al., 2003b). TrkA and TrkB receptors both underwent ubiquitination in response to neurotrophins, as detected with the P4D1 antibody (Figures 1A and 1B). To confirm the fidelity of the FK1 antibody, the state of ubiquitination of β-catenin was analyzed in PC12-615 cells. The FK1 antibody effectively detected polyubiquitinated β-catenin (Figure 1C). Therefore, these data demonstrated that Trk receptors are primarily multimonoubiquitinated in response to neurotrophins.

To define the TrkA domains required for the ubiquitination, we analyzed the behavior of several defined receptor mutants in HEK293 cells. Expression of a kinase-inactive TrkA receptor (K547R) resulted in a pronounced reduction of ubiquitinated receptor (Figure 1D). However, mutations of two other lysine residues in the juxtamembrane region of TrkA (K505A and K516A) did not affect ubiquitination. Also, mutations on the Shc binding site (Y490F or ΔNPQY motif) did not affect the ubiquitination pattern (data not shown). However, a PLC-γ binding site mutant of TrkA (Y785F) showed a prominent reduction in ubiquitination compared to the wild-type (Figure 1D). These data indicate that Trk tyrosine kinase activity and the Y785 residue are both required for the proper ubiquitination of TrkA receptor.

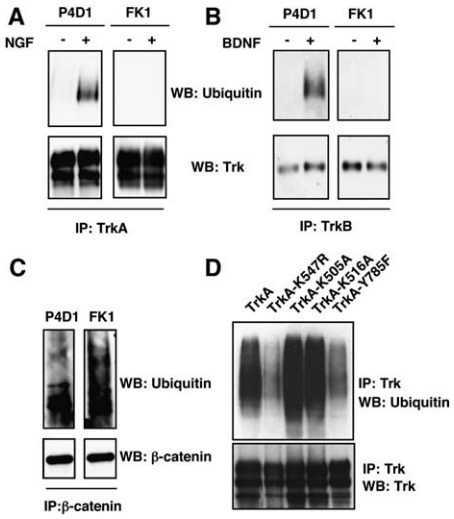

Figure 1. TrkA and TrkB Receptors Are Multimonoubiquitinated in Response to Neurotrophins

(A) Trk receptors undergo multimonoubiquitination in response to neurotrophins. PC12-615 cells were serum-starved for 12–16 hr before stimulation with NGF (100 ng/ml) for 10 min. Immunoprecipitation was performed for Trk receptors followed by Western blot with P4D1 antibody, which recognizes both multimonoubiquitinated and polyubiquitinated substrates, and FK1 antibody, which recognizes only polyubiquitinated substrates.

(B) Cortical neurons were starved in MEM, 2% glucose, 2 mM glutamine, and 10 μM MK-801 for 12–16 hr before stimulation with BDNF (50 ng/ml). Immunoprecipitation and Western blotting were performed as described in (A).

(C) PC12-615 cells were treated with lactacystin (2 μM) for 6 hr to block the proteasome and β-catenin was immunoprecipitated. Western blots were performed with P4D1 and FK1 antibodies.

(D) HEK293 cells were transiently transfected with different cDNAs for rat TrkA, and 2 days later, cells were lysed. Extracts were immunoprecipitated with pan-Trk antibodies and Western blots were performed with P4D1 antibodies. The same blot was reprobed with Trk antibodies to verify the equality of protein loading (bottom panel).

The E3 Ligase, Nedd4-2, Is Responsible for TrkA Ubiquitination

The sequence surrounding the Y785 residue in TrkA is PPXY^{785}, a motif that was previously described as a binding module for WW domains of the Nedd4 E3 ubiquitin ligase family (Ingham et al., 2004). The presence of the PPXY motif in TrkA receptor and the impairment in the ubiquitination in the TrkA-Y785F mutant led us to hypothesize that this family of E3 ubiquitin ligases may play a role in the ubiquitination of TrkA receptor.

Nedd4 proteins contain several WW domains and a catalytic C-terminal Homologous to E6-AP Carboxyl Terminal (HECT) domain. To test if TrkA is a target protein for the E3 ubiquitin ligase family and to investigate if there was an interaction between TrkA and Nedd4-2, we obtained a cDNA for Nedd4-2, a highly expressed member in the nervous system (Kumar et al., 1997). An association between TrkA and Nedd4-2 was easily observed after expression in HEK293 cells (Figure 2A). To confirm the involvement of the PPXY motif, we constructed mutants in TrkA in the PPXY sequence at P782S and Y785F. Both of these mutant receptors failed to interact with Nedd4-2 (Figure 2B, data not shown). To establish if the interaction was direct, recombinant GST fusion proteins containing each WW domain of Nedd4-2 were used in in vitro pulldown assays. Wild-type TrkA was efficiently recognized with GST-proteins containing WW3 and WW4 domains and to a lesser degree with the WW1 and WW2 domains (Figure 2C). However, no interaction was detected with TrkA-P782S mutation. Therefore, the PPXY motif of TrkA specifically binds to the WW domains of Nedd4-2, consistent with the binding properties of this E3 ligase (Hicke and Dunn, 2003; Staub et al., 1996).

To show that Nedd4-2 can ubiquitinate TrkA directly in vitro, we used recombinant GST-Nedd4-2 proteins and in vitro ^{35}S-labeled TrkA cytoplasmic domain as a substrate. The cytoplasmic domain of TrkA was ubiquitinated in the presence of wild-type Nedd4-2 protein, but not when GST protein alone or a Nedd4-2 mutant, C962S, was utilized (Figure 2D). The C962S mutant inactivates the E3 ligase activity in the HECT domain (Marchese et al., 2003). The reconstitution of ubiquitinated TrkA in this in vitro reaction with wild-type Nedd4-2 demonstrates a requirement for Nedd4-2 E3 ligase activity.

To address if the association of TrkA and Nedd4-2 is physiological, cell extracts from sensory neurons isolated from the DRG were subjected to immunoprecipitation with Nedd4-2 antibodies. TrkA receptors could be specifically recognized using Trk antibodies. An endogenous association between TrkA and Nedd4-2 proteins could be observed in primary DRG neurons (Figure 3A). To investigate the extent of Trk and Nedd4-2 colocalization, immunofluorescence analysis was performed on NGF-dependent DRG neurons. The results indicated there was partial colocalization of TrkA and Nedd4-2 (Figure 3B). Since these sensory neurons were grown

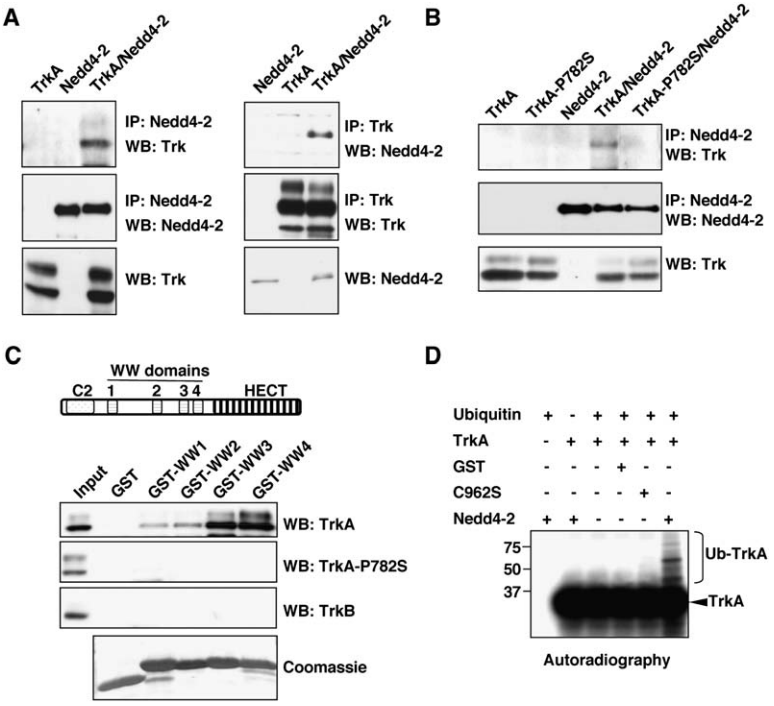

Figure 2. Nedd4-2 Binds and Ubiquitinates TrkA

(A) TrkA receptor interacts with Nedd4-2. Lysates from HEK293 cells transfected with TrkA receptor and FLAG-Nedd4-2 were immunoprecipitated with FLAG antibodies (left side, top and middle panels) or with Trk antibodies (right side, top and middle panels). Western blots were performed to detect the presence of Trk receptors (left side, top panel) or FLAG-Nedd4-2 (right side, top panel). Controls to detect the immunoprecipitation (middle panels) and expression levels were blotted with the corresponding antibody (bottom panels).

(B) TrkA-P782S mutant does not interact with Nedd4-2. Lysates from HEK293 cells transfected with TrkA or TrkA-P782S receptor and FLAG-Nedd4-2 were immunoprecipitated with FLAG antibodies. Western blots were performed to detect the presence of Trk receptors (top panel) and the same blot was reprobed with FLAG antibodies (middle panel). Comparable expression levels of TrkA and TrkA-P782S receptors are shown (bottom panel).

(C) WW domains of Nedd4-2 mediate the interaction with TrkA. Recombinant proteins containing the GST-WW domains (1-4) were incubated with lysates containing TrkA, TrkA-P782S, or TrkB and subjected to Western blotting analysis with anti-Trk antibodies. A Coomassie-stained gel of the input GST fusion protein is shown (bottom panel). Note the lack of association of TrkA-P782S and TrkB to the fusion proteins.

(D) In vitro reconstitution of TrkA ubiquitination by Nedd4-2. ^{35}S-labeled cytoplasmic TrkA was incubated with E1, E2 (UbcH5b), ubiquitin, and ATP substrate with GST, GST-Nedd4-2, or GST-Nedd4-2-C962S recombinant proteins followed by SDS-PAGE. In the presence of Nedd4-2, higher molecular weight bands (indicated) accompany TrkA proteins, representing ubiquitinated species. No ubiquitination of TrkA occurs when GST or GST-Nedd4-2-C962S is used instead of GST-Nedd4-2.

in the presence of NGF, the majority of TrkA receptors were found to be intracellular.

Nedd4-2 Causes Downregulation of TrkA Receptor

Activated Trk receptors are quickly removed from the cell surface after ligand binding. We found that increased expression of Nedd4-2 produced a negative effect upon the steady state level of TrkA receptors (Figure 4A, top panel). Quantification showed a marked decrease in the amount of TrkA with increasing concentrations of Nedd4-2 (Figure 4A). As a control, when the TrkA-P782S mutant was coexpressed with Nedd4-2, there was no effect upon the levels of the mutant TrkA receptor (Figure 4A). The HECT mutant, C962S, which abolishes E3 ligase activity, did not increase the downregulation of TrkA (Figure 4B). Therefore, the E3 ligase activity of Nedd4-2 was required for the downregulation of TrkA expression.

Unexpectedly, the levels of TrkB receptor were not affected by the expression of Nedd4-2 (Figure 4A). Western blot analysis using the anti-ubiquitin antibody P4D1 revealed an increase in the amount of ubiquitinated TrkA receptor with increasing levels of Nedd4-2 (Figure 4C, top panel). However, no effect was observed on the ubiquitination pattern of TrkA-P782S or TrkB in parallel experiments (Figure 4C). Therefore, increased ubiquitination by the E3 ligase activity of Nedd4-2 was specific for TrkA and not TrkB receptors. A previous study reported that a lysine residue (K485) was responsible for TrkA ubiquitination (Geetha et al. 2005). However, mutation of this amino acid K485R in TrkA still resulted in Nedd4-2-dependent ubiquitination (see Figure S1 in the Supplemental Data).

To determine the effect of ubiquitination upon Trk internalization, a cleavable biotinylation experiment was carried out using FLAG-tagged Trk receptors in primary neurons (Chen et al., 2005). After cell surface biotinylation, NGF and BDNF were administered for 30 min and biotinylated TrkA or TrkB receptors, respectively, were precipitated using streptavidin-conjugated beads. The level of internalized Trk receptors was not affected by the P782S mutation of TrkA (Figure 4D). Therefore, the ubiquitination of Trk receptors did not have a strong impact upon the internalization process.

Introduction of a Single Proline Residue in TrkB Can Promote Ubiquitination

To our surprise, the pattern of ubiquitination for TrkB was unaffected by increased Nedd4-2 levels (Figure 4C). Indeed, when immunoprecipitation experiments with TrkB and TrkC were performed, an association with Nedd4-2 was not observed (Figure 5A), indicating that Nedd4-2 was specific for the TrkA receptor. Likewise, no interaction between TrkB and Nedd4-2 WW domains were detected in the in vitro pulldown assays (Figure 2C). Closer inspection of the PPXY motif in TrkA and TrkB cytoplasmic domains reveals an indication that TrkB contains a serine residue, S813, instead of proline (Figure 5B).

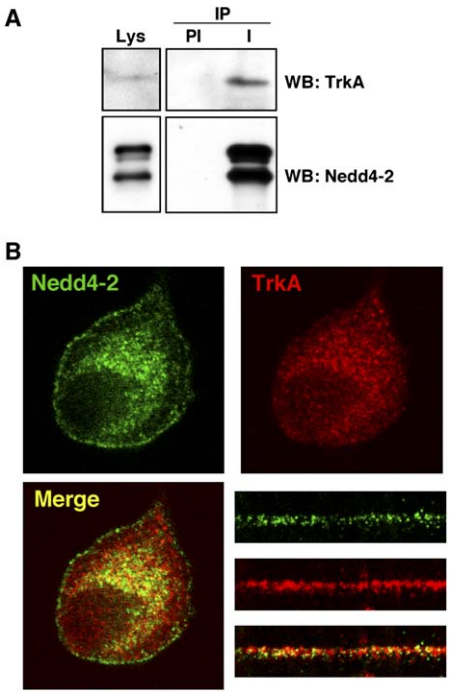

Figure 3. TrkA and Nedd4-2 Expression

(A) TrkA interacts with Nedd4-2 in DRG neurons. DRG neurons cultured in vitro for 6 days in the presence of NGF (50 ng/ml) were treated with lactacystin (2 μM) for 6 hr, and lysates were subjected to immunoprecipitation with preimmune (PI) or immune (I) Nedd4-2 antisera. Western blotting was performed with antibodies to detect the presence of TrkA receptors. Note the presence of TrkA receptors in the lane immunoprecipitated with Nedd4-2 I antisera but not in the PI lane.
(B) Colocalization of TrkA and Nedd4-2 in DRG neurons. Sensory neurons from the DRG cultured for 6 days in vitro in the presence of NGF were fixed, permeabilized, and blocked. Primary antibodies, monoclonal B3 (TrkA), and polyclonal Nedd4-2 were incubated O/N in blocking solution at 4ºC, followed by PBS washes and incubation with secondary antibodies conjugated with fluorescent probes. Nedd4-2 staining is shown in green and TrkA in red. Images were collected on a Leica confocal microscope.

To test if this serine is involved in Nedd4-2 binding, we introduced a proline residue at position S813 and investigated whether this mutated TrkB receptor could restore the interaction with Nedd4-2. The TrkB-S813P receptor was associated with Nedd4-2 by Western blot analysis (Figure 5C), and it was degraded in a Nedd4-2 dose-dependent manner (Figure 5D). At the same time, we observed that the mutant TrkB receptor became ubiquitinated when it was coexpressed with Nedd4-2 (Figure 5E). However, the mutation of S813P in TrkB did not alter the internalization of the receptor in response to BDNF, using the biotinylation assay as described above (Figure 4D, lower panel). Therefore, these data indicate that the presence of a PPXY motif on TrkB is sufficient to recruit Nedd4-2 and target the receptor for degradation by ubiquitination.

Nedd4-2 Binds Constitutively to Inactive TrkA Receptor

The effects of Nedd4-2 upon TrkA ubiquitination raise an important mechanistic question. How does Nedd4-2 bind specifically to TrkA receptors when an adjacent tyrosine (Y785) is also used by PLC-γ? To address this question, we carried out additional experiments to define more precisely the binding requirements of Nedd4-2 to TrkA. We took advantage of the finding of a direct association between the GST-WW3 and WW4 domains of Nedd4-2 to TrkA (Figure 2C). Using a nonphosphorylated and a phosphorylated peptide containing the tyrosine involved in the PLC-γ binding (Y785), we performed experiments to assess the association of the WW domains and TrkA. The interaction between GST-WW3 and TrkA was prevented in a dose-dependent manner only with the nonphosphorylated peptide (Figure 6A). However, the phosphorylated peptide did not disrupt the association of GST-WW3 and TrkA. Similar results were obtained when the GST-WW4 fusion protein was utilized (data not shown). Therefore, these data indicate Nedd4-2 preferentially associates with the unphosphorylated Y785 TrkA receptor.

To further confirm these data, we performed coimmunoprecipitation analysis with TrkA and PLC-γ. It is known that TrkA recruits PLC-γ once Y785 becomes phosphorylated (Obermeier et al., 1993). Therefore, according to the data presented above, Nedd4-2 and PLC-γ are capable of binding inactive and active TrkA receptors, respectively. To test this idea, we transfected HEK293 cells with TrkA along with increasing amounts of Nedd4-2. Endogenous PLC-γ was then immunoprecipitated, and the levels of TrkA receptors associated in absence or presence of Nedd4-2 were assessed by immunoblotting. We did not detect any significant difference in the overall level of TrkA receptors associated with PLC-γ when Nedd4-2 is present (Figure 6B). Therefore, these data indicate that Nedd4-2 can bind to inactive TrkA receptors.

Nedd4-2 Is Phosphorylated in Response to NGF

How does Nedd4-2 become activated? Recently, it has been reported that Nedd4-2 becomes serine/threonine phosphorylated in response to several different stimuli, such as aldosterone, vasopressin, insulin, and insulin growth factor (Snyder et al., 2004a; Bhalla et al., 2005). This event has been proposed to both modulate the activity of Nedd4-2 and to affect the affinity of different substrates for Nedd4 (Snyder, 2005). To assess if Nedd4-2 undergoes phosphorylation, we treated PC12-615 cells with NGF. After immunoprecipitation, increased phosphorylation of Nedd4-2 on serine/threonine residues was detected within 5 min of treatment (Figure 7A). The phosphorylation of Nedd4-2 by NGF was dependent upon Trk activity, since the Trk kinase inhibitor K252a prevented phosphorylation. The TrkA-dependent phosphorylation of Nedd4-2 was also verified in DRG sensory neurons (Figure 7B). Therefore, NGF leads to the rapid phosphorylation of Nedd4-2 in a TrkA-dependent manner. Future studies are required to identify the downstream protein kinases involved in NGF-mediated phosphorylation of Nedd4-2. Although it is not yet clear how phosphorylation affects Nedd4-2 E3 ligase activity or its affinity to TrkA receptors, it is possible that conformation changes in the WW or HECT domain by phosphorylation may promote increased ubiquitination of TrkA receptors.

Overexpression of Nedd4-2 Leads to Apoptosis in NGF-Dependent DRG Neurons

Sensory neurons from the dorsal root ganglion critically depend on neurotrophins for their survival during

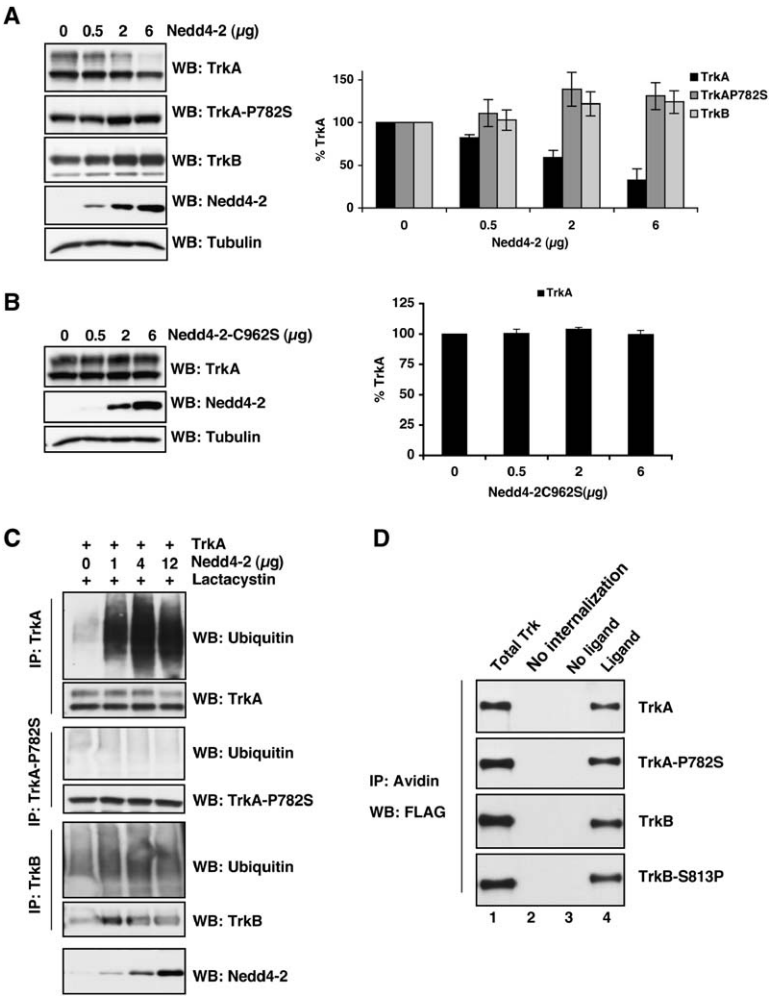

Figure 4. Nedd4-2 Downregulates TrkA Receptor by Ubiquitination

(A) Nedd4-2 expression has a dose-response effect on TrkA receptor levels. Lysates from HEK293 cells transiently transfected with TrkA, TrkA-P782S, or TrkB receptors and increasing concentrations of FLAG-Nedd4-2 were subjected to Western blotting analysis to detect the amount of Trk receptors. FLAG-Nedd4-2 protein levels from lysates are shown. Tubulin was used as a loading control (bottom panel). Quantification of Trk protein levels was carried out using Image J (NIH). The intensity of proteins from several independent Western blot experiments were quantified and normalized to the amount of Trk receptors in the absence of FLAG-Nedd4-2 (100%). Results are the mean ± SD of at least three independent experiments. Note the decrease in the percentage of TrkA with increasing amounts of Nedd4-2.

(B) TrkA downregulation requires the E3 ligase activity of FLAG-Nedd4-2. Lysates from HEK293 cells transiently transfected with TrkA receptor and increasing concentrations of FLAG-Nedd4-2-C962S were subjected to Western blotting analysis to detect the amount of TrkA receptor. FLAG-Nedd4-2 protein levels are shown. Tubulin was used as a loading control (bottom panel). Quantification of TrkA protein in the presence of FLAG-Nedd4-2-C962S was carried out as described in (B). Results are the mean ± SD of at least three independent experiments.

(C) Nedd4-2 expression leads to ubiquitination of TrkA receptor. Lysates from HEK293 cells transiently transfected with TrkA, TrkA-P782S, or TrkB receptors and increasing concentrations of FLAG-Nedd4-2 were immunoprecipitated with Trk antibodies and subjected to Western blotting analysis to detect ubiquitination of Trk receptors. Cells were treated with lactacystin (2 μM) to prevent the degradation of ubiquitinated TrkA receptors. The same blots were reprobed to show the amount of immunoprecipitated receptors. FLAG-Nedd4-2 protein levels from lysates are shown (bottom panel).

(D) Internalization of biotinylated Trk receptors in mass cultures of cortical neurons. Neurons expressing FLAG-Trk receptors were biotinylated and then incubated at 37°C for 30 min in media alone (lanes 1 and 3) or in media containing NGF (50 ng/ml: TrkA) or BDNF (50 ng/ml: TrkB) to allow for internalization of cell-surface proteins. The remaining cell surface biotin was cleaved by reducing its disulfide linkage (for all samples except lane 1, which represents total biotinylated Trk receptors). Lane 2 shows a control for the efficiency of the stripping procedure, in which cells were kept at 4°C after treatment with biotin and then subjected to biotin cleavage. Neurons were subsequently lysed, biotinylated proteins were precipitated with streptavidin beads, and complexes were immunoblotted with anti-FLAG antibodies.

development (Snider, 1994). These sensory neurons display differential dependencies upon NGF, BDNF, or NT-3 which rely upon expression of TrkA, TrkB, or TrkC receptors. To analyze the functional consequences of TrkA receptor ubiquitination, we generated a lentivirus expressing Nedd4-2 in order to infect NGF- or BDNF-dependent sensory neurons. Expression of the proteins from the lentivirus was observed 2 days after infection by following GFP-fluorescence and Nedd4-2 protein expression (data not shown).

Three days after infection with Nedd4-2 virus, NGF-dependent E16.5 sensory neurons became apoptotic (Figure 8A). Infection with a control virus did not produce any cytotoxicity. The apoptotic effect was specific for NGF-dependent neurons; infection of BDNF-dependent sensory neurons with Nedd4-2 virus did not lead to any cell death (Figure 8A). Quantification of multiple experiments indicated that the vast majority (98%) of NGF-dependent neurons infected with the Nedd4-2 virus underwent apoptosis compared with 8% of BDNF-dependent neurons (Figure 8B). The increased expression of Nedd4-2 was confirmed in parallel Western blot analysis using the Nedd4-2 antibody (Figure 8C).

To determine if the levels of activated Trk receptors were affected by the increased expression of Nedd4-2, we first performed immunofluorescence with phospho-specific TrkA and TrkB antibodies recently developed in our lab (Rajagopal et al., 2004). These antibodies recognize activated TrkA or TrkB receptors and do not display any crossreactivity (R.R., unpublished data). A significantly lower level of activated TrkA receptors was observed after 2 days in Nedd4-2 infected neurons (Figure 8D, white arrows), but not in TrkB receptors. To assess the level of TrkA receptors biochemically, we grew mass cultures of NGF-dependent sensory neurons and carried out immunoblot analysis after infection with

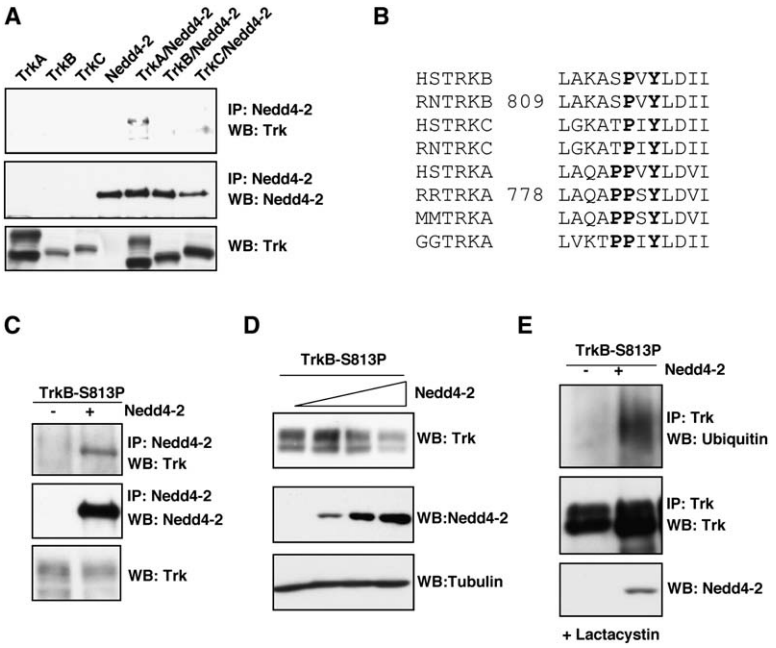

Figure 5. TrkB Containing a PPXY Motif Behaves Like TrkA

(A) TrkB and TrkC receptors do not interact with Nedd4-2. Cell extracts with TrkA, TrkB, or TrkC receptor and FLAG-Nedd4-2 were immunoprecipitated with FLAG antibodies. Western blots were performed to detect the presence of Trk receptors (top panel) and the same blot was reprobed with FLAG antibodies (middle panel). Expression levels of TrkA, TrkB, and TrkC receptors are shown (bottom panel).

(B) Alignment of Trk receptors at the PLC-γ site. The protein sequences surrounding the PLC-γ site from human (HS) and rat (RN or RR) species of TrkA, TrkB, and TrkC and the mouse (MM) and chicken (GG) sequences of TrkA were aligned. Note that all TrkA protein sequences share a motif, PPXY (in bold), that is not present in TrkB or TrkC.

(C) TrkB-S813P interacts with Nedd4-2. Lysates from HEK293 cells transfected with TrkB-S813P receptor and FLAG-Nedd4-2 were immunoprecipitated with FLAG antibodies. Western blots were performed to detect the presence of TrkB-S813P receptor (top panel) and the same blot was reprobed with FLAG antibodies (middle panel). Expression levels of TrkB-S813P receptor are shown (bottom panel).

(D) Nedd4-2 expression has a dose-response effect on TrkB-S813P protein levels. Lysates from HEK293 cells transiently transfected with TrkB-S813P receptor and increasing concentrations of FLAG-Nedd4-2 were subjected to Western blotting analysis to detect the amount of TrkB-S813P receptors (top panel). FLAG-Nedd4-2 protein levels from lysates are shown (middle panel). Tubulin was used as a loading control (bottom panel).

(E) TrkB-S813P receptor is ubiquitinated by Nedd4-2 expression. Lysates from HEK293 cells transiently transfected with TrkB-S813P receptor and FLAG-Nedd4-2 were immunoprecipitated with Trk antibodies and subjected to Western blotting analysis to detect ubiquitination of TrkB-S813P receptors (top panel). Cells were treated with lactacystin (2 μM) to prevent the degradation of ubiquitinated TrkB receptors. The same blot was reprobed to show the amount of immunoprecipitated receptor. Expression of FLAG-Nedd4-2 protein is shown (bottom panel).

the Nedd4-2 lentivirus. A reduction of phospho-TrkA receptors was also observed in Nedd4-2 infected neurons (Figure 8E). These data are similar to the effects of Nedd4-2 in the transfection experiments where Nedd4-2 expression decreased the levels of TrkA (Figure 4A). Also, these data indicate that Nedd4-2 is capable of influencing the survival of NGF-dependent sensory neurons through the levels of activated TrkA receptors.

To demonstrate further the physiological significance of the TrkA-Nedd4-2 interaction, a series of siRNA experiments were conducted on endogenous Nedd4-2 protein. Introduction of siRNA against Nedd4-2 in NGF- or BDNF-dependent sensory neurons resulted in a differential effect upon the activity of TrkA and TrkB receptors, as assessed by tyrosine phosphorylation. Lowering the level of Nedd4-2 protein led to an increase in phospho-TrkA levels (Figure 8F), consistent with decreased degradation of Trk receptor. In contrast, the levels of phospho-TrkB receptors were not affected, verifying the specificity of Nedd4-2 action on TrkA receptors. Therefore, changing the endogenous levels of Nedd4-2 in sensory neurons can differentially modulate the activity of Trk receptors.

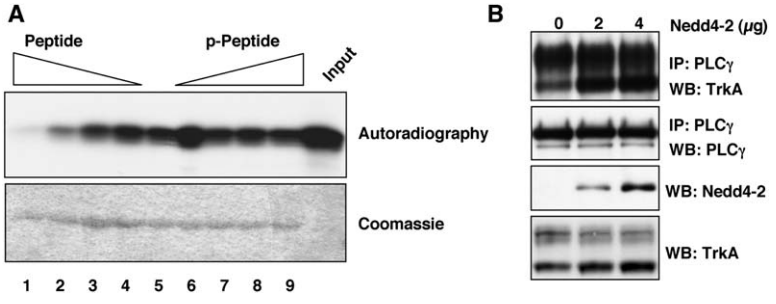

Figure 6. Requirements for Nedd4-2 Binding to TrkA

(A) Nedd4-2 binds to nonphosphorylated TrkA receptor. GST-WW3 fusion protein (5 μg) was preincubated with increasing concentrations of Y785 peptide (LQALAQAP PSYLDV) of pY785 peptide (LQALAQAPP SpYLDV) for 1 hr at 4ºC. In vitro-translated ^{35}S-labeled cytoplasmic TrkA was added to the previous mixture and incubated overnight at 4ºC. A 12% SDS-PAGE gel was loaded with the incubation reaction and autoradiography was performed. The amount of peptide used for each lane was as follows: 1,9: 2000 ng; 2,8: 500 ng; 3,7: 100 ng; 4,6: 20 ng; 5: 0.

(B) Nedd4-2 did not compete with PLC-γ binding to TrkA. Lysates from HEK293 cells transfected with TrkA and increasing concentrations of Nedd4-2 were immunoprecipitated with PLC-γ antibodies. Western blotting was performed to assess the amount of TrkA that was associated with PLC-γ. Note that there is no effect on the TrkA amount associated with PLC-γ in the presence and absence of Nedd4-2.

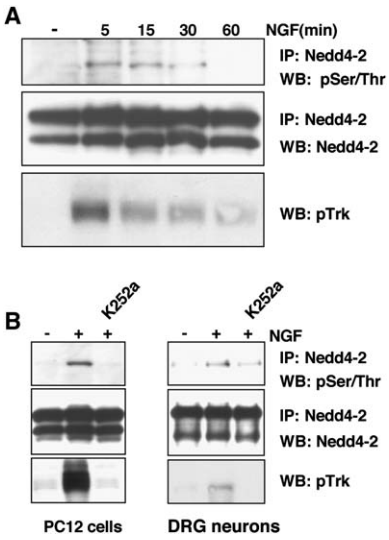

Figure 7. Nedd4-2 Is Phosphorylated in Response to NGF

(A) Time course of Nedd4-2 phosphorylation. PC12-615 cells were serum-starved with DMEM for 24 hr and stimulated with NGF (100 ng/ml). Nedd4-2 was immunoprecipitated from cell lysates and Western blotting was performed to assess the phosphorylation of Nedd4-2. A phosphospecific Ser/Thr polyclonal antibody (Cell Signaling) was used to detect phosphorylated Nedd 4-2.

(B) PC12 cells were treated as described in (A). DRG sensory neurons were NGF-deprived for 5 hr prior to stimulation. K252a (100 nM) was applied for 30 min to both cell types before NGF treatment (5 min, 100 ng/ml).

Discussion

In this report, we provide evidence that TrkA binds directly to Nedd4-2, an E3 ubiquitin ligase that promotes ubiquitination in an NGF-dependent manner. This event is specific to TrkA, as the closely related TrkB receptor does not undergo ubiquitination through Nedd4-2. We identified the sequence in TrkA (PPXY) responsible for Nedd4-2 E3 ubiquitin ligase binding. A single proline residue mutation substitution in the SPXY sequence of TrkB rendered this receptor susceptible to Nedd4-2 action. Increased expression of Nedd4-2 in DRG neurons led to a decrease in the levels of activated TrkA receptors without affecting TrkB receptors. As a consequence, NGF-dependent neurons became apoptotic. On the other hand, downregulation of Nedd4-2 by siRNA experiments in sensory neurons resulted in more active TrkA, but not TrkB, receptors.

Prior experiments in cerebellar granular neurons and immortalized HN10 cells suggested that sequences in the cytoplasmic domains of TrkA and TrkB promoted downregulation of each receptor (Sommerfeld et al., 2000). Although the structures of Trk receptors are highly similar, our study has identified a discrete sequence that accounts for functional differences between TrkA and TrkB, as manifested by the differential susceptibility to apoptosis (Figure 8).

With respect to other growth factor receptors, the EGF, PDGF, and CSF-1 tyrosine kinases all associate with c-Cbl, an E3 ubiquitin ligase containing a RING finger motif rather than a HECT domain (Marmor and Yarden, 2004). We initially assessed whether c-Cbl was associated with Trk, but extensive immunoprecipitation and dominant negative experiments indicated that c-Cbl was not involved in TrkA ubiquitination (data not shown). Instead, we have linked a previously unrelated family of ubiquitin E3 ligases, the Nedd4 family, to Trk receptor ubiquitination.

The Nedd4 protein was originally identified in the central nervous system as a neuronal precursor cell-expressed developmentally downregulated 4 gene (Kumar et al., 1992). It is the prototype of a large family of Nedd4 E3 ubiquitin ligase proteins, each containing conserved features. All of the members contain a C2 domain in the N-terminus, several WW domains, and a HECT catalytic domain at the C-terminus (Harvey and Kumar, 1999; Ingham et al., 2004; Rotin et al., 2000). The C2 domain is implicated in the protein localization whereas the WW domains are involved mainly in substrate recognition and binding. WW domains bind preferentially to proline-rich motifs—like PPXY, PPLP, PR, and phosphoserine/threonine (pS/pT)—that precede a proline residue (Ingham et al., 2004). The PPXY motif is preferred for the Nedd4 E3 ligases. The functions described for this family of E3 ligases include the regulation of epithelial sodium channels (Staub et al., 1996; Debonneville et al., 2001; Kamynina et al., 2001; Fotia et al., 2003; Snyder et al., 2004b; Zhou and Snyder, 2005), TGF-β signaling (Bai et al., 2004; Kuratomi et al., 2005; Yamashita et al., 2005), viral budding (Martin-Serrano et al., 2005; Martin-Serrano et al., 2001), and axonal guidance (Myat et al., 2002).

The mechanism by which Nedd family members induce their E3 ligase activity is not well understood. Nedd4-2, the best characterized member of the family, becomes serine/threonine (Ser/Thr) phosphorylated in response to different stimuli (Snyder, 2005; Bhalla et al., 2005). We have discovered that NGF could also stimulate the phosphorylation of Nedd4-2 (Figure 7). This event depended upon TrkA activation, suggesting that phosphorylation of Nedd4-2 may be involved in substrate binding and ubiquitination activities. Recent experiments indicate that phosphorylation of Itch, a similar protein to Nedd4-2, has profound effects on the activity of the protein (Gao et al., 2004; Chang et al., 2006). Serine and threonine phosphorylation has been proposed to change the conformation of Itch, such that its E3 ligase activity is enhanced (Gallagher et al., 2006).

In contrast to the findings reported here, a recent article indicated that p75 and an associated E3 ligase (TRAF6) were involved in ubiquitination of TrkA at a lysine residue (K485) in the juxtamembrane region of TrkA. This ubiquitination event was observed in PC12 cells and was correlated with endocytosis of the receptor (Geetha et al., 2005). These results are contrary to other published observations. For example, in studies of cultured sympathetic neurons, TrkA receptors undergo efficient internalization in the absence of p75 (Kuruvilla et al., 2004). Indeed, removal of TrkA juxtamembrane sequences containing the K485 residue did not have an effect upon NGF-dependent TrkA recycling (Chen et al., 2005) or degradation (Sommerfeld et al., 2000). Also, in HEK293 cells expressing TrkA, p75 had a negative effect upon TrkA ubiquitination instead of a positive effect, and delayed TrkA internalization in PC12 cells (Makkerh et al., 2005). Indeed, the TrkA-K485R mutant receptor undergoes substantial ubiquitination with Nedd4-2, in

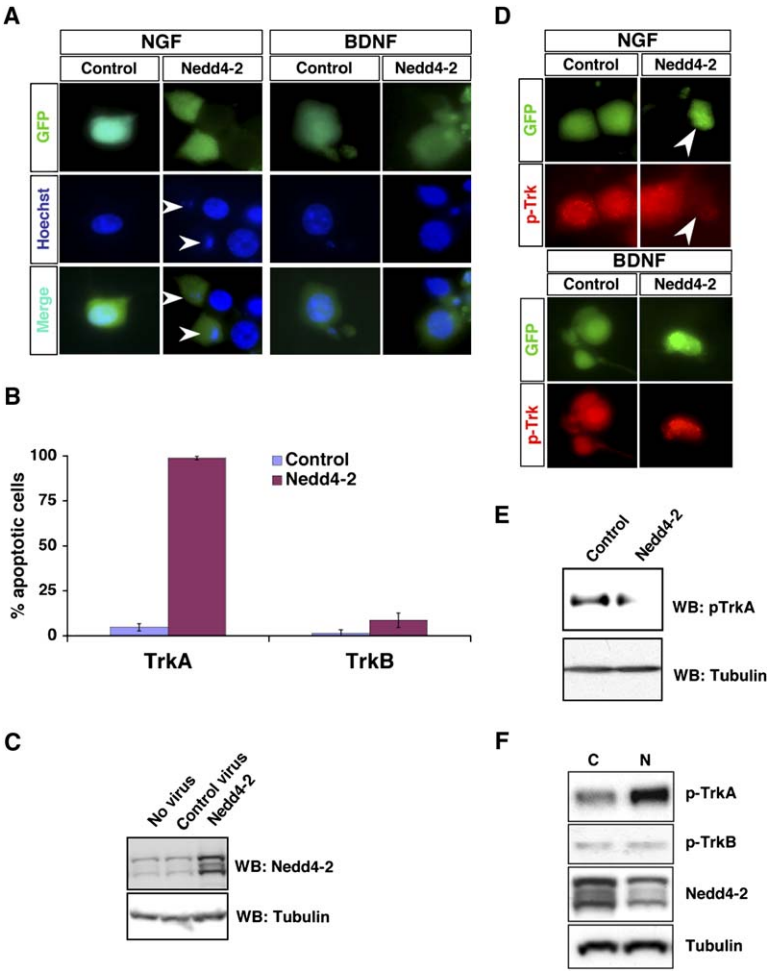

Figure 8. Expression of Nedd4-2 Causes Apoptosis of NGF-Dependent DRG Neurons

(A) NGF-dependent DRG neurons, but not BDNF-dependent neurons, undergo apoptosis upon Nedd4-2 overexpression. DRG neurons were grown in coverslips in the presence of NGF (50 ng/ml) or BDNF (25 ng/ml) for 5–6 days and infected with lentivirus expressing GFP (control) or GFP plus Nedd4-2. Three days later, coverslips were collected and stained with Hoechst 33258 for 5 min. Arrows indicate infected cells (GFP positive) with apoptotic nuclei. Pictures were taken with a microscope equipped with an Axiocam camera (Zeiss). Note that only NGF-dependent neurons infected with Nedd4-2 lentivirus are apoptotic (white arrows).

(B) Quantification of apoptotic neurons infected with lentivirus. GFP-positive neurons were scored 3 days after infection as apoptotic when the nuclei were fragmented or condensed. Results are the mean ± SD of three independent experiments after counting 100 neurons per experiment.

(C) Expression of Nedd4-2 in DRG infected neurons. DRG neurons were grown for 5–6 days in vitro in the presence of NGF and infected with empty virus or virus expressing Nedd4-2. Cells were collected in SDS protein loading buffer, boiled, and subjected to Western blot analysis to determine the expression of Nedd4-2 (top panel). Tubulin was used as a loading control (bottom panel).

(D) Nedd4-2 expression decreases activated TrkA receptors in DRG neurons. Infection of DRG neurons was carried out as described for (A). Immunofluorescence analyses were performed using pTrkA (Rajagopal et al., 2004) and p-TrkB specific antibodies (see Experimental Procedures) to detect the levels of activated receptors in the presence or absence of Nedd4-2 2 days after infection. Note that the presence of Nedd4-2 in NGF-dependent neurons leads to a decrease in the levels of pTrkA.

(E) Nedd4-2 expression decreases activated TrkA receptors in DRG neurons. Massive infection of NGF-dependent DRG neurons (80%–90% of cells) was carried out to check the levels of activated TrkA receptors 2 days after infection. Neurons were harvested in SDS loading buffer, and Western blotting analysis was performed with pTrkA-specific antibodies (top panel). Tubulin was used as a loading control (bottom panel). Note the decrease in the pTrkA signal in neurons infected with Nedd4-2 virus.

(F) Reduction of Nedd4-2 protein results in an increase in the levels of activated TrkA receptors. DRG neurons growing in the presence of NGF or BDNF were transfected with 20 nM final concentration of control (C) and Nedd4-2 siRNA (N). Two days later, cells were collected and subjected to Western blotting analysis to assess the levels of activated Trk receptors using phospho-tyrosine antibodies. An increase in the levels of activated TrkA receptors, but not activated TrkB receptors, was observed in neurons transfected with Nedd4-2 siRNA.

a very similar manner as wild-type TrkA (Figure S1). The physiological significance of this K485 ubiquitination event is not known and needs to be established in neurons.

Nedd4 proteins are dramatically downregulated 20-fold after embryonic development (Kumar et al., 1997). It is tempting to speculate that neuronal survival and death decisions by the availability of neurotrophins during development may be influenced by the expression of Nedd4 family members. Vertebrate members of the family include Smurf1, Smurf2, AIP4, Itch, WWP1, WWP2, and NEDL, each of which produces many spliced isoforms. Preliminary RT-PCR analysis indicates that multiple family members are expressed in brain and peripheral neurons, such as sympathetic and sensory neurons (data not shown). Determination of the impact of Nedd4 family members will therefore require further approaches to inactivate multiple Nedd4 genes, since several studies have indicated functional compensation by Nedd4 proteins (Myat et al., 2002; Wilkin et al., 2004; Yamashita et al., 2005). The identification of a specific E3 ubiquitin ligase molecule represents a potential mechanism to control the levels of Trk receptors in neurotrophin-responsive neurons.

Experimental Procedures

Materials

NGF was obtained from Harlan (Indianapolis, IN), and BDNF was from Peprotech (Rocky Hill, NJ). The following antibodies were used: Trk (C-14 and B-3), Akt, and P4D1 (mono- and polyubiquitin) were from Santa Cruz Biotechnology (Santa Cruz, CA); p-MAPK, p-Akt, and MAPK were from Cell Signaling Technology (Beverly,

MA); anti-FLAG was from Sigma (St. Louis, MO); FK1 (polyubiquitin) monoclonal antibody was from BIOMOL; β-catenin was from Transduction Labs; and GFP antibodies were purchased from Molecular Probes. *clasto*-Lactacystin β-Lactone was purchased from Calbiochem, and FK-009 (Z-VAD-FMK) was from MD Biosciences.

Plasmids

The Nedd4-2 WW domains were amplified by PCR using the human Nedd4-2 cDNA obtained from Kazusa DNA Research Institute. The Nedd4-2 mutation of the C962S residue that abolishes the ubiquitin E3 ligase activity and the K485R mutation in rat TrkA were constructed by PCR amplification. All PCR fragments were verified by DNA sequencing. Lentiviral vectors pWPI, psPAX2 and pMD.2G were used to produce recombinant lentiviruses.

DNA Transfections, Preparation of Cell Lysates, and Immunoblotting

Plasmid DNA was transiently transfected into HEK293 cells (2×10^6 cells/plate) using the Lipofectamine 2000 reagent. For PC12 cells and cortical neuron immunoprecipitations, $1-2 \times 10^7$ cells and 5×10^6 were used. For Western blotting, cells were lysed in 1% NP-40 lysis buffer and analyzed according to Arevalo et al. (2004).

Preparation of GST Fusion Proteins and In Vitro Binding Assays

The following GST-Nedd4-2 recombinant proteins were generated: GST-WW1 (aa 231–266); GST-WW2 (aa 406–438); GST–WW3 (aa 518–550); GST-WW4 (aa 569–601); GST-WW3-4HECT (aa 518–995) GST-WW3-4HECTC962S (aa 518–995). GST-fusion proteins (20 μg) immobilized on glutathione-agarose beads were incubated with 800 μg of the corresponding Trk-transfected HEK293 lysates. Western blotting was performed with antibodies to detect Trk, and Coomassie blue staining was used to verify equivalent protein loading.

Antibody Generation

Nedd4-2 polyclonal antibodies were generated using the fusion protein GST-WW1 as an antigen in rabbits. Purification of the antibodies was carried out by incubating twice with a GST affinity column to preclear the GST antibodies. The flow-through material was incubated with a GST-WW1 affinity column. The antibodies were eluted with 0.1 M Glycine pH 2.5 and neutralized with 1 M Tris pH 8.5.

Phospho-TrkB polyclonal antibodies were generated using a synthetic peptide (LQNLAKASPVpYLDIC) as an antigen in rabbits. Purification of the antibodies was carried out as described above, but using an affinity column with the nonphosphorylated peptide to preclear the antibodies and another with the phosphorylated peptide to obtain the phospho-TrkB antibodies.

In Vitro Ubiquitination Assay

In vitro ubiquitination was performed according to Fotia et al. (2004). Briefly, in vitro-translated ^{35}S-labeled TrkA cytoplasmic domain (TNT-coupled reticulocyte lysate system, Promega, Madison, WI) was incubated with 750 ng of GST, GST-WW3-4HECT, or GST-WW3-4HECTC962S recombinant proteins produced in bacteria in the presence of rabbit E1 (150 ng) and UbcH5b (300 ng) in 25 mM Tris-HCl, pH 7.5, 120 mM NaCl, 2 mM $MgCl_2$, 2 mM ATP, 500 μM dithiothreitol, and 500 ng/μl bovine ubiquitin (Sigma) for 2 hr at 30ºC. The reactions (15 μl) were stopped by the addition of protein loading buffer, resolved by SDS-PAGE, and visualized by autoradiography.

Biotinylation

Internalization of FLAG-tagged Trk and mutant Trk receptors was analyzed using a cell-surface biotinylation assay. Briefly, cortical neurons were transfected with Trk or mutant Trk receptors by electroporation (Amaxa, Cologne, Germany), grown in mass cultures for 3 DIV and then serum-starved for 8 hr. Neurons were subjected to biotinylation on ice with the reversible membrane-impermeable derivative of biotin (sulfo-NHS-S-S-biotin from Pierce; 1.5 mg/ml in PBS). Internalization was allowed to occur by incubation at 37ºC for 30 min with media containing neurotrophins, NGF (50 ng/ml), or BDNF (50 ng/ml). The remaining cell-surface biotin was cleaved by reducing its disulfide linkage with glutathione cleavage buffer and neurons lysed with RIPA buffer. Biotinylated proteins were precipitated using streptavidin-conjugated sepharose beads (Pierce, Rockford, IL), eluted from the beads, resolved by SDS-PAGE, and immunoblotted with an anti-FLAG antibody (Sigma).

DRG Neuron Culture

DRGs were dissected from E16.5 rat embryos, incubated, and dissociated with 0.25% trypsin in L-15 media for 45 min at 37ºC. Cells were plated on plating media (MEM, 10% FBS, 0.4% glucose, 2 mM glutamine, 100 U/ml Pen/Strep) and the corresponding neurotrophin, NGF (50 ng/ml) or BDNF (25 ng/ml) overnight on 12 mm glass coverslips or on plastic plates coated with Growth Factor Reduced Matrigel (BD Biosciences) as a substrate. On the next day, media was changed to NB media (Neurobasal media, B-27, 0.4% glucose, 2 mM glutamine), with NGF or BDNF and fluorodeoxyuridine (2.44 μg/ml) and uridine (2.44 μg/ml). NB media was replaced every 2–3 days.

siRNA Transfection

Control and Nedd4-2 siRNA (*SMART*pool reagent) were obtained from Dharmacon. Transfections were performed in sensory neurons growing for 4–5 days in vitro in the presence of NGF (50 ng/ml) or BDNF (25 ng/ml) in 24 well plates using a final concentration of 20 nM of the corresponding siRNA and HiPerFect reagent (Quiagen). Cells were harvested 2 days after transfection and Western blotting was performed.

Immunofluorescence

Cells were fixed with 4% paraformaldehyde (PFA) for 10 min; blocked/permeabilized with PBS containing 10% FBS, 0.1% Tween-20, and 0.1% Triton X-100; and were incubated with primary antibodies followed by the appropriate secondary antibodies. Images were collected on a Leica confocal microscope (Nussloch, Germany).

Lentivirus Generation

A FLAG-Nedd4-2 cDNA was cloned in pWPI vector. HEK293 cells (Invitrogen), plated in a 10 cm plate the day before, were transfected with 9 μg of pWPI or pWPI-FLAG-Nedd4-2 together with 6 μg of psPAX2 and 5 μg of pMD.2G plasmids using 30 μl of Lipofectamine 2000. Media was changed after 6 hr and collected 48 hr later. The collected virus was centrifuged at 3500 rpm for 10 min and filtered through a 0.45 μM filter.

Apoptotic Assay

Primary sensory neurons (DIV5–6) infected with lentivirus were subjected to fixation with 4% paraformaldehyde for 10 min at room temperature, then stained with Hoechst 33258 (1 μg/ml, Molecular Probes) at room temperature for 5 min. GFP-positive neurons were scored as apoptotic by the presence of fragmented or condensed nuclei.

Supplemental Data

The Supplemental Data for this article can be found online at http://www.neuron.org/cgi/content/full/50/4/549/DC1/.

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