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Necdin and TrkA Contribute to Modulation by p75NTR of Resistance to Oxidant Stress

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Abstract

The neurotrophin receptor p75NTR provides protection from oxidant stress induced by 6-hydroxydopamine (6-OHDA) and resultant cell death. In the absence of p75NTR, TrkA is upregulated and its signaling pathway effectors are increasingly activated. Necdin, a MAGE protein and known interactor of p75NTR and TrkA, is a potential mediator of this phenomenon. Decreased expression of necdin protein in p75NTR-negative PC12 cells decreased TrkA expression and increased PC12 cell resistance to 6-OHDA. Inhibition of JNK phosphorylation by SP600125 also resulted in increased resistance to 6-OHDA, suggesting that TrkA signaling underlies the susceptibility of these cells to oxidant stress.

Keywords

p75NTR; necdin; TrkA; oxidant stress; neurotrophin receptors; NGF

Introduction

Nerve growth factor (NGF) is a small, essential, neurotrophic signaling protein which is released from cells and binds to receptor-bearing neurons, affecting their survival, differentiation, and proliferation [1,2]. There are at least two known cell surface NGF receptors, the p75 neurotrophic receptor (NTR) and the tropomyosin-related kinase receptor A (TrkA). The p75NTR can signal with or without ligand [3,4], and appears to have a dual role in NGF-mediated signaling. It can signal independently of TrkA as a low-affinity NGF receptor or as part of a high-affinity NGF receptor complex with TrkA [5–7]. Both p75NTR and TrkA have been shown to induce [8,9] and prevent apoptosis [4,10,11] depending on NGF binding, interactor or adaptor protein binding, and the relative stoichiometry of p75NTR and TrkA.

The p75NTR receptor confers upon PC12 cells resistance to oxidant stress; conversely, loss of p75NTR expression results in diminished resistance to oxidant stress [12,13]. Reactive oxygen species- (ROS-) induced apoptosis in p75NTR-deficient cells could relate to a change in the TrkA:p75NTR stoichiometry, altered affinity of TrkA for NGF, and/or a coordinate increase in TrkA signaling. Conversely, while it is known that p75NTR-mediated resistance to oxidant stress is associated with enhanced recycling of oxidized glutathione to its reduced

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counterpart and signaling through the PI3K-pAkt pathway [12,13], the proximal mechanisms mediating this effect of p75NTR expression are not understood. Other effects of p75NTR and related “dependence receptors” have been shown to require the presence of proteins, variably called interactor or adaptor proteins, that bind to the intracellular domain of these receptors [14].

One such protein, necdin, is a melanoma antigen gene (MAGE) family member signaling protein that plays a role in terminal differentiation of postmitotic neurons. Interestingly, necdin binds to both p75NTR and TrkA and the association of TrkA with p75NTR to form the high-affinity NGF receptor has been found to be interrupted in sensory neurons in mice lacking the necdin gene [15]. The human necdin gene is not expressed in Prader-Willi syndrome, a neurogenetic disease arising from a partial deletion of paternal chromosome 15q. Mice deficient in necdin present a similar phenotype to humans with this syndrome (e.g., hyperphagia and linear growth deficiency); however, the specific mechanisms by which necdin deficiency might produce these effects have not yet been identified. Interestingly, the temporal and spatial pattern of developmentally regulated expression of necdin parallels that of p75NTR in wild type mice [16]. In addition, adolescents and young adults with Prader-Willi syndrome or obesity of other origins demonstrate deficient mitochondrial antioxidant reserve [17].

The present paper examines the effects of modulation of necdin expression on the resistance to oxidant stress of p75NTR-native and -deficient PC12 cells. Although p75NTR-positive PC12 cells were found to be more resistant to 6-OHDA than p75NTR-deficient PC12 cells, p75NTR-deficient PC12 cells were rendered resistant to 6-OHDA by necdin knockdown. p75NTR knockdown or intrinsic deficiency resulted in upregulation of TrkA and enhanced TrkA signaling. Suppression of TrkA signaling was as effective in restoring resistance of the cells to oxidant stress as necdin knockdown. These data suggest that a necdin-dependent shift from p75NTR to TrkA signaling results in loss of resistance to oxidant stress in PC12 cells.

Materials and methods

Cell lines

Generation and characterization of p75NTR-native and p75NTR-deficient PC12 cell lines have been described previously [8,12,13]. The p75NTR-deficient PC12 cell line was also transfected with an empty construct or a full-length p75NTR expression construct as we have described [12,18]. ATCC PC12 cells were obtained from the American Type Culture Collection (Rockville, MD). Murine primary cortical neurons were the kind gift of Rita Giuliani (Center for Neural Development and Disease, University of Rochester, Rochester, NY).

Cell culture

All PC12-derived cell lines were grown in DMEM (1x) with 4.5 g/L glucose and L-glutamine and without sodium pyruvate (Cellgro, Manassas, VA), supplemented with 10% w/v donor horse serum (Cellgro), 5% w/v fetal bovine serum (Cellgro) and 1% w/v penicillin-streptomycin (Cellgro). This medium was used for all subsequent cell culture experiments. All lines were plated at 10 000 cells/96-well plate or 10 cm plate and were grown in 5% CO_2 at 37°C.

RT-PCR

Cell pellets containing 3×10^6 cells were used for RNA isolation with the Qiagen RNeasy Mini Kit (Valencia, CA) with Step 3a used for lysis of the homogenate by Qiagen QIAshredder. Genomic DNA was minimized by using DNase I (Invitrogen, Carlsbad, CA) as instructed by the manufacturer. The reverse transcriptase reaction was performed using the SuperScript III First-Strand Synthesis System (Invitrogen) with or without reverse transcriptase in the reaction.

Primers specific to necdin (forward: gctggtgcagaaggcgcacga, reverse: gctggtacttcaggtaattc) were used to amplify a 455bp fragment by polymerase chain reaction (T_m= 58, 35 cycles).

siRNA treatment

Necdin siRNA (Santa Cruz Biotechnology, Santa Cruz, CA), p75NTR siRNA (Integrated DNA Technologies, Coralville, IA) and control scrambled siRNA (Santa Cruz Biotechnology) were thawed in a tissue culture hood, diluted into 50 μL medium, and incubated at room temperature for 5 min. Lipofectamine 2000 (Invitrogen) was added to a total of 50 μL of culture medium as per the manufacturer's instructions (0.25 μL Lipofectamine/well of a 96-well plate; 30 μL Lipofectamine/10 cm plate) and allowed to sit for 5 min at room temperature in a tissue culture hood. The siRNA and Lipofectamine solutions were then combined, mixed, and allowed to incubate at room temperature together for 20 min. This mixture was then diluted and added to the cells to a final working siRNA concentration of 20 μM (10 μM each when two siRNAs were used simultaneously). The siRNA treatment was performed overnight (18–24 h) at 37° C, at which time some cells were harvested for protein (10 cm plates) and the rest (96-well plates) were treated with various concentrations of 6-OHDA.

Custom fluorescently-tagged p75-Cy5, necdin-Cy3 and control scrambled-Cy3 siRNA were purchased from Qiagen. All siRNAs were used as described above at a final concentration of 20 μM overnight at 37°C. Cells were fixed with 4% paraformaldehyde (Sigma-Aldrich) for 1 hour at room temperature co-treated with 10 μg/mL Hoechst 33342 stain (Fluka). A standard mercury lamp was used with appropriate filters to capture an image with a gain of 2x (unless otherwise stated). The intensity (time of exposure) was kept constant for each set of samples. The overlap between the Hoechst 33342 stain and the released fluorescent tags was displayed in yellow and counted vs cells with only the nuclei stained blue expressed as a percent of the total.

JNK phosphorylation inhibitor treatment

Following siRNA treatment, the JNK phosphorylation inhibitor SP600125 (BIOMOL, Plymouth Meeting, PA) was added to the cells at a concentration of 10 μM for 1 h at 37°C immediately prior to 6-OHDA treatment.

6-OHDA treatment

The 96-well plates of PC12 cells treated with siRNA were subsequently treated with 0–500 μM 6-OHDA (Sigma-Aldrich, St. Louis, MO). Briefly, 10 mg of 6-OHDA was diluted into 200 μL of saline containing 100 μg/mL L-ascorbate (Sigma-Aldrich) for stabilization of 6-OHDA. This solution was then diluted into culture medium to a final working concentration of between 0 and 500 μM, added to the siRNA-treated cells on 96-well plates, and incubated at 37°C for 24 h.

Alamar blue assay

The metabolic viability of PC12 cells treated with siRNA and then 6-OHDA was determined using the Alamar blue assay (Invitrogen Biosource, Carlsbad, CA). Alamar blue dye was diluted to 10% v/v in cell culture medium and cells were treated for 1 h at 37°C, at which time the fluorescence in each well was quantified with a Molecular Devices SpectraMax M5 plate reader at an excitation wavelength of 530 nM and an emission wavelength of 590 nM. Six measurements were taken per well and the average fluorescence was calculated.

Trypan blue exclusion assay

The Trypan blue exclusion assay was used as a second measurement of cell viability following siRNA and 6-OHDA treatment. Trypan blue (0.4%; Invitrogen Gibco, Carlsbad, CA) was

diluted to 50% v/v in cell culture medium and added to the PC12 cells. The stained cells were subsequently counted manually with a minimum of 4 repeats each.

Protein isolation

PC12 cells were washed with Hank's Balanced Salt Solution (Cellgro) and then trypsinized (Cellgro) for 5 min at 37°C. The cells were then pipetted into a conical tube, centrifuged at 250 × g for 3 min and the supernatant was removed. The pellet was resuspended in 100 μL RIPA buffer [10 mM Tris, pH 8 (MP Biomedicals, Solon, OH); 150 mM NaCl (EMD Biosciences, Madison, WI); 0.1% v/v Nonidet P-40 (Sigma-Aldrich); 0.5% w/v sodium deoxycholate (Sigma-Aldrich); 0.1% w/v sodium dodecyl sulfate (Bio-Rad, Hercules, CA)] supplemented with 4 μg/mL aprotinin (Sigma-Aldrich), 1mM phenylmethanesulphonylfluoride (G-Biosciences, Maryland Heights, MO) and 1 mM sodium orthovanadate (Sigma-Aldrich). This solution was then sonicated through 10 cycles with a Branson Sonifier 450 on a constant output setting of 2 and incubated at 4°C for 30 min, vortexing every 10 min. The resulting mixture was centrifuged at 10 000 × g for 15 min to pellet the protein and the supernatant was removed. The protein concentration was measured with a protein assay kit (Bio-Rad).

Western blotting

Gradient separating and stacking acrylamide gels were poured [7.5–12% gradient separating gel: 7.5%–12% v/v acrylamide/bis (Bio-Rad); 375 mM Tris, pH 8.8 (MP Biomedicals, Solon, OH); 0.05% w/v ammonium persulfate (VWR, Batavia, IL); 0.05% v/v TEMED (Bio-Rad); 4% stacking gel: 4% v/v acrylamide/bis; 375 mM Tris, pH 6.8; 0.05% w/v ammonium persulfate; 0.1% TEMED]. Protein samples were denatured and loaded onto the gels at 50–150 μg protein/lane and were run at 60 V for 30 min and then 90 V for 2 h. The gel was then transferred onto a nitrocellulose membrane at 90 volts for 1.5 h on ice. The membrane was then blocked for 1 h at room temperature in blocking buffer [5% dry milk (Bio-Rad); 1x PBS (Sigma-Aldrich)] on an orbital shaker. Primary antibodies, all purchased from Santa Cruz, except for anti-p75NTR (Promega, Madison, WI), were added at dilutions ranging from 1:200–1:1000 and the membranes were allowed to incubate at 4°C overnight on an orbital shaker. Membranes were washed twice for 10 min with washing buffer [0.1% v/v Tween-20 (Fisher Scientific, Hampton, NH) diluted in 1x PBS] while shaking. The secondary antibody was added in a ratio of 1:5000 in blocking buffer and the membrane and overlying solution were incubated at room temperature for 1 h on an orbital shaker. The membranes were washed with washing buffer 4 times for 10 min each at room temperature on an orbital shaker. The membranes were then placed on Saran Wrap™ and a chemiluminescent solution (Santa Cruz Biotechnology) was added. The excess solution was wiped away and the membranes were exposed onto Kodak Biomax film (Kodak, Rochester, NY). The films were then digitally scanned as TIFs.

Western blot band quantification and statistical analysis

Scion Imaging Software was used to quantify the TIF images. Background measurements were subtracted from each band density and the results were normalized to an actin loading control. A univariate ANOVA (2-way) with a *post hoc* Fisher's LSD test was used to determine the statistical significance of the difference between pairs of samples at each concentration of 6-OHDA (Figure 3, Figure 4, Figure 5 and Figure 8), or pairs of Western blot bands (Figure 6, Figure 7 and Figure 9). Statistical significance was assigned to differences with p values of no greater than 0.01. LD_{50} and IC_{50} values were calculated by using a non-linear regression analysis (Graphpad Prism 5, Graphpad Software Inc., La Jolla, CA). A Student's T-test was used to determine the statistical significance between pairs of samples at each LD_{50}/IC_{50} and for transfection efficiency of siRNA.

Results

Necdin mRNA is present in the p75NTR-native and p75NTR-deficient cell lines

The PC12 p75NTR-native and –deficient cell lines were shown by RT-PCR to express necdin (Fig. 1a–b, +RT). Primary cortical neurons were known to express necdin [19] and were used as a positive control. ATCC PC12 cells were used as a negative control, as they were known to lack necdin expression [14, 15]. The –RT lanes did not have reverse transcriptase during the production of cDNA and were used as controls to exclude genomic DNA contamination of the samples. Since there were no bands in the –RT lanes, the bands amplified are indicative of the presence of necdin mRNA in the PC12 p75NTR-native and -deficient cell lines.

Susceptibility of p75NTR-deficient PC12 cells to cell death induced by 6-OHDA is dependent upon necdin

Our previous studies demonstrated the protective effects of p75NTR against and the relative susceptibility of p75NTR-deficient PC12 cells to oxidative cell death [12,13]. Necdin, a known p75NTR and TrkA interactor, was examined to determine its role in 6-OHDA-induced oxidative cell death. Necdin expression was suppressed using siRNA and metabolic viability was determined using Alamar Blue after treatment with 6-OHDA. A scrambled siRNA construct was used as a control and all siRNA treatments resulted in a baseline decrease in cell viability of approximately 20%. The relative knockdowns of necdin and p75 in p75NTR-native PC12 cells are shown in Fig. 1c–d. Scrambled siRNA had no effect on the resistance of PC12 cells with native levels of p75NTR to 6-OHDA relative to p75NTR-deficient cells (Fig. 3). Necdin siRNA had no effect on the metabolic viability of p75-native PC12 cells indicating that suppression of necdin expression in the presence of p75NTR does not affect cell survival. Interestingly, necdin knockdown confers 6-OHDA resistance to p75NTR-deficient PC12 cells resulting in an IC_{50} comparable to that of p75NTR-native cells. Taken together, these results confirm that p75NTR expression confers resistance to 6-OHDA-induced cell death upon PC12 cells, and demonstrates that this resistance can be mimicked in p75NTR-deficient PC12 cells by suppression of necdin expression.

The PC12 p75NTR-native and p75NTR-deficient cell lines transfect equally with necdin siRNA

Since the necdin mRNA knockdown (55%) was not as effective as the p75NTR mRNA knockdown (85%), they were both fluorescently-tagged. Both PC12 p75-native and p75-deficient cells were treated with p75 siRNA-Cy5, necdin siRNA-Cy3, scrambled siRNA-Cy3 or p75 siRNA-Cy5-NL (Fig. 2, NL = no lipofectamine). All cells were also treated with Hoechst staining so that the number of cells transfected could be counted and expressed as a percent of the total. The p75 siRNA knocked down around 85% of the p75 in the PC12 p75-native cells as expected. The p75 siRNA knocked down p75 in around 50% of the PC12 p75-deficient cells although the total fluorescence was greatly diminished in intensity (the gain was increased 4x) as they have such a low concentration of the p75 protein. The necdin siRNA was successfully transfected into at least 90% of the PC12 p75-native and p75-deficient cells (Fig. 2). The scrambled siRNA did not yield fluorescence (even with an 8x gain) and was used as a negative control for specificity of the knockdowns. Lastly, p75 siRNA-Cy5-NL was used without lipofectamine to show that the fluorescent tag is only released upon transfection into the PC12 cells.

Susceptibility of stable p75NTR knockdown PC12 cells to cell death induced by 6-OHDA is dependent upon necdin

As the p75NTR-deficient PC12 cell line is a spontaneously arising mutant cell line and not a p75NTR knockout, p75NTR was knocked down in p75NTR-native PC12 cells to confirm that the effects depicted in Fig. 3 are indeed specifically due to decreased expression of p75NTR.

As expected, the p75NTR knockdown caused an increase in susceptibility to 6-OHDA-induced cell death as compared to p75NTR-native PC12 cells (Fig. 4). In p75NTR-native PC12 cells, necdin knockdown did not affect susceptibility to 6-OHDA, just as was the case for the comparison of p75NTR-native to spontaneously p75NTR-deficient cells (Fig. 3). Similarly, p75NTR knockdown increased sensitivity to 6-OHDA, and subsequent necdin knockdown raised the IC_{50} and restored resistance to 6-OHDA. In aggregate, these results support the notion that p75NTR-induced resistance to 6-OHDA can be mimicked in p75NTR-knockdown cells by suppression of necdin expression. They suggest that susceptibility to 6-OHDA resulting from downregulation of p75NTR may relate to release of necdin that ordinarily might be sequestered via binding to p75NTR.

Cell membrane integrity of p75NTR-native and p75NTR-deficient PC12 cells treated with 6-OHDA

As an anatomic reflection of cell viability, Trypan blue staining was performed and cells permeable to Trypan blue were counted by hand and subtracted from the total number of cells to derive “percent viable cells”. As shown in Fig. 5, PC12 cells expressing native levels of p75NTR were more Trypan blue impermeable following 6-OHDA treatment than the analogous p75NTR-deficient PC12 cells. Necdin knockdown did not affect the Trypan blue permeability of p75NTR-native PC12 cells, but did affect the Trypan blue permeability of p75NTR-deficient PC12 cells by increasing the LD_{50} 6-OHDA concentration. Once again, the effect of necdin knockdown on PC12 cell sensitivity to 6-OHDA depended on the abundance of p75NTR.

TrkA signaling increases in response to a decrease in p75NTR when necdin is present

Western blotting was used to determine the effect of p75NTR and/or necdin expression on signaling through TrkA and is shown in Fig. 6. These results also verify that necdin siRNA treatment knocked down necdin protein levels. Since necdin is an interactor with both p75NTR and TrkA, phosphorylated and total TrkA protein levels were also determined (Fig. 6). Surprisingly, total TrkA doubled when p75NTR-deficient cells were used and a concomitant and equivalent increase in phosphorylated TrkA was also observed. Note that, although the fraction of TrkA that was phosphorylated did not change, the absolute cellular concentration of phosphorylated TrkA increased commensurate with the increase in total TrkA. This TrkA increase was abolished when necdin was knocked down.

TrkA signaling resulted in activation of JNK and ERK (Fig. 6). Activated (i.e., cleaved) caspase 3 also increased in p75NTR-deficient, necdin-native PC12 cells and decreased when necdin was knocked down. The knockdown of both p75NTR and necdin resulted in an increase in active (i.e., phosphorylated) Bcl-2 (Fig. 7), suggesting that altered expression and activation of anti-apoptotic proteins may at least partially underlie the effects of necdin knockdown on resistance to oxidative cell death. The p75NTR-deficient PC12 cell line consistently manifested a Bid protein with a higher molecular weight than that found in the other cell lines (Fig. 7), most likely a reflection of the phosphorylation of Bid in these cells. Necdin knockdown decreased the expression of Bid, consistent with the observed decreased incidence of cell death and increased phosphorylation of Bcl-2. Surprisingly, in some Western blots, cellular content of Bax, a pro-apoptotic protein, appears to be decreased in the p75NTR-deficient cells. However, this decrease was not consistent on multiple repetitions.

Inhibition of TrkA signaling prevents the increase in 6-OHDA sensitivity seen with p75NTR deficiency

The increase in TrkA signaling seen with p75NTR deficiency was accompanied by an increase in 6-OHDA sensitivity and incidence of cell death after 6-OHDA treatment. An inhibitor of JNK phosphorylation, SP600125, was used to determine if the sensitivity to 6-OHDA was

related to the TrkA-dependent increase in JNK signaling. The results shown in Fig. 8 were obtained with PC12 cells studied under the same conditions as depicted in Fig. 1, with the addition of SP600125. As predicted, the inhibitor restored resistance to 6-OHDA to p75NTR-deficient cells (Fig. 8) and inhibited JNK phosphorylation (Fig. 9). Thus, the 6-OHDA sensitivity seen in p75NTR-deficient and -knockdown PC12 cells can be reversed by either transfection with p75NTR (12), downregulation of necdin, or inhibition of JNK phosphorylation.

Discussion

The expression of p75NTR by PC12 cells confers upon them resistance to 6-OHDA treatment by a mechanism that involves decreased TrkA expression and downstream enhancement of recycling of oxidized glutathione to its reduced form [12]. The effectors that link increased p75NTR expression and decreased TrkA expression to increased reducing potential are not known. Conversely, it is not known through what effectors suppression of p75NTR expression leads to increased TrkA expression and decreased reducing potential. Here we present evidence to link the interactor, necdin, to enhanced TrkA signaling and consequent susceptibility of oxidant-induced cell death. Our data show that necdin knockdown prevents this increase in TrkA activation and cell death only in cells that are p75NTR-deficient, that this increased TrkA activation leads to activation of ERK, JNK, and caspase 3, and that the sensitivity to 6-OHDA seen in p75NTR-deficient cells can be reversed by either necdin knockdown or prevention of JNK phosphorylation.

Although TrkA is best known to prevent apoptosis [20] and is generally shown to increase cell survival in a variety of cell types and conditions [21,22], TrkA also has also been observed to induce apoptosis in medulloblastomas and neuroblastomas through a mechanism that involves NGF ligand binding and signaling through MEK, MAPK, and p38 [23–25].

Disruption of the gene for the MAGE homolog, necdin, leads to a decrease in TrkA signaling [15] and deletion of the same gene leads to Prader-Willi syndrome [26]. Interestingly, necdin appears to bind specifically to p75NTR and TrkA individually and in their heterocomplex [15]. Loss of p75NTR may allow for the increased binding of necdin to TrkA, facilitating, in turn, activation of TrkA by NGF. Accordingly, necdin knockdown decreases this activation. It is tempting to relate the effects of necdin knockdown specifically on p75NTR-deficient cells to the emergence of the Prader-Willi phenotype as the nervous system develops and progressively down-regulates p75NTR [16,27].

The increased TrkA phosphorylation observed in p75NTR-deficient cells was accompanied by increased JNK signaling, in turn leading to caspase 3 cleavage and apoptosis [28,29]. Although TrkA has not previously been shown to increase JNK-mediated apoptosis, p75NTR-dependent JNK signaling in response to cellular stress has been shown to lead to apoptosis [30–32]. In our model, inhibition of JNK activation by SP600125 blocked oxidant stress-induced cell death. This suggests that the JNK activation seen in p75NTR-deficient PC12 cells is causally related to the sensitivity of these cells to 6-OHDA.

The phosphorylation of Bid in p75NTR-deficient PC12 cells would likely result in an increase in Bax activation. Our finding of decreased Bax expression in these cells is therefore perplexing. It is possible that Bax downregulation in p75NTR-deficient PC12 cells is a secondary, compensatory phenomenon.

A complete understanding of the relationship between p75NTR and TrkA is still emerging. Both TrkA and p75NTR have been shown to promote either cell survival or cell death depending on the specific cell type and milieu. There is some evidence to suggest that the relative level of expression of the two receptors is crucial to determination of the downstream

consequences of ligand-receptor binding. The absence of one or the other receptor, resulting in a shift in p75NTR and TrkA equilibrium, may alter, not only the specific signaling pathway triggered by the remaining receptor, but also the effect that triggering of a particular signaling pathway has on the cell. Although the TrkA and p75NTR signaling pathways are independent of one another, both receptors bind extracellular NGF and intracellular necdin. Furthermore, JNK can be activated by either receptor-ligand pair, making it difficult to distinguish additivity from synergy between the two signaling pathways. It is clear, however, that receptor interactors, like necdin, play a critical role in modulating the cellular events that follow activation of successive signaling effectors in a given pathway.

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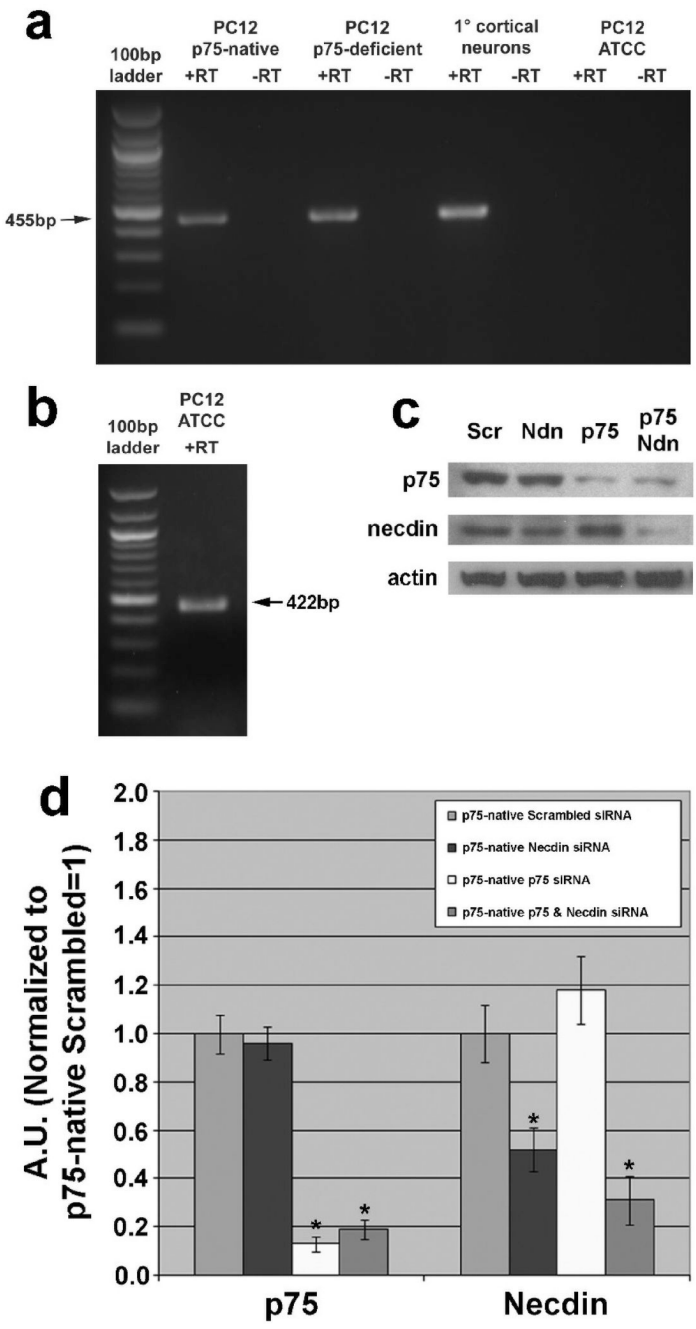

Figure 1. Necdin mRNA and protein are present in PC12 p75NTR-native and p75NTR-deficient cells. siRNA to p75NTR and necdin, respectively, decrease p75NTR and necdin protein content of the cells

a) RT-PCR: A polymerase chain reaction amplifying a 455bp fragment of necdin was carried out with cDNA from 4 independent samples. This reverse transcriptase reaction was performed using mRNA isolated from PC12 p75NTR-native, PC12 p75NTR-deficient, ATCC PC12 and primary cortical neuronal cells as described in the Materials and Methods. The primary cortical neurons were used as a positive control and the ATCC PC12 cells were used as a negative control for the presence of necdin mRNA. The absence of reverse transcriptase (−RT) was used as a control to show there was no genomic DNA contamination in the mRNA samples

prior to making the cDNA. b) A second polymerase chain reaction amplifying a 422bp fragment of p75 was carried out with cDNA from ATCC PC12 cells as a control to show that there is sufficient cDNA present in the sample. c–d) Western blot: Optical density values were determined for each band using Scion Imaging as described in Materials and Methods and normalized to the optical density of the analogous actin band. The number thus obtained for each protein in p75NTR-native PC12 cells was set to 1 and the other values were expressed as a function of the control scrambled siRNA condition. The p75NTR-native PC12 cells treated with scrambled siRNA (Scr), necdin siRNA (Ndn), p75NTR siRNA (p75) or both necdin and p75NTR siRNA (p75 Ndn) are depicted in a representative blot and in the plotted average data. *$P < 0.01$ (Univariate 2-way ANOVA, $n = 3$, westerns on different samples).

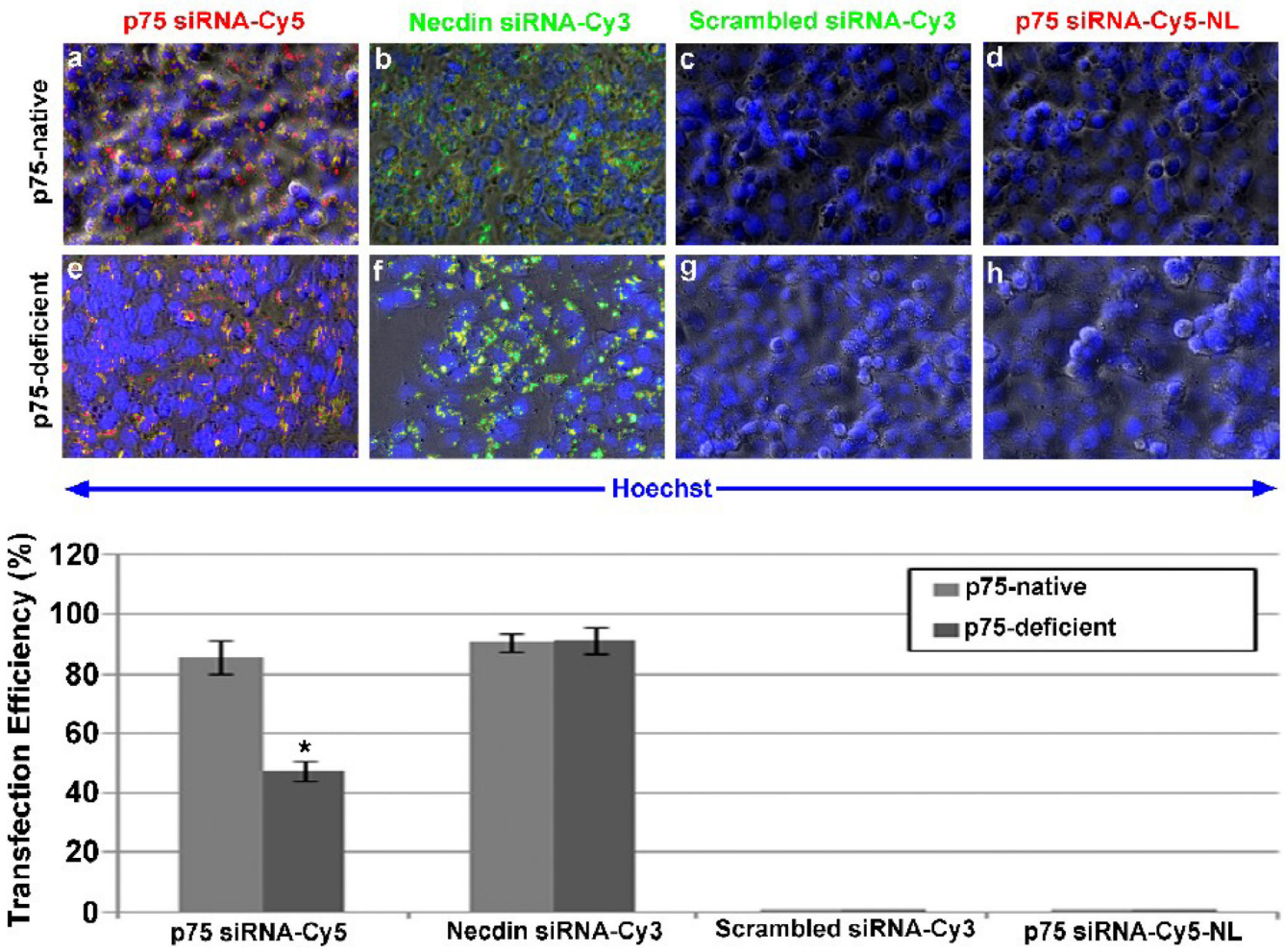

Figure 2. The transfection efficiency of p75 siRNA and necdin siRNA are relatively similar in PC12 p75NTR-native cells

All siRNAs were fluorescently-tagged with either Cy3 (green, necdin & scrambled siRNA) or Cy5 (red, p75 siRNA). A mercury lamp along with Hoescht staining of the nuclei allowed for quantification of the number of p75-native and p75-deficient cells in which the targets were knocked down and the fluorescent tags were released. The scrambled siRNA was used as a negative control as it was not expected to knockdown anything or release its fluorescent tag. The p75 siRNA-Cy5 was used without lipofectamine as an additional negative control to show that the fluorescent signal only correlates with knockdown. The overlap (shown in yellow) was only statistically significant when comparing the p75 knockdown in both p75-native and p75-deficient cells. *P<0.01 (Student's T-test).

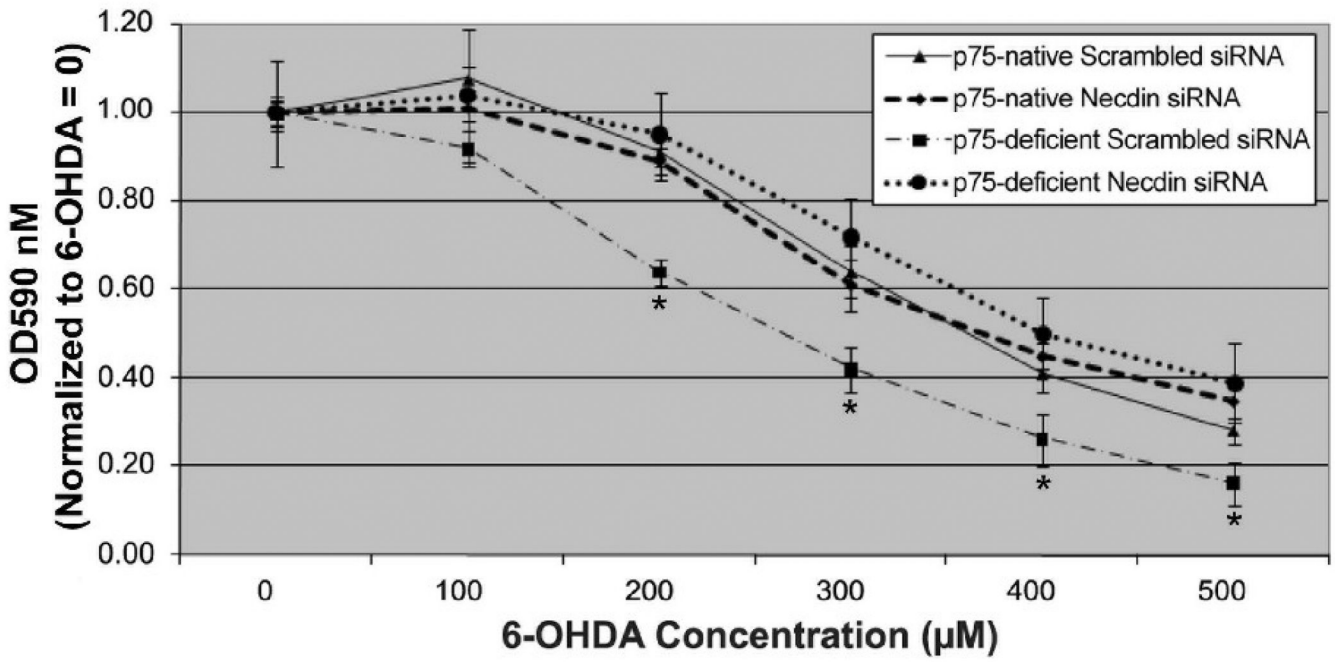

Figure 3. The effect of necdin siRNA on metabolic viability following 6-OHDA treatment of p75NTR-native and p75NTR-deficient PC12 cells

Alamar blue staining was performed as described in Materials and Methods and $OD_{590\ nm}$ values obtained at each concentration of 6-OHDA were normalized to the value obtained in the absence of 6-OHDA for all conditions. Both p75NTR-native and p75NTR-deficient cell lines were treated with necdin siRNA or a scrambled control siRNA along with 6-OHDA. *P < 0.01 (Univariate 2-way ANOVA, n = 16, 4 separate runs with 4 repeats each). The IC_{50} values were: 351 μM ± 28.6 μM (p75NTR-native/scrambled siRNA), 343 μM ± 31.2 μM (p75NTR-native/necdin siRNA), 249 μM ± 14.7 μM (p75NTR-deficient/scrambled siRNA**) and 401 μM ± 48.6 μM (p75NTR-deficient/necdin siRNA). **P<0.01 (Student's T-test).

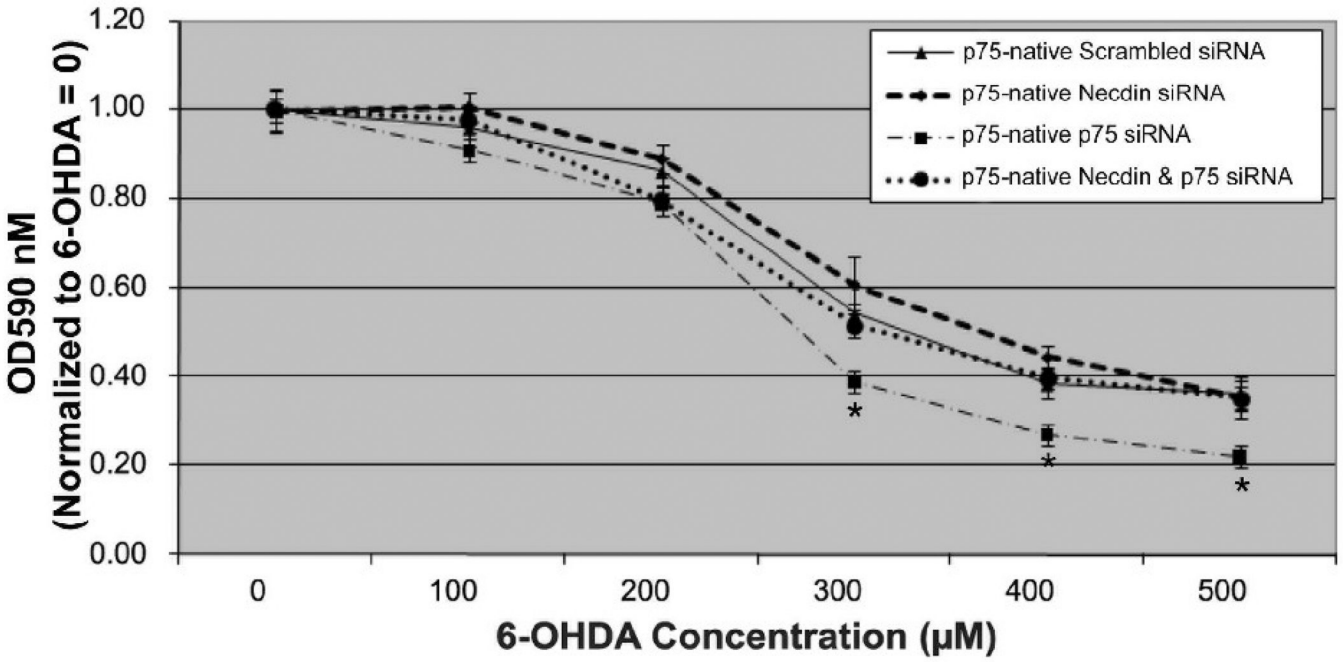

Figure 4. The effect of necdin and p75NTR siRNAs on metabolic viability following 6-OHDA treatment of p75NTR-native PC12 cells

Alamar blue staining was performed as described in Materials and Methods and values obtained at each concentration of 6-OHDA were normalized to the value obtained in the absence of 6-OHDA for all conditions. p75NTR-native cells were treated with necdin siRNA, p75NTR siRNA, or a scrambled control siRNA along with 6-OHDA treatment. *$P < 0.01$ (Univariate 2-way ANOVA, $n = 8$, 2 separate runs with 4 repeats each). The IC50 values (see Experimental) were: 328 μM ± 26.2 μM (p75NTR-native/scrambled siRNA), 349 μM ± 46.6 μM (p75NTR-native/necdin siRNA), **262 μM ± 12.4 μM (p75NTR-native/p75NTR siRNA) and 330 μM ± 18.9 μM (p75NTR-native/necdin and p75NTR siRNA). **$P<0.01$ (Student's T-test).

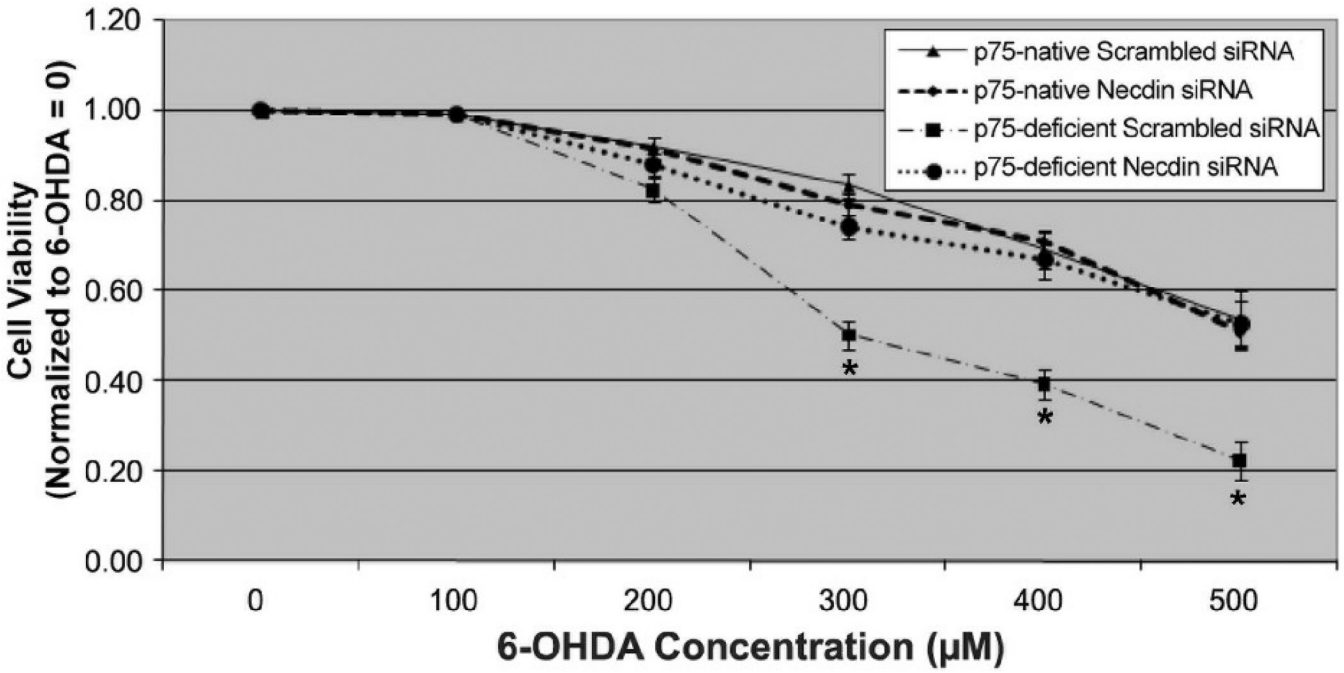

Figure 5. The effect of necdin siRNA on cell membrane integrity following 6-OHDA treatment of p75NTR-native and p75NTR-deficient PC12 cells

Trypan blue exclusion determinations were performed as described in Materials and Methods and values obtained at each concentration of 6-OHDA were normalized to those obtained in the absence of 6-OHDA for all conditions. Both p75NTR-native and p75NTR-deficient cell lines were treated with necdin or a scrambled control siRNA along with 6-OHDA treatment. *P < 0.01 (Univariate 2-way ANOVA, n = 8, 2 separate runs with 4 repeats each). The LD_{50} values (see Experimental) were: 513 μM ± 32.0 μM (p75NTRnative/scrambled siRNA), 499 μM ± 37.5 μM (p75NTR-native/necdin siRNA), 302 μM ± 11.7 μM (p75NTR-deficient/scrambled siRNA**) and 494 μM ± 43.3 μM (p75NTR-deficient/necdin siRNA). **P<0.01 (Student's T-test).

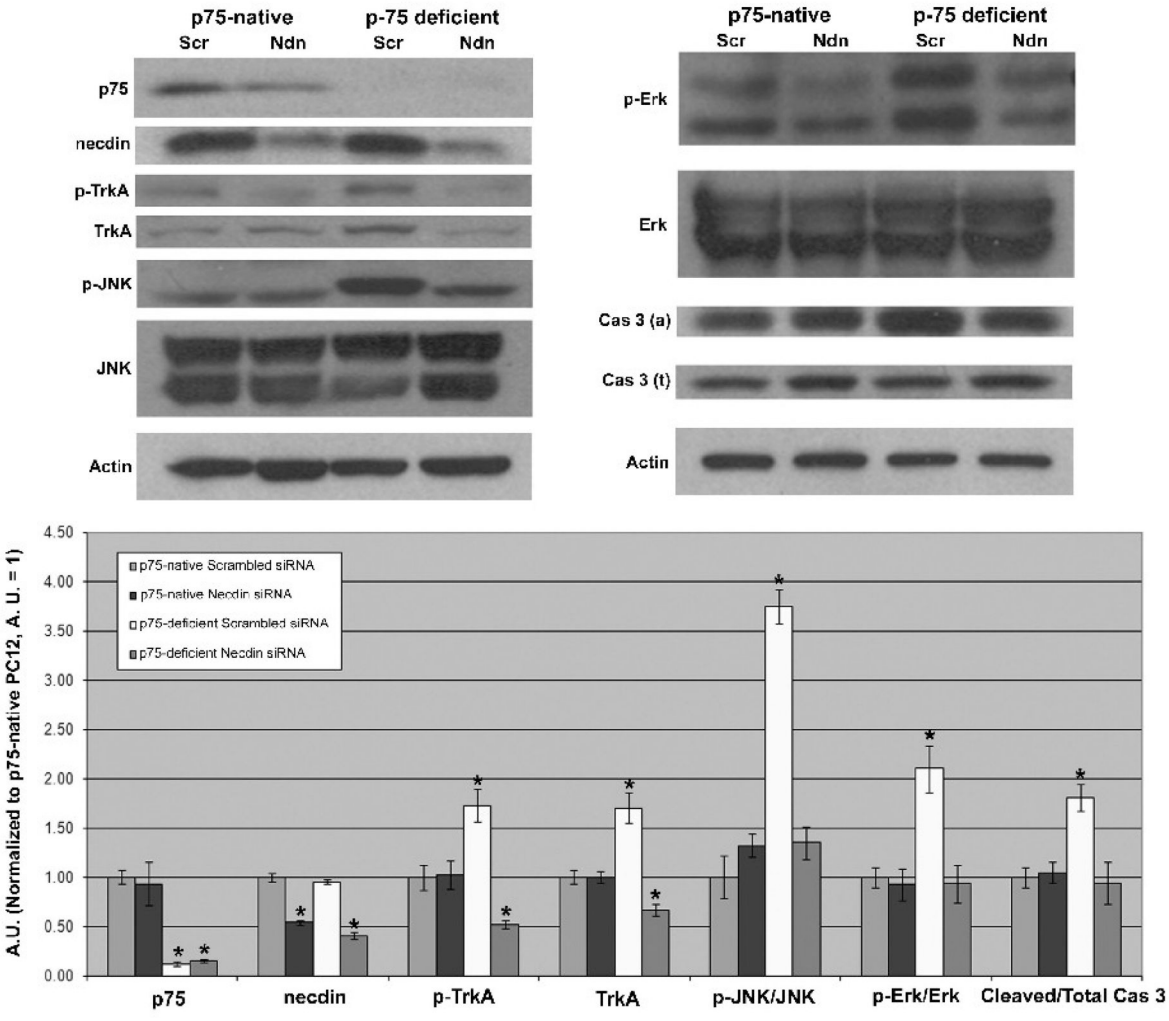

Figure 6. Western blotting and quantification of TrkA signaling effectors in p75NTR-native and p75NTR-deficient cell lines treated with scrambled control siRNA (Scr) or necdin siRNA (Ndn)

Optical density values were determined for each band using Scion Imaging as described in Materials and Methods and normalized to the optical density of the analogous actin band. The number thus obtained for each protein in p75NTR-native PC12 cells was set to 1 and the values for other cells were expressed as a function of those obtained for this control condition. The p75NTR-native PC12 cells treated with scrambled siRNA , with necdin siRNA , p75NTR-deficient cells treated with scrambled siRNA, and necdin siRNA are depicted in a representative blot and in the plotted average data. The pTrkA and TrkA bands are both in the neighborhood of 140kDa (the 110kDa bands did not appear). *P < 0.01 (Univariate 2-way ANOVA, n = 3–5, Western blots on different samples).

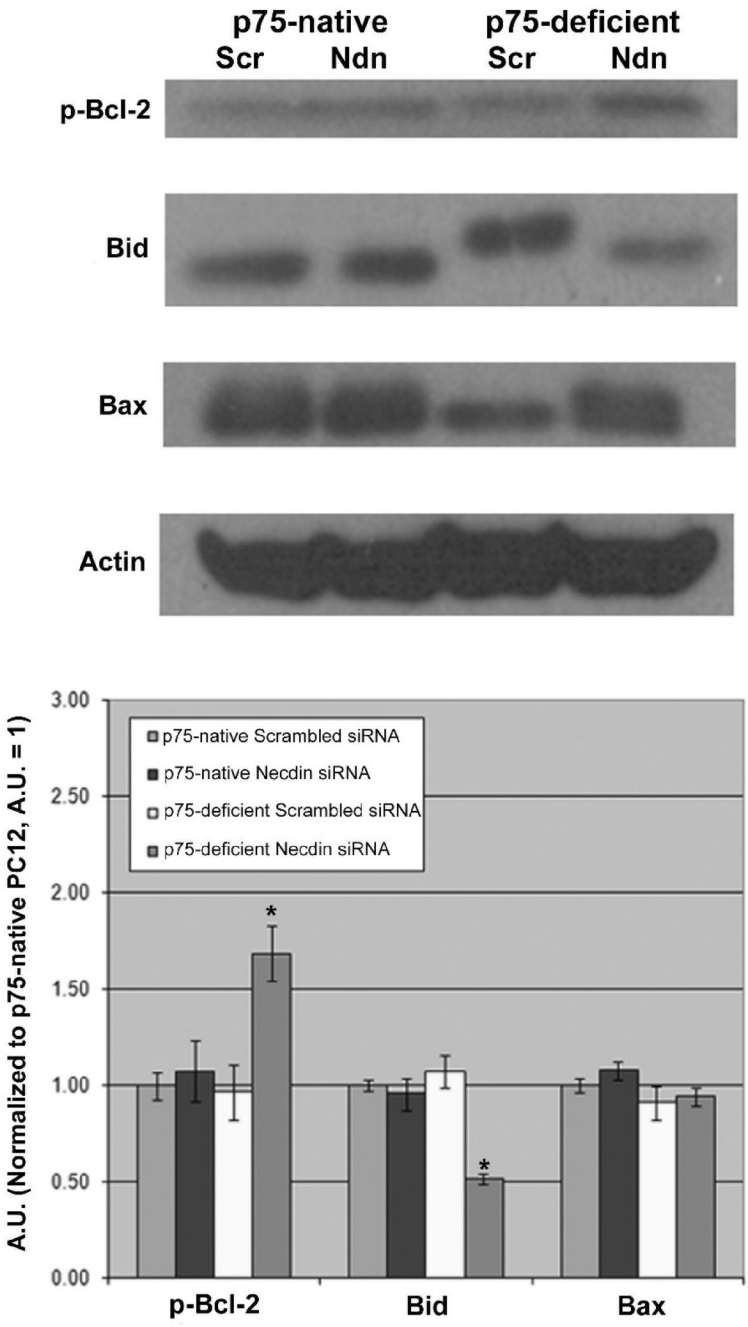

Figure 7. Western blotting and quantification of pro- and anti-apoptotic proteins in p75NTR-native and p75NTR-deficient cell lines treated with scrambled control siRNA (Scr) or necdin siRNA (Ndn)

Optical density values were determined for each band using Scion Imaging as described in Materials and Methods and normalized to the optical density of the analogous actin band. The number thus obtained for each protein in p75NTR-native PC12 cells was set to 1 and the values for other cells were expressed as a function of the values obtained for this control condition. The p75NTR-native PC12 cells treated with scrambled siRNA, with necdin siRNA, p75NTR-deficient cells treated with scrambled siRNA, and necdin siRNA are depicted in a representative blot and in the plotted average data. *P < 0.01 (Univariate 2-way ANOVA, n = 3, Western blots on different samples).

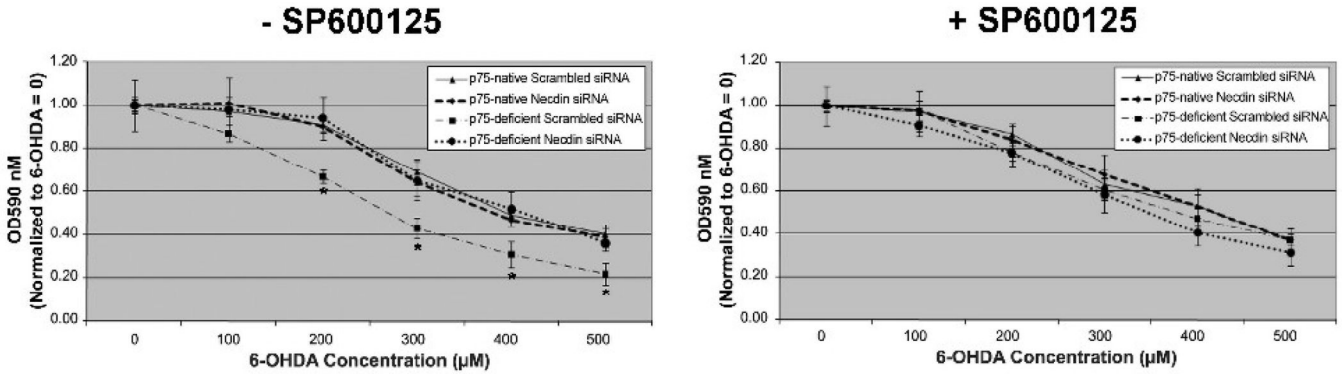

Figure 8. The effect of necdin siRNA and a JNK phosphorylation inhibitor, SP600125, on cell metabolic viability following 6-OHDA treatment of p75NTR-native and p75NTR-deficient PC12 cells

Alamar blue staining was performed as described in Materials and Methods and values obtained at each concentration of 6-OHDA were normalized to the value obtained in the absence of 6-OHDA for all conditions. p75NTR-native and p75NTR-deficient cell lines were treated with necdin siRNA or a scrambled control siRNA with or without SP600125 and 6-OHDA treatment. *P values < 0.01 (Univariate 2-way ANOVA, n = 8, 2 separate runs with 4 repeats each). Without SP600125 treatment the IC_{50} values were: 371 μM ± 22.4 μM (p75NTR-native/scrambled siRNA), 350 μM ± 32.6 μM (p75NTR-native/necdin siRNA), 237 μM ± 16.4 μM (p75NTR-deficient/scrambled siRNA**) and 378 μM ± 36.9 μM (p75NTR-deficient/necdin siRNA). With SP600125 treatment the IC50 values (see Experimental) were: 392 μM ± 37.8 μM (p75NTR-native/scrambled siRNA), 408 μM ± 51.7 μM (p75NTR-native/necdin siRNA), 347 μM ± 41.2 μM (p75NTR-deficient/scrambled siRNA) and 346 μM ± 34.1 μM (p75NTR-deficient/necdin siRNA). **P<0.01 (Student's T-test).

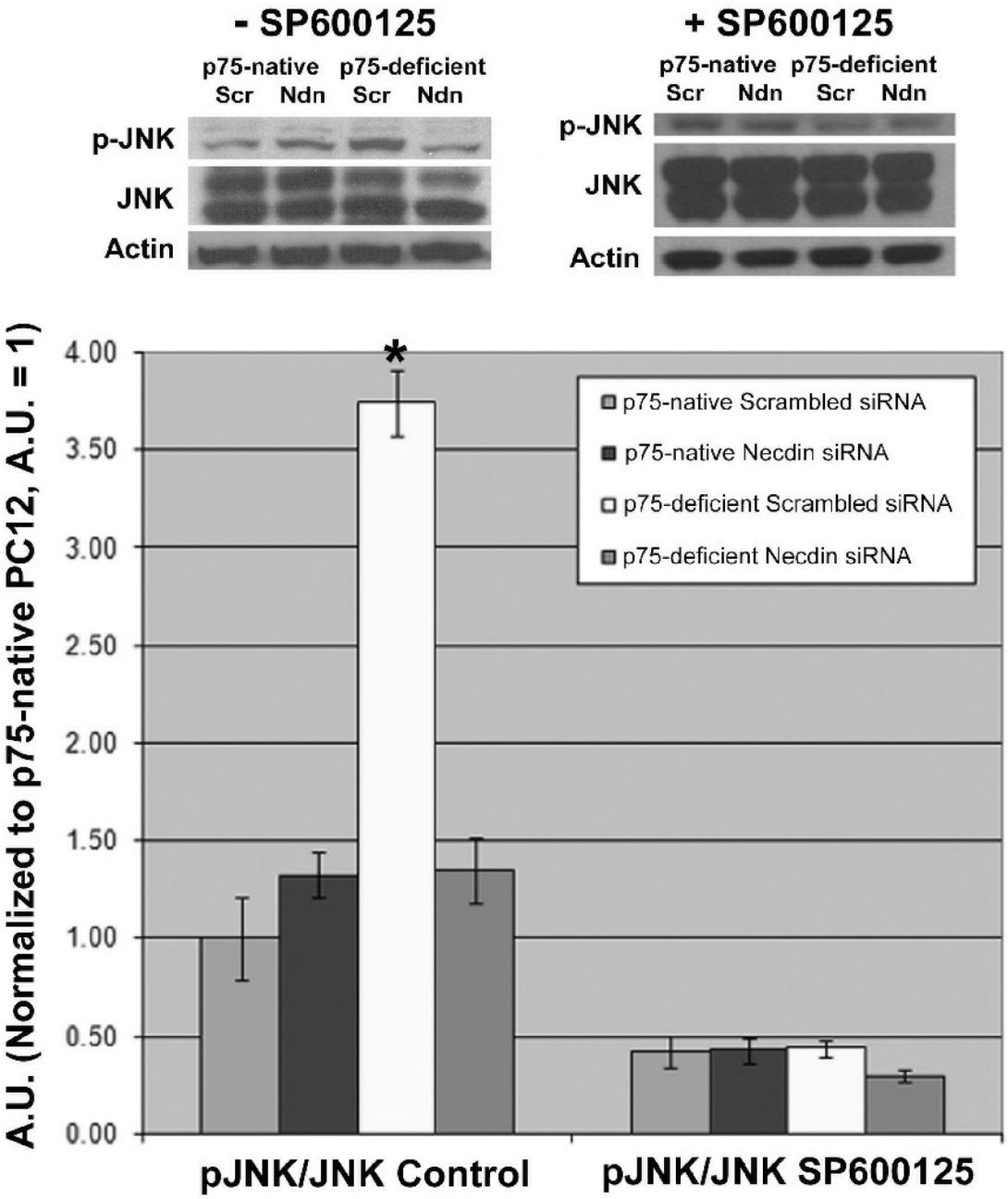

Figure 9. Western blotting and quantification of the p75NTR-native and p75NTR-deficient cell lines treated with scrambled control siRNA (Scr) or necdin siRNA (Ndn) +/− SP600125

Optical density values were determined for each band using Scion Imaging as described in Materials and Methods and normalized to the optical density of the analogous actin band. The number thus obtained for each protein in p75NTR-native PC12 cells was set to 1 and the values for other cells were expressed as a function of the control condition. The p75NTR-native PC12 cells treated with scrambled siRNA, with necdin siRNA, p75NTR-deficient cells treated with scrambled siRNA, and necdin siRNA are depicted in a representative blot and in the plotted average data. *$P < 0.01$ (Univariate 2-way ANOVA, $n = 3$, Western blots on different samples).