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Nerve Growth Factor Receptor TrkA, a New Receptor in Insulin Signaling Pathway in PC12 cells

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Running title**:** *TrkA, a New Receptor in Insulin Signaling*

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Background: TrkA is a transmembrane receptor tyrosine kinase for nerve growth factor.
Result: TrkA forms a molecular complex with insulin receptor and IRS-1 to induce Akt and Erk5 phosphorylation.
Conclusion: NGF-TrkA receptor influences insulin signaling.
Significance: TrkA receptor is involved in insulin signaling and NGF may regulate neuronal glucose uptake as neurons are insulin insensitive.

SUMMARY
TrkA is a cell surface transmembrane receptor tyrosine kinase for nerve growth factor (NGF). TrkA has an NPXY motif and kinase regulatory loop similar to insulin receptor (INSR) suggesting that NGF→TrkA signaling might overlap with insulin→INSR signaling. During insulin or NGF stimulation TrkA, insulin receptor substrate-1 (IRS-1), INSR (and presumably other proteins) forms a complex in PC12 cells. In PC12 cells, tyrosine phosphorylation of INSR and IRS-1 is dependent upon the functional TrkA kinase domain. Moreover, expression of TrkA kinase–inactive mutant blocked the activation of Akt and Erk5 in response to insulin or NGF. Based on these data, we propose that TrkA participates in insulin signaling pathway in PC12 cells.

Nerve growth factor (NGF) regulates survival and differentiation of neurons in the central and peripheral nervous systems (*1*, *2*). NGF is structurally related to brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (*3*). Neurotrophins can bind to high affinity receptor tyrosine kinase Trk and a low affinity $p75^{NTR}$ receptor. Trk receptor consists of three family members, including TrkA, TrkB and TrkC. TrkA binds specifically to NGF, TrkB to BDNF and TrkC to NT-3 (*4*).

NGF causes TrkA dimerization and autophosphorylation (*5*), which recruits downstream signaling proteins including phospholipase C (PLC)-γ1 (*6*), Shc, FRS2 (*7*) and phosphatidylinositol 3-kinase (PI-3K) (*8*). TrkA is also polyubiquitinated, which mediates its internalization into the signaling vesicles (*9-11*), where it activates the mitogen-activated protein kinase (ERK/MAPK) that promotes cell differentiation (*12*). TrkA is deubiquitinated by the proteasome prior to its trafficking to lysosomes for degradation (*13*).

Insulin plays a crucial role in brain functions (*14*) such as memory improvement (*15, 16*) and energy metabolism (*17, 18*). Insulin binds to the insulin receptor (INSR) on the cell surface, leading to its autophosphorylation (*19*). The activated IR then binds and phosphorylates the intracellular substrates such as the insulin receptor substrate (IRS) family proteins. Tyrosine phosphorylated IRS-1 interacts with SH_2 domain of various signaling proteins, including the 85 kDa regulatory subunit of the PI 3-kinase (p85•p110) that stimulates the production of PIP3P. PIP3P recruits phosphoinositide dependent kinase 1 (PDK1) and protein kinase B (Akt) to the membrane where PDK1 phosphorylates and activates AKT (*20*). Akt activation leads to increase in glucose uptake. In addition to PI 3-kinase, IRS-1 also interacts with sequestosome 1/p62, a scaffolding protein that is involved in the activation of Akt, Glut4 translocation and glucose uptake (*21*). IRS-1 also interacts with growth factor receptor binding protein 2 (Grb-2), leading to MAPK activation, which mediates cell survival and mitogenesis (*22, 23*).

Neurons are classified as insulin insensitive, as insulin is incapable of increasing glucose uptake in neurons (*24*), compared to muscle and fat cells (*25*). In this study, we report that NGF or insulin induces TrkA to form a molecular complex with INSR and IRS-1. The tyrosine phosphorylation of the INSR and IRS-1 requires functional TrkA kinase in PC12 cells. In addition, TrkA influences insulin signaling through the activation of Akt and Erk5, which reveals a novel overlapping signaling mechanism between NGF → TrkA and insulin → INSR.

EXPERIMENTAL PROCEDURES

Antibodies, Reagents and Constructs - Anti-TrkA (C-14), TrkA (E-6) that recognizes the phosphorylation at tyrosine 496, pIRS-1 (Tyr 632) and anti-INSR were obtained from Santa Cruz Biotechnology, Santa Cruz, CA; Anti-IRS-1, ant-HA was purchased from Millipore, Temecula, CA. Antibodies against pAkt (S473), pINSR (Tyr1146), pINSR (Tyr1150/1151), total Akt antibody, Erk5 were purchased from Cell signaling Technology (Danvers, MA). Anti-phosphotyrosine (PY20) was from BD Transduction Laboratories. The insulin receptor SiRNA was purchased from Santa Cruz Biotechnology, Santa Cruz, CA and TrkA SiRNA from OriGene Technologies, Inc (Rockville, MD). K252a was purchased from Biomol Research Laboratories Inc (Pennsylvania, PA). NGF (2.5S) was from Bioproducts for Science (Indianapolis, IN). Anti-rabbit IgG and anti-mouse IgG-HRP linked secondary antibody were from GE Healthcare UK Ltd., and enhanced chemiluminescence (ECL) was from Thermo Scientific, IL. Protein A sepharose beads, insulin and all other reagents were obtained from Sigma (St Louis, MO). The HA-tagged rat wild-type TrkA and K547A inactive kinase receptor constructs were generated as previously described (7).

Cell Culture - PC12 rat pheochromocytoma cells were maintained in Dulbecco's modified Eagle media (DMEM) with 10% heat-inactivated horse serum, 5% fetal bovine serum and antibiotics (100 units/ml streptomycin and penicillin). Parental L6 cells were maintained in DMEM supplemented with 10% fetal bovine serum and penicillin/streptomycin. Cells were incubated in a humidified atmosphere containing 5% CO_2 and 95% air at 37ºC. Cells were transfected with using the cationic lipid method by using Lipofectamine™ 2000 transfection reagent (Invitrogen, Carlsbad, CA). The cells were deprived of serum in culture medium overnight at 37ºC before cell lysis.

Immunoprecipitation and Western Blotting Analysis - Cells were stimulated with insulin

(100 nM) or NGF (100 ng/ml) according to the experimental design. The cells were lysed with Triton lysis buffer (50 mM HEPES [pH 7.6], 150 mM NaCl, 20 mM sodium pyrophosphate, 10 mM NaF, 20 mM beta-glycerophosphate, 1% Triton, 1 mM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, and 10 μg/ml leupeptin and aprotinin). Protein was estimated by the Bradford procedure (Bio-Rad) using bovine serum albumin (Sigma, St Louis, MO) as standard. Cell lysates (1 mg) were diluted in lysis buffer and incubated with 4 μg of primary antibody. The immunoprecipitates were collected with protein A Sepharose beads (Sigma, St. Louis, MO) overnight at 4°C and then washed three times with phosphate-buffered saline (PBS). Samples were boiled in sodium dodecyl-sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and resolved on 10% SDS-PAGE, transferred onto polyvinylidene fluoride (PVDF) membranes (Millipore, Temecula, CA), and analyzed by western blotting with the appropriate antibodies such that the immune complex was detected by enhanced chemiluminescence.

Neurite Outgrowth - PC12 cells were grown on collagen and polylysine-coated 24-well plate, treated with NGF or insulin, and 200 cells were scored for the presence or absence of neuritis (*11*).

Glucose Uptake Assay - The glucose uptake in PC12 cells was measured by the method described previously (26, 27).

RESULTS AND DISCUSSION

TrkA receptor possess NPQY and YSTDYY motifs similar to INSR - Amino acid sequence alignment showed that TrkA receptors have NPQY motif similar to NPEY in insulin receptors (INSR) (solid box in Fig.1). NPEY motif in the juxtamembrane domain of INSR is an IRS-1 binding site (*28-30*). This motif is also involved in binding several adaptor proteins such as Shc and Frs2 through their phosphotyrosine binding (PTB) domains (30, *31*). In addition, TrkA receptor has YSTDYY similar to YETDYY in INSR (dashed line box in Fig.1). The triple tyrosine residues in INSR are essential to interact with IRS-2 (*32*). Autophosphorylation of the triple tyrosine amino acids enhances the catalytic activity of the receptor tyrosine kinases (*33*).

TrkA receptor and IRS-1 form a molecular complex - As TrkA receptors have NPQY motifs similar to NPEY in INSR, we sought to investigate whether TrkA can associate with IRS-1. PC12 cells were stimulated with either insulin (100 nM) for 10 min or NGF (100 ng/ml) for 1, 10 and 15 min. TrkA or IRS-1 was immunoprecipitated and western blotted with anti-IRS-1 and anti-TrkA. Insulin and NGF stimulation (10 and 15 min) induced the interaction between TrkA and IRS-1 (Fig. 2A). The same lysates were also used to check whether TrkA is tyrosine phosphorylated by insulin and NGF stimulation. The receptor was immunoprecipitated and blotted with TrkA (E-6) antibody that recognizes the phosphorylation of tyrosine residue at 496 of TrkA. Stimulation of PC12 cells with insulin or NGF induced tyrosine phosphorylation of TrkA receptor (Fig. 2B). The lysates were also western blotted with TrkA and IRS-1 antibody to check their expression level (Fig. 2C). These results suggest that stimulation of PC12 cells with insulin or NGF leads to the association of TrkA receptor with IRS-1 and tyrosine phosphorylation of TrkA. These results are in parallel to previous findings suggesting that IRS-1 and IRS-2 could be substrates of TRK-T1 and TrkA (*34*).

TrkA receptor interacts and phosphorylates INSR - Because insulin stimulation induced the phosphorylation of TrkA receptor (Fig. 2B), TrkA receptor may interact with INSR. To investigate this possibility, we performed coimmunoprecipitation experiments in cells treated with either NGF, insulin or both for 10 and 15 min. INSR or TrkA was immunoprecipitated and western blotted with TrkA or INSR antibody. Stimulation with NGF, insulin or both induced the association of TrkA with INSR (Fig. 3A). However the interaction might be indirect through other unknown proteins. In order to avoid artifacts due to the antibody, the expression of TrkA or INSR was reduced by using corresponding SiRNA and the interaction of TrkA with INSR was determined. When TrkA or INSR was knocked-down the

interaction was lost as shown in Figure 3B and 3C. Since NGF induced the interaction, we aimed to determine whether NGF would lead to tyrosine phosphorylation of INSR. PC12 cell lysates treated with either NGF, insulin or both were immunoprecipitated with INSR and immunoblotted for phospho INSR antibody. Interestingly, NGF phosphorylated the INSR at tyrosine 1146 and tyrosine 1150/1151 in the kinase activation loop similar to insulin (Fig. 3D). The lysates were also checked for phospho INSR, total INSR and TrkA expression (Fig. 3E). These results indicate that NGF induced the interaction of TrkA with INSR and tyrosine phosphorylation of INSR. As both NGF and insulin can induce the phosphorylation of TrkA (Fig. 2B) and INSR (Fig. 3E), we want to confirm whether NGF and insulin action is only through their respective receptors. We knocked down TrkA or INSR expression by SiRNA and stimulated it either with insulin or NGF as shown in Figure 3F and 3G. When INSR expression was depleted insulin failed to activate TrkA and similarly NGF did not activate INSR in absence of TrkA.

NGF is known to induce differentiation of PC12, here we want to determine whether insulin can mediate the differentiation as well. PC12 cells were treated with either NGF, insulin or both for 3 days. The majority of cells treated only with NGF and along with insulin developed a network of neurites. Insulin treated cells failed to develop neurites compared to NGF. The cells were scored for neurite outgrowth, and the percentage of cells with neurites was determined (Fig. 3H).

Functional TrkA kinase is required for the activation of INSR and IRS-1 - Since NGF induced the tyrosine phosphorylation of INSR, we sought to determine whether TrkA kinase domain is essential for the activation of INSR. Therefore, PC12 cells were transfected with HA tag wild-type TrkA or kinase-inactive (KD) form of TrkA and stimulated with insulin, NGF or both and phosphorylation of INSR was determined by western blotting. INSR was phosphorylated at tyrosine 1146 and 1150/1151 on insulin, NGF or both in presence of wild-type TrkA, but the phosphorylation was much reduced in the presence of kinase-inactive (KD) TrkA (Fig. 4A). The expression of total INSR and the expression of TrkA constructs were also verified (Fig. 4A). TrkA interacts with IRS-1 as well, so we also explored whether the IRS-1 activation is dependent upon the TrkA kinase domain. IRS-1 was tyrosine phosphorylated in the presence of insulin and NGF in presence of wild-type TrkA, whereas over-expression of kinase-inactive (KD) TrkA decreased the activation of IRS-1 (Fig. 4B). Thus, the tyrosine phosphorylation of INSR and IRS-1 is dependent upon the kinase domain of the TrkA receptor. Similar results were also obtained in L6 rat muscle cells (Fig. 4C and 4D).

INSR and IRS-1 interacts with TrkA receptor in a phosphotyrosine-dependent manner - Because the TrkA kinase domain is essential for the tyrosine phosphorylation of INSR and IRS-1, we sought to determine whether the association of the TrkA receptor with INSR and IRS-1 is dependent upon the activation of TrkA. PC12 cells were co-transfected with HA tag wild-type TrkA or kinase-inactive (KD) TrkA. The cells were treated with insulin (100 nM) or NGF (100 ng/ml) for 10 min, followed by immunoprecipitation of HA and western blotted with INSR, IRS-1, pTrkA (Tyr 496) and HA antibodies. We observed that wild-type TrkA was only tyrosine phosphorylated and interacted with INSR and IRS-1 upon insulin or NGF stimulation, whereas the kinase inactive form of TrkA was not phosphorylated and did not interact with INSR and IRS-1 (Fig. 5A). In parallel, PC12 cells were pretreated with K252a (100 nM), the kinase inhibitor which impairs the tyrosine phosphorylation of TrkA receptor (*35, 36*) for 1 h prior to the stimulation with insulin or NGF for 10 min. We performed immunoprecipitation with anti-TrkA followed by western blotting with INSR, IRS-1, pTrkA (Tyr 496) or total TrkA antibody. K252a attenuated the interaction of TrkA with INSR and IRS-1 as well as the activation of TrkA receptor induced by insulin or NGF (Fig. 5B). Together, these data indicate that the interaction of TrkA receptor with INSR and IRS-1 is dependent upon the phosphorylation of TrkA.

TrkA influences the insulin signaling - Insulin signaling plays a role in the activation of two distinct signaling cascades, such as phosphoinositol 3-kinase (PI 3-kinase)/Akt (protein kinase B) and mitogen activated protein kinase (MAPK) pathway. To examine the effects of TrkA on insulin signaling, we determined the activity of Akt, and Erk5 as downstream targets. PC12 cells were stimulated with insulin for 10 min or NGF for 1, 10 or 15 min. Equivalent amount of cell lysates were western blotted with phospho Akt (S473 or T308), stripped and reprobed with total Akt antibody. Both insulin and NGF induced the activation of Akt (Fig. 6A). Since the functional TrkA kinase domain is essential to interact with INSR and IRS-1, we sought to explore whether it influences the Akt activation as well. HA-wild-type TrkA or kinase-inactive (KD) TrkA were transfected, treated with insulin or NGF and the phosphorylation of Akt was determined by western blot. Overexpression of kinase-inactive TrkA caused a marked reduction in the phosphorylation of Akt on insulin or NGF stimulation compared to the wild-type TrkA (Fig. 6B). We next set out to examine the effect of TrkA on the activation of Erk5. PC12 cells were stimulated with NGF, insulin or both and the activation of Erk5 was determined. Erk5 was immunoprecipitated and western blotted with PY20 antibody that recognizes the tyrosine phosphorylation. Erk5 was tyrosine phosphorylated upon insulin and/or NGF treatment (Fig. 7A). Tyrosine phosphorylation of TrkA was abrogated by treating the cells with inhibitor K252a prior to the addition of insulin or NGF and Erk5 activation was analyzed. K252a severely blocked the NGF and insulin-induced activation of Erk5 (Fig. 7B). These results indicate that TrkA kinase activity is required for Akt and Erk5 activation. We also examined the effect of insulin, NGF or both on glucose uptake. The cells were incubated with 2-deoxy-D-[3H]glucose, and the uptake of glucose was measured. Insulin alone did not show significant change in glucose uptake compared to control. In contrast, NGF alone or along with insulin significantly elevated the glucose uptake (Fig. 7C). Altogether, these findings reveal that TrkA participates in insulin receptor signaling pathway.

Our studies revealed a novel function for the TrkA tyrosine kinase in regulating insulin signaling. We provide evidence that TrkA, INSR and IRS1 (and presumably other proteins) form a complex that permits cross-phosphorylation. The activated NPEY motif in the juxtamembrane domain of INSR interacts with phosphotyrosine binding domain of IRS-1 (*28, 29*). The juxtamembrane domain of TrkA receptor also has NPQY motif similar to INSR. NGF stimulation is capable of phosphorylating the tyrosine residues at kinase regulatory loop, YETDYY in INSR and tyrosine 632 of IRS-1, which is essential for binding and activating p85 subunit of PI-3K similar to insulin. Insulin binding to the INSR is known to activate PI-3K, Akt (*20*) and MAPK (*23*) downstream signaling cascades. The Akt pathway mediates 'metabolic' effect (*20*) and MAPK pathway mediates 'mitogenic' responses of insulin (*22*). Our results suggest that activation of Akt and Erk5 by insulin or NGF is dependent upon the phosphorylation of TrkA. We propose that TrkA participates in insulin signaling pathway to regulate cell survival and mitogenesis.

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FOOTNOTES

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The abbreviations used are: NGF, nerve growth factor; INSR, insulin receptor; IRS-1, insulin receptor substrate-1; TrkA, tropomyosin-receptor-kinase A; Akt, protein kinase B; MAPK, mitogen activated protein kinase.

FIGURE LEGENDS

FIGURE 1. **Amino acid sequence similarities between human insulin receptor (INSR) and NGF receptor TrkA.** The solid box represents the NPXY motif and the dashed line box represents the triple Tyr (Y) aminoacids in INSR and TrkA receptor.

FIGURE 2. **TrkA receptor interacts with IRS-1.** PC12 cells were either stimulated with insulin (100 nM) for 10 min or with NGF (100 ng/ml) for 1, 10 or 15 min. *A*, The cells were lysed and immunoprecipitated (IP) with anti-TrkA or anti-IRS-1 and western blotted with anti-IRS-1 or anti-TrkA. *B*, The lysates were immunoprecipitated with TrkA antibody and western blotted for TrkA (E-6) antibody

that recognizes the phospho-tyrosine 496 of TrkA. *C,* The lysates were western blotted with anti-IRS-1 or anti-TrkA. Experiments were replicated three times with similar results.

FIGURE 3. **INSR interacts with TrkA receptor.** *A,* PC12 cells were either stimulated with NGF (100 ng/ml), insulin (100 nM) or both for 10 min and 15 min. The cell lysates were immunoprecipitated (IP) with anti-INSR or anti-TrkA and western blotted with anti-TrkA or anti-INSR. *B,* PC12 cells were transfected with control or TrkA SiRNA and stimulated with either NGF, insulin or both. The cells were lysed and immunoprecipitated (IP) with anti-INSR and western blotted with anti-TrkA or anti-INSR. *C,* Cells were transfected with control or INSR SiRNA and stimulated as above. TrkA was immunoprecipitated and immunoblotted with TrkA or INSR antibody. *D,* PC12 cells treated with NGF, insulin or both were immunoprecipitated with INSR and blotted for phospho INSR antibody that recognizes the Tyrosine 1146 or 1150/1151 of INSR. *E,* The lysates were blotted for phospho INSR (Tyr 1146) or (Tyr 1150 or 1151), total INSR and TrkA. *F,* PC12 cells were transfected with control or INSR SiRNA and stimulated with or without insulin for 15 min. TrkA was immunoprecipitated and western blotted for anti-TrkA (E-6) that recognizes the phospho-tyrosine 496 of TrkA. *G,* Cells were transfected with control or TrkA SiRNA and treated with NGF for 15 min. The cell lysates were immunoprecipitated (IP) with anti-INSR and blotted for phospho INSR (Tyr1146) antibody. *H,* The PC12 cells were treated either with NGF (50 ng/ml) or insulin (100 nM) followed by assessment of neurite outgrowth three days post addition of NGF or insulin. The cells were counted and the percentage of cells with neurites was determined (Values are expresses as mean ± S.D.). Experiments were replicated three times with similar results.

FIGURE 4. **Activation of INSR and IRS-1 requires functional TrkA kinase.** *A,* PC12 cells were transfected with HA-wild-type TrkA or kinase-inactive TrkA (KD) followed by stimulation of insulin (100 nM) or NGF (100 ng/ml) or both for 10 min. The lysates were western blotted with phospho INSR antibody that recognizes the Tyrosine 1146 or 1150/1151 of INSR and total INSR. The expression of wild-type TrkA or kinase inactive (KD) was verified by blotting with HA tag. *B,* The phosphorylation of IRS-1 was determined by blotting the PC12 cell lysates with phospho IRS-1 antibody that recognizes the tyrosine 632 and total non-phospho IRS-1. *C,* L6 cells were cotransfected with HA-wild-type or kinase-inactive TrkA (KD) and treated with insulin or NGF or both as above. The lysates were western blotted with phospho INSR (Tyr1146 and Tyr1150/1151) antibody and total INSR. *D,* L6 cell lysates were blotted with phospho and non-phospho IRS-1 antibody. Experiments were replicated three times with similar results.

FIGURE 5. **Kinase activity of TrkA is required for the interaction with INSR and IRS-1.** *A,* PC12 cells were transfected with HA-wild-type TrkA or kinase-inactive TrkA followed by insulin (100 nM) or NGF (100 ng/ml) stimulation for 10 min. The lysates were immunoprecipitated with anti-HA followed by western blotting with INSR-β, IRS-1, pTrkA, or HA tag antibody. The lysates were western blotted with INSR-β, IRS-1 and HA antibody to verify the protein expression levels. *B,* PC12 cells were pretreated with K252a (100 nM) for 1 h prior to stimulation with insulin or NGF for 10 min. The cell lysates were immunoprecipitated with anti-TrkA and western blotted with INSR-β, IRS-1, pTrkA and TrkA antibody. Cell lysates was analyzed by blotting with INSR-β, IRS-1 or TrkA antibody. Experiments were replicated three times with similar results.

FIGURE 6. **Functional TrkA kinase is required for Akt activation.** *A,* PC12 cells were either stimulated with insulin (100 nM) for 10 min or with NGF (100 ng/ml) for 1, 10 or 15 min. Equivalent cell lysates were western blotted with phospho (S473 or T308) and non-phospho Akt antibody. *B,* Cells were cotransfected with HA-wild-type TrkA or kinase-inactive TrkA and treated with insulin (100 nM) or NGF (100 ng/ml) for 10 min. The lysates were western blotted with phospho and non-phospho Akt antibody. Experiments were replicated three times with similar results.

FIGURE 7. **Erk5 activation and glucose uptake in PC12 cells.** *A,* PC12 cells were stimulated with NGF (100 ng/ml), insulin (100 nM) or both for 10 min and 15 min. The activation of Erk5 was determined by immunoprecipitating the lysates with Erk5 and western blotting with PY20 antibody that recognizes the tyrosine phosphorylation and Erk5. *B,* Cells were pretreated with K252a (100 nM) for 1 h prior to stimulation with insulin or NGF for 10 min. Erk5 was immunoprecipitated in the lysates and western blotting with PY20 or Erk5 antibody. *C,* PC12 cells were stimulated with NGF (100 ng/ml), insulin (100 nM) or both for 20 min, and the rates of 2-deoxy-D-[3H]glucose uptake were determined. Each bar in the graph indicates the percentage change relative to the control cells. Differences from the control value treated with NGF, both NGF and insulin are statistically significant (*, $p< 0.001$). Error bars indicate S.D. Experiments were replicated three times with similar results.

Fig. 1

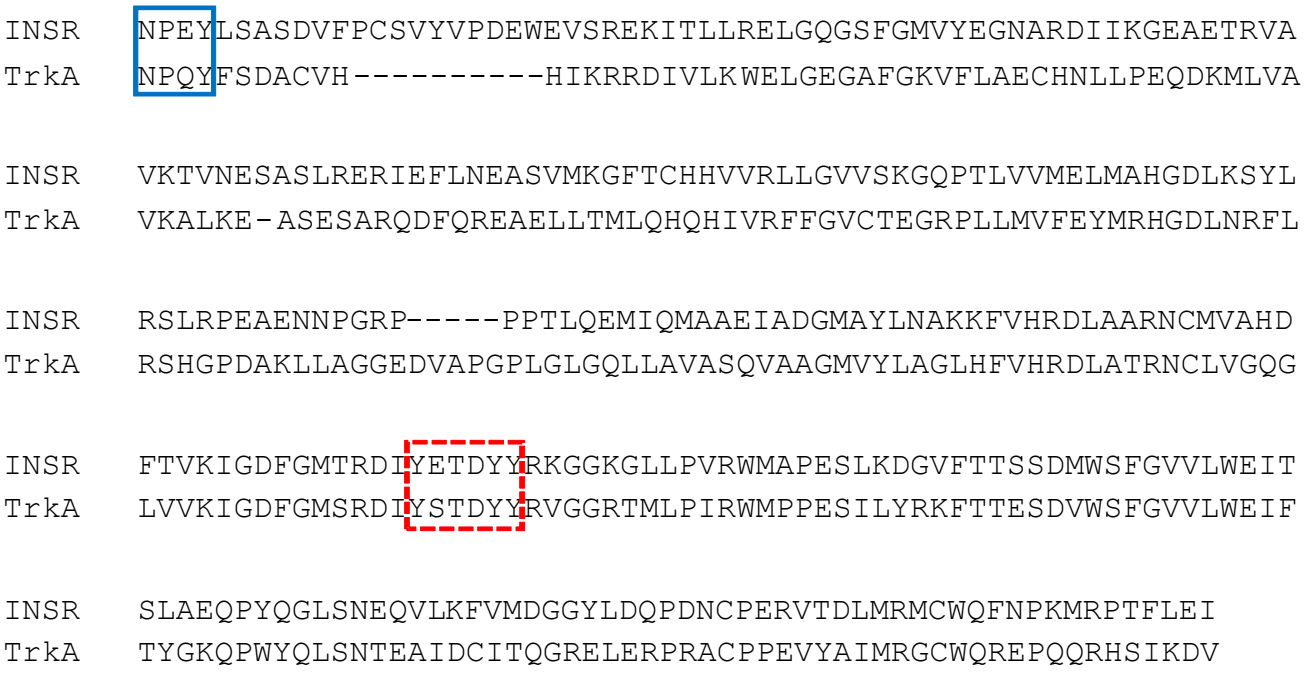
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Fig. 2

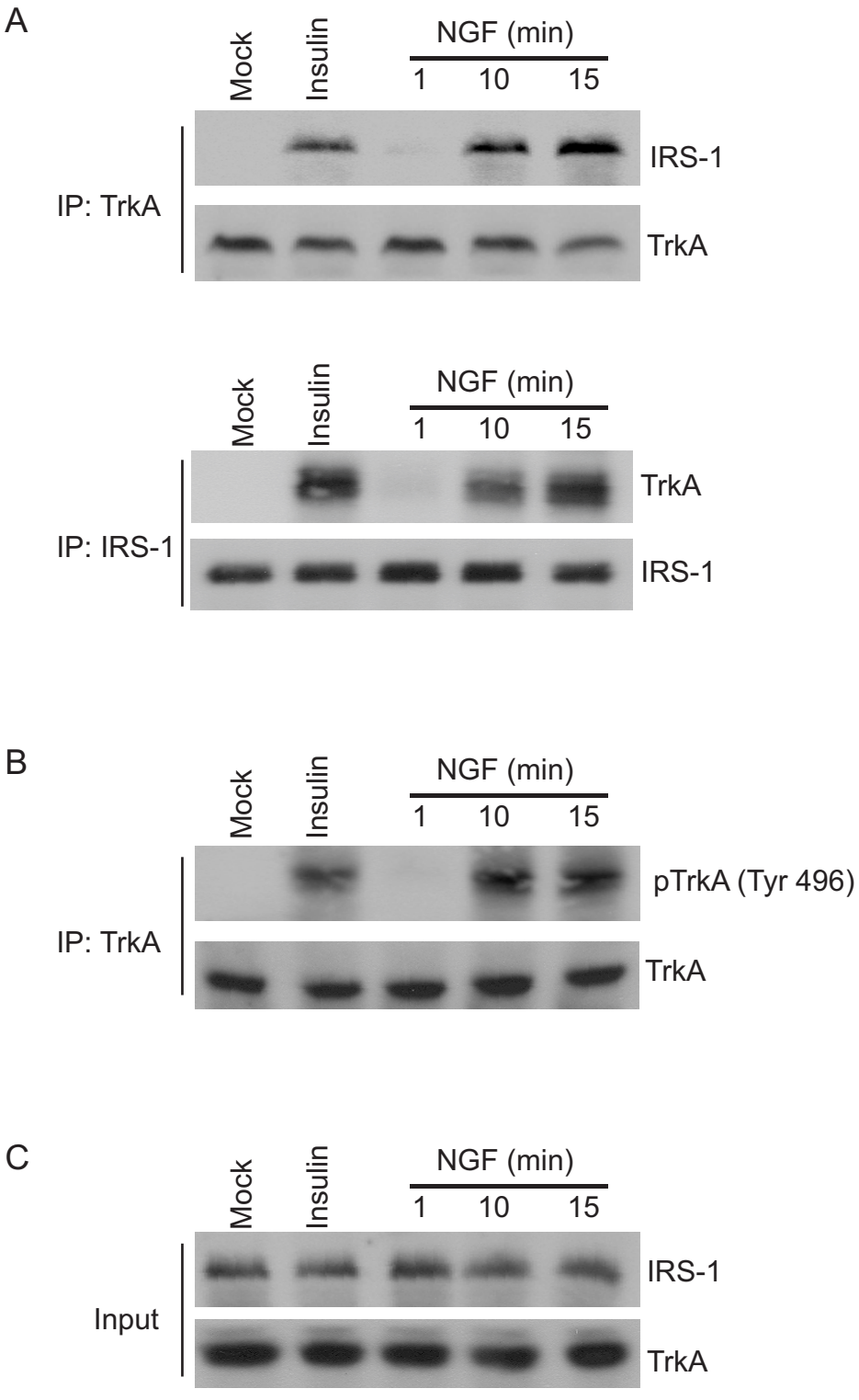


Fig. 3

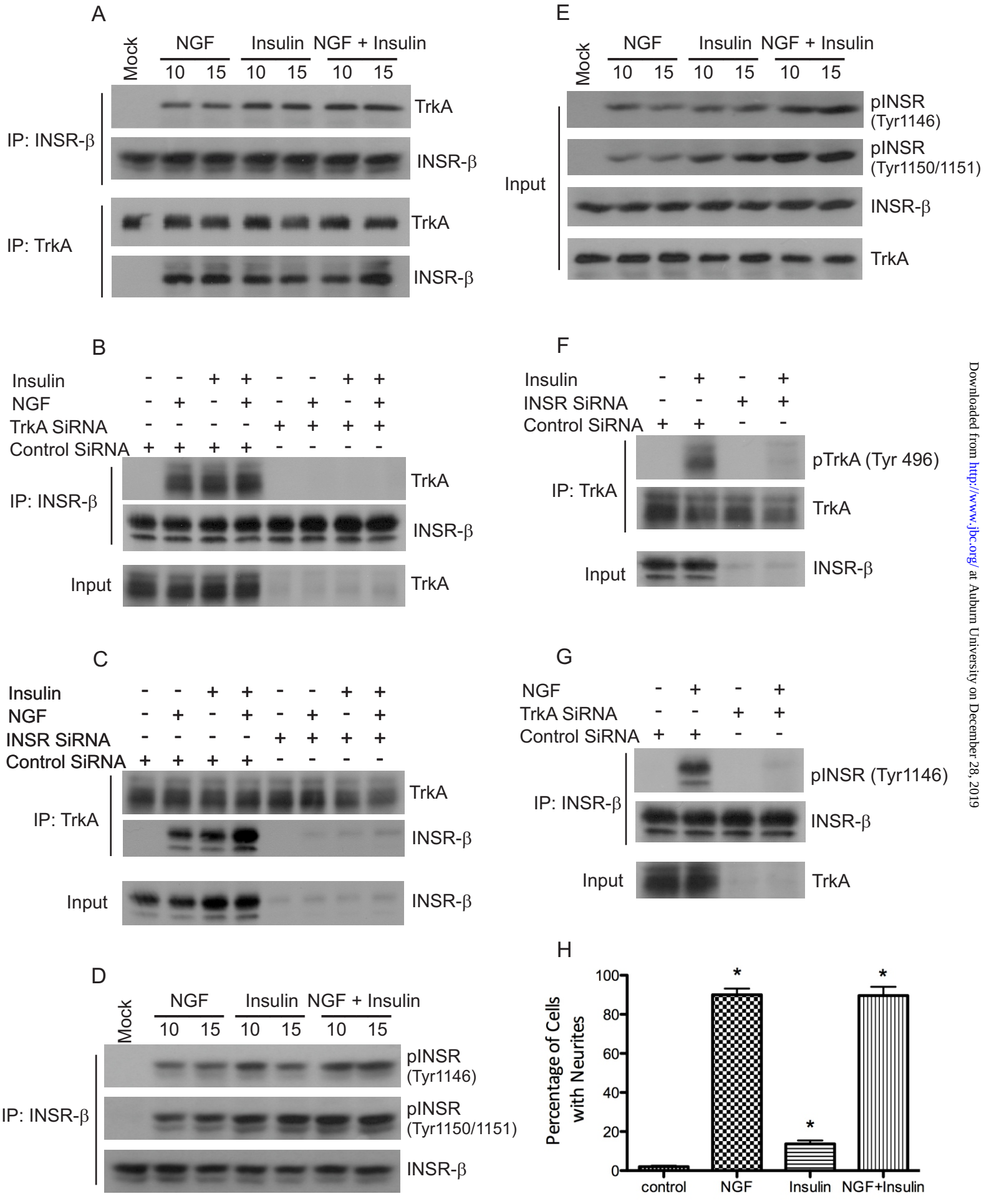


Fig. 4

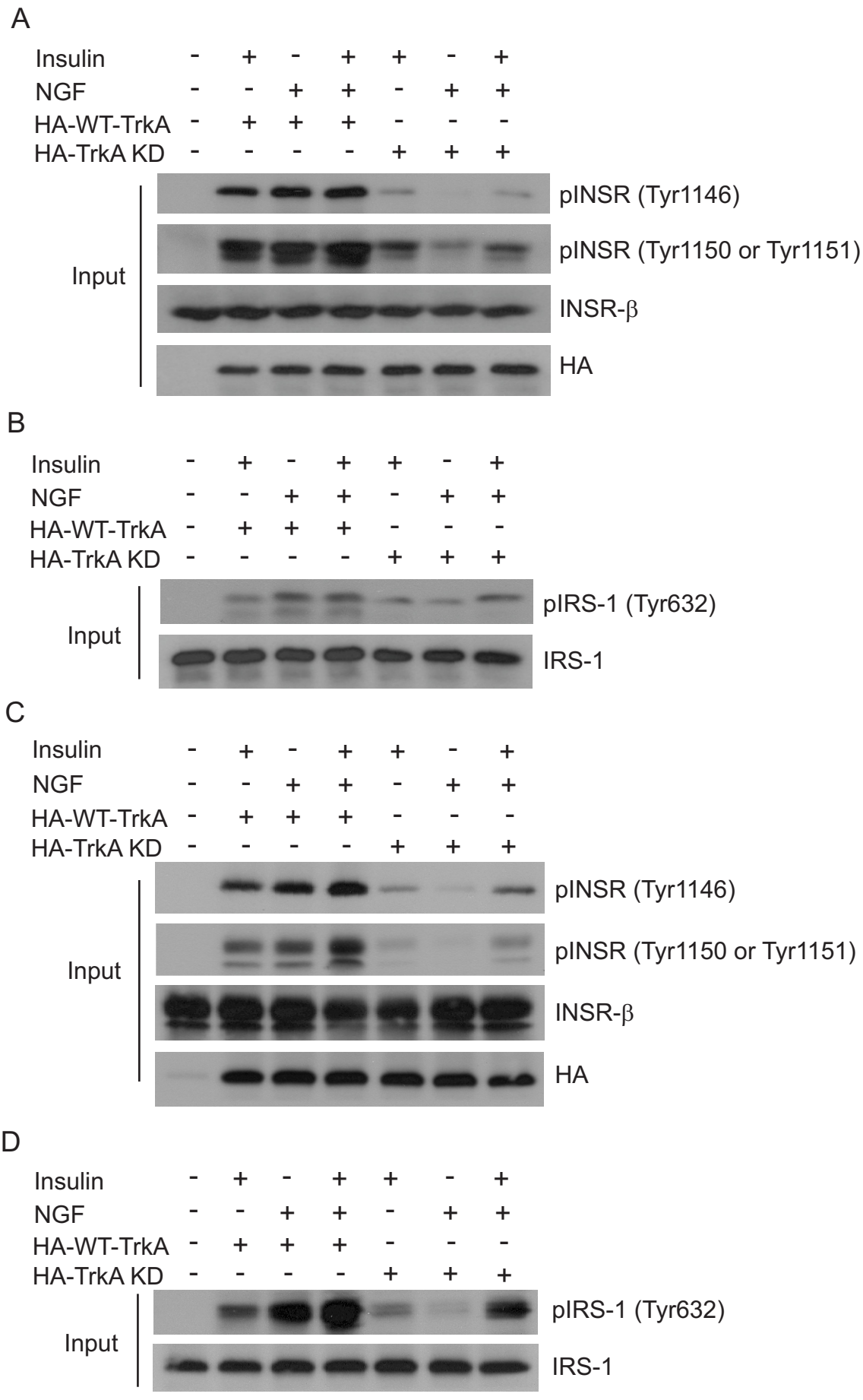


Fig. 5

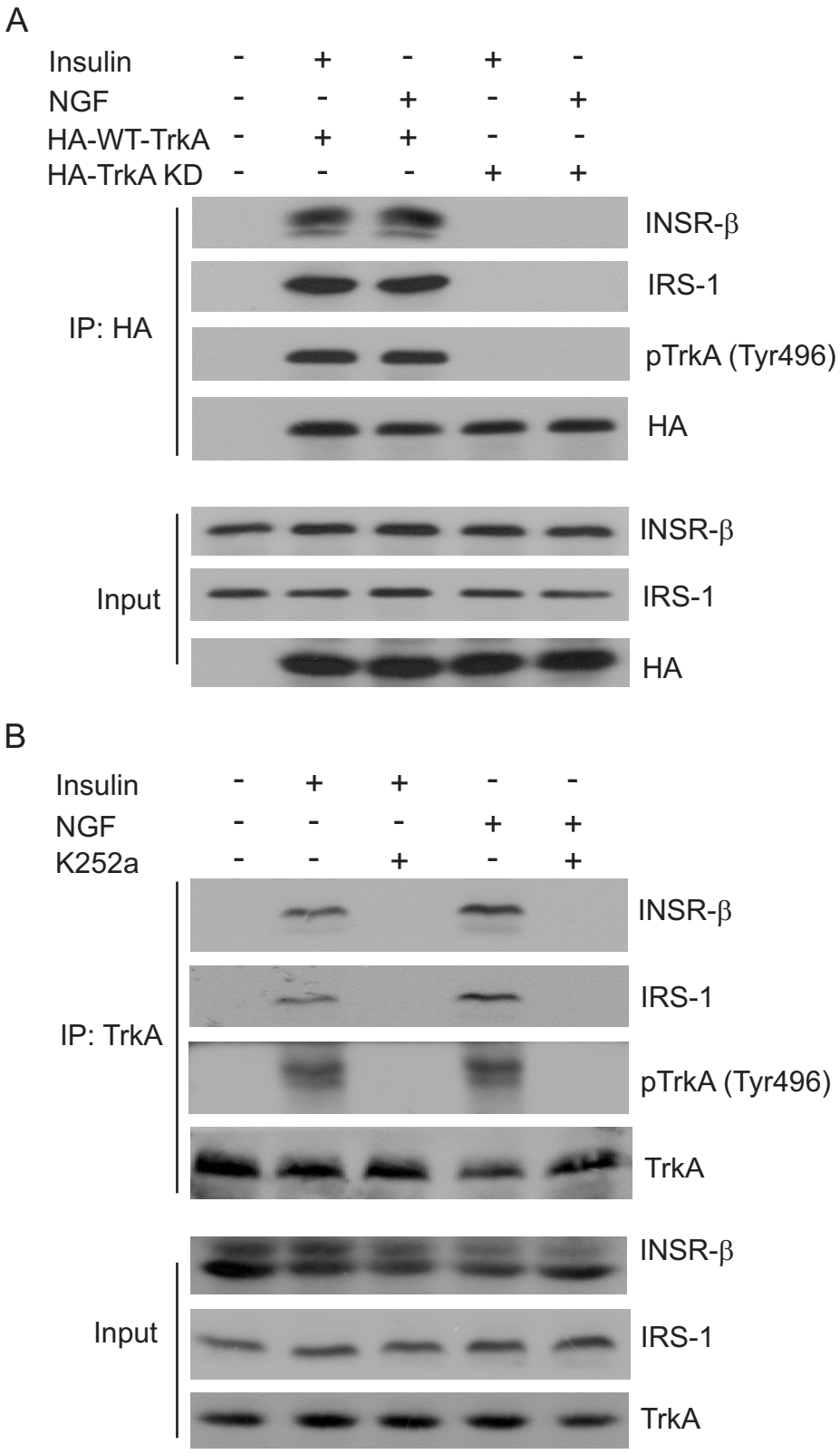


Fig. 6

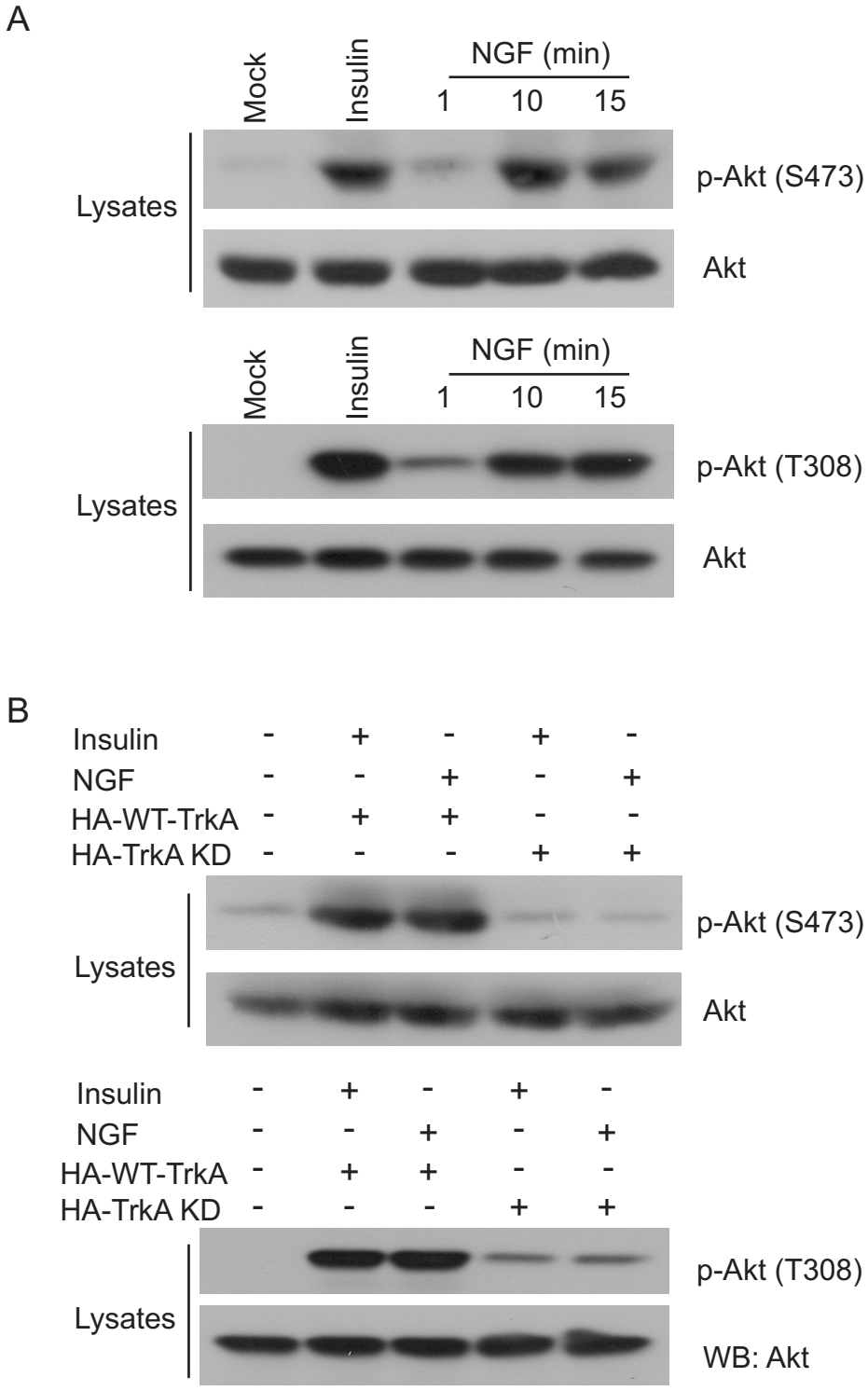
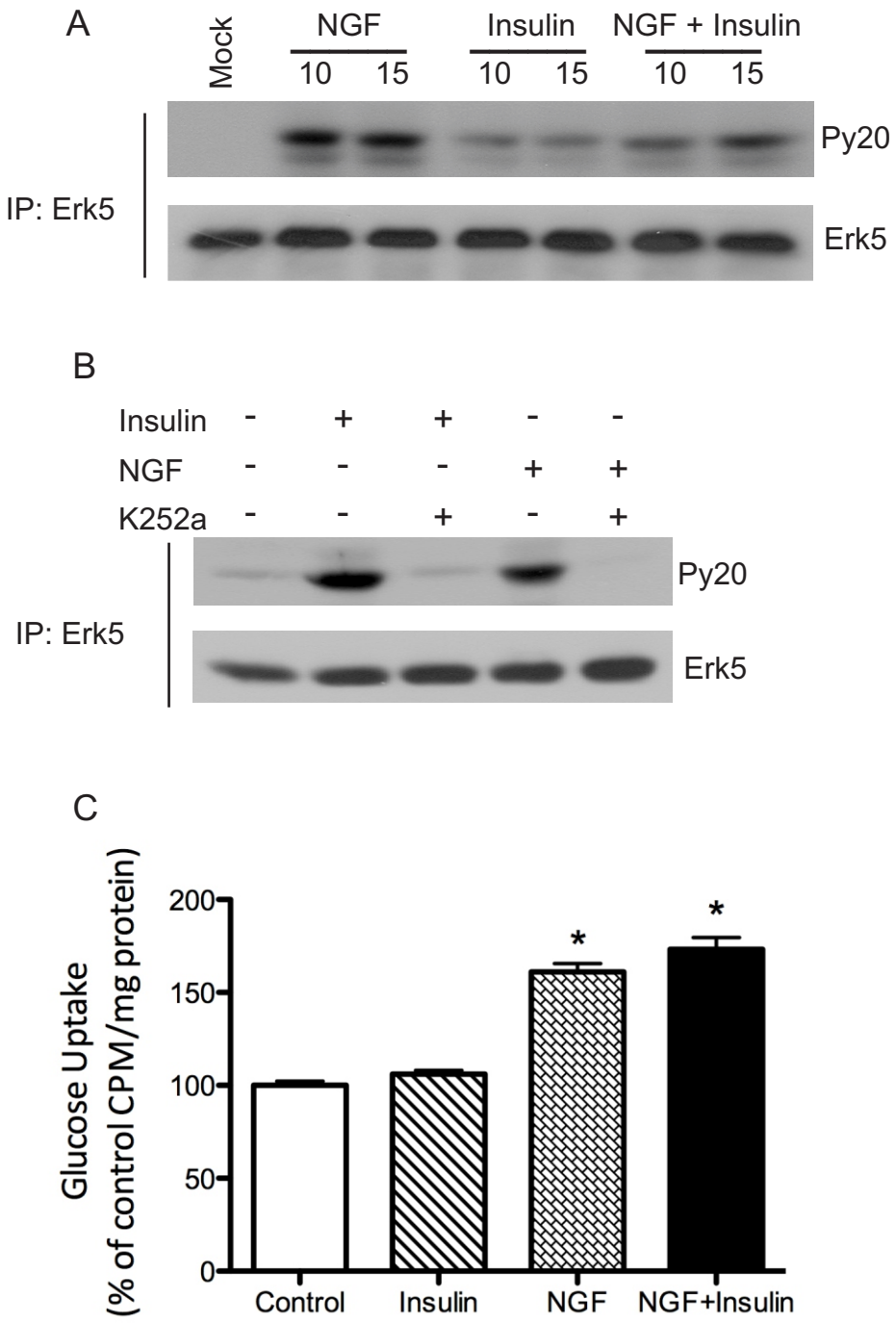


Fig. 7



Nerve Growth Factor Receptor TrkA, a New Receptor in Insulin Signaling Pathway in PC12 cells

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