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NGF-induced Tyro3 and Axl function as survival factors for differentiating PC12 cells

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ABSTRACT

Tyro3 and Axl, two members of the TAM family of receptor tyrosine kinases, play important regulatory roles in a variety of tissues, including the central nervous, reproductive, immune, and vascular systems. We have found that expression of Tyro3 and Axl on PC12 cells is upregulated by nerve growth factor (NGF). PI3K inhibitor LY294002, which is known to inhibit NGF-induced PC12 differentiation, blocked up-regulation of Tyro3 and Axl. NGF regulates Tyro3 and Axl expression by activating their transcription. Both Tyro3 and Axl were associated with the NGF receptor, and protected PC12 cells from stress or toxin-induced cell death. Gas6, a common ligand for both Tyro3 and Axl, was able to replace NGF to support PC12 growth in serum-free medium, and to prevent cell death following serum deprivation. In summary, both Tyro3 and Axl receptors are upregulated by NGF on the differentiating PC12, where they collaborate with TrkA to support neuronal differentiation and survival.

The neurotrophins play important roles in development of the nervous system and promotion of neuronal survival. This family consists of four members, i.e., nerve growth factor (NGF), brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), and neurotrophin-4/5 (NT-4/5) [1]. Each of the mammalian neurotrophins has been shown to activate one or more of the three members of the tropomyosin-related kinase (Trk) family of receptor tyrosine kinases (TrkA, TrkB, and TrkC). TrkA is a receptor for NGF, TrkB is a receptor for BDNF and NT-4, and TrkC is a receptor for NT-3 [1–3]. The major signaling pathways activated by Trk receptors are Ras, PLC-γ1, PI3-kinase (PI3K) [2]. PI3K is essential for NGF-induced differentiation and survival of PC12 cells [4–6], which are derived from a pheochromocytoma of the rat adrenal medulla and provide an excellent model for studies of neuronal cell differentiation [7].

A newly identified family of receptor tyrosine kinases, called the TAM family, has been shown to provide growth trophic support for a variety of cell types [8–13]. This family is comprised of three members, Tyro3, Axl, and MerTK [8,14]. Two related proteins, Gas6 and protein S, have been identified as ligands for these receptors [15,16]. The three members of the TAM family are frequently co-expressed [9,10]. However, Tyro3 is preferentially expressed during neurogenesis in the central nervous system where it is concentrated in neurons of the cerebral cortex and hippocampus [17,18]. Binding of the Gas6 ligand protects cortical neurons from beta-amyloid induced apoptosis [19], and attenuates serum starvation-induced cell death in hippocampal and gonadotropin-releasing neurons [11,20]. To date, however, there is no evidence demonstrating that the TAM family receptors function in coordination with other neurotrophic factors in the support of neuronal growth and differentiation.

We demonstrate here that NGF induces both Tyro3 and Axl in PC12 cells through activation of the TrkA receptor. Following induction, Tyro3 and Axl co-localize with TrkA on the cell surface, where they cooperate with TrkA in neuronal differentiation. Furthermore, activation of Tyro3 and Axl by Gas6 protects PC12 cells from death induced by serum starvation and nerve growth factor-deprivation.

Materials and methods

Cell culture and transfection. PC12 pheochromocytoma cells were maintained in RPMI medium 1640 (Invitrogen, Carlsbad, CA) containing 5% FBS at 37 °C in 5% CO_2. For NGF or Gas6 protection assays, the cells were incubated in media containing either NGF (10 ng/ml) or recombinant mouse Gas6 (100 ng/ml, R&D).

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For signal transduction inhibition experiments, the cells were cultured in NGF or Gas6 containing medium in the presence of one of the following inhibitors: LY294002 (50 μM, Cell signaling) or TrkA/Fc chimera (1 μg/ml) (R&D) or PD98059 (50 μM, Sigma–Aldrich). For overexpression experiments, PC12 cells were transiently transfected with the indicated expression plasmids (Supplemental methods) using lipofectamine-2000 reagent (Invitrogen, Carlsbad, CA) following the manufacturer's procedure.

Western blotting. Protein lysates prepared using a protease inhibitor cocktail (Sigma–Aldrich), were separated by electrophoresis, and transferred to nitrocellulose membranes. The membranes were blocked with 5% nonfat dry milk in PBS containing 0.2% Tween 20 for 1 h, and then incubated overnight at 4 °C with the indicated primary antibody. Membranes were then treated with IRDye^{TM}800 (green) or IRDye^{TM}700 (red) conjugated affinity purified anti-mouse, anti-rabbit or anti-goat secondary IgG for 1 h, followed by three washes with PBS containing 0.1% Tween 20 and two washes with PBS alone. Fluorescent bands were visualized using an LI-COR Odyssey infrared double-fluorescence imaging system (American Company LI-COR).

Full methods are available in the online version of the paper.

Data analysis. Statistical analysis of the results was performed using the Student's *t* test for unpaired samples or One Way ANOVA using Prism4.0 software (GraphPad Software, San Diego, CA). A *p*-value of <0.05 was considered statistically significant.

Results and discussion

Tyro3 and Axl are upregulated during the NGF-induced differentiation of the PC12 cells

PC12 cell can be differentiated into cholinergic neurons upon NGF treatment [7]. Cell differentiation can be detected as early as 6 h after NGF treatment and this differentiation progresses out to 24 h (Fig. S1A). The neuron-specific marker, β-III-tubulin, was expressed in approximately 95% of the PC12 cells by 24 h (Fig. S1B). While TAM receptors were not evident on untreated PC12 cells, they appeared on the cell surface after 24 h of NGF treatment where they were concentrated in regions of the membrane associated with neurite outgrowth (Fig. S2A, arrows). Tyro3 and Axl mRNA was also induced in the cells as determined by real-time PCR analysis (Fig. 1A). Expression of Tyro3 and Axl increased in a timecourse out to 24 h of NGF treatment concomitant with neuronal differentiation; during this time period, the level of the control housekeeping protein GAPDH remained constant (Fig. 1B and Fig. S2B). In contrast to Tyro3 and Axl, the third member of the TAM family MerTK was not induced in response to NGF (data not shown).

NGF stimulates Tyro3 and Axl expression at the transcriptional level

We constructed two GFP-reporter vectors driven by either a 3.7 kb-Tyro3 promoter or a 3.2 kb-Axl promoter. These two promoter-reporter plasmids were independently transfected into PC12 cells and the cell were treated for 24 h with NGF. GFP protein levels were then analyzed by Western blot as a readout of promoter activity. As expected, GFP protein was present in the pCMV-GFP-transfected cells independent of the NGF stimulation (Fig. 1E). However, GFP expression driven by the Tyro3 or Axl promoters was present only in the NGF-treated cells (Fig. 1C, lanes 4–6 and 1D, lanes 5–8), but not in the unstimulated cells (Fig. 1C, lanes 1–3 and 1D, lanes 1–4). These results provide evidence that NGF induces Tyro3 and Axl by increasing transcription from their promoters.

A TrkA/Fc decoy receptor inhibits Tyro3 and Axl expression and PC12 differentiation

NGF triggers PC12 differentiation through the TrkA receptor [21–23]. To test if the TrkA receptor–NGF interaction is required for induction of Tyro3 and Axl, we used a soluble TrkA/Fc decoy pseudo-receptor to block NGF binding to the endogenous TrkA receptor. TrkA/Fc inhibited neurite outgrowth and expression of choline acetyl transferase (ChAT) (Fig. S3A-a), and peripherin (Fig. S3B, lanes 7–9). As with these markers of neuronal differentiation, TrkA/Fc also abolished NGF-induced Tyro3 and Axl expression (Fig. 2C, lanes 7–9), suggesting that NGF-mediated induction of Tyro3 and Axl requires its binding to TrkA.

NGF-mediated PC12 cell differentiation and induction of Tyro3 and Axl requires the PI3-kinase pathway but not the ERK signaling pathway

The effects of NGF on neuronal survival and PC12 neurite outgrowth reported involve the PI3-kinase pathway [24,25]. To determine which signaling pathway(s) may be involved in NGF-mediated induction of Tyro3 and Axl, we tested well-studied kinase inhibitors directed at different pathways. PC12 cells were

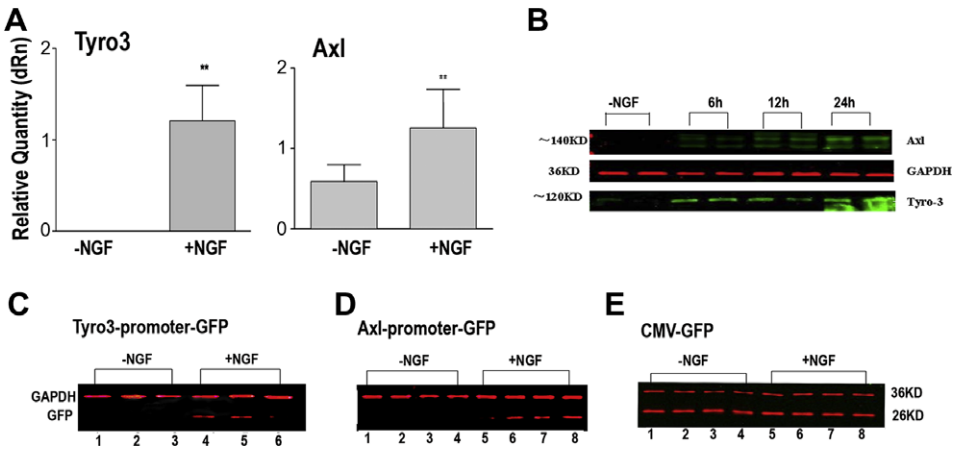

Fig. 1. NGF induces Tyro3 and Axl expression in differentiating PC12 cells increasing their gene transcription. (A) SYBR green Real Time PCR was performed on RNA isolated from the PC12 cells that had been treated with NGF for 24 h. The data are presented as relative quantity of the Tyro3 and Axl mRNA to that of the housekeeping gene, GAPDH (mean ± SEM) ($n = 4$, $^{**}P < 0.01$). (B) Expression of both Tyro3 and Axl proteins during differentiation was analyzed by Western blot (C–E). Expression of GFP for the reporter vectors assayed by Western blotting using anti-GFP in the PC12 cells transfected with the indicated constructs.