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·Original Article·

Expressions of Axl and Tyro-3 receptors are under regulation of nerve growth factor and are involved in differentiation of PC12 cells

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Abstract: Objective Tyro-3 and Axl receptors are expressed in brain in a region-specific manner and their bioactivities in the central nervous system remain still elusive. The aim of the present study was to investigate their functions in neuronal differentiation. **Methods** PC12 cells overexpressing Tyro-3 or Axl were established by transfection with full-length CMV-Tyro-3-eCFP or CMV-Axl-eGFP plasmid, respectively. CMV-eGFP plasmid served as a control vector. After that, the fluorescence intensity and distributions of green fluorescent protein (GFP) and cyan fluorescent protein (CFP) in the cells with or without nerve growth factor (NGF) treatment were real-time monitored. **Results** Expressions of Tyro-3 and Axl receptors were under the regulation of NGF and associated with neuronal differentiation. This was not observed in CMV-eGFP-transfected PC12 cells. Besides, confocal microscopy revealed that NGF affected intracellular localization of full-length Axl-eGFP and Tyro-3-eCFP in PC12 cells. Moreover, the development of outgrowth of differentiated PC12 cells under stimulation of NGF was promoted by overexpression of Tyro-3 or Axl. **Conclusion** Expressions of Tyro-3 and Axl receptors are under the regulation of NGF and are involved in NGF-induced neuronal differentiation of PC12 cells.

Keywords: Axl; Tyro-3; nerve growth factor; PC12 cells; differentiation

1 Introduction

The Tyro-3 subfamily of receptor protein tyrosine kinases (RPTKs) is comprised of Rse/Tyro-3, Axl/UFO and Mer/Eyk, as identified by Cary Lai and Greg Lemke in 1991[1]. The 3 receptors share a ligand-binding ectodomain, a single membrane-spanning domain and a cytoplasmic tyrosine kinase domain[2]. *TYRO-3* gene is expressed during neurogenesis in the central nervous system (CNS) and exhibits distinct and highly regionalized patterns of expression in the adult brain. In human, the highest level of *TYRO-3* mRNA expression is observed in the brain[3]. In mouse, Tyro-3 is expressed at high levels in cerebral cortex and hippocampus. Moreover, the highest levels of Tyro-3 expression in the brain are associated with neurons[4]. The region-specific expression pattern of Tyro-3 suggests that Tyro-3 may play an important role in the development and the biological functions of CNS. Different from Tyro-3 predominantly expressed in the brain, Axl is found at the highest levels in heart and skeletal muscle, and at a low but relatively constant level in the brain throughout development[5]. Structures of Tyro-3 and Axl are similar, sharing 35% and 63% sequence identities in the extracellular and the intracellular domains, respectively[3]. In addition, evidence has shown that Tyro-3 and Axl are closely related with each other in mediating cell-cell communication in many organ systems[6,7]. However, it has not yet

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been clarified how these receptors play physiological and biological roles in the CNS.

Nerve growth factor (NGF) has been indicated to regulate the expressions of Tyro-3 and Axl in PC12 cells, a cell line that can differentiate into cholinergic neurons under NGF induction and serves as a useful model system for neurobiological and neurochemical studies[8]. It has also been ascertained that Gas6, a native ligand of Tyro-3 and Axl, rescues differentiated PC12 cells from serum starvation- and NGF withdrawal-induced apoptosis. The interaction between the high affinity receptor of NGF, TrkA, and Tyro-3/Axl, has also been found[9]. NGF is an important neurotrophic factor secreted mainly from the nervous system, and its dysfunction is associated with some neurodegenerative diseases such as Alzheimer's disease (AD)[10,11]. Another important reason is that expression and the targets of NGF are regionalized in the same brain fields as that of Tyro-3 receptors. Here, the effects of NGF on intracellular localization of full-length Axl-eGFP and Tyro-3-eCFP in PC12 cells were investigated by confocal microscopy. The effects of NGF on the development of the outgrowth of differentiated PC12 cells were also studied.

2 Material and methods

2.1 Cell culture and treatment PC12 pheochromocytoma cells were cultured in RPMI medium 1640 (Gibco, Grand Island, NY, USA) supplemented with 5% fetal bovine serum at 37 °C in a humidified atmosphere containing 5% CO_2. One day before transfection, the cultured cells were dissociated by 0.125% trypsin and plated onto poly-*L*-lysine-coated 12-mm round glass coverslips at a density of 1×10^4 cells/coverslip. Then the PC12 cells were transiently transfected with full-length CMV-Tyro-3-eCFP, CMV-Axl-eGFP or CMV-eGFP expression plasmid as described previously[9], using Lipofectamine 2000 reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. For expression regulating experiments, the transfected cells were washed once with serum-free 1640 medium at 6 h after transfection, and then incubated with serum-free medium containing 10 ng/mL NGF. The control cells were still incubated in complete medium after being washed.

2.2 Fluorescent or confocal microscopy Fluorescent or confocal microscopy was conducted at 7 h, 9 h, 11 h, and 24 h post transfection (NGF treatment for 1 h, 3 h, 5 h and 18 h, respectively). The poly-*L*-lysine-coated coverslips plated with transfected cells were placed on slides with a drop of 75% glycerol in phosphate buffered saline (PBS), and then subjected to fluorescent or confocal microscopy observation. Subcellular localization was observed with 1-mm z-axis steps using a 100× oil immersion objective.

2.3 5-Bromodeoxyuridine (BrdU) incorporation assay BrdU (Sigma-Aldrich, St. Louis, MO, USA) and NGF were added simultaneously to the cultures, and incubated for 24 h. After 24 h of BrdU incorporation, cells were fixed with 4% paraformaldehyde for 20 min and treated consecutively with 1mol/L HCl for 10 min, 2 mol/L HCl for 30 min, and 0.1 mol/L sodium borate for 12 min to denature nuclear DNA into single strand term. After being washed with PBS, cells were incubated with 3% normal goat serum for 30 min, followed by incubation with mouse anti-BrdU primary monoclonal antibody (1:1 000; Sigma-Aldrich, St. Louis, MO, USA) at 4 °C overnight. After that, cells were incubated with TRITC-conjugated goat anti-mouse IgG (1:200; Jackson, USA). The coverslips were then mounted with mounting medium (Vector, USA), and fluorescent images were viewed under a fluorescent microscope (Olympus DP71, Japan).

2.4 Western blotting Western blotting was conducted to assess the relative quantification of protein. Protein lysates were prepared from experimental cells by using the Cellytic buffer containing protease inhibitor cocktail (Sigma-Aldrich, St. Louis, MO, USA). Proteins were analyzed by 6% SDS-PAGE, and electrophoretically transferred to nitrocellulose membranes. Membranes were blocked with 5% nonfat dry milk in 1×PBS for 1 h, and further incubated overnight at 4 °C with rabbit anti-TrkA (1:1 000; Chemicon) and mouse anti-GFP antibodies (1:2 000; Sigma, USA), respectively. The primary antibody-labeled membranes were then treated with IRDye™800 (green)- or IRDye™700 (red)-conjugated affinity purified anti-rabbit or anti-mouse IgG (1:10 000; Rockland Immunochemicals, USA) for 1 h. After that, the membranes were washed 3 times with PBS containing 0.1% Tween and twice with PBS alone. The protein bands were visualized by

the Odyssey infrared double-fluorescence imaging system (American Company LI-COR Biosciences, Lincoln, NE, USA).

2.5 Data analysis All the experiments were performed in at least 3 independent assays. Data were presented as means±SEM, and analyzed with Student's *t*-test for unpaired samples using Prism4.0 software (GraphPad Software, San Diego, CA, USA). $P < 0.05$ was considered to be statistically significant.

3 Results

To determine whether NGF could be a modulator for expressions of Tyro-3 and Axl in differentiating PC12 cells, PC12 cells transfected with full-length Tyro-3-eCFP and full-length Axl-eGFP constructs were subjected to medium with or without NGF (10 ng/mL) after discard of transfection mixture, at 6 h post transfection. Cells under transfection for

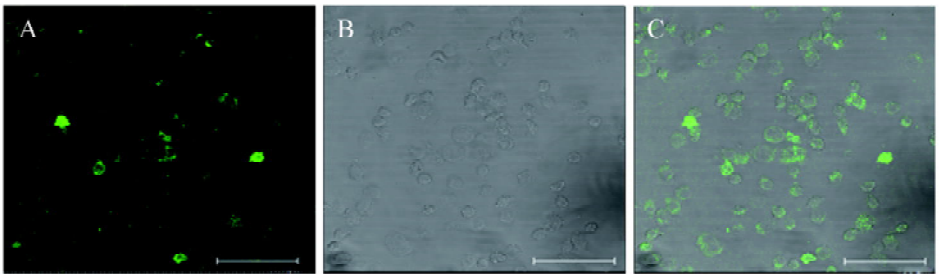

Fig. 1 A representative confocal microscopy observation of PC12 cells transfected with CMV-Axl-eGFP. A: A photo of green fluorescent protein (GFP) expression. B: A phase contrast image in the same visual field of A. C: The merged image. Most of the cells were successfully transfected with CMV-Axl-eGFP plasmid. Scale bar: 100 μm.

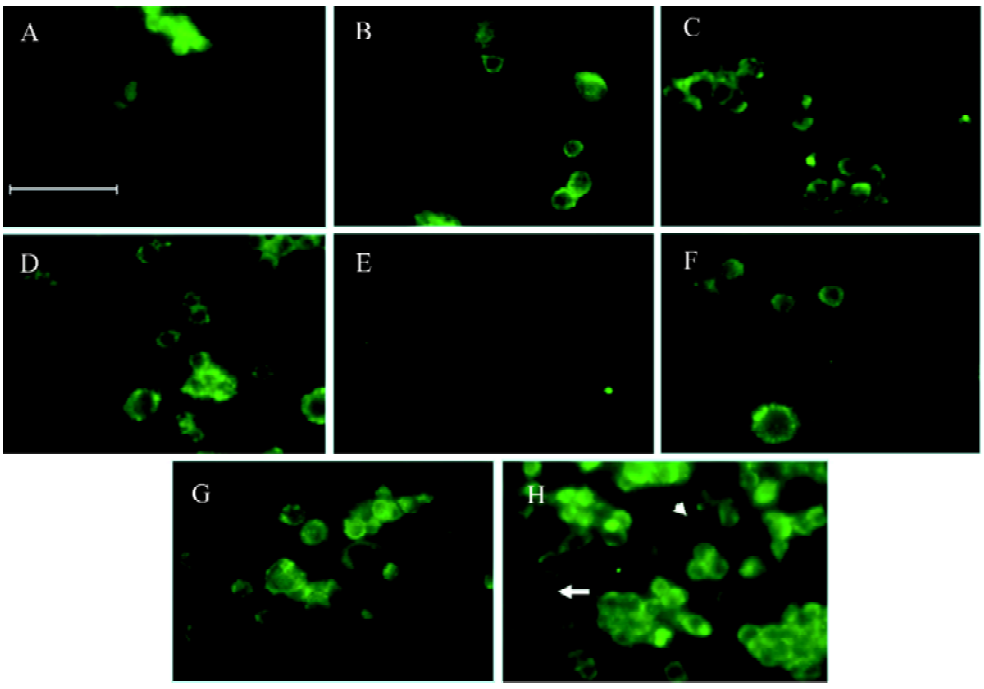

Fig. 2 PC12 cells transfected with full-length Axl-eGFP expression vector in the absence of NGF (A-D) were detected with fluorescent microscope at different time points (A, 7 h; B, 9 h; C, 11 h; D, 24 h) after transfection. The counterparts in the presence of NGF (E-H) were also observed at the corresponding time points (E, 7 h, NGF treatment for 1 h; F, 9 h, NGF treatment for 3 h; G, 11 h, NGF treatment for 5 h; H, 24 h, NGF treatment for 18 h) after transfection. Scale bar: 20 μm.

24 h were observed by confocal microscopy. Transfection efficiency of these 2 constructs were tested (Fig. 1).

As shown in Fig. 1, approximately over 85% of the cultures were successfully transfected with plasmids and there was no difference between NGF-treatment and non-treatment. Green fluorescence intensity of cells transfected with Axl-eGFP chimera were detected by fluorescence microscopy at different time points (Fig. 2). As compared with the counterparts with no NGF treatment (Fig. 2A-D), NGF treatment for 1 h (Fig. 2E), 3 h (Fig. 2F), 5 h (Fig. 2G), and 18 h (Fig. 2H) gradually increased the fluorescent intensity in cells, respectively. Cultures treated with NGF for 5 h exhibited dramatically strong expression of these constructs (Fig. 2G) when compared with their counterparts in the absence of NGF (Fig. 2C). In addition, NGF treatment for 18 h promoted the development of PC12 cell outgrowth, as shown by the arrows in Fig. 2H. Most importantly, neurite outgrowth was obvious at 10 h of NGF treatment (Fig. 3, arrows), which developed earlier than that of wild type cultures[9]. Cells transfected with Tyro-3-eCFP showed the same results as Axl-eGFP-transfected cultures (data not shown).

Western blotting showed that at 24 h after transfection, Tyro-3-eCFP and Axl-eGFP expressions were significantly increased in cultures with NGF treatment, compared with those in untreated cultures (Fig. 4).

To exclude the possibility that the increase in gene expression might be due to the cell apoptosis during NGF-induced cell differentiating, the BrdU incorporation method was employed to discriminate the newborn cells. It was found that most of the cells with a higher fluorescent intensity were newborn cells. Nevertheless, there were also some BrdU-negative cells including those with a lower fluorescent intensity and those with neurites as indicated by the arrows in Fig. 5.

The intracellular localization of Axl-eGFP and Tyro-3-eCFP chimera under the condition of NGF treatment or non-treatment was also observed. Confocal microscopy indicated that in the absence of NGF, Axl-eGFP expression was targeted essentially to the cellular periphery in the form of discrete bright punctate structures, with relatively fainter diffuse intracellular signals (Fig. 6A-D). Characteristic patterns of signal distribution in cultures in the presence of NGF were presented in Fig. 6E-H. Axl-eGFP exhibited membrane and intracellular expressions at much higher levels in the pres-

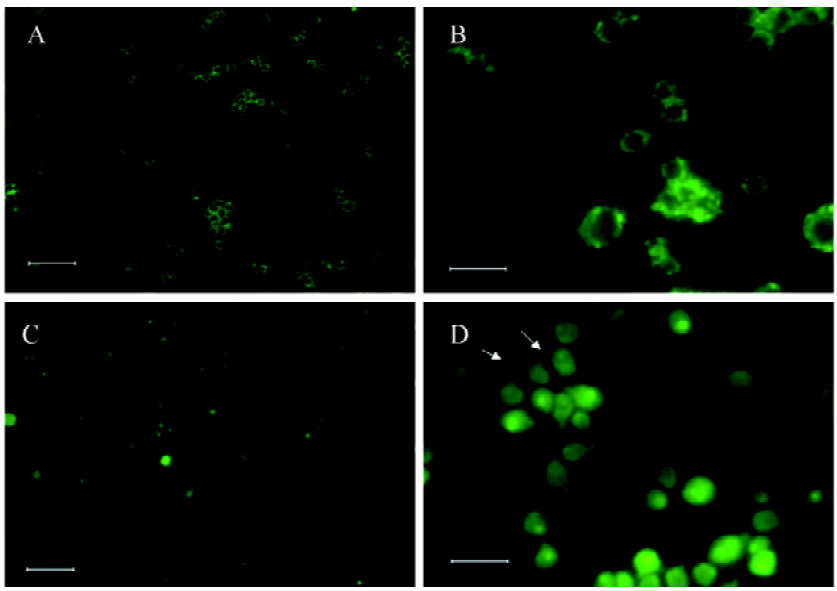

Fig. 3 Live PC12 cells transfected by Axl-eGFP construct with NGF treatment for 10 h were observed by fluorescent microscopy. In the absence of NGF (A, B), GFP protein was mainly distributed on the membrane and expression was relatively fainter compared with that in the presence of NGF (C, D). Besides, some cells with NGF treatment developed neurite outgrowth (arrows in D). Scale bar for A and B, 100 μm. Scale bar for C and D, 30 μm.

ence of NGF than in the absence of NGF. Interestingly, perinuclear localization of GFP signals were observed after addition of NGF in cultures (arrows in Fig. 6E-H). Confocal microscopy analysis of Tyro-3-eCFP construct came to the same results (data not shown). These data indicate that NGF not only increases expression but also affects cellular distributions of Tyro-3 and Axl in differentiating PC12 cells. Besides, the same pattern of subcellular localization of endogenous Tyro-3 and Axl protein were found in differentiating PC12 cells[9].

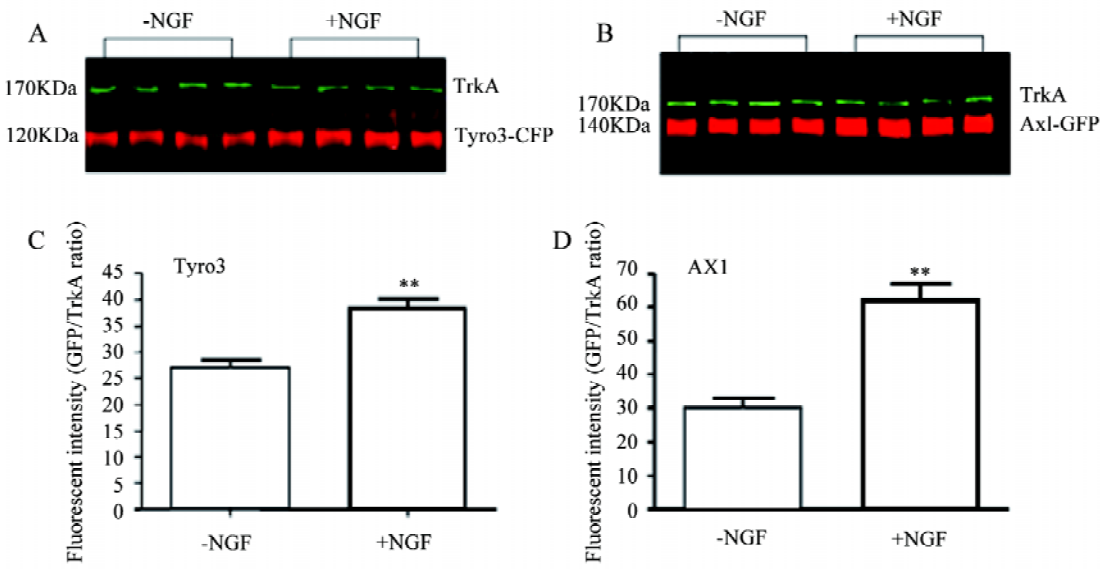

Fig. 4 Expressions of the fusion of full-length Tyro-3-eCFP (A) and full-length Axl-eGFP (B) in the presence or absence of NGF in PC12 cells at 24 h post transfection. The relative fluorescent intensities of Tyro-3-eCFP (C) and Axl-eGFP (D) were calculated as the ratios of CFP or GFP intensity to TrkA intensity, respectively, using Odyssey imaging system. $^{**}P < 0.000\ 1$. Same results were obtained from repeated independent experiments.

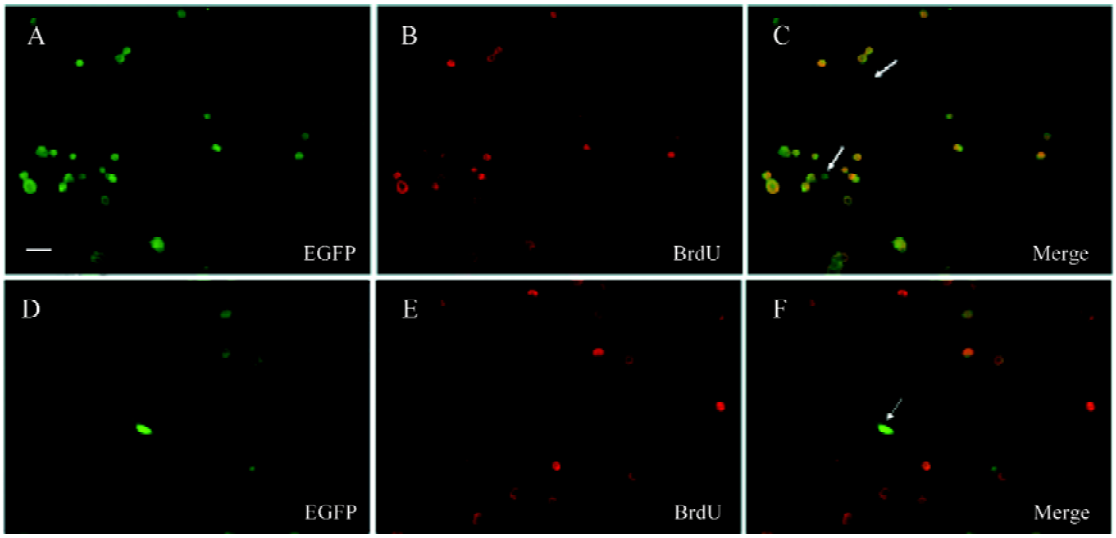

Fig. 5 BrdU incorporation staining showed the expression of GFP (A, D) and incorporated BrdU (B, E) in the PC12 cells treated by NGF for 24 h. C and F were the merged images. Arrows in C indicated the cells that did not uptake BrdU and showed a relatively weaker intensity. Some cells with a higher fluorescent intensity did not incorporate BrdU either (arrow in F). Scale bar: 10 μm.

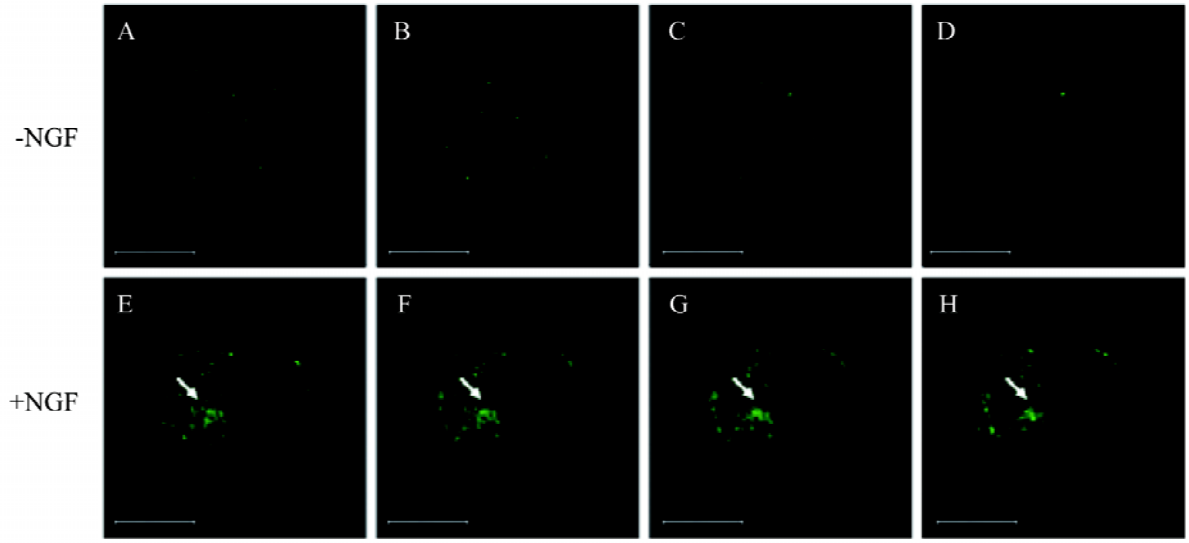

Fig. 6 NGF increases subcellular distribution of full-length Axl-eGFP in PC12 cells. PC12 cells were transfected with full-length Axl-eGFP vector, followed by NGF treatment (E-H) or non-treatment (A-D). At 24 h post transfection, cells spreading on poly-*L*-lysine-coated coverslips were washed with PBS and scanning fluorescent images were obtained with confocal microscopy. Successive portraits of transfected cells in the presence of NGF (E-H, pinhole, approximately 1 airy/unit) showed a higher fluorescent intensity than that of cells in the absence of NGF (A-D). Scale bar: 10 μm.

4 Discussion

Our previous studies have demonstrated that the expressions of endogenous Axl and Tyro-3 receptors are specific for NGF-induced differentiated PC12 cells. Besides, the expressions of endogenous Tyro-3 and Axl proteins are under regulation of NGF in a time-dependent manner in these differentiating cells. Furthermore, we have firstly discovered that Tyro-3 and Axl are also expressed on the membrane of differentiating PC12 cells where the interaction between Tyro-3/Axl and TrkA occurs. This interaction exerts neurotrophic effects on differentiating PC12 cells[9]. In the present study, the effects of exogenous constructs on the differentiation of PC12 cells were detected, to determine whether overexpression of Tyro-3 or Axl can affect the differentiation of PC12 cells under NGF stimulation.

The present results further verify our discovery that expressions of Axl and Tyro-3 receptors are under the regulation of NGF and are associated with the procedure of neuronal differentiation. Transfection experiments using CMV-GFP expression vector were performed to confirm the specificity of this regulatory effects of NGF and here we reveal that Tyro-3 and Axl are indeed the downstream of NGF rather than a result of increased intake of corresponding gene expression vectors after reagent treatment (data not shown). Therefore, it is reasonable to assume that NGF regulates the expressions of Tyro-3 and Axl at the post-translational level. As a matter of fact, our previous study found that NGF also regulated expressions of Tyro-3 and Axl at the transcriptional level[9] .

As we know, the regulatory effects of NGF on neuronal differentiation and development are exerted through the binding of NGF to its high affinity receptor TrkA, which is also a member of receptor tyrosine kinases family[12]. It is well known that NGF triggers PC12 differentiation through the TrkA receptor. Next, we want to know how NGF regulates the expressions of *Tyro-3* and *Axl* genes and whether TrkA interacts with Tyro-3 and Axl in a direct manner. A striking finding of confocal microscopy analysis is that Axl-eGFP and Tyro-3-eCFP accumulate in perinuclear areas which appear to be the place of endosome localization in cytoplasm after NGF treatment. It has been indicated that NGF binding to wild type TrkA induces internalization and degradation of this receptor in endosome[13]. Our observations and the interac-

tion between TrkA and Axl/Tyro-3 mentioned in our published data[9] support our hypothesis that increased post-translational expressions of Tyro-3 and Axl receptors may result directly from the activation of the NGF/TrkA signaling pathway. Indeed, the expressions of Tyro-3 and Axl in differentiating PC12 cells could be deleted by inhibition of TrkA binding to NGF[9].

Although accumulative lines of evidence illustrate that insufficiency of NGF plays an important role in the process of cholinergic neuronal loss and degeneration and is a main event of the neurodegenerative diseases such as AD, there are some studies demonstrating that NGF level is increased in the brain of AD patients[14-17]. These findings suggest that there is dysfunction of receptors in aging brain. In addition, altered histology, increased apoptosis and neuronal degeneration have also been found in the CNS in elder *Tyro-3*-knockout mice[6,7]. These studies together with our observation suggest that dysfunction of Tyro-3 receptor signaling may be involved in the pathogenesis of neurodegenerative diseases such as AD, given that Tyro-3 receptor-expressing neurons in brain are located exactly in regions targeted by NGF[18,19]. In summary, our studies demonstrate for the first time that expressions of Tyro-3 and Axl receptors are under the regulation of NGF and are involved in NGF-stimulated neuronal differentiation.

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Axl 和 Tyro-3 受体表达受神经生长因子调控并参与 PC12 细胞的分化

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摘要：目的 Axl 和 Tyro-3 受体在脑内有区域性的分布，但两者在中枢神经系统中的生物学功能尚不明确。本研究旨在探讨Axl和Tyro-3受体在神经元分化中的作用。**方法** PC12细胞分别转染CMV-Axl-eGFP、CMV-Tyro-3-eCFP 和 CMV-eGFP 质粒后，给予神经生长因子(nerve growth factor, NGF)诱导，观察绿色荧光蛋白和青色荧光蛋白的表达和分布。**结果** Axl-eGFP 和 Tyro-3-eCFP 的表达随着NGF作用时间的延长而逐渐上调，并且荧光蛋白在细胞内的定位也发生变化。作为对照组，CMV-eGFP 转染的 PC12 细胞并没有出现此变化。此外，过表达 Axl 和 Tyro-3 能够促进 PC12 细胞的突起生长。**结论** Axl 和 Tyro-3 受体的表达受 NGF 调控，其过表达可能参与了 PC12 细胞的分化。

关键词： Axl；Tyro-3；神经生长因子；PC12 细胞；分化