Leukemia inhibitory factor receptor signaling negatively modulates NGF-induced neurite outgrowth in PC12 cells and sympathetic neurons

Yu Pong Ng*, Wei He*, and Nancy Y. Ip

Department of Biochemistry, Biotechnology Research Institute and Molecular Neuroscience Center, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China.

* These authors contributed equally to this work

Correspondence should be addressed to: Prof. Nancy Y. Ip, Department of Biochemistry, Hong Kong University of Science and Technology, Clear Water Bay, Hong Kong, China. Phone: 852-2358-7304 Fax: 852-2358-2765 E-mail: BOIP@UST.HK

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Summary

Nerve growth factor (NGF) is required for the development of sympathetic neurons and subsets of sensory neurons. Our current knowledge on the molecular mechanisms underlying the biological functions of NGF is in part based on the studies with PC12 rat pheochromocytoma cells, which differentiate into sympathetic neuron-like cells upon NGF treatment. Here we report that the expression of leukemia inhibitory factor receptor (LIFR), one of the signaling molecules shared by several neuropoietic cytokines of the IL-6 family, is specifically upregulated in PC12 cells following treatment with NGF. Attenuation of LIFR signaling through stable transfection of antisense or dominant negative LIFR constructs enhances NGF-induced neurite extension in PC12 cells. On the contrary, overexpression of LIFR retards the growth of neurites. More importantly, while NGF-induced Rac1 activity is enhanced in AS-LIFR and DN-LIFR expressing PC12 cells, it is reduced in LIFR expressing PC12 cells. Following combined treatment with NGF and CNTF, sympathetic neurons exhibit attenuated neurite growth and branching. On the other hand, in sympathetic neurons lacking LIFR, neurite growth and branching is enhanced when compared to wild type controls. Taken together, our findings demonstrate that LIFR expression can be specifically induced by NGF and, besides its known function in cell survival and phenotype development, activated LIFR signaling can exert negative regulatory effect on neurite extension and branching of sympathetic neurons.

Abbreviation: NGF, nerve growth factor; EGF, epidermal growth factor; LIF, leukemia inhibitory factor; OSM, oncostatin M; LIFR, LIF receptor; CNTF, ciliary neurotrophic factor
Introduction

Nerve growth factor (NGF) is the prototype of the neurotrophin family, which also includes brain-derived neurotrophic factor (BDNF), neurotrophin-3 (NT-3), NT-4/5 in the mammals and NT-6, NT-7 in the fish species (1, 2, 3). NGF was originally identified based on its ability to support the survival and development of sympathetic and specific subsets of sensory neurons. Intense studies on the molecular mechanisms of NGF functions have been conducted using the rat adrenal pheochromocytoma cell line, PC12, which has been widely used as a model system to study neuronal differentiation. Upon NGF treatment, PC12 cells exhibit morphological and biochemical changes and undergo differentiation to a sympathetic neuron-like phenotype. NGF-induced neurite outgrowth is characterized by the formation of filamentous actin containing spikes within minutes, followed by the extension of neurite processes with growth cone-like structures (4). These differentiated cells also express a wide variety of neuronal markers, including voltage-gated sodium channels, enzymes for synthesis of neurotransmitters and components of the neuronal cytoskeleton (5-9). Previous in vitro and in vivo studies have revealed some of the key signaling molecules, such as Erks, that are involved in the signaling cascade leading from the activation of NGF receptor to the acquisition of a neuronal phenotype (10). However, the precise mechanisms of signal transduction involved in the regulation of neurite outgrowth are just beginning to be unraveled.

In addition to the neurotrophins, the neuopoietic cytokines or the interleukin-6 (IL-6) family, represent another group of neurotrophic factors. Members of this family include IL-6, IL-11, leukemia inhibitory factor (LIF), oncostatin M (OSM), ciliary neurotrophic factor (CNTF), cardiotrophin-1 (CT-1), and cardiotrophin-like cytokine/cardiotrophin-like factor complex (CLC/CLF) (11-14). These cytokines exhibit a wide range of biological functions in the regulation of various complex cellular processes such
as hematopoiesis and neuronal differentiation. While IL-6 and IL-11 induce homodimerization of gp130 to activate the signaling cascade, other members of the IL-6 family induce heterodimerization of gp130 and LIFR. Homodimerization of gp130 or heterodimerization of gp130 and LIFR results in the activation of receptor pre-associated Janus kinases (Jak1, Jak2 and Tyk2) (15, 16). Activated Jaks phosphorylate the tyrosine residues within the cytoplasmic domains of gp130 and LIFR. These phosphotyrosine residues in turn act as docking sites for the signal transducers and activators of transcription (STAT) transcription factors, such as STAT3 (17, 18). Phosphorylated STAT3 forms a homodimer or a heterodimer with STAT1, which, upon translocation into the nucleus, regulates the expression of cytokine responsive genes.

One of the distinctive features of neuronal cells is their capability to extend or retract neurites and adjust the direction of growth cone in response to environmental cues. Accumulating evidence demonstrates that neurotrophins are involved in the dynamic regulation of the neuronal cytoskeleton and neuroplasticity (19, 20). NGF has been shown to collaborate with the neuropoietic cytokines, such as LIF and CNTF, to influence the differentiation program of neuronal progenitors (21-23). These studies suggest that the neuronal differentiation program involves sequential and cooperative actions of different types of neurotrophic factors. However, how these collaborations exert effects on the process of neuronal differentiation, such as the dramatic morphological changes of neuronal cells, remains elusive. Here we report that LIFR, one of the common signaling molecules shared by several IL-6 family cytokines, is specifically upregulated by NGF in PC12 cells. While attenuation of LIFR signaling enhances neurite outgrowth, overexpression of LIFR inhibits the extension of neurites. Thus, NGF-induced LIFR signaling exerts negative regulatory effects on the neurite outgrowth during neuronal differentiation. More importantly, LIFR signaling modulates
the activation of Rac1 during the initiation phase of neuronal differentiation, and controls
the expression of some neuronal cytoskeletal protein, such as β-tubulin III. The inhibitory
effects of LIFR signaling on neurite outgrowth can similarly be observed with primary
cultures of sympathetic neurons upon treatment with CNTF. Moreover, developing
sympathetic neurons lacking LIFR exhibit enhanced extension and branching of neurites.
Taken together, our findings demonstrate that the LIFR signaling induced by NGF exert
negative regulatory effects on the outgrowth and branching of neurites in developing
sympathetic neurons.
Experimental Procedures

Cell culture and transfection

PC12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with heat-inactivated horse serum (6 % v/v), heat-inactivated fetal bovine serum (6 % v/v), penicillin (50 U/ml), and streptomycin (100 µg/ml, Invitrogen). Cells were routinely grown on 100-mm diameter tissue culture dishes (Falcon) at 37 °C in a humidified atmosphere with 7.5 % CO₂ and medium was changed every three days. For NGF-induced differentiation, cells were plated at a density of 1x10⁵ cells/dish one day before treatment with the appropriate growth factors, and serum concentration was reduced to 1 %. NGF (Alomone labs), EGF (gift from Prof. W. K. Wong, The Hong Kong University of Science and Technology) or LIF (Invitrogen) was added at the concentration of 50 ng/ml. Half of the culture medium was changed every three days supplemented with additional NGF or EGF. For the studies on STAT3 phosphorylation, cells were washed twice with DMEM, and then starved in DMEM for 4 hr. Rat CNTF (50 ng/ml, purified in our laboratory) or mouse LIF (50 ng/ml) were applied to the cultures for 15 min as indicated.

Transient transfection was carried out using LipofectAMINE PLUS (Invitrogen) reagents according to the manufacturer’s instructions. For stable transfection, PC12 cells were incubated in the standard culture medium for 48 hr after transfection and cells were passaged at 1:20 dilution followed by addition of G418 at 400 µg/ml. After a three-week selection, single colony was isolated and screened for the presence of appropriate constructs. Positive clones were maintained in the culture medium with G418 at 200 µg/ml.

For the preparation of primary culture of sympathetic neurons, superior cervical ganglia (SCG) were dissociated from postnatal day 1 mice and cultured as described
previously (24) with slight modification in the medium consisting of Neurobasal/B27 medium (Invitrogen), NGF (10 ng/ml), penicillin (50 U/ml) and streptomycin (100 µg/ml). Cultures were maintained at 37 °C in a humidified atmosphere with 5 % CO₂. Nonneuronal cells were eliminated by treatment with cytosine arabinoside (10 µM) on culture day 1 and 3.

**Animals**

LIFR +/- mice, generated on the B6,129/J genetic background and C57BL6 STOCK (for expanding the LIFR +/- mice), were obtained from The Jackson Laboratory (Bar Harbor, ME). Mice were bred to allow for the generation of homozygote, heterozygote, and wild-type littermates. The genotyping of mice carrying LIFR mutations was performed as described previously (25). Mice were maintained at the Animal Care Facility of HKUST. SCGs were collected from E18 mice and dissociated as described above.

**Construction of expression plasmids**

Full-length rat LIFR cDNA was amplified from rat brain cDNA library and subcloned into the mammalian expression vector pcDNA3 (Invitrogen). DNA sequencing was performed using a ABI310 Genetic Analyzer (Applied Biosystems). Antisense LIFR insert was amplified using full-length rat LIFR-pcDNA3 vector as the template. 5’ primer 5’-TAGGCGGCCGCACTGGGAGCTTTCTC-3’ contains a NotI site, and 3’ primer 5’-AGAGAATTCACCTCCCAGGTGGCATTG-3’ contains an EcoRI site. PCR product was digested with NotI/EcoRI restriction enzymes and then subcloned into pcDNA3 vector. For the construction of dominant negative LIFR-GFP fusion protein expression vector, cDNA sequence for the extracellular and transmembrane region of rat LIFR was amplified using full-length rat LIFR-pcDNA3 vector as the template. 5’ primer 5’-ACTGAATTCGGACAATGGGAGCTTTCTC-3’ contains an EcoRI site, and 3’ primer
5'-TCTGGATCCCCATAGCAGAGGATGC-3’ contains a BamHI site. PCR product was digested with EcoRI/BamHI restriction enzymes and subcloned into an EGFP expression vector pEGFP-N1 (Clontech), with the coding sequence for EGFP protein fused in frame after the sequence for transmembrane region of rat LIFR.

RT-PCR and Southern blot analysis

Total RNA of PC12 cells was extracted using acid guanidinium thiocyanate method as previously described (26). For the synthesis of cDNA, 5 µg total RNA was mixed with 0.5 µM oligo-dT primer (Invitrogen) and denatured at 70 °C for 10 min. After cooling on ice, reverse transcription mixture (1× PCR buffer, 2.5 mM MgCl2, 0.5 mM dNTPs, 10 mM DTT and 10 U/µl Superscript II reverse transcriptase) was added. Reaction was carried out at 42 °C for 50 min and terminated at 70 °C for 15 min. One tenth of the cDNA mixture was used as the template for the subsequent PCR amplification. PCR primers and conditions for the amplification of gp130, LIFR and CNTFR fragments were according to the previous report (27). Primers for LIF: 5' primer 5'-AGTCAACTGGCTCAACTCAACG-3' and 3' primer 5'-CTGGACCACCGCACTAATGACT-3'; OSM: 5' primer 5'-CTTCCCCAGTGAGGACATGCTC-3' and 3' primer 5'-CATGAAGCGGTGATAGC-3'; and GAPDH: 5' primer 5'-TGATGCTGGTGCTGATGTCG-3' and 3' primer 5'-TCCTTGAGGGCATAGGC-3'. PCR products were separated on 1 % agarose gel and then transferred onto nylon membranes. DNA blots were hybridized with appropriate probes and exposed to Kodak XAR-5 X-ray film at –80 °C overnight.

Preparation of cell extracts and Western blot analysis

PC12 cells were harvested and lysed in RIPA lysis buffer: 150 mM NaCl, 1 % (v/v) Nonidet P-40, 0.1 % (w/v) SDS, 2 µg/ml aprotinin, 0.5 % deoxycholic acid, 1 mM PMSF, 5 mM benzamidine, 1 mM sodium orthovanadate (NaOV) and 10 µg/ml soybean
trypsin inhibitor (Sigma) in 50 mM phosphate buffer, pH8.0. The proteins separated by SDS-PAGE gel electrophoresis were then transferred onto a nitrocellulose membrane (Micron). Following blocking with 0.1 % (v/v) Tween-20 and 5 % (w/v) non-fat dry milk in Tris-buffered saline at room temperature for 1 hr, the membrane was incubated with primary antibody (1:500 or 1:1,000) at 4 °C overnight, HRP-conjugated secondary antibody (1:2,000) for 1 hr, and detected using Enhanced Chemiluminescence (ECL) Western Blot System (Amersham). Primary antibodies used included anti-LIFR (Santa Cruz), β-tubulin III (Sigma), anti-phosphothreonine PAK (Cell Signaling) and anti-PAK1 (Cell Signaling).

**STAT3 phosphorylation assay**

Cultured cells were starved for 4 hr in serum-free DMEM culture medium without antibiotics. After starvation, 50 ng/ml factors were applied and cells were incubated at 37 °C for 15 min. Cells were then lysed in RIPA buffer and insoluble material was pelleted. Protein concentrations of supernatants were assayed using the Bradford method (Bio-Rad). Total cell lysates (50 µg) was subjected to SDS-PAGE followed by Western blotting using an anti-phosphotyrosine STAT3 (Tyr705) antibody (Cell Signaling). After stripping membranes in a stripping buffer (0.1 M β-mercaptoethanol, 62.5 mM Tris, pH6.7 and 2 % SDS), a STAT3 specific antibody (Santa Cruz) was used for the detection of total STAT3 expression.

**Rac1 and RhoA assays**

Measurement of Rac1 and RhoA activities was performed using commercial kits (Upstate). LIFR mutant PC12 cells were seeded in 100-mm culture dishes at a density of $3 \times 10^6$ cells/plates for Rac1 activity assay and $6 \times 10^6$ cells/plate for RhoA activity assay. After 24-hr culture, cells were changed to serum-free DMEM for 4 hr. Cells were then treated with NGF (50 ng/ml) for the indicated times and lysed for 30 min with the cell
lysis buffer (25 mM HEPES, pH7.5, 150 mM NaCl, 10 mM MgCl₂, 1 % Nonidet P-40, 1 mM EDTA, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM NaOV and 1 mM sodium fluoride). Cell lysates were then centrifuged at 14,000 g for 10 min at 4 °C. The supernatants were incubated with agarose-GST-PBD for Rac1 activity assay or agarose-GST-RSK for RhoA activity assay with continuous rotation for 1 hr at 4 °C. After the beads were washed three times with the cell lysis buffer, the bound proteins were eluted by boiling in 2 x protein loading buffer and separated by 15 % SDS-PAGE. The separated proteins were detected by immunoblotting with a mouse monoclonal anti-Rac1 (1:1,000) or rabbit polyclonal anti-RhoA antibody (1:1,000) followed by 1 hr incubation of HRP-conjugated secondary antibody (1:2,000) and detected using Enhanced Chemiluminescence (ECL) Western Blot System (Amersham). Densitometry analysis was performed using Adobe Photoshop 7.0 software (Adobe Systems Incorporated), and the amounts of active Rac1 and RhoA were normalized by total amounts of Rac1 and RhoA in cell lysates, respectively.

**Immunocytochemical staining**

PC12 cells were fixed by 4 % paraformaldehyde for 30 min at room temperature. After blocking with 4 % FBS in PBS containing 0.4 % Triton X-100 for 20 min at room temperature, cells were incubated with an anti-Rac1 monoclonal antibody (Upstate, 1:1,000) at 4 °C overnight. Cells were washed with PBS three times. F-actin was stained with rhodamine-conjugated phalloidin (1:500), which was added together with the secondary antibody, FITC-conjugated anti-mouse antibody (Amersham, 1:1,000). After washing with PBS, cells were mounted with Mowiol and analyzed under Olympus confocal microscope.
Quantification of neurite outgrowth and cell size

In the morphological study of LIFR stable cell lines, the length of the longest neurite of individual cells was recorded using MetaMorph Ver 5.0r1 software (Universal Imaging Corp.), while cell size was determined using the longest diameter of the cell. For each measurement, at least 80 cells per dish were counted from randomly selected fields and n=3 dishes. Each experiment was repeated at least three times. For cultured SCG neurons, length of the longest neurite and total neurite length were measured to quantify the neurite outgrowth. The total number of tip ends was counted to represent the number of branching from individual neurons. For each measurement, 50 cells per dish was counted, and n=3 dishes. Each experiment was repeated at least 3 times. In the study using LIFR knockout mice, eight mice from each group were studied in 5 separate preparations. The length of the longest neurite, total neurite length and tip addition of 50 cells were recorded for each culture.
Results

NGF specifically upregulated LIFR expression in PC12 cells

To investigate whether the expression of the CNTF receptor complex was affected by neuronal differentiation, we examined the mRNA expression of CNTFR, gp130 and LIFR in PC12 cells maintained in growth medium supplemented with high serum or low serum which was conducive for neuronal differentiation. RT-PCR analysis revealed the reduction of LIFR mRNA in low serum condition, while CNTFRα and gp130 remained relatively unchanged (Fig. 1a). LIFR transcript and protein expression were induced after treatment with NGF for 7 days, and reached the highest level on day 14 (Fig. 1b&c). The expression of gp130 and CNTFR, however, remained unchanged in response to NGF (data not shown). While EGF also induced Erk phosphorylation (10, 28), it did not induce LIFR expression after treatment for 14 days (data not shown). In addition to LIFR, the expression of LIF and OSM was also induced after 7 days of NGF treatment (Fig. 1b). Interestingly, we found that phosphorylation of STAT3 on tyrosine705 residue was also induced by NGF after 8-14 days of treatment, with a temporal profile similar to that observed with increased LIFR expression (Fig. 1d). While PC12 cells normally do not respond to CNTF or LIF, a 7-day priming with NGF resulted in tyrosine phosphorylation of STAT3 upon treatment with CNTF or LIF (Fig. 1e). Similar STAT3 activation was not observed with PC12 cells pre-treated with EGF (Fig. 1e).

Construction of PC12 cell lines stably expressing antisense-LIFR and dominant negative-LIFR

To explore the putative function of LIFR signaling in NGF-induced PC12 differentiation, we stably transfected antisense (AS)-LIFR and dominant negative (DN)-LIFR constructs into PC12 cells (Fig. 2a). AS-LIFR expressing stable clones were
screened based on the reduced basal level of protein expression of LIFR in PC12 cells cultured in growth medium. At least two individual AS-LIFR stable clones were selected for further experiments with similar results and representative data from one of the AS-LIFR clones is presented in this study. Unlike S-LIFR PC12 cells, NGF-primed AS-LIFR PC12 cells did not exhibit STAT3 tyrosine phosphorylation in response to CNTF treatment, indicating that the lack of LIFR expression in these cells abolished the cytokine response (Fig. 2b). We have employed an additional approach to attenuate the LIFR signaling in PC12 cells by transfecting a dominant negative LIFR construct such that the heterodimerization and the downstream JAK-STAT signaling pathway would not be activated in response to cytokine treatment (Fig. 2a). PC12 cells stably transfected with DN-LIFR were selected based on the expression of DN-LIFR-GFP fusion protein as revealed under the fluorescence microscope. At least two individual DN-LIFR stable clones were selected for further experiments, and representative data from one of the clones is presented in this study. The expression of DN-LIFR was confirmed by Western blotting using an anti-LIFR antibody (data not shown). Expression of the truncated LIFR-GFP fusion protein partially inhibited CNTF induced STAT3 tyrosine phosphorylation (~60%) in PC12 cells (Fig. 2c).

**AS-LIFR and DN-LIFR PC12 cells showed enhanced NGF-induced neurite outgrowth and increased β-tubulin III expression**

Unlike parental or S-LIFR PC12 cells, AS-LIFR PC12 cells extended short processes in the absence of NGF and exhibited larger cell diameter (Fig 3a-c). Upon treatment with NGF, AS-LIFR cells rapidly extended longer neurites when compared to S-LIFR cells (Fig. 3a&c). The observed morphological difference in both S-LIFR and AS-LIFR cells persisted after NGF application for 7 days. Prior to NGF treatment, DN-LIFR cells were morphologically indistinguishable when compared to the mock
transfectants (Fig. 3a). Like AS-LIFR cells, DN-LIFR cells also extended longer neurites after being treated with NGF for 2 days. However, the morphological difference between mock and DN-LIFR cells was less obvious after 7 days of NGF treatment (Fig. 3a&d). The extent of neurite outgrowth in AS-LIFR cells following combined treatment with NGF and LIF was similar to that observed with NGF alone (Fig. 3e).

PC12 cells expressing LIFR mutants were treated with NGF for 14 days and the expression of β-tubulin type III was examined by Western blot analysis. AS-LIFR cells expressed a higher basal level of β-tubulin III when compared to S-LIFR control (Fig. 3f) and the expression was maintained throughout the course of NGF treatment. Unlike AS-LIFR cells, the induction of β-tubulin III expression in S-LIFR cells was more delayed (Fig. 3g).

**Blockade of LIFR signaling enhanced basal and NGF-induced Rac1 activation**

As the small GTPase Rac1 is required for the NGF-induced neurite outgrowth, we next examined whether Rac1 activation was involved in the spontaneous and enhanced neurite outgrowth observed in AS-LIFR and DN-LIFR cells. Consistent with previous reports (29), NGF induced rapid activation of Rac1, with the maximum activity detected at 3 min (Fig. 4a). In AS-LIFR cells, NGF induced further elevation of Rac1 activity at 3 min (~3-fold increase when compared to S-LIFR). Similar enhancement of Rac1 activity was observed with DN-LIFR cells (data not shown). The subcellular distribution of Rac1 and F-actin following NGF treatment of PC12 cells was also examined. When S-LIFR PC12 cells were treated with NGF for 3 min, both Rac1 and F-actin were induced to translocate to the protrusions. Prior to NGF addition, Rac1 and F-actin were already located at the protrusions in AS-LIFR cells (Fig. 4b) and further NGF-induced Rac1 translocation was not observed in these cells. Since the basal level of Rac1 activity was higher in AS-LIFR than S-LIFR cells (Fig. 5a), we examined whether such elevated level
of basal Rac1 activation might lead to spontaneous neurite outgrowth in AS-LIFR cells after replating of the cells in the absence of NGF. PC12 cells were removed from the plates and allowed to settle on another tissue culture plate for the time period indicated in Fig. 5b. The distribution of Rac1 and F-actin in S-LIFR and AS-LIFR cells was examined after replating. While S-LIFR cells remained round in shape after replating, short protrusions extended from AS-LIFR cells after 1 hr of replating. Interestingly, Rac1 and F-actin were found to translocate to the tips of protrusions in replated AS-LIFR cells in the absence of NGF (Fig. 5b), in a manner similar to that observed in PC12 cells following NGF treatment (Fig. 4b). The extension of these short processes occurred in parallel with the translocation of Rac1 and F-actin.

**Overexpression of LIFR retarded the NGF-induced neurite outgrowth**

As both AS-LIFR and DN-LIFR constructs could enhance the NGF-induced neurite outgrowth in PC12 cells, we examined whether overexpression of LIFR could antagonize this effect. We co-transfected rat LIFR construct with GFP construct and stable clones were selected based on the expression of LIFR and GFP expression as revealed under the fluorescence microscope. At least two stable clones with high basal LIFR expression were selected for further analysis, and representative data from one of the clones is presented in this study. We found that LIFR cells could respond to CNTF stimulation by STAT3 tyrosine phosphorylation in the absence of NGF priming (Fig. 6a). Unlike the mock transfectants, these LIFR overexpressing cells only extended short neurites upon NGF treatment (Fig 6b&c). Similar inhibitory effects on neurite outgrowth were obtained with PC12 cells stably expressing human LIFR construct (data not shown). In the presence of exogenous LIF, the inhibition of NGF-induced neurite outgrowth observed in LIFR overexpressing cells was not affected (Fig. 6d). Together with our finding on the induction of LIF expression following NGF treatment, these results
indicate that the retardation of NGF-induced neurite outgrowth in LIFR overexpressing cells is mediated by an autocrine mode of action.

**Overexpressing LIFR showed enhanced RhoA activity and decreased β-tubulin III expression**

During the initiation phase of NGF-induced differentiation of PC12 cells, RhoA activity was inhibited by NGF (30, 31). We found that in LIFR overexpressing PC12 cells, basal level of Rac1 activity was reduced while RhoA activity in both control and NGF-treated cultures was enhanced (Fig. 7a-c), consistent with the possibility that the inhibition of neurite outgrowth in these cells was mediated by an increase in Rho activity and inhibition of Rac1. Unlike mock transfectants, which showed an induction of β-tubulin III expression upon NGF treatment, PC12 cells overexpressing LIFR did not exhibit an enhanced expression of this neuronal marker (Fig. 7d).

**Activation of neuropoietic cytokine signaling suppressed neuritic growth and branching of cultured sympathetic neurons**

Primary culture of sympathetic neurons prepared from superior cervical ganglia (SCG) is widely used for the study of the regulation of cholinergic phenotype by neuropoietic cytokines (32). When SCG neurons were plated on a poly-D-lysine coated tissue culture dish, extension of neurites can be observed within one day in the presence of NGF. We examined whether activation of the LIFR pathway could inhibit neurite extension of sympathetic neurons, in a manner similar to that observed with LIFR overexpressing PC12 cells. When compared to treatment with NGF alone, sympathetic neurons treated with both CNTF and NGF exhibited reduction in neurite length on day 1 (Fig. 8a&b). The level of phosphorylated PAK protein, effector target of Rac1, was also lower in these neurons following combined treatment with NGF and CNTF (Fig. 8c). Interestingly, while the inhibitory effect of CNTF on neurite growth was not obvious by
day 2, we observed a significant decrease in average branch tip numbers in NGF and CNTF-treated sympathetic neurons (Fig. 8d&e).

**Increased neuritic growth and branching in sympathetic neurons of LIFR mutant mice**

To examine the *in vivo* role of LIFR on neurite outgrowth of sympathetic neurons, we examined the neurite extension of SCG neurons prepared from LIFR-deficient mice. Consistent with the above observations that addition of CNTF inhibited neurite outgrowth in sympathetic neurons, a small but statistically significant increase in neurite growth was detected in SCG neurons lacking LIFR. We also compared the extent of neuritic branching in sympathetic neurons isolated from LIFR mutant mice and wild type controls. A significant increase in average branch tip numbers was observed in developing sympathetic neurons that lack LIFR (Fig. 9).
Discussion

The present study provides evidence for a key modulatory role of LIFR signaling during the process of neuronal differentiation. We report here that attenuation of LIFR signaling by stably expressing antisense LIFR or dominant negative LIFR results in enhanced initiation and extension of neurites in PC12 cells. Increased Rac1 activation, both at basal level and upon NGF treatment, is detected in these stably transfected cells. On the contrary, neurite outgrowth and Rac1 activation are suppressed in PC12 cells stably expressing LIFR. During the later stage of NGF-induced differentiation in PC12 cells, increased LIFR expression allow the differentiating neurons to respond to neuropoietic cytokines such as CNTF. NGF also specifically induces the expression of neuropoietic cytokines, such as LIF and OSM, raising the possibility of an autocrine mode of action in PC12 cells during neuronal differentiation. Taken together with the induction of IL-6 expression after NGF priming (33, 34), activation of both gp130 and LIFR signaling can be observed during the process of NGF-induced neuronal differentiation. Consistent with a negative modulatory role of LIFR signaling, treatment of sympathetic neurons with CNTF inhibits the extension of neurites and branching induced by NGF. More importantly, sympathetic neurons prepared from LIFR mutant mice exhibit increased extension and branching of neurites, in a manner similar to that observed with PC12 cells expressing LIFR mutants. Taken together, our findings reveal an important role for LIFR signaling in modulating the neurite outgrowth and branching of sympathetic neurons during development.

Early studies on the functions of neuropoietic cytokines in the nervous system have identified their involvement in the promotion of survival of different neuronal cell types, maintenance of neural stem cells in an undifferentiated state, as well as regulation of differentiation of both peripheral and spinal cord neurons (13, 35-37). In addition,
these cytokines have also been reported to modulate neuronal phenotype and function by controlling the expression of neurotransmitters and neuropeptides, and regulate the development of glial cells in the CNS (13, 35-37). In the present study, the basal mRNA expression of endogenous LIFR is low during the early phase of neuronal differentiation in PC12 cells. It is possible that such attenuated LIFR signaling might facilitate the initiation of neuritic spikes and allow PC12 cells to enter into committed neuronal differentiation state induced by NGF. Interestingly, LIFR expression is also reduced in the region immediately proximal and distal to the lesion site of nerve injury, perhaps providing a conducive environment for regeneration (38). It is well documented that heterodimerization of LIFR and gp130 leads to STAT3 activation and mediates the actions of several neurotrophic cytokines, such as CNTF and LIF (1). Inhibition of STAT3 activation will likely lead to similar functional consequences as reduced expression of LIFR. Consistent with the present study, mutations in gp130 cytoplasmic region resulting in inhibition of STAT3 activation could also initiate neurite outgrowth (34). However, using a PC12 variant cell line, PC12-E2, Wu et al. demonstrated that sustained signaling of STAT3 triggered by IL-6 was effective in inducing neuronal differentiation (39, 40). It is possible that the signals required for differentiation in this variant was different and reflected another cytokine-dependent neuronal differentiation mechanism during a particular stage of neuronal development.

Our study also sheds light on the molecular mechanisms through which LIFR signaling regulates neurite outgrowth. PC12 cells respond to NGF stimulation by neurite initiation and elongation. The precise molecular mechanisms underlying these morphological changes, however, are just beginning to be unraveled. A recent study on NGF-induced neurite initiation in PC12 cells suggests that small GTPases Rac1 and RhoA are required for the initiation of neurite outgrowth (29-31). Activation and
localization of Rac1, together with inactivation of RhoA, play crucial roles in inducing neurite outgrowth of PC12 cells. In the present study, PC12 cells stably expressing AS-LIFR extend short protrusions even without NGF treatment. The less pronounced basal level of neurite initiation observed in DN-LIFR PC12 cells might be due to the incomplete blockade of LIFR signaling or alternatively, different receptor-receptor interactions on the cell surface based on the formation of ligand-free dimers between CNTFR and gp130/LIFR (41, 42). The enhanced Rac1 activity observed in AS-LIFR and DN-LIFR cells is consistent with the central tenet that Rac1 activity plays a critical role in the initiation of neurite outgrowth. It is noteworthy that the expression of PAK1, one of several effector targets of Rac1, is also higher in AS-LIFR and DN-LIFR PC12 cells (unpublished observations). Thus, down-regulation of LIFR during the initiation of neurite outgrowth can regulate activation of small GTPases like Rac1 and RhoA, and their effectors such as PAK1, which leads to neurite extension. Reduced basal Rac1 activity and decrease in NGF-induced Rac1 activation, together with increased RhoA activity, in LIFR PC12 cells provide further evidence that LIFR signaling modulates neurite outgrowth through inhibition of Rac1 signaling and enhanced RhoA activation.

Axon growth, guidance and branching are essential features of the precise wiring in the nervous system. It has been suggested that progressive loss of combined Rac activity affects multiple aspects of axon development, leading first to defects in axon branching, then guidance and finally growth (43). We have demonstrated that NGF and CNTF-treated sympathetic neurons led to a lower level of PAK1 activation, which might account for the slower axonal growth and reduced number of branches. For sympathetic neurons, one source of neuroepoietic cytokines is the target of sympathetic innervation, such as submaxillary glands, where cholinergic differentiation occurs (44). Activation of LIFR at the time of target innervation thus help to eliminate excess branches and trigger a
“stop” signal for the axon outgrowth by regulating small GTPase activity and suppressing the expression of neuronal cytoskeletal proteins. The finding that NGF was present in submaxillary glands further suggesting that NGF signaling and neuropoietic cytokine signaling might contribute to the wiring at the site of target innervation (45). While the structures of the CNS appear normal in LIFR deficient mice, analysis of the sections of spinal cord taken from E18.5 LIFR mutant mice reveal abnormal phenotype with thickened processes in the area between neuronal bodies (25). Interestingly, we also observe that neurite length and branching are enhanced in sympathetic neurons lacking LIFR expression. Moreover, when primary cultures of sympathetic neurons are treated with NGF and CNTF, we observe a reduction in neurite length and branching of axons. This is consistent with previous findings implicating an important role of CNTF in dendritic retraction following treatment of cultured rat sympathetic neurons with OP-1 (46, 47).

We have provided evidence that LIFR signaling, besides the known functions in supporting survival and controlling phenotypic switch, also regulates morphological changes during neuronal differentiation through regulation of Rac1 and RhoA activity and controlling the expression of neuronal cytoskeletal proteins such as β-tubulin III. We have observed a significant reduction of neurite branching in sympathetic neurons treated with CNTF, and conversely, an increased neurite branching sympathetic neurons lacking LIFR. Taken together with the observations in PC12 cells, it is an intriguing possibility that the induction of LIFR signaling exerts an inhibitory effect on neurite outgrowth, leading to branching elimination in the developing neurons. Thus, in addition to inhibiting neurite outgrowth, LIFR activation might function to reduce neurite branching during development and help to establish the precise wiring of the nervous system.
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Figure Legends

Figure 1. **LIFR expression and STAT3 phosphorylation in PC12 cells treated with NGF.** a. PC12 cells were cultured in normal high serum medium (GM) or low serum medium (DM) for 1 day. Total RNA was isolated and subjected to RT-PCR analysis of LIFR, gp130 and CNTFRα. Glyceraldehyde-3-phosphorylate dehydrogenase (GAPDH) levels served as control. PC12 cells were treated with 50 ng/ml NGF for the indicated number of days and total cell lysates were subjected to RT-PCR analysis for LIFR, LIF and OSM (b) or Western blot analysis using LIFR antibody (c). Actin level served as a loading control. d. Phosphorylation of STAT3 on tyrosine residue was examined in PC12 cells treated with NGF for 2-14 days. Antibody specific for the phosphorylated Tyr705 form of STAT3 was used. e. PC12 cells were primed with 50 ng/ml NGF or EGF in low serum medium for 7 days. Cells were then washed and treated with CNTF or LIF (50 ng/ml) for 15 min. STAT3 phosphorylation was detected as described in d. The blot was stripped and rebotted with STAT3 antibody and LIFR antibody.

Figure 2. **AS-LIFR and DN-LIFR expressing PC12 cells.** a. Schematic diagram of antisense and dominant negative rat LIFR constructs, depicting the domains of antisense LIFR (AS-LIFR) and dominant negative LIFR (DN-LIFR). Sig. pep. indicates signal peptide; CBD1, cytokine binding domain I; Ig, Immunoglobulin-like domain; CBD2, cytokine binding domain II; Fibronectin type III, fibronectin-type III domain; Transmembrane; transmembrane domain; Cytoplasmic, cytoplasmic domain. Open bars in the CBDs, conserved cysteine residues; Black bars, conserved WSXWS motif. b. Stable clones of PC12 cells transfected with S-LIFR or AS-LIFR constructs were selected. S-LIFR and AS-LIFR PC12 cells were primed with NGF (50 ng/ml) for 7 days. Activation of LIFR upon CNTF (50 ng/ml) treatment in NGF-primed S-LIFR and AS-
LIFR cells (b) or mock and DN-LIFR cells (c) was detected using antibody against phosphorylated Tyr705 form of STAT3. The blot was stripped and reblotted with anti-STAT3 antibody.

Figure 3. **NGF-induced neurite outgrowth and β-tubulin III expression in AS-LIFR and DN-LIFR expressing PC12 cells.** a. Stable clones of PC12 cells transfected with mock (pEGFP-N1), S-LIFR, AS-LIFR, and DN-LIFR constructs were subjected to NGF (50 ng/ml) treatment. Cells were observed under the microscope with phase contrast after 0, 2 or 7 days of NGF treatment. Scale bar, 50 μm. Quantitation of the longest diameter (b) and length of the longest neurite (c) after NGF treatment was determined for S-LIFR and AS-LIFR cells. *, p<0.01. d. Quantitation of the length of the longest neurite in mock and DN-LIFR cells following NGF treatment. *, p<0.01. e. Quantitation of the length of the longest neurite in PC12 cells expressing LIFR mutants following treatment with NGF or NGF and LIF (50 ng/ml) for 7 days. The values represent the average ± S.E.M., n=3 dishes. Basal level (f) and NGF-induced expression (g) of β-tubulin III in S-LIFR and AS-LIFR PC12 cells.

Figure 4. **NGF-induced Rac1 activity in AS-LIFR and DN-LIFR cells.** a. S-LIFR and AS-LIFR PC12 cells were exposed to NGF (50 ng/ml) at the times indicated. Activated Rac1 was measured using agarose bead-PAK and the amount of activated Rac1 and total Rac1 was determined using Rac1 specific antibody. b. Subcellular distribution of endogeneous Rac1 and F-actin in NGF-stimulated S-LIFR and AS-LIFR PC12 cells. After stimulation with NGF for 3 min, cells were fixed and stained with Rac1 specific antibody (green) and rhodamine-conjugated phalloidin (red). Merged images are on the right panels. The results shown are representative of four independent experiments. Scale bar, 50 μm.
Figure 5. **Basal Rac1 activity in AS-LIFR and DN-LIFR cells.** a. The basal Rac1 activity in PC12 cells (including S-LIFR and AS-LIFR cells, mock and DN-LIFR) was determined. Activated Rac1 was measured using agarose bead-PAK and the amount of activated Rac1 and total Rac1 was determined using anti-Rac1 antibody. b. Subcellular distribution of endogenous Rac1 and F-actin in S-LIFR and AS-LIFR PC12 cells after replating. After the indicated times of replating, cells were fixed and stained with a Rac1 specific antibody (green) and rhodamine-conjugated phalloidin (red). Merged images are on the right panels. The results shown are representative of three independent experiments. Scale bar, 50 μm.

Figure 6. **NGF-induced neurite outgrowth in PC12 cells overexpression LIFR.** Stable clones of PC12 cells transfected with pcDNA3 (mock) or LIFR expression constructs were selected. a. Mock transfectants and LIFR cells kept in normal medium were washed twice with DMEM alone and starved for 4 hr. Cells were then treated with 50 ng/ml CNTF for 15 min. LIFR expression in the representative clones was shown by Western blot analysis with antibody against LIFR. STAT3 phosphorylation was detected using antibody against phosphorylated Tyr705 form of STAT3. The blot was stripped and rebotted with STAT3 antibody. b. Stable clones of PC12 cells transfected with pcDNA3 (mock) or LIFR constructs were treated with 50 ng/ml NGF. Cells were observed at the indicated times under the microscope with phase contrast. Scale bar, 50 μm. c. Quantitation of the length of the longest neurite in mock and LIFR cells following NGF treatment. *, p<0.01. d. Quantitation of the length of the longest neurite in mock and LIFR cells following treatment with NGF or NGF and LIF (50 ng/ml) for 7 days.

Figure 7. **RhoA and Rac1 activities in LIFR PC12 cells.** Stable clones of PC12 cells transfected with pcDNA3 (mock) or LIFR were treated with 50 ng/ml NGF. a. Basal
level of Rac1 activity in mock and LIFR cells. Total Rac1 expression was determined by Rac1 antibody. Basal level of RhoA activity (b) or after treatment with 50 ng/ml NGF for the times indicated (c) were measured in mock and LIFR cells. Total RhoA was determined by RhoA antibody. d. Following treatment with 50 ng/ml NGF for the times indicated, total cell lysates from mock and LIFR PC12 cells were subjected to immunoblotting with antibody against β-tubulin III. The blots were stripped and rebotted with antibodies against actin.

Figure 8. **Primary cultures of SCG neurons treated with NGF and CNTF.** Postnatal day 1 superior cervical ganglia were collected and primary sympathetic neuron culture was prepared. Cultures were treated with NGF supplemented with or without CNTF. a&b. Morphological depiction of representative neurons on day 1 and quantification of length of the longest neurite. c. Western blot analysis of p-thr PAK and total PAK1 in these cultures; actin served as control. d. Morphological depiction of representative neurons on day 2 and quantification of branch tips in these cultures (e). *, p<0.01. The values represent the average ± S.E.M., n = 3 dishes.

Figure 9. **Primary cultures of SCG neurons prepared from LIFR deficient mice showed enhance neurite growth.** Embryonic day 18 superior cervical ganglia were collected from LIFR deficient mice and wild type littermates. Dissociated neuronal cultures were prepared and treated with or without NGF. a. Morphological representation of neurons from wild type (LIFR+/+) and LIFR deficient (LIFR-/-) mice on day 1. b. Quantitative analysis of these neurons in terms of the length of the longest neurite, total neurite length and tip addition. *, p<0.01.
Fig. 1

(a) Western blot analysis showing the expression levels of LIFR, gp130, CNTFRα, and GAPDH in GM and DM conditions. LIFR expression is not detectable in GM, but is present in DM. gp130 and CNTFRα show similar expression patterns in both conditions. GAPDH serves as a loading control.

(b) Time course of LIFR, LIF, OSM, and GAPDH expression levels in untreated and NGF-treated cells. LIFR expression is not detectable in untreated cells but is induced by NGF treatment. LIF and OSM show comparable expression levels in both conditions.

(c) NGF treatment in cell extracts at different time points (0, 2, 4, 8, 10, 14 days). LIFR expression is not detectable in the absence of NGF, but is induced by NGF treatment. Actin serves as a loading control.

(d) Time course of p-tyr STAT3 and STAT3 expression levels in NGF-treated cells. p-tyr STAT3 is induced by NGF treatment, while STAT3 expression remains relatively constant.

(e) Effect of CNTF and LIF on p-tyr STAT3, STAT3, and LIFR expression in unprimed and NGF-treated cells. EGF treatment results in no significant change in expression levels.
Fig. 2

(a) Schematic diagram of LIFR and its mutants. Sig. Pep. stands for signal peptide, CBD1 and CBD2 for cysteine-rich domains, Ig for immunoglobulin-like domain, and cytoplasmic for the cytoplasmic region.

(b) Western blot analysis of S-LIFR and AS-LIFR with antibodies against p-tyr STAT3 and STAT3. CNTF treatment showed an increase in p-tyr STAT3 and STAT3 levels.

(c) Western blot analysis of Mock and DN-LIFR with antibodies against p-tyr STAT3 and STAT3. CNTF treatment showed an increase in p-tyr STAT3 and STAT3 levels in Mock, but not in DN-LIFR.
Fig. 3

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Fig. 5

(a) Western blot analysis showing GTP-Rac1, Total Rac1, and Actin levels across different conditions: S-LIFR, AS-LIFR, Mock, and DN-LIFR.

(b) Immunofluorescence images showing Rac1 (green), F-actin (red), and merged images for S-LIFR and AS-LIFR at 1 hr, 2 hr, 4 hr, and 12 hr.
Fig. 7

(a) GTP-Rac1
(b) GTP-RhoA
(c) GTP-RhoA
(d) β-tubulin III
Fig. 8

(a) Neurites with NGF and NGF+CNTF. 

(b) Neurite length comparison between NGF and NGF+CNTF. 

(c) Western blot analysis showing p-thr PAK, PAK1, and Actin levels in NGF and NGF+CNTF. 

(d) Stained neurites with NGF and NGF+CNTF. 

(e) Tip addition comparison between NGF and NGF+CNTF. 

* indicates statistical significance.
Fig. 9

a

LIFR^{+/+}

LIFR^{-/-}

b

<table>
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<th>Neurite Length (µm)</th>
<th>Total Length (µm)</th>
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<td>200 ± 10</td>
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Yu Pong Ng, Wei He and Nancy Y. Ip

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