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 interleukin-6 family, is specifically up-regulated 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, whereas NGF-induced Rac1 activity is enhanced in antisense-LIFR and dominant negative-LIFR expressing PC12 cells, it is reduced in LIFR expressing PC12 cells. Following combined treatment with NGF and ciliary neurotrophic factor, 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 with 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 effects on neurite extension and branching of sympathetic neurons.

Leukemia Inhibitory Factor Receptor Signaling Negatively Modulates Nerve Growth Factor-induced Neurite Outgrowth in PC12 Cells and Sympathetic Neurons*

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LIFR Signaling Inhibits Neurite Outgrowth

the dynamic regulation of the neuronal cytoskeleton and neurolapoticity (19, 20). NGF has been shown to collaborate with the neuroplasticity 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 up-regulated by NGF in PC12 cells. Whereas 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 exerts 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 (5%, v/v), heat-inactivated fetal bovine serum (6%, v/v), penicillin (50 units/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 3 days. For NGF-induced differentiation, cells were plated at a density of 1 × 105 cells/dish 1 day before treatment with the appropriate growth factors, and serum concentration was reduced to 1%. NGF (Alomone labs) 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 subsequent PCR amplification. PCR primers and conditions for the amplification of gp130, LIFR, and CNTFR fragments were according to the previous report (27). Primers used were: LIF, 5′-AGTCAACTGCTCAGATCAAGC-3′ and 3′-CTGGAGCCAGCACTAATGAC-5′; 5′-CTGGACCCAGCACTAATGAC-3′ and 3′-CATGAACGGGTATGACG3′-; and 5′-GACGTATGGCCTC-3′ and 3′-GACGTATGGCCTC-3′. PCR products were separated on a 1% agarose gel and then transferred onto nylon membranes. DNA blots were hybridized with appropriate probes and exposed to X-ray film at –80 °C.

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, 2.5 mM MgCl2, 0.5 mM dithiothreitol, and 10 units/µl microsome II reverse transcriptase) and centrifuged at 4°C for 10 min. Both supernatant and pellets were subjected to Western blotting using an anti-β-tubulin III antibody (Sigma). STAT3 specific antibody (Cell Signaling) was used for the detection of total STAT3 expression. The Bradford method (Bio-Rad) was used to determine the protein concentration. Total cell lysates (50 µg) were subjected to SDS-PAGE followed by Western blotting using an anti-phosphotyrosine STAT3 (Tyr-705) antibody (Cell Signaling). After stripping membranes in a stripping buffer (0.1 M β-mercaptoethanol, 62.5 mM Tris, pH 6.7, 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, seeded in 100-mm tissue culture dishes at 105 cells/plate, were cultured for 24 h. Cells were treated with NGF (10 ng/ml) for the indicated times and lysed for 30 min with the cell lysis buffer (25 mM HEPES, pH 7.5, 150 mM NaCl, 10 mM MgCl2, 1% Nonidet P-40, 1 mM Na3VO4, and 2 µl/ml leupeptin, 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/glutathione S-transferase/p21 binding domain for Rac1 activity assay or agarose/glutathione S-transferase/Rhotekin Rho binding domain for RhoA activity.
activity assay with continuous rotation for 1 h at 4 °C. After the beads were washed three times with the cell lysis buffer, the bound proteins were eluted by boiling in 2× 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-Rhod antibody (1:1,000) followed by a 1-h incubation of horse-radish peroxidase-conjugated secondary antibody (1:2,000) and detected using the ECL Western blot system (Amersham Biosciences). Densitometry analysis was performed using Adobe Photoshop 7.0 software (Adobe Systems Inc.), 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 with 4% paraformaldehyde for 30 min at room temperature. After blocking with 4% fetal bovine serum in phosphate-buffered saline 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) followed by a 1-h incubation of fluorescein isothiocyanate-conjugated secondary antibody (1:2,000) and detected using the ECL Western blot system (Amersham Biosciences). Densitometry analysis was performed using Adobe Photoshop 7.0 software (Adobe Systems Inc.), and the amounts of active Rac1 and RhoA were normalized by total amounts of Rac1 and RhoA in cell lysates, respectively.

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 version 5.0r1 software (Universal Imaging Corp.), whereas 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 were counted, and n = 3 dishes. Each experiment was repeated at least three 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 Up-regulated 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 that was conductive for neuronal differentiation. Reverse-transcription PCR analysis revealed the reduction of LIFR mRNA in low serum condition, whereas 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. 1, b and c). The expression of gp130 and CNTFR, however, remained unchanged in response to NGF (data not shown). Whereas EGF also induced Erk phosphorylation (10, 28), it did not induce LIFR expression after treat-
ment 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 phospho-
ylation of STAT3 on tyrosine 705 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).

Whereas 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
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. 3, a–c). Upon treatment with NGF, AS-LIFR cells rapidly extended longer neurites when compared with S-LIFR cells (Fig. 3, a and 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 with 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. 3, a and 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 with 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).
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 with 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. Because the basal level of Rac1 activity was higher in AS-LIFR than S-LIFR cells (Fig. 5a), we examined whether such elevated levels 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. Whereas S-LIFR cells remained round in shape after replating, short protrusions extended from AS-LIFR cells after 1 h 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. 6, b and c). Similar inhibitory effects on neurite outgrowth were obtained with PC12 cells stably expressing the 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, the basal level of Rac1 activity was reduced, whereas RhoA activity in both control and NGF-treated cultures was enhanced (Fig. 7, a–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 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-L-lysine-coated tissue culture dish, extension of neurites can be observed within 1 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 with treatment with NGF alone, sympathetic neurons treated with both CNTF and NGF exhibited reduction in neurite length on day 1 (Fig. 8, a and b). The level of phosphorylated PAK protein, effector target of Rac1, was also lower in these neurons following the combined treatment with NGF and CNTF (Fig. 8c). Interestingly, whereas the inhibitory effect of CNTF on neurite growth was not obvious by

![Fig. 6. NGF-induced neurite outgrowth in PC12 cells overexpression LIFR. Stable clones of PC12 cells transfected with pDNA3 (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 h. 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 the phosphorylated Tyr-705 form of STAT3. The blot was stripped and reblotted with antibody against -Tubulin III.](image-url)
day 2, we observed a significant decrease in average branch tip numbers in NGF and CNTF-treated sympathetic neurons (Fig. 8, d and 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 central nervous system (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 a 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 neuropoietic 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 and Bradshaw (39, 40) demonstrated that sustained signaling of STAT3 triggered by IL-6 was effective in inducing neuronal differentiation. It is possible that the signals required for differentiation in this variant were 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 because of 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. Thus, down-regulation of LIFR during the

\[ a \] Y. P. Ng, W. He, and N. Y. Ip, unpublished observations.
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 neurotrophic cytokines is the target of sympathetic innervation, such as submaxillary glands, where cholinergic differentiation occurs. Cytokines is the target of sympathetic innervation, such as submaxillary glands, where cholinergic differentiation occurs.

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 in 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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REFERENCES
1. Ip, N. Y., and Yancopoulos, G. D. (1996) Annu. Rev. Neurosci. 19, 491–515
2. Chaos, M. V. (2000) Nat. Rev. Neurosci. 1, 399–399
3. Huang, E. J., and Reichardt, L. F. (2003) Annu. Rev. Biochem., in press
4. Greene, L. A., and Tischler, A. S. (1976) Proc. Natl. Acad. Sci. U. S. A. 73, 2424–2428
5. Kalman, D., Wong, B., Horvai, A. E., Cline, M. J., and O’Lague, P. H. (1990) Science 244, 355–366
6. Reeben, M., Neuman, T., Polgi, J., Palm, K., Paalne, V., and Saarma, M. (1995) J. Neurosci. Res. 40, 177–188
7. Pengrae, J. L., and Rylett, R. J. (1996) Brain Res. Mol. Brain Res. 42, 25–34
8. Zurcher, E., Han, S. Y., Pessoa-Brandao, L., Butterfield, L., and Heasley, L. E. (2002) J. Biol. Chem. 277, 4110–4118
9. Tordosok, B., Angelastro, J. M., and Greene, L. A. (2002) J. Neurosci. 22, 8971–8980
10. Markus, A., Patel, T. D., and Snider, W. D. (2003) Curr. Opin. Neurobiol. 12, 523–531
11. Taga, T., and Kishimoto, T. (1997) Annu. Rev. Immunol. 15, 797–819
12. Turner, A. M., and Bartlett, P. F. (2000) J. Neurochem. 76, 839–849
13. Heinrich, P. C., Behrmann, I., Haan, S., Herrmanns, H. M., Muller-Newen, G., and Schaper, F. (2003) Biochem. J. 374, 1–20
14. Elson, G. C., Lefevre, E., Guillet, C., Chevalier, S., Plun-Favreau, H., Froger, J., Saud, I., de Cognac, A. B., Delinaite, Y., Bonsejofy, J. F., Gauchat, J. F., and Gaecan, H. (2000) Nat. Neurosci. 3, 867–872
15. Ihle, J. N., Wittbuhr, B. A., Quelle, F. W., Yamakami, K., and Silvennoinen, O. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 7699–7704
16. Rane, S. G., and Reddy, E. P. (2000) Oncogene 19, 5662–5679
17. Levy, D. E., and Darnell, J. E., Jr. (2003) J. Biol. Chem. 277, 615–622
18. Ohnati, T., Ishihara, K., Atsumi, T., Nishida, K., Kaneko, Y., Miyata, T., Itoh, S., Narumiya, M., Maeda, H., Fukada, T., Itoh, M., Okano, H., Hibi, M., and Hiranoe, T. (2000) Immunity 12, 95–105
19. Gallo, G., and Letourneau, P. C. (2000) J. Neurobiol. 44, 159–173
20. McAllister, A. K., Katz, L. C., and Lo, D. C. (1999) Nat. Neurosci. 2, 288–292
21. Ip, N. Y., Boulton, T. G., Li, Y., Verdi, J. M., Sirven, A. J., Besse, D. M., and Anderson, D. J. (1996) J. Biol. Chem. 271, 700–708
22. Wu, Y. Y., and Bradshaw, R. A. (2000) J. Biol. Chem. 275, 2147–2156
23. He, W., Gong, K., Zhu, G., Smith, D. K., and Ip, N. Y. (2002) Proc. Natl. Acad. Sci. U. S. A. 99, 13023–13032
24. Wong, V. Y., Pearsall, D., Arriaga, R., Ip, N. Y., Staihi, N., and Lindsay, R. M. (1995) J. Biol. Chem. 270, 531–536
25. Vargiu, B., and Guo, X. (1999) J. Biol. Chem. 274, 1457–1463
26. Ip, F. C., Fu, A. K., Tsim, K. W., and Ip, N. Y. (1995) J. Biol. Chem. 270, 2393–2400
27. Zhao, A., Zhang, W., and Liu, R. (1995) J. Neurosci. Res. 42, 1457–1461
28. Greene, L. A., and Tischler, A. S. (1976) J. Neurosci. Res. 68, 1–20
29. Pessina, L., and Mccarthy, S. M. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 9013–9020
30. Fassler, R., and Patterson, P. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7109–7113
31. Visweswariah, S. K., Chinnaiyan, A. M., and Weiss, S. (2001) J. Neurosci. 21, 7642–7653
32. Moon, C., Yao, J. Y., Matarazzo, V., Sung, Y. K., Kim, E. J., and Ronnett, G. V. (1999) Proc. Natl. Acad. Sci. U. S. A. 96, 18977–18983
33. Lemmon, M. R., and Patterson, P. H. (1994) Proc. Natl. Acad. Sci. U. S. A. 91, 7109–7113
34. Wu, Y. Y., and Bradshaw, R. A. (1996) J. Biol. Chem. 271, 13023–13032
35. Wu, Y. Y., and Bradshaw, R. A. (2000) J. Biol. Chem. 275, 2147–2156
36. Ip, N. Y., Boulton, T. G., Li, Y., Verdi, J. M., Sirven, C. J., and Anderson, D. J. (1996) J. Biol. Chem. 271, 13023–13032