Effects of the gp130 cytokines ciliary neurotropic factor (CNTF) and interleukin-11 on pituitary cells: CNTF receptors on human pituitary adenomas and stimulation of prolactin and GH secretion in normal rat anterior pituitary aggregate cultures

C Perez Castro, A Carbia Nagashima, M Páez Pereda[1],
V Goldberg[2], A Chervin[3], G Carrizo[3], H Molina[4], U Renner[1],
G K Stalla[1] and E Arzt

Laboratorio de Fisiología y Biología Molecular, Departamento de Biología, FCEN, Universidad de Buenos Aires, Buenos Aires, Argentina

[1]Max-Planck Institute of Psychiatry, Department of Endocrinology, Kraepelinstrasse 10, 80804 Munich, Germany

[2]Instituto de Investigaciones Médicas, Facultad de Medicina, Universidad de Buenos Aires, Donato Alvarez 3150, 1427 Buenos Aires, Argentina

[3]Hospital Santa Lucía, Av. San Juan 2021, 1232 Buenos Aires, Argentina

[4]Hospital Ramos Mejía, 1221 Buenos Aires, Argentina

(Requests for offprints should be addressed to E Arzt, Laboratorio de Fisiología y Biología Molecular, Departamento de Biología, FCEN, Universidad de Buenos Aires, Ciudad Universitaria, Pabellon II, 1428 Buenos Aires, Argentina; Email: earzt@bg.fcen.uba.ar)

Abstract

Two of the most potent cytokines regulating anterior pituitary cell function are leukemia inhibitory factor (LIF) and interleukin (IL)-6, which belong to the cytokine family using the common gp130 signal transducer. Recently, the expression and action of two other members of this family, IL-11 and ciliary neurotrophic factor (CNTF), on different cell lines has also been demonstrated. We studied the expression of the specific receptor subunits for CNTF in mammotropic, non-functioning and somatotropic tumors and the action of CNTF and IL-11 in the regulation of hormone secretion in these and normal pituitary cells. The mRNA for the α chain specific for the CNTF receptor was detected by Northern blot in tumors secreting prolactin (PRL) and GH and in non-functioning tumors. We found that both IL-11 and CNTF exerted a similar stimulatory effect on GH mRNA expression in somatotropic monolayer cell cultures from acromegalic tumors, but these cytokines had no significant influence on GH secretion. CNTF stimulates prolactin secretion in lactotropic monolayer cell cultures from patients with prolactinoma. In monolayer cell cultures from normal rat anterior pituitary, IL-11 and CNTF had no significant effect on the release of either GH or PRL, or on GH mRNA. However, when the cells were cultured in aggregate cultures, in which the three-dimensional structure of the cells is reconstituted, both cytokines, in doses at which they had no effect on monolayer cultures, significantly stimulated both PRL and GH secretion. These data show that IL-11 and CNTF may act as regulatory factors in anterior pituitary cells, in which the three-dimensional structure of the gland is of critical importance.

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Introduction

The interleukin (IL)-6 or gp130 cytokine family is composed of IL-6, leukemia inhibitory factor (LIF), IL-11, oncostatin M, ciliary neurotrophic factor (CNTF) and cardiotropin-1 (Kishimoto *et al.* 1995). All these cytokines bind specific receptors that use the gp130 protein as initial cellular signal transducer (Kishimoto *et al.* 1994). Originally IL-6 and LIF, and more recently IL-11 and CNTF, have been shown to be important for the autocrine/paracrine loops that cytokines conform to in the pituitary gland (Ray & Melmed 1997, Arzt *et al.* 1999).

IL-6 and LIF, in addition to their receptors, are expressed in pituitary cell lines on which they regulate hormone secretion and proliferation (Arzt *et al.* 1993, Matsumoto *et al.* 1993, Akita *et al.* 1995, Kobayashi *et al.* 1997, Gloddek *et al.* 1999). Furthermore, their expression and action have been described in great detail in normal anterior pituitary cells (Spangelo *et al.* 1989, 1990, Vankelecom *et al.* 1989, Yamaguchi *et al.* 1990, Lyson & McCann 1991, Ohmichi *et al.* 1992, Akita *et al.* 1995, Stefana *et al.* 1996, Wang *et al.* 1996) and human tumor pituitary cells, further underlying their role in pituitary pathophysiology (Jones *et al.* 1991, 1993, 1994, Tsagarakis *et al.* 1992, Velkeniers *et al.* 1994). Very recently, the

Table 1 Clinical features of human pituitary adenomas

	Invasion	Tumor size	Immunohistochemistry
Clinical symptoms			
Acromegaly (*n*=18)	11 Non-invasive 7 Invasive	2 Microadenomas 16 Macroadenomas	9 GH-reactive 9 GH- and PRL-reactive
Prolactinoma (*n*=6)	3 Non-invasive 3 Invasive	6 Macroadenomas	6 PRL-reactive
Non-functioning (*n*=3)	2 Invasive 1 ND	3 Macroadenomas	3 Unreactive

ND, Not determined.

stimulation of mRNA expression of POMC by IL-6 has been described (Páez Pereda *et al.* 2000*b*).

The action and expression of two other members of this family, IL-11 and CNTF, on different cell lines have also been demonstrated. IL-11 mRNA was detected by RT-PCR and IL-11 receptor (IL-11-R) mRNA by Northern blot in AtT-20 cells (Auernhammer & Melmed 1999). In these cells, IL-11 stimulates the secretion of ACTH and the expression of POMC (Auernhammer & Melmed 1999). In folliculostellate (FS) cells (TtT/GF cell line) and lactosomatotropic cells (GH3 cell line), the expression and action of IL-11 and CNTF have been demonstrated (Perez Castro *et al.* 2000). The mRNA for the α chain specific for the IL-11-R and CNTF receptor (CNTF-R) are expressed on both cell types. IL-11 and CNTF dose-dependently stimulate the proliferation of FS and lactosomatotropic GH3 cells. In addition, CNTF stimulates production of growth hormone (GH) and prolactin (PRL) by GH3 cells and IL-11 stimulates the secretion of the angiogenic factor, vascular endothelial growth factor (VEGF), by FS cells. Furthermore, both GH3 and FS cells express CNTF mRNA.

Expression of CNTF-R and IL-11-R has been demonstrated by Northern blot in normal cells (Auernhammer & Melmed 1999, Perez Castro *et al.* 2000), and expression of IL-11-R has been reported by RT-PCR in corticotropic and non-functioning human tumors (Auernhammer & Melmed 1999). Functional studies in normal and tumoral cells have not been undertaken to date. In the present paper we describe the expression of the specific α subunit of the CNTF-R in mammotropic, nonfunctioning and somatotropic tumors, and the action of CNTF and IL-11 in the regulation of hormone secretion in these and normal pituitary cells.

Materials and Methods

Materials

Cell culture reagents and materials were purchased from Flow Laboratories (Meckenheim, Germany), Gibco Brl Life Technologies (Eggenstein, Germany), Seromed (Berlin, Germany), Sigma Chemical Co. (St Louis, MO, USA), Falcon (Heidelberg, Germany) or Nunc (Wiesbaden, Germany).

Patients

Patients with pituitary adenomas and clinical symptoms of acromegaly, prolactinoma, or non-functioning tumors were diagnosed by plasma pituitary hormone testing and magnetic resonance imaging as previously described (Arzt *et al.* 1992, Páez Pereda *et al.* 1996, 2000*a*). Clinical data of the patients and histological characterization of the tumors are provided in Table 1. Tumor tissue was obtained by trans-sphenoidal surgery. The purity of the samples was assessed by immunohistochemistry for the different pituitary hormones.

Animals

Male Sprague–Dawley rats (200–250 g) were kept in an environment of constant temperature, humidity and day–night cycle; food and water were available *ad libitum*. Glands were obtained within minutes of the animals' death by decapitation and were treated as indicated below. All experimental procedures were approved by the Ethics Committee on Animal Care and Use, University of Buenos Aires, Argentina, following the Guidelines on the Handling and Training of Laboratory Animals published by the Universities' Federation.

Cell culture

Human pituitary adenoma or rat normal pituitary cell cultures were performed as previously described (Páez Pereda *et al.* 1996). Briefly, normal pituitary or adenoma tissue was rinsed three times in PBS and then dissected into small pieces. Tissue fragments were incubated in preparation buffer with the addition of 1000 U/ml collagenase (Worthington Biochemical Corporation, Freehold, NJ, USA), 2 g/l hyaluronidase, 4 g/l BSA, 10 mg/l DNAse II and 1 g/l soybean trypsin inhibitor,

with gentle rocking at 37 °C. Cell dispersion was aided by pippeting after 30 min of incubation. The cell suspension was centrifuged and cells resuspended in culture medium (Dulbecco's modified Eagle's medium (DMEM) pH 7·3 containing 10% fetal calf serum (FCS), 2·2 g/l NaHCO$_3$, 10 mM HEPES, 2 mM glutamine, 10 ml/l non-essential amino acids, 10 ml/l minimal essential medium vitamins, 5 mg/l insulin, 5 mg/l transferrin, 2·5 mg/l amphotericin–B, 105 U/l penicilin/streptomycin, 20 mg/ml sodium selenite, and 30 pM tri–iodothyronine (Henning, Berlin, Germany). Cell viability was routinely greater than 90% as assessed by acridine orange/ethidium bromide staining.

Cells were plated on six-well plates at a density of 3×10^5 cells/ml for RNA determinations or on 24–well plates at a density of 2×10^5 cells/ml for secretion experiments and incubated for 3 days under an atmosphere of 5% CO$_2$ at 37 °C. After cell adhesion to the plates had been verified, the culture medium was replaced by stimulation medium (DMEM pH 7·3 containing 2·2 g/l NaHCO$_3$, 10 mM HEPES, 2 mM glutamine, and 1 g/l BSA) and cells were cultured for 24 h. After 24 h in serum-free medium, the indicated reagents were added to the cultures and the culture continued for the indicated times. At the end of the incubation, medium was removed for determination of hormones and cells were homogenized for extraction of total RNA.

Aggregate cell cultures

Anterior pituitary lobes were carefully separated from the neurointermediate lobe and dispersed into single cells as described above. Aggregate cultures were performed as previously described (Allaerts & Denef 1989, Vankelecom *et al.* 1989). Briefly, the pituitary cells were allowed to re-associate into aggregates in Petri dishes (35 mm diameter) on a gyratory shaker at 65 r.p.m. in a humidified CO$_2$–air incubator at 37 °C. The number of cells per Petri dish was 1×10^6 in 2 ml culture medium. After 2 days of gyratory shaking, cell debris was removed from the spontaneously formed rat pituitary cell aggregates, by passing the suspension through a 100 µm filter. The cell aggregates were re-suspended in fresh culture medium. The initial number of cells per Petri dish did not affect the responsiveness of the aggregates (within a range of 5×10^5-2×10^6/2 ml medium).

After an additional culture period of 2 days on the gyratory shaker, aggregates were washed twice with medium without FCS and then were transferred to a chamber and stimulated for 12 and 24 h in medium without FCS. Viability and integrity of the cells in the aggregates were carefully checked by acridine orange/ethidium bromide staining every 2 days and at the end of the experiments.

Cell stimulation

In the light of our previous experience in studies of the effects of interleukin (Arzt *et al.* 1999, Perez Castro *et al.* 2000), recombinant rat (rr) and human (rh) CNTF (R&D-Systems Inc., Minneapolis, MN, USA), and rhIL-11 (Roche Molecular Biochemicals, Mannheim, Germany) were dissolved in 0·05% BSA (crystallized and lyophilized, 99% purity) and used at the indicated doses, the same diluent was used as control in all experiments referred to as 'basal'. The preparations were free of endotoxin.

For tumor experiments, the small amount of tissue available limited the number of parallel experiments that could be run on each tumor.

Hormone determination

Hormones were measured by RIA as previously described (Arzt *et al.* 1992). For rat PRL and rat GH, reagents were kindly provided by Dr Parlow (National Hormone and Pituitary Program, Torrance, CA, USA). The RIA for human GH is commercially available (DPG Diagnostics, Los Angeles, CA, USA); the inter- and intra-assay coefficients of variation were 3·4% and 1·5% respectively. Human PRL was measured with a commercial IRMA (DRG instruments, Hamburg, Germany) that detects PRL in the range 1–200 µg/l with an intra-assay error of 2% and an inter-assay coefficient of variation less than 4%.

Northern blot

At the end of the stimulation time, Northern blot was performed as previously described (Arzt *et al.* 1992, Perez Castro *et al.* 1999). Unless stated, reagents were from Sigma, Boehringer (Mannheim, Germany) or Pharmacia (Uppsala, Sweden). Briefly, total RNA, isolated by the guanidine isothiocyanate–phenol–chloroform extraction method, was denatured with glyoxal, electrophoresed on a 1·2% agarose gel and transferred overnight to a nylon membrane. Filters were baked for 2 h at 80 °C and stained with methylene blue. They were prehybridized for 1 h at 60 °C (50% formamide, 5 × SSPE, 5 × Denhardt's solution, 0·1% SDS, 100 µg/ml denatured salmon sperm DNA) and then the probe was added for 12 h. Blots were washed at increasing salt and temperature stringency with a final wash of 30 min at 60 °C in 0·1 × SSC containing 0·1% SDS. Dried filters were exposed to Kodak XAR5 film at −70 °C with intensifying screens for 6 days. A 1·56 kb huCNTF-R α chain cDNA fragment (Davis *et al.* 1991), a 0·55 kb hGH (obtained from the American Type Culture Collection) and a 1 kb PstI fragment of β actin cDNA (Arzt *et al.* 1994) were labeled with a random-priming kit using [α-^{32}P]dCTP (specific activity 2–4×10^8 c.p.m./µg). The autoradiograms were scanned with an LKB ultroscan II laser densitometer. The blots

A.

CNTF-R (α) -

B.

CNTF-R (α) -

C.

CNTF-R (α) -
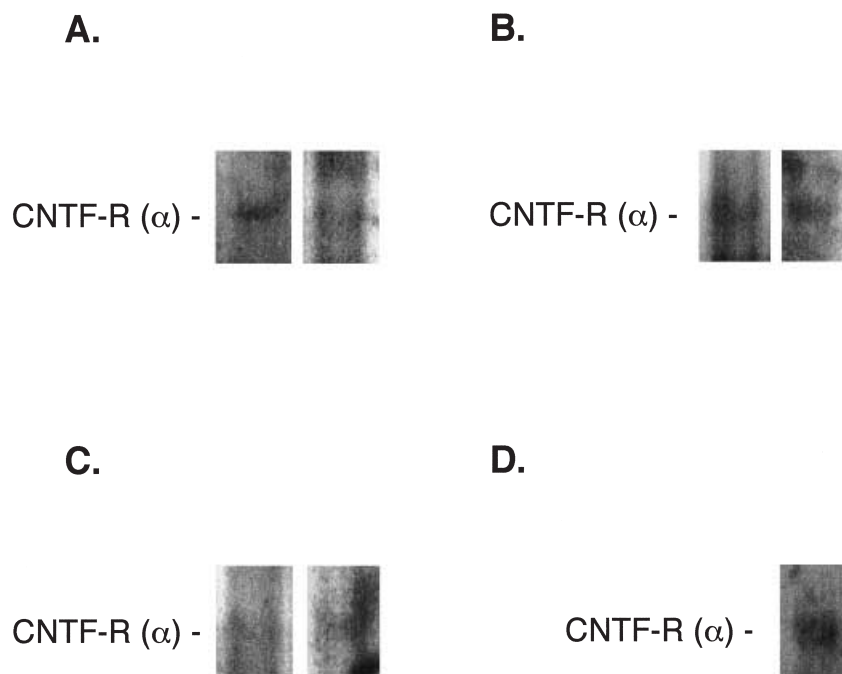
D.

CNTF-R (α) -

Figure 1 Expression of CNTF-R α (2 kb) mRNA in human pituitary adenoma cells. Northern blot analysis is shown using 30 μg RNA per lane as stated in Materials and Methods. A single band corresponding to the CNTF-R mRNA (2 kb) is shown in two representative examples from a total of the indicated number for each adenoma type. (A) Mammotropic cells ($n=4$); (B) somatotropic cells ($n=7$); (C) non-functioning anterior pituitary cells ($n=3$); (D) GH3 cell line as a positive control.

were reprobed after eluting the first probe with 5 mM Tris–HCl pH 8·0, 2 mM EDTA, 0·1 × Denhardt's solution, at 65 °C for 2 h. After the previous signal had been removed and confirmed by re-exposure of the filter, the blots were prehybridized and hybridized following the methods described above. A control with the fragment of β actin cDNA as probe was performed in each blot.

Statistics

Statistics were performed by ANOVA in combination with Scheffé's test. Data are shown as means ± S.E.M.

Results

Expression of α CNTF-R in pituitary adenomas

As stated in the introduction, it has recently been demonstrated that normal pituitary cells express both IL-11-R and CNTF-R and that human pituitary tumors express IL-11-R. We first examined whether the CNTF-R is also expressed in human tumors. The mRNA for the α chain specific for the CNTF-R is expressed in human pituitary adenoma cells as a single band of 2 kb similar to that known to be expressed in GH3 cells (Fig. 1). The mRNA

for the α chain specific for the CNTF-R was detected by Northern blot in tumors secreting PRL (Fig. 1A) or GH (Fig. 1B) or in non-functioning tumors (Fig. 1C) and in the GH3 tumor cell line as a positive control (Fig. 1D). All the adenomas ($n=14$) that were investigated expressed CNTF-R mRNA to different extents.

IL-11 and CNTF stimulation of GH mRNA in cell cultures from acromegalic patients

In order to study the possible role of CNTF and IL-11 in the regulation of GH expression in human pituitary adenomas, we cultured cells obtained from acromegalic patients, *ex vivo* in monolayer cultures, and stimulated with the cytokines for 24 h. We found that both IL-11 (5 nM) and CNTF (2 nM) exerted a similar stimulatory effect on the expression of GH mRNA (Fig. 2).

Effect of IL-11 and CNTF on GH and PRL secretion in pituitary tumors

In adenoma cultures we evaluated the effects of IL-11 and CNTF at the hormone secretion level. In prolactinoma cells, CNTF stimulated the secretion of PRL (Fig. 3A). In contrast, IL-11 and CNTF had no effect, even in high
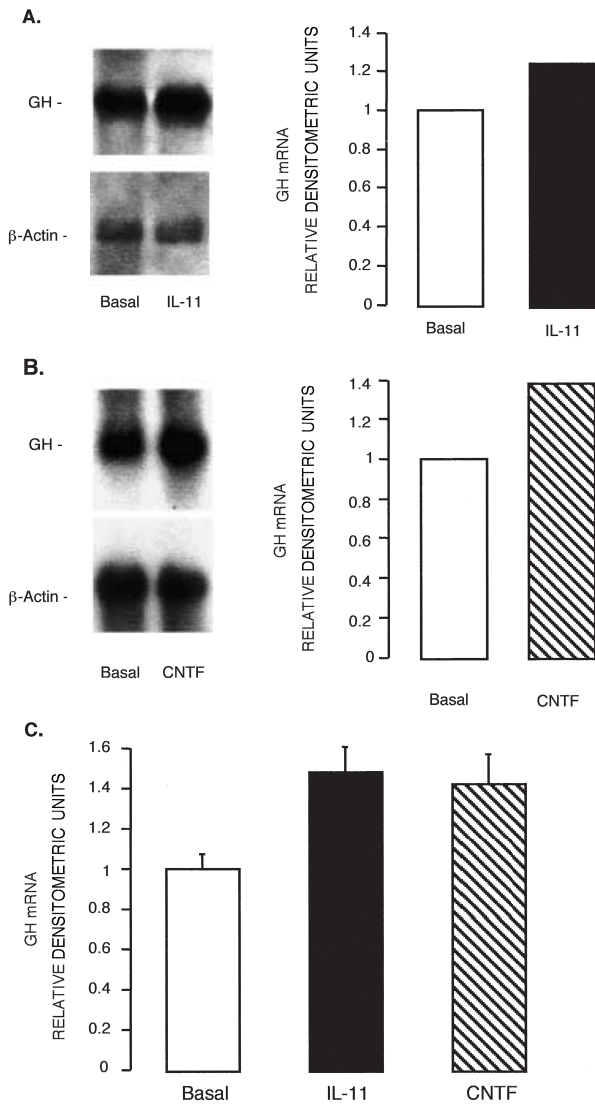
Figure 2 Effect of rhIL-11 and rhCNTF on GH mRNA expression in somatotropic human pituitary adenoma cells. Two different representative examples from a total of six adenomas analyzed with comparable results are shown. Northern blot analyses used 30 µg RNA per lane as stated in Materials and Methods. A single band corresponding to GH mRNA (1 kb) or β actin is shown. The cells were seeded at 6×10^5 cells/well and treated with rhIL-11 (5 nM) (A) or rhCNTF (2 nM) (B) for 24 h. Densitometric units relative to constitutive β actin expression are shown. (C) Mean densitometric analysis of GH mRNA content in the six adenomas; data are expressed as a ratio of autoradiographic signals of GH mRNA, relative to β actin mRNA used as loading control, with S.E.M.

Figure 3 Effect of rhIL-11 or rhCNTF on GH secretion in somatotropic and prolactinoma human pituitary adenoma cells in monolayer cultures. Cells were seeded at 2×10^5 cells/well in 24-well plates. After serum depletion for 24 h, treatments with rhIL-11 or rhCNTF for 24 h were performed in fresh serum-depleted medium. (A) After 24 h of CNTF treatment (1·2 nM) the supernatants were collected and PRL was measured by IRMA. Values represent the means ± S.E.M. of one of two separate adenomas, with four wells per treatment group. *$P<0·05$ compared with basal (ANOVA with Scheffé's test). (B) After 24 h treatment with rhIL-11 (5 nM) or rhCNTF (2 nM), the supernatants were collected and GH was measured by RIA. Values represent the means ± S.E.M. of one of eight separate adenomas, with four wells per treatment group. The other experiments gave comparable results. There were no significant difference between treatments (ANOVA with Scheffé's test). Other doses of IL-11 (1·25 and 2·5 nM) and CNTF (0·4 and 1·2 nM) that were also tested did not show any effect. Data from another two tumors with higher basal values and similar results are not shown.

doses, on GH secretion by acromegalic cell cultures (Fig. 3B). This lack of action was evident even in four cases included in Fig. 2, in which we observed a simultaneous stimulation of GH mRNA expression and lack of action on GH secretion.
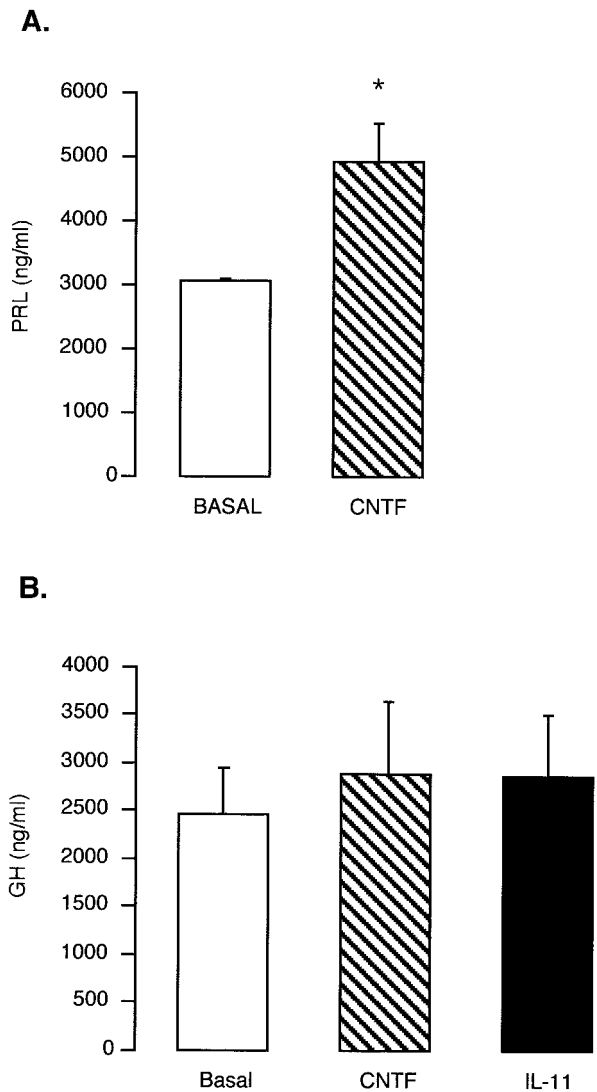
Effect of CNTF and IL-11 on the release of GH and PRL from normal rat pituitary cells

Both IL-11 and CNTF treatments for 24 h, even with high doses, had no significant effect on the release of either GH or PRL in monolayer cell cultures from normal rat anterior pituitary glands (Fig. 4). In these cultures the mRNA for GH also was not stimulated by CNTF or IL-11 (Fig. 5). However, when the cells were cultured in aggregate cultures (in which the three-dimensional structure of the cells is reconstituted), both cytokines, in doses at which they had no effect on monolayer cultures, significantly stimulated the secretion of both PRL and GH (Fig. 6).

Discussion

The expression of CNTF-R has previously been described in anterior pituitary cell lines and normal rat anterior pituitary cultures. The present observation that these receptors are expressed on lactotropic, somatotropic and non-functioning pituitary adenomas shows that these receptors are also expressed on human pituitary cells and underlines their putative involvement in pituitary pathophysiology.

Both CNTF and IL-11 significantly stimulate the expression of GH mRNA in monolayer cultures of cells obtained from acromegalic patients, and CNTF stimulates the release of PRL in cells from patients with prolactinoma. Interestingly, GH secretion was not stimulated in the same conditions. We had previously reported that CNTF induced a slight but consistent stimulation of GH and PRL secretion by the GH3 tumor cell line (Perez Castro *et al.* 2000). Interestingly, neither CNTF nor IL-11 stimulated GH mRNA and the release of GH and PRL from normal rat anterior pituitary cells dispersed in monolayer cultures, but they significantly stimulated the release of the hormones in the same cells cultured as aggregates. The necessity for a three-dimensional structure of the cultures for the effective action of these cytokines on normal anterior pituitary cells appears to be critical. This may involve the FS supporting cells, which have been shown to be present in aggregate cell cultures by immunohistochemistry (Allaerts & Denef 1989) and have been postulated to be an important component of the integration of information in the anterior pituitary autocrine/paracrine loops (Allaerts & Denef 1989). The fact that an FS cell line expresses receptors for CNTF and IL-11 and also proliferates and secretes VEGF in response to these cytokines strongly supports this notion (Perez Castro *et al.* 2000). In addition, we have shown that the matrix metalloproteinases (MMPs) are highly expressed in both normal and adenomatous pituitary cells, and that the level of digestion of the extracellular matrix by these MMPs enzymes is important for the availability of factors neces-
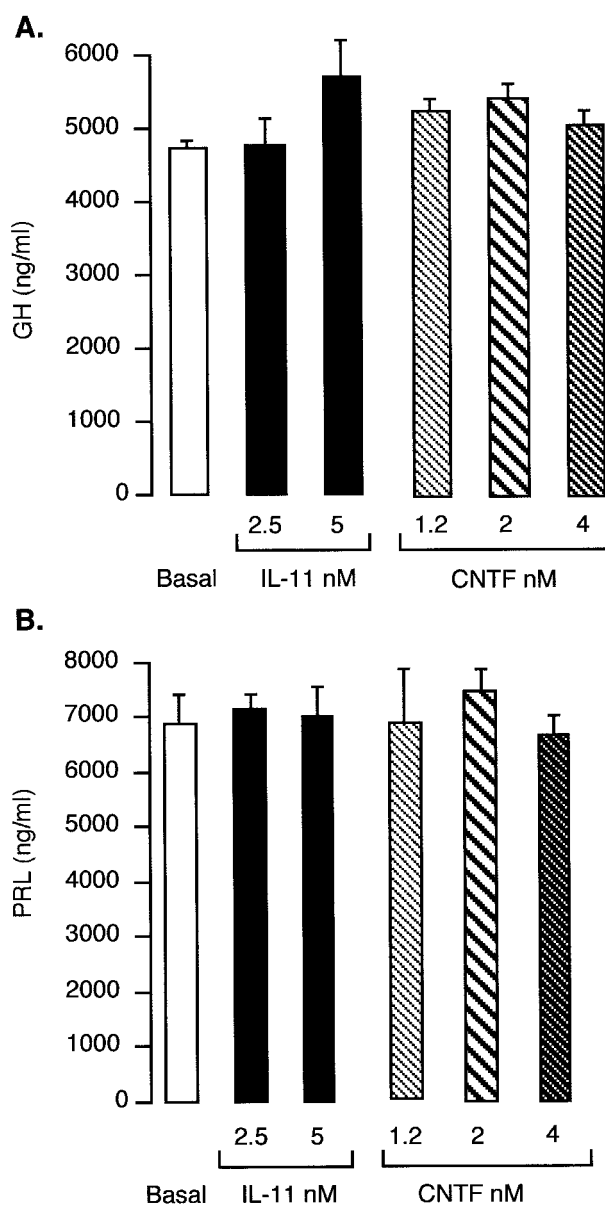

Figure 4 Effect of rhIL-11 or rrCNTF on the secretion of (A) GH and (B) PRL in normal rat anterior pituitary cells in monolayer cultures. Cells were seeded at 1×10^5 cells/well in 24-well plates. After serum depletion for 24 h, treatments with rhIL-11 (2·5 and 5 nM) or rrCNTF (1·2, 2 and 4 nM) for 24 h were performed in fresh serum-depleted medium. After 24 h the supernatants were collected and GH and PRL were measured by RIA. Values represent the means ± S.E.M. of one of four independently performed experiments, with four wells per treatment group. The other experiments showed comparable results. There was no significant difference between treatments (ANOVA with Scheffé's test).

sary for proliferation and hormone secretion (Páez Pereda *et al.* 2000*a*). In cultures from normal rat anterior pituitary cells, this action through MMPs will only occur in

A.

GH -

β-Actin -
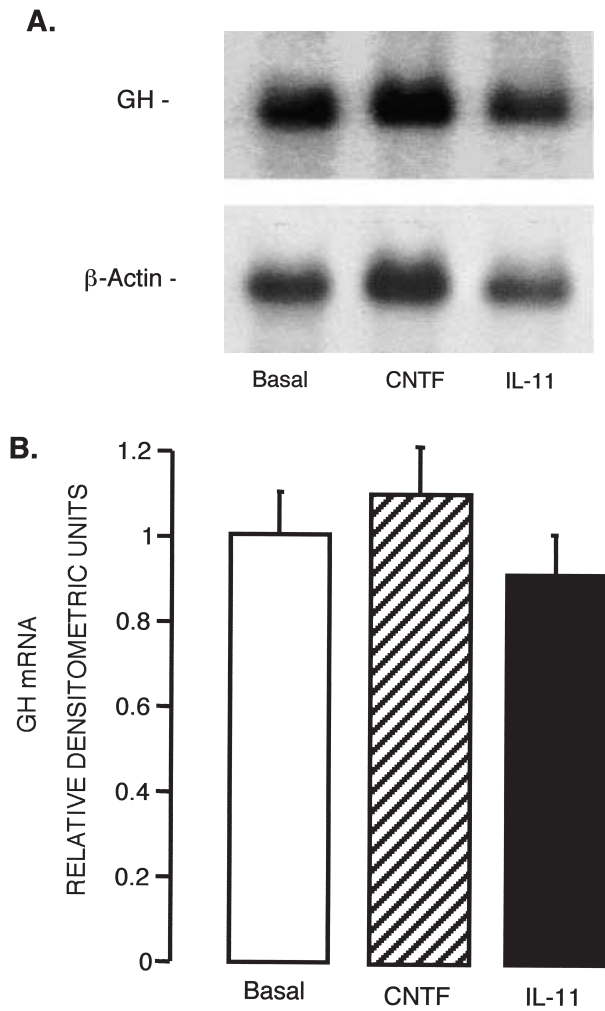
Basal   CNTF   IL-11

B.

Figure 5 Effect of rhIL-11 and rhCNTF on the expression of GH mRNA in normal rat anterior pituitary cells in monolayer culture. (A) Northern blot analysis using 30 μg RNA per lane as stated in Materials and Methods. A representative example from three independent experiments that gave comparable results is shown. The cells from 10 pituitaries were seeded at 6×10^5 cells/well and treated with rhIL-11 (5 nM) or rhCNTF (2 nM) for 24 h. A single band corresponding to GH mRNA (1 kb) or β actin is shown. (B) Mean ± S.E.M. densitometric analysis of the GH mRNA content of three independent experiments as described in (A). Data are expressed as a ratio of autoradiographic signals of GH mRNA relative to β actin mRNA used as loading control.

A.

B.

Figure 6 Effect of rhIL-11 or rrCNTF on the secretion of (A) GH and (B) PRL in normal rat anterior pituitary cells in aggregate cultures. Cell aggregates were cultured in 24-well plates. Treatments with rhIL-11 (5 nM) or rrCNTF (2 nM) for 24 h were performed in fresh serum-depleted medium. After 24 h, the supernatants were collected and PRL and GH were measured by RIA. Values represent the means ± S.E.M. of one of four independently performed experiments, with four wells per treatment group. The other experiments and experiments with 12 h stimulation showed comparable results. *$P<0.05$, ***$P<0.001$ compared with the corresponding basal value (ANOVA with Scheffé's test).

aggregate cultures, and not when the cells are cultured as monolayers. The more physiological configuration of cells in aggregate cultures can also result in a better expression or accessibility of receptors. Thus the three-dimensional structure of the tissue, the interaction of hormone-secreting cells with the extracellular matrix, and the supporting FS cells, are critical for the action of IL-11 and CNTF in the regulation of GH and PRL secretion by anterior pituitary cells.
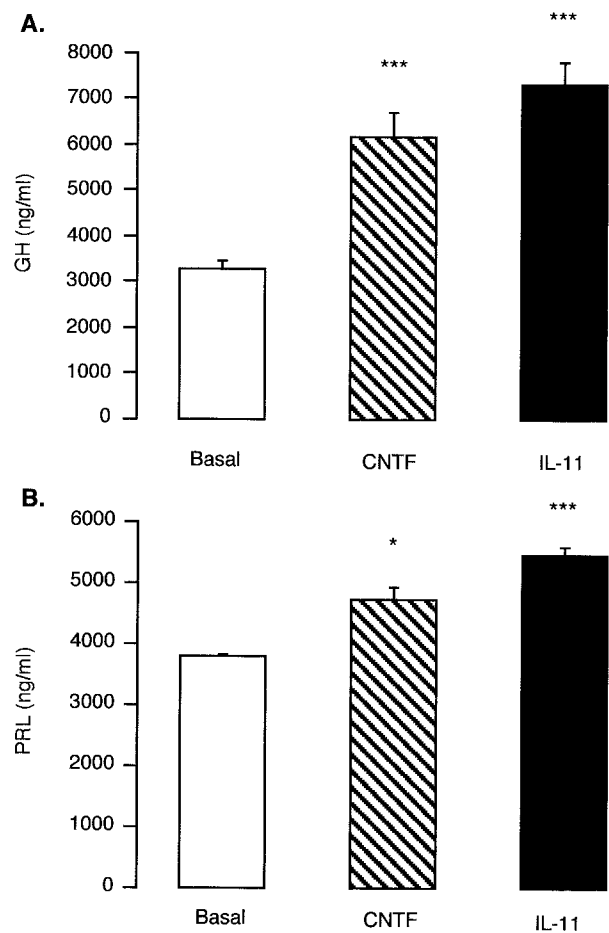
A similar process could be involved in the secretory mechanisms that follow CNTF or IL-11 stimulation of human somatotropic adenomas. Similarly, we have previously shown that the action of IL-6 in inducing c-fos in human adenoma cell cultures only occurs in explant cultures (where the structure of the tissue is maintained) and not in monolayer cultures (Páez Pereda *et al.* 1996). In pituitary adenomas, the interaction with FS cells in the transition zone surrounding the tumoral cells, which is extremely rich in FS cells (Marin *et al.* 1992, Farnoud *et al.* 1994), could be critical for regulation by these

cytokines. In cultures of cells from GH-secreting adenomas, this seems to be important for the secretion, but not the transcriptional step of regulation. Post-translational mechanisms modifying the levels of translation of mRNA have been shown to occur in different models of secretagog stimulation, for example for the action of IL-1 in skeletal muscle (Cooney *et al*. 1999) and the expression of genes during development (Kuge & Richter 1995). The inhibition of lipoprotein lipase by GH in human adipose tissue occurs during translation or post-translational processing (or both) of the enzyme (Ottosson *et al*. 1995). Similar to the effect of CNTF and IL-11 on GH mRNA/ protein secretion in human adenomas, GH induces hepatic lipase mRNA but the translation is impaired in hypothyroid rats (Neve *et al*. 1997), in which the mechanism is also unknown but speculated to occur at the (post) translational level. The GHRH-induced release of GH by human GH-secreting cells is transmitted through protein kinase A, which activates nonselective cation currents and induces membrane depolarization, an increase in intracellular Ca^{2+} and GH secretion. In human adenomas with a constitutively active gsp mutation, GHRH does not increase the non-selective cation current, which may account for the oversecretion of GH in these adenomas (Yasufuku-Takano *et al*. 1999). Further studies on the coupling of the receptors for CNTF with the signals leading to hormone secretion are needed in order to understand the mechanisms leading to the different regulation among transcription and secretion. The understanding of the cellular and molecular mechanisms leading to the lack of secretion of GH after CNTF or IL-11 stimulation in somatotropic human adenomas will be of great utility for further understanding the hormone oversecretion in these adenomas.

As has been extensively shown for LIF and IL-6, IL-11-R and CNTF-R are not only expressed, but are also functional, in anterior pituitary cell lines, normal anterior pituitary cells and human adenomas. Through these receptors these cytokines are able to regulate the secretion of hormone and VEGF and the proliferation of these cells. These cytokines, acting through the gp130 transducer, may be involved in the progression of pituitary pathogenesis.

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