Evidence for a negative feedback in the control of eel growth hormone by thyroid hormones

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Abstract

The regulation of growth hormone (GH) by thyroid hormones (THs) has been shown to present species variation. We investigated the regulation of GH in the eel, a representative of an ancient group of teleosts. *In vivo* administration of triiodothyronine (T₃) or thyroxine (T₄) significantly reduced pituitary and serum GH levels, as measured by homologous RIA. In order to investigate the ability of THs to regulate GH production directly at the pituitary level, we used a long-term, serum-free primary culture of eel pituitary cells. Both T₃ and T₄ inhibited GH release in a concentration-dependent manner, producing up to 50% inhibition at 10 nM, with an ED₅₀ of <0.2 nM, within the range of their physiological circulating levels. Other hormones also acting via the nuclear receptor superfamily, such as sex steroids (testosterone, estradiol and progesterone) and corticosteroid (cortisol), had no effect on GH release *in vitro*, underlining the specificity of the regulatory effect of THs on GH. Measurement of both GH

release and cellular content for calculation of GH production *in vitro* indicated that THs not only inhibited GH release but also GH synthesis. Dot-blot assay of GH messenger RNA (mRNA) using an homologous eel cDNA probe showed a decrease in GH mRNA levels in cells cultured in the presence of T₃, as compared with control cells. This demonstrated that the inhibition of T₃ on GH synthesis was mediated by a decrease in GH mRNA steady state levels. In conclusion, we demonstrate inhibitory regulation of eel GH synthesis and release by THs, exerted directly at the pituitary level. These data contrast with the rat, where THs are known to have a stimulatory effect and suggest that the pattern observed here in an early vertebrate and also found in birds, reptiles and some mammals including humans, may represent an ancestral and more generalized vertebrate pattern of TH regulation of pituitary GH.

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Introduction

Thyroid hormones (THs) and growth hormone (GH) are thought to play synergistic roles in the control of growth and development in vertebrates (McNabb 1992). Several physiological crosslinks have been demonstrated between thyrotropic and somatotrophic axes at peripheral and central levels. In humans (both normal and GH deficient), GH has been shown to act on thyroid function by inducing extrathyroidal conversion of thyroxine (T₄) to the biologically active hormone, triiodothyronine (T₃) (Grunfeld *et al.* 1988). GH has also been reported to increase circulating T₃ levels by stimulating peripheral 5'-monodeiodination in birds (Kühn *et al.* 1987) and teleosts (European eel: de Luze & Leloup 1984, rainbow trout: MacLatchy *et al.* 1992). At the pituitary level, thyrotropin-releasing hormone (TRH) was shown to be able to stimulate not only thyrotropin (TSH) but also GH release in various vertebrates including mammals, birds, reptiles,

amphibians (Harvey 1990a) and teleosts (Peng & Peter 1997). Moreover, corticotropin-releasing hormone (CRH) has been shown not only to stimulate corticotropin release but also to act as a GH-releasing factor and TSH-releasing factor in reptiles (Denver & Licht 1989) and in teleosts (Larsen *et al.* 1998, Rousseau *et al.* 1999).

It is well established that THs are required for normal pituitary GH secretion in man and several other mammalian species (for review: Valcavi *et al.* 1992, Müller *et al.* 1999). In the rat and human, hypothyroidism results in impaired basal and stimulated GH secretion and reduced pituitary GH content, effects which are overcome by restoration of euthyroidism (Williams *et al.* 1985). However, various reports including data in humans indicated that THs may also exert inhibitory effects on GH. Children with thyrotoxicosis have normal GH responses to provocative stimuli (Katz *et al.* 1969) but diminished 24-h GH secretion (Finkelstein *et al.* 1974). In hyperthyroid patients, sleep-related GH release is decreased

(Sazaki *et al.* 1985). The action of T_3 varies across species with reported stimulation in rats (Evans *et al.* 1982), and inhibition in humans (Cattini *et al.* 1986) and in bovines (Silverman *et al.* 1988). This suggests that the well-documented positive regulation by T_3 of rat GH may not be a common feature among mammalian species.

In birds, T_3 exerts a strong inhibitory effect on GH. *In vivo*, hypothyroidism is a potent stimulator of GH secretion, and exogenous THs markedly suppress GH release (Harvey 1990b, Harvey *et al.* 1991). A negative effect of T_3 on GH synthesis and release has also been documented *in vitro* in birds (Denver & Harvey 1991) and similarly, T_3 is reported to exert inhibitory effects on GH release *in vitro* in chelonid reptiles (turtles: Denver & Licht 1988). These data from cattle, humans, birds and reptiles suggest that the more generalized vertebrate pattern of thyroidal regulation of pituitary GH may be inhibitory (Denver & Harvey 1991). In teleosts, the few data available on the action of THs on GH production are contradictory, reporting no effect (Nishioka *et al.* 1985, Luo & McKeown 1991, Moav & McKeown 1992), inhibitory (Baker & Ingleton 1975) or stimulatory (Moav & McKeown 1992, Farchi-Pisanty *et al.* 1995, Melamed *et al.* 1995).

Here, we investigated the action of THs on GH release and synthesis in the European eel. The phylogenetic position of the eel, as a member of the Elopomorphs group, an ancient group among teleosts, may provide information on ancestral endocrine regulatory processes in vertebrates (De Pinna 1996). We used a long-term, serum-free system of primary cultures of eel pituitary cells (Rousseau *et al.* 1998) to investigate the direct pituitary effect of THs on eel GH *in vitro*. The effects of THs on GH were followed at the protein GH level by homologous RIA of GH cell content and release and at the messenger RNA (mRNA) level by dot-blot using an homologous complementary DNA (cDNA) probe for eel GH. The ability of THs to inhibit GH was validated by an *in vivo* study, analyzing their effects on GH serum level and pituitary content.

Materials and Methods

Fish

Female European eels, *Anguilla anguilla* L., were netted in ponds in the north and west of France. Animals were transferred to the laboratory and kept in running aerated freshwater for a short time (1 to a few weeks) until experimentation. Animal manipulations were performed according to the recommendations of the French ethical committee and under the supervision of authorized investigators.

Hormones

T_3 , T_4 , cortisol, estradiol, progesterone, testosterone and somatostatin (SRIH-14) were obtained from Sigma Chemical Co. (Saint-Quentin Fallavier, France).

In vivo experiments

Hormonal treatments were administered to female silver eels, netted at the time of the downstream migration (November) in ponds in the north of France (Peronne). Eels were transferred to the laboratory and kept in 100 l aquaria (8 eels/aquarium) in running aerated freshwater ($12 \pm 2^\circ\text{C}$). Since at the silver stage eels have initiated a period of physiological fasting, fish were not fed during the experiment. Fish (8 eels/group) received one perivisceral injection per week of T_3 or T_4 ($2 \mu\text{g/g}$ body weight) suspended in saline (0.15 M NaCl) or of saline alone (controls) for 3 months (December–February). No mortality occurred during the experiment. Body weights ($282 \pm 11 \text{ g}$, $288 \pm 10 \text{ g}$, $285 \pm 10 \text{ g}$ for controls, T_3 - and T_4 -treated groups respectively at the beginning of the experiment) were reduced by about 10% at the end of the experiment, with no significant variations between groups ($254 \pm 10 \text{ g}$, $263 \pm 9 \text{ g}$, $258 \pm 10 \text{ g}$ respectively). The day after the last injection, eels were killed by decapitation. Sera were obtained from blood samples allowed to clot overnight. Pituitaries were quickly removed and kept frozen in 0.15 M NaCl until extraction.

Primary cultures of eel pituitary cells and in vitro treatments

In vitro experiments were performed on pituitary cells from batches of yellow (sedentary growth stage) or silver (migratory stage) female eels (body weight 200–300 g) netted the year round in ponds in the west or north of France. A batch of about 100 eels was used for each cell preparation. Each experiment was repeated in three independent studies (performed on different cell preparations from various eel batches). Similar results were obtained with pituitary cell cultures from yellow and silver eels. Results of representative experiments are shown.

Dispersion of pituitary cells was performed using an enzymatic and mechanical procedure as previously described (Rousseau *et al.* 1998). The number of viable cells, as ascertained by Trypan Blue (Sigma Chemical Co.) exclusion test, represented more than 90% of the total dispersed cells. Cells were cultured on poly-L-lysine (Sigma Chemical Co.) -precoated plates (Costar, Cambridge, MA, USA) in a serum-free medium (CM; medium 199 with Earle's salt, sodium bicarbonate, 100 U/ml penicillin, 100 $\mu\text{g/ml}$ streptomycin, and 250 ng/ml fungizone; Life Technologies, Cergy-Pontoise, France) at 18°C under 3% CO_2 and saturated humidity. Cells were plated on 96-well plates (Costar) at a density of 62 500 cells/well for measuring GH by RIA (Montero *et al.* 1998, Rousseau *et al.* 1998), or on 12-well plates at a density of 500 000 cells/well for measuring mRNAs by dot-blot (Huang *et al.* 1999a). After 1 day of culture to allow attachment, medium was changed, and treatments were started (day 0).

Stock solutions (10^{-3} M) were diluted in 0.1 M acetic acid (T_3 and T_4), 100% ethanol (cortisol, estradiol,

progesterone, testosterone) or in pure sterile water (SRIH-14). They were further diluted with CM just before culture medium renewal. The final concentration of acetic acid or ethanol in culture wells never exceeded 0.2%; control wells were treated with the same concentration of acetic acid or ethanol in CM. Media were collected and treatments were renewed at each sampling time for up to 12 days. Collected media were kept frozen (-20°C) until RIA of GH. Cultures were stopped before the addition of hormones (day 0) and at the end of the culture for measuring GH cell content or mRNA levels for GH.

Radioimmunoassays

Pituitaries were extracted by sonication (Bioblock Scientific, Illkirch, France) in 0.15 M NaCl, and for measurement of GH cellular contents from *in vitro* experiments, cells were submitted to osmotic shock (distilled water) and two repeated cycles of freezing and thawing (Rousseau *et al.* 1998, Huang *et al.* 1999b).

GH levels from pituitary extracts, sera, cellular contents, and culture media were assayed using an homologous RIA for eel GH (Marchelidon *et al.* 1996). Results from *in vivo* studies were expressed as μg /pituitary and ng/ml . For *in vitro* studies, total GH released into the culture medium was determined as cumulative GH release between each sampling point. GH synthesis in culture was calculated as described (Rousseau *et al.* 1998, Huang *et al.* 1999b) and reported as the sum of the cumulative release for 12 days and the variation of cell contents between day 0 and day 12. Results were expressed as nanograms GH per 62 500 cells.

GH cDNA probe

A European eel pituitary cDNA library constructed into $\lambda\text{gt}10$ vector (Quérat *et al.* 1990) was used to amplify a GH cDNA by PCR. Two primers were designed based on the nucleotide sequences of the Japanese eel GH cDNA (primer 1: 5'-GTTAACC GAGCACAGCA CCT-3'; primer 2: 5'-AGGTCCTCCA CTTTGTGCA TGTC-3') (Saito *et al.* 1988). The conditions for PCR were as follows: 1 μl amplified library lysate was mixed with 0.4 μM of each primer, 200 μM deoxynucleotriphosphates, 10 mM Tris-HCl, 1.5 mM MgCl_2 , 50 mM KCl, pH 8.3 and 1 unit *Taq* DNA polymerase (Boehringer-Mannheim, Meylan, France) in a final volume of 50 μl . Cycling parameters were 3 min denaturation at 94°C followed by 30 cycles of 1 min denaturation at 94°C , 1 min annealing at 53°C and 1 min elongation at 72°C , the final elongation step was extended to 10 min. PCR products were separated by 1.5% agarose gel electrophoresis, bands excised, purified with JET quick gel extraction kit (GENOMED, Bad Oeynhausen, Germany) and subcloned into pGEMT Easy (Promega-France,

Charbonnières, France). PCR products of three independent PCR amplifications were cloned, sequenced on both strands using a dye terminator cycle sequencing kit (Amersham Life Science-France, Les Ulis, France) and sequence identity performed using the BLAST program (Genbank, NCBI, USA). One major fragment of 481 bp was amplified from the pituitary cDNA ligated into $\lambda\text{gt}10$ vector using primer 1 and primer 2, subcloned and sequenced. Comparison of the nucleotide sequences showed a 98% nucleotide identity with the Japanese eel GH cDNA (Saito *et al.* 1988).

Dot-blot assay of GH mRNAs

Cells were collected and total RNAs were extracted according to the method described by Huang *et al.* (1999a). Cells were scraped in ice-cold PBS (Life Technologies) using a cell scraper (Costar), centrifuged (12 000 g) for 5 s at 4°C , the supernatant was removed, and 45 μl TE (10 mM Tris and 1 mM EDTA, pH 7.2) buffer were added. Cells were then kept at -80°C until RNA extraction. Total RNA was extracted by adding 5 μl 5% Nonidet P-40 (Sigma Chemical Co.) to disrupt cell walls, centrifuged (12 000 g) for 15 min at 4°C , and the supernatant containing total RNA was collected and denatured at 60°C for 15 min after adding 30 μl 20 $\times$ SSC (standard saline citrate) and 20 μl 37% formaldehyde. RNA was blotted onto Hybond-N+, Nylon membrane (Amersham Pharmacia Biotech, Les Ulis, France) through a Hybri-blot manifold (Life Technologies). The membrane was air-dried and RNAs were immobilized by baking at 80°C for 2 h.

The PCR-derived cDNA fragment of eel GH was [^{32}P]dCTP-labeled by random priming with the NEBlot-Kit (New England Biolabs, Hitchin, Herts, UK), to a specific activity of 1×10^9 c.p.m./ μg and used as a probe. The membrane was prehybridized for 5 h at 42°C in hybridization solution (ULTRAhybTM, Ambion, Clinisciences, Montrouge, France) and then hybridized with the ^{32}P -labeled eel GH cDNA overnight at 42°C . After high stringency washing (0.1 $\times$ SSC and 0.1% SDS for 30 min at 60°C), the membranes were autoradiographed. Scanning densitometry and data processing were performed using a PhosphorImager (Molecular Dynamics, Sunnyvale, CA, USA). The membranes were stripped by boiling in 0.1% SDS and subsequently hybridized under the same conditions with ^{32}P -labeled eel β -actin probe.

Statistical analysis

For *in vitro* experiments, replicates of six wells (62 500 cells/well) for RIA studies or of four wells (500 000 cells/well) for mRNA studies, were used for each treatment, and the means $\pm$ S.E.M. are given. For *in vivo* experiments, groups of eight eels were used for each treatment, and the means $\pm$ S.E.M. are given. Homogeneity

of variance was assessed by Bartlett's test, and data compared by one-way ANOVA followed by Student-Newman-Keul's multiple comparison test, using InStat (GraphPad Software, Inc., San Diego, CA, USA).

Results

In vivo effects of T_3 and T_4 on pituitary GH content and serum GH level (Fig. 1)

GH pituitary content was high in control eels ($102 \pm 16 \mu\text{g/pituitary}$), in agreement with previous studies (Marchelidon *et al.* 1996). Chronic treatment with T_3 or T_4 significantly ($P < 0.05$) reduced eel pituitary GH content by about 50% of control values (Fig. 1a). Serum GH level ($3.4 \pm 0.6 \text{ ng/ml}$) was also significantly ($P < 0.05$) reduced by 50% with T_3 or T_4 treatment (Fig. 1b).

Kinetics of in vitro effects of T_3 and T_4 on GH release - comparison with SRIH effect (Fig. 2)

We investigated whether the inhibitory effects of T_3 and T_4 on GH levels could result from a direct pituitary action. Pituitary cells were cultured in serum-free medium to avoid interaction with hormones and growth factors contained in serum (Rousseau *et al.* 1998). In control wells, sustained GH release was observed throughout the duration of culture (Fig. 2). The total amount of GH released into the medium over 12 days ($18 \pm 1 \mu\text{g}/62\,500$ cells) represented 170% of the initial cell content (day 0: $11 \pm 1 \mu\text{g}/62\,500$ cells), reflecting active synthesis of GH during the culture, as reported previously (Rousseau *et al.* 1998, 1999).

Addition of T_3 or T_4 (100 nM) significantly decreased GH release; the inhibitory effects of T_3 and T_4 were significant after 48 h of incubation ($P < 0.05$) and were maintained up to the end of the culture on day 12 ($P < 0.001$; Fig. 2). Total GH release over 12 days of culture was decreased by 50% by T_3 and T_4 ($P < 0.001$).

The addition of SRIH (100 nM) significantly decreased GH release at each time period of incubation over 12 days ($P < 0.001$), in agreement with our previous studies (Rousseau *et al.* 1998). After 12 days of culture, GH release was reduced by 95% as compared with controls, an inhibitory effect much stronger than that produced by T_3 or T_4 ($P < 0.001$; Fig. 2).

Measurement of GH cellular content at the beginning and at the end of the culture, and of total GH release, allowed us also to estimate the effect of T_3 on GH synthesis *in vitro*. Control cells showed a sustained synthesis of GH over 12 days of culture. Culture in the presence of 100 nM T_3 induced a significant reduction in GH synthesis, with a value after 12 days ($4 \pm 0.5 \mu\text{g}/62\,500$ cells) which was significantly lower ($P < 0.001$) than in control cells ($10 \pm 1 \mu\text{g}/62\,500$ cells).

Figure 1 Effect of *in vivo* treatments with T_3 or T_4 on GH pituitary content (a) and serum level (b). Eels received one weekly injection of 2 μg T_3 or T_4 in saline/g body weight for 3 months. Controls were injected with saline alone (0.15 M NaCl). GH was measured by homologous RIA. The means $\pm$ S.E.M. are given ($n = 8$ eels/group). * $P < 0.05$ vs controls (by variance analysis).

Concentration-dependence of T_3 and T_4 effects on GH release in vitro - comparison with SRIH (Fig. 3)

Cultured pituitary cells were incubated with various concentrations of T_3 or T_4 (0.001 to 1000 nM) for 12 days. The release of GH in the medium was decreased in a concentration-dependent manner by both T_3 and T_4 (Fig. 3). Both T_3 and T_4 produced similar maximal inhibitory effects (about 45% of control values) and maximal inhibition was produced at T_3 or T_4 concentrations of

Figure 2 Kinetics of the effects of thyroid hormones (T_3 or T_4) and somatostatin (SRIH) on GH release by primary culture of eel pituitary cells. Cells were cultured in the absence (controls) or presence of 100 nM T_3 , T_4 or SRIH. Culture media and hormone treatments were renewed at each sampling time. Total GH release was calculated by cumulating GH released between each sampling time up to 12 days. GH was measured by homologous RIA. The means $\pm$ S.E.M. are given ($n=6$ wells/group; 62 500 cells/well). * $P<0.05$ and *** $P<0.001$ vs controls.

≥ 10 nM. The median efficient dose (ED_{50}) was <0.2 nM for both hormones, with no significant differences between T_3 and T_4 .

For comparison, the effects of various concentrations of SRIH (0.001 to 1000 nM) were tested in the same conditions. SRIH induced a concentration-dependent inhibition of GH release up to a maximal effect (5% of control values) significantly greater than the suppression by T_3 or T_4 ($P<0.001$) (Fig. 3).

Specificity of T_3 and T_4 effects on GH release - comparison with effects of other hormones in vitro (Fig. 4)

To test the specificity of TH action on GH release, we compared the effects of different concentrations (0.001 to 1000 nM) of various steroid hormones which also act via nuclear receptors. In contrast to the concentration-dependent inhibitory effects of T_3 and T_4 , corticosteroid (cortisol) and sex steroids (testosterone, estradiol and progesterone) had no significant effect on GH release at any concentration tested, up to 12 days of culture (Fig. 4).

Effect of T_3 on GH mRNA levels in vitro (Fig. 5)

In order to further investigate the inhibitory action of T_3 on GH synthesis, we analyzed whether it implicated an action at the level of transcription.

Cell cultures were stopped for mRNA measurement before (day 0) or after various days in the presence or not of 100 nM T_3 (Fig. 5a,b). GH mRNA levels increased

Figure 3 Concentration-dependent inhibitory effects of T_3 , T_4 and SRIH on GH release by primary culture of eel pituitary cells. Cells were cultured for 12 days in the absence (controls) or in the presence of various concentrations of T_3 , T_4 or SRIH (0.001 to 1000 nM). Culture media and hormone treatment were renewed every third day. Total GH release was calculated by cumulating GH released every third day up to 12 days. Results are expressed as percentage of mean control values (13 500 $\pm$ 690 ng). The means $\pm$ S.E.M. are given (6 wells/group; 62 500 cells/well). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ vs controls. ^a $P<0.01$ and ^b $P<0.001$: significant differences between SRIH- and T_3 / T_4 -inhibitory effects, at the same hormone concentration.

Figure 4 Comparative effects of T_3 , T_4 and other hormones that act via the nuclear receptor superfamily (corticosteroid: cortisol and sex steroids: testosterone, estradiol, progesterone) on GH release by primary culture of eel pituitary cells. Cells were cultured for 12 days in the absence (controls) or in the presence of various concentrations (0.001 to 1000 nM) of T_3 , T_4 , or of steroids. Culture media and hormone treatment were renewed every third day. Total GH release was calculated by cumulating GH released every third day up to 12 days. Results are expressed as a percentage of mean control values (8750 $\pm$ 450 ng). The means $\pm$ S.E.M. are given ($n=6$ wells/group; 62 500 cells/well). * $P<0.05$ and *** $P<0.001$ vs controls.

Figure 5 Effect of T_3 on GH mRNA levels in primary culture of eel pituitary cells. Cells were cultured for 1 and 2 days (a) or for 8 days (b) in the absence (controls) or presence of 100 nM T_3 . GH mRNA levels and β -actin mRNA levels were measured by dot-blot, using homologous cDNA probes for eel GH and β -actin. The means $\pm$ S.E.M. are given ($n=4$ wells/group; 500 000 cells/well). * $P<0.05$ and *** $P<0.001$ vs respective controls.

significantly with culture time in control cells (Fig. 5a; $P<0.05$ between day 2 and day 0 controls). The presence of T_3 blocked this increase in GH transcription, and this inhibitory effect was significant after 2 days of culture (Fig. 5a; $\times 0.75$ control values, $P<0.05$). The inhibitory effect of T_3 was specifically exerted on GH mRNA levels (Fig. 5b; $\times 0.5$ control values after 8 days of treatment, $P<0.001$), no change in β -actin mRNA levels being observed (Fig. 5b).

Discussion

Chronic administration of thyroid hormones (T_3 or T_4) significantly decreased both GH pituitary content and serum concentration, indicating that THs inhibit GH

synthesis and release *in vivo* in the eel. The *in vivo* experiment was performed at the silver stage, when eels are physiologically fasting, in order to avoid possible indirect effects of THs on food intake and metabolism. Accordingly, no significant differences in body weight were observed between control and T_3 - or T_4 -treated fish at the end of the experiment. However, *in vivo* inhibitory effects of THs on GH could still reflect indirect actions such as changes in GH response to fasting (Marchelidon *et al.* 1996) or other indirect pathways.

In order to investigate whether THs were acting directly at the pituitary level to inhibit GH production, we used a serum-free primary culture of pituitary cells. T_3 and T_4 decreased GH release *in vitro* in a concentration-dependent manner with a maximal suppression of about 50%, similar to that observed *in vivo*. These data contrast with our earlier studies in which we demonstrated that THs had no effect on luteinizing hormone (LH) production by gonadotropes under the same culture conditions (Huang *et al.* 1998), assessing the specificity of TH inhibitory effect on somatotrophs. We further investigated the specificity of TH action by comparing the activities of various hormones also acting via the same molecular family of nuclear receptors, such as sex steroids and corticosteroids (Glass 1994). None of the steroids tested (androgen: testosterone; estrogen: estradiol; progestagen: progesterone and glucocorticosteroid: cortisol) had any significant effect on GH release, in contrast to our earlier studies in which we demonstrated stimulatory effects of testosterone and cortisol on LH (Huang *et al.* 1999a).

In the eel, T_3 and T_4 had similar maximal inhibitory effects on GH release *in vitro* at concentrations within the physiological range (4 to 8 nM) (de Luze & Leloup 1984). These inhibitory effects of TH could be observed throughout the 12-day period of culture, indicating no desensitization. This suggests that THs may exert a physiological and chronic role in the negative regulation of GH levels in the eel. Support for this hypothesis comes from studies reporting significant inhibition of GH release by pituitaries cultured for 6 days in the presence of a high dose (1 μ g/ml) of T_4 (Baker & Ingleton 1975). Our data on dispersed cultured cells indicate that the effect of TH acts directly on pituitary cells and is not mediated by the hypothalamic axonal endings which directly innervate the adenohypophysis in teleosts. These data are thus the first demonstration of an inhibitory effect of TH on GH release *in vivo* and *in vitro* in a teleost species. Indeed, previous studies in teleosts failed to show any significant alterations of GH in response to T_3 *in vivo* (rainbow trout: Moav & McKeown 1992) and *in vitro* (tilapia: Nishioka *et al.* 1985, carp: Luo & McKeown 1991).

These inhibitory effects of TH on GH in the eel are similar to those previously demonstrated in tetrapod vertebrates. In the chicken, exogenous TH markedly suppressed circulating GH levels, while thyroidectomy or goitrogen treatment resulted in a rise in the basal GH level

(Harvey *et al.* 1988). T_3 also suppressed basal as well as TRH- or growth hormone-releasing hormone (GHRH)-induced GH secretion *in vivo* in euthyroid and hypothyroid chicken (Scanes & Harvey 1989), an inhibitory effect also evidenced *in vitro* (Harvey 1990b). In chelonian reptiles, THs were shown to act directly at the level of the pituitary to inhibit basal and TRH-induced GH secretion *in vitro* (Denver & Licht 1988). In humans, although THs are required for normal GH secretion (Valcavi *et al.* 1992, Müller *et al.* 1999), they have also been shown to reduce it *in vivo* in hyperthyroid patients (Sazaki *et al.* 1985) and in adolescents with thyrotoxicosis (Finkelstein *et al.* 1974). T_3 has also been shown to inhibit GH release *in vitro* from fetal pituitary cells (Mulchahey *et al.* 1988) and from pituitary tumor cells of acromegalic patients (Adams *et al.* 1981). In contrast, in the rat, positive effects of THs on GH secretion have been emphasized *in vivo* (Peake *et al.* 1973, Coiro *et al.* 1979, Martin *et al.* 1985) and *in vitro* (Hinkle & Goh 1982).

In the eel, comparison of T_3 and T_4 dose-dependent effects *in vitro* indicated that both hormones had a similar maximal effect and ED_{50} , suggesting that they were acting through similar mechanisms. In teleost pituitaries, nuclear TH binding sites have been described which bind T_3 with approximately 20–50 times greater affinity than T_4 , properties closely resembling those of mammalian TH nuclear receptors (Bres & Eales 1990). These data suggest that T_4 , because of its weaker affinity for the receptor, may act after deiodination into T_3 (McNabb 1992). Local deiodination of T_4 into T_3 is regulated by type II and type I 5'-deiodinases in rat anterior pituitary cells (Köhrle *et al.* 1995). Although deiodinase activities have been characterized in various tissues in teleosts, including liver, kidney, gill and brain (Eales *et al.* 1993), the existence of such enzymes in the pituitary has not yet been determined. The high capacity of T_4 to inhibit GH release in the eel *in vitro* likely reflects the presence of an active pituitary deiodinase. A similar conclusion was previously raised in view of the similar efficiency of both T_3 and T_4 to inhibit TSH mRNA levels in eel pituitary cells, in the same culture conditions (Pradet-Balade *et al.* 1997). In the turtle, T_3 and T_4 also had similar inhibitory effects on GH secretion *in vitro* (Denver & Licht 1988). In contrast, in the domestic fowl, T_3 , but not T_4 , has been shown to inhibit GH secretion *in vitro* (Harvey *et al.* 1991). In the rat, in hyperthyroid states, GH mRNA levels are stimulated by T_3 generated by type I 5'-deiodinase, whereas in hypothyroidism, when type II 5'-deiodinase activity is markedly increased, GH mRNA is more responsive to T_4 (Volpato & Nunes 2001). This suggests inter-species variability in pituitary deiodinase activity, which may modulate the direct action of T_4 .

In the eel, evaluation of GH synthesis *in vitro* indicated that TH not only reduced GH release but also significantly inhibited GH synthesis. This corroborates the inhibitory effect of TH on both pituitary GH content and release

Figure 6 Multiple cross-links between the somatotrope and the thyrotrope axes in the eel. Brain neurohormones, somatostatin (SRIH) and corticotropin-releasing hormone (CRH) control both pituitary growth hormone (GH) and thyrotrope hormone (TSH). TSH stimulates the synthesis of T_4 by the thyroid, while GH activates the peripheral deiodination of T_4 into biologically active T_3 . Both T_3 and T_4 exert negative feedbacks on pituitary GH and TSH.

in vivo. Similarly, in the domestic fowl, T_3 not only inhibited GH release, but also blocked the accumulation of newly synthesized GH through a direct action on the pituitary (Denver & Harvey 1991). In the present study, we investigated whether the inhibitory effect of T_3 on GH synthesis could be mediated by an inhibition of GH mRNA levels. After cloning an homologous eel GH cDNA probe, we demonstrated that T_3 significantly decreased GH mRNA levels in eel cultured pituitary cells. These data indicate that the T_3 inhibitory effect may, at least partly, be mediated by a decrease in GH mRNA steady state levels. In contrast to the present study in the eel, data in other teleost species indicated a stimulatory effect of T_3 on GH mRNA levels. In the carp, T_3 increased GH mRNA levels in pituitary fragments *in vitro* (Farchi-Pisanty *et al.* 1995). In the rainbow trout, one-week T_3 treatment *in vivo* increased steady state levels of GH mRNA but produced no alterations in pituitary or plasma GH levels (Moav & McKeown 1992). Species-related variations in the effects of T_3 on GH mRNA levels were also observed among mammals. In the rat, T_3 was shown to stimulate GH mRNA levels in cultured pituitary tumor cell lines (Samuels & Shapiro 1976). In contrast, T_3 reduced GH mRNA levels in bovine pituitary cells *in vitro* (Silverman *et al.* 1988), a situation similar to that found for human GH (Valcavi *et al.* 1992). In the rat, the positive effect of T_3 on GH mRNA levels has been attributed to a direct stimulatory effect on GH gene transcription,

mediated by the presence of positive thyroid responsive elements (TRE) (Theill & Karin 1993). In contrast, T₃ blocks human GH gene transcription even after transfection into rat pituitary cells (Cattini *et al.* 1986, Morin *et al.* 1990), indicating a regulation opposite to that of the rat GH gene. In an analysis of rat GH promoter constructs, Brent *et al.* (1991) were able to show that varying the position of TRE induced positive or negative regulation by T₃.

In conclusion, our study of a primitive teleost demonstrates negative regulation of GH expression and release exerted by THs acting directly on pituitary cells *in vitro*. This inhibition, confirmed by *in vivo* studies, may represent an ancestral negative feedback of THs on GH production, reflecting the crosslink between the thyrotrope and somatotrope axes (Fig. 6). It is clear that variation in the effects of THs on GH regulation are observed amongst vertebrate species. The negative regulation demonstrated here in an early vertebrate is also observed in birds, reptiles and some mammals including humans. This may represent an ancestral and more generalized vertebrate pattern of TH regulation of pituitary GH.

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