

# Thyrotrophin receptor protein expression in normal and adenomatous human pituitary

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## Abstract

Thyrotrophin (TSH) synthesis and secretion is under the positive control of thyrotrophin releasing hormone and under the negative control of the thyroid hormones. However, it is hypothesised that TSH has a direct effect on the regulation of its own synthesis through an intrapituitary loop mediated by pituitary TSH receptors (TSH-R). The aim of this investigation was to study the expression of TSH-R in normal human pituitary at mRNA and protein levels, and to compare the pattern of protein expression between different pituitary adenomas. Using RT-PCR we were able to detect TSH-R mRNA in the normal pituitary, and immunohistochemical studies showed TSH-R protein expression in distinct areas of the anterior pituitary. Double immunostaining with antibodies against each of the intrapituitary hormones and

S100 revealed that TSH-R protein is present in thyrotrophs and folliculostellate cells. Examination of 58 pituitary adenomas, including two clinically active and two clinically inactive thyrotroph adenomas, revealed TSH-R immunopositivity in only the two clinically inactive thyrotroph adenomas.

This study shows, for the first time, the presence of TSH-R protein in the normal anterior pituitary and in a subset of thyrotroph adenomas. The expression of TSH-R in the thyrotroph and folliculostellate cell subpopulations provides preliminary evidence of a role for TSH in autocrine and paracrine regulatory pathways within the anterior pituitary gland.

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## Introduction

Thyrotrophin (TSH) is the major regulator of thyroid hormone synthesis and secretion (Magner 1990). Despite the fact that the primary site of action of TSH is the thyroid gland, there is accumulating evidence that there is also extrathyroidal TSH receptor (TSH-R) expression. The extrathyroidal manifestations of Graves' disease, in which TSH-R is considered to represent a major auto-antigen (Burman & Baker 1985), prompted the search and detection of the TSH-R ectodomain in retroocular fibroblasts (Heufelder *et al.* 1993) and orbital preadipocyte fibroblasts (Bahn *et al.* 1998), and a TSH-R variant in extraocular muscle (Paschke *et al.* 1994). TSH-R has also been found to be expressed in thymus, kidney and adrenal cortex (Dutton *et al.* 1997), cardiac muscle (Drvota *et al.* 1995), and adipose tissue (Endo *et al.* 1995).

The cloning of a brain-derived TSH-R, and its localisation in the hypothalamus provided a hint of an additional feedback mechanism in the neuroendocrine control of the

thyroid gland, in which TSH is directly involved in the regulation of thyrotrophin releasing hormone (TRH) secretion (Bockmann *et al.* 1997). On the other hand, it is possible that TSH could exert a tight regulatory control at the level of the pituitary gland.

In this study, we investigated the expression of TSH-R mRNA and protein in the normal human pituitary. In addition we examined the expression of TSH-R in a broad range of different pituitary adenomas.

## Materials and Methods

### Materials

Cell and primary cell culture materials and reagents were obtained from Gibco (Karlsruhe, Germany), Falcon (Heidelberg, Germany), Nunc (Wiesbaden, Germany), and Sigma Chemicals (St Louis, MO, USA). Forskolin and IBMX were obtained from Sigma.

Human standard pituitary TSH (IRP 80/558) was obtained from the National Institute for Biological Standards and Controls (Potters Bar, Herts., UK), and recombinant human TSH was obtained from GENZYME GmbH (Alzenau, Germany).

#### Human tissue preparation

Samples from five human pituitary glands and two thyroid glands were obtained from autopsy cases without any evidence of endocrinological disease, with a post mortem delay of between 8 and 12 h. Additionally, 58 pituitary tumours were obtained after operation: 9 acromegalic-associated adenomas (ACRO), 8 prolactinomas (PROL), 5 Cushing's adenomas (CUSH), 4 thyrotrophinomas (TSH-oma), 19 gonadotrophic adenomas (GONA) and 13 null cell adenomas (NULL). The 4 TSH-omas were further divided into 2 clinically active and 2 clinically inactive TSH-omas. The patients suffering from clinically active TSH-omas had elevated serum TSH levels and clinical signs of hyperthyroidism, and were treated pre-operatively with sandostatin. The patients with the hormone inactive TSH-omas were characterised by normal tri-iodothyronine (T<sub>3</sub>) and thyroxine (T<sub>4</sub>) levels, and the type of adenoma was determined postoperatively by immunohistochemistry. Samples were snap-frozen and stored at -80 °C.

#### RNA extraction and RT-PCR

RNA was extracted from 3 human anterior pituitaries and 2 thyroid glands. One microgram was reverse transcribed as previously described (Pagotto *et al.* 1995). Two microlitres from the transcription reaction were amplified by PCR (94 °C for 45 s, 58 °C for 45 s, 72 °C for 1 min; 35 cycles; PCR reaction (PCR buffer, MgCl<sub>2</sub>, dNTP mix, and Taq-polymerase (MBI Fermentas, Vilnius, Lithuania) in a total volume of 30 µl) using DNA primers complementary to the extracellular portion of the human TSH-R (sense primer: 5'-GCCATCAGGAGGAGGACTT-3'; nucleotides 192-210; antisense primer 5'-CAGGTGTTTCTTGCTATC-3'; nucleotides 856-873; fragment size 681 bp). Fifteen microlitres of the reaction products were separated on a 1.2% agarose gel and visualised by staining with ethidium bromide. Human TSH-R cDNA and reverse transcribed RNA from thyroid tissue were amplified as positive controls.

The integrity of the RNA from each sample was confirmed by PCR for human β-actin (sense primer: 5'-CTAGAAGCATTGCGGTGGACGATG-3' and antisense primer: 5'-ACGGGGTCACCCACACTG TGC-3'; fragment size 660 bp).

To screen rat and mouse pituitary cell lines for TSH-R mRNA, another set of TSH-R primers was designed complementary to the extracellular portion of the rat TSH-R (sense 5'-GCGTTACTGCTCTTCCTTCC-3';

nucleotides 710-730, antisense 5'-GACTTTCTTTGACGCAGGTT-3'; nucleotides 922-941; fragment size 231 bp).

#### Immunohistochemistry (IHC)

Mouse monoclonal antibody (ab) raised against the C-terminal domain of human TSH-R (amino acids 604-764) (Loosfelt *et al.* 1992, Mizukami *et al.* 1994), and rabbit antiserum raised against the extracellular human TSH-R domain (amino acids 357-372) (Patibandla *et al.* 1997) were used at final dilutions of 1:100 and 1:250 respectively.

Monoclonal mouse ab were used to assess the expression of pituitary. Antibodies were from Immunotech (Karlsruhe, Germany), unless otherwise stated, and were diluted as follows: anti-follicle-stimulating hormone (anti-FSH) 1:800, anti-luteinising hormone (anti-LH) 1:800, anti-TSH 1:800, anti-prolactin (anti-PRL) 1:400, anti-alpha subunit 1:500, anti-adrenocorticotrophin (anti-ACTH) 1:100 (Dako Diagnostika, Hamburg, Germany) and anti-growth hormone (anti-GH) 1:800 (generous gift of Dr C J Strasburger, Department of Medicine, University of Munich, Germany). Rabbit antiserum against the folliculostellate cell marker, S100, (Biogenesis, Poole, Dorset, UK) was used at 1:20 dilution.

Sections (8 µm) were cut on a cryostat, fixed in freshly prepared 4% paraformaldehyde for 5 min, and stored in 96% ethanol. IHC was performed as previously described (Lange *et al.* 1994) with minor modifications. In brief, sections were incubated in 0.3% hydrogen peroxide in phosphate-buffered saline (PBS) for 15 min to block endogenous peroxidase activity, followed by incubation in horse serum diluted 1:10 in PBS to eliminate nonspecific binding. The sections were incubated with the primary ab diluted in PBS overnight at 4 °C. After washing in PBS, sections were incubated in biotinylated horse anti-mouse or goat anti-rabbit immunoglobulin G (Vector, Burlingame, CA, USA; 1:300 dilution) for 30 min and in avidin-biotin-peroxidase complex (Vectastain Elite Kit, Vector) for 30 min. Immunoreactivity (ir) was visualised using 1 mg/ml diaminobenzidine (DAB) as chromogen and 0.01% hydrogen peroxide as substrate. Between all steps of the staining procedure, the sections were washed three times in PBS for 5 min. Peroxidase reaction was stopped, and after 5 min in distilled water, sections were counterstained in Toluidine blue, dehydrated and mounted with Entellan.

IHC with antibodies against intrapituitary hormones was performed as described above. Sections were incubated in avidin-biotin-alkaline phosphatase complex (Vectastain Elite Kit, Vector). All buffers used were phosphate-free. Immunoreactivity was detected after incubation in Vector Red (Vector) for 15 min, with 10 mM levamisole (Sigma) added to block endogenous alkaline phosphatase activity.

Double IHC for the TSH-R and S100 colocalisation was carried out by performing first the IHC for the TSH-R, visualising with DAB, and then the IHC for S100, visualising with Vector Red, according to the protocols described above. All the buffers used were phosphate-free.

Controls for TSH-R and pituitary hormone immunostaining were performed by omitting the primary ab.

#### Primary cell culture and cell lines

Rat anterior pituitary cell culture was performed as previously described (Stalla *et al.* 1988). Male Sprague-Dawley rats were decapitated, and fragments of 10 anterior pituitary glands were mechanically and enzymatically dispersed (4 g/l collagenase, 0.01 g/l DNAase II, 0.1 g/l soybean trypsin inhibitor and 1 g/l hyaluronidase II). The pituitary cell viability was 95% as determined by acridine orange/ethidium bromide staining. Cells were diluted with cell culture medium to a density of  $2 \times 10^5$  cells/ml, distributed in 24-well tissue culture plates, and maintained at 37 °C for 3–4 days before being treated and assayed.

The mouse folliculostellate cell line, TtT/GF (Inoue *et al.* 1992), kindly provided by Prof. Kinji Inoue (Department of Regulation Biology, Saitama University, Urawa, Japan), and the rat somatomammotroph tumour cell line, GH3, were used for functional assays. TtT/GF and GH3 cells were grown in 24-well tissue culture plates at 37 °C and 5% CO<sub>2</sub> in Dulbecco's modified Eagle's medium supplemented with 2% fetal calf serum (FCS) and 10% FCS respectively, 2.2 g/l NaHCO<sub>3</sub>, 10 mM HEPES, 2 nM glutamine, 2.5 mg/l amphotericin B, 105 U/l penicillin-streptomycin, 5 mg/l insulin, 5 mg/l transferrin, 20 mg/l sodium selenite and 30 pM T<sub>3</sub>.

#### cAMP radioimmunoassay

Radioimmunological cAMP determination was performed with a commercial RIA kit from NENTM Life Science Products Inc. (Boston, MA, USA). In brief, rat pituitary, TtT/GF and GH3 cells were cultivated until confluency, washed and stimulated with standard human TSH or recombinant human TSH at concentrations of 20, 200, 500 and 1000 ng/ml. Forskolin (5 µM) was used as a positive control. The phosphodiesterase inhibitor, IBMX (5 mM), was added to all stimulation solutions. The supernatants were collected and assayed after 4 h of incubation.

#### Cell proliferation assay

GH3 and TtT/GF cell proliferation in response to TSH stimulation was measured using the WST-1 proliferation assay (Roche Molecular Biochemicals, Mannheim, Germany) which is based on the gratification of mitochondrial succinate dehydrogenase activity. The cell cultures

were incubated for three days in medium containing 2% serum and 100 µU/ml or 1 mU/ml human standard TSH. WST-1 reagent, made up following the manufacturer's instructions, was added to the cell cultures. After a 2-h incubation period, the absorbencies were determined at 440 nm using an ELISA plate reader.

#### Statistics

Each of the experiments was repeated three times. The individual experiments were performed with quadruplicate wells. ANOVA in combination with Scheffe's test was used for statistics. The data are expressed as means ± s.d.

## Results

#### RT-PCR

RT-PCR analysis of all three normal pituitary glands resulted in the amplification of the predicted 681 bp band corresponding to the TSH-R (Fig. 1a). Two thyroid glands were used as a positive control. However, it is well known that TSH-R is expressed in mesenchymal cells such as fibroblasts (Heufelder *et al.* 1993). RT-PCR studies are limiting as they cannot be used to determine the exact cell types involved; therefore IHC was performed in order to further localise the TSH-R protein within individual cell subpopulations in normal and adenomatous human pituitary.

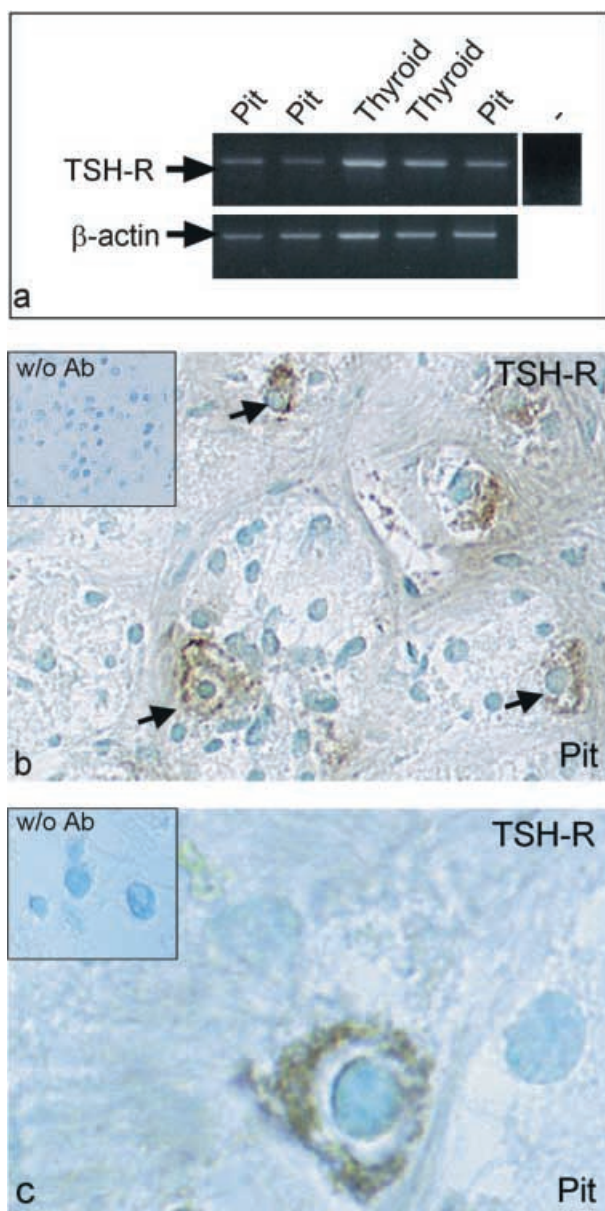
#### IHC of normal pituitary

TSH-R ir was detected in the anterior lobe of all 5 normal pituitaries by both monoclonal and polyclonal anti-TSH-R antibodies, being present in a small number of cells mainly located in the periphery, and in some fibroblasts. TSH-R ir was observed in the cytoplasm and the cell membrane (Fig. 1b,c).

To further characterise TSH-R positive areas, IHC was performed for each of the pituitary hormones in an adjacent section, and for S100 on the same section. TSH-R expression was found in cells immunopositive for TSH (Fig. 2a,b). However, the number of TSH-R expressing cells was smaller than the number of thyrotrophs. Cell counting revealed that 40% of TSH immunopositive cells show TSH-R ir. No TSH-R ir was detectable in cell populations immunopositive for ACTH, GH, FSH, LH and PRL. TSH-R ir was also found in cells immunopositive for the folliculostellate cell marker, S100 (Fig. 2c).

#### IHC of pituitary adenomas

Of the 58 pituitary adenomas analysed, endocrine cells of ACRO, PROL, CUSH, GONA, NULL and two out



**Figure 1** TSH-R expression in normal human anterior pituitary at mRNA (a) and protein (b,c) levels. (a) RT-PCR for TSH-R and  $\beta$ -actin in 3 normal human pituitaries (Pit) using 2 human thyroid glands as positive controls, and water instead of template as negative (-) control. (b) Some anterior pituitary cells (arrows) show TSH-R ir (objective 20 $\times$ ). (c) Higher magnification of a TSH-R immunopositive cell reveals the distribution of ir on the cell membrane and in the cytoplasm (objective 100 $\times$ ). In the small icons up-left of each photo is the tissue without the primary antibody (w/o Ab). In this study the monoclonal antibody directed against the C-terminal of the TSH-R was used.

of four of the TSH-omas were negative for TSH-R ir (Fig. 2h). These two TSH-omas were clinically active, since the two patients had high concentrations of

circulating thyroid hormones ( $T_3$ : 14.5 pmol/l and 14.8 pmol/l, normal values: 4.0–7.8; free  $T_4$ : 45.3 pmol/l and 45.8 pmol/l, normal values: 13–23) but normal/slightly reduced levels of TSH (0.21 mU/l and 0.28 mU/l; normal values: 0.27–4.20). The other two TSH-omas revealed a significant number of TSH-R immunopositive cells (Fig. 2f). These two adenomas were characterised as clinically nonfunctioning pituitary adenomas (the two patients had TSH $\beta$ ,  $T_3$  and free  $T_4$  values within the normal range).

To further assess the distribution of hormone producing cells in each tumour, and to detect the areas of normal pituitary tissue, immunohistochemical examination was performed on each tumour for all the pituitary hormones. IHC of the four TSH-omas revealed that the two clinically active tumours expressed lower levels of TSH ir (Fig. 2g) compared with the two clinically nonfunctioning TSH-omas, where TSH ir was very intense (Fig. 2e).

#### Functional analysis

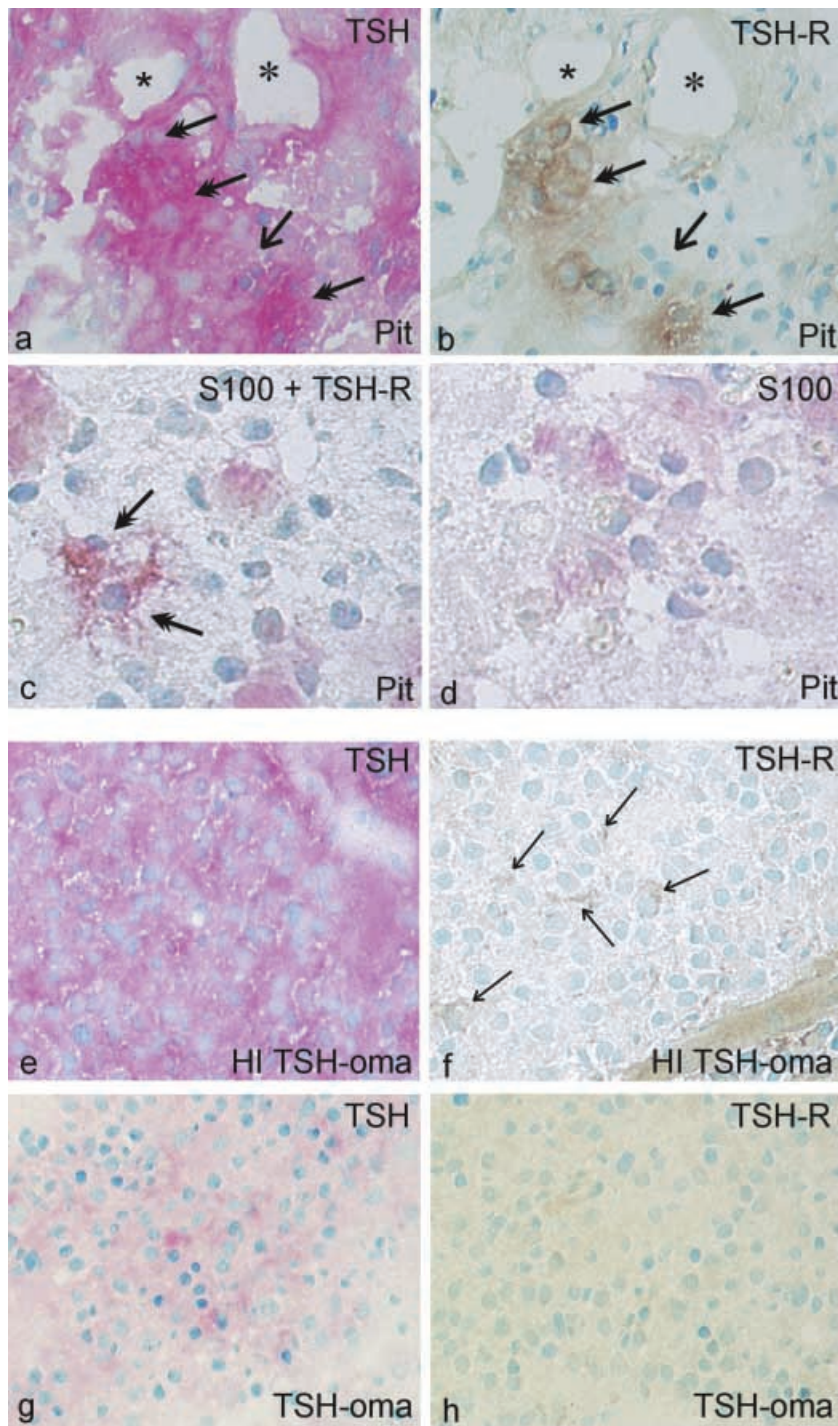
PCR studies in pituitary cell lines showed TSH-R mRNA in the rat somatomammotroph tumour cell line, GH3, and the mouse folliculostellate cell line, TtT/GF (data not shown). Therefore, we used these two cell lines as models to perform cAMP and proliferation assays. Following stimulation with standard or recombinant human TSH, no increase in cAMP levels was detected in the case of GH3 cells. In contrast, TtT/GF cells showed a modest increase in cAMP levels after stimulation with 1000 ng/ml human TSH (Fig. 3). The same experiment was repeated in rat pituitary primary cell culture and gave negative results.

The WST-1 proliferation assay failed to reveal any changes in proliferation after TSH stimulation in rat pituitary primary culture and in TtT/GF and GH3 cells (data not shown).

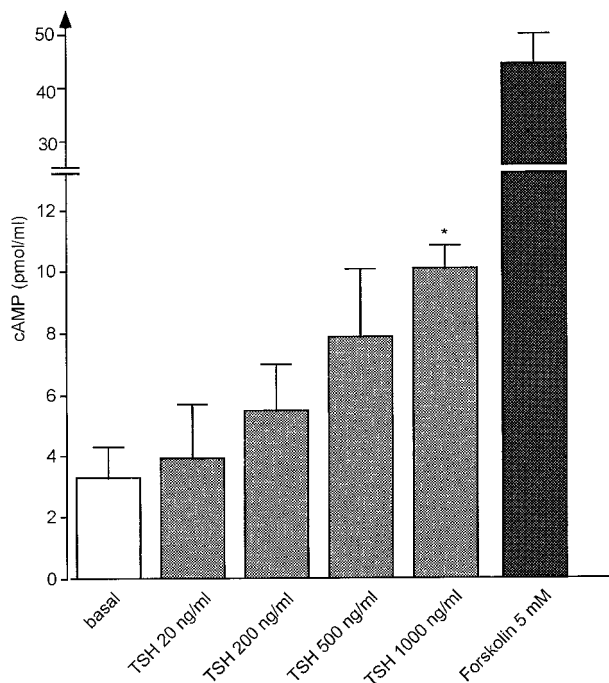
#### Discussion

In this study, we demonstrate the presence of TSH-R at both mRNA and protein levels in the human anterior pituitary. Moreover, we show by immunohistochemical analysis that TSH-producing cells and folliculostellate cells are the sole source of TSH-R ir.

Analysis of TSH-R ir in 58 pituitary adenomas revealed positive staining in only two TSH-omas, both of which were clinically inactive. The number of TSH-R immunopositive cells was lower than the number of TSH immunopositive cells. On the other hand, the number of TSH-R immunopositive thyrotrophs was lower in the TSH-omas than in the normal pituitary, in which almost half of the TSH immunopositive cells were displaying TSH-R ir. It is of interest to note that the other two TSH-omas, which were negative for TSH-R, were



**Figure 2** TSH (a) and TSH-R (b) immunostaining in two parallel sections of normal anterior pituitary. Double-headed arrows depict cells which show ir for both TSH and TSH-R, while the single-headed arrows show cells immunopositive for TSH but not for TSH-R. The different types of asterisks represent corresponding regional formations (objective  $20\times$ ). (c) Colocalisation of TSH-R with the folliculostellate cell marker S100 (objective  $40\times$ ). Double-headed arrows depict cells in which S100 and TSH-R colocalise. (d) S100 alone in an adjacent section, as a control. TSH (e, g) and TSH-R (f, h) immunostaining in a representative hormonally inactive thyrotroph adenoma (HI TSH-oma), and in a clinically active thyrotroph adenoma (TSH-oma). (e, f) HI TSH-oma has high TSH ir, while the TSH-R is concentrated in few cells indicated by arrows. (g, h) Clinically active TSH-oma showing weak TSH ir and no TSH-R ir (objective  $20\times$ ). In this study the monoclonal antibody directed against the C-terminal of the TSH-R was used.



**Figure 3** Increase in cAMP after stimulation of the TtT/GF folliculostellate cell line with standard human TSH. Forskolin (5 mM) served as a positive control. \* $P < 0.01$  vs basal stimulation.

associated with TSH-dependent hyperthyroidism. Compared with the two clinically inactive TSH-omas, IHC for TSH revealed a smaller number of TSH immunopositive cells, perhaps due to the fact that the patients had received octreotide treatment. It is possible that this treatment is responsible for the absence of TSH-R immunoreactivity in these two TSH-omas. TSH-omas have been reported to display different TSH isoform composition when compared with the normal pituitary, and with other thyrotrophinomas (Magner *et al.* 1992). Taking into account the fact that TSH synthesis and secretion by TSH-omas escape from the classical feedback control of TRH and thyroid hormones (Beck-Peccoz *et al.* 1996), it is possible that TSH plays a role in regulating its own synthesis.

The concept of pituitary hormone autoregulation has already been reported for other pituitary hormones. Previous studies have demonstrated the expression of prolactin receptor (Jin *et al.* 1997) in the normal and adenomatous human pituitary gland. Prolactin receptor mRNA was found to be present in all pituitary cell types and in all types of pituitary adenomas. Prolactinomas showed the highest levels of prolactin receptor expression, and the authors suggested that the regulatory function of prolactin receptor has greater influence on prolactin-secreting cells than any other cell type. Other studies have shown the presence of growth hormone receptor (Mertani *et al.* 1995), suggesting that growth hormone may have an autocrine action on its own production, and possibly a

paracrine action on the synthesis of PRL, FSH and LH. In contrast, TSH-R is concentrated only in the thyrotroph cell subpopulation and in a subset of thyrotrophinomas; therefore we conclude that TSH has an autocrine role in the regulation of its own expression and posttranslational modification.

In addition, in this study we have identified TSH-R in cells immunopositive for the folliculostellate cell marker, S100. While this manuscript was in preparation, Prummel and Brokken (1999) reported the presence of TSH-R in folliculostellate cells. Folliculostellate cells comprise 5–10% of the anterior pituitary cell population, and although their role is still controversial (Allaerts *et al.* 1990), there is increasing evidence suggesting an important role in the regulation of pituitary function (Schwartz & Cherny 1992). During the last decade, cell culture studies have demonstrated that folliculostellate cells are involved in the paracrine regulation of hormone secretion through the release of several cytokines and growth factors (Renner *et al.* 1996). Our demonstration of TSH-R expression in the folliculostellate cell population provides further evidence of a possible role for TSH in intrapituitary regulation, through the release of growth factors.

In view of this possible function of TSH, we attempted to assess whether the pituitary TSH-R is able to stimulate cAMP production, which is known to be the most important TSH-R secondary messenger system in the thyrocyte (Van Sande *et al.* 1990, Schaaf *et al.* 1997). We used two cell lines, known to express TSH-R mRNA, and rat pituitary cells in primary culture to examine whether stimulation of the intrapituitary TSH-R is capable of altering cAMP levels. Our results show that TSH has no effect on cAMP when applied to cultured GH3 cells or rat pituitary cells in primary cell culture. In contrast, TtT/GF cells responded with a small increase in cAMP levels following stimulation with TSH. However, the effect was observed after stimulation with physiologically high concentrations of TSH, and therefore the physiological significance of this finding is questionable. Proliferation assays showed that, unlike the thyroid TSH-R, the intrapituitary TSH-R has no proliferative effect in any of the cell cultures tested. Of note, similar results have been reported for TSH-R in astrocytes, where TSH failed to stimulate cAMP synthesis and phosphatidylinositol hydrolysis, was not mitogenic, and exerted its effect by stimulating arachidonate release and by activating the MAP kinase pathway (Tournier *et al.* 1995). Whether the intrapituitary TSH-R is coupled to similar alternative pathways must be addressed in subsequent studies.

In conclusion, we demonstrate TSH-R expression at both mRNA and protein levels in the human pituitary gland and in TSH immunopositive pituitary adenomas. These data provide new evidence for a short-loop homeostatic mechanism in which the pituitary thyrotroph may 'sense' the quantity and quality of circulating TSH and adjust TSH secretion and bioactivity in response to the

endocrine needs of the individual. This effect may involve an alteration of the glycoprotein composition and thus bioactivity of the nascent TSH. Moreover, our demonstration of TSH-R expression by folliculostellate cells favours a paracrine role for TSH within the pituitary. Further studies are needed to explore the significance of the TSH-R and the paracrine and autocrine effects of TSH in the normal and adenomatous pituitary gland.

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