

Salivary gland is capable of GH synthesis under GHRH stimulation

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Abstract

Twelve female rats weighing ~ 150 g received in the submaxillary gland a pellet capable of releasing $3.5 \mu\text{g}$ GHRH/h for 60 days. Another eight sex- and weight-matched animals received placebo pellets in the same place. After two months the animals were killed, heart blood was collected and pituitary and submaxillary glands were carefully dissected. Pituitary GH content in both placebo- and GHRH-treated animals showed similar values, but plasma GH and IGF-I levels were significantly lower in the animals carrying GHRH pellets ($P < 0.03$);

these animals also had a significantly higher GH content in the submaxillary gland (19.2 ± 8 ng/mg protein) compared with the placebo-treated group (1.1 ± 0.3 ng/mg protein). GH mRNA was present only in the submaxillary gland of GHRH-treated rats as determined by PCR-Southern blot and by *in situ* hybridization methods. It is concluded that high local GHRH levels are capable of inducing transdifferentiation in submaxillary gland cells to synthesize GH.

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Introduction

Previous data from our studies have shown that parotid gland tissue is capable of partially assuming pituitary functions when influenced by hypothalamic hormones both *in vivo* (Álvarez-Vega *et al.* 1991, Granados *et al.* 1993) and *in vitro* (Fernández *et al.* 1994). Thus, parotid cells when submitted to hypothalamic extracts were able to synthesize and secrete at least luteinizing hormone (LH), follicle-stimulating hormone, thyrotrophin and growth hormone (GH). In these studies, LH secretion by parotid tissue was found to be related to the dose of hypothalamic extract used and to the presence of LH releasing hormone (LHRH); a dramatic reduction in LH values was detected when the hypothalamic extract used had previously been treated with a specific antibody against LHRH.

This study has been designed in order to see if GH releasing hormone (GHRH) by itself is capable of inducing morphofunctional changes in submaxillary glands when administered *in vivo* at high continuous concentrations.

Materials and Methods

Twenty female Wistar rats weighing 157 ± 3 g (range 142–173 g) were used. Animals were kept in Macrolon cages under controlled conditions of temperature

(21 ± 2 °C) and light (12 h light/12 h darkness), with tap water and rat chow (Panlab, Barcelona, Spain) available *ad libitum*. The studies were conducted in accordance with the principles and procedures outlined in the NIH Guide for the Care and Use of Laboratory Animals. All animals were implanted with a slow release pellet (Innovative Research of America, Sarasota, FL, USA) in the right submaxillary gland under light ether anaesthesia. Group A (12 rats) received a pellet loaded with 5 mg GHRH 1–29 NH₂ (GRF; Bachem, Bubendorf, Switzerland) allowing a continuous peptide release for 60 days ($3.5 \mu\text{g}/\text{h}$). Group B (8 rats) received placebo pellets. Animals were weighed each week.

After 60 days, the submaxillary glands were dissected out under ether anaesthesia. Glands were halved: one half was immediately frozen in liquid nitrogen and the other half was kept at -20 °C. Blood was drawn from the heart, the rats were killed by decapitation and their pituitary glands carefully removed and also kept frozen at -80 °C until analysed.

Protein estimation

Protein content was determined in homogenates of pituitary and submaxillary glands by the Brilliant Blue method (Bradford 1976). The homogenates were obtained by sonication (Branson sonifier 450), the tissues were processed in saline solution and then centrifuged for

15 min at 3000 r.p.m. at 4 °C. The colouring reagent was added to the supernatant and the sample read in a Spectrophotometer (U-1100 Hitachi). A reference curve was prepared with bovine serum albumin (Sigma, St Louis, MO, USA).

Hormone determination

For the GH determinations, the reagents provided by the NIADDK (Baltimore, MD, USA) were used as previously described (Lima *et al.* 1993). The iodination of rat GH I-6 was performed using lactoperoxidase (Thorell & Johansson 1971). The standard used was rat GH r-p2 and the sensitivity of the curve was 25 pg/ml. The intra- and interassay coefficients of variation were 5.7% and 9.4% respectively. Insulin-like growth factor I (IGF-I) was measured using the antibody (UB2-495) provided by the NIADDK. The IGF-I used for iodination (by the Chloramine T method) and standard curve dilution was A 52-EDP-186 (Lilly Company, Indianapolis, IN, USA). The assay was performed following the method of Daughaday *et al.* (1980, 1982) as previously described by our group (Hermanussen *et al.* 1996). The sensitivity of the curve was 10 pg/ml, and the intra- and interassay coefficients of variation were 7.8% and 12.9% respectively. Plasma GH was measured directly and plasma IGF-I was estimated after acid-ethanol extraction. For the estimation of both GH and IGF-I content, halved submaxillary and pituitary glands were homogenized in saline and centrifuged. Measurements were carried out in the supernatant. GH was determined directly and IGF-I after acid-ethanol extraction.

Extraction of GH mRNA and cDNA synthesis

A portion of the rat submaxillary glands, kept at -80 °C and from rats treated with GHRH or placebo pellets were homogenized and mRNA was extracted using poly-dT coupled particles (Quick-Prep mRNA micro purification kit; Pharmacia, Biotech, Uppsala, Sweden). Pituitary glands from normal animals were used as a positive control of the amplified product.

The extracted mRNA was used for the synthesis of cDNA using 200 IU Moloney murine leukaemia virus reverse transcriptase, 20 mM Tris-HCl pH 8.3, 50 mM KCl, 5 mM MgCl₂, 1 mM dNTP, 30 U RNAase inhibitor, 10 mM dithiothreitol, 1×10^{-6} M oligonucleotide 3' and was then used in the amplification reaction. This entire extracted sample was submitted to a cycle of 37 °C for 50 min, 42 °C for 15 min and 95 °C for 5 min.

PCR and Southern blot

Two specific primers, upstream 5' TTGCTTCGCTT CTCGCTGCT 3' (1820-1840 nt), downstream 5' AAGCGGCGACACTTCATGAC 3' (2342-2361 nt)

(25 pmol each) for the GH gene (Barta *et al.* 1981) were used for the amplification of GH cDNA by PCR, in the presence of 1 U Taq polymerase, 18.6 mM Tris-HCl, 45.9 mM KCl, 3 mM MgCl₂, and 2 mM dNTP. Thirty 1-min cycles at 94 °C, 55 °C, 72 °C and a final 10-min cycle at 72 °C were run.

Results were visualized in 1% agarose gel electrophoresis with ethidium bromide staining under UV light.

The agarose gel was blotted onto a Hybond-N⁺ nylon membrane (Amersham Life Science, Amersham, Bucks, UK) under 0.5 M NaOH overnight. The prehybridization was performed at 57 °C for 3 h in 50 × Denhardt's solution (1% Ficoll, 1% polyvinylpyrrolidone and 1% BSA), 20 × SSPE, 10% SDS and 10 mg/ml denatured salmon sperm DNA. Hybridization was carried out in the same buffer as that in the prehybridization step using a ³²P-labelled specific probe for 20 h at 57 °C. After the hybridization, the membrane was washed five times in 6 × SSC for 20 min at 57 °C. The blots were exposed overnight to X-ray film at -80 °C with an intensifying screen and developed by standard procedures.

In situ hybridization

Halved pituitary glands of control rats and the remaining portion of the submaxillary glands of the rats treated with the GHRH or placebo pellets, were kept at -80 °C and were used for *in situ* hybridization. The tissues were cut in a cryostat (Slee, Mainz, Germany) in sections of 7-10 µm.

A specific synthetic probe for GH (5' GACTGGATG AGCAGCAGCGAGAAGCG 3') was non-radioactively labelled by the incorporation of digoxigenin. After hybridization the oligonucleotide-labelled probe was found to target nucleic acids in the pituitary or in the submaxillary gland as determined by enzyme-linked immunoassay using an antibody conjugate (anti-digoxigenin alkaline phosphate conjugate, anti-DIG-AP). A subsequent enzyme-catalysed colour reaction with 5-bromo-4-chloro-3-indolyl-phosphate (BCIP) (Boehringer Mannheim, Barcelona, Spain) and nitroblue tetrazolium salt (NTB) (Boehringer Mannheim) produced an insoluble blue precipitate, which indicates hybrid molecules.

To avoid the possibility of non-specific (proper) signals, some sections were treated with ribonucleases (Ribonuclease A, Ribonuclease T₁, Sigma) before hybridization. Another control was performed avoiding the use of anti-DIG-AP.

Statistical evaluation

Data are presented as means ± s.e.m. Comparisons between the groups were made by Student's *t*-test analysis for unpaired means.

Results

Body weight at the beginning of the experiment was 157 ± 3.3 g in group B and 157.5 ± 2.9 g in group A rats. From the 4th week onwards, a significant attenuation of weight increase was observed in rats in group A. At the end of the experiment the placebo group weighed 274 ± 8.4 g and the GHRH-treated animals weighed 257 ± 6 g ($P < 0.01$).

GH content of pituitary glands of both GHRH- and placebo-treated rats showed similar values (185 ± 39 $\mu\text{g}/\text{mg}$ vs 197 ± 25 $\mu\text{g}/\text{mg}$) but plasma levels were significantly lower in the animals implanted with GHRH pellets (5.5 ± 0.8 vs 24 ± 9 ng/ml, $P < 0.03$). This diminution was also observed in the IGF-I plasma levels of GHRH-treated rats (1.1 ± 0.07 vs 1.6 ± 0.1 $\mu\text{g}/\text{ml}$, $P < 0.01$) (Fig. 1).

In the 8 animals in group B, the GH content of the submaxillary glands was 1.1 ± 0.3 ng/mg protein whereas in 7 of the 12 rats treated with GHRH pellets, GH content was 19.2 ± 8 ng/mg ($P < 0.01$) (Fig. 2). There was no significant difference in the IGF-I content of placebo- or GHRH-treated rats (group B, 3.6 ± 0.6 ng/mg protein; group A, 3.1 ± 0.4 ng/mg protein).

mRNA for GH was absent in the glands from placebo-treated rats but was present in tissue from GHRH-treated rats. In the 7 animals showing significant GH content, a good correlation was found with the semiquantitative mRNA determination (Fig. 3). GH mRNA was confirmed by Southern blot in the glands of GHRH-treated rats.

In situ hybridization for GH in all the pituitaries used was positive, and the signal disappeared when the tissues were pretreated with ribonucleases, or were not treated with anti-DIG-AP. The rats implanted with the GHRH pellet and showing high GH content in the submaxillary gland by RIA or PCR-Southern blot presented specific GH hybridization, which was always absent in controls with a placebo pellet (Fig. 4).

Discussion

Morphofunctional changes are also possible in adult tissues, as has been shown by several authors (Potter *et al.* 1986, Bellows *et al.* 1990, Kasper *et al.* 1990) and also our group (Fernández *et al.* 1994).

Parotid gland tissue submitted to the influence of crude hypothalamic extracts was able to assume some pituitary functions including GH synthesis (Granados *et al.* 1995). This was also true when the parotid gland cultures were treated with synthetic hypothalamic hormones, although in this case the effect was less marked (Fernández *et al.* 1994), probably indicating the existence of unknown enhancers in the crude hypothalamic extract.

Since hypothalamic hormones have also been shown to play a role in pituitary differentiation (Khorram *et al.* 1983,

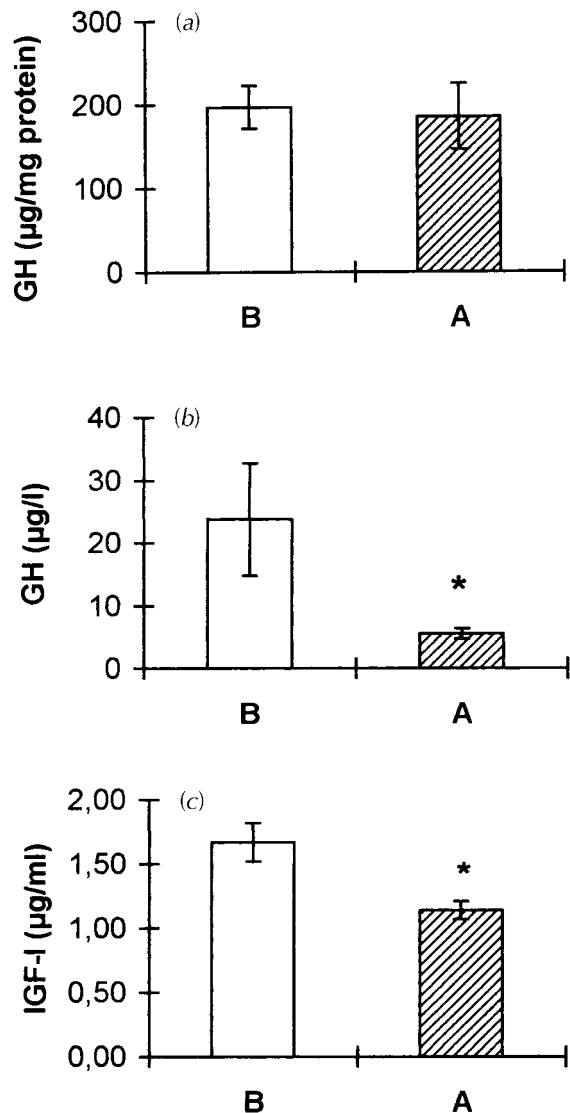


Figure 1 (a) Pituitary GH content, (b) plasma GH levels and (c) plasma IGF-I levels in GHRH-treated (group A, $n=12$) and placebo-treated (group B, $n=8$) rats. * $P < 0.03$ in (b), * $P < 0.01$ in (c).

Heritier & Dubois 1994) the present study was performed to confirm the possible transdifferentiating effect of GHRH on submaxillary glands *in vivo*. Using continuous release pellets charged with GHRH, nearly 60% of the treated animals showed, two months later, GH content and GH mRNA in the submaxillary gland tissue as determined by PCR-Southern blot and by *in situ* hybridization. Animals treated with placebo were negative for GH mRNA in the two systems used, although low GH-like contents were detected by RIA. Since salivary glands are known to produce at least some peptide hormones (Hauser-Kronberger *et al.* 1992) and especially

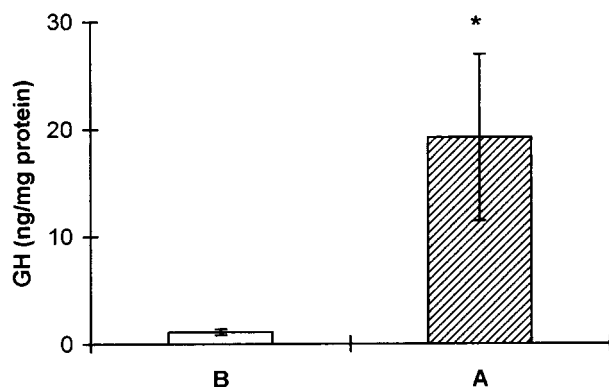


Figure 2 GH content in the submaxillary gland of GHRH-treated (group A) and placebo-treated (group B) rats. * $P < 0.01$.

growth factors (Thesleff *et al.* 1988, Ryan *et al.* 1992, Amano *et al.* 1993), the possibility of a persisting genomic situation allowing GH synthesis cannot be ruled out, although the absence of GH mRNA by PCR-Southern blot and *in situ* hybridization in the placebo-treated animals does not support this theory. However, the high local content of growth factors, and especially epidermal

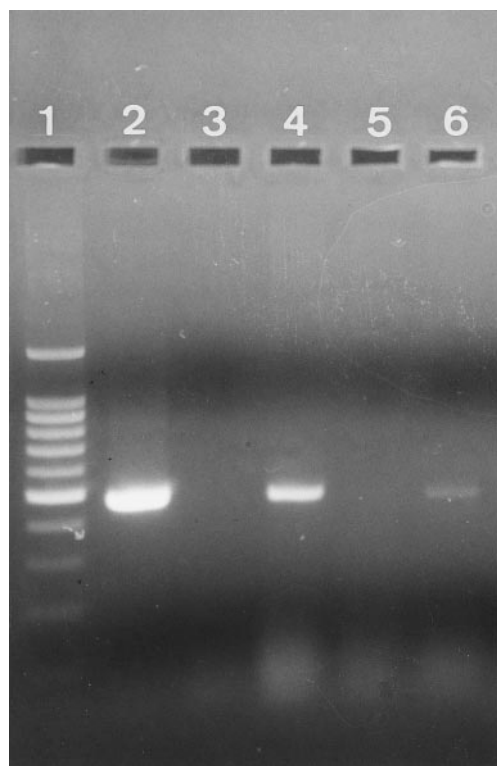


Figure 3 GH cDNA obtained after PCR in submaxillary glands of GHRH-treated rats (lanes 4 and 6), and the absence of GH cDNA in placebo-treated rats (lanes 3 and 5). Lane 2, positive control in pituitary; lane 1, molecular weight markers.

growth factor from the submaxillary gland could at least play an important role in the GHRH-induced GH synthesis (Ikeda *et al.* 1984).

Another possibility arises in which high GHRH levels could induce GH synthesis in tissues other than the pituitary, as was originally suggested by Leveston *et al.* (1981). This was also observed in some cases of GHRH-producing tumours, but in these cases the GH was thought to be an artefact (Caplan *et al.* 1978). Pancreatic tumours which cause acromegaly, in which GHRH was isolated for the first time (Guillemin *et al.* 1982, Rivier *et al.* 1982), could also have produced GH.

GHRH is not only capable of stimulating GH synthesis and release at the pituitary level (Devesa *et al.* 1992) but also enhances somatotrophic proliferation (Sano *et al.* 1988), resulting in pituitary hyperplasia through c-fos expression (Billestrup *et al.* 1987). However, Melmed *et al.* (1985) considered that it was highly unlikely that GHRH stimuli alone were sufficient to initiate the multistep process of pituitary tumorigenesis, since the pituitaries of patients with ectopic GHRH production have hyperplasia but not adenoma (Sano *et al.* 1988). Our data indicate that very high local GHRH levels in the submandibular salivary gland, as obtained with the GHRH pellets, are capable of stimulating GH synthesis after 2 months, in accordance with an evident action of GHRH.

This could perhaps explain an apparent discrepancy. Various cases of acromegaly due to ectopic GHRH secretion have been published (Guillemin *et al.* 1982, Rivier *et al.* 1982, Sano *et al.* 1988). In all of them the source of the high plasma GH levels responsible for the disease was assumed to be the hyperplastic pituitary. When GHRH, at four different dosages for 1 month, was given continuously to human volunteers by the s.c. route using portable infusion pumps, no modification or an increase in the GH secretion pattern appeared. If this infusion was maintained for 6 months or more, as was the case in a group of 5 children with idiopathic GH deficiency, there was an initial important increase in growth velocity. However, a very dramatic reduction in this parameter was observed after 4 months, with complete cessation after 6 months (Tresguerres *et al.* 1993). These data indicate a desensitizing effect of the continuously administered GHRH as is already known for LHRH (Knobil 1980) albeit with a much longer latency period. The down-regulation was confirmed in dogs using slow release GHRH microcapsules. One month following the injection a marked reduction in plasma GH levels was observed as compared with placebo-treated animals, together with an almost complete depletion of the pituitary GH content (J Devesa, L Limaand and V Arce, unpublished observation). Rats submitted to continuous GHRH administration showed, after three weeks, a marked pituitary hyperplasia but the GH content was not increased (Ariznavarreta *et al.* 1994). All these data support the existence of a down-regulatory mechanism in pituitary

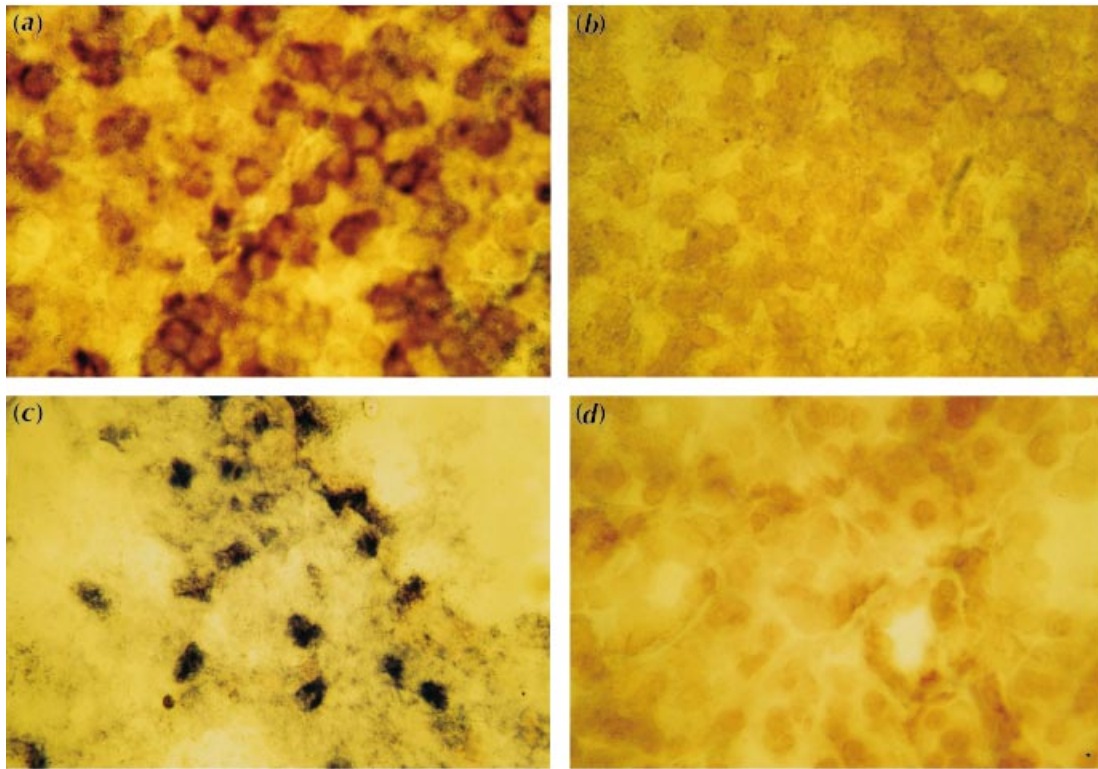


Figure 4 (a) *In situ* hybridization of a normal pituitary. (b) Absence of signal in a pituitary when treated with ribonucleases. (c) Positive staining of a submaxillary gland from a GHRH-treated rat. (d) Absence of response in a submaxillary gland from a placebo-treated rat. Scale bar represents 115 μ m.

GH secretion when submitted to continuous GHRH stimulation. If this is the case, one possible explanation for the acromegalies in ectopic GHRH-secreting tumours could be that at least part of the GH production originates in the tumour itself, as our data with the pellets suggest.

The high amounts of GHRH released at the submaxillary level also seem to exert a negative feedback on GH secretion, since GH and IGF-I plasma levels are significantly reduced and body weight is lower. This effect of GHRH, as the inductor of GH formation, could be produced through the synthesis of Pit-1 (Fox 1990, Delhase *et al.* 1993) in the salivary gland and other tissues, but this theory needs to be investigated further.

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