

# Immunocytochemical evidence for growth hormone-releasing hormone in the tanycytes of the median eminence of the rat

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*The current study was performed to analyse the potential existence and structure of a GHRH-transporting tuberoinfundibular system in the rat median eminence. The immunocytochemical analysis using anti-GHRH revealed an intense immunoreaction in the ependymary cells, tanycytes, at the level of the floor of the infundibular recess forming part of the median eminence. The basal processes of these cells course towards the external layer of the median eminence and reach the growth hormone-releasing hormone (GHRH) fibres of the tuberoinfundibular tract and this reaction was increased after intraventricular treatment with colchicine. Thus, these observations suggest the existence of a second or alternative cerebrospinal fluid-mediated route of GHRH transport to the median eminence and implicate the involvement of tanycytes in the regulation of this novel transport system.*

**key words:** hypothalamus, neuropeptides, ependymary cells, immunocytochemistry, colchicine

## INTRODUCTION

Tanycytes may serve a neuroendocrine transport function based on their specialised structures, which include apical microvilli on the ventricular surface and long basal processes that terminate in blood vessels or in the glia limitans. Although the precise mechanisms by which tanycytes modulate hypothalamic secretory axons have not yet been elucidated, tanycytes are believed to possess guiding functions for hypothalamic axons and to be involved in transport mechanisms between the ventricle and blood vessels of the portal system [69]. Furthermore, tanycytes in the arcuate nucleus and median eminence release trophic factors that regulate hormone secretion by hypothalamic neurones [25]. Supporting this idea, transected axons of

adult hypothalamo-neurohypophyseal neurones regenerate along tanycytic processes [14].

Recently, in several animal species, biologically relevant proteins have been detected in tanycytes by immunohistochemistry or *in situ* hybridisation, including glutamate transporters GLT-1 and GLAST [3],  $\alpha$ -MSH [15], glucose transporter-1 [24], aquaporin-9 [21], type 2 iodothyronine deiodinase [22], insulin-like growth factor binding protein-2 [9], connective tissue growth factor [36], and several receptors for somatostatin [29], GABA [51], glutamate [34, 65], activin type I [52], insulin growth factor I [11, 26], erbB3 and erbB4 [64].

Given that receptors for different mediators of pituitary growth hormone (GH) secretion, such as GHRH

(growth-hormone releasing hormone), somatostatin and IGF-I, have been localised to the tanycytes of the median eminence [2, 11, 26, 29], it is reasonable to hypothesise that tanycytes may play a role in the regulation of GH secretion. Although several publications demonstrate the presence of receptors for regulatory peptides in tanycytes, the immunohistochemical analyses of these peptides in tanycytes have received much less study. Thus, we were prompted to perform the current investigation to analyse the GHRH-transporting tuberoinfundibular system in the rat median eminence and its different components, particularly the tanycytes and ependymal cells.

## MATERIAL AND METHODS

### Maintenance and use of animals

Twenty Sprague-Dawley rats (10 of each sex) were used for this study. Five of each sex were injected intraventricularly (lateral ventricle) with 90 µg of colchicine dissolved in 15 µl of saline. The remaining animals (5 of each sex) were used as controls and received intraventricular injections with 15 µl of saline (lateral ventricle) under ketamine anaesthesia, according to the established standards of Pellegrino and Cushman [48]. Animals were handled according to the guidelines of the European Communities Council directive (86/609/EEC) and current Spanish legislation for the use and care of laboratory animals (BOE 67/8509-12,1998).

### Generation of anti-GHRH antibodies

To obtain the primary antibodies against rat GHRH, rat GHRH (amino acids 1–43) was conjugated to bovine thyroglobulin using the coupling reagent carbodiimide. This complex, containing 1.2 mg of rat GHRH, was emulsified with 4 mg of complete Freund adjuvant and 4 mg of dried mycobacterium tuberculosis bacilli and injected subcutaneously in adult male rabbits. Each animal also received 0.5 ml of pertussis vaccine with the primary immunisation. Booster injections were given at fortnightly intervals. The specificity of the serum was tested against pancreatic polypeptide, insulin, gastrin, glucagon, somatostatin, arginine-vasopressin, luteinising hormone-releasing hormone, cholecystokinin, β-endorphin, secretin and gastrin inhibitory peptide. Based on radioimmunoassay, the cross reaction was 100% for rat GHRH (amino acids 1–43), 0.04% for human GHRH (aa 1–44 isoform) and 0.037% for human GHRH (aa 1–29 isoform). Thus, all results indicate that the anti-serum is highly specific for rat GHRH, as was observed by Fernández et al. [23].

### Preparation of histological sections and immunostaining

Twenty-four hours post-treatment, the animals were sacrificed. After removal, the brains were fixed in Bouin-Hollande fluid for 5 days and then embedded in paraffin. Serial frontal sections (5 µm) were made and processed by the PAP enzymatic method with rabbit serum anti-rat GHRH (dilution 1:1000), swine serum anti-rabbit immunoglobulin (Dako z-196, dilution 1:100) and PAP complex (Dako z-113, dilution 1:200). Saline tris 0.05M (pH: 7.4 with 8% NaCl) was used for the dilutions and washes. Pre-absorption tests with rat GHRH and human GHRH and substitution tests of the primary serum with normal rabbit serum were performed. After either the substitution test or preabsorption with rat GHRH, the immunoreaction was completely abolished.

### Morphometric analysis of GHRH immunostaining

The preparations obtained were studied using a Zeiss-axiophot microscope. The number of GHRH-immunoreactive endings contacting with the third ventricle was calculated by analysing 25 serial sections per animal (each 25 µm) in the transitional cranio-caudal zone at the level of the infundibular recess (125 sections per sex and group).

In order to determine the number of GHRH-immunoreactive tanycytes (only tanycytes in which the cell nuclei was apparent were scored), we studied 25 sections of the median eminence in the cranio-caudal transitional zone [21] (each 50 µm, mean diameter of cell nuclei: 6.74 µm), per animal. The values obtained are shown in Table 1. Table 2 shows the values obtained in the count of GHRH-reactive tanycytes after applying the correction factor (CF: 0.88) according to the Abercrombie [1] formula: Section thickness/Object length + Section thickness. For both the parameters considered, we analysed statistical values per section and per animal (expressed as the arithmetical mean ± the standard deviation).

**Table 1.** Mean values (±SD) of morphometric analysis of anti-GHRH immunoreactive tanycytes

Group	Sex	Tanycyte/slide	Tanycyte/animal
Control	Males	2.21 ± 1.87	25.61 ± 7.62
	Females	1.86 ± 0.84	24.62 ± 6.39
Colchicine	Males	8.75 ± 3.18*	126.30 ± 20.16*
	Females	6.94 ± 3.25*	118.57 ± 18.37*

\*p < 0.01

**Table 2.** Mean values ( $\pm$ SD) morphometric analysis of anti-GHRH immunoreactive tanyocytes with Abercrombie's correction factor (1)

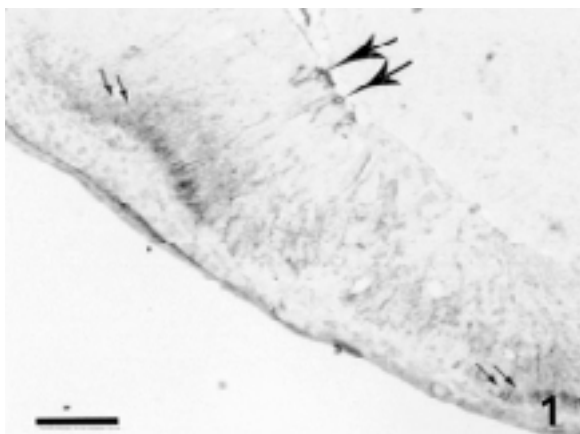
Group	Sex	Tanyocytes/animal
Control	Males	22.57 $\pm$ 6.71
	Females	21.70 $\pm$ 5.63
Colchicine	Males	111.30 $\pm$ 17.77
	Females	104.49 $\pm$ 19.71

Calculation of the GHRH-immunoreactive tanyocytes was performed with an Apple digital planimeter connected to an RCA video system. The values obtained were compared statistically using the ANOVA-test. Values of  $p < 0.05$  were considered as significant.

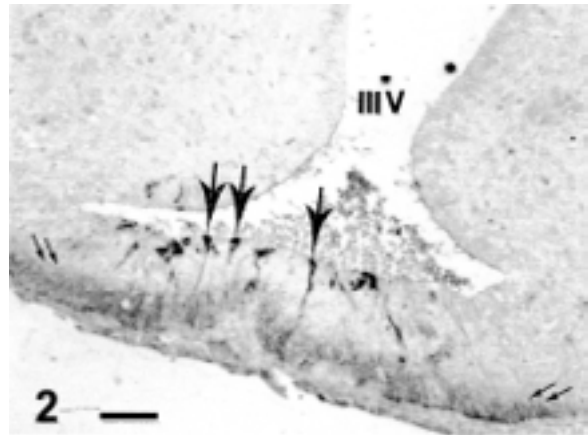
### RESULTS

The hypothalamus of control and colchicine-treated rats contained GHRH-reactive neurones situated in the arcuate nucleus, in the lateral basal hypothalamus and in the perifornical region. GHRH-immunoreactive fibres were noted in the external layer of the median eminence (Fig. 1, 2), forming part of the tuberoinfundibular system.

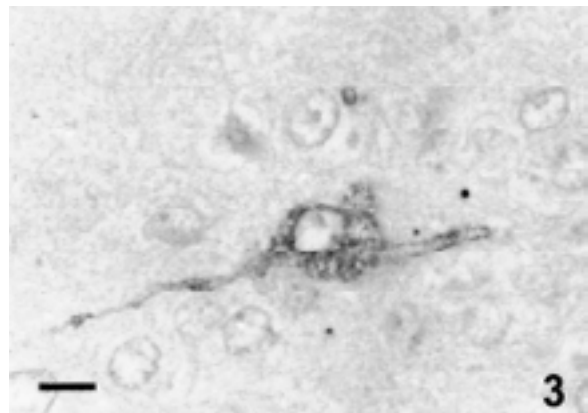
Most anti-GHRH reactive neurones were spindle-shaped or round, with two prominent prolongations (Fig. 3). Occasionally, in the arcuate nucleus, bipolar cells were observed; they exhibited a medial prolongation directed towards the lumen of the third ventricle, and another lateral one projecting towards other zones, possibly to the GHRH-tuberoinfundib-



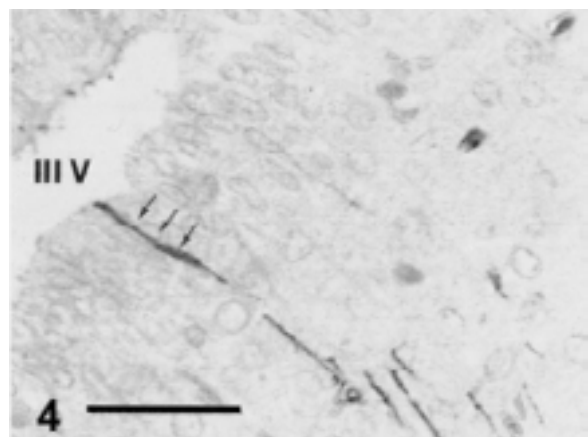
**Figure 1.** Frontal section of the cranial region of the median eminence of an untreated control animal demonstrating GHRH-immunoreactive fibres located in the external layer of the median eminence (thin arrows) and somata of GHRH-immunoreactive tanyocytes (arrows). Bar = 50  $\mu$ m.



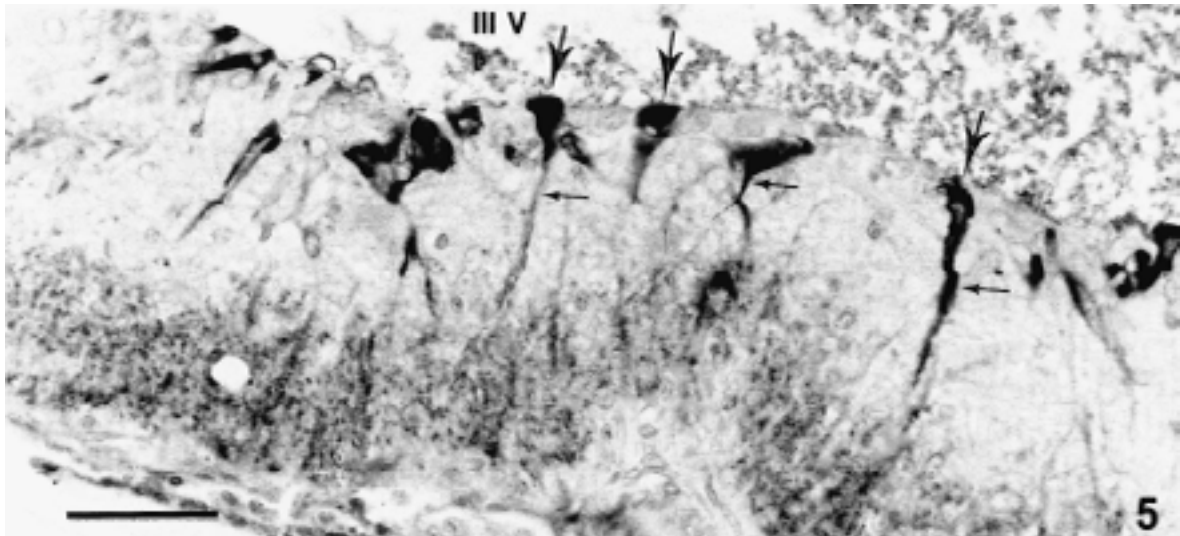
**Figure 2.** Frontal section of the median eminence at the cranio-caudal transition level of a colchicine-treated animal displaying the fibres of the tuberoinfundibular system in its external zone (thin arrows) and tanyocytes (head arrows) at the level of the floor of the infundibular recess that are immunoreactive with anti-GHRH (III V — third ventricle). Bar = 50  $\mu$ m.



**Figure 3.** GHRH-reactive neurone located in the lateral basal hypothalamus of a colchicine-treated rat. Bar = 10  $\mu$ m.



**Figure 4.** Anti-GHRH immunoreactive fibres (arrows) that reach the lateral wall of the third ventricle (III V) and penetrate between ependymary cells. Image obtained from the anterior caudal portion of the median eminence of a colchicine-treated animal. Bar = 50  $\mu$ m.



**Figure 5.** Magnification anti-GHRH staining of the somata of the tanycytes (arrow bead) and their basal processes (thin arrows) (III V — third ventricle). Bar = 50  $\mu$ m.

ular system. In the colchicine-treated animals, GHRH immunoreactive endings were also seen in the lateral wall of the third ventricle at the level of the arcuate nucleus. After passing between two ependymary non-immunoreactive cells, these fibres contacted the lumen of the third ventricle (Fig. 4).

Analysis of anti-GHRH staining of the median eminence from both animal groups revealed specialised ependymary cells, whose morphology suggested that they were GHRH-containing tanycytes (Fig. 1, 2, 5). These cells were localised in the floor of the infundibular recess at the level of the cranial region and the transition of the cranial to the caudal regions of the median eminence, as described by Rodriguez et al. [56]. Moreover, these cells continued until the beginning of the hypophyseal stalk. Caudally to these immunoreactive cells, neither fibres nor reactive ependymary cells were observed. In the untreated control animals, only isolated tanycyte somata were seen and these stained weakly (Fig. 1). As expected, in the animals treated with colchicine, the anti-GHRH immunoreaction was stronger than in the control animals. Many basal processes were observed (Fig. 2, 5), together with an increase in the number of the GHRH-immunoreactive tanycytes when compared to the untreated control animals ( $p < 0.01$ ) (Table 1, 2). Colchicine treatment appeared to slightly alter the shape of the immunoreactive tanycytes, although they were typically well-stained and visibly projected from the floor of the infundibular recess to the external layer of the median eminence (Fig. 1–3), intermingling with other immunoreactive fibres possibly belonging to the tuberoin-

fundibular system. The appearance of reactive tanycytes in the dorsal and angular walls of the infundibular recess was very rare.

These findings were similar for both male and female rats. No differences in the morphological and immunohistochemical features between the sexes were observed. Interestingly, anti-GHRH immunoreactive tanycytes were more numerous in males than in females (Table 1, 2), although the differences were not statistically significant.

## DISCUSSION

Since the discovery and synthesis of GHRH [27, 54], many studies have focused on the identification of GHRH-producing neurones and their intrahypothalamic location in teleost fish [45, 46], rats [6, 9, 16, 32, 38, 41], cats [10], primates [4, 5, 37] and human [7, 8, 39, 49, 63]. Although the projections of the GHRH-tuberoinfundibular system in these various species have been documented, none of these studies has described the tanycyte system as one containing GHRH-reactive cells.

Several reasons may explain why the present findings have been overlooked or not detected by previous studies. First, some of the earlier studies were performed in humans or in animal species other than the rat and, thus, anatomical or technical differences may have obscured the detection of GHRH-reactive tanycytes in these other systems. In the published studies of the rat, differences in methodology may explain the failure of other researchers to detect GHRH staining in tanycytes. Some investigations have relied on anti-human or porcine GHRH serum [35,

41]; others used vibratome sections to analyse the reactive neuronal somata and fibres of the tubero-infundibular system [18, 20, 31, 66], while others analysed the co-expression of GHRH with other substances [16, 17, 40, 44, 53]. Vibratome preparations may have masked these results because the tanycyte processes would have been hidden behind the immunoreactive fibres. None of the published studies considered the possibility that tanycytes contain immunoreactive GHRH and none was conducted with paraffin sections immunostained for GHRH after colchicine treatment as in the current study.

As a classic inhibitor of neuronal secretion and re-uptake, colchicine administration has been used extensively as a tool to facilitate detection of low-abundance peptides or hormones in different cellular types, which under normal conditions might not display sufficient immunoreactivity for detection. Thus, colchicine is commonly used to increase the intensity of the immunoreaction, especially in the studies of the central nervous system [42, 58, 59, 61]. In our studies, we did not observe any evidence of an inflammatory reaction in response to treatment with colchicine, as described by Hasson and Norstrom [30] and Norstrom et al. [43] and, thus, can exclude the possibility that our findings regarding GHRH-staining in tanycytes reflect a secondary effect of this substance. This fact is also confirmed by the detection of anti-GHRH immunoreactive-tanycytes in the untreated animals, further dismissing any doubts that our observations represent artefacts resulting from colchicine administration at the level of the lateral ventricle.

The increased immunoreactivity for GHRH after treatment with colchicine was most likely the result of the slowing of intracellular transport and inhibition of exocytosis, similar to what has been described by our laboratory in neuronal and pituitary cells [12, 13, 47, 58, 59] and by other authors in the median eminence [60, 62, 68].

Although the transport through the tanycytes of the median eminence has been observed by several researchers [55, 57, 63], no clear evidence exists that this actually occurs. In fact, the possibility of this transport system has been refuted by several publications [50, 67]. The release of GHRH toward the CSF is possible, although a detailed characterisation of the peptide in the CSF of the rat similar to that described in humans [33] would be required to corroborate such a hypothesis.

Guldner and Wolff [28] have described ultrastructural images of synapse-like connections between

axonal profiles and tanycyte processes and studying the neuronal regulation of GH secretion, Daikoku et al. [19] have reported similar images, although these connections were observed in unstained sections. Given that expression of IGF-I binding protein-2 [11], receptors for IGF-I [2, 11, 26] and somatostatin [29] have been observed in the luminal membrane of tanycytes of the median eminence, the immunodetection of GHRH in the tanycytes reported by the present study suggests that tanycytes of median eminence may participate in the regulation of GH secretion.

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