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Localization and Role of Galanin in the Thyroid Gland of *Podarcis sicula* Lizard (Reptilia, Lacertide)

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ABSTRACT Galanin (GAL) is a 29-amino acid residue neuropeptide, which was initially isolated from porcine intestine extracts and since then, widely found in a variety of vertebrate organs, in correlation with multiple neuro-hormonal actions exerted and so receiving a constantly growing attention. Moreover, although the studies undertaken so far suggest a local intrathyroidal peptidergic regulatory action, the exact role of GAL on thyroid gland remains to be established. The aim of this study was to determine in the lizard, *Podarcis sicula*, (1) the presence of GAL immunoreactivity in the thyroid gland and (2) the short- and long-term effects of in vivo GAL administration by intraperitoneal injection on thyroid gland physiology. First of all, the presence of GAL in the thyroid gland of *P. sicula* was demonstrated by immunohistochemical technique (avidin–biotin–peroxidase complex—ABC method). Second, the role of GAL in the control of thyroid gland activity was studied in vivo using light microscopy (LM) technique coupled to a specific radioimmunoassay for thyroid-stimulating hormone (TSH) and thyroid hormones (T_4 and T_3). Prolonged GAL administration [(0.4 mg/100 g body wt)/day] increased T_4 and T_3 release, but decreased the plasma concentration of TSH. In addition, using LM clear signs of stimulation of the thyroid gland were observed. These findings suggest that systemic administration of GAL was able to stimulate the thyroid gland of the lizard both at morphological and physiological level. *J. Exp. Zool. 311A:199–206, 2009.* © 2009 Wiley-Liss, Inc.

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Galanin (GAL) is a 29-amino acid residue neuropeptide, which was initially isolated from porcine intestine extracts (Tatemoto et al., '83) and since then widely found in a variety of vertebrate organs. The amino acid compositions and sequences of GAL are known in mammals (Rökaeus and Carlquist, '88), in birds (Kohchi and Tsutsui, 2000), amphibians (Chartrel et al., '95) and fishes (Anglade et al., '94). Moreover, GAL structure has also been identified in alligator and tortoise (Wang and Conlon, '94; Wang et al., '99), demonstrating that the structure of GAL in alligator was more similar to that of sheep (three amino acid substitutions) (Wang and Conlon, '94), whereas it differed from that of tortoise by five amino acid residues (Wang et al., '99). The most extensive studies on GAL distribution have been carried out on mammalian organs, in fact, high concentrations of GAL were found in the nervous system, especially in the hypothalamus, the median eminence and hypophyseal portal blood (Tatemoto et al., '83; Rökaeus et al., '84; Skofitsch and Jacobowitz, '85; Lopez et al., '90; Lopez et al., '91; Giustina et al., '94; Dutriez et al., '97; Landry et al., 2000), in the endocrine system, particularly

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in the adrenal gland (Rokaeus and Carlquist, '88; Holst et al., '91; Mazzocchi et al., '92; Malendowicz et al., '94; Hochól et al., 2000), in the cardiovascular (Revington et al., '90; Johansson and Rokaeus, '95), gastrointestinal (Melander et al., '85; Rattan, '93; Botella et al., '95) and in the reproductive systems (Bauer et al., '86; Bek et al., '88; Papka and Traurig, '89; Shew et al., '92; Torsello et al., '92; Fox et al., '94; Lakomy et al., '95). In nonmammalian vertebrates, GAL-like-immunoreactivity (GAL-like ir) has been found in the CNS of lamprey (Jiménez et al., '96), dogfsh (Vallarino et al., '91), teleosts (Batten et al., '90a,b; Holmqvist and Ekström, '91; Olivereau and Olivereau, '91; Anglade et al., '94; Rodríguez-Gómez et al., 2000), amphibians (Wolfbauer and Skofitsch, '89; McKeon et al., '90; Lázár et al., '91;Olivereau and Olivereau, '92; Pieribone et al., '94; González-Nicolini et al., '98), reptiles (Jiménez et al., '94; Alponti et al., 2006) and birds (Józsa and Mess, '93). In the oviduct of the lizard *P. sicula,* GAL seemed to interact with estrogen, vasoactive intestinal polypeptide and oviposition (Lamanna et al., '99a,b). GAL caused vasoconstriction and occasionally activated the gut wall of the estuarine crocodile, *Crocodylus porosus* (Kagstrom et al., '98). There were no changes in the GAL innervation of the gut or on GAL-induced intestinal motility between fasting and digesting Burmese python, *Python molurus bivittatus* (Holmberg et al., 2003). Central injection of GAL stimulates food intake in goldfish, *Carassius auratus* (DePedro et al., '95), while it does not affect feeding in the neonatal chick (Ando et al., 2000).

Present evidences assign a variety of physiological functions to GAL in mammals, including the regulation of intestinal motility, the control of endocrine and exocrine pancreatic secretions and modulation of hormone secretion from pituitary and adrenal glands. Besides, in the male and female mammalian species, GAL seems to play an estrogen-mediated role in reproductive functions.

Until now, comparative studies about the physiological roles of GAL in nonmammalian vertebrates, instead, have been considerably less; our understanding of GAL roles and its regulation in nonmammalian vertebrates are very limited. At our knowledge the biological effects of GAL have not yet been studied in nonmammalian species.

The data on GAL effects on thyroid gland in mammalian are largely still lacking. GAL-immunoreactive fibers were observed around blood vessels and thyroid follicles in mice and rats only (Grunditz et al., '87; Ahrèn, '91). Hence, although the studies undertaken so far suggest a local intrathyroidal peptidergic regulatory action, the exact role of GAL on thyroid gland remains to be established. However, there is no knowledge of the physiological role played by GAL in the thyroid gland of reptiles.

The aim of this study was to determine in the lizard, *P. sicula*, (1) the presence of GAL immunoreactivity in the thyroid gland and (2) the short- and long-term effects of in vivo GAL administration by intraperitoneal injection on thyroid gland physiology in winter when the gland is in functional stasis.

MATERIALS AND METHODS

Animals and housing conditions

Adult male lizards of *P. sicula* (weighing 13–15 g) were live-captured in the neighborhood of Naples both in June ($n = 20$), when the thyroid gland was in full functional activity and in December ($n = 80$), when the thyroid gland showed clear signs of functional stasis (Sciarrillo et al., 2000). After capture, the animals were housed in large soil-filled terraria containing heather, and exposed to natural temperature and photoperiod. Water dishes were present in the terraria, and the animals were fed on live fly larvae daily. Captivity lasted 20 days to reverse capture-related stress (Manzo et al., '94). All animals have been captured with the authorization of 06/01/2000 no. SCN/2D/2000/9213 of Italian Ministry of Environment.

Experimental procedure

Only the animals live-captured in December, received i.p. injections of GAL (Rat, Sigma Chem. Co., St. Louis, MO). GAL was dissolved in reptilian physiological solution (NaCl 0.75%) with an injection volume of 0.1 mL. Injections were administered between 8.00 and 8.30 a.m.. The specimens were divided into four groups, each consisting of 20 animals in order to obtain an adequate plasma volume.

Group 1. The animals received a single i.p. injection of GAL (0.4 mg/100 g wt) and were sacrificed 2 hr later.

Group 2. Untreated animals (controls) received a single i.p. injection of reptilian physiological solution and sacrificed 2 hr later.

Group 3. The animals received an i.p. injection of GAL (0.4 mg/100 g body wt)/day for four consecutive days and were sacrificed 2 hr after the last injection.

Group 4. Untreated animals (controls) received an i.p. injection of reptilian physiological solution for four consecutive days and were sacrificed 2 hr after the last injection.

The animals were anaesthetized by hypothermia, chilling them in chipped ice. Blood samples were collected by intracardiac puncture and put into heparinized tubes. Blood collection lasted less than 3 min; plasma obtained centrifuging (2,500 *g* for 10 min at 4°C) the blood samples, was stored at −20°C until assay.

Light microscopy

Immediately after collection of blood samples, the animals were decapitated, and the thyroid glands were removed and fixed in Bouin's fixative and processed for light microscopy (LM). Serially cut paraffin sections (7 μm) were stained by Galgano I stain (Beccari and Mazzi, '66).

Observations were performed using a Zeiss Axioskop microscope; images were captured with a camera attached to an IBM computer running the Kontron Elektronik KS 300 image analysis system and then they were processed by Adobe Photoshop.

The height of the follicular cells was measured in 30 cells every 3 slides, always on the second section of normal and treated specimens using a digital system of imagine (KS 300).

Hormone assay

Plasma levels of T_3 and T_4 were determined by radioimmunoassay (RIA) (Sciarrillo et al., 2008). T_3 sensitivity was 0.1 ng/mL with an accuracy of about 97%. The range of intraassay variance in 20 assays was 1.0–2.6%, whereas the interassay variance ranged between 3.9 and 5.7% in 12 assays. T_4 sensitivity was 0.45 ng/mL with an accuracy close to 100%; the mean intra and interassay coefficients of variance were 4.6 and 4.3%, respectively. A logit–log curve fit using a % B/Bo calculation was used. T_4 and T_3 concentrations were determined by computing the % B/Bo for each sample and then finding the results on the standard curve. Cross-reactivity for T_4 in the T_3 RIA (1.3%) was not considered for data calculations, neither was that for T_3 in the T_4 RIA (0.1%).

Plasma TSH was determined by immunoradiometric assay (Sciarrillo et al., 2008). In the TSH procedure, sample serum and standards were added to anti-ligand-coated tubes. The Tracer/Capture Reagent, a blend of ligand-tagged TSH-specific antibody and 125I labelled TSH (10 μCi) was added to each tube. A cubic spline function with the zero standard as one of the standard points was used for calculations. The minimum detectable dose was 0.01 μIU/mL, with an accuracy close to 100%, and the mean intra and inter-assay coefficients of variance of 5.0 and 7.5%, respectively.

Immunohistochemistry

The sections of the thyroid gland of control *P. sicula* animals live-captured in June were examined by immunohistochemistry with the avidin–biotin-peroxidase complex (ABC) method (Hsu et al., '81) to localize the GAL. Briefly, the fixed tissues were embedded in paraffin wax. Sections of each tissue were cut at 5–7 μm, mounted on glass slides and dried overnight at 37°C. They were then deparaffinized in xylene, rehydrated through a graded ethanol series and washed in phosphate-buffered saline (PBS). PBS was used for all subsequent washes and for antisera dilutions. Next, the sections were quenched sequentially in 3% hydrogen peroxide for 15 min and blocked with 6% milk protein in PBS for 1 hr at room temperature; they were after incubated with an antiserum against porcine GAL raised in rabbit (1:500) (Biogenesis, Poole, England) overnight at 4°C in a moist chamber. After several washes to remove excess antibody, the sections were incubated with 1:200 goat anti-rabbit biotinylated antibody (Vector Laboratories, Burlingame, CA) for 1 hr. They were then processed by the ABC method (Vector Laboratories) for 30 min at room temperature. Diaminobenzidine (Vector Laboratories) was used as the final chromogen for revealing bound GAL antibody. Negative controls for each tissue section were prepared by substituting the primary antiserum with the isotype-matched nonimmune rabbit IgG. All the samples were processed under the same conditions.

Statistical analysis

All data were expressed as means ± standard error of mean (SEM). The control and the experimental data of all the groups were tested together for significance using one-way analysis of variance (ANOVA), followed by Duncan's test for multigroup comparison and Student's *t*-test for between group comparison. Differences were considered significant at $P < 0.05$.

RESULTS

GAL-like immunoreactivity localization in P. sicula thyroid gland

The thyroid gland of the lizard *P. sicula* untreated (control) specimens is a single discrete ribbon-like structure, which transversely crosses the middle of the trachea. It is formed by follicles that are connected by an interfollicular connective tissue containing blood vessels. A superficial connective capsule envelops the gland and sends branches that form a network that surrounds the follicles. In June, thyroid gland showed a cuboidal follicular epithelial cells with medium-sized colloidal mass. The distribution of GAL-like ir in the thyroid gland of *P. sicula* was examined by immunohistochemistry. GAL-like ir was especially distributed in the apical cytoplasm of the follicular cells (Fig. 1a). Moreover, GAL-like ir were observed around blood vessels and follicles (Fig. 1b). Control sections exhibited no GAL staining (Fig. 1c).

Morphological observations of GAL-treated thyroid gland

In December, the thyroid follicles of control groups (Group 2 and 4) showed a low follicular epithelium (1.15±0.03 μm) and they did not present reabsorbing vacuoles in the colloid (control group) (Fig. 2a).

All samples treated with GAL i.p. injections revealed clear morphological signs of stimulation of thyroid gland. In Group 1, the follicles were irregular in shape with a very high epithelium (8.91±0.05 μm, $P<0.05$) and the roundish nuclei localized at the basis of the follicular cells. The colloid filled up the follicular lumen, showing numerous reabsorption vacuoles (Fig. 2b).

In Group 3 (Fig. 2c), there was the highest stimulation of the thyroid gland, which revealed an increase in follicular epithelium (15.2±0.01 μm, $P<0.05$). Data about the height of follicular epithelium are shown in Figure 3.

Hormonal variations of the T_3, T_4 and TSH

GAL administration increased plasma levels of both thyroid hormones. Specifically, T_3 plasma levels significantly increased ($P<0.05$) from a value of 1.23±0.05 ng/mL in the control specimens (Group 2) to a value of 3.44±0.05 ng/mL in the specimens treated with a single GAL injection (Group 1) until the highest value of 8.23±0.03 ng/mL is reached in the specimens treated with four GAL injections (Group 3). Moreover, T_4 plasma levels significantly increased ($P<0.05$) from a value of 2.31±0.02 ng/mL in the control specimens (Group 2) to a maximum value of 9.89±0.03 ng/mL in the specimens of Group 3, following a trend similar to that of T_3 (Fig. 4).

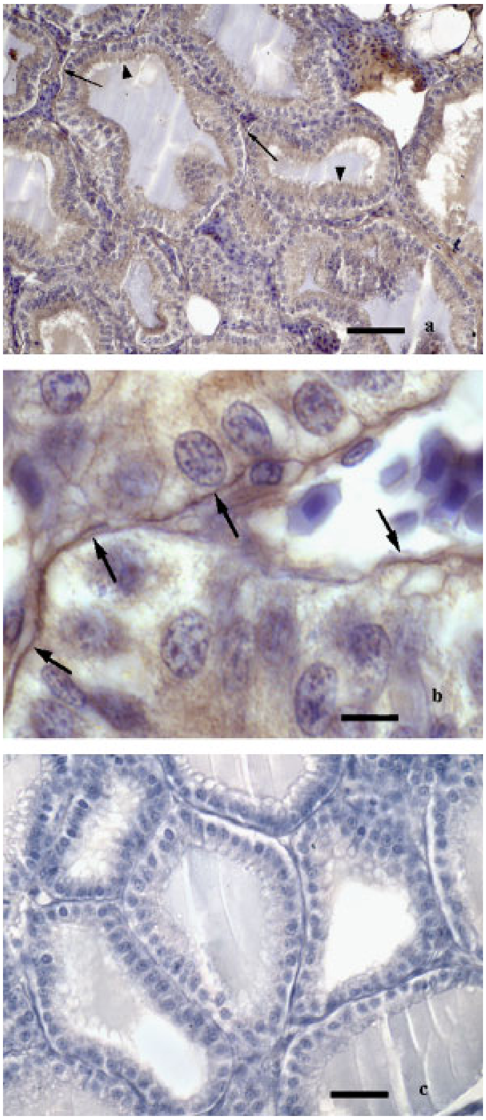

Fig. 1. Localization of galanin in *P. sicula* thyroid gland during summer. (**a**) Immunostaining for galanin was present around blood vessels (arrows) and in the apical cytoplasm of thyroidal cells (arrowhead) (scale bar: 20 μm); (**b**) higher magnification showing immunostaining for galanin especially present around blood vessels (arrows) (scale bar: 90 μm); (**c**) negative control of ABC reaction showing no signal (scale bar: 40 μm).

On the contrary, after GAL administration, TSH plasma levels showed a significant decrease ($P<0.05$). It decreased from 3.13±0.01 μIU/mL in the control group (Group 2) to 1.10±0.03 μIU/mL in Group 3 (Fig. 5).

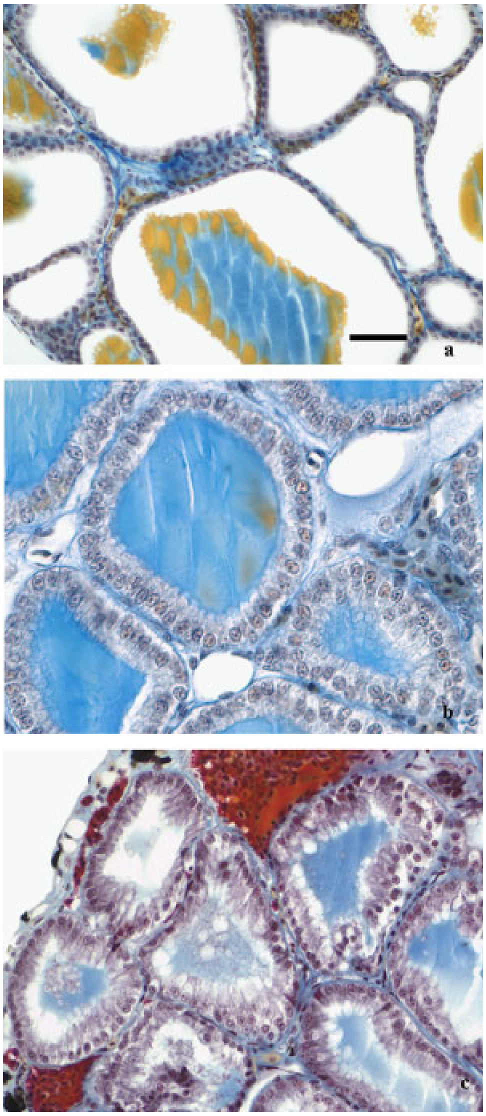

Fig. 2. Section of thyroid of untreated (control) and galanin-treated *P. sicula* (stain Galgano I; scale bar: 20 µm). (**a**) Control specimen of group 2 showing the typical low follicular epithelium characterizing the functional stasis during winter; (**b**) specimen treated with one injection of galanin (0.4 mg/100 g body wt); the follicular epithelium is higher than in control specimens; (**c**) specimen treated with four injections of galanin (0.4 mg/100 g body wt); note the follicular epithelium which is very high and the colloid that appears very vacuolized.

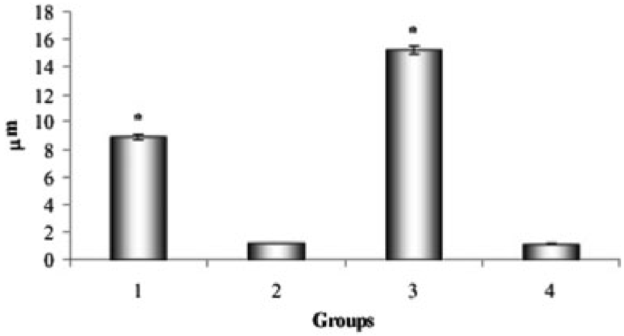

Fig. 3. Variations of epithelium height of the follicular cells of the thyroid gland in *P. sicula* subjected to different experimental treatment. Group 1: specimen treated with one injection of galanin (0.4 mg/100 g body wt); group 2: control specimen received a single intraperitoneal reptilian physiological solution injection; group 3: specimen treated with four injections of galanin (0.4 mg/100 g body wt); group 4: control specimen received four intraperitoneal reptilian physiological solution injections. Values are shown as means ± SEM. ∗Significant at $P < 0.05$ compared with the control.

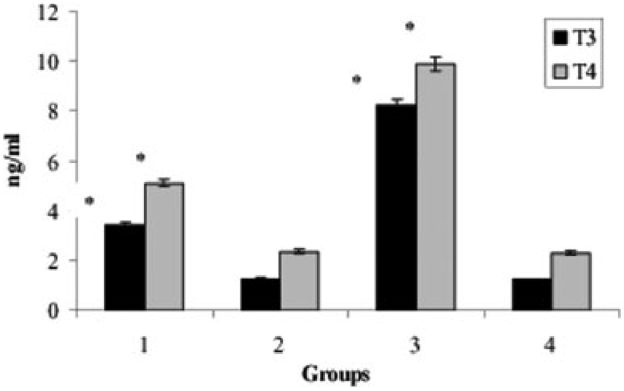

Fig. 4. Variations of T_3, T_4 levels in the plasma of *P. sicula* subjected to different experimental treatment. Group 1: specimen treated with one injection of galanin (0.4 mg/100 g body wt); group 2: control specimen received a single intraperitoneal reptilian physiological solution injection; group 3: specimen treated with four injections of galanin (0.4 mg/100 g body wt); group 4: control specimen received four intraperitoneal reptilian physiological solution injections. Values are shown as means ± SEM. *Significant at $P < 0.05$ compared with the control.

DISCUSSION

GAL is a phylogenetically old and well-conserved peptide and has been found in several phyla, such as insects, amphibians, fishes, reptiles, birds and mammals. GAL has, since its discovery, been the subject of a constantly growing attention, due to its wide spread pattern of distribution that correlates with multiple neuro-hormonal actions exerted in a variety of species.

In this article we have demonstrated, for the first time, the presence of GAL-like immunoreactivity in the thyroid gland of *P. sicula* lizard by immunohistochemistry using an antiserum against porcine GAL. It has been demonstrated that the structure of GAL in alligator is more similar to that of sheep (three amino acid substitutions) (Wang and Conlon, '94), while it differs from that of tortoise by five amino acid

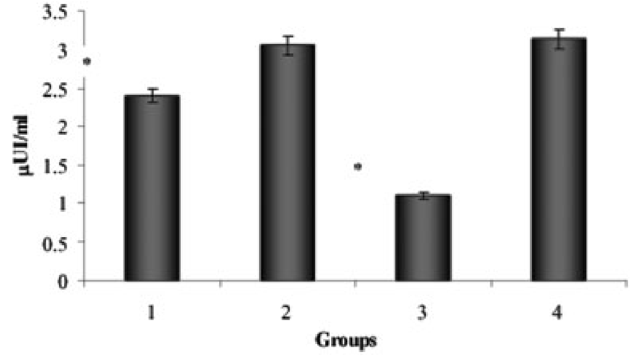

Fig. 5. Variations of TSH levels in the plasma of *P. sicula* subjected to different experimental treatment. Group 1: specimen treated with one injection of galanin (0.4 mg/100 g body wt); group 2: control specimen received a single intraperitoneal reptilian physiological solution injection; group 3: specimen treated with four injections of galanin (0.4 mg/100 g body wt); group 4: control specimen received four intraperitoneal reptilian physiological solution injections. Values are shown as means ± SEM. *Significant at $P<0.05$ compared with the control.

residues (Wang et al., '99). The anti-GAL serum used in the this study has been used to identify GAL-ir in mammalian and nonmammalian vertebrates, including the turtle *Mauremys caspica* (Jiménez et al., '94) and the snake *Bothrops jararaca* (Alponti et al., 2006). In addition, Western blot analysis using an antibody directed against porcine GAL had been performed on lizard *P. sicula* gastrointestinal tract and oviduct extracts that recognized a single protein band corresponding to a molecular weight (3.4 kDa) similar to that porcine GAL (Lamanna et al., '99a,b).

Particularly, the distribution of GAL-like ir in the *P. sicula* thyroid gland was observed both in the apical portion of follicular cells that is formed by cytoplasm and around blood vessels surrounding thyroid follicles, suggesting that this peptide, released by thyroid cells and probably by nerve fibers, may be involved in the regulation of local blood flow and follicular cell activity.

Second, we aimed to study the GAL roles on thyroid gland activity by the use of *P. sicula* lizard as the animal model. Our results demonstrated that, in *P. sicula* lizard, GAL may affect the activity of pituitary–thyroid axis. Specifically, we have demonstrated that GAL administration by intraperitoneal injections was able to increase strongly both the thyroid hormone (T_3 and T_4) plasma levels, whose levels raised about five fold with respect to control specimens. On the contrary, we showed a decrease of TSH plasma levels, whose levels lowered about three-fold in the specimens receiving prolonged GAL treatment. The TSH plasma level decrease was probably determined by a negative feedback induced by increased plasma levels of both thyroid hormones. The statistically significant increase in both thyroid hormone (T_3 and T_4) plasma levels agrees with the morphological features of the thyroid tissue, showing clear signs of stimulation, like an increase in follicular epithelium. The rapidity of the action (after 2 hr time interval) may suggest that GAL released by thyroid follicular cells and/or by fibers around blood vessels inside the thyroid gland can directly affect the intrathyroidal regulation of thyroid function and physiology. Besides, to verify this hypothesis, studies are in progress in our laboratory on thyroidal cells, to investigate the presence and the type of GAL receptors involved in thyroid hormones secretion.

The present data complete a previous study investigating the role of GAL on hypothalamic–pituitary–thyroid axis, in fact several lines of in vivo studies have indicated that GAL might regulate many pituitary secretions such as thyroid-stimulating hormone (TSH) (for review, see Vrontakis et al., '91). The physiological effects of GAL in the regulation of TSH have been shown to be diverse (Hooi et al., '90; Arvat et al., '95; Hyde et al., '96). Evidence indicates that GAL and its receptors are synthesized in the anterior pituitary of the rat (Wynick et al., '93). It has been shown that GAL regulates TSH secretion in rats (Hooi et al., '90; O'Halloran et al., '90) and humans (Arvat et al., '95). In fact, GAL injected into the third cerebral ventricle of rats produced highly significant, dose-related decreases in TSH levels , whereas in the human subjects TSH secretion was not altered by GAL.

Summarizing our results, we may propose a GAL action modality: the GAL-like-immunoreactive fibers around blood vessels might directly stimulate follicular cell activity that show an increase of epithelium height and a release of thyroid hormones, whose plasma levels strongly increased. In turn, thyroid hormones decreased TSH plasma levels through a negative feedback. In conclusion, our results indicate that GAL may play a stimulatory role on the thyroid activity in reptiles suggesting that, on the basis of GAL high identity, its effect may be very similar also in other vertebrates such as mammals.

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