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Biologia. — *GnRH-like immunoreactive substances in some different tissues of the lizard,* Podarcis s. sicula *Raf, during the sexual cycle.* Nota di GAETANO CIARCIA (*) e VIRGILIO BOTTE (**), presentata (***) dal Corrisp. G. CHIEFFI.

ABSTRACT. - The distribution of immunoreactive GnRH-like substances in various tissues of the lizard (*Podarcis s. sicula*) in relation to different phases of the sexual cycle are described.

GnRH-like substances in the hypothalamic extracts decrease during the reproductive period. In the extrahypothalamic tissue, the levels of immunoreactive GnRH-like substances are highest in early spring and summer, and decrease in autumn, winter and late spring. In the other tissues (gonads, liver and small intestine), a more constant immunoreactivity has been observed.

All tissue extracts stimulate the release of luteininzing hormone (LH) from chicken anterior pituitary cells. In testis this phenomenon is more evident in early spring and summer; in the other tissues, two peaks of activity can be detected, in spring and in autumn. These findings suggest that GnRH-like substances could be involved in the regulation of various aspects of reproductive processes in the lacertid *Podarcis s. sicula* Raf.

KEY WORDS: Reptiles; Gonadotropin-releasing hormones; Reproduction GnRH-like substances.

RIASSUNTO. - *Sostanze GnHR-simili in vari tessuti della lucertola* Podarcis s. sicula *Raf. durante il ciclo sessuale.* È stata determinata la presenza e la concentrazione di sostanze immunologicamente simili al GnRH in vari organi del lacertide *Podarcis s. sicula*, utilizzando tre anticorpi specifici per tratti diversi di questo peptide.

Negli estratti di ipotalamo e di encefalo (privo di ipotalamo) i risultati delle reazioni immunologiche indicano la presenza di tali sostanze che vanno incontro a definire variazioni di concentrazione nel corso del ciclo riproduttivo (nell'ipotalamo diminuiscono in primavera; nelle regioni extraipotalamiche dell'encefalo sono più elevate all'inizio della primavera ed in estate). Negli altri organi esaminati (testicolo, ovario, fegato ed intestino) sono state evidenziate, essenzialmente, delle componenti che potrebbero avere sequenze di amminoacidi in parte simili a quelle del GnRH; esse, tuttavia, non mostrano delle chiare modificazioni nel corso dell'anno.

Gli estratti di tutti gli organi esaminati posseggono la capacità di indurre *in vitro* il rilascio di LH dall'ipofisi di pollo. Tale proprietà risulta però piuttosto variabile nei diversi periodi dell'anno e spesso non in accordo con i dati ottenuti con le indagini immunologiche.

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Introduction

In vertebrates, the hypothalamic gonadotropin-releasing hormone (GnRH) was initially thought to be a unique molecular form, but subsequent studies established that there is a considerable diversity in the structure of this decapeptide in relation to the species. GnRH or GnRH-like molecules, which differ from the mammalian hypothalamic hormone, have been also found in various extrahypothalamic tissues such as brain, gonads, placenta and pancreas, and some pancreatic and mammary gland tumors (see for rewiew, Sherwood, 1986; Millar and King, 1987).

Multiple forms of GnRH within brain tissue of a single species have been described in birds (King and Millar, 1982; Miyamoto et al., 1984; Sherwood et al., 1988), reptiles (Powell et al., 1985; Sherwood and Whittier, 1988), amphibians (Sherwood et al., 1986) and fishes (King and Millar, 1985). Among reptiles, considerable structural diversity of brain GnRHs has been observed in different species (Powell *et al.*, 1985, 1986; Sherwood and Whittier, 1988). For example, in the brain of the lizard, *Podarcis s. sicula* three GnRH molecules have been identified, none of which showed a structure identical to mammalian GnRH (Powell et al., 1986). These findings could explain conflicting results as the effect of mammalian GnRH when tested, *in vivo* and *in vitro*, in reptilian systems (Licht *et al.*, 1984; Licht and Porter, 1985; Lance *et al.*, 1985).

In each species, therefore, the determination of GnRH molecular forms synthesized in neural and non-neural tissues and the evaluation of their behaviour during the sexual cicle should be preliminary to any functional study. In this context, we have looked for the presence of immunoreactive GnRH-like substances in various tissues of the lizard *Podarcis s. sicula* during different phases of the reproductive cycle. The LH-releasing activity of tissues *in vitro* bioassay was also tested.

Materials and methods

Podarcis s. sicula Raf., (160 males and as many females) were obtained from the outskirts of Naples in the following periods of the year:

1. Winter (January-February). The lizards were in semi-hibernation. Their gonads and genital tracts were completely quiescent. In the testes, seminiferous tubules contained the complete series of germ cells that had been produced in the previous autumn (Galgano and D'Amore, 1960).

2. Early spring (late March and April). The lizards had emerged from semi-hibernation. In the males, the new spermatogenetic wave and the development of the epididymis and secondary sexual characters (SSC) were evident, and the males were often engaged in fights with con-specifics to affirm territoriality. In the females, the ovary and the oviducts were in a phase of slow growth.

3. Late spring (May and the first week of June). This is the breeding period. In both sexes, the gonads, genital tracts and SSC were fully active. The ovary and oviducts had reached full maturity. Mating and deposition of eggs were common.

4. Summer (July). The breeding season had terminated. In both sexes the gonads, genital tracts and SSC were quiescent. No sexual behaviour was observed. In this phase, the animals are not responsive to stimulation by high temperature or photoperiod (*refractoriness*: Licht *et al.*, 1969; Angelini *et al.*, 1976).

5. Autumn (October). Some spermatogenetic activity was resumed, but it was unrelated to genital duct and SSC development. In the female, both the ovary and oviduct were quiescent.

Tissue extraction. For each reproductive stage, twenty or more lizards of both sexes were killed. Brain (dissected into hypothalamic and extrahypothalamic regions), gonads, liver and small intestine were rapidly dissected. Tissues samples of each stage were pooled, lyophilized. The powders were then homogenized in ice-cold 2 N acetic acid, and centrifuged for 1 h at 18,000 x g at 4°C. The supernatants were lyophilized and kept at -20°C. Before the assays, the samples were reconstituted in 3-4 ml of water, sonicated, and centrifuged. The supernatants were used as indicated below.

Radioimmunoassay with region-specific GnRH antisera. GnRH immunoreactivity was measured according to the methods of Millar *et al.* (1984). Mammalian GnRH (R.C. de L. Milton, Department of Chemical Pathology, University of Cape Town) was used as standard and for preparation of ^{125}I-GnRH (Millar *et al.*, 1984). Antisera 80/1,1076 and IJ-29 were produced against GnRH. The sensitivities (half maximal displacement of iodinated peptide) in the RIA with antisera 80/1,1076,IJ-29 were 32.7, 15.7 and 79 pg of GnRH, respectively. Specificities of the antisera were as follows: antiserum 80/1 required pGlu^1 and Gly^{10}NH$_2$ (King et al., 1983), *antiserum 1076* required residues in the region Trp^3 to Prp^9 of GnRH (King and Millar, 1982), and *antiserum IJ-29* required the COOH-terminal region Leu^7 to Gly^{10}NH$_2$ of GnRH (Copeland *et al.*, 1979). Samples were assayed in serial doubling diluitions in posphate-buffered saline containing 0.1% gelatin. The results have been expressed as pg of immunoreactive GnRH-like substance/mg tissue dry weight.

Peptidases and binding substances. Preliminary experiments were performed to test for the presence of peptidases and binding substances which would interfere with the radioimmunoassays. To test for peptidase activity, tissue extracts were incubated with ^{125}I-GnRH at 20°C. Aliquots were removed at 30 min intervals for 3 h and incubated with excess antiserum 1076 as in the radioimmunoassay. Since the binding of labelled GnRH remained stable during the 3 h incubation, it could be excluded that there was peptidase activity. Moreover, extracts incubated with ^{125}I-GnRH in the absence of antiserum did not display any binding. Therefore the presence of binding substances other than GnRH can be excluded.

LH-releasing activity. Tissue extracts were used in a chicken pituitary cell bioassay (each pool assayed in triplicate) as indicated by Millar and King (1983). The LH content was measured by RIA as reported by Follet *et al.* (1972). Chicken

pituitary cells were used rather than mammalian pituitary cells as the former responde to a much greater structural variation in GnRH than do the mammalian cells (Millar and King, 1983).

Results

Table 1 shows the results of radioimmunoassay determinations using different antisera. With 80/1 antiserum, GnRH-like substances were detected in brain tissue only (hypothalamic and extrahypothalamic regions). The concentration of immunoreactive substances underwent a seasonal variation. In the hypothalamus, they decreased in spring; in the extrahypothalamic regions, their level was highest in early

Table 1. - *Quantitation of GnRH immunoreactivity in* Podarcis s. sicula *Raf. tissues using antisera 80/1, 1076 and IJ-29.*

Season	Tissues	Immunoreactive GnRH (pg/mg dry wt.)		
		80/1	1076	IJ-29
Winter	Hypothalamus	8.6	1.2	11.5
	Extrahypothalamic brain	7.6	0.9	8.4
	Testis	— (*)	—	3.4
	Ovary	—	—	2.9
	Liver	—	0.4	1.6
	Small intestine	—	0.7	0.8
Early spring	Hypothalamus	—	—	—
	Extrahypothalamic brain	29.2	1.3	4.6
	Testis	—	—	1.5
	Ovary	—	—	0.9
	Liver	—	0.7	2.9
	Small intestine	—	1.2	1.9
Late spring	Hypothalamus	2.0	—	14.2
	Extrahypothalamic brain	6.0	1.0	13.5
	Testis	—	0.4	1.3
	Ovary	—	—	—
	Liver	—	—	1.1
	Small intestine	—	—	—
Summer	Hypothalamus	5.2	—	—
	Extrahypothalamic brain	69.0	—	6.4
	Testis	—	—	—
	Ovary	—	—	—
	Liver	—	—	0.7
	Small intestine	—	1.3	0.9
Autumn	Hypothalamus	13.7	—	—
	Extrahypothalamic brain	6.7	1.7	7.5
	Testis	—	—	1.2
	Ovary	—	—	24.7
	Liver	—	—	—
	Small intestine	—	2.0	—

(*) Below the limits of sensitivity

spring and summer. In the hypothalamus, only winter extract reacted with antiserum 1076, whereas in the extrahypothalamic regions this reactivity was more constant since it was absent only from summer extract. In the hypothalamus, IJ-29 immunoreactive substance were evident in winter and late spring; in the extraypothalamic brain they were detected in all seasons.

In the other tissues (gonads, liver ad small intestine), a positive immunoreactivity was mainly observed with IJ-29 antiserum. A reactivity with IJ-29 antiserum was detected throughout the year, except summer, in the testicular extracts and in autumn, winter and early spring ovary extracts. No reactivity was detected in autumn and late spring small intestine extracts and in autumn liver extracts.

Table 2 shows the LH-releasing activity of testis, ovary, liver and small intestine

Table 2. *LH-releasing activity in Podarcis s. sicula Raf. testis, ovary, liver ad small intestine extracts.*

Season	Tissue	Dose	LH release (mean ± SEM)*	
			(μg/ml)	(pg/mg dry wt.)
	Gln^8-GnRH	10^{-10}M	0.029 ± 0.004	
		10^{-9}M	0.189 ± 0.011	
		10^{-8}M	0.293 ± 0.034	
		10^{-7}M	0.247 ± 0.052	
Winter	Testis		0	0
	Ovary		0	0
	Liver		0.021 ± 0.004	33.09 ± 6.29
	Small intestine		0.034 ± 0.009	53.44 ± 14.75
Early spring	Testis		0.045 ± 0.020	123.28 ± 54.78
	Ovary		0.14 ± 0.006	25.45 ± 10.90
	Liver		0.29 ± 0.008	58.00 ± 16.00
	Small intestine		0.055 ± 0.009	130.95 ± 21.46
Late spring	Testis		0.031 ± 0.006	46.26 ± 8.95
	Ovary		0.145 ± 0.040	216.41 ± 59.70
	Liver		0.076 ± 0.020	113.43 ± 29.85
	Small intestine		0.023 ± 0.006	43.39 ± 11.32
Summer	Testis		0.040 ± 0.002	444.44 ± 22.22
	Ovary		0	0
	Liver		0	0
	Small intestine		0	0
Autumn	Testis		0.013 ± 0.003	45.61 ± 10.52
	Ovary		0.102 ± 0.003	300.00 ± 8.82
	Liver		0.148 ± 0.030	493.33 ± 100.00
	Small intestine		0.133 ± 0.040	443.39 ± 133.33

* Refer to three determinations.

extracts in the chicken pituitary cell bioassay. Releasing-activity was shown by all tissues types. In testis, this was higher in early spring and in summer; in the ovary, in late spring and autumn. In this period, high activity was also observed in liver and small intestine extracts.

Discussion

In a previous study, three GnRH-like forms have been identified and characterized in the extracts of *Podarcis s.sicula* Raf. brain (Powell *et al.*, 1986). The first one eluted on HPLC in the same position as Gln^8-GnRH and reacted with 80/1 antiserum. This constituted the major immunoreactive peak. The second eluted on HPLC as Trp^7-Leu^8 - GnRH and reacted also with 80/1 antiserum. These substances did not react with 1076 antiserum. From the HPLC a third peak was obtained which reaeted with 1076 antiserum (third form).

Our radioimmunoassays confirm the presence of three GnRH-like forms in hypothalamus and extrahypothalamic brain (Powell *et al.*, 1986). The putative first and second forms, as evaluated by reactivity to 80/1 antiserum, undergo seasonal variations since they are not detectable in the hypothalamus during the spring, and are more concentrated in early spring and summer extrahypothalamic brain. The third putative form reactived with 1076 antiserum seems to be present in the hypothalamus during the winter, whereas in the extrahypothalamic brain it is detectable throughout the year.

The absences of any type of GnRH-like immunoreactivity in early spring hypothalamus is somewhat intriguing, unless the peptides are released, as soon as they are synthesized, to stimulate the pituitary gland gonadotropic cells. In this period, in fact, the gonads and genital tracts rapidly resume their activity. On the contrary, high GnRH concentrations have been found in the hypothalamus of reproductively active *Xenopus laevis* (King and Millar, 1979).

The presence and fluctuations of GnRH-like substance in the extrahypothalamic brain are in agreement with previous biochemical and immunohistochemical studies. It has been shown that neurons containing LHRH immunoreactive substances are distributed in septo-preoptic regions of lizard brains (see for review, Nozaki *et al.*, 1984). Their axons, moreover, have been identified in the median eminence and in the telencephalon (Doerr-Schott and Dubois, 1978). This anatomical relationship led to the notion that GnRHs exert other functions besides the LH-releasing activity. In mammals, it has been suggested that GnRHs are involved in regulating sexual behaviour (Nozaki *et al.*, 1984).

The findings obtained with testes, ovary, liver and small intestine seem to indicate that the third form of lizard GnRH could be synthesized by testis all year, except summer, and by ovary, liver and small intestine during different periods of the year. At present, it is difficult to interprete these findings. Moreover, the positive cross-reactivity of different non-neural tissues to IJ-29 antiserum indicates the

presence in all these tissues of proteins and/or peptides that share some aminoacid sequences with GnRH. The physiological meaning of this phenomenon merits an investigation. In gonads, at least, there is evidence of direct involvement of GnRH in the regulation of steroid biosynthesis and spermatogenesis (Hsueh and Erickson, 1979; Sharpe and Cooper, 1982; Pierantoni *et al.* 1984; Minucci *et al.* 1986).

The results of the bioassay, reported in Table 2, show that gonads, liver and small intestine contain some LH-releasing activity *in vitro*. In several cases, this finding was not correlated to immunoreactivity determination (see, for example, late spring ovary). These discrepancies could be tentatively explained assuming the presence of substances that do not react with the known GnRH antisera but are able to induce LH release. In several vertebrate brains, some peptides, unrelated to the common GnRH, induce chicken pituitary to release LH (J.A.King, personal comunication).

In conclusion, our data, although preliminary, provide evidence that seasonal variations occur in the type and quantity of GnRH-like substances synthesized by both neural and non-neural tissues of the lizard *Podarcis s. sicula*. The specific roles of various GnRHs in this species have not been investigated, but it cannot be ruled out that the reported modifications are in some way related to specific implications in the regulation of various sexual cycle phases.

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