

Relationships among GnRH, substance P, prostaglandins, sex steroids and aromatase activity in the brain of the male lizard *Podarcis sicula sicula* during reproduction

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The release of PGF_{2α} and PGE₂, progesterone, androgens and oestradiol *in vitro*, and the aromatase activity in the brain of the male lizard *Podarcis sicula sicula* during three different phases of the reproductive period were evaluated. In addition, the effects of salmon GnRH, substance P, salmon GnRH antagonist, substance P antagonist, PGF_{2α}, PGE₂ and acetylsalicylic acid on the release of prostaglandins and sex steroids and on aromatase activity in the brain were evaluated during the same three phases. PGF_{2α}, oestradiol and aromatase activity were higher during the refractory phase, androgens during the fighting phase, and progesterone during the mating phase, while PGE₂ was lower during the refractory phase. Treatment with salmon GnRH increased PGF_{2α}, oestradiol and aromatase activity, but decreased the amount of androgens released. Substance P decreased PGF_{2α}, oestradiol and aromatase activity, but increased the amount of androgens released. PGF_{2α} increased oestradiol and aromatase activity, but decreased the amount of androgens released. Acetylsalicylic acid decreased PGF_{2α}, oestradiol and aromatase activity, but increased the amount of androgens released. These data suggest that salmon GnRH and substance P have different roles in reproductive processes, with opposite mechanisms, in the central nervous system of this male lizard: salmon GnRH seems to be involved in regulating the refractory phase, while substance P plays a role in regulating the fighting phase.

Introduction

The presence of salmon GnRH (sGnRH) (Powell *et al.*, 1985, 1986) and substance P (Lembeck *et al.*, 1985; Bello *et al.*, 1991) has been documented in some lizard species. These neuropeptides can act on gonadotrophin secretion and steroidogenesis in several species, including reptiles (Ciarcia *et al.*, 1986; Varriale *et al.*, 1986; Arisawa *et al.*, 1990; Pitzel *et al.*, 1991). This may not be true for all species since in some reptiles sGnRH is not present (Tsai and Licht, 1993) and since studies *in vivo* have shown that various forms of GnRH are inactive in some species (Licht and Porter, 1987). However, in other studies carried out in various organs of the anuran water frog *Rana esculenta* (Gobbetti and Zerani, 1991, 1992a, b) and the urodele crested newt *Triturus cristatus* (Gobbetti and Zerani, 1992b; Gobbetti *et al.*, 1992), GnRH was found to induce an increase in the concentration of prostaglandins, which, in turn, regulated the secretion of sex steroids. These findings suggest that the stimulatory role of this neuropeptide on steroidogenesis is mediated by prostaglandins at least in some amphibians. In particular, Gobbetti *et al.* (1992) suggested that in *Triturus cristatus* the regulation of reproductive activity by the central nervous system depends on the relationships between GnRH, prostaglandins and sex steroids.

In reptiles, prostaglandins influence the expression of reproductive behaviour (Tokarz and Crews, 1981; Whittier and Crews, 1986) but nothing is known about the relationships among neuropeptides, prostaglandins and sex steroids in the central nervous system of reptiles. It is still unclear whether the lizard brain synthesizes or accumulates (or both) progesterone, androgens and oestradiol despite the demonstration of aromatase activity in the brain of several species (Callard *et al.*, 1978; Zerani *et al.*, 1992). *De novo* steroid biosynthesis has also been found in cultures of rat brain cells, which supports the concept that steroids are synthesized in brain cells (Jung-Testas *et al.*, 1989).

In this study we compared the effects *in vitro* of substance P, sGnRH, an antagonist of substance P, an sGnRH antagonist and prostaglandins on the release of prostaglandins and sex steroids by, and aromatase activity in, the brain of the male lizard *Podarcis sicula sicula* during reproduction.

Materials and Methods

Animals

The reproductive cycle of the male lizard *P. s. sicula* that lives around Naples (Campania, Italy, 25–75 m above sea level) is briefly described below. The annual breeding activity is

restricted to spring. The gonads and the secondary sexual characteristics develop at the beginning of the spring and are functional until June, when a refractoriness to the still favourable ambient cues begins, followed by complete regression of gonads and secondary sexual characteristics (Angelini *et al.*, 1980, 1981, 1986). The reproductive behaviour of the male is characterized by fighting behaviour in March and April and by mating in May (Ciarra *et al.*, 1986).

Incubation in vitro

Adult male lizards of this population were captured in April (fighting phase), May (mating phase) and June (refractory phase). Immediately after capture, the animals were killed by decapitation, the brains were rapidly removed and pituitaries were gently removed. The brains were placed in cold Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St Louis, MO) containing 10 mmol Hepes l^{-1} , 1 mg penicillin ml^{-1} and 2 mg streptomycin ml^{-1} , and transferred to the laboratory. For animals captured in each month, 110 brains were randomly and separately placed in wells of multiple tissue culture plates (Becton Dickinson, Clifton, NJ).

Each set of wells for incubation was divided into 11 experimental groups (each consisting of two wells). The experimental groups comprised one brain incubated with (i) DMEM alone, (ii) DMEM plus substance P (75 ng; SP, S6883, Sigma Chemical Co.), (iii) DMEM plus sGnRH (50 ng; LHRH salmon, L4897, Sigma Chemical Co.), (iv) DMEM plus an antagonist of substance P (125 ng; [D-Pro², D-Trp^{7,9}]-substance P, S0145, Sigma Chemical Co.), (v) DMEM plus sGnRH antagonist (100 ng; [D-Phe^{2,6}, Pro³]-LHRH, L4261, Sigma Chemical Co.), (vi) DMEM plus substance P (75 ng) plus sGnRH (50 ng), (vii) DMEM plus substance P (75 ng) plus antagonist of substance P (125 ng), (viii) DMEM plus sGnRH (50 ng) plus sGnRH antagonist (100 ng), (ix) DMEM plus PGF_{2α} (200 ng; Sigma Chemical Co.), (x) DMEM plus PGE₂ (300 ng; Sigma Chemical Co.), (xi) DMEM plus acetylsalicylic acid (ASA; 1 μg; Sigma Chemical Co.).

The final volume of each well was 1 ml. Culture plates were wrapped with aluminium foil and incubated in a shaking water bath (32°C), set at 30 revolutions min^{-1} . The medium of one well of each experimental group was removed after 1 h and 3 h of incubation. The incubation medium samples and the brains were immediately stored at $-80^{\circ}C$ until use. In addition, the experiments were repeated in the absence of brains. Tests on five parallel incubation sets were carried out. Preliminary evidence led to the choice of incubation conditions and the minimum effective dose of the substances used in the present study (data not shown).

Determination of aromatase activity

Aromatase activity was determined in the same brains used for incubation *in vitro*, as reported by Schumacher and Balthazar (1987) and Zerani *et al.* (1992). The brains were weighed and homogenized in cold buffer (50 μl mg^{-1} fresh tissue; 20 mmol K_2HPO_4 l^{-1} , 1 mmol EDTA l^{-1} , 3 mmol NaN_3 l^{-1} , 10% glycerol (v/v), 10 mmol β-mercaptoethanol l^{-1} , pH 7.4). Homogenates were immediately frozen in an

acetone-dry ice bath ($-80^{\circ}C$). For the assay, 200 μl of the total homogenate was thawed, and immediately 500 μl of the homogenization buffer containing 200 000 d.p.m. of [1,2,6,7-³H]testosterone (Amersham International, Amersham; specific activity 70–105 Ci $mmol^{-1}$) and 2.5 mg NADPH₂ ml^{-1} were added to the incubation tubes. Homogenates were put in a shaking bath (20°C) at 30 revolutions min^{-1} for 15 min. The samples were immediately stored at $-20^{\circ}C$. Steroids were extracted with 5 ml diethyl ether. Extracts were dried under a flow of nitrogen and resuspended in 500 μl of radioimmunoassay buffer (74.5 mmol Na_2HPO_4 l^{-1} , 12.5 mmol EDTA- $Na_2 \cdot 2H_2O$ l^{-1} , 0.1% gelatine, 0.1% NaN_3 , pH 7.5). Radioimmunoassay buffer (500 μl) containing oestradiol-specific antiserum in excess (compounds that crossreact more than 0.1%: oestrone, 1.6%; oestradiol, 0.5%) was added to duplicate samples (200 μl). The mixtures were incubated at 4°C for 16 h. The [³H]oestradiol-bound antiserum was separated with 500 μl of a charcoal-dextran (0.45% and 0.5%, respectively) suspension and added to 3 ml of Aqualyte (J. T. Baker, Deventer). The radioactivity was counted in a liquid scintillation system LS 1801 (Beckman Instruments, Fullerton, CA). The radioactivity of the control tubes (blanks) containing buffer, NADPH₂ and tracer, but no tissue homogenate, was subtracted from the final values.

Determination of prostaglandins and sex steroids

A radioimmunoassay (Gobbetti *et al.*, 1993) was used to detect PGF_{2α} and PGE₂, progesterone, androgens and oestradiol in incubation media. Intra-assay and interassay coefficients of variation and minimum detectable doses were: PGF_{2α}, 10%, 16%, 11 pg; PGE₂, 9%, 13%, 8 pg; progesterone, 9%, 12%, 7 pg; androgens, 8%, 12%, 13 pg; and oestradiol, 6%, 12%, 7 pg, respectively. The recoveries of added tritiated hormones were: PGF_{2α} 81.6%; PGE₂, 79.3%; progesterone, 86.3%; testosterone, 85.1%; and oestradiol, 88.6%, respectively. PGF_{2α} progesterone, testosterone and oestradiol antisera were provided by G. F. Bolelli and F. Franceschetti (CNR Physiopathology of Reproduction Service, University of Bologna, Bologna). The PGE₂ antiserum was purchased from Cayman Chemical Co. (Ann Arbor, MI). As testosterone was not separated from 5α-dihydrotestosterone and therefore the antiserum used was not specific, the data are expressed as androgens. Tritiated prostaglandins and sex steroids were purchased from Amersham; non-radioactive compounds were supplied by Sigma Chemical Co.

Statistical analysis

Analysis of variance (ANOVA) followed by Duncan's multiple range test was used to analyse the data (Duncan, 1955; Sokal and Rohlf, 1981). Correlation coefficients were calculated as described by Scossiroli and Palenzona (1979).

Results

Incubation of brains

A comparison of the basal release of the various hormones measured from lizard brains *in vitro* reveals that during the

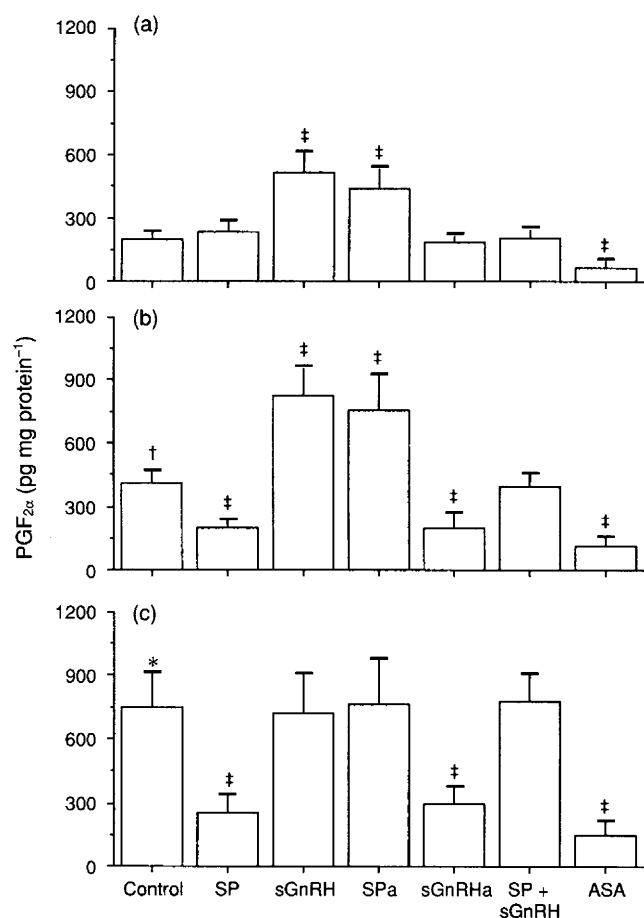


Fig. 1. Effects of substance P (SP), salmon GnRH (sGnRH), SP antagonist (SPa), sGnRH antagonist (sGnRHa), SP plus sGnRH, and acetylsalicylic acid (ASA) on the release *in vitro* of $\text{PGF}_{2\alpha}$ from the brain of the male lizard *Podarcis sicula sicula*, at different reproductive phases: (a) the fighting phase (April); (b) the mating phase (May); and (c) the refractory phase (June). Values are means of five determinations \pm SD. * $P < 0.01$ versus April and May, † $P < 0.01$ versus April, ‡ $P < 0.01$ versus control (Duncan's multiple range test).

refractory phase (June), the amount of $\text{PGF}_{2\alpha}$ and oestradiol released was significantly higher ($P < 0.01$) than during the fighting (April) and mating (May) phases (Figs 1, 2); the release of these two hormones was positively correlated ($r = 0.82$; d.f. = 13; $P < 0.001$). Conversely, the release of PGE_2 and androgens were least ($P < 0.01$) in the refractory phase, while androgen release was at its greatest ($P < 0.01$) during the fighting phase (Figs 3, 4). There was therefore an inverse correlation between androgens and oestradiol ($r = -0.88$; d.f. = 13; $P < 0.001$) and between androgens and $\text{PGF}_{2\alpha}$ ($r = -0.85$; d.f. = 13; $P < 0.001$). During the mating phase the amount of $\text{PGF}_{2\alpha}$ and oestradiol released was greater ($P < 0.01$) than in the fighting phase, and the amount of progesterone released was higher ($P < 0.01$) at this time than in the other two phases (Fig. 5).

A comparison of the effects of the various agents on the release of hormones measured from the lizard brain *in vitro* reveals that during the mating and refractory phases substance P decreased ($P < 0.01$) the amount of $\text{PGF}_{2\alpha}$ and oestradiol released (Figs 1, 2) and increased ($P < 0.01$) the

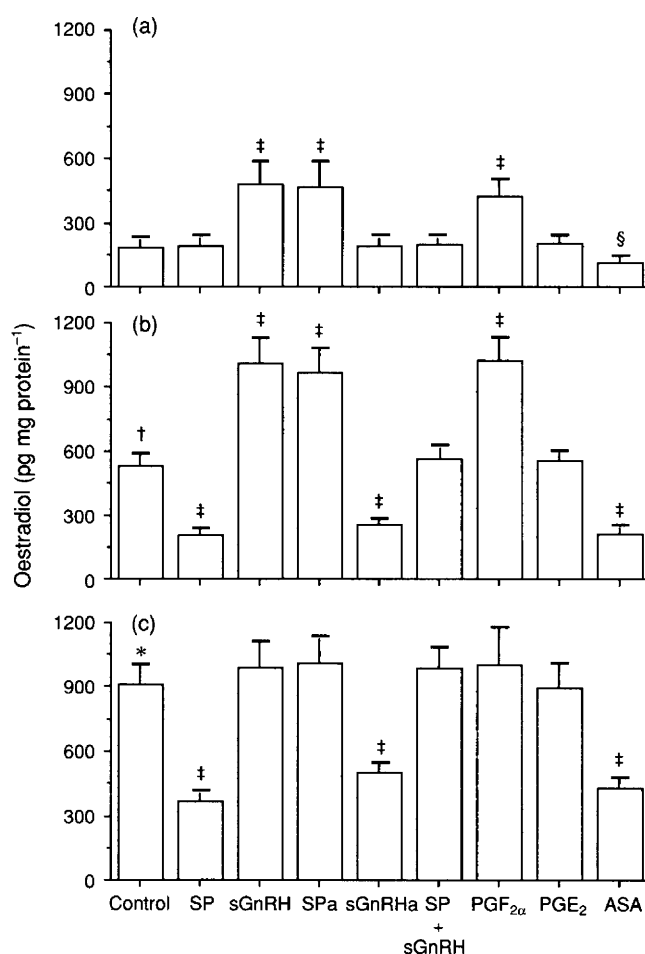


Fig. 2. Effects of substance P (SP), salmon GnRH (sGnRH), SP antagonist (SPa), sGnRH antagonist (sGnRHa), SP plus sGnRH, $\text{PGF}_{2\alpha}$, PGE_2 and acetylsalicylic acid (ASA) on the release *in vitro* of oestradiol from the brain of the male lizard *Podarcis sicula sicula*, at different reproductive phases: (a) the fighting phase (April); (b) the mating phase (May); and (c) the refractory phase (June). Values are means of five determinations \pm SD. * $P < 0.01$ versus April and May, † $P < 0.01$ versus April, ‡ $P < 0.01$, § $P < 0.05$ versus control (Duncan's multiple range test).

release of androgens (Fig. 4). Conversely, during the fighting and mating phases sGnRH increased ($P < 0.01$) the release of $\text{PGF}_{2\alpha}$ and oestradiol (Figs 1, 2) and decreased ($P < 0.01$) the release of androgens (Fig. 4). These two neuropeptides alone and in combination increased ($P < 0.01$) the release of PGE_2 and progesterone during the mating phase (Figs 3, 5). Substance P plus its antagonist and sGnRH plus sGnRH antagonist did not affect the basal release of any hormones (data not shown).

$\text{PGF}_{2\alpha}$, PGE_2 , progesterone, androgens and oestradiol were not detected in incubation samples in the absence of brain tissue (data not shown).

Aromatase activity

The quantity of labelled oestradiol produced from tritiated testosterone was greater ($P < 0.01$) in the refractory phase

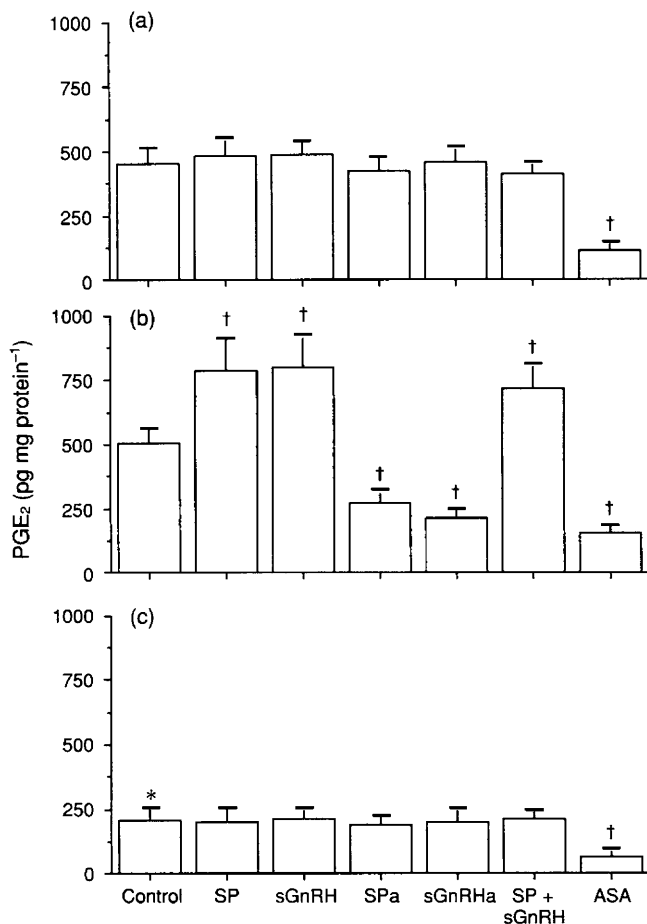


Fig. 3. Effects of substance P (SP), salmon GnRH (sGnRH), SP antagonist (SPa), sGnRH antagonist (sGnRHs), SP plus sGnRH, and acetylsalicylic acid (ASA) on the release *in vitro* of PGE₂ from the brain of the male lizard *Podarcis sicula sicula*, at different reproductive phases: (a) the fighting phase (April); (b) the mating phase (May); and (c) the refractory phase (June). Values are means of five determinations \pm SD. * $P < 0.01$ versus April and May, † $P < 0.01$ versus control (Duncan's multiple range test).

than in the other two phases, and higher ($P < 0.01$) in the mating than in the fighting phase. This finding correlated significantly with the changes in the basal release of oestradiol ($r = 0.94$; d.f. = 13; $P < 0.001$) and PGF_{2 α} ($r = 0.92$; d.f. = 13; $P < 0.001$), and was negatively correlated with the basal release of androgens ($r = -0.84$; d.f. = 13; $P < 0.001$) (Fig. 6).

The quantity of labelled oestradiol produced from tritiated testosterone was decreased ($P < 0.01$) by substance P in the mating and refractory phases. Conversely, in the fighting and mating phases sGnRH increased ($P < 0.01$) the production of tritiated oestradiol. The antagonists of substance P and sGnRH showed opposite effects. The effects of PGF_{2 α} on the production of [³H]oestradiol were similar to those found for sGnRH. ASA decreased the conversion of [³H]testosterone into [³H]oestradiol in all phases (Fig. 6). Substance P plus sGnRH, substance P plus substance P antagonist, sGnRH plus sGnRH antagonist and PGE₂ did not affect [³H]oestradiol values (data not shown).

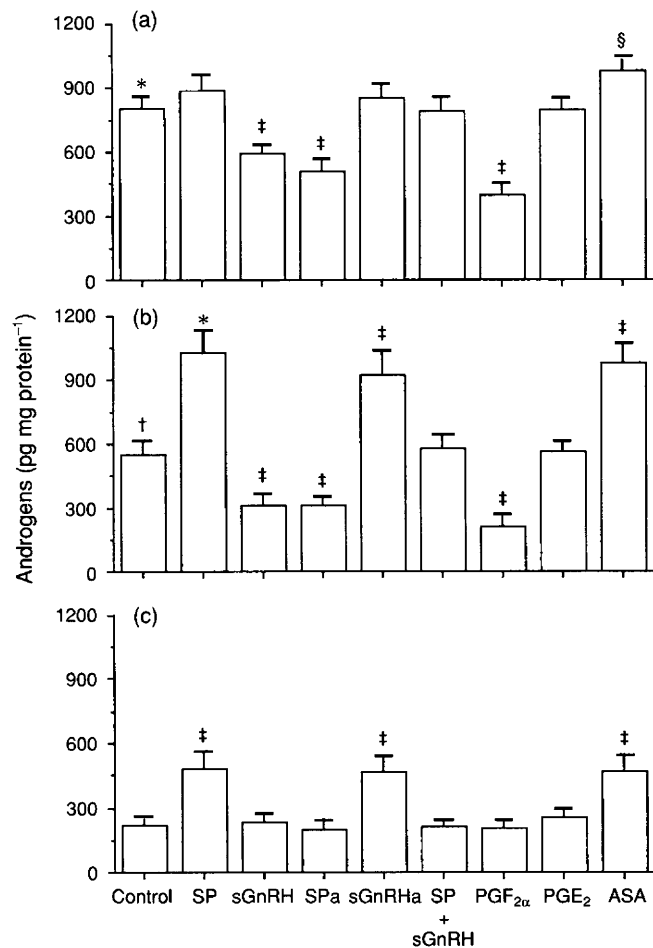


Fig. 4. Effects of substance P (SP), salmon GnRH (sGnRH), SP antagonist (SPa), sGnRH antagonist (sGnRHs), SP plus sGnRH, PGF_{2 α} , PGE₂, and acetylsalicylic acid (ASA) on the release *in vitro* of androgens from the brain of the male lizard *Podarcis sicula sicula*, at different reproductive phases: (a) the fighting phase (April); (b) the mating phase (May); and (c) the refractory phase (June). Values are means of five determinations \pm SD. * $P < 0.01$ versus May and June, † $P < 0.01$ versus June, ‡ $P < 0.01$, § $P < 0.05$ versus control (Duncan's multiple range test).

Discussion

This is the first report that shows that during the various phases of the reproductive period of the male lizard *P. s. sicula* different amounts of prostaglandins and sex steroids and various degrees of aromatase activity occur in the brain. In particular, PGF_{2 α} and oestradiol release, and aromatase activity exhibit maximum values during the refractory phase (June), confirming for this reptile the relationship between PGF_{2 α} and oestradiol that has been found in two amphibian species: the anuran water frog *Rana esculenta* (Gobbetti and Zerani, 1991, 1992a, b) and the urodele crested newt *Triturus cristatus* (Gobbetti and Zerani, 1992b; Gobbetti *et al.*, 1992). This relationship between these two hormones is supported by the positive correlation among PGF_{2 α} , oestradiol and aromatase activity. The high amount of oestradiol released could be responsible for interrupting the reproductive processes (refractory phase), when the environmental cues are still favourable

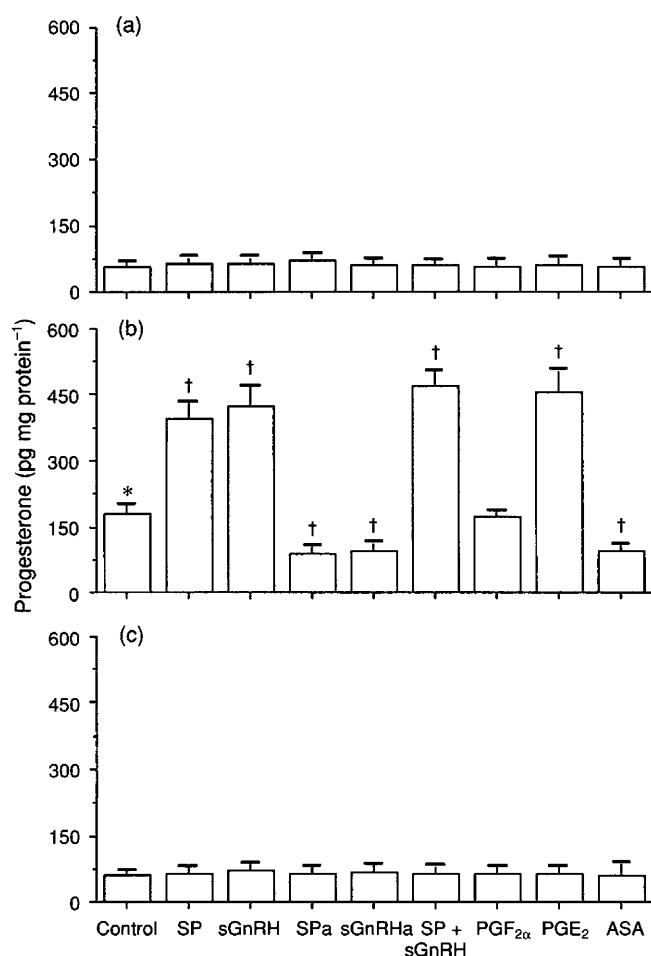


Fig. 5. Effects of substance P (SP), salmon GnRH (sGnRH), SP antagonist (SPa), sGnRH antagonist (sGnRHa), SP plus sGnRH, PGF_{2α}, PGE₂, and acetylsalicylic acid (ASA) on the release *in vitro* of progesterone from the brain of the male lizard *Podarcis sicula sicula*, at different reproductive phases: (a) the fighting phase (April); (b) the mating phase (May); and (c) the refractory phase (June). Values are means of five determinations \pm SD. * $P < 0.01$ versus April and June, † $P < 0.01$ versus control (Duncan's multiple range test).

for reproduction. This adaptive mechanism would prevent the occurrence of newly hatched lizards in early autumn, as suggested by Angelini *et al.* (1986). The lizard brain *in vitro* released the greatest amounts of androgens during the fighting phase (April), confirming the role of androgens in regulating the aggressive behaviour of males, which in this phase are engaged in fights with the other males (Ciarcia *et al.*, 1986). The physiological significance of the different amounts of PGE₂ and progesterone released during the various phases of reproduction is, at present, difficult to interpret.

Various forms of GnRH have been found in reptiles: sGnRH (Licht and Porter, 1987), chicken GnRH-I (King and Millar, 1987), and chicken GnRH-II (Sherwood and Whittier, 1988). These three forms of GnRH have been found in the lizard *P. s. sicula* (Powell *et al.*, 1986) and sGnRH causes an increase in the plasma concentration of testosterone in the male of this lizard (Ciarcia *et al.*, 1989), while in the female an analogue of GnRH exhibits a direct effect on progesterone release by follicles

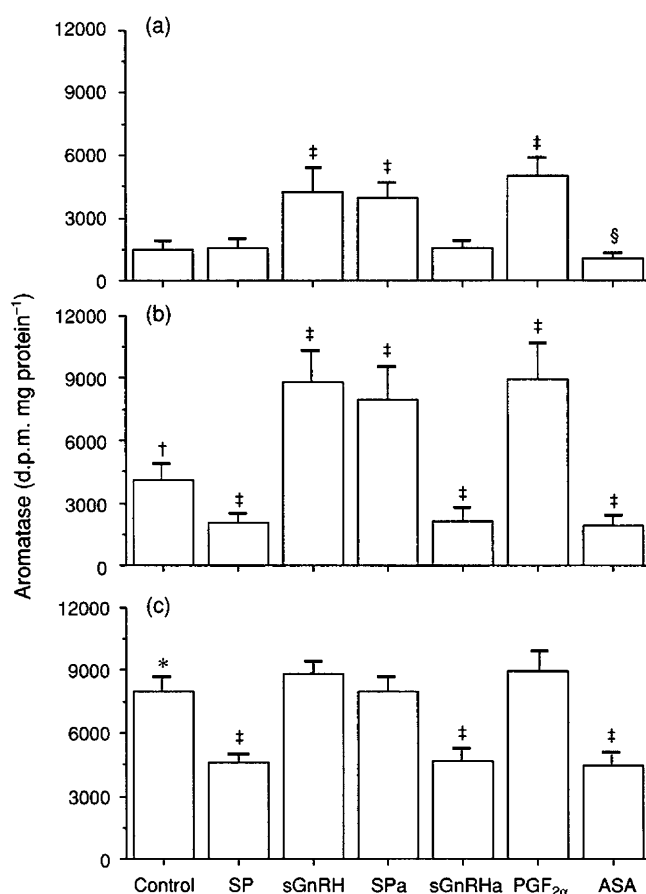


Fig. 6. Effects of substance P (SP), salmon GnRH (sGnRH), SP antagonist (SPa), sGnRH antagonist (sGnRHa), PGF_{2α} and acetylsalicylic acid (ASA) on aromatase activity (conversion of [³H]testosterone into [³H]oestradiol) *in vitro* in the brain of the male lizard *Podarcis sicula sicula*, at different reproductive phases: (a) the fighting phase (April); (b) the mating phase (May); and (c) the refractory phase (June). Values are means of five determinations \pm SD. * $P < 0.01$ versus April and May, † $P < 0.01$ versus April, ‡ $P < 0.01$, § $P < 0.05$ versus control (Duncan's multiple range test).

(Varriale *et al.*, 1986). Substance P has been found in several reptiles, such as *Alligator mississippiensis* (Wang *et al.*, 1992), *Galloti galloti* (Bello *et al.*, 1991), *Chrysemys picta* (Koste *et al.*, 1980), *Lacerta* sp. and *Testudo kleinmani* (Lembeck *et al.*, 1985) but nothing is known about the function of this neuropeptide in the regulation of prostaglandin and sex steroid concentrations in reptiles.

The present data on the effects *in vitro* of sGnRH and substance P in the brain of *P. s. sicula* suggest an opposite mechanism of action for these two neuropeptides in the regulation of release of prostaglandins and sex steroids. sGnRH induces an increase in oestradiol release and a decrease in the release of androgens probably by increasing the release of PGF_{2α}, which then activates aromatase activity. In contrast, SP induces a decrease in oestradiol release and an increase in the release of androgens by decreasing the release of PGF_{2α}, which attenuates aromatase activity (as confirmed by ASA treatment). These opposite mechanisms attributed to sGnRH and SP are validated by the opposite effects of their antagonists and by the lack of effect following the treatment with substance P

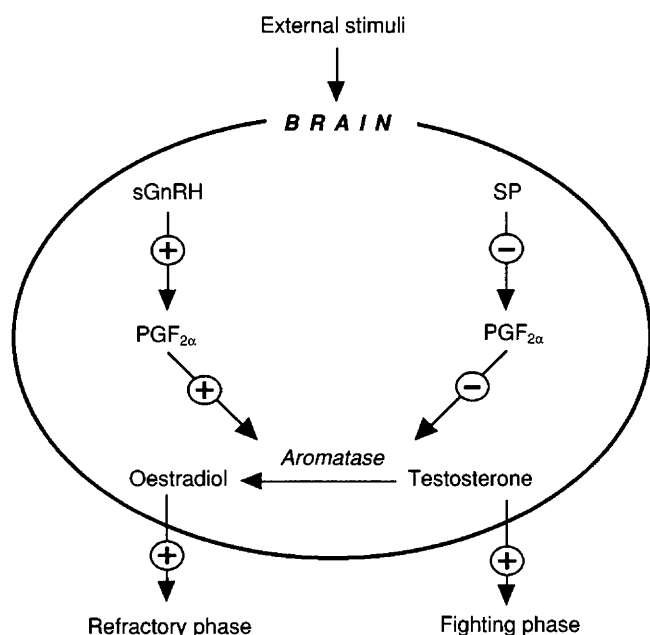


Fig. 7. Possible mechanisms of action of salmon GnRH (sGnRH) and substance P (SP) in the regulation of reproduction in the brain of the male lizard *Podarcis sicula sicula*.

plus sGnRH. These findings suggest different roles for these two neuropeptides in the reproductive processes of the male *P. s. sicula*.

The regulation of the refractory phase seems to involve the release of oestradiol by sGnRH, which is one of the factors that induces the cessation of reproductive activity, as reported above (Angelini *et al.*, 1986). sGnRH fails to increase $\text{PGF}_{2\alpha}$, oestradiol and aromatase activity in the refractory phase, probably because in this phase the lizard brain expresses the highest values for $\text{PGF}_{2\alpha}$, oestradiol and aromatase activity, so that the additional stimulus does not affect these patterns. This hypothesis is supported by the results obtained by the sGnRH antagonist, which in the refractory phase decreases $\text{PGF}_{2\alpha}$, oestradiol and aromatase activity. SP could play a role in regulating the fighting behaviour of *P. s. sicula* by releasing androgens, which are the main factor inducing this reproductive behaviour, as reported above (Ciarcia *et al.*, 1986). Just as sGnRH does not exhibit effects *in vitro* in the refractory phase, substance P fails to decrease $\text{PGF}_{2\alpha}$ and aromatase activity and to increase androgens in the fighting phase, probably because in this phase the brain expresses the lowest values for $\text{PGF}_{2\alpha}$ and aromatase activity and the highest amounts of androgens so that the additional stimulus of substance P is ineffective.

Finally, sGnRH and substance P cause an increase in progesterone release in the mating phase (May), and this effect is mediated by PGE_2 . However, at present, the significance of this phenomenon is unclear.

In summary, sGnRH and SP could have different roles in reproductive processes, with opposite mechanisms, in the central nervous system of the male *P. s. sicula* (see Fig. 7). Substance P appears to be involved in beginning reproductive behaviour (fighting phase), and sGnRH in terminating it (refractory phase).

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