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Hormonal and Cellular Brain Mechanisms Regulating the Amplexus of Male and Female Water Frog (*Rana esculenta*)

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Abstract

The role of nitric oxide (NO) synthase, prostaglandin E_2–9-ketoreductase, and aromatase brain activities in regulating frog amplexus was assessed in the water frog (*Rana esculenta*). Plasma concentrations of testosterone were higher, and concentrations of 17β-oestradiol lower, in amplexing males than in unamplexing males; while concentrations of testosterone and PGE_2 were lower, and those of 17β-oestradiol and $PGF_{2\alpha}$ higher, in amplexing females compared to unamplexing females. Hormone release rescued from frog brains *in vitro* mirrored plasma hormone measures. Brain aromatase activity was lower in amplexing males; NO synthase was lower and PGE_2–9-ketoreductase and aromatase were higher in amplexing females. In male brains, PGE_2–9-ketoreductase inhibitor decreased $PGF_{2\alpha}$ release and increased that of PGE_2; aromatase inhibitor decreased 17β-oestradiol and increased testosterone release. In female brains, NO donor and PGE_2–9-ketoreductase inhibitor increased testosterone and PGE_2 release and decreased that of 17β-oestradiol and $PGF_{2\alpha}$; NO synthase inhibitor decreased testosterone release and PGE_2 and increased 17β-oestradiol and $PGF_{2\alpha}$ release; $PGF_{2\alpha}$ decreased testosterone release and increased 17β-oestradiol release; aromatase inhibitor decreased 17β-oestradiol release and increased testosterone release. In female brains, NO donor and PGE_2–9-ketoreductase inhibitor decreased PGE_2–9-ketoreductase and aromatase activities; $PGF_{2\alpha}$ increased aromatase activity; NO synthase inhibitor increased PGE_2–9-ketoreductase and aromatase activity. The data suggest that, in amplexing female brains, external and/or internal stimuli inhibit NO synthase, decreasing NO and activating PGE_2–9-ketoreductase; in turn, $PGF_{2\alpha}$ increases aromatase activity and 17β-oestradiol release; while, in amplexing male brains, stimuli inhibit aromatase activity, thereby increasing testosterone production.

Behaviour and steroid hormones have a dynamic interaction; steroid hormones activate behaviour by modulating neural circuits, while circulating steroid concentrations are rapidly changed by behavioural stimuli (1). Reproductive behaviour can also be induced in several vertebrates by aromatase, the enzyme which converts testosterone into 17β-oestradiol (2). However, the neuroendocrine control of reproduction is still unclear in amphibians, and there is conflicting evidence about the role of oestrogens and androgens in regulating amphibian reproductive processes (3–8). Recently, we (9) reported that prostaglandin (PG) E_2–9-ketoreductase (PGE_2–9-K), the enzyme which converts PGE_2 into $PGF_{2\alpha}$, and $PGF_{2\alpha}$ itself stimulates brain aromatase activity to induce male courtship in the urodele crested newt, *Triturus carnifex*. We found that in this male newt, nitric oxide (NO) has a role during courtship, since reproductive success was characterized by the highest brain activity of NO synthase (NOS), the enzyme producing NO, which progressively increases over the various courtship phases (10).

In the present work, the possible role of NOS, PGE_2–9-K, and aromatase brain activities in the control of breeding behaviour (amplexus) was studied in the anuran water frog, *Rana esculenta*.

Materials and methods

Animals

The reproductive cycle and the courtship of the water frog *R. esculenta* population living in the Colfiorito pond (Umbria, Italy, 870 m above sea level) is here described. Frogs breed in May–June (reproduction), when the

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temperature increases; at the beginning of summer, breeding is interrupted (refractory); the recrudescence of sex organs starts in the middle of summer and continues over the autumn (recovery); the frogs disappear into ground shelters to avoid the very cold winter months until the next spring (hibernation); at the beginning of spring, the frogs return to the pond (pre-reproduction). The breeding behaviour of this species is very simple: the male calls until a female contacts him; he then quickly clasps the female jumping on her back leading to amplexus (11).

At the beginning of June, amplexing and unamplexing male and female frogs were captured and killed in the field by decapitation. Amplexing females, at autopsy, were divided into preovulatory (absence of eggs in the oviduct) and ovulatory (eggs in the oviduct); unamplexing females, at autopsy, were divided into preovulatory (ovary with full-grown follicles) and postovulatory (ovary without full-grown follicles). The males were divided into unamplexing, amplexing with preovulatory female, and amplexing with ovulatory female. Brains and blood samples were rapidly removed and transferred to our laboratory. Blood samples were centrifuged at $1500 \times g$ for 30 min and plasma samples kept at $-20\,^{\circ}C$.

In-vitro incubations

The frog brains were placed in cold Dulbecco's modified Eagle's medium (DMEM; Sigma Chemical Co., St. Louis, MO, USA) containing 10 mM Hepes, 0.1 mg penicillin G/ml and 0.2 mg streptomycin/ml. For each group the brains were randomly distributed each in one incubation well (Multiple tissue culture plates, Becton Dickinson Co, USA). Each set of wells was divided into six experimental groups. The experimental groups were one brain incubated with: (1) DMEM alone; (2) DMEM plus a NO donor (sodium nitroprusside, 20 μM, Sigma); (3) DMEM plus $PGF_{2\alpha}$ (200 ng, Sigma); (4) DMEM plus a NOS inhibitor (Nω-nitro-L-arginine methyl ester, 1 μM, Sigma); (5) DMEM plus a PGE_2–9-K inhibitor (palmitic acid, 10 μM, Sigma); or (6) DMEM plus an aromatase inhibitor (4-hydroxyandrostenedione, 7.5 μM, Sigma). The final volume of each well was 1.5 ml. Culture plates were wrapped with aluminium foil and incubated at room temperature ($\approx 19\,^{\circ}C$). Medium was removed from each well after 60 min of incubation and immediately stored at $-20\,^{\circ}C$. In addition, the whole experiment was repeated with incubation sets without brains. Tests on five parallel incubation sets were carried out. Preliminary evidence led to our choosing the incubation conditions and the minimum effective doses of the substances utilized in the present *in-vitro* study (data not shown).

NOS activity determination

NOS activity was determined in the brains used for *in-vitro* incubations, by monitoring the conversion of [^{3}H]L-arginine into [^{3}H]L-citrulline, with a modified method previously described (12–14). The brain tissues were weighed and homogenized in 2 ml of cold fresh homogenating buffer (50 mM Tris, 1 mM EDTA and 1 mM EGTA, pH 7.4) and centrifuged at $20\,000 \times g$ for 60 min at 4 °C. Twenty-five μl of supernatant and 100 μl of incubation buffer (1.5 mM NADPH, 1 mM $CaCl_2$) containing 100 000 d.p.m. [2,3–^{3}H]L-arginine (sp. act 30–40 Ci/mmol, Sigma) were added to the incubation tube. After 30 min incubation at room temperature ($\approx 19\,^{\circ}C$), the enzymatic reaction was stopped by addition of 2 ml of blocking buffer (20 mM HEPES, 2 mM EDTA, pH 5.5). The mixture was applied to a pre-equilibrated column (20 mM sodium acetate, 2 mM EDTA, 0.2 mM EGTA, pH 5.5; 1 cm diameter) containing 1 ml of Dowex AG50 W-X8 (Sigma) and the material was eluted with 2 ml of water. [^{3}H]L-Citrulline was quantified in a liquid scintillation system LS 1801 (Beckman Instr., Fullerton, CA, USA). Additional determinations were performed in the presence of excess of NOSi to verify the specificity of the assay for production of [^{3}H]L-citrulline by NOS (data not shown). Protein concentration was determined by a commercial assay kit (Bio-Rad, Hercules, CA, USA).

PGE_2–9-K activity determination

PGE_2–9-K activity was determined in the brains, used for *in-vitro* incubations, by monitoring the conversion of [^{3}H]PGE_2 into [^{3}H]$PGF_{2\alpha}$, with a method previously described (9). The brain tissues were weighed and homogenized in fresh cold buffer (100 μl/mg fresh tissue; 20 mM K_2HPO_4, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, pH 7.4). One hundred μl of homogenate and 50 μl of the homogenating buffer containing 200 000 d.p.m. [5,6,8,11,12,14,15-^{3}H]PGE_2 (sp. act 140–170 Ci/mmol, Amersham Int., Buckinghamshire, UK) and $NADPH_2$ (3 mg/ml) were added to the incubation tube. After 60 min incubation, at room temperature ($\approx 19\,^{\circ}C$), the enzymatic reaction was stopped by the addition of 100 μl 0.1 M HCl. PGs were extracted with 5 ml of diethyl ether, dried under a nitrogen flow and resuspended in 250 μl of fresh RIA buffer (74.5 mM Na_2HPO_4, 12.5 mM EDTA-Na_2, 0.1% gelatin, pH 7.5). Fresh RIA buffer (150 μl) containing $PGF_{2\alpha}$ specific antiserum(see below) was added to the duplicate samples (100 μl). The mixtures were incubated at 4 °C for 16 h. The [^{3}H]$PGF_{2\alpha}$-bound antiserum was separated with 250 μl of a charcoal dextran suspension, and the radioactivity was counted in a liquid scintillation system LS 1801 (Beckman). Additional determinations were performed in the presence of excess of PGE_2–9-Ki to verify the specificity of the assay for production of [^{3}H]$PGF_{2\alpha}$ by PGE_2–9-K (data not shown). Protein concentration was determined by a commercial assay kit (Bio-Rad).

Aromatase activity determination

Aromatase activity was determined in the brains, used for *in-vitro* incubations by monitoring the conversion of [^{3}H]testosterone into [^{3}H]17β-oestradiol, with a method previously described (8). The brain tissues were weighed and homogenized in fresh cold buffer (100 μl/mg fresh weight tissue; 20 mM K_2HPO_4, 1 mM EDTA, 10 mM β-mercaptoethanol, 10% glycerol, pH 7.4). One hundred μl of homogenate were thawed, and immediately 50 μl of the homogenating buffer containing 100 000 d.p.m. [1,2,6,7-^{3}H]testosterone (sp. act 70–105 Ci/mmol, Amersham Int.) and $NADPH_2$ (2.5 mg/ml) were added to the incubation tube. After 60 min of incubation at room temperature ($\approx 19\,^{\circ}C$), the samples were immediately stored at $-20\,^{\circ}C$. Steroids were extracted with 5 ml of diethyl ether. Extracts were dried under a nitrogen flow and resuspended in 250 μl of fresh RIA buffer. Fresh RIA buffer (150 μl) containing 17β-oestradiol specific antiserum (see below) were added to the duplicate samples (100 μl). The mixtures were incubated at 4 °C for 16 h. The [^{3}H]17β-oestradiol-bound-antiserum was separated with 250 μl of a charcoal-dextran suspension and the radioactivity was counted in a liquid scintillation system LS 1801 (Beckman). Additional determinations were performed in the presence of excess of ARi to verify the specificity of the assay for production of [^{3}H]17β-oestradiol by AR (data not shown). Protein concentration was determined by a commercial assay kit (Bio-Rad).

Hormone determination

On each plasma and medium sample, testosterone, 17β-oestradiol, PGE_2, and $PGF_{2\alpha}$ were measured by a RIA method previously reported (8, 9). Because the antiserum against testosterone is not specific, this hormone was separated by partition chromatography of the diethyl ether extract in celite according to Wingfield and Farner (15). The parallelism among the standard curve in buffer, a standard curve in incubation medium (then extracted), and a serial dilution of a single incubation medium sample (extracted) was constant. The intra-and interassay coefficients and the minimum detectable dose were: testosterone, 9.5%, 14%, 12 pg; 17β-oestradiol, 11%, 19%, 18 pg; PGE_2, 7.5%, 12%, 15 pg; $PGF_{2\alpha}$, 8%, 14%, 18 pg. Testosterone and 17β-oestradiol antisera were kindly provided by Dr G. F. Bolelli (CNR-Normal and Pathologic Cytomorphology Institute, Bologna, Italy) and Dr F. Franceschetti (CNR-Physiopathology of Reproduction Service, University of Bologna, Italy), PGE_2 antiserum was purchased from Cayman Chem. (USA) and $PGF_{2\alpha}$ antiserum from Sigma. Tritiated hormones were purchased from Amersham Int., non-radioactive ones from Sigma.

Statistical analysis

The data were submitted to ANOVA followed by Duncan's multiple range test, correlation coefficents were calculated (16, 17).

Results

Plasma hormone concentrations

Male

Plasma concentrations of testosterone were significantly higher ($P < 0.01$) in males amplexing with an ovulatory female than in males amplexing with a preovulatory female or in unamplexing males; testosterone concentrations were also higher ($P < 0.01$) in males amplexing with a preovulatory female than in unamplexing males (Fig. 1). By contrast, concentrations of 17β-oestradiol were lower ($P < 0.01$) in males amplexing with an ovulatory female than in males amplexing with a preovulatory female or in unamplexing

males, and lower ($P<0.01$) in males amplexing with a preovulatory female than in unamplexing males (Fig. 1). Concentrations of PGE_2 and $PGF_{2\alpha}$ were not different (Fig. 1). Testosterone concentrations were negatively correlated ($P<0.001$) with those of 17β-oestradiol (Table 1).

Female

Plasma concentrations of testosterone and PGE_2 were both significantly lower ($P<0.01$) in amplexing ovulatory females than in amplexing preovulatory, unamplexing preovulatory, and unamplexing postovulatory females; concentrations of testosterone and PGE_2 were lower ($P<0.01$) in amplexing preovulatory females than in unamplexing preovulatory and unanplexing postovulatory females (Fig. 1). Concentrations of 17β-oestradiol and $PGF_{2\alpha}$ were higher ($P<0.01$) in amplexing ovulatory females than in amplexing preovulatory, unamplexing preovulatory, and unamplexing postovulatory females and were higher ($P<0.01$) in amplexing preovulatory females than in unamplexing preovulatory and unamplexing postovulatory females (Fig. 1). Testosterone concentrations were negatively correlated ($P<0.001$) with those of 17β-oestradiol and $PGF_{2\alpha}$, and positively correlated ($P<0.001$) with those of PGE_2; 17β-oestradiol concentrations were positively correlated ($P<0.001$) with those of $PGF_{2\alpha}$, and negatively correlated ($P<0.001$) with those of PGE_2; concentrations of $PGE_{2\alpha}$ were negatively correlated ($P<0.001$) with those of $PGF_{2\alpha}$ (Table 1).

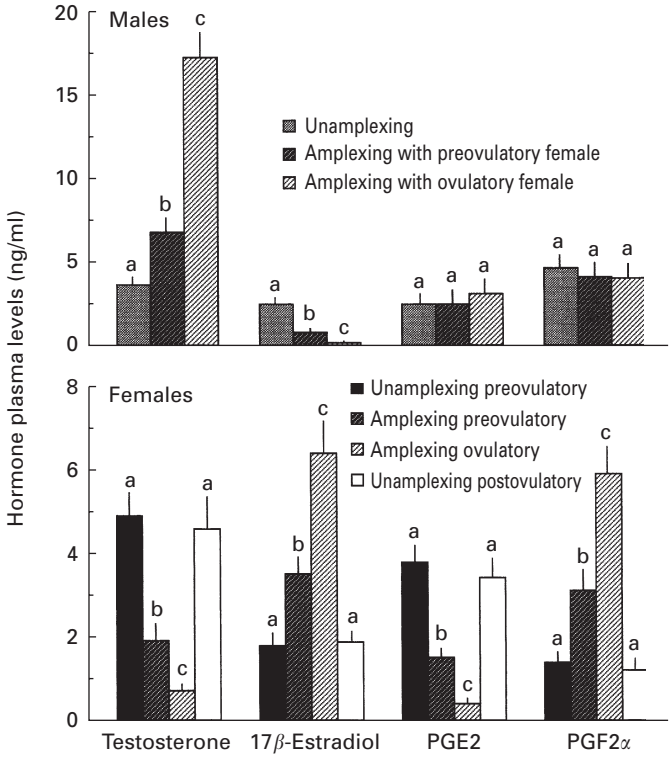

FIG. 1. Testosterone, 17β-oestradiol, PGE_2, and $PGF_{2\alpha}$ plasma concentrations in amplexing and unamplexing male and female water frog, *Rana esculenta*. Means ± SD; n = 15 each; groups with different letters are significantly different ($P<0.01$).

TABLE 1. Correlation Coefficients Among Plasma Hormone Levels, *In-Vitro* Bain Hormone Releases, and *In-Vitro* Brain Enzyme Activities By Male and Female Water Frog, *Rana esculenta*, During Amplexus.

			Male	Female
Plasma hormone levels				
Testosterone	*vs*	17β-Oestradiol	−0.683*	−0.535*
Testosterone	*vs*	PGE_2	0.256	0.554*
Testosterone	*vs*	$PGF_{2\alpha}$	−0.187	−0.611*
17β-Oestradiol	*vs*	PGE_2	−0.204	−0.562*
17β-Oestradiol	*vs*	$PGF_{2\alpha}$	0.125	0.599*
PGE_2	*vs*	$PGF_{2\alpha}$	0.204	−0.626*
Brain hormone releases				
Testosterone	*vs*	17β-Oestradiol	−0.922*	−0.751*
Testosterone	*vs*	PGE_2	0.113	0.773*
Testosterone	*vs*	$PGF_{2\alpha}$	0.057	−0.725*
17β-Oestradiol	*vs*	PGE_2	−0.139	−0.817*
17β-Oestradiol	*vs*	$PGF_{2\alpha}$	0.304	0.842*
PGE_2	*vs*	$PGF_{2\alpha}$	−0.452	−0.908*
Brain enzyme activities				
NOS	*vs*	PGE_2-9-K	0.243	−0.833*
NOS	*vs*	AR	0.114	−0.804*
PGE_2-9-K	*vs*	AR	−0.324	0.849*

Plasma hormone levels: n = male 45, female 60; brain hormone releases and brain enzyme activities: n = male 15, female 20; *$P<0.001$.

In-vitro brain hormone releases

Testosterone, 17β-oestradiol, PGE_2, and $PGF_{2\alpha}$ were not detectable in incubations without brain (data not shown).

Male

(Table 2). The basal release of testosterone, 17β-oestradiol, PGE_2, and $PGF_{2\alpha}$ from frog brains incubated *in vitro* showed similar trends and correlations to those found for plasma concentrations (Tables 1, 2). In the presence of a PGE_2–9-K inhibitor, the release of $PGF_{2\alpha}$ was reduced ($P<0.01$) and that of PGE_2 was increased ($P<0.01$) compared to medium-alone in all groups considered (Table 2). In the presence of an aromatase inhibitor, the release of 17β-oestradiol was reduced ($P<0.01$) and that of testosterone was increased ($P<0.01$) compared to medium-alone in all groups considered (Table 2).

Female

(Table 3). As in males, the basal release of testosterone, 17β-oestradiol, PGE_2, and $PGF_{2\alpha}$ from frog brains incubated *in vitro* showed similar trends and correlations to those found for plasma concentrations (Tables 1, 3). In the presence of a NO donor or in the presence of a PGE_2–9-K inhibitor, the release of testosterone and PGE_2 was increased ($P<0.01$), while the release of 17β-oestradiol and $PGF_{2\alpha}$ was decreased ($P<0.01$) compared to medium-alone in all groups considered; NOS inhibitor (NOSi) decreased ($P<0.01$) testosterone and PGE_2 releases and increased ($P<0.01$) those of 17β-oestradiol and $PGF_{2\alpha}$ with respect to medium-alone in all groups considered except in amplexing ovulatory females; $PGF_{2\alpha}$ decreased ($P<0.01$) testosterone release and increased ($P<0.01$) that of 17β-oestradiol with respect to medium-alone in all groups considered except in amplexing ovulatory females. In the presence of an aromatase inhibitor, the release of 17β-oestradiol was decreased ($P<0.01$) and that of testo-

TABLE 2. *In-Vitro* Effects Of NO Donor (NOd), $PGF_{2\alpha}$, NOS Inhibitor (NOSi), PGE_2-9-Ketoreductase Inhibitor (PGE_2-9-Ki), and Aromatase Inhibitor (ARi) On Basal Release Of Testosterone, 17β-Oestradiol, PGE_2, and $PGF_{2\alpha}$ By the Brain Of Male *Rana esculenta*, During Amplexus.

	Unamplexing	Amplexing with preovulatory female	Amplexing with ovulatory female
Testosterone (pg/mg protein)			
Medium-alone	121 ± 21^a	274 ± 38^c	426 ± 75^d
NOd	134 ± 17^a	238 ± 41^c	461 ± 81^d
$PGF_{2\alpha}$	139 ± 20^a	232 ± 30^c	497 ± 65^d
NOSi	110 ± 19^a	223 ± 42^c	452 ± 88^d
PGE_2-9-Ki	128 ± 22^a	260 ± 40^c	449 ± 84^d
ARi	621 ± 143^b	684 ± 156^b	705 ± 127^b
17β-Oestradiol (pg/mg protein)			
Medium-alone	185 ± 33^a	82 ± 15^c	39 ± 6^d
NOd	166 ± 42^a	91 ± 17^c	34 ± 7^d
$PGF_{2\alpha}$	170 ± 29^a	75 ± 15^c	32 ± 9^d
NOSi	194 ± 47^a	84 ± 20^c	31 ± 5^d
PGE_2-9-Ki	176 ± 38^a	80 ± 13^c	33 ± 7^d
ARi	14 ± 5^b	17 ± 4^b	15 ± 6^b
PGE_2 (pg/mg protein)			
Medium-alone	153 ± 32^a	179 ± 27^a	160 ± 33^a
NOd	162 ± 35^a	172 ± 34^a	121 ± 26^a
NOSi	183 ± 22^a	190 ± 38^a	182 ± 27^a
PGE_2-9-Ki	506 ± 69^b	458 ± 91^b	481 ± 72^b
ARi	167 ± 24^a	150 ± 34^a	160 ± 33^a
$PGF_{2\alpha}$ (pg/mg protein)			
Medium-alone	235 ± 48^a	287 ± 47^a	211 ± 40^a
NOd	262 ± 55^a	259 ± 53^a	254 ± 66^a
NOSi	299 ± 63^a	220 ± 71^a	287 ± 79^a
PGE_2-9-Ki	72 ± 14^b	84 ± 18^b	69 ± 16^b
ARi	284 ± 51^a	279 ± 44^a	218 ± 37^a

Means $\pm$ SD; n = 5 each; groups with different letters are significantly different ($P < 0.01$).

sterone was increased ($P < 0.01$) with respect to medium-alone in all groups.

In-vitro brain enzyme activities

Male

(Table 4) The basal aromatase activity was significantly lower ($P < 0.01$) in males amplexing with an ovulatory female than in males amplexing with a preovulatory female or in unamplexing males; aromatase activity was also lower in males amplexing with a preovulatory female than in unamplexing males ($P < 0.01$) (Table 4). The basal activities of NOS and PGE_2–9-K did not vary between groups. NOSi decreased ($P < 0.01$) NOS activity, PGE_2–9-Ki decreased PGE_2–9-K activity ($P < 0.01$), and aromatase inhibitor decreased aromatase activity ($P < 0.01$) with respect to medium-alone in all groups.

Female

(Table 5) The basal activity of NOS was significantly lower ($P < 0.01$) and the basal activities of PGE_2–9-K and aromatase were higher in amplexing preovulatory females and amplexing ovulatory females than in unamplexing preovulatory females and unamplexing postovulatory females ($P < 0.01$); NOS activity was lower ($P < 0.01$) and PGE_2–9-K and aromatase activities were higher ($P < 0.01$) in amplexing ovulatory females than in amplexing preovulatory females. The activity levels of NOS were negatively correlated ($P < 0.001$) with those of PGE_2–9-K and aromatase; PGE_2–9-*K* values were positively correlated ($P < 0.001$) with those of aromatase (Table 1). NO donor and PGE_2–9-K inhibitor decreased PGE_2–9-K and aromatase activities with respect to medium-alone in all groups considered ($P < 0.01$); the decrease of PGE_2–9-K activity induced by PGE_2–9-K inhibitor was greater than that induced by the NO donor ($P < 0.01$). $PGF_{2\alpha}$ increased aromatase activity in all groups except amplexing ovulatory females ($P < 0.01$). The NOS inhibitor decreased ($P < 0.01$) NOS activity in all groups and increased PGE_2–9-K and aromatase activities in all groups except amplexing ovulatory females ($P < 0.01$). Aromatase inhibitor decreased aromatase activity in all groups ($P < 0.01$); the decrease of aromatase activity induced by the aromatase inhibitor was greater than those induced by the NO donor and PGE_2–9-K ($P < 0.01$).

Discussion

Nitric oxide is an important intra- and intercellular messenger that regulates a wide range of physiological events (18, 19). The functions attributed to NO include the modulation of endocrine activity (20, 21), and in particular, it has been suggested that NO is an important modulator of hypothalamic releasing factors (22–24). Mani *et al.* (25) has reported that NO-induced LHRH release plays a crucial role in the mediation of sexual behaviour, including lordosis, in female

TABLE 3. *In-Vitro* Effects Of NO Donor (NOd), $PGF_{2\alpha}$, NOS Inhibitor (NOSi), PGE_2-9-Ketoreductase Inhibitor (PGE_2-9-Ki), and Aromatase Inhibitor (ARi) On Basal Release Of Testosterone, 17β-Oestradiol, PGE_2, and $PGF_{2\alpha}$ By the Brain Of Female *Rana esculenta*, During Amplexus.

	Unamplexing preovulatory	Amplexing preovulatory	Amplexing ovulatory	Unamplexing postovulatory
Testosterons (pg/mg protein)				
Medium-alone	544 ± 47^a	285 ± 21^d	95 ± 10^c	463 ± 51^a
NOd	952 ± 96^b	863 ± 90^b	783 ± 97^b	931 ± 83^b
$PGF_{2\alpha}$	106 ± 17^c	89 ± 12^c	82 ± 11^c	95 ± 14^c
NOSi	112 ± 13^c	101 ± 17^c	96 ± 12^c	84 ± 11^c
PGE_2-9-Ki	819 ± 85^b	752 ± 92^b	789 ± 85^b	904 ± 81^b
ARi	903 ± 98^b	778 ± 88^b	817 ± 94^b	935 ± 99^b
17β-Oestradiol (pg/mg protein)				
Medium-alone	189 ± 44^a	369 ± 62^d	623 ± 74^c	231 ± 51^a
NOd	94 ± 19^b	103 ± 21^b	86 ± 14^b	99 ± 15^b
$PGF_{2\alpha}$	756 ± 75^c	687 ± 66^c	644 ± 83^c	711 ± 57^c
NOSi	627 ± 82^c	702 ± 73^c	761 ± 70^c	658 ± 74^c
PGE_2-9-Ki	79 ± 15^b	87 ± 17^b	80 ± 20^b	95 ± 15^b
ARi	68 ± 17^b	79 ± 18^b	84 ± 16^b	77 ± 17^b
PGE_2 (pg/mg protein)				
Medium-alone	362 ± 75^a	125 ± 24^d	42 ± 16^c	346 ± 69^a
NOd	661 ± 94^b	613 ± 85^b	594 ± 88^b	655 ± 98^b
NOSi	59 ± 16^c	65 ± 12^c	39 ± 12^c	58 ± 14^c
PGE_2-9-Ki	634 ± 83^b	669 ± 91^b	608 ± 98^b	654 ± 91^b
ARi	355 ± 61^a	132 ± 32^d	54 ± 19^b	347 ± 86^a
$PGF_{2\alpha}$ (pg/mg protein)				
Medium-alone	215 ± 18^a	363 ± 42^d	684 ± 66^c	201 ± 22^a
NOd	116 ± 9^b	123 ± 12^b	108 ± 13^b	131 ± 22^b
NOSi	616 ± 74^c	532 ± 66^c	725 ± 83^c	620 ± 58^c
PGE_2-9-Ki	124 ± 13^b	136 ± 19^b	131 ± 17^b	122 ± 15^b
ARi	199 ± 22^a	401 ± 50^d	711 ± 59^c	215 ± 19^a

Means $\pm$ SD; n = 5 each; groups with different letters are significantly different ($P < 0.01$).

rats. Nitric oxide may also be involved in the regulation of sexual behaviour in male mice, since mice with disruption of neuronal NOS exhibit inappropriate sexual behaviour and increased aggressiveness (26). In amphibians, NO has been studied only in the urodele crested newt *T. carnifex*: during courtship, brain NO is an internal cellular stimulus in the male, while no function has been described to NO in the female (10).

The present data show that NO plays a role at the level of the CNS in another species of amphibian, the anuran water frog, *R. esculenta*, during reproductive behaviour. In fact, the lowest brain NOS activity was found in amplexing ovulatory females, while this brain enzymatic activity did not differ between amplexing and unamplexing males. This pattern is the opposite of that found in the above reported newt, where NO apparently regulates only male reproductive behaviour (10).

Our results clarify the possible mechanism by which NO exerts its role in the female brain during reproductive behaviour. We hypothesize that female amplexus requires a low NOS activity; this decreased enzymatic activity disinhibits PGE_2–9-K with the consequent production of $PGF_{2\alpha}$ which, in turn, increases the 17β-oestradiol levels throughout the activation of aromarase; this steroid favours female amplexus. This hypothesis is supported by the measured hormone plasma concentrations and by the *in-vitro* hormone brain basal releases. Amplexing-ovulatory females released the highest amounts of $PGF_{2\alpha}$ and 17β-oestradiol, and the lowest amounts of testosterone and PGE_2. The above-reported mechanism is also supported by the basal pattern of *in-vitro* enzyme activities, since PGE_2–9-K and aromatase showed the highest, and NOS the lowest, activities in the brain of amplexing-ovulatory females. *In-vitro* experiments demonstrate that, in the brain, NO inhibits the activity of PGE_2–9-K and aromatase with a consequent decrease of $PGF_{2\alpha}$ and 17β-oestradiol releases. On the other hand, NOS inhibitor enhanced these two enzyme activities with the consequent increase of these two hormones. In addition, $PGF_{2\alpha}$ increased and PGE_2–9K inhibitor decreased 17β-oestradiol release *in vitro*.

The present data regarding 17β-oestradiol plasma concentrations are similar to those found in two populations of *R. esculenta* during reproduction (27), where these authors reported that amplexing females have higher 17β-oestradiol concentrations than unamplexing females.

A similar cellular mechanism, here proposed for female amplexus, was recently proposed by us (28) for the short captivity stress in the same population of *R. esculenta*. We reported that this kind of stress enhances the circulating $PGF_{2\alpha}$, activating the PGE_2–9-K in the brain; the $PGF_{2\alpha}$, in turn, activates the gonad and interrenal aromatase, increasing the plasmatic 17β-oestradiol, which represents the first hormonal response to short captivity stress.

As regards the male, the mechanism proposed for the female cannot be valid. In fact, NOS and PGE_2–9-K do not seem to be involved in male amplexus, since these two enzyme

TABLE 4. *In-Vitro* Effects Of NO Donor (NOd), $PGF_{2\alpha}$, NOS Inhibitor (NOSi), PGE_2-9-Ketoreductase Inhibitor (PGE_2-9-Ki), and Aromatase Inhibitor (ARi) On Basal Activity Of NOS (Conversion Of [^{3}H]L-Arginine Into [^{3}H]L-Citrulline), PGE_2-9-Ketoreductase (Conversion Of [^{3}H]PGE_2 Into [^{3}H]$PGF_{2\alpha}$), and Aromatase (Conversion Of [^{3}H]Testosterone Into [^{3}H]17β-Oestradiol) By the Brain Of Male *Rana esculenta*, During Amplexus.

	Unamplexing	Amplexing with preovulatory female	Amplexing with ovulatory female
NO synthase			
[^{3}H]-Citrulline (d.p.m./mg protein)			
Medium-alone	15 841 ± 2323[a]	14 915 ± 2374[a]	17 642 ± 2036[a]
NOd	16 325 ± 2411[a]	17 524 ± 2365[a]	18 021 ± 2145[a]
$PGF_{2\alpha}$	15 368 ± 2288[a]	13 587 ± 1966[a]	16 521 ± 1856[a]
NOSi	432 ± 61[b]	521 ± 54[b]	627 ± 78[b]
PGE_2-9-Ki	14 028 ± 2308[a]	15 637 ± 2285[a]	15 597 ± 1854[a]
ARi	17 020 ± 2412[a]	16 483 ± 2435[a]	16 664 ± 2365[a]
PGE_2-9-ketoreductase			
[^{3}H]$PGF_{2\alpha}$ (d.p.m./mg protein)			
Medium-alone	10 279 ± 1304[a]	13 381 ± 1142[a]	12 432 ± 1024[a]
NOd	12 592 ± 1224[a]	10 325 ± 985[a]	13 055 ± 1138[a]
$PGF_{2\alpha}$	11 037 ± 1511[a]	11 731 ± 1005[a]	11 586 ± 1085[a]
NOSi	10 784 ± 1064[a]	9249 ± 1204[a]	12 154 ± 965[a]
PGE_2-9-Ki	789 ± 165[b]	892 ± 258[b]	627 ± 146[b]
ARi	11 238 ± 1038[a]	11 469 ± 1157[a]	9388 ± 1234[a]
Aromatase			
[^{3}H]17β-oestradiol (d.p.m./mg protein)			
Medium-alone	19 429 ± 1635[a]	6423 ± 732[c]	904 ± 185[d]
NOd	18 084 ± 1846[a]	7125 ± 815[c]	743 ± 162[d]
$PGF_{2\alpha}$	19 684 ± 1628[a]	6256 ± 902[c]	637 ± 135[d]
NOSi	18 412 ± 1776[a]	5869 ± 759[c]	917 ± 179[d]
PGE_2-9-Ki	20 563 ± 1671[a]	6758 ± 624[c]	811 ± 138[d]
ARi	354 ± 65[b]	325 ± 82[b]	381 ± 77[b]

Means ± SD; n = 5 each; groups with different letters are significantly different ($P < 0.01$).

TABLE 5. *In-Vitro* Effects Of NO Donor (NOd), $PGF_{2\alpha}$, NOS Inhibitor (NOSi), PGE_2-9-Ketoreductase Inhibitor (PGE_2-9-Ki), and Aromatase Inhibitor (ARi) On Basal Activity Of NOS (Conversion Of [^{3}H]L-Arginine Into [^{3}H]L-Citrulline), PGE_2-9-Ketoreductase (Conversion Of [^{3}H]PGE_2 Into [^{3}H]$PGF_{2\alpha}$), and Aromatase (Conversion Of [^{3}H]Testosterone Into [^{3}H]17β-Oestradiol) By the Brain Of Female *Rana esculenta*, During Amplexus.

	Unamplexing preovulatory	Amplexing preovulatory	Amplexing ovulatory	Unamplexing postovulatory
NO synthase				
[^{3}H]-Citrulline (d.p.m./mg protein)				
Medium-alone	13 456 ± 1532[a]	6842 ± 925[c]	1215 ± 120[d]	11 354 ± 2156[a]
NOd	12 689 ± 1603[a]	7421 ± 834[c]	1093 ± 151[d]	12 687 ± 1672[a]
$PGF_{2\alpha}$	15 236 ± 1845[a]	6218 ± 759[c]	1302 ± 183[d]	14 368 ± 1934[a]
NOSi	567 ± 84[b]	564 ± 96[b]	428 ± 74[b]	435 ± 91[b]
PGE_2-9-Ki	14 032 ± 1732[a]	6984 ± 855[c]	1402 ± 231[d]	11 567 ± 2035[a]
ARi	13 915 ± 2018[a]	6139 ± 912[c]	1347 ± 189[d]	12 003 ± 1759[a]
PGE_2-9-ketoreductase				
[^{3}H]$PGF_{2\alpha}$ (d.p.m./mg protein)				
Medium-alone	9236 ± 754[a]	20 354 ± 1603[e]	31 457 ± 2345[e]	11 125 ± 861[a]
NOd	3369 ± 235[b]	5632 ± 531[f]	6002 ± 512[f]	3562 ± 286[b]
$PGF_{2\alpha}$	10 035 ± 811[a]	22 905 ± 1567[e]	32 186 ± 2408[e]	10 308 ± 905[a]
NOSi	30 125 ± 2158[c]	35 294 ± 2671[c]	33 429 ± 2815[c]	32 741 ± 2654[c]
PGE_2-9-Ki	1127 ± 95[d]	1039 ± 84[d]	1168 ± 97[d]	984 ± 99[d]
ARi	8956 ± 937[a]	21 451 ± 1657[e]	33 354 ± 2772[c]	11 318 ± 920[a]
Aromatase				
[^{3}H]17β-oestradiol (d.p.m./mg protein)				
Medium-alone	12 325 ± 2104[a]	27 236 ± 3012[e]	42 354 ± 6245[c]	14 068 ± 2225[a]
NOd	2645 ± 311[b]	2836 ± 399[b]	2364 ± 416[b]	2185 ± 524[b]
$PGF_{2\alpha}$	38 421 ± 5212[c]	37 687 ± 4860[c]	46 384 ± 7052[c]	39 058 ± 5047[c]
NOSi	40 215 ± 4621[c]	38 624 ± 5274[c]	43 567 ± 4676[c]	37 110 ± 5312[c]
PGE_2-9-Ki	2354 ± 445[b]	2569 ± 484[b]	2813 ± 531[b]	2587 ± 468[b]
ARi	754 ± 101[d]	823 ± 93[d]	864 ± 112[d]	912 ± 90[d]

Means ± SD; n = 5 each; groups with different letters are significantly different ($P < 0.01$).

activities showed no change in amplexing and unamplexing males, similarly to PGE_2, and $PGF_{2\alpha}$, which do not change either. On the other hand, brain aromatase could be involved in the regulation of male amplexus, since its activity was lowest in males amplexing with ovulatory female, and that testosterone plasma concentrations and *in-vitro* brain basal release were highest in the same males.

In this context, it should be recalled that testosterone plasma concentrations of amphibian males engaged in sexual behaviour (chorusing, amplexus, courtship) show contrasting patterns among the species studied up to date (6, 8, 29–34).

In conclusion, a possible brain cellular mechanism for the amplexus regulation of male and female water frog, *R. esculenta*, is schematized in Fig. 2. In the male brain, external (male clasping?) and/or internal stimuli inhibit NOS activity, decreasing NO and activating PGE_2–9-K; the consequent $PGF_{2\alpha}$ increase enhances 17β-oestradiol throughout the activation of AR; this steroid leads to female amplexus.

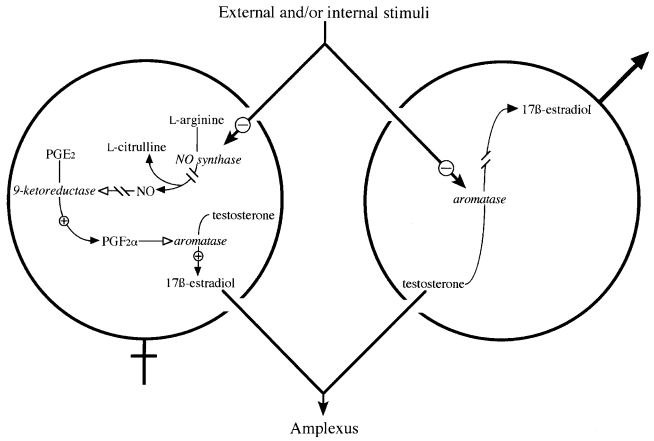

FIG. 2. Proposed schema of the hormonal and cellular brain mechanisms regulating the amplexus of male and female *Rana esculenta*.

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References

1 Nelson RJ. *An Introduction to Behavioral Endocrinology.* Sunderland, MA: Sinauer Associates Inc. Publishers, 1995.
2 Callard GV. Androgen and estrogen actions in the vertebrate brain. *Am Zool* 1983; **23:** 607–620.
3 Moore FL. Differential effects of testosterone plus dihydrotestosterone on male courtship of castrated newts, *Taricha granulosa. Horm Behav* 1978; **11:** 202–208.
4 Andreoletti GE, Malacarne G, Vellano C. Androgen control of male sex behavior in the crested newt (*Triturus cristatus carnifex* Laur.): castration and sex steroid administration. *Horm Behav* 1983; **17:** 103–110.
5 Deviche P, Moore F. Steroidal control of sexual behavior in the rough-skinned newt (*Taricha granulosa*): effects of testosterone, estradiol, and dihydrotestosterone. *Horm Behav* 1988; **22:** 26–34.
6 Orchinik M, Licht P, Crews D. Plasma steroid concentrations change in response to sexual behavior in *Bufo marinus. Horm Behav* 1988; **22:** 338–350.
7 Townsend DS, Palmer B, Guillette LJ Jr. The lack of influence of exogenous testosterone on male parental behavior in neotropical frog (*Eleutherodactylus*): a field experiment. *Horm Behav* 1991; **25:** 313–322.
8 Zerani M, Amabili F, Gobbetti A. Plasma testosterone, and 17β-estradiol concentrations, and aromatase activity during the courtship in male *Triturus carnifex*. *Horm Behav* 1992; **26:** 56–61.
9 Gobbetti A, Zerani M. Prostaglandin E_2–9-ketoreductase and prostaglandin $F_{2\alpha}$ activate brain aromatase to induce courtship in the male crested newt, *Triturus carnifex. Horm Behav* 1995; **29:** 267–277.
10 Zerani M, Gobbetti A. NO sexual behaviour in newts. *Nature* 1996; **382:** 31.
11 Wells KD. The courtship of frogs. In: Taylor DH, Guttman SI, eds. *The Reproductive Biology of Amphibians,* New York: Plenum Press, 1977: 233–262.
12 Gobbetti A, Zerani M. Cellular mechanism of Substance P in the regulation of corticosteroid secretion by newt adrenal gland. *Biochem Biophys Res Commun* 1997; **233:** 395–400.
13 Burnett AL, Ricker DD, Chamness SL, Maguire MP, Crone JK, Bredt DS, Snyder SH, Chang TSK. Localization of nitric oxide synthase in the reproductive organs of the male rat. *Biol Reprod* 1995; **52:** 1–7.
14 Bush PA, Gonzalez NE, Griscavage JM, Ignarro LJ. Nitric oxide synthase from cerebellum catalyzes the formation of equimolar quantities of nitric oxide and citrulline from L-arginine. *Biochem Biophys Res Commun* 1992; **185:** 969–966.
15 Wingfield J, Farner DS. The determinations of five steroids in avian plasma by radioimmunoassay and competitive protein binding. *Steroids* 1975; **26:** 311–327.
16 Duncan DB. Multiple range and multiple F test. *Biometrics* 1955; **11:** 1–42.
17 Sokal RR, Rohlf FJ. *Biometry. The Principles and Practice of Statistics in Biological Research, 2 edn.* San Francisco: WH Freeman, 1981.
18 Moncada S, Palmer RMJ, Higgs EA. Nitric oxide: physiology, pathophysiology, and pharmacology. *Pharmacol Rev* 1991; **43:** 109–142.
19 McCall T, Vallance P. Nitric oxide takes centre-stage with newly defined roles. *Trends Pharmacol Sci* 1992; **13:** 1–6.
20 Bredt DS, Hwang PM, Snyder SH. Localization of nitric oxide synthase indicating a neural role for nitric oxide. *Nature* 1990; **347:** 768–770.
21 Bhat GK, Mahesh VB, Lamar CA, Ping L, Aguan K, Brann DW. Histochemical localization of nitric oxide neurons in the hypothalamus: association with gonadotropin-releasing hormone neurons and co-localization with N-methyl-D-aspartate receptors. *Neuroendocrinol* 1995; **62:** 187–197.
22 Ceccatelli S, Ulthing AL, Zhang X, Gustafsson L, Villar M, Hokfelt T. Nitric oxide synthase in the rat anterior pituitary gland and the role of nitric oxide in regulation of luteinizing hormone secretion. *Proc Natl Acad Sci USA* 1993; **90:** 11292–11296.
23 Moretto M, Lopez FJ, Negro-Vilar A. Nitric oxide regulates luteinizing hormone-releasing hormone secretion. *Endocrinol* 1993; **133:** 2399–2402.
24 Rettori V, Belova NH, Dees WL, Nyberg CL, Gimeno M, McCann SM. Role of nitric oxide in the control of luteinizing hormone-releasing hormone release *in vivo* and *in vitro. Proc Natl Acad Sci USA* 1993; **90:** 10130–10134.
25 Mani SK, Allen JMC, Rettori V, McCann SM, O'Malley BW, Clark JH. Nitric oxide mediates sexual behavior in female rats. *Proc Natl Acad Sci* 1994; **91:** 6468–6472.
26 Nelson RJ, Demas GE, Huang PL, Fishman MC, Dawson VL, Dawson TM, Snyder SH. Behavioural abnomalities in male mice lacking neuronal nitric oxide synthase. *Nature* 1995; **378:** 383–386.
27 Carnevali O, Mosconi G, Sabbieti MG, Murri CA, Villani P, Polzonetti-Magni AM. Some aspects of the reproductive biology of *Rana esculenta* at sea-level and montane habitats. *Amphibia-Reptilia* 1993; **14:** 381–388.
28 Gobbetti A, Zerani M. Possible mechanism for the first response to short captivity stress in the water frog, *Rana esculenta. J Endocrinol* 1996; **148:** 233–239.
29 Siboulet R. Seasonal variations of plasma testosterone and dihydrotestosterone in the mauritanian toad. *Gen Comp Endocrinol* 1981; **43:** 71–75.
30 Licht P, McCreery BR, Barnes R, Pang R. Seasonal and stress related changes in plasma gonadotropins, sex steroids, and corticosterone in the bullfrog, *Rana catesbeiana*. *Gen Comp Endocrinol* 1983; **50:** 124–145.
31 Mendonça MT, Licht P, Ryan MG, Barnes R. Changes in hormone levels in relation to breeding behavior in male bullfrogs (*Rana esculenta*)

at the individual and population levels. *Gen Comp Endocrinol* 1985; **58:** 270–279.

32 Itoh M, Ishii S. Changes in plasma levels of gonadotropins and sex steroids in the toad, *Bufo japonicus*, in association with behavior during breeding season. *Gen Comp Endocrinol* 1990; **80:** 451–464.

33 Houck LD, Mendonça MT, Lynch TK, Scott DE. Courtship behaviour and plasma levels of androgens and corticosterone in male marbled salamanders, *Ambystoma opacum* (Ambystomidae). *Gen Comp Endocrinol* 1996; **104:** 243–252.

34 Harvey LA, Propper CR, Woodley SK, Moore MC. Reproductive endocrinology of the explosively breeding desert spadefoot toad, *Scaphiopus couchii. Gen Comp Endocrinol* 1997; **105:** 102–113.