Kisspeptin regulates steroidogenesis and spermiation in anuran amphibian

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Abstract

Kisspeptin (Kp) system has a recognized role in the control of gonadotropic axis, at multiple levels. Recently, a major focus of research has been to assess any direct activity of this system on testis physiology. Using the amphibian anuran, *Pelophylax esculentus*, as animal model, we demonstrate – for the first time in non-mammalian vertebrate – that testis expresses both Kiss-1 and Gpr54 proteins during the annual sexual cycle and that *ex vivo* 17B-estradiol (E₂, 10⁻⁶M) increases both proteins over control group. Since the interstitium is the main site of localization of both ligand and receptor, its possible involvement in the regulation of steroidogenesis has been evaluated by *ex vivo* treatment of testis pieces with increasing doses of Kp-10 (10⁻⁶–10⁻⁴M). Treatments have been carried out in February – when a new wave of spermatogenesis occurs – and affect the expression of key enzymes of steroidogenesis inducing opposite effects on testosterone and estradiol intratesticular levels. Morphological analysis of Kp-treated testes reveals higher number of tubules with spermatozoa detached from Sertoli cells than control group and the expression of connexin 43, the main junctional protein in testis, is deeply affected by the treatment. In spite of the effects on spermatozoa observed *ex vivo*, *in vivo* administration of Kp-10 has been unable to induce sperm release in cloacal fluid. In conclusion, we demonstrate Kp-10 effects on steroidogenesis with possible involvement in the balance between testosterone and estradiol levels, and report new Kp-10 activities on spermatozoa–Sertoli cell interaction.


Introduction

The gonadotropic axis is under the control of gonadotropin-releasing hormone (GnRH) whose release is upstream regulated by kisspeptins (Kps) (Messager et al. 2005). Kps are a group of peptides derived from Kiss1 gene (Kotani et al. 2001) able to activate Kiss1 receptor (GPR54, Thompson et al. 2004). The central Kp/GPR54 signaling system has been deeply characterized in mammals, especially as a potent trigger of GnRH secretion during puberty onset (Pinilla et al. 2012, Herbison 2016). Conversely, gene knockout studies have revealed that kiss/gpr54 signaling is not absolutely required for zebrafish reproduction in both sexes (Tang 2015), due to compensation mechanisms capable to stimulate the gonadotropic axis in the absence of Kiss1 signaling (Liu 2017).

However, in Gpr54 knockout mice, the re-expression of GPR54 exclusively in GnRH neurons reactivates the neuroendocrine axis, but does not completely restore gonadal functions, strongly suggesting that peripheral Kp/GPR54 signaling regulates gonadal physiology (León et al. 2016). Accordingly, as in the hypothalamus, in testis a similar Kp/GPR54 signaling system has been reported in primates, rodents and amphibians (Meccariello et al. 2014, Chianese et al. 2016) for review), becoming part of the intricate intratesticular network of regulators governing testis physiology (Pierantoni et al. 2002a). Although several studies have reported the expression of both ligand and receptor in the male reproductive organs of several vertebrates, humans included (Meccariello et al. 2014, Chianese et al. 2016, Wabab et al. 2016), the possible physiological role of intragonadal Kp signaling is still controversial (Mei et al. 2013, Meccariello et al. 2014, Chianese et al. 2016).

In testis, the interstitial compartment is mainly involved in testosterone production thanks to the contribution of Leydig cells (Chen et al. 2009) in which Kp has been localized (Anjum et al. 2012, Salehi et al. 2015). Studies carried out on the Leydig cell line MA-10 – that also expresses Gpr54 mRNA – do not reveal any effect of Kp-10 (Mei et al. 2013), the active
metabolite produced by the proteolytic cleavage of the largest precursor protein Kp-145 (Oakley et al. 2009). Unlike rodents, in rhesus monkey in vivo administration of Kp-10 stimulates testosterone production and exhibits synergistic activity with human chorionic gonadotropin (hCG) on steroid biosynthesis in acyltreated monkeys, thus suggesting a direct effect on testis (Irfan et al. 2014). Conversely, the in vitro Kp-10 treatment fails to have any effect (Tariq & Shabab 2016). In germ cell compartment, many traits of a Kp-dependent physiological autocrine/paracrine network are still elusive, especially in mammals, since the only clear evidence has been provided in spermatozoa (SPZ), with a suggested role of Kp in sperm motility and fertilization capacity (Pinto et al. 2012, Hsu et al. 2014).

Interestingly, studies in non-mammalian vertebrates have been devoted to clarify Kp role in both spermatogenesis and steroidogenesis (Ten-A-Sempere et al. 2012). In the pubertal teleost, Scomber japonicus, peripheral injection of Kp-15 accelerated spermatogenesis and induced sperm production (Selvaraj et al. 2013a,b). Accordingly, in the anuran amphibian Pelophylax esculentus, Kp role has been deeply investigated in testis. P. esculentus shows a very peculiar organization of testis, since germ cells at the same maturational stage are clustered in cysts – formed by Sertoli cell cytoplasmic protrusions – and develop during the annual sexual cycle (Rastogi et al. 1976, Pierantoni et al. 2002b). In particular, in March–April (breeding season), SPZ are released and spermatogonia (SPG) start proliferating after the winter stasis (December–February), when environmental and hormonal conditions are not advantageous to reproduction. Meiotic stages only appear in May–July (post-reproductive period), when spermatogenesis actively proceeds; lastly post-meiotic cells richly populate testis during resumption (September–November) (Rastogi et al. 1976, Pierantoni et al. 2002b). Interestingly, the use of this non-mammalian animal model has contributed to unravel important aspects of Kp-dependent intratesticular signaling. Notably, gpr54 mRNA was localized in the interstitial compartment and in proliferating germ cells and its expression was indicated to be estradiol-dependent (Chianese et al. 2013). Furthermore, Kp-10 was able to modulate the expression of estrogen receptor alpha (era) (Chianese et al. 2013), erb, proliferating cell nuclear antigen (pcna) and GnRH system – both ligands and receptors (Chianese et al. 2015), since the suggested idea of Kp as a testicular bioregulator of germ cell progression.

 Starting from this evidence, we localized both Kiss-1 ligand and Gpr54 receptor in frog testis during the annual sexual cycle. Then, at the onset of a new cycle of spermatogenesis (February), we investigated a possible role exerted by Kp-10 on steroidogenesis and evaluated the possibility that Kps may regulate the detachment of SPZ from Sertoli cells.

Materials and methods

Chemicals and antibodies

Kp-10 (Metastin 45-54 (H-YNWNSFGLRF-NH2)) of human origin, a Kp form with a potency similar to Xenopus-Kp-10 for bullfrog Gpr54 (Moon et al. 2009), was purchased from DBA Italia, Milan, Italy; Kp-234, a specific Gpr54 antagonist (Roseweir et al. 2009) was purchased from DBA Italy; 17B-estradiol (E2) was purchased from Sigma-Aldrich S.R.L.; ICI 182780, a high-affinity estrogen receptor antagonist, was purchased from AstraZeneca, UK; and buseralen, a GnRH agonist, was a gift from Dr J Sandow, Hoechst, Frankfurt, Germany.

For Western blot and immunohistochemistry the following antibodies were used: goat polyclonal anti-Kiss-1 antibody (raised against a peptide mapping at the C-terminus of Kiss-1 of human origin; sc-18134) and rabbit polyclonal anti-Gpr54 antibody (raised against amino acids 141–342 mapping within an internal region of Gpr54 of human origin sharing 78% similarity with P. esculentus Gpr54; sc-134499); both were purchased from Santa Cruz Biotechnology and were validated for Western blot and immunohistochemistry (present data, Supplementary Figs 1 and 2, see section on supplementary data given at the end of this article; Hsu et al. 2014, Ciaramella et al. 2016). Mouse monoclonal anti-α-tubulin (T6199) was purchased from Sigma-Aldrich.

Animal and tissue collection

Adult P. esculentus male frogs (n = 5) were collected monthly in the neighborhood of Naples (Italy) except in August. To minimize the stress, the animals were anesthetized in ethyl 3-aminobenzoate methanesulfonate salt, MS222 (Sigma-Aldrich) and immediately killed after capture. Testes were removed, rinsed in Krebs Ringer Buffer for amphibians (KRB, 68 mM NaCl, 1 mM KCl, 1.17 mM MgSO4, 1.2 mM KH2PO4, 25 mM NaHCO3, 2.5 mM CaCl2, 10 mg/L gelatin, pH 7.4) and processed for qPCR, Western blot and immunohistochemistry, or in vitro incubated with Kp-10 and E2 as reported below. Brain was also removed from frogs of February and processed for validation of antibody as positive control. Experiments were performed under the guidelines established in the National Institute of Health Guide for Care and Use of Laboratory Animals and were approved by the Italian Ministry of Education, University and Research.

Ex vivo incubation of testis

Experiment 1: Male frogs (n = 15) collected in February were used for treatment with E2. Testes were cut into halves and were incubated for 1 h in KRB alone (control group, C), in KRB/ E2 10−6M (E2 group), in KRB/ICI 182780, 10−5M for 30 min – preliminarily – then in KRB/E2 10−6M and ICI 182780 10−5M (El group). E2 and ICI 182780 doses and incubation times were chosen on the basis of previous studies in frogs (Cobellis et al. 1999). After the treatment, testes were stored at −80°C and then processed for protein extraction.

Experiment 2: Testes were removed from n. 60 animals collected in pre-reproductive period (February) and divided
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O−6. whose boundary as reference gene was previously (et al.; 2013, 2015). For each dose and time point, 4 testes were stored at −80°C and used for expression analysis; 4 halves of testes were fixed in Bouin’s fluid and processed for standard histological analysis; and 4 halves of testes were used for steroid dosage.

In vivo treatments and protein extraction from SPZ

Male frogs (n = 3 each treatment) collected in December were injected in the dorsal sac with 100 µL of KRB (control group, C); KRB/Kp-10 at 10−6 M (treated group, Kp-10); KRB/Kp-234 at 10−5 M and after 30 min with KRB/Kp-10 at 10−6 M (treated group, Kp-10 + A); KRB/GnRH agonist/buserelin at 10−5 M (treated group, bus), a well-known treatment capable to induce spermiation in frogs (Minucci et al. 1989). After 2 h from the injection, cloacal fluid was collected and analyzed under light microscopy (CTR500; Leica) for the detection of SPZ. Frogs were then sacrificed as previously described and testes were removed for histological analysis. Two positive controls were used: human and rat SPZ. Human semen samples were provided by one healthy volunteer donor and processed for SPZ isolation as previously described (Meccariello et al. 2008); rat SPZ were collected in PBS pH 7.4 from epididymis conveniently removed and cut into few pieces and centrifuged at 1000 g for 15 min at 4°C. Freshly collected SPZ from frog, rat and human were immediately processed for protein extraction as previously described (Meccariello et al. 2007, 2008).

Total RNA extraction and cDNA preparation

The extraction of total RNA from frog testis (n = 4) was performed using Trizol reagent (Life Technologies) following the manufacturer’s instructions. DNasel (10 U/sample) (Amersham Pharmacia Biotech, UK) treatment at 37°C for 30 min ensured the elimination of any genomic DNA contaminations. RNA purity and integrity were determined by spectrophotometer analyses at 260/280 nm and by electrophoresis. The reverse transcription of a total RNA pool was then carried out using 5 µg total RNA, 0.5 µg oligo dT18, 0.5 mM dNTP mix, 5 mM DTT, 1× first strand buffer (Life Technologies), 40 U RNase Out (Life Technologies), 200 U SuperScript-III RnaseH− Reverse Transcriptase (Life Technologies) in a final volume of 20 µL, following the manufacturer’s instructions. As negative control, total RNA not treated with reverse transcriptase was used.

Quantitative real-time RT-PCR (qPCR)

In brief, cDNA was diluted 1:5 in water and subjected to qPCR using 10 pmol of oligonucleotide primers designed on P. esculentus nucleotide sequence as previously described (Chianese et al. 2014). Primers were designed on Rana rugosa nucleotide were used to amplify androgen receptor (ar) sequence: S: 5′-ccagaggtgagcgcttt-3′ and AS: 5′-agcagaggtctcctcataa-3′, predicted amplification size 156bp. The cloning of ar partial cDNA was carried out in pGEM-T Easy Vector (Promega), transforming DH5a high efficiency competent cells and sequencing the insert on both strands by Primm Sequence Service (Primm srl, Naples Italy). P. esculentus AR sequence revealed 96% and 98% nucleotide and amino acid identity, respectively, vs R. rugosa androgen receptor sequences. T (°C) annealing for all primers was 60°C. A negative control in which cDNA was replaced by water was also included. Signals were normalized toward the reference gene fp1 whose boundary as reference gene was previously reported (Chianese et al. 2011, 2012). Assay included a melting curve analysis for which all samples displayed single peaks for each primer pairs. Data were then reported as mean fold change ± s.d. over the minimal value arbitrarily assigned to control group.

Protein extraction and Western blot analysis

Total proteins were extracted from testes and brain (n = 5), from E2 or E2/ICI-treated testes, and then processed for Western blot analysis as previously reported (Ciaramella et al. 2016). Anti-Kiss-1 and anti-Gpr54 antibodies were both diluted 1:500 and validated as shown in Supplementary Figs 1 and 2; mouse monoclonal anti-α-tubulin diluted 1:15,000 was used to quantify protein content. Western blot signals were scanned and protein levels were plotted as quantitative densitometry analysis of signals. Data were expressed as mean of Kiss-1 or Gpr54/tub ratio fold increase ± s.d.

Immunohistochemistry analysis

Frog testes (n = 5) collected in February, July and November were fixed in Bouin’s fluid for histological observation and embedded in paraffin using standard procedures. Thus, sections (5 µm) were used to analyze Kiss-1 and Gpr54 distribution in testis. Endogenous peroxidase activity was inhibited in methanol and 0.3% H2O2, for 20 min. To prevent background staining, sections were washed in PBS 0.01 M, pH 7.1, and incubated in PBS containing 3% BSA and 1:30 goat serum (PBS/BSA/serum, PBS-BS) in a moist chamber at room temperature. Slides were incubated with the previously described anti-Kiss-1/GPR54 antibodies diluted 1:100 in PBS-BS overnight at 4°C in a moist chamber. Another section on the same slide was incubated only with PBS-BS as an immunohistochemical control. After three washes in PBS and 0.3% Triton X-100, sections were incubated with biotinylated rabbit anti-goat IgG (E0466) or goat anti-rabbit IgG (E0432), for Kiss-1 and Gpr54 detection, respectively (DAKO), and diluted 1:100 in PBS-BS for 1 h at room temperature. Subsequently, the sections were incubated with Vectastain ABC Reagents (Vector Laboratories, Inc., Burlingame, CA, USA) for 30 min at room temperature. Immunoreaction products were detected with 3,3′-diaminobenzidine tetrahydrochloride.
(Sigma-Aldrich) in Tris–HCl 0.05 mM buffer according to the manufacturer’s protocol.

To check the specificity of the immunoreactions, controls were treated omitting incubation with primary or secondary antiserum or incubating section with PBS-BS alone during the procedures. For antiKISS-1, preadsorption of antiserum with the corresponding epitope was also carried out (details in Supplementary Figs 1 and 2). The sections were observed under a light microscope, and images were captured using a high-resolution digital camera (DC300F; Leica).

**Histological analysis and count of tubules containing detached and released SPZ**

Testes from Kp-10 dose–response experiments and in vivo treatment were fixed in Bouin’s fluid and embedded in paraffin following standard procedures, and slides (5 µm) were then stained with standard hematoxylin–eosin and observed under a light microscope. Therefore, one section/treatment/time point/animal approximately every 50 µm, except 10⁻⁹ M Kp-10 treatment, was analyzed and the following elements were counted: the number of total cross sections of seminiferous tubules; the number of cross sections of seminiferous tubules containing only SPZ detached from Sertoli cells; and the number of cross sections of seminiferous tubules containing SPZ completely released in the lumen. Data were reported as the % of tubules with released SPZ or tubules with detached SPZ over the total of tubules counted for animals ± s.d.

**Steroid detection**

**Steroid extraction from testis**

Testes (n = 5) were homogenized in 70% methanol and extracted with 2 × 7 mL diethyl ether. After drying, each extract was dissolved in PBS (0.1 M, pH 7)–0.2% gelatine for hormone determination (Pierantoni et al. 1984).

**EIA assay**

Intratesticular E₂ and testosterone (TS) levels were quantified using commercially available EIA kits according to the directions provided by the manufacturer (Cayman and DRG Diagnostics GmbH, Germany, respectively). The detection limits of kits were approximately 20 pg/mL and 83 pg/mL, for E₂ and TS, respectively. The intra- and inter-assay coefficients of variation, determined at multiple points, were 7.4% and 6.3%, and 5.7% and 1.6%, for E₂ and TS, respectively. All determinations were made in triplicate from each sample, expressed in pg steroid/mg/weight of testes. Then, for each experimental group we calculated the ratio E₂/TS and normalized each ratio vs control.

**Statistical analysis**

One-way ANOVA followed by Duncan’s test for multi-group comparison or Student’s t test were used where appropriate.

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**Results**

**Frog testis expresses both Kiss-1 and Gpr54**

With the aim to shed light on the role of Kp system in frog spermatogenesis we analyzed the expression of Kiss-1 and Gpr54 in testis during the annual sexual cycle by Western blot. Kiss-1 signal shows high levels in March–April (P < 0.01) in conjunction with a new spermatogenic wave (Rastogi et al. 1976), and then it decreases in summer to increase again during the resumption (September–November) and the winter stasis (December–February) (P < 0.01) (Fig. 1A). The profile of Gpr54 shows clear peaks of expression in April, during the reproductive period, when SPZ are released (P < 0.01), in June–October – with the appearance of...
meiotic and early post-meiotic stages – and in February again – when spermatogonia proliferation resumes. Lowest expression rates have been detected during the winter stasis (Rastogi et al. 1976, Pierantoni et al. 2002b) (Fig. 1B).

By immunohistochemistry Kiss-1 and Gpr54 proteins were localized in frog testis of February, July and November animals (Fig. 2). The analysis revealed the presence of Kiss-1 in the interstitial compartment in all the observed periods (Fig. 2A, B, C, D, E and F), whereas in November a weak immunolabeling was also detected in germinal compartment especially in cysts of secondary spermatocytes (IISPC) (Fig. 2E). In all the analyzed periods, Gpr54 protein was mainly detected in the interstitium (Fig. 2G, H, I, J, K and L). In February, Gpr54 was also localized in peritubular myoid cells, Sertoli cells surrounding actively proliferating primary spermatogonia (ISPG), ISPG and cysts of secondary spermatogonia (IISPG) (Fig. 2G and H). In July, Gpr54 was localized in cysts of primary spermatocytes (ISPC) and IISPC (Fig. 2I and J), whereas in November the immunolabeling was detected in post-meiotic germ cells such as spermatids (SPT) and SPZ (Fig. 2K and L). Immunohistochemical signals completely disappeared in the control sections obtained by omitting one step of reaction to demonstrate the specificity of the immunoreactivity (Fig. 2B, D, F, H, J and L insets).

To corroborate Gpr54 immunolocalization in post-meiotic cells, we carried out a Western blot analysis on proteins extracted from frog and rat SPZ: the latter used as positive control. Gpr54 signal appeared in both lanes, even if with greater intensity in rat SPZ protein extracts (Fig. 2M).

Further details concerning antisera validation for both Western blot and immunohistochemistry are shown in Supplementary Figs 1 and 2.

**Figure 2** Sections of *P. esculentus* testis collected in February (A, B, G, H), July (C, D, I, J) and November (E, F, K, L) analyzed by immunocytochemistry for Kiss-1 (A, B, C, D, E and F) or Gpr54 (G, H, I, J, K and L). Kiss-1 signal almost exclusively localized in the interstitial compartment (i). Instead, Gpr54 also followed the progression of spermatogenesis since the signal marked ISPG and IISPG cysts (*), but also Sertoli (s) and peritubular myoid cells (m) (February G, H), cysts of ISPC and IISPC (Δ) (July I, J), and post-meiotic cells such as SPT and SPZ (▲) (November K, L). The specificity of the immunolocalization was carried out by omitting one step of reaction (Kiss-1: B, D, F insets; Gpr54: H, J, L insets). Scale bars = 20 µm. Expression of Gpr54 protein in frog and rat SPZ analyzed by Western blot (M).
E₂ modulates the expression of both Kiss-1 and Gpr54 in frog testis

A possible estrogen regulation of both Kiss-1 and Gpr54 protein expression was assayed in frog testis by in vitro treatment with E₂. Interestingly, such a treatment increased the expression levels of both ligand and receptor vs control (P < 0.01). A combined treatment with the estrogen receptor antagonist ICI 182780 reduced Kiss-1 and Gpr54 levels to control values (Fig. 3).

Kp effects on key enzymes of steroidogenesis

To give insight in the direct functional role of Kp system in basic testicular activities we evaluated in vitro the possible effect of increasing doses of Kp-10 on the expression of 3β-HSD, cyp17 and cyp19, key enzymes in steroidogenesis.

After 1 h treatment (Fig. 4A) the expression of 3β-HSD significantly decreased at all Kp-10 doses compared to the control (P < 0.01), whereas the expression of cyp17 and cyp19 significantly increased with major effects at high Kp-10 doses (P < 0.01). After 4 h treatment (Fig. 4B) 3β-HSD expression was also strongly stimulated by Kp-10. Differently from 1 h observations, the expression of cyp17 only increased at high Kp-10 doses (10⁻⁷ and 10⁻⁶ M) (P < 0.01). Cyp19 expression increased at all Kp-10 doses with a major effect at 10⁻⁷ M (P < 0.01).

Default expression rate of 3β-HSD, cyp17 and cyp19 throughout the treatment has been detected comparing the expression rate of the selected genes between 1 and 4 h KRB-incubated control group (Fig. 4C). The expression rate of 3β-HSD and cyp17 in 1 h control group was significantly higher than that in 4 h control group (P < 0.01); conversely, the expression rate of cyp19 in 1 h control group was significantly lower than that in 4 h control group (P < 0.01).

Kisspeptin regulates the expression of ar and the intratesticular levels of testosterone and E₂

Possible effect of Kp-10 treatment has been analyzed on ar expression (Fig. 5A). After 1 h treatment ar expression significantly decreased at all Kp-10 doses (P < 0.01); conversely, 4 h treatment dose-dependently increased ar levels compared to the control (P < 0.01). Default expression rate of ar during the treatment was significantly higher in 1 h control group than in 4 h control group (P < 0.01).

In support to these data, we measured intratesticular levels of both testosterone and E₂ after Kp-10 in vitro treatment by EIA method (Fig. 5B). After 1 h treatment, Kp-10 significantly decreased testosterone levels compared to the control at all tested Kp-10 doses (P < 0.01 vs control), with major effects at lower doses (P < 0.01 10⁻⁹ and 10⁻⁸ M vs 10⁻⁷ and 10⁻⁶ M Kp-10); on the other hand, E₂ levels increased at 10⁻⁹ M Kp-10 (P < 0.05 vs control) and then decreased at higher Kp-10 doses (P < 0.05 vs control). Conversely, 4 h treatment failed to have any significant effect on both testosterone and E₂. The ratios between E₂ and testosterone at 1 h normalized vs control group were as follows: control group 1; Kp-10 (10⁻⁹ M) 3.21; Kp-10 (10⁻⁸ M) 1.19; Kp-10 (10⁻⁷ M) 0.46; Kp-10 (10⁻⁶ M) 0.65.
Possible role of Kp-10 in spermiation

In February, at the end of the winter stasis, frog testis is populated by ISPG that start to proliferate forming germinal cysts and by SPZ that have to be released during the breeding season (Rastogi et al. 1976). Usually, during spermiation, SPZ are detached from Sertoli cells and concentrate in the lumen of seminiferous tubules. In testis treated with increasing doses of Kp-10 for 1 and 4 h (Fig. 6A1–A4 and B1–B4) a massive detachment of SPZ from Sertoli cells was observed, but most of them were just detached from Sertoli cells and do not concentrate in the middle lumen of seminiferous tubules. Thus, we counted the number of tubules with released SPZ or with detached SPZ over the total tubules counted for each section, after 1 and 4 h treatment (Fig. 6A5 and B5). While in the control sections the lumen of seminiferous tubule cross sections appeared empty (Fig. 6A1 and B1) being characterized by ISPG and SPZ, after 1 h treatment $10^{-8}$, $10^{-7}$ and $10^{-6}$ M Kp-10 doses strongly increased the number of tubules with detached SPZ (Fig. 6A5) that appeared untidily distributed in the seminiferous tubule ($P < 0.01$ vs control) (Fig. 6A2–A4). In addition, $10^{-7}$ M Kp-10 also induced the formation of a bolus of SPZ ($P < 0.01$ vs control) in parallel to the increase of tubules containing detached SPZ ($P < 0.01$ vs control) (Fig. 6A3).

Similarly, after 4 h treatment all Kp-10 doses significantly induced the detachment of SPZ (Fig. 6B2–B5) compared to the control section (Fig. 6B1), but in $10^{-7}$ and $10^{-6}$ M Kp-10 treated testis many tubules appeared enriched with SPZ ready to be released ($P < 0.01$ vs control) (Fig. 6B3, B4 and B5), in parallel to a significant increase of B tubules (Fig. 6B5, $P < 0.01$ vs control).

Kp-10 treatment also affected the expression of cx43, reported to have a prominent role to maintain the...
connections between Sertoli and germ cells – including spermatozoa – in *P. esculentus* (Palmiero et al. 2003). After 1 and 4 h treatment, $10^{-8}$ M Kp-10 dose significantly decreased *cx43* compared to the control ($P<0.01$), whereas both $10^{-7}$ and $10^{-6}$ M Kp-10 increased it ($P<0.01$) (Fig. 6A6 and B6).

To ascertain any role of Kp on SPZ, during the winter stasis (December) Kp-10 effects were observed *in vivo* in parallel to those of buserelin, a well-known GnRH agonist capable to induce spermatiation (Minucci et al. 1989). While SPZ were collected in cloacal fluid of buserelin-injected animals, no SPZ were collected from the cloacal fluid of Kp-10-injected animals (Fig. 7). Morphological analysis of the testes of control group showed empty tubules with prevalence of quiescent ISPG and SPZ ready to be released under suitable hormonal and environmental conditions (Fig. 7A1). In buserelin-treated animals most tubules were devoid of SPZ attached to Sertoli cells, and the number of tubules containing released SPZ in the middle of the lumen was higher than that in control group (Fig. 7A4 and A5) (buserelin vs control $P<0.01$); by contrast, in Kp-10-treated frogs most tubules showed detached SPZ (Fig. 7A2 and A5) ($P<0.01$ vs control group). Kp-10 effect was completely counteracted by the combined treatment with Kp-234 (Kp-10 + A), suggesting a direct effect on Gpr54 receptor (Fig. 7A3 and A5).

Figure 6: Histological analysis of Kp-10-treated frog testis of February, after staining with standard hematoxylin–eosin. Incubation time were 1 h (A1–A4) and 4 h (B1–B4). Representative histological analysis of control testis (A1, B1), $10^{-8}$ M Kp-10 (A2, B2), $10^{-7}$ M Kp-10 (A3, B3) and $10^{-6}$ M Kp-10 (A4, B4) incubations. White arrows, fully released SPZ; black arrows, detached SPZ. Count of tubules with released SPZ (white bars) or detached SPZ (black bars) over the total number of tubules/section/animal at 1 h (A5) and 4 h treatment (B5). Control testis incubated with KRB (C); −8/−6, Kp-10 concentrations (−logM). Data are reported as the % of tubules containing released (detached) SPZ over the total of tubules counted for animals ± s.d. (n=3–4); at each treatment dose, asterisks (letters) indicate the statistically significant differences ($P<0.01$) among tubules containing released SPZ (detached SPZ) vs control. Direct actions of Kp-10 on the expression of *cx43* in frog testis of February after 1 h (A6) and 4 h treatment (B6) by qPCR. Quantitative PCR data, normalized against *fp1*, are reported as normalized fold expression (nfe) ± s.d. over the value one arbitrarily assigned to the sample C. Different letters indicate statistically significant differences ($P<0.01$). Data are representative of three separate experiments at least ($n=4$).
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April, when the expression rate of both proteins is high, throughout the annual cycle the expression peaks of Kiss-1 precede those of Gpr54, with opposite expression profile detected in post-reproductive period (May–July) and in late resumption – early winter stasis (November–December). Nevertheless, Kiss-1 and Gpr54 expression profiles parallel the fluctuations of steroid hormones that characterize the sexual cycle of frogs (Fasano et al. 1993, Cobellis et al. 1997, Polzonetti et al. 1998). In fact, overlapping profiles between Kiss-1 and testosterone levels occur throughout the annual cycle with highest levels registered in reproductive period and resumption, when testosterone drives sperm release and post-meiotic stages maturation, respectively (Rastogi et al. 1976). The expression profile of Gpr54, instead, parallels the biosynthesis of estradiol, except in the breeding season (April) when its expression peak parallels the increase of testosterone levels that supports sperm release. The high expression levels of both Kiss-1 and Gpr54 at the end of the winter stasis (February), a critical period for the onset of a new reproductive cycle under the hormonal control of estradiol that recruits quiescent spermatogonia for proliferation in cooperation with intratesticular activity of GnRH (Cobellis et al. 2002, 2003) and Kp-10 (Chianese et al. 2015), deserve particular attention. These observations corroborate the evidence that in frog, in the same timeframe, Kp-10 has the ability to promote the transcription of its own receptor similar to estradiol (Chianese et al. 2013). Consistently, in vitro treatment of frog testis collected in February just revealed estradiol-dependent expression of both Kiss1 and Gpr54 proteins, according to previous observations concerning the expression rate of gpr54 mRNA in frog (Chianese et al. 2013) and data from mammalian brain (Pinilla et al. 2012, Herbison 2016). However, estradiol effects were fully counteracted by ICI 182780, thus involvement of nuclear estrogen receptors may be suggested.

At present, most Kp functions in reproduction, including puberty onset and the switch on/off of steroid biosynthesis, have been supposed to be the direct consequence of its hypothalamic activity on GnRH-secreting neurons (Pinilla et al. 2012). Nevertheless, the localizations of Kiss1 and Gpr54 proteins suggest autocrine and paracrine activities inside the testis due to intratesticular Kp production. In fact, interstitial Leydig cells are the main producers of Kiss1 throughout the annual sexual cycle, as reported in mouse and primates (Anjum et al. 2012, Mei et al. 2013, Hsu et al. 2014, Salehi et al. 2015, Wang et al. 2015, Irfan et al. 2016). In parallel, immunoreactivity for Gpr54 has been detected in interstitial compartment, confirming previous data concerning the localization of gpr54 mRNA by in situ hybridization (Chianese et al. 2013). Thus, autocrine activity on Leydig cells may occur. Conversely, Gpr54 immunoreactivity has been observed in the germinal compartment in both somatic and germ cells suggesting the involvement of Kp signaling in

Discussion

Although the characterization of Kp system in the brain is one of the major finding in reproductive endocrinology of the last 20 years, data concerning the synthesis and the activity of Kp inside testis are really poor. Here we demonstrate, for the first time in a non-mammalian vertebrate, the intratesticular production of Kiss-1 protein during the annual sexual cycle and confirm the presence of Gpr54 previously reported at mRNA level only (Chianese et al. 2013). The fluctuations of Kiss-1/ Gpr54 proteins during the annual sexual cycle of frogs open new perspective in understanding the possible role in testis physiology. With the exception of February and

Figure 7 Histological analysis of frog testis injected in December with KRB alone (C; A1), Kp-10 (10^{-8} M; A2), Kp-10 + A (frogs were firstly injected with 10^{-8} M Kp-234; after 30 min they were injected with 10^{-8} M Kp-10; A3) and buserelin (10^{-5} M; A4) after staining with standard hematoxylin–eosin. White arrows, fully released SPZ; black arrows, detached SPZ. Count of tubules with released SPZ (white bars) or with detached SPZ (black bars) on the total number of tubules/section/animal. Data are reported as the % of tubules containing released (detached) SPZ over the total of tubules counted for animals ± s.d. (n = 3–4). Asterisks (letters) indicate the statistically significant differences (P < 0.01) among tubules containing released (detached SPZ) vs control.

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intratesticular paracrine communication (i.e. Leydig vs Sertoli cells, Leydig vs germ cells and Sertoli vs germ cells). The localization of Gpr54 in Sertoli cells is not surprising since it has been detected in adult testes of rhesus monkey and common marmoset monkey (Irfan et al. 2016) but not in rodents (Mei et al. 2013, Hsu et al. 2014). Conversely, at present, the frog is the only specie that expresses Gpr54 – both protein and mRNA – in peritubular myoid cells and spermatogonia (present data and Chianese et al. 2013). This observation confirms recent data concerning the requirement of Kp signaling for spermatogenesis onset and progression in frog (Chianese et al. 2013, 2015), a situation that in mammals mimics puberty onset. However, Kp half-live in biological tissues has been reported to be very low (Liu et al. 2013), and the presence of different shorter Kp fragments, with specific inter- and intra- cellular localizations and expression profiles (and functions as consequence), has been reported in the brain (Franceschini et al. 2013). At present, the biochemical characterization and purification of any Kiss-1 cleavage peptides have never been reported from the gonad of amphibians. Furthermore, the anti-Kiss1 antiserum used in this study is directed towards the C-terminal common portion of all Kiss-1 products, including both the larger form and the cleavage products, raising the possibility that different Kiss-1 peptides may exert different biological activities throughout the annual cycle of frogs.

Since the major site of localization of Gpr54 is the interstitium, the possible role of Kp system in the direct regulation of steroidogenesis has been examined by ex vivo treatment of testis with increasing doses of Kp-10. Interestingly, we demonstrated that the expression of key enzymes for the biosynthesis of testosterone and estradiol were differently regulated by Kp-10 after 1 h of treatment, with direct consequences on the intratesticular levels of both testosterone and estradiol. Consistently, Kp-10 administration in adult male rhesus monkeys treated with GnRH-antagonist sustains the hCG-induced production of testosterone (Irfan et al. 2014); by contrast, no effects on testosterone biosynthesis have been detected following in vitro treatment of adult testis in monkey (Tariq & Shabab 2016) nor in cultured Leydig cells (Mei et al. 2013, Wang et al. 2015) or testis explants in mice (Mei et al. 2013). In this study we demonstrate that at 1 h of treatment, Kp-10 directly decreases intratesticular testosterone levels vs control group with higher effects observed at the lowest doses (10^{-8}–10^{-5}M) and have double effects on estradiol levels, being stimulatory at 10^{-9}M dose only and inhibitory at the highest ones. In parallel, decreased expression levels of 3β-HSD and increases of cyp19, the enzyme that irreversibly converts testosterone into estradiol, have been observed. Interestingly, at 10^{-7} and 10^{-6}M Kp-10 doses, testosterone levels are significantly lower than those in control group, but are higher than those in 10^{-9} and 10^{-8}M doses and do not fully parallel the expression profile of 3β-HSD and ar mRNA, that are comparable at all doses or decreased, respectively. Similarly, for estradiol levels, the expression of cyp17, cyp19, era and erb mRNA increases (present data and Chianese et al. 2013, 2015) in parallel to the decrease of estradiol levels observed at 10^{-8}–10^{-6}M doses. Conversely, at longer treatment time (4h), steroid levels do not display any statistically significant difference, whereas the expression rates of all the considered steroidogenesis enzymes and of ar, era and erb mRNA are higher than those in control group (present data and Chianese et al. 2013, 2015). Intriguingly, data in frog revealed a possible involvement of Kp in the balance between testosterone tone and its irreversible conversion into estradiol. In fact, (i) at each dose, Kp-10 effects on testosterone and estradiol are opposite; (ii) estradiol–testosterone ratio revealed that 10^{-9} and 10^{-8}M Kp-10 doses move hormonal balance toward E2 whereas 10^{-7} and 10^{-6}M Kp-10 doses toward testosterone ones. Thus, lower Kp-10 doses may promote estradiol-dependent intratesticular activities and highest doses the testosterone-dependent ones. Accordingly, in the same time frame, Kp-10 treatment induced the progression of spermatogenesis (Chianese et al. 2015) with lowest doses increasing mitotic stages – well-known estradiol-dependent stages (Cobellis et al. 2002) – and highest doses promoting the progression toward meiotic stages (Chianese et al. 2015) and the release of spermatozoa in the lumen (present data), well-known testosterone-dependent process in mammals as for the progression of germ cells beyond meiosis (Sharpe 1994).

Frog SPZ expressed Gpr54, a condition functionally correlated to fertilization process and sperm hypermotility in human and mouse SPZ, respectively (Pinto et al. 2012, Hsu et al. 2014). In frog, pituitary gonadotropins and estradiol signaling are critical to gain successful spermiation and sperm transport toward cloaca (Cobellis et al. 2005, 2008). Here, morphological analysis of Kp-10-treated testes revealed a large number of tubules with SPZ detached from Sertoli cells, and the detection of Gpr54 in both Sertoli cells and peritubular myoid cells supports a possible involvement in sperm release. Accordingly, fully released SPZ occur in the lumen of animals treated at higher Kp-10 doses. Indeed, in vivo Kp-10 caused the detachment of SPZ from Sertoli cells, but failed to induce the transport of SPZ in cloacal fluids as buserelin, used as positive control, did. Thus, Kp-10 involvement in the reshuffle of junction proteins between Sertoli and sperm cells may be hypothesized. Consistently, Kp-10-dependent changes in the expression of cx43, the main junction protein connecting Sertoli cells to germ cells and spermatozoa in P. esculentus (Palmiero et al. 2003), have been here reported. In particular, 10^{-6}M Kp-10 dose decreases the expression of cx43 whereas higher Kp-10 doses increase it. This aspect fully fits the steroid-dependent expression
of çx43 (Izzo et al. 2009). In fact, 10⁻⁸ M dose decreases the intratesticular level of estradiol, a steroid that usually exerts positive effects on çx43 expression. By contrast, highest Kp-10 doses may promote the testosterone-dependent activities, including the expression of çx43 (Izzo et al. 2009). In addition, the involvement of local GnRH signaling system – that in frog comprises two ligands and three receptors (Chianese et al. 2011) and has a recognized role in spermatogenesis (Minucci 1989) – may be suggested. In fact, in February, gnrhs and gnrhrs mRNA have been detected in SPZ attached to Sertoli cells (Chianese et al. 2012) and high Kp-10 doses positively affect the expression rates of GnRH-II and GnRH-Rs (Chianese et al. 2015).

Taken together, our data demonstrate the existence of a complete Kp system in the testis of a non-mammalian specie and a double functional role in the modulation of spermatogenesis in frog. First, as autocrine factors for Leydig cells, Kp signaling modulates steroid biosynthesis. Second, as paracrine factor, Kp produced for Leydig cells, Kp signaling modulates steroidogenesis. Second, as paracrine factor, Kp produced by the interstitium targets spermatogonia and sperm cells to affect spermatogenesis onset/progression and sperm functions, respectively.

Supplementary data
This is linked to the online version of the paper at http://dx.doi.org/10.1530/REP-17-0030.

Declaration of interest
The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported; there is any financial or other potential conflict of interest.

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