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Kisspeptin drives germ cell progression in the anuran amphibian *Pelophylax esculentus*: A study carried out in *ex vivo* testes

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ABSTRACT

Kisspeptin, *via* Gpr54 receptor, regulates puberty onset in most vertebrates. Thus, the direct involvement of kisspeptin activity in testis physiology was investigated in the anuran amphibian, *Pelophylax esculentus*. In this vertebrate *gpr54* mRNA has been localized in both interstitial compartment and spermatogonia (SPG), whereas SPG proliferation requires the cooperation between estradiol and testicular Gonadotropin releasing hormone (Gnrh). In the pre-reproductive period, dose response curve to assess the effects of Kisspeptin-10 (Kp-10) was carried out *in vitro* (dose range: 10^{-9}–10^{-6} M; incubation times: 1 and 4 h); proliferative activity and germ cell progression were evaluated by expression analysis of *proliferating cell nuclear antigen* (*pcna*), *estrogen receptor beta* (*erβ*), Gnrh system (*gnrh1*, *gnrh2*, *gnrhr1*, *r2*, *r3*) and by the count of empty, mitotic and meiotic tubules. All selected markers were up regulated at 4 h Kp-10 incubation. Histological analysis also proved the increase of mitotic activity and the progression of spermatogenesis. Besides Kp-10 modulation of testicular Gnrh system, *in vitro* treatment with 17β-estradiol (10^{-6} M) ± the antagonist ICI182-780 (10^{-5} M) revealed *gnrh2* and *gnrhr3* estrogen dependent expression.

In the reproductive period, testes were incubated for 1 and 4 h with Kp-10 (10^{-7} M) or Kp-10 (10^{-7} M) + kisspeptin antagonist [Kp-234 (10^{-6} M)]. Results obtained in the pre-reproductive period were confirmed and Kp-234 completely counteracted Kp-10 effects.

In conclusion, Kp-10 modulated the expression of *pcna*, *erβ*, *gnrhs* and *gnrhrs*, inducing the progression of the spermatogenesis.

1. Introduction

Over the past decade, our understanding of the physiology of the gonadotropic axis has been enriched by the discovery of kisspeptins, highly conserved hypothalamic RF-amide neuropeptides encoded by the *kiss-1* gene (Ukena and Tsutsui, 2005). They actively take part in the development and regulation of reproductive functions through the binding to Gpr54 receptor (Thompson et al., 2004). Kisspeptins elicit the increase of plasma levels of gonadotropins [Luteinizing Hormone (LH) and Follicle Stimulating Hormone (FSH)] (Dhillo et al., 2005; Shahab et al., 2005), upstream regulating Gonadotropin-releasing hormone (Gnrh) secretion (Novaira et al., 2009; Oakley et al., 2009).

Kisspeptin signaling has been characterized at hypothalamic level (Oakley et al., 2009) and its involvement in sex steroid negative/positive feedback actions on Gnrh and gonadotropin secretion has also been reported (Oakley et al., 2009; García-Galiano et al., 2012). From fish to humans kisspeptin system has been linked to the onset of puberty (Beck et al., 2012; Biran et al., 2008; Navarro et al., 2004; Selvaraj et al., 2013; Seminara et al., 2003; Teles et al., 2011; Tena-Sempere et al., 2012). Targeted disruption of either ligand or receptor in animal models and mutations in human *kiss-1*/*gpr54* genes lead to hypogonadotropic hypogonadism and lack of sexual maturation (d'Anglemont de Tassigny et al., 2007; De Roux et al., 2003; Seminara et al., 2003; Topaloglu et al., 2012). In particular, mutant mice show impaired gametogenesis and altered reproductive organ development thus suggesting a potential role of kisspeptin system in cell mobility and proliferation events that are necessary for normal postnatal development of the gonad (Funes et al., 2003).

Besides detectable expression of *kiss-1*/*gpr54* genes has been demonstrated in vertebrate gonads (Anjum et al., 2012; Chianese

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et al., 2013; Kotani et al., 2001; Oakley et al., 2009; Terao et al., 2004), as well as in spermatozoa (Hsu et al., 2014; Pinto et al., 2012), the discovery of their direct role in testis physiology requires further investigations. The involvement of kisspeptin/Gpr54 system in estrogen dependent testicular signaling recently rose from a study conducted in a non mammalian vertebrate, the anuran amphibian *Pelophylax esculentus* – previously known as *Rana esculenta* – once again confirming the importance of non mammalian vertebrate animal models in the discovery of conserved physiological mechanisms (Chianese et al., 2013, 2011a). *P. esculentus* is a seasonal breeder characterized by a cystic organization of the testis in which clusters of germ cells at the same developmental stage proceed in germinal cysts delimited by Sertoli cells (Pierantoni et al., 2002b; Rastogi et al., 1976). In this animal model, estrogen dependent expression of *gpr54* was reported in mitotic germ cells and in interstitial compartment whereas Kisspeptin-10 (Kp-10) was able to modulate the expression of estrogen receptor alpha (*erα*) (Chianese et al., 2013). Since estrogens regulate mitotic events in both vertebrate testis and spermatogonial cell lines (Carreau et al., 2001; Cobellis et al., 2002; Minucci et al., 1989; Pierantoni et al., 2002a; Sirianni et al., 2008), observations in frog have suggested that kisspeptin, *via gpr54*, might be an important player in the modulation of such estrogen dependent events. In frog, the proliferation of primary spermatogonia (ISPG) requires a crosstalk between estradiol and testicular Gnrh activity *via* Fos protein activation (Cobellis et al., 2003, 2002; Pierantoni et al., 2002a). Interestingly, a complex Gnrh system has recently been described in *P. esculentus*, since two Gnrh molecular forms (*gnrh1* and *gnrh2*) and three Gnrh receptors (*gnrhr1*, *r2*, *r3*) fluctuate in the testis during the annual sexual cycle (Chianese et al., 2012).

Thus, to explore the physiological role of kisspeptin in the testis of *P. esculentus*, we analyzed *in vitro* the potential direct effects of Kp-10, *via gpr54*, on the upsurge of the spermatogenesis in pre-reproductive (February) and reproductive (March) periods. Besides changes in testis histology, the expression of the following targets was evaluated: (1) *proliferating cell nuclear antigen* (*pcna*), as marker of S phase of the cell cycle (Mathews et al., 1984); (2) Gnrh system – both ligands and receptors – as key signaling pathway in the recruitment of ISPG (Cobellis et al., 2003); (3) estrogen receptor beta (*erβ*) expression, as marker of germ cell progression (Carreau et al., 2003). Estrogen dependent and independent modulation of the above described markers was also evaluated. Thus, we demonstrated that Kp-10 may locally work in the testis as a regulator of germ cell progression.

2. Materials and methods

2.1. Animal and tissue collection

Adult *P. esculentus* male frogs (20–25 g) were collected in the neighborhood of Naples (Italy) during February and March, pre-reproductive and reproductive periods, respectively (Rastogi et al., 1976). To minimize the stress, animals were anesthetized in ethyl 3-aminobenzoate methanesulfonate salt, MS222 (Sigma–Aldrich, Milan, Italy) and immediately killed after capture. Testes were removed, rinsed in Krebs buffer for amphibians (KRB, 68 mM NaCl, 1 mM KCl, 1.17 mM $MgSO_4$, 1.2 mM KH_2PO_4, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 10 mg/l gelatin, pH 7.4) and processed for qPCR, Western blot and immunohistochemistry, *in vitro* incubation with Kp-10 and morphological analysis as reported below. 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 Ministry of Education, University and Research, Italy.

2.2. Protein extraction and Western blot analysis for Pcna detection

Total proteins were extracted from testes of animals collected in February and March ($n = 5$) as previously reported (Cobellis et al., 2002). Proteins were separated using 10% SDS–polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride filters (GE Healthcare) by TransBlot Turbo Transfer System (BioRad) in order to evaluate Pcna immunoreactivity. Filters were treated with blocking solution (5% non-fat powdered milk, 0.25% Tween-20 in Tris-buffered saline, TBS, pH 7.6) for 3 h to prevent non specific binding and then incubated with a mouse monoclonal primary antibody raised against rat Pcna diluted 1:500 (sc-56; Santa Cruz Biotechnology, Inc.) in TBS 3% non-fat powdered milk solution overnight at 4 °C on an orbital shaker. Filters were washed in TBS-0.25% Tween 20, incubated with 1:1000 horseradish peroxidase-conjugated IgG (DAKO Corp.) in TBS-1% normal swine serum (NSS; DAKO) and then washed three times in TBS-0.25% Tween-20. The immune complexes were detected using the ECL-Western blotting detection system (Amersham Pharmacia Biotech) following the manufacturer's instructions. The membranes, stripped at 60 °C for 30 min in stripping buffer (100 mM β-mercaptoethanol, 2% SDS, and 62.5 mM Tris–HCl, pH 7.6), were reprobed with Erk2 (Extracellular regulated kinase 2) antibody (anti-Erk2 diluted 1:1000; sc-154; Santa Cruz Biotechnology, Inc.) 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 Pcna/Erk2 ratio ± SD.

2.3. Immunohistochemistry analysis for Pcna detection

Frog testes ($n = 5$) collected in February and March were fixed in Bouin's fluid for histological observation and embedded in paraffin using standard procedures. Thus, sections were used to analyze Pcna distribution in seminiferous epithelium. Endogenous peroxidase activity was inhibited in methanol and 0.3% H_2O_2 for 20 min. To prevent background staining, sections (5 μm) were washed in phosphate-buffered saline (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-Pcna diluted 1:100 (sc-56; Santa Cruz Biotechnology, Inc.) 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 goat antimouse IgG (sc-2039; Vector Laboratories, Milan, Italy), diluted 1:200 in PBS-BS for 1 h at room temperature. Subsequently, the sections were incubated with Vectastain ABC reagents (Vector Laboratories, Inc., Burlingame, CA) 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 the primary antiserum incubation step during the procedures. Sections were observed under a light microscope (CTR500; Leica) and images were captured using a high resolution digital camera (DC300F; Leica).

2.4. In vitro incubations of frog testis

2.4.1. Experiment (1)

A dose response experiment was carried out *in vitro* on testes from 44 animals collected in the pre-reproductive period (February). The range of Kp-10 doses (from 10^{-9} to 10^{-6} M) and the incubation times (1 and 4 h) were chosen on the basis of dose response

experiments previously carried out in amphibians (Chianese et al., 2013; Moon et al., 2009) and mammals (Luque et al., 2011; Mei et al., 2013).

The testes from 4 animals were processed as follows: left testes were immediately frozen in liquid nitrogen and stored at −80 °C (fresh control, C0); right testes were fixed in Bouin's fluid and processed for histological analysis. The testes from the remaining 40 animals were removed and divided into halves taking care to group together all left and right testes that were processed for 1 h treatment (left testis) and 4 h treatment (right testis) as follows: control group (C), testes incubated in KRB (n = 8/time point); treatment group (Kp-10), testes incubated in KRB and increasing doses (10^{-9}–10^{-6} M) of Kp-10 [Metastin 45–54 (H-YNWNSFGLRF-NH_2) of human origin, a kisspeptin form exhibiting a potency similar to Xenopus-Kp-10 for bullfrog Gpr54 (Moon et al., 2009), DBA Italia, Milan, Italy] (n = 8/time point/each dose); for each dose and time point, 4 testes were stored at −80 °C and used for expression analysis and 4 testes were fixed in Bouin's fluid and processed for histological analysis.

2.4.2. Experiment (2)

Male frogs (n = 15) collected in February were used for treatment with 17β-estradiol (E_2). Six untreated testes collected from different animals were immediately frozen in liquid nitrogen, stored at −80 °C and used as fresh control (C0). The remaining testes were cut into halves and were incubated for 1 h in KRB alone (control group, C), in KRB/E_2 10^{-6} M (E_2 group), in KRB/ICI 182780 10^{-5} M [Zeneca Pharmaceuticals, Macclesfield, UK (ICI group)] (n = 6/treatment). Lastly, testes preliminarily incubated in KRB/ICI 182780 10^{-5} M (n = 6) were incubated in KRB/E_2 10^{-6} M and ICI 182780 10^{-5} M (E_2 + ICI group). E_2 and ICI 182780 doses and incubation times were chosen on the basis of previous studies (Cobellis et al., 1999). After the treatment, testes were stored at −80 °C and then processed for RNA extraction and qPCR as reported below to assess the expression of *pcna*, *erβ*, *gnrhs* and *gnrhrs*.

2.4.3. Experiment (3)

In the reproductive period (late March), Kp-10 (10^{-7} M) was antagonized for 1 and 4 h with Gpr54 antagonist, Kisspeptin-234 trifluoroacetate salt (Kp-234; Roseweir et al., 2009). In detail, testes were collected from 28 animals taking care to group together all left and right testes; then, each testis was divided into halves; untreated left testes (n = 4), immediately frozen in liquid nitrogen, were stored at −80 °C and used as fresh control (C0); 4 untreated right testes were fixed in Bouin's fluid and processed for histological analysis. The remaining testes (n = 8/treatment/time point) were incubated for 1 h treatment (left testes) and 4 h treatment (right testes) as follows: control group (C) testes incubated in KRB alone; Kp-10 group (Kp-10), testes incubated in KRB/Kp-10 10^{-7} M; Kp-10 + Kp-234 group (Kp-10 + A), testes incubated in KRB/Kp-234 10^{-6} M alone for 30 min and subsequently incubated in KRB/Kp-10 10^{-7} M and Kp-234 10^{-6} M. For each dose and time point, testes were stored at −80 °C and used for expression analysis.

2.5. Total RNA extraction and cDNA preparation

The extraction of total RNA from frog testis was performed using Trizol reagent (Life Technologies, Paisley, UK) following the manufacturer's instructions. DNaseI (10U/sample) (Amersham Pharmacia Biotech, UK) treatment at 37 °C for 30 min ensured the elimination of any genomic DNA contaminations. The reverse transcription of a total RNA pool was then carried out using 5 μg total RNA, 0.5 μg oligo $dT_{(18)}$, 0.5 mM dNTP mix, 5 mM DTT, 1× first strand buffer (Life Technologies), 40U RNase Out (Life Technologies), 200U 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.

2.6. Expression analysis of gnrhs, gnrhrs, pcna and erβ in Kp-10 and E_2 treated testes

Quantitative expression of *gnrh1*, *gnrh2*, *gnrhr1*, *gnrhr2* and *gnrhr3* was carried out on Kp-10 and E_2 treated testes by qPCR using primers and protocols as previously reported (Chianese et al., 2012, 2011b). Quantitative PCR efficiencies – calculated by standard dilution curves – were: *gnrh1* 100.6%, *gnrh2* 98.6%, *gnrhr1* 97.02%, *gnrhr2* 101.3%, *gnrhr3* 99.85%.

The isolation of a cDNA fragment 236 bp long of *pcna* from *P. esculentus* testis was obtained by standard RT-PCR using primers designed upon *Lithobates catesbeianus* cDNA (forward primer: 5′-gactagtgcagggctccatc-3′, reverse primer: 5′-ggcagcacatttcaggattt-3′; *T* (°C) annealing 55 °C) [Accession number in GenBank AY787854.1]. A cDNA fragment 223 bp long of *erβ* from *P. esculentus* testis was obtained by standard RT-PCR using primers designed upon *Rana rugosa* cDNA (forward primer: 5′-aatgcgtggaaggaattttg-3′, reverse primer: 5′-gctatgacccagaccagtcc-3′; T (°C) annealing 56 °C) [Accession number in GenBank: FJ828859.1]. PCR amplificates were cloned in pGEM-T Easy vector and sequenced on both strands. Sequence analysis revealed nucleotide and amino acid identity of 97% to *L. catesbeianus pcna* and of 96% to *R. rugosa erβ*. Thereafter, the same primer couples was used for qPCR analysis to assess the expression of *pcna* and *erβ* in testes treated with Kp-10; qPCR efficiencies were: 99.7% for *pcna* and 105.4% for *erβ*.

Relative gene expression, corrected for respective PCR efficiency, was normalized toward the reference gene *fp1* whose boundary as reference gene was previously reported (Chianese et al., 2012, 2011b) according to the comparative Cq method with the formula $2^{-\Delta\Delta Cq}$. 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 value one arbitrarily assigned to KRB incubated control group.

2.7. Histological analysis and count of empty, mitotic and meiotic tubules in Kp-10 dose response experiment

For histological observations, frog testes were fixed in Bouin's fluid, dehydrated in ethanol, cleared in xylene and embedded in paraffin. Slides (5 μm) were, then, stained with standard hematoxylin–eosin and observed under a light microscope (Leica 165 CTR500, Leica Microsystem, Wetzlar, Germany); images were captured using a high resolution digital camera (Leica DC300F). 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 isolated ISPG, SPZ and less than 3 cysts of secondary SPG (IISPG) (empty tubules); more than 3 cysts of IISPG and less than 3 cysts of primary spermatocytes (ISPC) (mitotic tubules); more than 3 cysts of ISPC (meiotic tubules). Data were reported as the % of empty or mitotic or meiotic tubules on the total of tubules counted for animals ± S.D.

2.8. Statistical analysis

ANOVA test followed by Duncan's test for multi-group comparison was performed where appropriate.

3. Results

3.1. Molecular and histological evaluation of proliferative activity in testis

Proliferative activity in testis was evaluated by means of *pcna* expression analyzed by qPCR. *Pcna* mRNA expression was higher in pre-reproductive (February) than in reproductive (March) period ($P < 0.01$) (Fig. 1A). In support to this molecular evaluation, Pcna presence in February and March testis was also ascertained by immunohistochemistry. In February, when most tubules are rich in ISPG and spermatozoa (SPZ) tightly associated to Sertoli cells (Fig. 1B), Pcna signal was localized in the nucleus of proliferating germ cells, in particular ISPG (Fig. 1C). In March, when testis was mainly populated by cysts of ISPG and IISPG and the first meiotic stages appeared (Fig. 1E), Pcna marked the nucleus of IISPG, besides ISPG (Fig. 1F). Absence of signal obtained omitting one step of the reaction demonstrated the specificity of the immunoreactivity (Fig. 1D, 1G).

3.2. Induction of germ cell proliferation by Kp-10 in the pre-reproductive period

To investigate the physiological role of Kp-10 in testicular proliferative events, we evaluated *in vitro* the possible effect of increasing doses of Kp-10 on the expression of *pcna*, a marker of cell cycle (S phase) (Mathews et al., 1984) and of *erβ*, a marker of germ cell progression (Carreau et al., 2003).

At 1 h treatment, only 10^{-6} M Kp-10 stimulated Pcna mRNA and protein ($P < 0.01$) (Fig. 2A). After 4 h treatment, also 10^{-7} M Kp-10 had a stimulatory effect on *pcna* transcriptional levels ($P < 0.01$) (Fig. 2B).

After 1 h treatment, all Kp-10 doses stimulated *erβ* expression ($P < 0.01$ *vs* control values) (Fig. 2C). After 4 h treatment, Kp-10 induced *erβ* expression in a dose dependent manner with highest expression rates registered at both 10^{-7} and 10^{-6} M (Fig. 2D).

3.3. Kp-10 effect on Gnrh system expression in the pre-reproductive period

A time and dose dependent modulation for each component of Gnrh system was shown in testis after Kp-10 treatment. One hour treatment with 10^{-8} and 10^{-7} M Kp-10 had an inhibitory effect on *gnrh1* and *gnrh2* expression, as compared with both fresh and control groups ($P < 0.01$); such an effect persisted for *gnrh1* also at 10^{-6} M Kp-10, becoming stimulatory for *gnrh2* ($P < 0.01$). The lowest Kp-10 dose (10^{-9} M) did not have effect on *gnrh1* whereas decreased *gnrh2* expression ($P < 0.01$) (Fig. 3A). Differently from ligands, *gnrhr* expression was stimulated by Kp-10, at 10^{-7} (in particular for *gnrhr2* and *gnrhr3*) and 10^{-6} M ($P < 0.01$), (Fig. 3A). For each component of Gnrh system, 4 h treatment at 10^{-7} and 10^{-6} M of Kp-10 had a stimulatory effect ($P < 0.01$); such an effect was also observed on *gnrh2*, *gnrhr1* and *gnrhr3* expression at 10^{-8} M ($P < 0.01$). No effect was shown at 10^{-9} M (Fig. 3B).

3.4. Morphological analysis of Kp-10 treated testes

We analyzed the morphology of Kp-10 treated testis counting empty, mitotic and meiotic tubules (see Table 1 for details).

In the control section, after 1 h incubation with KRB, the majority of seminiferous tubule cross sections (about 90%) appeared empty (Fig. 4A1) being characterized by ISPG and SPZ (Fig. 4A2). The treatment with 10^{-8} as well as 10^{-7} M Kp-10 did not significantly change the percentage of empty tubules, thus suggesting

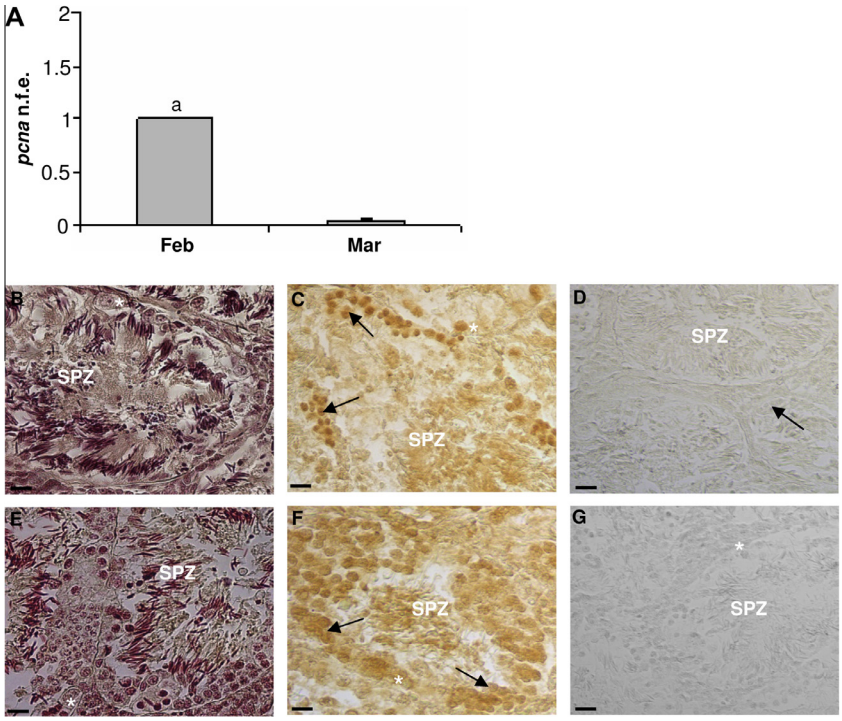

Fig. 1. Basal *pcna* expression in fresh control testis of February and March analyzed by qPCR (A); morphological analysis of February (B) and March (E) frog testis by means of standard hematoxylin–eosin staining supported by immunocytochemistry analysis for Pcna presence in February (C) and March (F) frog testis, with respective controls of immunoreactions (D, February; G, March). "a" letter indicates statistically significant difference ($P < 0.01$). Data, normalized against *fp1*, are reported as mean fold change ± S.D. over the value one arbitrarily assigned to February and are representative of three separate experiments at least ($n = 6$). n.f.e., normalized fold expression. Scale bar, 20 μm. Asterisk indicates ISPG, black arrow indicates cysts of IISPG and SPZ indicates spermatozoa.

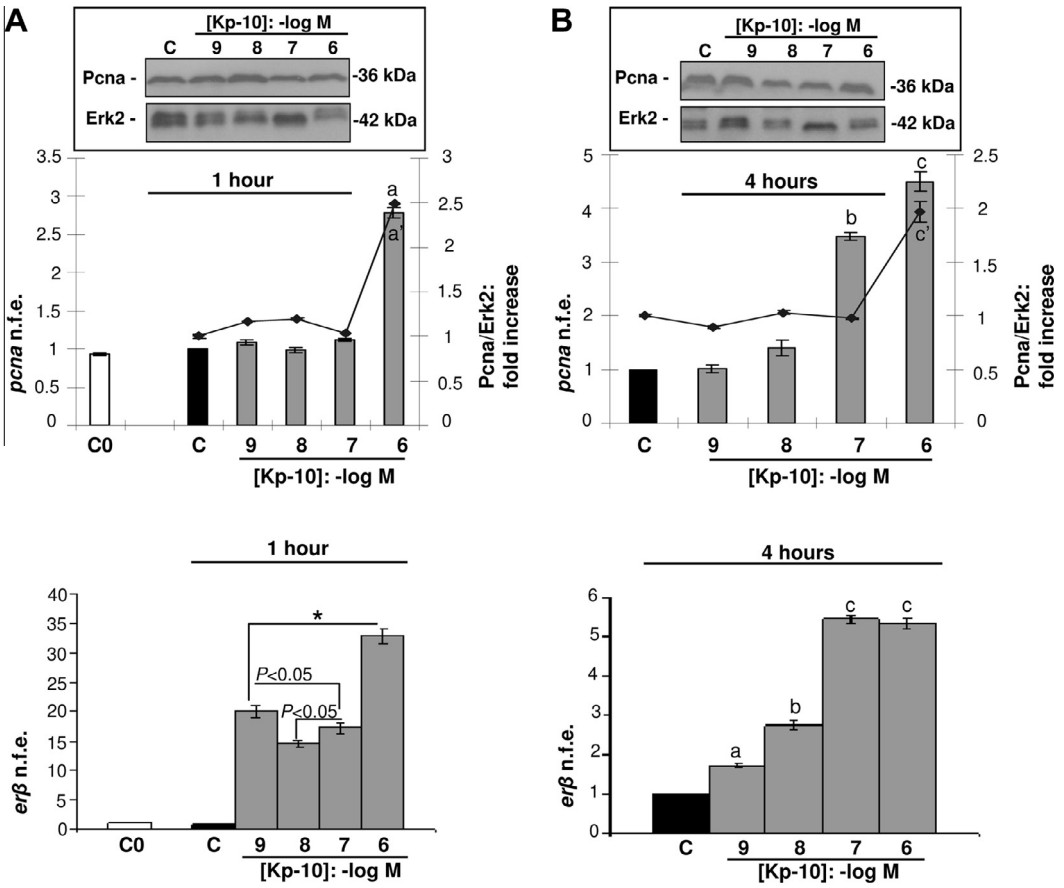

Fig. 2. Direct actions of Kp-10 on the expression of Pcna mRNA and protein (A, B) and on *erβ* mRNA (C, D) in frog testis of February. Kp-10 incubation time were 1 h (A, C) and 4 h (B, D). Inserts in (A) and (B): Western blot analysis for Pcna and Erk2. Bars: mRNA levels, lines: protein levels. C0, fresh control, white bar; C, control testis incubated with KRB, black bar; numbers 9–6, Kp-10 concentrations (−log M), grey bar. Different letters indicate statistically significant differences ($P < 0.01$). Asterisk indicates statistically significant differences between control group and all Kp-treated testis at 1 h for *erβ* analysis. Quantitative PCR data, normalized against *fp1*, are reported as mean fold change ± S.D. over the value one arbitrarily assigned to the sample C. n.f.e., normalized fold expression. Western blot data were expressed as mean Pcna/Erk2 ratio ± SD. Data are representative of three separate experiments at least ($n = 6$).

the failure of such doses to affect germ cell progression. Interestingly, 10^{-6} M caused a strong increase of the number of mitotic tubules ($P < 0.01$) (Fig. 4A1). These tubule cross sections appeared enriched with cysts of IISPG indicating high germ cell proliferation (Fig. 4A3). At 4 h the pattern of proliferation changed since the only ineffective Kp-10 dose was 10^{-8} M (Fig. 4B1). Indeed, 10^{-7} M increased the percentage of mitotic tubules as compared with controls and 10^{-8} M Kp-10 treated group ($P < 0.01$), thus significantly reducing the percentage of empty tubules ($P < 0.01$) (see also histological data depicted in Fig. 4B2 and B3). Noticeably, treatment with 10^{-6} M Kp-10 further decreased the percentage of empty tubules ($P < 0.01$). This reduction was balanced by the increase of both mitotic and meiotic tubules ($P < 0.01$) (Fig. 4B1), the latter characterized by numerous cysts of ISPC characterized by a dark stained nucleus (Fig. 4B4).

3.5. E_2 effect on the expression of pcna, erβ and gnrhs and gnrhrs in the pre-reproductive period

E_2 treatment did not affect the expression of *pcna*, *gnrh1*, *gnrhr1* and *gnrhr2* (Fig. 5A, B, D, E). Conversely, the treatment significantly increased the expression of *gnrh2*, *gnrhr3* (Fig. 5C and F) and *erβ* (not shown) ($P < 0.01$). Pre-treatment with the estrogen receptor antagonist, ICI 182780, counteracted the estradiol dependent effects.

3.6. Kp-10 effects on pcna, erβ and gnrh system in the reproductive period

On the basis of dose response experiment carried out in February, we chose 10^{-7} M Kp-10 for treatment with Gpr54 antagonist, Kp-234 (Roseweir et al., 2009) using testes of late March.

One and 4 h treatment with 10^{-7} M Kp-10 significantly increased Pcna expression (both mRNA and protein) as compared with fresh and control groups ($P < 0.01$) (Fig. 6A and B). Both effects were completely counteracted by Kp-234, indicating a Gpr54 mediation.

A similar positive effect was observed upon *erβ* expression. In fact, at 1 h as well as 4 h treatment, 10^{-7} M Kp-10 significantly increased *erβ* expression as compared with both fresh and control groups ($P < 0.01$) (Fig. 6C and D). All effects were counteracted by Kp-234.

Also Gnrh system was affected by Kp-10 treatment. In particular, after 1 h treatment, 10^{-7} M Kp-10 decreased the expression of both ligands ($P < 0.01$) and increased those of *gnrhr2* and *gnrhr3* ($P < 0.01$) while *gnrhr1* expression was not affected (Fig. 7A). Conversely, after 4 h treatment, the same dose of Kp-10 up regulated the components of the Gnrh system ($P < 0.01$) (Fig. 7B). These effects were completely counteracted by Kp-234, indicating a Gpr54 mediation.

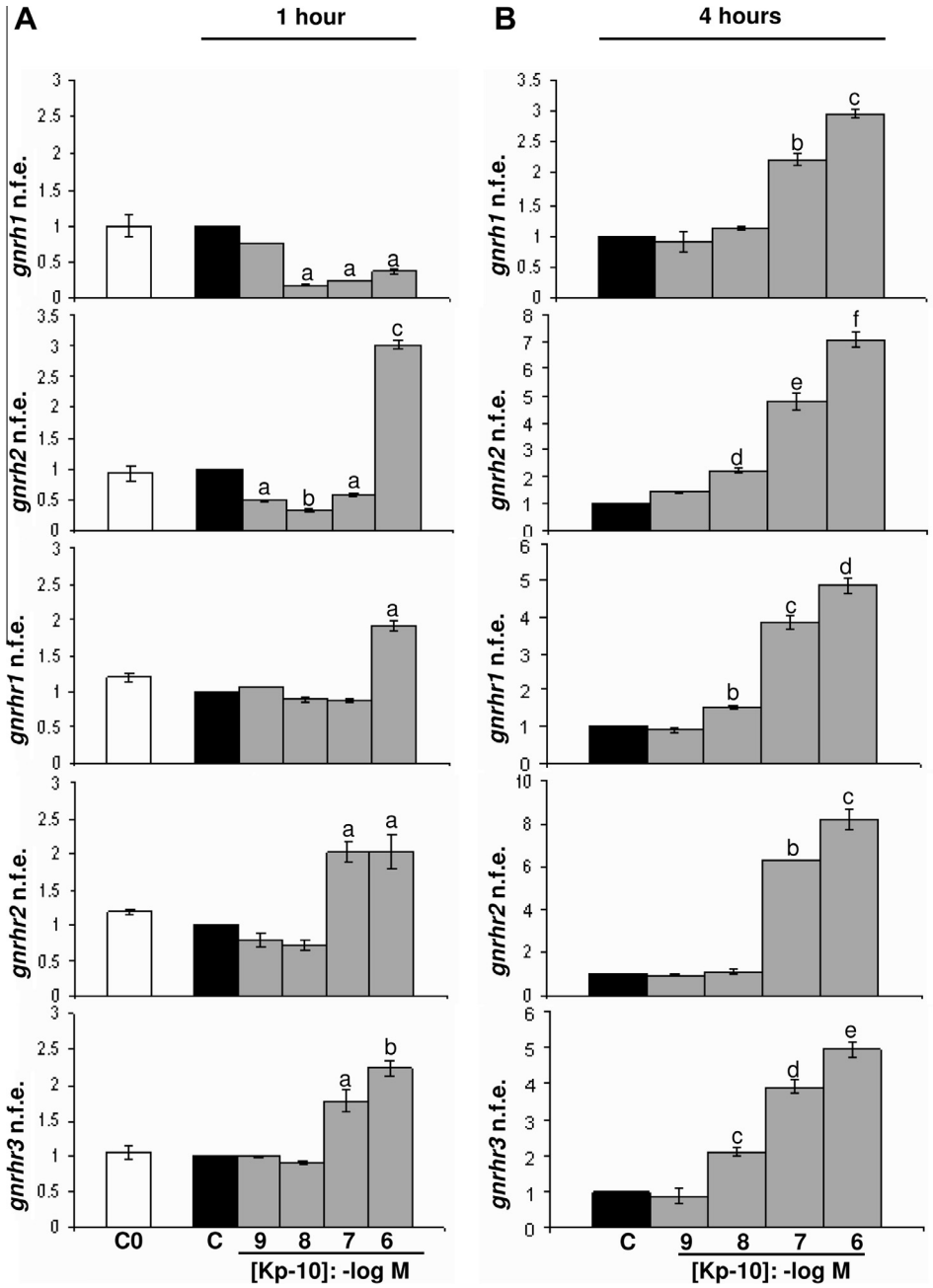

Fig. 3. Direct actions of Kp-10 on the expression of *gnrh1*, *gnrh2*, *gnrhr1*, *gnrhr2* and *gnrhr3* in frog testis of February analyzed by qPCR. Kp-10 incubation time were 1 h (A) and 4 h (B). C0, fresh control, white bar; C, control testis incubated with KRB, black bar; numbers 9–6, Kp-10 concentrations (−log M), grey bar. Different letters indicate statistically significant differences ($P < 0.01$). Data, normalized against *fp1*, are reported as mean fold change ± S.D. over the value one arbitrarily assigned to the C and are representative of three separate experiments at least ($n = 6$). n.f.e., normalized fold expression.

4. Discussion

In the testis, germ cells undergo an intricate program of proliferation and differentiation in order to produce functional gametes. In vertebrates, the onset of a new spermatogenic wave depends on a complex network of endocrine, paracrine and autocrine factors and the list of local bio-regulators is still growing (Battista et al., 2012; Meccariello et al., 2014; Pierantoni et al., 2009).

Nowadays, the involvement of kisspeptin system in the control of reproductive function emerges, but the direct role of kisspeptin system in testicular physiology is controversial. Only recently, a study carried out in pituitary gonadotropin-clamped monkey reported the kisspeptin dependent modulation of Leydig cell activity (Irfan et al., 2013), whereas no effects have been reported in mouse testis explants (Mei et al., 2013). Here, we investigated the direct involvement of kisspeptin in the onset of the new spermatogenic wave, occurring during February–March, in the seasonal breeder, *P. esculentus*, at present the first vertebrate in which *gpr54* mRNA has been localized in both interstitial compartment and proliferating SPG (Chianese et al., 2013).

We carried out a dose response curve to assess the effects of Kp-10 *in vitro* and evaluated testicular activity by means of expression analysis of *pcna*, a marker of S phase of the cell cycle (Mathews et al., 1984), whose expression in frog is intensive in pre-reproductive and in reproductive periods (Chieffi et al., 2000; Raucci and Di Fiore, 2007). In pre-reproductive period, as soon as the first mitotic

Table 1
Count of empty, mitotic and meiotic tubules in Kp-10 treated testis of frogs collected in February.

Incubation time	Treatment	Total counted tubules/animal: mean value ± SD (n = 8 sections/3–4 testes)	% Empty tubules ± SD	% Mitotic tubules ± SD	% Meiotic and mitotic tubules ± SD
1 h	Control	170 ± 17.9	89.87% ± 5.11	5.73% ± 4.36	4.33% ± 1.85
	Kp-10 10^{-8} M	222 ± 57.2	77.33% ± 2.86	12.5% ± 2.95	10.53% ± 3.33
	Kp-10 10^{-7} M	156 ± 26.2	84.35 ± 2.62	6.58 ± 3.14	9.08 ± 0.54
	Kp-10 10^{-6} M	191 ± 51.1	13 ± 5.94	71.23 ± 6.13	15.75 ± 3.63
4 h	Control	158 ± 40.8	81.4 ± 8.9	10.46 ± 7.3	8.1 ± 2.7
	Kp-10 10^{-8} M	152 ± 29	68.87 ± 3.82	16.2 ± 3.42	14.93 ± 1.99
	Kp-10 10^{-7} M	209 ± 22	19.03 ± 3.21	61.17 ± 8.43	19.7 ± 6.26
	Kp-10 10^{-6} M	218 ± 54.8	5.5 ± 3.5	55.77 ± 8.39	38.7 ± 9.8

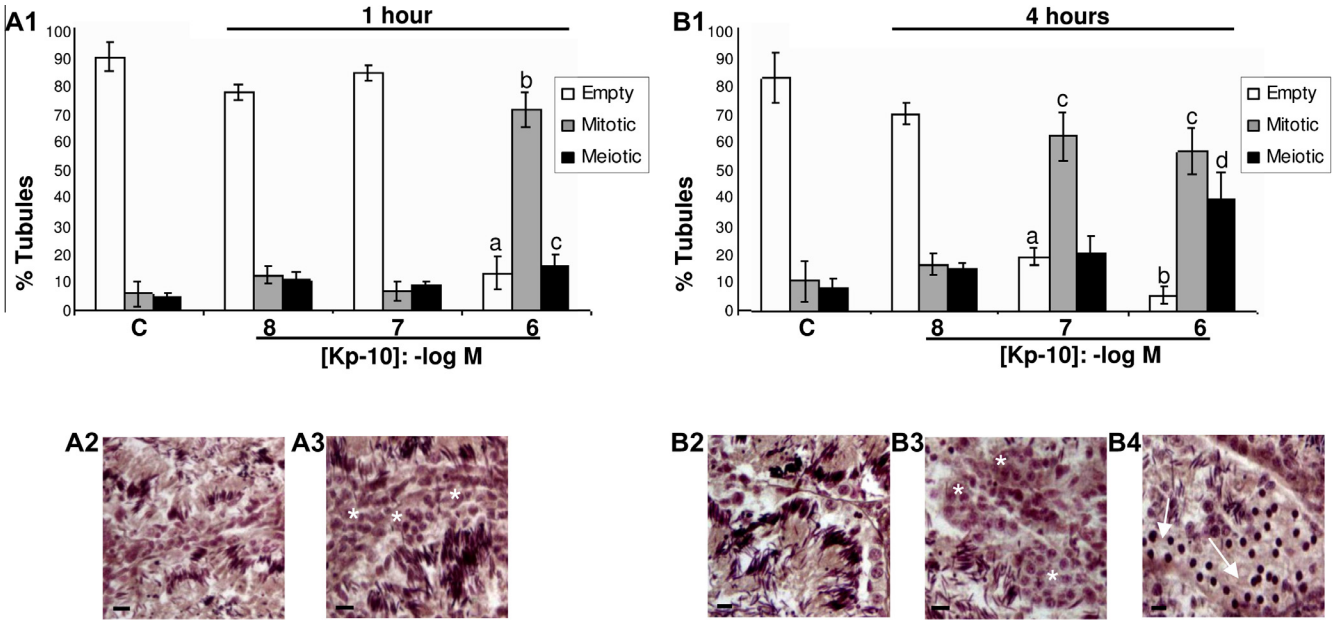

Fig. 4. Histological analysis of Kp-10 treated frog testis of February, after staining with standard hematoxylin–eosin. Incubations times were 1 h (A1–A3) and 4 h (B1–B4). C, control testis incubated with KRB; numbers 8–6, Kp-10 concentrations (−log M). Count of empty, mitotic and meiotic tubules (A1–B1). Empty tubules, cross sections of seminiferous tubules containing isolated ISPG, SPZ and less to 3 cysts of IISPG; mitotic tubules, cross sections of seminiferous tubules containing more than 3 cysts of IISPG and less to 3 cysts of ISPC; meiotic tubules, cross sections of seminiferous tubules containing more than 3 cysts of ISPC. Different letters indicate statistically significant differences ($P < 0.01$). Data are reported as the % of empty or mitotic or meiotic tubules on the total of tubules counted for animals ± S.D. (n = 3–4). Representative histological analysis of empty and IISPG rich tubules after 1 h KRB (A2) and 10^{-6} M Kp-10 (A3) incubations, respectively. Representative histological analysis of empty, IISPG and ISPC cyst rich tubules after 4 h KRB (B2), 10^{-7} M Kp-10 (B3) and 10^{-6} M Kp-10 (B4) incubations, respectively. Asterisk indicates cysts of IISPG, white arrow indicates cysts of ISPC.

stages were observed, only 10^{-6} M Kp-10 dose induced Pcna expression (both mRNA and protein) after 1 h incubation, whereas both 10^{-7} and 10^{-6} M doses increased Pcna expression after 4 h incubations. Accordingly, histological analysis demonstrated that, after 1 h incubation, 10^{-6} M Kp-10 strongly increased the number of mitotic cysts and decreased the number of empty tubules. After 4 h incubation, both *pcna* mRNA and the number of mitotic cysts increased using 10^{-7} and 10^{-6} M Kp-10 doses. Furthermore, 10^{-6} M dose had a significant effect on Pcna protein increase and also upon the appearance of meiotic stages. Surprisingly, the up regulation achieved using 10^{-7} M did not result into a Pcna protein increase. We have no explanation for the moment, but changes in mRNA stability or processing rate into mature mRNA might explain this issue. However, it is interesting to emphasize that at shorter incubation time only the highest Kp-10 dose stimulated mitotic stages; conversely, at longer incubation time, 10^{-7} M Kp-10 stimulated mitotic stages and highest Kp-10 dose induced the appearance of meiotic stages. Accordingly, the up regulation of *erβ* expression, evaluated as marker of germ cell progression in amphibians (Caneguim et al., 2013; Stabile et al., 2006), confirmed the motion of spermatogenesis after Kp-10 treatment.

Chronic intraperitoneal administration of Kp-10 and subcutaneous administration of Kp-54 have been reported to induce dose dependent degenerative changes in maturing and adult rat testes, respectively (Ramzan and Qureshi, 2011; Thompson et al., 2006), but intraperitoneal administration increased the coefficient of efficiency of spermatogonial mitosis (Ramzan and Qureshi, 2011). Thus, since the time and dose dependent deleterious effects of kisspeptin reported in rodents and dose response effects registered in frogs collected in February (present data), during the reproductive period we incubated testis only with Kp-10 10^{-7} M and counteracted its action with Kp-234, a specific Gpr54 antagonist.

Kp-10 dependent induction of Pcna at both mRNA and protein levels was confirmed in reproductive period at 1 and 4 h incubation times, when a prevalence of mitotic and meiotic cysts was observed in testis. Furthermore, the effects observed using 10^{-7} M Kp-10 were fully counteracted by the antagonist, clearly indicating the involvement of Gpr54. Interestingly, during the

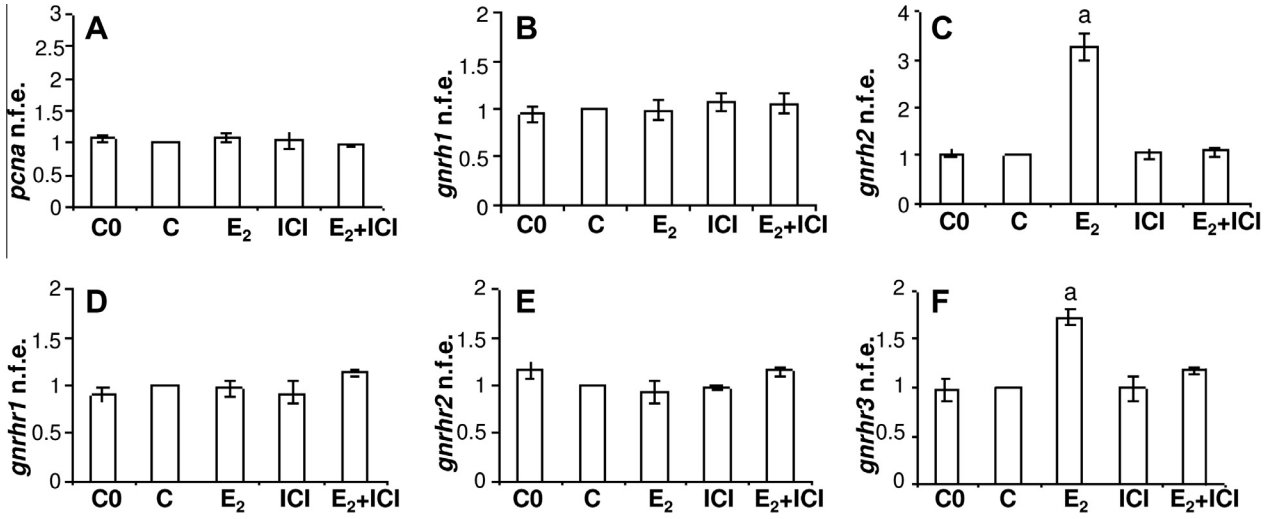

Fig. 5. Direct actions of estradiol on the expression of *pcna*, *gnrh1*, *gnrh2*, *gnrhr1*, *gnrhr2* and *gnrhr3* in frog testis of February analyzed by qPCR. C0, fresh control; C, control testis incubated with KRB; E_2, testis incubated with 10^{-6} M estradiol; ICI, testis incubated with 10^{-5} M ICI 182780, an estrogen receptor antagonist; E_2 + ICI, testis incubated with both estradiol and ICI 182780. Different letters indicate statistically significant differences ($P < 0.01$). Incubation time: 1 h. Data, normalized against *fp1*, are reported as mean fold change ± S.D. over the value one arbitrarily assigned to the C and are representative of three separate experiments at least ($n = 6$). n.f.e., normalized fold expression.

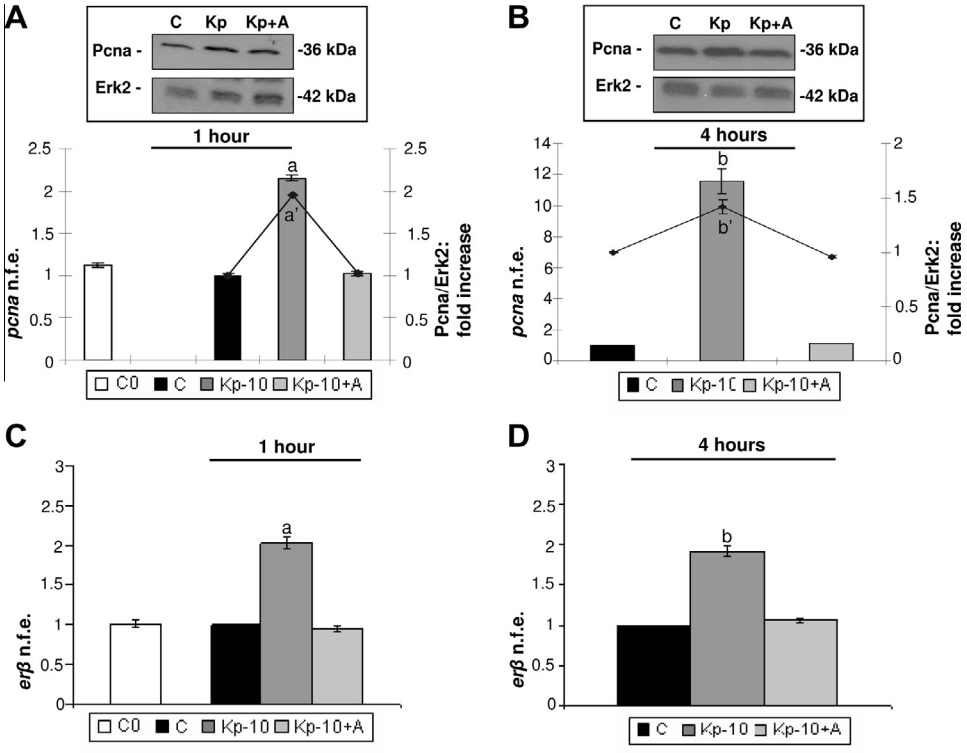

Fig. 6. Direct actions of Kp-10 ± the antagonist Kp-234 on the expression of Pcna mRNA and protein (A, B) and on *erβ* mRNA (C, D) in frog testis of March analyzed by qPCR. Incubations times were 1 h (A, C) and 4 h (B, D). Inserts in (A) and (B): Western blot analysis for Pcna and Erk2. Bars: mRNA levels, lines: protein levels. C0, fresh control; C, control testis incubated with KRB; Kp-10, testis incubated with Kp-10 (10^{-7} M); Kp-10 + A, testis incubated with Kp-10 (10^{-7} M) in combination with Kp-234 (10^{-6} M). Different letters indicate statistically significant differences ($P < 0.01$). Quantitative PCR data, normalized against *fp1*, are reported as mean fold change ± S.D. over the value one arbitrarily assigned to the sample C. n.f.e., normalized fold expression. Western blot data were expressed as mean Pcna/Erk2 ratio ± SD. Data are representative of three separate experiments at least ($n = 6$).

reproductive period, *gpr54* expression in the testis was up regulated by Kp-10 treatment (Chianese et al., 2013). It is important to note that the different effects of Kp-10 obtained in pre-reproductive and reproductive periods on the expression of *pcna* might be the consequence of the different basal expression of *pcna* (Raucci and Di Fiore, 2007) and/or of the different testicular cell composition typical of these periods (from empty tubules to mitotic/meiotic cysts rich tubules).

In the hypothalamus as well as in Gnrh secreting cell lines, kisspeptins modulate the expression of Gnrh (Clarkson and Herbison,

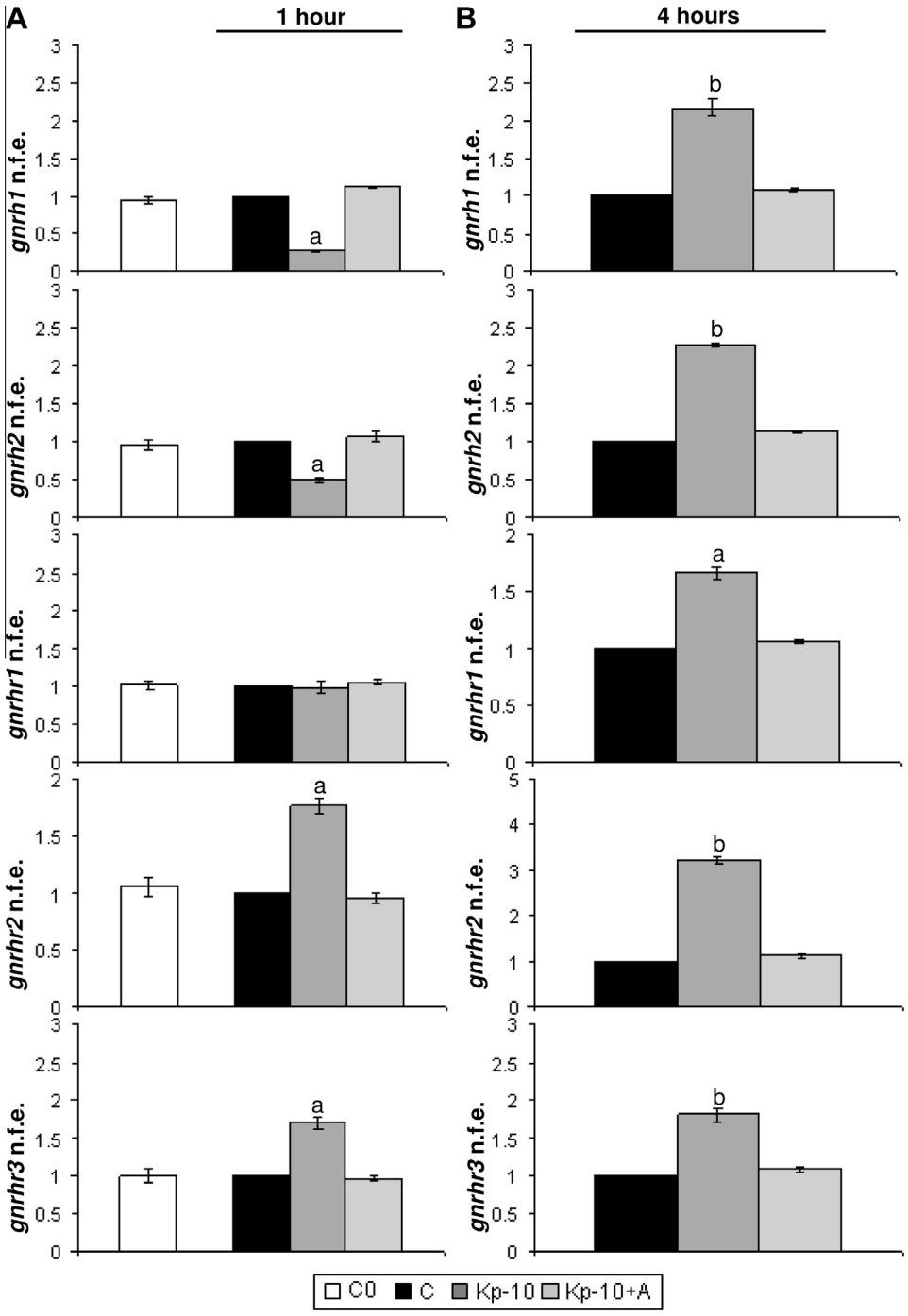

Fig. 7. Direct actions of Kp-10 ± the antagonist Kp-234 on the expression of *gnrh1*, *gnrh2*, *gnrhr1*, *gnrhr2* and *gnrhr3* in frog testis of March analyzed by qPCR. Incubations times were 1 h (A) and 4 h (B). C0, fresh control; C, control testis incubated with KRB; Kp-10, testis incubated with kisspeptin (10^{-7} M); Kp-10 + A, testis incubated with Kp-10 (10^{-7} M) in combination with Kp-234 (10^{-6} M). Different letters indicate statistically significant differences ($P < 0.01$). Data, normalized against *fp1*, are reported as mean fold change ± S.D. over the value one arbitrarily assigned to the C and are representative of three separate experiments at least ($n = 6$). n.f.e., normalized fold expression.

2009; Colledge, 2009; Novaira et al., 2009; Oakley et al., 2009), centrally regulating reproductive functions and affecting puberty onset (Beck et al., 2012; Biran et al., 2008; Navarro et al., 2004; Seminara et al., 2003; Teles et al., 2011; Tena-Sempere et al., 2012). Extra-pituitary functions of Gnrh as positive/negative modulator of cell proliferation have been reported in various tumor and non tumor cells (Park et al., 2013). Focusing in males, Gnrh involvement in paracrine Sertoli/Leydig cell communication has been suggested in both mammals and lower vertebrates (Pierantoni et al., 2002a; Sharpe, 1986), with Gnrh acting as local bio-regulator of steroidogenesis, germ cells progression, SPG multiplication, sperm release and fertilization (Deragon and Sower, 1994; Pierantoni et al., 2002a, 1984a, 1984b; Meccariello et al., 2014; Minucci et al., 1989; Morales et al., 2002). Furthermore, estrogens and "testicular" Gnrh act in concert to promote spermatogonial mitosis (Cobellis et al., 2003, 2002) in the frog, *P. esculentus*. Since only recently, two Gnrh molecular forms and three Gnrh receptors have also been cloned (Chianese et al., 2011b)

and characterized in frog testis during the annual reproductive cycle (Chianese et al., 2012), Kp-10 effects on testicular Gnrh system were also evaluated.

As far as we know, present data in *ex vivo* testis, show for the first time that Kp-10 treatment, *via* Gpr54, modulated the expression of testicular Gnrh system, affecting both ligands and receptors in a dose and time dependent manner in pre-reproductive period. Similar effects on testicular GnRH system also occurred in reproductive period for Kp-10 10^{-7}M dose and were fully counteracted by Kp-234. Interestingly, in pre-reproductive period, ligand and receptor expression closely paralleled *pcna*, *erβ* expression and the increase of mitotic and meiotic percentage of tubules at 4 h incubation. With respect to results obtained after 1 h incubation, the proliferative effect observed using Kp-10 treatment may proceed *via gnrh2* activity and may require the involvement of estradiol signaling. In fact, 1) *gnrh2* expression was shown to be estradiol dependent (present data); 2) estrogens induced *gpr54* expression and 3) Kp-10 increased *erα*/*erβ* and *gpr54* as well as *pcna* expression (Chianese et al., 2013 and present data). Notably, also *gnrhr3* expression increased after estrogen and Kp-10 stimulation. Accordingly, *gnrhr3* mRNA basal expression level quickly peaked in reproductive and post-reproductive periods (Chianese et al., 2012) whereas gnrh2 has been localized in both interstitial and germinal compartments (Di Matteo et al., 1996). Taken together the above considerations strongly suggest that Kp-10, *via gpr54*, might be locally involved in the modulation of estradiol/Gnrh testicular interplay (Pierantoni et al., 2002a) in order to promote the upsurge of the spermatogenesis and spermatogonial mitosis. However, Kp-10 also modulated the expression of *gnrh1*, *gnrhr1 and gnrhr2*, in estradiol independent mechanisms, thus suggesting that a complex interplay exists between kisspeptins and testicular *gnrh* system to regulate locally the gonadal activity.

Besides the explanation of mechanisms concerning germ cell progression specifically in amphibians, present results – for the first time – indicate that kisspeptins, not only in brain, but also in testis modulate the Gnrh system. With respect to the role that kisspeptins exert within the male gonad, a new role emerges in the participation of mechanisms regulating germ cell progression.

Declaration of interest

The authors have nothing to disclose.

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