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In vitro spermatogenesis – optimal culture conditions for testicular cell survival, germ cell differentiation, and steroidogenesis in rats

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Although three-dimensional testicular cell cultures have been demonstrated to mimic the organization of the testis *in vivo* and support spermatogenesis, the optimal culture conditions and requirements remain unknown. Therefore, utilizing an established three-dimensional cell culture system that promotes differentiation of pre-meiotic murine male germ cells as far as elongated spermatids, the present study was designed to test the influence of different culture media on germ cell differentiation, Leydig cell functionality, and overall cell survival. Single-cell suspensions prepared from 7-day-old rat testes and containing all the different types of testicular cells were cultured for as long as 31 days, with or without stimulation by gonadotropins. Leydig cell functionality was assessed on the basis of testosterone production and the expression of steroidogenic genes. Gonadotropins promoted overall cell survival regardless of the culture medium employed. Of the various media examined, the most pronounced expression of *Star* and *Tspo*, genes related to steroidogenesis, as well as the greatest production of testosterone was attained with Dulbecco's modified eagle medium + glutamine. Although direct promotion of germ cell maturation by the cell culture medium could not be observed, morphological evaluation in combination with immunohistochemical staining revealed unfavorable organization of tubules formed *de novo* in the three-dimensional culture, allowing differentiation to the stage of pachytene spermatocytes. Further differentiation could not be observed, probably due to migration of germ cells out of the cell colonies and the consequent lack of support from Sertoli cells. In conclusion, the observations reported here show that in three-dimensional cultures, containing all types of rat testicular cells, the nature of the medium *per se* exerts a direct influence on the functionality of the rat Leydig cells, but not on germ cell differentiation, due to the lack of proper organization of the Sertoli cells.

Keywords: testis, spermatogenesis, cell culture, culture medium, Leydig cells, testosterone, stem cell niche

INTRODUCTION

Male infertility, a common disorder, is associated with a wide spectrum of spermatogenic failures, an increasing number of which are iatrogenic effects of clinical treatment (1). Treatment of children with cancer, including radiotherapy and high-dose chemotherapy, can severely damage the immature gonads and lead to infertility later in life (2). Since long-term survival of pre-pubertal patients with cancer has risen by as much as 80% during recent decades (3–5), more infertile patients can be expected in the future.

One approach to developing ways to rescue the fertility of these and other infertile patients is *in vitro* characterization of spermatogenesis, utilizing systems that mimic the natural situation as closely as possible and provide functional testicular cells for analyses (6).

In three-dimensional cultures of murine Sertoli, Leydig, peritubular, and germ cells stimulated with gonadotropins, pre-meiotic germ cells differentiate into postmeiotic spermatids, but with very low efficiency (6–8). Clearly, the optimal conditions for such cultures remain to be elucidated. In three-dimensional cultures containing all murine testicular cells, testosterone production by the Leydig cells was enhanced in response to stimulation by hCG for as long as 16 days (6). It remains to be determined whether similar Leydig cell function can be achieved with testicular cells from other species, including humans, under the same conditions.

To date, only traditional media, i.e., Dulbecco's modified eagle medium (DMEM) medium, F12, and minimal essential medium (MEM), have been employed for culturing testicular cells (9, 10). Even though it is well established that gonadotropins play a pivotal role in spermatogenesis and that functioning Leydig and other somatic cells are important for the spermatogenic process (7, 11–13), optimal culture conditions for the different types of testicular cells, and for appropriate paracrine interactions between these cells have not yet been determined.

Accordingly, in the present investigation we attempted to create an optimal culture system, of endocrine and paracrine stimulation focusing on the nutritional requirements for appropriate development of three-dimensional cultures of rat testicular cells. More specifically, we assessed germ cell differentiation, tubule formation, Leydig cell functionality, and cell survival in cultures hosting

all of the testicular cells, i.e., Sertoli, Leydig, peritubular, and germ cells.

MATERIALS AND METHODS

ANIMALS

Male Sprague-Dawley rats at 7 days of *post-partum* (d*pp*) age were purchased from Charles River (Sulzfeld, Germany) and transported to Karolinska Institutet (Stockholm, Sweden) together with their mothers. Each experiment involved testicular material from several different litters of these pups. Their use and handling was pre-approved by the ethics committee for experimental laboratory animals at Karolinska Institutet (N489/11).

TISSUE AND CELL PREPARATION

The rat pups were sacrificed by decapitation and their testes immediately placed in DMEM containing glutamine (P/N 41966, Gibco, CA, USA) and supplemented with 1% penicillin/streptomycin (pen/strep; P/N 15070, Gibco). Single-cell suspensions were obtained by the three-step enzymatic digestion described previously (14). In brief, the first digestion was performed with Collagenase/Dispase (P/N 269638, Roche, Switzerland, Basel; final concentration: 0.04/0.32 U/ml) in DMEM for 10 min at 32°C with shaking at 120 rpm, followed by centrifugation at $100 \times g$ for 2 min. The resulting supernatant was centrifuged again at $200 \times g$ for 8 min and the cell pellet thus obtained re-suspended in DMEM and stored on ice.

The second digestion was accomplished with Collagenase/Dispase + DNAse (P/N 104159, Roche; final concentrations: 0.04/0.32 and 48 U/ml, respectively) in DMEM for 15 min at 32°C with shaking at 120 rpm, followed by centrifugation at $100 \times g$ for 2 min. Centrifugation of the supernatant for 8 min at $200 \times g$ provided the second cell pellet, which was also re-suspended in DMEM and stored on ice.

The third digestion of remaining tissue involved Collagenase/Dispase + DNAse + Collagenase IV (P/N C-1889, Sigma-Aldrich, St. Louis, USA; final concentrations: 0.04/0.32, 48 and 50 U/ml, respectively) in DMEM for 20 min at 32°C with shaking at 120 rpm, followed by collection and re-suspension of the third cell pellet in the same manner as above. All three cell suspensions were pooled, centrifuged at $200 \times g$ for 8 min, re-suspended in 1 ml DMEM, counted in a Bürker chamber, and examined for viability by trypan blue staining (P/N 15250061, Gibco; 1:20 dilution).

CELL CULTURES

As stated in **Table 1**, the different media tested here were DMEM + glutamine or without glutamine (DMEM − glutamine; P/N 21969, Gibco), DMEM + Glutamax (P/N 31966, Gibco), DMEM/F12 (P/N 21331, Gibco), F12 (P/N 21765, Gibco), and MEM (P/N 21430, Gibco). Pre-pubertal rat testicular cells were cultured in an agarose-medium matrix in accordance with previous reports (7). In brief, this matrix was prepared by mixing autoclaved 0.7% SeaKem® LE agarose (P/N 50004, Lonza, Basel, Switzerland) or 0.7% LMP agarose (P/N 15517022, Invitrogen, CA, USA) with the relevant culture medium (supplemented with 1% pen/strep) at a ratio of 1:1 to give a final agarose concentration of 0.35% agarose.

Table 1 | Schematic illustration of the experimental conditions employed to characterize the effects of the culture medium and gonadotropins on three-dimensional cultures of testicular cells.

Medium	Supplement			
	AA (%)	NEAA (%)	rFSH (IU/l)	hCG (IU/l)
DMEM (high glucose, +pyruvate, +l-glutamine; P/N 41966, Gibco)	–	–	5.0	5.0
	–	–	–	–
DMEM (high glucose, +pyruvate, −l-glutamine; P/N 21969, Gibco)	–	–	5.0	5.0
	–	–	–	–
DMEM (high glucose, +pyruvate, +Glutamax; P/N 31966, Gibco)	–	–	5.0	5.0
	–	–	–	–
F12 (+l-glutamine; P/N 21765, Gibco)	–	–	5.0	5.0
	–	–		
	4.0	–	5.0	5.0
	–	–		
	–	4.0	5.0	5.0
	–	–		
	4.0	4.0	5.0	5.0
	–	–		
DMEM/F12 (without l-glutamine; P/N 21331, Gibco)	–	–	5.0	5.0
	–	–	–	–
MEM (without l-glutamine; P/N 21430, Gibco)	–	–	5.0	5.0
	–	–	–	–

AA, amino acids; NEAA, non-essential amino acids; rFSH, recombinant follicle-stimulating hormone; hCG, human chorionic gonadotropin; IU/l, international units per liter; M, molar mass (kg/mol); DMEM, Dulbecco modified Eagle's medium; MEM, minimal essential medium; − = none.

These cultures were exposed to recombinant follicle-stimulating hormone [rFSH; P/N Gonal F 75 IE, Merck, Frankfurt, Germany; final concentration: 5I U/l (international units per liter)] and human chorionic gonadotropin (hCG; P/N Pregnyl 5000 IE, Merck Sharpe and Dohme, NJ, USA; final concentration: 5I U/l) as also described in **Table 1**. The influence of amino acids on testosterone production were evaluated by adding essential amino acids (AA; P/N 11130-036, Gibco) or non-essential amino acids (NEAA; P/N 11140-035, Gibco) separately to F12 medium at a final concentration of 4%, similar to their concentrations in DMEM.

The single-cell suspensions (1.0×10^6 cells/ml) were inoculated into the agarose-medium matrix before it solidified. To study cell migration, individual cell colonies, containing 50–100 cells each, were aspirated into a 22S-gage Hamilton syringe (P/N 80665/00, Hamilton Bonaduz AG, Bonaduz, Switzerland), placed separately onto six-well culture dishes (Gibco) containing DMEM (a high concentration of glucose + pyruvate, + L-glutamine; P/N 41966, Gibco) and cultured for as long as 5 days without changing the medium. All cell cultures were maintained at 35°C under 5% CO_2 and performed in triplicates.

IMMUNOHISTOCHEMICAL, IMMUNOFLUORESCENT, AND MORPHOLOGICAL ANALYSES

Testicular tissue and cell cultures were fixed in 4% paraformaldehyde (PFA; P/N15812-7, Sigma-Aldrich) overnight at 4°C, followed by serial dehydration in 30, 50, and 70% aqueous ethanol (24 h at each concentration) at room temperature (RT). Thereafter, the samples were placed for 6 h each in 80, 96, and 99.6% ethanol at RT, followed by soaking in 100% butyl acetate for 6 h at RT (P/N 45860, Sigma-Aldrich). Subsequently, these samples were embedded in paraffin (Paraplast X-TRA®; P/N P3808, Sigma-Aldrich) at 61°C overnight in standard fashion; cut into 5–20 μm slices using a Biocut sectioning machine (Reichert-Jung, NY, USA) and then placed on microscope slides (P/N10143352, Superfrost Plus, Thermo Scientific, MA, USA).

For immunohistochemical (IHC) and immunofluorescent (IF) staining, these samples were next de-paraffinized with xylene (P/N 02080, HistoLab, Gothenburg, Sweden) for 10 min and then serially rehydrated with 99.6, 96, and 70% aqueous ethanol, each step being performed twice for 5 min. After washing twice with phosphate-buffered saline (PBS, pH 7.4; P/N 14190-094, Gibco), antigen retrieval was achieved either by incubation with 0.1% sodium citrate (P/N S4641, Sigma-Aldrich) and 0.1% Triton X-100 (P/N 11869, Merck) in PBS for 8 min at RT or by heating for 15 min in 0.1 M sodium citrate buffer (P/N S4641, Sigma-Aldrich; pH 6) in a microwave oven at 600 W. Blocking was performed for 20 min at RT with 5% goat serum (P/N S-1000, VECTOR, CA, USA) or 5% donkey serum (P/N 017-000-121, Jackson ImmunoResearch, West Grove, PA, USA), depending on the secondary antibody employed, in 0.1% BSA (Bovine serum albumin; P/N A4503, Sigma-Aldrich) in PBS.

Rabbit polyclonal anti-Ddx4 antibody (also known as Vasa; P/N ab13840, Abcam, Cambridge, UK, 1:200 dilution, final concentration 5 μg/ml) in PBS containing 0.1% BSA was used for IHC staining, with non-specific rabbit IgGs (P/N ab27478, Abcam, final concentration 5 μg/ml and P/N sc-2027, Santa Cruz, CA, USA, final concentration 5 μg/ml) as negative controls. Polyclonal rabbit anti-Ap-2gamma (Ap-2γ; P/N sc-8977, Santa Cruz, 1:100 dilution, final concentration 2 μg/ml in PBS containing 0.1% BSA) was utilized for immunofluorescence staining, again with rabbit IgGs (P/N sc-2027, Santa Cruz, final concentration 2 μg/ml) as negative controls.

After incubation with the primary antibodies or control IgGs at 4°C overnight and three subsequent washes at RT with PBS, samples were stained immunohistochemically with biotinylated goat anti-rabbit IgG secondary antibodies (P/N ab64256, Abcam, final concentration 5 μg/ml) at RT for 2 h; then, washed three times with PBS, incubated with ABC reagents (P/N PK-6100, VECTOR); and developed with DAB (Diaminobenzidine; SK-4100, VECTOR). These slides were counterstained with hematoxylin (Mayer's Hemalaun solution; P/N 1092491000, Merck), serially dehydrated with increasing aqueous ethanol solutions and then 100% xylene, and mounted with Entellan® new (P/N 1079610100, Merck). For IF staining, samples were incubated with a Cy^3-conjugated donkey anti-rabbit IgG secondary antibody (P/N 711-166-152, Jackson ImmunoResearch, West Grove, PA, USA, 1:600 dilution, final concentration 2.5 μg/ml) at RT for 1 h and the slides then counterstained and mounted with VECTASHIELD mounting medium containing DAPI (P/N H-1500, VECTOR).

For IF double-staining, paraffin-embedded samples on slides were first de-paraffinized with xylene for 10 min and then gradually rehydrated with 99.6, 96, and 70% ethanol, each step being performed twice for 5 min as described above. Staining was achieved employing the protocol described by van den Driesche and colleagues (15). In brief, for antigen retrieval, slides were treated with 0.01 M sodium citrate buffer, pH 6.0, containing 0.05% Tween 20 (P/N 8.17072.1000, Merck) at 96°C for 20 min in a water bath and thereafter blocked with 3% H_2O_2 (P/N 1.07209.0250, Merck) dissolved in methanol (P/N 1.06009.2511, Merck) for 30 min at RT. After two 5-min washes in Tris-buffered saline (TBS; P/N sc-24951, Santa Cruz), the sections were again blocked using 20% chicken serum (P/N C5405, Sigma-Aldrich) in TBS containing 5% BSA (P/N 001-000-161 Jackson ImmunoResearch) (TBS/NChS/BSA).

Subsequently, rabbit polyclonal primary antibodies against Ddx4 (P/N ab13840, Abcam, 1:200 dilution, final concentration 5 μg/ml), rabbit monoclonal primary antibodies against vimentin (P/N ab92547, Abcam, 1:200 dilution, final concentration 5 μg/ml), rabbit polyclonal primary antibodies against 3βHSD (P/N sc-28206, Santa Cruz, 1:200 dilution, final concentration 1 μg/ml) or rabbit IgGs (negative control) (P/N ab27478, Abcam, final concentration 5 μg/ml), all diluted in TBS/NChS/BSA, were incubated with the samples at 4°C overnight. The slides were then washed in TBS three times for 5 min each, followed by incubation with peroxidase-conjugated chicken secondary anti-rabbit antibody (P/N sc-2963, Santa Cruz, 1:200 dilution, final concentration 2 μg/ml) in TBS/NChS/BSA for 30 min at RT. After again washing in TBS three times for 5 min each, the Tyramide Fl kit (Perkin-Elmer-TSA plus Fluorescein System; P/N NEL741001KT, Perkin Elmer Life Sciences, Boston, USA) was employed in accordance with the manufacturer's instructions. After washing once more with TBS, the sections were blocked again with 3% H_2O_2 in TBS–Tween for 30 min at RT, followed by blocking in TBS/NChS/BSA for 30 min at RT.

Thereafter, the sections were incubated with polyclonal rabbit primary anti-Ki67 antibodies (P/N ab27478, Abcam, dilution 1:200, final concentration 5 μg/ml) or rabbit IgGs (negative control) (P/N ab27478, Abcam, final concentration 5 μg/ml), both diluted in TBS/NChS/BSA, at 4°C overnight. Following washing with TBS, the samples were then incubated with peroxidase-conjugated chicken secondary anti-rabbit antibody (P/N sc-2963, Santa Cruz, 1:200 dilution, final concentration 2 μg/ml) dissolved in TBS/NChS/BSA for 30 min at RT. After again washing with TBS, the Tyr–Cy5 system (Perkin-Elmer-TSA plus Cyanine3 System; P/N NEL744001KT, Perkin Elmer Life Sciences) was applied in accordance with the manufacturer's protocol and the slides subsequently mounted in VECTASHIELD mounting medium containing DAPI (P/N H-1500, VECTOR).

The different types of male germ cells were identified on the basis of morphological characteristics described previously: spermatogonia: round to oval nucleus with densely stained chromatin; leptotene spermatocytes: round with chromatin "speckled" nucleus; early pachytene spermatocytes: slightly larger nucleus containing chromatin cords throughout (16).

All stained sections were examined under an Eclipse E800 microscope (Nikon, Japan, Tokyo) and photographed with a 12.5 million-pixel cooled digital color camera system (Olympus DP70, Tokyo, Japan).

STAINING OF APOPTOTIC CELLS

To evaluate the influence of the various media on the viability of testicular cells *in vitro,* apoptosis was assessed using the TUNEL (Terminal deoxynucleotidyl transferase dUTP nick end-labeling) assay kit (DeadEnd™ Colorimetric Tunel System, P/N G7130, Promega, WI, USA) in accordance with the protocol provided. In brief, cell cultures were fixed in 4% PFA (P/N 8187081000, Merck) overnight at 4°C, followed by serial dehydration in 30, 50, and 70% aqueous ethanol for 24 h each. The samples were then transferred into 80, 96, and 99.6% ethanol for 6 h each at RT, followed by soaking in 100% butyl acetate for 6 h at RT (P/N 45860, Sigma-Aldrich) and, thereafter, routine embedding in paraffin (Paraplast X-TRA®; P/N P3808, Sigma-Aldrich) at 61°C overnight. After being cut into 5–20 μm slices using a Biocut sectioning machine (Reichert-Jung, NY, USA) and placed on microscope slides (P/N 10143352, Superfrost Plus, Thermo Scientific, MA, USA), the paraffin-embedded samples were de-paraffinized with xylene for 10 min; serially rehydrated with 99.6, 96, and 70% aqueous ethanol, with each step being performed twice for 5 min, and then washed twice with PBS.

Thereafter, the samples were treated with proteinase K (20 μg/ml in PBS) for 20 min at RT; washed again with PBS; and then incubated with biotinylated nucleotide mix + rTDT enzyme + buffer at 37°C for 1 h (adding only biotinylated nucleotide mix to the negative control). After terminating the reaction with stopping buffer (provided with the kit) and washing in PBS, endogenous peroxidase was blocked using 0.3% hydrogen peroxide (also supplied with the kit) in PBS for 15 min at RT. The samples were then incubated with streptavidin–HRP (from the kit) for 30 min at RT, stained with DAB (from the kit); counterstained with hematoxylin (Mayer's Hemalaun solution; P/N 1092491000, Merck); dehydrated with increasing concentrations of aqueous ethanol and then 100% xylene; and mounted with Entellan® new (P/N 1079610100, Merck). By examining at least 500 cells in each sample under an ECLIPSE E800 microscope (Nikon), the percentage of TUNEL-positive (i.e., apoptotic) cells was finally determined. The apoptotic frequency is expressed relative to the corresponding frequency on the first day of culturing, in order to minimize the effect of possible differences in culturing techniques.

TESTOSTERONE ASSAY

Testosterone production following 0, 1, 7, and 14 days of culture was employed as a measure of the influence of various media on the functionality of Leydig cells. First, testosterone was extracted by adding 0.5 ml ethyl acetate (P/N 1096232500, Merck) to the culture samples, each in a 1.5 ml Eppendorf tube, followed by vigorous automatic shaking for 15 min. After centrifugation for 2 min at 16000 × *g*, the resulting supernatant was re-subjected to the same procedure. The two ethyl acetate extracts were combined and evaporated overnight; the pellet obtained dissolved in PBS and the COAT-A-COUNT® kit (P/N TKTT2, Siemens, Germany, Munich) used to quantify testosterone in accordance with the manufacturer's protocol.

RNA EXTRACTION AND cDNA SYNTHESIS

Employing samples collected at the time-points designated and stored thereafter at −80°C, RNA was extracted as described previously (17). In brief, each sample was lysed with TRIzol® reagent (P/N 15596018, Invitrogen) and disrupted for 30 s in an ULTRA-TURRAX T25 homogenizer (JANKE and KUNKEL, Staufen, Germany). Following addition of chloroform (P/N 1024452500, Merck) and centrifugation at 16000 × *g* for 10 min at 4°C, a half volume of ethanol 100% was added to the aqueous upper phase containing the RNA and the sample then applied to the spin column of the RNeasy Mini Kit (P/N 74104, Qiagen, Venlo, Netherlands) in accordance with the manufacturer's protocol. The RNA thus isolated was treated with DNase 1 Amplification Grade (P/N AMPD1, Sigma-Aldrich) to eliminate contamination by DNA and thereafter 0.6 μg RNA from each sample were used to synthesize 20 μl cDNA with the IScript™ cDNA synthesis kit (P/N 170-8891, Bio-Rad, CA, USA) as instructed by the manufacturer.

ANALYSIS OF GENE EXPRESSION

The influence of the various culture media on steroidogenesis and male germ cell differentiation was examined by analyzing relative gene expression by quantitative PCR (qPCR).

To assess steroidogenic gene expression, the iQ SYBER® Green Super mix (P/N 170-8882, Bio-Rad) was employed as instructed and qPCR performed with the iCycler iQ multicolor RT-PCR detection system (Bio-Rad). The qPCR program was initiated with denaturation (3 min at 96°C); followed by 40 cycles of denaturation (96°C for 10 s) and annealing/elongation (60°C for 45 s). Two genes expressed specifically by rat Leydig cells – i.e., those encoding steroidogenic acute regulatory protein (*Star*) and peripheral benzodiazepine receptor or translocator protein (*Tspo*) – were examined, with beta actin (*Actb*) as the endogenous control. The qPCR efficiencies for *Star*, *Tspo*, and *Actb* were 87.6, 85.8, and 94.2%, respectively. All primer sequences and product sizes are documented in **Table 2**. The mean gene expression for the triplicates run in each medium was calculated by the ddCt procedure and then normalized to the mean level of *Actb* mRNA (dCt). Freshly isolated cells inoculated into agarose without gonadotropins were snap frozen immediately and the gene expression in each sample presented relative to the corresponding expression in these day-0 cells [fold-change ($2^{-\mathrm{ddCT}}$)].

In the case of male germ cell differentiation *in vitro*, TaqMan® probes and TaqMan® Gene Expression Master Mix (P/N 4369510, Applied Biosystems, Life technologies, CA, USA) were employed using the protocol suggested. In brief, utilizing the iCycler iQ multicolor RT-PCR detection system (Bio-Rad), the qPCR program started with 2 min at 50°C; then 10 min at 95°C; followed by 45 cycles of two steps; 15 s at 95°C and 1 min at 60°C. Six genes, expressed specifically in connection with germ cell differentiation were investigated, i.e., *Kit, Zbtb16* (zinc finger- and BTB-domain containing 16), *Dazl* (deleted in azoospermia-like), *Boll* [Boule-like (*Drosophila*)], *Crem* (cAMP responsive element modulator), and *Prm1* (protamine 1). The TaqMan® probes utilized and assay numbers are listed in **Table 3**. The mean gene expression for the

Table 2 | The primers and conditions used for qPCR.

Gene	Primer sequence 5′–3′	Amplicon size (bp)	Conditions
Star	Fw: CTGCTAGACCAGCCCATGGAC	90	40 cy
	Rev: TGATTTCCTTGACATTTGGGT		60°C
Tspo	Fw: GCTATGGTTCCCTTGGGTCT	195	40 cy
	Rev: GGCCAGGTAAGGATACAGCA		60°C
Actb	Fw: TGAAGATCAAGATCATTGCTC	120	40 cy
	Rev: ACTCATCGTACTCCTGCTTGC		60°C

Bp, basepair; cy, cycles.

Table 3 | The assay and conditions used for qPCR.

Gene	TaqMan® assay number	Conditions
Kit	Rn00573942_m1	45 cy
		60°C
Zbtb16	Rn01418644_m1	45 cy
		60°C
Dazl	Rn01757162_m1	45 cy
		60°C
Boll	Rn01441407_m1	45 cy
		60°C
Crem	Rn01538528_m1	45 cy
		60°C
Prm1	Rn02345725_g1	45 cy
		60°C
Actb	Rn00667869_m1	45 cy
		60°C

Cy, cycles.

three triplicates run in each medium was calculated by the ddCt procedure and normalized to the corresponding mean level of *Actb* mRNA (dCt). The gene expression in each sample is presented relative to the corresponding expression in DMEM + glutamine [fold-change (2^{-ddCT})].

STATISTICAL ANALYSES

Gene expression, apoptotic frequency, and testosterone production were calculated as the means ± standard deviations (SD) for the triplicates run under each condition. Student's *t*-test, One-way ANOVA and One-way RM ANOVA were applied to compare the differences between experimental conditions (SigmaPlot 11.0; Systat Software Inc., CA, USA). Following the Shapiro–Wilk test for normality, pairwise multiple comparisons were performed with the "Holm–Sidak" procedure as stated in the Figure legends (SigmaPlot 11.0; Systat Software Inc.). A difference was considered to be statistically significant if the p value was ≤ 0.05.

RESULTS

INFLUENCE OF THE VARIOUS CULTURE MEDIA AND GONADOTROPINS ON THE CAPACITY OF LEYDIG CELLS IN A THREE-DIMENSIONAL CULTURE TO PRODUCE ANDROGENS

Comparison of the production of testosterone during the first 24 h of *in vitro* culture with stimulation by gonadotropins revealed significantly lower testosterone levels with F12, DMEM/F12, and MEM media than with DMEM + glutamine (**Figure 1**). According to the supplier, DMEM + glutamine contains higher levels of amino acids than F12 and DMEM/F12, but addition of NEAA or AA to the F12 medium did not elevate testosterone production to the same level as with DMEM + glutamine (**Figure 1A**). For all media examined, testosterone production was stimulated by gonadotropins, as expected (**Figure 1A**).

INFLUENCE OF THE VARIOUS CULTURE MEDIA ON THE EXPRESSION OF STEROIDOGENIC GENES BY LEYDIG CELLS IN THREE-DIMENSIONAL CULTURES

As assessed by qPCR, within 1 day of stimulation by gonadotropins the relative up-regulation of *Star* expression was fivefold with DMEM + glutamine, threefold with F12, threefold with F12/NEAA, twofold with F12/AA, onefold with DMEM/F12, and fourfold with MEM (**Figure 1B**). The increase with DMEM + glutamine was significantly higher than with all of the other culture media except MEM. Moreover, after 1 day of stimulation with gonadotropins, the relative expression of *Tspo* was also up-regulated (DMEM + glutamine, threefold; F12, threefold; F12/NEAA, threefold; F12/AA, onefold; DMEM/F12, onefold; and MEM, onefold) (**Figure 1C**). This elevation was significantly greater with DMEM + glutamine than F12/AA, DMEM/F12 or MEM. Thus, with DMEM + glutamine, up-regulation of both *Star* and *Tspo* was most pronounced, in agreement with the observation that testosterone production was highest in the same medium.

INFLUENCE OF THE VARIOUS CULTURE MEDIA ON TESTOSTERONE PRODUCTION BY LEYDIG CELLS IN THREE-DIMENSIONAL CULTURES

The functionality of the Leydig cells in the mixture of testicular cells was assessed on the basis of testosterone production after 1, 7, and 14 days of culture, both in the presence and absence of gonadotropins. There was a significant difference between stimulated and un-stimulated cells at all three time-points with DMEM + glutamine, DMEM without glutamine (−glutamine), or DMEM + Glutamax. After 1 day of stimulation this production was highest with DMEM + glutamine, followed by DMEM + Glutamax, and the lowest level with DMEM − glutamine (**Figure 2A**), but there was no significant difference between these three media in this respect following stimulation for 7 or 14 days (**Figures 2B,C**). At the same time, DMEM + Glutamax promoted the capacity of basal (unstimulated) Leydig cells to produce testosterone after 1, 7, and 14 days to a greater extent than DMEM+ or −glutamine (**Figures 2A–C**). Moreover, the levels of testosterone after 1 day of culture in all three media with gonadotropins (DMEM + glutamine: 79 ± 11 nmol/l; DMEM − glutamine: 39 ± 4 nmol/l; DMEM + Glutamax: 58 ± 12 nmol/l) as well as in DMEM + Glutamax without stimulation (20 ± 6 nmol/l), were higher than after 7 days (DMEM + glutamine: 14 ± 4 nmol/l; DMEM − glutamine: 10 ± 2 nmol/l; DMEM + Glutamax: 15 ± 3 nmol/l; DMEM + Glutamax without stimulation: 4 ± 1 nmol/l) or 14 days (DMEM + glutamine: 16 ± 6 nmol/l; DMEM − glutamine: 12 ± 3 nmol/l; DMEM + Glutamax: 16 ± 5 nmol/l; DMEM + Glutamax without stimulation: 6 ± 2 nmol/l) of culture (**Figures 2A–C**).

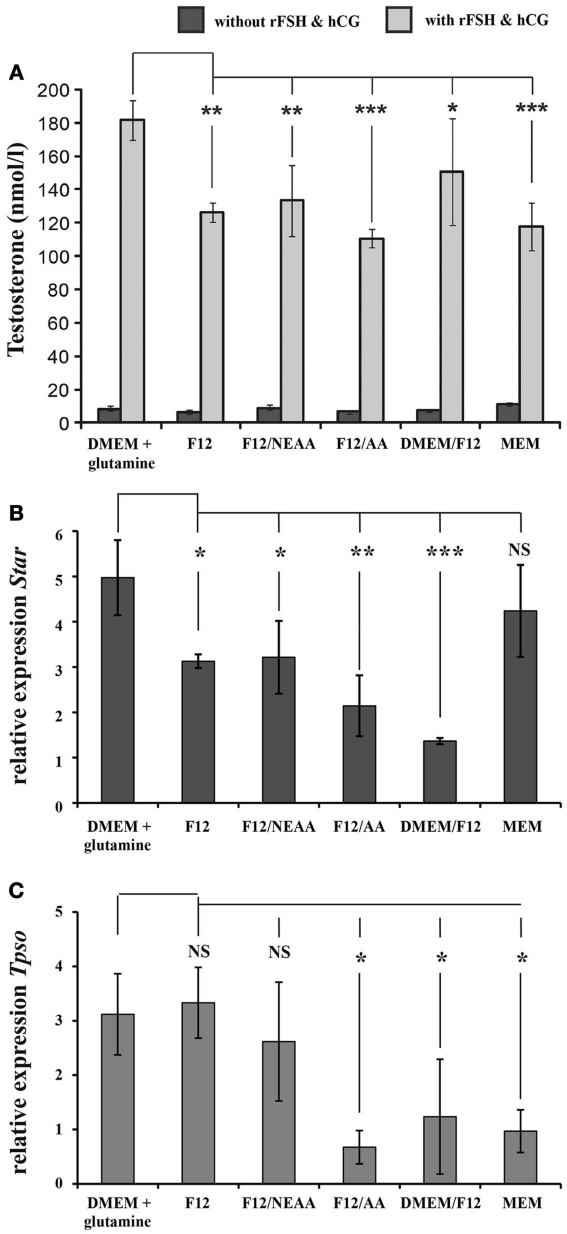

FIGURE 1 | The influence of various culture media on the capacity of the Leydig cells in three-dimensional cultures of rat testicular cells to produce testosterone and express steroidogenic genes. **(A)** On the *X*-axis are the different culture media employed [DMEM + glutamine (GL), F12, F12 + NEAA (non-essential amino acids), F12 + AA (essential amino acids), F12/DMEM, and MEM (minimal essential medium)], and the *Y*-axis depicts the concentration of testosterone (evaluated by radioimmunoassay and expressed in nanomoles/liter) in the medium of cells cultured for 1 day. The relative expression of **(B)** *Star* (Steroidogenic Acute Regulatory Protein) and **(C)** *Tspo* (Translocator Protein) (determined by qPCR analysis with *Actb* as an internal control) by testicular cell suspensions from 7 d*pp* rats cultured for 1 day in six different media in the presence (light columns) or absence (dark columns) of hCG and FSH. The mean relative expression for triplicates was calculated by the ddCt procedure. One-way ANOVA with the Shapio–Wilk test for normality was applied to compare the different experimental conditions. NS: non-significant; $*p < 0.05$, $**p < 0.01$, $***p < 0.001$ in comparison to the value with DMEM + glutamine.

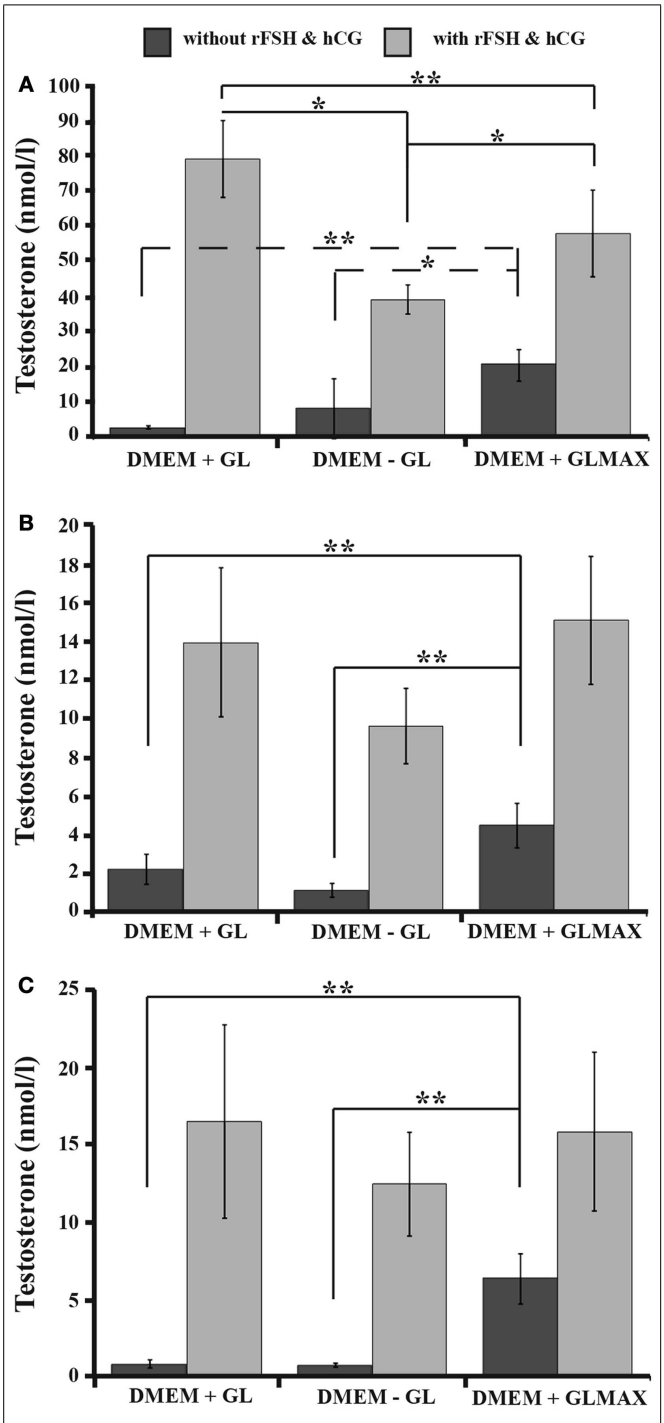

FIGURE 2 | The influence of different DMEM culture media on testosterone production by the Leydig cells in three-dimensional cultures of rat testicular cell. The cells were cultured for 14 days in DMEM + glutamine (GL), DMEM − glutamine (GL), or DMEM + Glutamax (GLMAX) (presented on the *X*-axis) either with (light columns) or without (dark columns) hCG and rFSH stimulation. The concentration of testosterone in the culture medium following 1 day **(A)**, 7 days **(B)**, and 14 days **(C)** of culture (determined by radioimmunoassay and expressed in nanomoles/liter) is shown on the *Y*-axis. One-way RM ANOVA with the Shapio–Wilk test for normality was applied to compare the different experimental conditions. $*p < 0.05$, $**p < 0.01$.

GONADOTROPINS PROTECT RAT TESTICULAR CELLS IN THE DIFFERENT CULTURE MEDIA FROM APOPTOSIS

Cell proliferation, expressed as the percentage of Ki67 positive cells (%) after 1 day of culture was 3.2 ± 0.8 with DMEM + glutamine, 3.9 ± 0.9 with DMEM + Glutamax, and 2.6 ± 2.6 with F12, with no significant differences. Nor did the relative numbers of different cell types immediately following the enzymatic digestion and after 1 day of culture differ between the culture media examined (DMEM + glutamine: 82 ± 17% Ddx4-positive cells, 32 ± 10% Vimentin-positive cells, 2 ± 1% 3 βHSD-positive cells; DMEM + Glutamax: 77 ± 13% Ddx4-positive cells, 28 ± 10% Vimentin-positive cells, 3 ± 2% 3 βHSD-positive cells; F12: 92 ± 6% Ddx4-positive cells, 26 ± 9% Vimentin-positive cells, 1 ± 1% 3 βHSD-positive cells). Application of the TUNEL assay revealed a significantly lower rate of apoptosis following 7 days of culture with than without gonadotropins in DMEM + glutamine (15 vs. 33%) or DMEM + Glutamax (10 vs. 24%) (**Figure 3A**), but no such difference was observed in the case of the F12 medium. Without stimulation, the cells in DMEM + glutamine exhibited a higher apoptotic rate (33%) than those in DMEM + Glutamax (24%) or F12 (20%), whereas there was no such difference when these three media were supplemented with gonadotropins (**Figure 3A**).

MEDIUM-RELATED EFFECTS ON THE DIFFERENTIATION OF PRE-PUBERTAL RAT MALE GERM CELLS *IN VITRO*

When expression of *Zbtb16* (also known as *Plzf*), *Kit*, *Dazl*, *Boll*, *Crem*, and *Protamine* by cells cultured with hCG and FSH was evaluated by qPCR, the expression of *Zbtb16* in DMEM + glutamine was observed to be significantly higher (2.5-fold) after 21 days than after 0 and 7 days, with no such changes in the case of DMEM + Glutamax or F12 and no significant differences between these three different media (**Figure 3B**). With DMEM + glutamine or DMEM + Glutamax, *Kit* expression was down-regulated after 7 (threefold) and 21 days (fivefold) in culture, whereas in cells cultured in F12 this expression remained constant during the entire experimental period (**Figure 3C**). Expression of *Dazl* by cells cultured in DMEM + glutamine or DMEM + Glutamax was significantly down-regulated (10-fold) after 7 and 21 days with a similar, although not significant tendency in the case of F12 (11- and 3-fold down-regulation after 7 and 21 days, respectively) (**Figure 3D**). After 7 days, only cells in DMEM + Glutamax demonstrated down-regulation (2.5-fold) of *Crem* expression (**Figure 3E**), while after 21 days, expression of *Crem* was significantly higher with F12 than DMEM + glutamine (threefold) or DMEM + Glutamax (twofold) (**Figure 3E**). No expression of *Boll* or *Protamine* was detected under any of the experimental conditions (data not shown).

MORPHOLOGICAL EVALUATION AND IMMUNOHISTOCHEMICAL AND FLUORESCENT STAINING

Morphological evaluation and IHC and IF staining revealed colony formation in the three-dimensional cultures of rat testicular cells (**Figure 4A**), with undifferentiated spermatogonia being detected in these colonies (**Figures 4B–D**). Following 3 days in culture, the colonies formed by un-stimulated cells were already less compact than those formed in the presence of gonadotropins (data not shown). Active cell migration toward colonies could be observed (**Figures 4E–G**). However, the total number of viable cell colonies was low.

Morphological evaluation of colonies formed in the three-dimensional culture (**Figures 4H,M**), as well as in conventional two-dimensional cultures (**Figure 4I**) revealed migration of cells from the inner side to the outer side of the colonies. These migrating cells could be identified as germ cells by IHC staining for Ddx4, a marker specific for germ cells (**Figures 4J–L**).

More detailed morphological analysis after 21 days *in vitro* showed small structures containing a mixture of Sertoli (**Figure 4N**) and peritubular cells (**Figure 4N**), as well as male germ cells in different stages of differentiation up to early pachytene spermatocytes (**Figure 4N**).

DISCUSSION

The major novel observations documented here are as follows: (1) the culture medium *per se* exerts a direct influence on the functionality of the rat Leydig cells, but not on germ cell differentiation in three-dimensional cultures; (2) rat germ cells migrating from the inner side to the outer side of the cell colonies suggest an unfavorable organization of tubules formed *de novo* in the three-dimensional culture; (3) undifferentiated rat spermatogonia differentiate up to the stage of pachytene spermatocytes in a similar time-period to the situation *in vivo* in three-dimensional cultures.

After 7 days of culture in three different media, less extensive apoptosis was observed among cells in the presence than in the absence of rFSH and hCG, in agreement with earlier findings in literature (18–20). The nature of the medium *per se* exerted no significant impact on overall cell survival.

Although in our three-dimensional cultures stimulation with gonadotropins promoted Leydig cell functionality (as reflected in testosterone production) after 1, 7, and 14 days regardless of the medium, DMEM + glutamine was clearly most effective in this respect after 1 day of stimulation. Thus, at this early time-point, the level of testosterone in the culture medium appeared to be related to the levels of glutamine [an important source of energy, as well as a precursor for protein synthesis (21–24)], since the other culture media examined contain less glutamine or none at all. In addition, cells cultured in DMEM + glutamine exhibited the most pronounced up-regulation of *Star* and *Tspo*, which transfer cholesterol (the precursor for testosterone) across an aqueous phase from the outer to the inner mitochondrial membrane (25–28) and are thereby essential for the steroidogenic process. Thus, the presence of glutamine in the culture medium may be essential for the synthesis of the enzymes and other proteins required for testosterone production.

Furthermore, since DMEM + glutamine medium contains higher levels of amino acids both (essential and non-essential) than F12, this difference was eliminated by adding essential or non-essential amino acids to the F12 medium. However, such supplementation did not increase testosterone production to a level similar to that obtained with DMEM + glutamine and addition of both kinds of amino acids to F12 resulted in a low pH and thereby a cytotoxic environment (data not shown). Moreover compared to

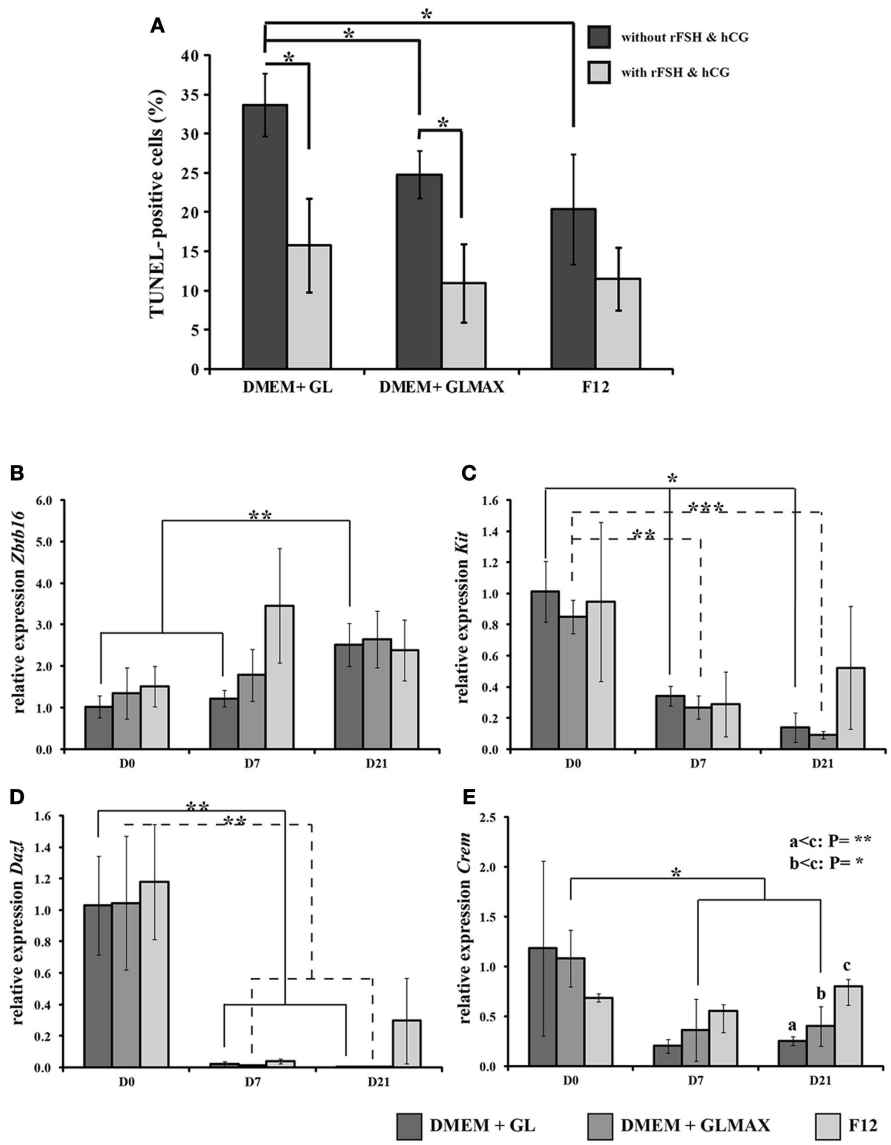

FIGURE 3 | The influence of different culture media on cell survival and expression of genes related to germ cell differentiation in a three-dimensional cultures of rat testicular cells. **(A)** The cells were cultured for 7 days in DMEM + glutamine (GL), DMEM + Glutamax (GLMAX), or F12 (presented on the *X*-axis) either with (light columns) or without (dark columns) hCG and rFSH stimulation. The percentage of apoptotic (TUNEL-positive) cells, normalized to the 1-day value, is shown on the *Y*-axis. **(B–E)** The cells were cultured for 0, 7, and 21 days. The graphs depict the relative expression of rat *Zbtb16* (also known as *Plzf*) **(B)**, *Kit* **(C)**, *Dazl* **(D)**, and *Crem* **(E)** (determined by qPCR analysis with *Actb* as an internal control) by cells cultured in DMEM + glutamine (DMEM + GL), DMEM + Glutamax (DMEM + GLMAX), or F12. On the *X*-axis, the different periods of culture [0 (D0), 7 (D7), and 21 (D21) days] are depicted and the *Y*-axis shows the mean relative expression of replicates calculated by the ddCt procedure. Student's *t*-test was applied to compare the different experimental conditions. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

DMEM + glutamine, the relative levels of expression of *Star* and *Tspo* were lower in cells cultured in F12 supplemented with essential amino acids, and expression of *Star* was lower when F12 was supplemented with non-essential amino acids medium. Of course, DMEM + glutamine and F12 also differ with respect to the levels of several other components, such as vitamins and inorganic salts, which might explain their different effects.

Analysis of the relative expression of genes associated with male germ cell differentiation (i.e., *Zbtb16*, *Kit*, and *Dazl* in spermatogonia, *Dazl* and *Boll* in spermatocytes, and *Crem* and *Protamine* in spermatids) by cells cultured in DMEM + glutamine, DMEM + Glutamax, or F12 supplemented with gonadotropins demonstrated that none of these media alone promoted robust spermatogenesis after 21 days of culture. The overall down-regulation of these genes might reflect the increase in the number of apoptotic cells with culture time, which could also explain at least partially the low efficiency of the three-dimensional culture system employed.

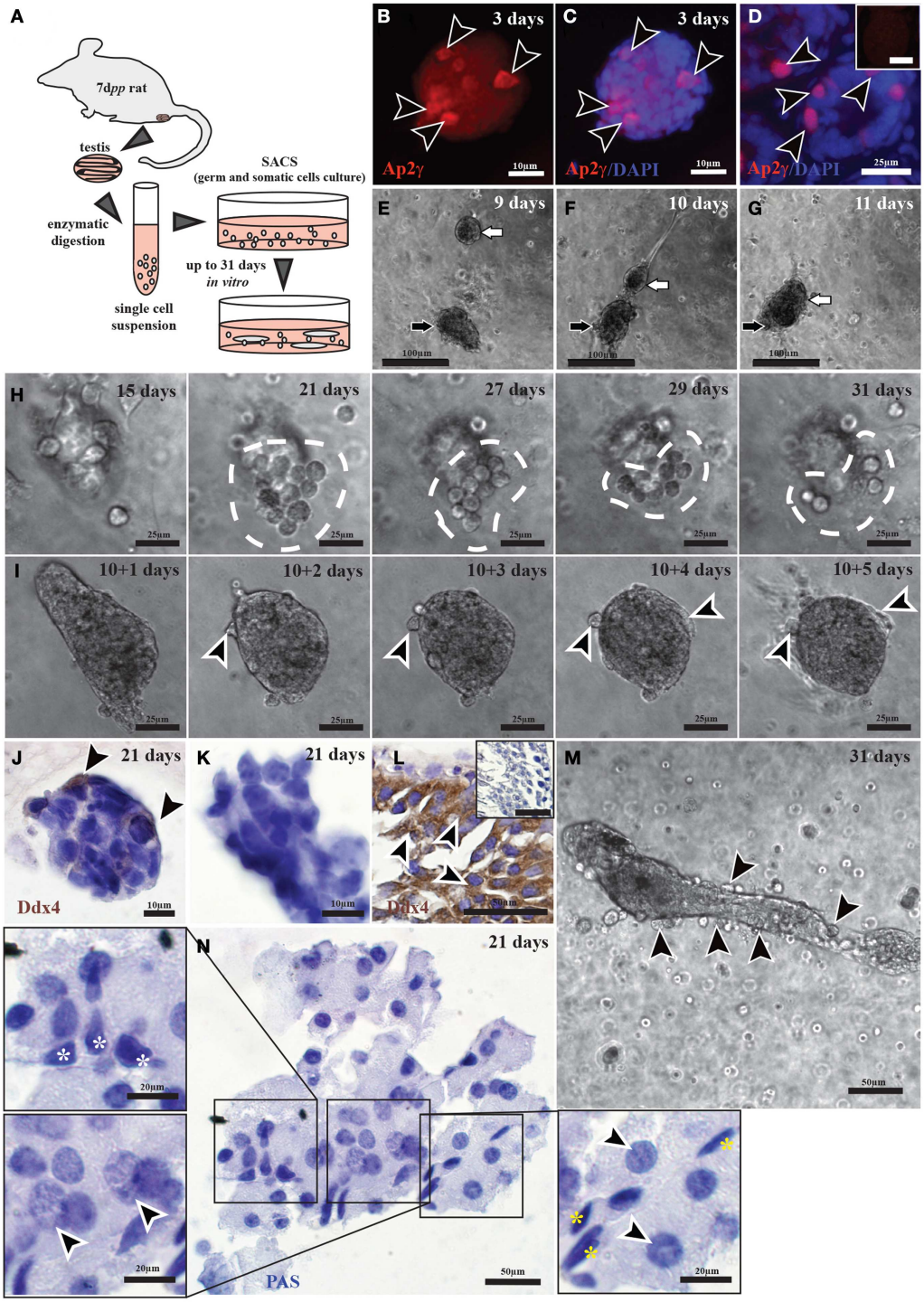

FIGURE 4 | Tubule formation by and germ cell differentiation of pre-pubertal rat testicular cells in three-dimensional cultures. **(A)** A schematic overview of the experimental conditions. **(B–D)** Immunofluorescent staining of undifferentiated spermatogonia (Ap-2γ) in cell colonies originated from culturing cells from 7-day-old rats for 3 days **(B,C)**, as well as from 8-day-old rats as a positive control **(D)** [Ap2γ: red staining (black arrow heads); DAPI: blue staining]. The negative control with IgGs is shown as small insert in **(D)**. **(E–G)** 9- **(E)**, 10- **(F),** and 11-day cultures **(G)** showing two colonies (the black and the white arrows) migrating toward one another. **(H,I,M)** Active migration of cells out of colonies cultured for as long as 31 days [white dashed line in **(H)**, black arrows heads in **(M)**] or following incubation of isolated cell colonies in liquid medium for 5 days [black arrow heads in **(I)**]. **(J–L)** Immunohistochemical staining for germ cells (Ddx4) in colonies originating from 7-day-old rats and cultured for 21 days **(J,K)**, as well as from 60-day-old rat testis [positive control; **(L)**] [Ddx4: brown staining (black arrow heads); Hematoxylin: blue staining]. **(N)** Cells cultured for 21 days exhibit morphologies similar to those of peritubular cells (yellow stars), Sertoli cells (white stars), leptotene spermatocytes, and early pachytene spermatocytes (black arrow heads).

However, there were certain differences in the expression of *Crem* by cells in the different media after 21 days. *Crem* is expressed primarily by spermatocytes, but also by Sertoli cells, although the latter expression appears not be necessary for spermatogenesis (29–32). *Crem* acts downstream of cAMP signaling (33) and its activation modulates the cAMP response element, thereby altering gene expression (32, 33). Interestingly, the different isoforms of the Crem protein act as a master switch for the regulation of various genes during spermatogenesis (30–32, 34).

After 21 days in culture, the cells in only a few of the colonies formed still exhibited an intact morphology, most having decreased in size. However, all colonies with intact cells contained a mixture of somatic (Sertoli and peritubular cells) and germ cells (differentiated as far as pachytene spermatocytes). Thus, undifferentiated spermatogonia, the only germ cells present in the testes of 7 d*pp* rats had differentiated as far as to the stage of pachytene spermatocytes, a level of differentiation similar to the situation *in vivo* at the age of 25–28 d*pp*. These observations indicate that at least a partially functional microenvironment supporting germ cell survival and differentiation was obtained.

Suitable support for germ cells through the formation and proper orientation of Sertoli and peritubular cells is needed for completion of spermatogenesis (13, 35, 36). As shown earlier, when utilized as feeders for germ cells or embryonic stem cells *in vitro*, Sertoli cells tend to be unorganized in contrast to their highly polarized orientation *in vivo* (35–37). Such disorganization presumably disallows the crucial support of the blood–testis barrier as a result of missing or premature junctional complexes between Sertoli cells (36). Such lack of support leads to meiotic arrest, with the meiotic germ cells going into apoptosis.

In our three-dimensional cultures, germ cells were seen to migrate out of the cell colonies formed and thereafter disintegrate and die within a couple of days due to the lack of support from the Sertoli cells. Strategies for obtaining the proper polarized orientation of the Sertoli cells and thereby establishing an appropriate niche for germ cell differentiation *in vitro* warrant more detailed investigations.

In conclusion, the present study demonstrates that although the nature of the culture medium *per se* does not influence the overall viability of rat testicular cells *in vitro*, it does influence the functionality of rat Leydig cells in three-dimensional cultures. Cells cultured in DMEM + glutamine medium displayed more testosterone production and higher expression of *Star* and *Tspo* than any of the other cell culture media examined. This might reflect the higher concentration of glutamine in this medium, but further studies concerning the influence of glutamine on Leydig cell functions, as well as on other endocrine/paracrine pathways in such complex three-dimensional cultures containing all types of testicular cells are required.

Differentiation of germ cell up to the stage of pachytene spermatocytes, i.e., similar to the situation *in vivo*, could be detected in a few small colonies hosting a mixture of somatic and germ cells. However, the crucial structural support provided by the Sertoli and peritubular cells in the seminiferous tubules *in vivo* could not be duplicated and none of the media examined provided a robust system for male germ cell differentiation *in vitro*. Thus, additional work on this question remains to be done.

AUTHOR CONTRIBUTIONS

Ahmed Reda: study design, data acquisition, analysis and interpretation, drafting the article, and final approval of the submitted version. Mi Hou, Luise Landreh, Kristín Rós Kjartansdóttir: data acquisition and analysis, drafting the article, and final approval of the submitted version. Konstantin Svechnikov, Olle Söder: data analysis and interpretation, drafting the article, and final approval of the submitted version. Jan-Bernd Stukenborg: study design, data acquisition, analysis and interpretation, drafting the article, and final approval of the submitted version.

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REFERENCES

1. Martin-du Pan RC, Campana A. Physiopathology of spermatogenic arrest. *Fertil Steril* (1993) **60**(6):937–46.
2. Jahnukainen K, Stukenborg JB. Clinical review: present and future prospects of male fertility preservation for children and adolescents. *J Clin Endocrinol Metab* (2012) **97**(12):4341–51. doi:10.1210/jc.2012-3065
3. Holoch P, Wald M. Current options for preservation of fertility in the male. *Fertil Steril* (2011) **96**(2):286–90. doi:10.1016/j.fertnstert.2011.06.028
4. Molgaard-Hansen L, Glosli H, Jahnukainen K, Jarfelt M, Jonmundsson GK, Malmros-Svennilson J, et al. Quality of health in survivors of childhood acute myeloid leukemia treated with chemotherapy only: a NOPHO-AML study. *Pediatr Blood Cancer* (2011) **57**(7):1222–9. doi:10.1002/pbc.22931
5. Robison LL, Green DM, Hudson M, Meadows AT, Mertens AC, Packer RJ, et al. Long-term outcomes of adult survivors of childhood cancer – results from the childhood cancer survivor study. *Cancer* (2005) **104**(11):2557–64. doi:10.1002/cncr.21249
6. Stukenborg JB, Schlatt S, Simoni M, Yeung CH, Elhija MA, Luetjens CM, et al. New horizons for in vitro spermatogenesis? An update on novel three-dimensional culture systems as tools for meiotic and post-meiotic differentiation of testicular germ cells. *Mol Hum Reprod* (2009) **15**(9):521–9. doi:10.1093/molehr/gap052
7. Stukenborg JB, Wistuba J, Luetjens CM, Elhija MA, Huleihel M, Lunenfeld E, et al. Coculture of spermatogonia with somatic cells in a novel three-dimensional soft-agar-culture-system. *J Androl* (2008) **29**(3):312–29. doi:10.2164/jandrol.107.002857
8. Abu Elhija M, Lunenfeld E, Schlatt S, Huleihel M. Differentiation of murine male germ cells to spermatozoa in a soft agar culture system. *Asian J Androl* (2012) **14**(2):285–93. doi:10.1038/aja.2011.112
9. Movahedin M, Ajeen A, Ghorbanzadeh N, Tiraihi T, Valojerdi MR, Kazemnejad A. In vitro maturation of fresh and frozen-thawed mouse round spermatids. *Andrologia* (2004) **36**(5):269–76. doi:10.1111/j.1439-0272.2004.00617.x
10. Sato T, Katagiri K, Gohbara A, Inoue K, Ogonuki N, Ogura A, et al. In vitro production of functional sperm in cultured neonatal mouse testes. *Nature* (2011) **471**(7339):504–7. doi:10.1038/nature09850
11. Reuter K, Schlatt S, Ehmcke J, Wistuba J. Fact or fiction: in vitro spermatogenesis. *Spermatogenesis* (2012) **2**(4):245–52. doi:10.4161/spmg.21983
12. Stukenborg JB, Kjartansdóttir KR, Reda A, Colon E, Albersmeier JP, Söder O. Male germ cell development in humans. *Horm Res Paediatr* (2014) **81**(1). doi:10.1159/000355599

13. Reuter K, Ehmcke J, Stukenborg JB, Simoni M, Damm OS, Redmann K, et al. Reassembly of somatic cells and testicular organogenesis in vitro. *Tissue Cell* (2014) **46**(1):86–96. doi:10.1016/j.tice.2013.12.001
14. Hou M, Andersson M, Zheng C, Sundblad A, Soder O, Jahnukainen K. Immunomagnetic separation of normal rat testicular cells from Roser's T-cell leukaemia cells is ineffective. *Int J Androl* (2009) **32**(1):66–73. doi:10.1111/j.1365-2605.2007.00819.x
15. van den Driesche S, Walker M, McKinnell C, Scott HM, Eddie SL, Mitchell RT, et al. Proposed role for COUP-TFII in regulating fetal Leydig cell steroidogenesis, perturbation of which leads to masculinization disorders in rodents. *PLoS One* (2012) **7**(5):e37064. doi:10.1371/journal.pone.0037064
16. Russel L, Shinha Hitaim A, Clegg E. *Histological and Histopathological Evaluation of the Testis*. St. Louis: Cache River Press (1990).
17. Wang L, Stegemann JP. Extraction of high quality RNA from polysaccharide matrices using cetyltrimethylammonium bromide. *Biomaterials* (2010) **31**(7):1612–8. doi:10.1016/j.biomaterials.2009.11.024
18. Billig H, Furuta I, Rivier C, Tapanainen J, Parvinen M, Hsueh AJ. Apoptosis in testis germ cells: developmental changes in gonadotropin dependence and localization to selective tubule stages. *Endocrinology* (1995) **136**(1):5–12. doi:10.1210/en.136.1.5
19. Tapanainen JS, Tilly JL, Vihko KK, Hsueh AJ. Hormonal control of apoptotic cell death in the testis: gonadotropins and androgens as testicular cell survival factors. *Mol Endocrinol* (1993) **7**(5):643–50. doi:10.1210/me.7.5.643
20. Steinberger E. Biological action of gonadotropins in the male. *Pharmacol Ther B* (1976) **2**(4):771–86.
21. Prusiner S, Miller RE, Valentine RC. Adenosine 3′:5′-cyclic monophosphate control of the enzymes of glutamine metabolism in *Escherichia coli*. *Proc Natl Acad Sci U S A* (1972) **69**(10):2922–6. doi:10.1073/pnas.69.10.2922
22. Tate SS, Meister A. Regulation of rat liver glutamine synthetase: activation by alpha-ketoglutarate and inhibition by glycine, alanine, and carbamyl phosphate. *Proc Natl Acad Sci U S A* (1971) **68**(4):781–5. doi:10.1073/pnas.68.4.781
23. Engstrom W, Zetterberg A. The relationship between purines, pyrimidines, nucleosides, and glutamine for fibroblast cell proliferation. *J Cell Physiol* (1984) **120**(2):233–41. doi:10.1002/jcp.1041200218
24. Wasa M, Bode BP, Abcouwer SF, Collins CL, Tanabe KK, Souba WW. Glutamine as a regulator of DNA and protein biosynthesis in human solid tumor cell lines. *Ann Surg* (1996) **224**(2):189–97. doi:10.1097/00000658-199608000-00012
25. Clark BJ, Wells J, King SR, Stocco DM. The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR). *J Biol Chem* (1994) **269**(45):28314–22.
26. Hasegawa T, Zhao L, Caron KM, Majdic G, Suzuki T, Shizawa S, et al. Developmental roles of the steroidogenic acute regulatory protein (StAR) as revealed by StAR knockout mice. *Mol Endocrinol* (2000) **14**(9):1462–71. doi:10.1210/mend.14.9.0515
27. Li H, Yao Z, Degenhardt B, Teper G, Papadopoulos V. Cholesterol binding at the cholesterol recognition/interaction amino acid consensus (CRAC) of the peripheral-type benzodiazepine receptor and inhibition of steroidogenesis by an HIV TAT-CRAC peptide. *Proc Natl Acad Sci U S A* (2001) **98**(3):1267–72. doi:10.1073/pnas.98.3.1267
28. Li H, Papadopoulos V. Peripheral-type benzodiazepine receptor function in cholesterol transport. Identification of a putative cholesterol recognition/interaction amino acid sequence and consensus pattern. *Endocrinology* (1998) **139**(12):4991–7. doi:10.1210/endo.139.12.6390
29. Wistuba II, Ashfaq R, Maitra A, Alvarez H, Riquelme E, Gazdar AF. Fragile histidine triad gene abnormalities in the pathogenesis of gallbladder carcinoma. *Am J Pathol* (2002) **160**(6):2073–9. doi:10.1016/S0002-9440(10)61157-1
30. Ruppert S, Cole TJ, Boshart M, Schmid E, Schutz G. Multiple mRNA isoforms of the transcription activator protein CREB: generation by alternative splicing and specific expression in primary spermatocytes. *EMBO J* (1992) **11**(4):1503–12.
31. Lamas M, Monaco L, Zazopoulos E, Lalli E, Tamai K, Penna L, et al. CREM: a master-switch in the transcriptional response to cAMP. *Philos Trans R Soc Lond B Biol Sci* (1996) **351**(1339):561–7. doi:10.1098/rstb.1996.0055
32. Walker WH, Habener JF. Role of transcription factors CREB and CREM in cAMP-regulated transcription during spermatogenesis. *Trends Endocrinol Metab* (1996) **7**(4):133–8. doi:10.1016/1043-2760(96)00035-5
33. Behr R, Weinbauer GF. Germ cell-specific cyclic adenosine 3′,5′-monophosphate response element modulator expression in rodent and primate testis is maintained despite gonadotropin deficiency. *Endocrinology* (1999) **140**(6):2746–54. doi:10.1210/endo.140.6.6764
34. Hecht NB. Molecular mechanisms of male germ cell differentiation. *Bioessays* (1998) **20**(7):555–61. doi:10.1002/(SICI)1521-1878(199807)20:7<555::AID-BIES6>3.3.CO;2-J
35. Gassei K, Schlatt S, Ehmcke J. De novo morphogenesis of seminiferous tubules from dissociated immature rat testicular cells in xenografts. *J Androl* (2006) **27**:611–8. doi:10.2164/jandrol.05207
36. Pan F, Chi L, Schlatt S. Effects of nanostructures and mouse embryonic stem cells on in vitro morphogenesis of rat testicular cords. *PLoS One* (2013) **8**(3):e60054. doi:10.1371/journal.pone.0060054
37. Ehmcke J, Hubner K, Scholer HR, Schlatt S. Spermatogonia: origin, physiology and prospects for conservation and manipulation of the male germ line. *Reprod Fertil Dev* (2006) **18**(1–2):7–12. doi:10.1071/RD05119

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Central pathways integrating metabolism and reproduction in teleosts

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Energy balance plays an important role in the control of reproduction. However, the cellular and molecular mechanisms connecting the two systems are not well understood especially in teleosts. The hypothalamus plays a crucial role in the regulation of both energy balance and reproduction, and contains a number of neuropeptides, including gonadotropin-releasing hormone (GnRH), orexin, neuropeptide-Y, ghrelin, pituitary adenylate cyclase-activating polypeptide, α-melanocyte stimulating hormone, melanin-concentrating hormone, cholecystokinin, 26RFamide, nesfatin, kisspeptin, and gonadotropin-inhibitory hormone. These neuropeptides are involved in the control of energy balance and reproduction either directly or indirectly. On the other hand, synthesis and release of these hypothalamic neuropeptides are regulated by metabolic signals from the gut and the adipose tissue. Furthermore, neurons producing these neuropeptides interact with each other, providing neuronal basis of the link between energy balance and reproduction. This review summarizes the advances made in our understanding of the physiological roles of the hypothalamic neuropeptides in energy balance and reproduction in teleosts, and discusses how they interact with GnRH, kisspeptin, and pituitary gonadotropins to control reproduction in teleosts.

Keywords: neuropeptide, metabolism, energy balance, fish, reproduction

INTRODUCTION

A close connection between energy balance and reproduction has been well documented in mammals (1). Energy balance is maintained by a process that controls food consumption, energy expenditure, and energy storage. A number of hypothalamic neuropeptides including orexin, ghrelin, neuropeptide-Y (NPY), melanin-concentrating hormone (MCH), pituitary adenylate cyclase-activating polypeptide (PACAP), proopiomelanocortin (POMC)-derived peptides, cholecystokinin (CCK), chicken gonadotropin-releasing hormone-II (cGnRH-II), 26RFamide (26RFa), galanin (GAL), and cocaine- and amphetamine-regulated transcript (CART) have been implicated in the regulation of feeding behavior and energy balance. On the other hand, peripheral hormones such as leptin and ghrelin provide information about the availability of stored metabolic foods.

Initiation of reproduction is affected by the amount of body energy reserves and is responsive to diverse metabolic factors. The neuroendocrine mechanisms responsible for the association between energy balance and fertility are represented by metabolic hormones and neuropeptides that affect the hypothalamic center controlling the expression and release of gonadotropin-releasing hormone (GnRH) (2, 3). Therefore, adequate body energy stores are crucial for full activation of the hypothalamus–pituitary–gonadal (HPG) axis at puberty and its proper functioning in adulthood (4). Generally high amount of food supply favor reproduction, while low food supply inhibits the reproductive system (1). During energetic challenges, the physiological mechanisms that partition energy into various activities tend to favor the processes for the survival of the individual over the processes for growth, longevity, and reproduction (5). Therefore, the reproductive system is suppressed by energetic challenges. At the same time it is also true that when the reproductive system is highly activated, animal primates reproduction rather than feeding. Many factors such as starvation, eating disorders, excessive exercise, cold exposure, and lactation act on both food intake and reproduction by increasing hunger and/or food ingestion and by suppressing reproductive processes (5, 6).

Most feeding-related neuropeptides in mammals have also been identified in fish species (7), suggesting that the regulatory system of feeding has been well conserved from fish to mammals. On the other hand, as the links between energy balance and reproduction have been demonstrated in several vertebrates (8), this might also exist in teleosts. Indeed, seasonal changes in feeding often coincide with spawning migration and reproduction in fish, suggesting association between nutrition and reproduction (9).

This review focuses on the role of the neuropeptides that regulate feeding and energy balance on reproduction in teleosts, and discusses if the metabolic control of reproduction is conserved from fish to mammals.

REGULATION OF REPRODUCTION IN TELEOSTS

In teleosts, as in other vertebrates, reproduction is coordinated by the HPG axis. The hypothalamus produces GnRH, which regulates the synthesis and release of gonadotropins (GTHs), follicle-stimulating hormone (FSH), and luteinizing hormone (LH), from the pituitary. The GTHs act on the gonads to stimulate gonadal development through the secretion of sex steroid hormones. These steroids, in turn, feedback to the brain and the

pituitary to complete the HPG axis and to regulate the reproductive cycle (10, 11). Thus, hypothalamic GnRH is considered as the key player in the regulation of reproduction in teleosts. Furthermore, recent findings of kisspeptin and gonadotropin-inhibitory hormone (GnIH) added new players in the reproductive system, which stimulate and inhibit mostly GnRH neurons, respectively.

GONADOTROPIN-RELEASING HORMONE (GnRH)

In the early 1970s, two research groups simultaneously reported the isolation of a LH-releasing factor from the hypothalamus of pigs and sheep (12, 13), and named it LH-releasing hormone (LHRH). Later, this decapeptide was also found to stimulate FSH release, and accordingly re-named GnRH. The GnRH isolated from mammals is also functional in fish, and stimulates the release of GTH in the carp (14). The first fish GnRH was identified in salmon, and named as salmon GnRH (sGnRH) (15). To date, 15 different forms of GnRH have been identified in vertebrates (13, 15–25), among them 10 original forms in fish species: salmon, sea bream, whitefish, medaka, catfish, herring, dogfish, and lamprey (lamprey I, II, and III). Most vertebrates possess two, and some teleosts have three, forms of GnRH in the brain (23, 25–31). Based on phylogenetic analysis, recent classification defines the species-specific (hypophysiotropic) form as GnRH1, while the most evolutionarily conserved chicken GnRH-II as GnRH2 (32). The third form is GnRH3 (33), which is present only in the brain of certain teleost species (31, 34).

Distribution of three different forms of GnRH in the brain was first reported in a perciform fish, the sea bream (35). GnRH1 neurons are generally present in the region from the ventral forebrain–preoptic area (POA) to basal hypothalamus, whereas GnRH2 neurons are restricted to the dorsal mesencephalon. GnRH3 neurons are located in the caudal-most olfactory bulb as a ganglion and along the terminal nerve in most fish species that possess three GnRH forms (31, 36). On the other hand, in the sea bream and the European sea bass, the distribution of GnRH1 and GnRH3 cells overlap in the olfactory bulbs, ventral telencephalon, and POA (37–40). Similar results were reported in several other fish species (41–45). In the sea bass brain, GnRH1 neuronal fibers are observed in the ventral surface of the forebrain, associated with the ventral telencephalon, POA, and the hypothalamus, whereas GnRH2 and GnRH3 neuronal fibers show profuse distributions throughout the brain (40).

The function of GnRH in the central regulation of LH release has been recognized in all orders of teleosts. Although the assay for FSH peptide is lacking for most fish species, studies in the rainbow trout (46–48) and the Coho salmon (49) show that GnRH also stimulates FSH release in salmonids. However, the different patterns of fiber projections of each GnRH form suggest different physiological function of each GnRH form in the brain (31). GnRH1 neurons are generally present in the ventral forebrain–POA–hypothalamus and send neuronal fibers directly into the pituitary, which represents its primary role in the stimulation of GTH secretion. The physiological significance of GnRH1 as a regulator of GTH secretion and gametogenesis has been established in several teleosts (28, 50–54).

GnRH2 neurons are exclusively present in the midbrain. The absence or low levels of GnRH2 peptide in the pituitary has been demonstrated in several *perciformes* (50, 51, 55–57) and pleuronectiformes species (28, 58), suggesting that GnRH2 is not directly involved in GTH secretion. Rather, its wide fiber projection throughout the brain suggests that GnRH2 has neuromodulatory functions (30). However, in some fish species including the goldfish, GnRH2 seems to act as a hypophysiotropic GnRH together with GnRH3 (59).

GnRH3 has been shown to control reproductive behaviors in several fish species. GnRH3 stimulates nest-building behavior in the male dwarf gourami (60), homing migration in the sockeye salmon (61), and aggressive and nest-building behaviors in the male Nile tilapia (62), which suggests probable neuromodulatory roles of GnRH3. The neuromodulatory role of GnRH3 was confirmed by electrophysiological studies in the retina of goldfish (63, 64) and olfactory receptor cells of the mudpuppy (65). The neuromodulatory function of GnRH3 has also been demonstrated in the rainbow trout (66, 67) and the dwarf gourami [reviewed by Oka (68)]. Fish species such as some salmonids and the zebrafish possess only two forms of GnRH (GnRH2 and GnRH3). In these species, GnRH3 expressed in the basal forebrain acts as a hypophysiotropic GnRH (45, 69–71).

KISSPEPTIN

Kisspeptin is a neuropeptide that plays an important role in reproduction through the stimulation of GnRH neurons by activating GPR54 in mammals (72, 73). In teleosts, two kisspeptin genes, namely *kiss1* and *kiss2*, have been identified in several fish species (74–77), whereas placental mammals possess only the *kiss1* gene. Similarly, two kisspeptin receptor genes, named *kiss1r* and *kiss2r*, were also identified in several fish species (76, 78), suggesting two Kiss/Kissr systems in teleosts. However, this situation is not common among all fish species. Only one kisspeptin gene, *kiss2*, and one receptor, *kiss2r*, are present in some fish species including the Senegalese sole (79), orange-spotted grouper (80), grass puffer (81), and the Atlantic halibut (82), indicating that the *kiss1* and *kiss1r* genes have been lost during evolution in these species (82). Both *kiss1* and *kiss2* mRNAs are expressed in the brain and the gonads in several fish species (74, 76–78, 83). On the other hand, kisspeptin and kisspeptin receptor are also expressed in the fish pituitary, suggesting local actions of kisspeptin in the pituitary (76, 78, 81). In the medaka brain, two populations of *kiss1* neurons are found in the hypothalamus, one in the nucleus ventral tuberis (nVT) and the nucleus posterioris periventricularis (NPPv) (74, 84), while neurons in the dorsal zone of the periventricular hypothalamus (Hd) express *kiss2*. In the zebrafish all hypothalamic populations express *kiss2* mRNA (74). A recent study showed that zebrafish Kiss2 neuronal fibers are found widely in the subpallium, POA, ventral and caudal hypothalamus, and the mesencephalon (85). The fact that all three GnRH neuron types express kisspeptin receptors in the Nile tilapia (86) suggests that the role of Kiss2 neurons in the regulation of the HPG axis is via the activation of the GnRH systems. The *kiss1* neurons are exclusively localized in the habenula in the zebrafish (74), and send fibers only to the ventral part of the interpeduncular nucleus (85, 87). The habenula Kiss1 system is thus implicated in the modulation of serotonergic system rather than the HPG axis in the zebrafish (87).

The role of kisspeptin in the onset of puberty and sexual maturation is conserved among vertebrates including fish. In the zebrafish, both *kiss1* and *kiss2* mRNA levels are increased significantly at the start of the pubertal phase together with GnRH2 and GnRH3 mRNAs (74). Significant positive correlation is observed between the levels of *kiss2* mRNA and those of *gnrh1* mRNA during the spawning period in the grass puffer (81). Kiss2 but not Kiss1 stimulates GTH synthesis and release in the sea bass and the zebrafish (74, 75). Administration of Kiss2–10 peptide increases GnRH1 mRNA levels in the sexually mature female orange-spotted grouper (80), indicating that *Kiss2* most probably plays an important role in the regulation of reproductive functions through the stimulation of GnRH1 secretion.

The information of the interaction between kisspeptin neuronal fibers and GnRH cell bodies had been limited in teleosts due to the lack of specific antibody to kisspeptins. A recent study using an antibody to prepro-Kiss2 proved that Kiss2 neuronal fibers make close contacts with POA GnRH (GnRH3) neurons in the zebrafish (85), suggesting that Kiss2 directly act on GnRH neurons. Moreover, kisspeptin receptor expression in the three GnRH neuronal populations (86) in tilapia suggests that kisspeptin directly stimulates not only GnRH1 neurons to induce LH secretion, but also GnRH2 and GnRH3 neurons to activate other aspects of the reproduction such as sexual behavior.

GONADOTROPIN-INHIBITORY HORMONE (GnIH)

GnIH or RFamide-related peptide (RFRP), which has a characteristic C-terminal LPXRFa motif (X = L or Q), is a hypothalamic neuropeptide that was originally identified from the quail as a neuropeptide that inhibits gonadotropin release from the pituitary (88). Extensive studies revealed that GnIH functions at the level of GnRH neurons and at the level of pituitary gonadotropes to suppress reproduction in avian and mammalian species [see reviews in Ref. (89, 90)]. GnIH in the teleost species has been named LPXRFamide peptide based on the amino acid sequence of the C-terminal motif. All precursors of teleost GnIH identified so far encode three GnIH orthologs (LPXRFa-1, -2, and -3), while only goldfish LPXRFa-3 has been purified as a mature peptide.

As in birds and in mammals, teleost GnIH neurons are located in the hypothalamus, in particular in the NPPv, and send neuronal fibers throughout the brain and to the pituitary (91, 92). The physiological function of teleost GnIH in the control of reproduction is complicated. *In vivo* studies using the goldfish show that GnIH decreases plasma LH levels as in avian and mammalian species (93, 94). On the other hand, GnIH significantly increases pituitary levels of mRNAs for LHβ and FSHβ in a reproductive state-dependent manner *in vivo*, whereas general suppression of LHβ and FSHβ mRNA levels is observed *in vitro* in a study (93). This differential *in vivo* effect of GnIH in different seasons can be explained by the differential action of GnIH on the gonads (95). GnIH does not affect plasma estradiol levels in the female goldfish, but increases plasma testosterone levels in the male goldfish (96). GnIH injections into the female goldfish suppress pituitary LHβ and FSHβ and hypothalamic GnRH mRNA levels (95). In addition, GnIH suppresses GnRH-induced increase in LHβ mRNA levels *in vitro* (95). Therefore, in the goldfish, the inhibition of the HPG axis at the level of hypothalamic GnRH neurons and pituitary gonadotropes appears as an evolutionarily conserved function of GnIH. On the contrary, goldfish GnIH peptides stimulate the synthesis and release of LH and FSH in cultured pituitary cells of the grass puffer and the sockeye salmon, respectively (92, 97). Therefore, as in mammals (98), the stimulatory or inhibitory action of GnIH in fish is probably species dependent or species-specific GnIH peptide might be necessary for an inhibitory action.

More recently, it has been shown that medaka LPXRFa-2 (GnIH-2) peptide decreases the firing frequency of non-hypophsiotropic terminal nerve GnRH3 neurons in the dwarf gourami (99). Since GnRH3 controls nest-building, aggression, and homing migration (60–62), GnIH-2 might negatively regulate reproductive behaviors.

METABOLIC NEUROPEPTIDES INVOLVED IN REPRODUCTION

A number of hypothalamic neuropeptides have been identified in fish species (7), and found to be involved in the control of food intake as well as reproduction (**Table 1**). To understand the overall metabolic control of reproduction, the involvement of metabolic neuropeptides in the regulation of GnRH and GTHs must be taken into consideration. However, compared to mammals, the information related to the role of metabolic neuropeptides in the regulation of reproduction is still limited in fish.

OREXIN

Orexin has two well conserved molecular forms, a 33-amino acid peptide known as orexin A (OXA) and a 28-amino acid peptide known as orexin B (OXB) derived from the same precursor [see review in Ref. (150)]. Orexin was first identified as a ligand of an orphan receptor, and consequently found to stimulate feeding in mammals (151). The orexin's orexigenic action is also observed in teleosts, including the goldfish and the ornate wrasse (101, 152).

In mammals, orexin is known to stimulate the HPG axis via GnRH secretion (153–155). In the goldfish, an interaction between orexin and hypophysiotropic GnRH (GnRH2) has also been proposed. Intracerebroventricular administrations of OXA inhibit spawning behavior and lower GnRH2 mRNA levels, while treatment with GnRH decreases OXA mRNA levels (102). These results suggest that, unlike in mammals, orexins might act as inhibitory agents in the control of GnRH at least in some fish species. In addition, OXA is detected in the pituitary of the medaka (156) and the Japanese sea perch (157), whilst OXB is detected in the pituitary of the Nile tilapia (158), suggesting orexin's local action at the level of pituitary. Thus orexin, an orexigenic neuropeptide, inhibits the HPG axis at the hypothalamus GnRH level and possibly also at the pituitary level, in fish.

NEUROPEPTIDE-Y (NPY)

NPY which is composed of 36 amino acid residues, was first identified in the porcine brain (159), and was found to function as a powerful appetite enhancer in mammals (160). In fish species, NPY also show powerful orexigenic activity in the goldfish (103, 123, 161–164), trout (104), puffer fish (105, 165), and the zebrafish (106).

Centrally or peripherally injected NPY increases plasma LH levels in the goldfish, common carp, rainbow trout, and in the sea bass (107, 109, 111), indicating that NPY stimulates teleost reproduction as was shown in mammals (166).

Table 1 | Neuropeptides and their functions in representative fish species.

Neuropeptide	Species	Function	Reference
Orexin	Goldfish	Increase food intake	(100)
	Ornate wrasse	Increase food intake	(101)
	Goldfish	Inhibit spawning behavior, decrease GnRH2 mRNA level	(102)
NPY	Goldfish	Increase food intake	(103)
	Rainbow trout	Increase food intake	(104)
	Puffer fish	Increase food intake	(105)
	Zebrafish	Increase food intake	(106)
	Goldfish	Stimulate GnRH and LH release	(107, 108)
	Common carp	Increase plasma LH level	(109)
	Sea bream	Stimulate GnRH release	(110)
	Sea bass	Increase plasma LH level	(111)
PACAP	Goldfish	Decrease food intake	(112)
	Goldfish	Stimulate LH release	(113, 114)
	Tilapia	Stimulate GTH subunit mRNA expression	(115)
	Blue gourami	Stimulate FSHβ mRNA expression	(116, 117)
GnRH2	Goldfish	Decrease food intake	(102, 118)
	Zebrafish	Decrease food intake	(119)
	Goldfish	Stimulate LH release	(120)
26RFa	Mouse	Increase food intake	(121)
	Goldfish	Increase plasma LH level	(122)
Galanin	Goldfish	Increase food intake	(123)
	Tench	Increase food intake	(124)
	(Rat)	Stimulate GnRH release	(125)
MCH	Goldfish	Decrease food intake	(126)
	Goldfish	Stimulate LH release	(127)
α-MSH	Goldfish	Decrease food intake	(128)
	Rainbow trout	Decrease food intake	(129)
	(Mouse)	Stimulate GnRH neurons	(130)
CART	Goldfish	Decrease food intake	(131, 132)
	(Rat)	Stimulate GnRH release	(133, 134)
CCK	Goldfish	Decrease food intake	(135)
	Goldfish	Stimulate LH release	(136)
Nesfatin-1	Goldfish	Decrease food intake	(137, 138)
	Goldfish	Decrease plasma LH level	(139)
Leptin	Goldfish	Decrease food intake	(140)
	Rainbow trout	Decrease food intake	(141)
	Sea bass	Stimulate LH release	(142)
	Rainbow trout	Stimulate LH release	(143)
Ghrelin	Goldfish	Increase food intake	(144–146)
	Rainbow trout	Decrease food intake	(147)
	Goldfish	Stimulate LH release	(148)
	Common carp	Stimulate LH release	(149)

(Continued)

In the brown trout (167) and the rainbow trout (168), NPY neuronal fibers project to the areas where hypophysiotropic GnRH neurons exist, particularly in the ventral telencephalon, POA, and in the basal hypothalamus. Furthermore, double immunolabeling reveals close appositions of NPY fibers with GnRH cells in the POA of the ayu (*Plecoglossus altivelis*) (169) and the Siberian sturgeon (170), suggesting the direct action of NPY in the regulation of GnRH neurons. Indeed, NPY stimulates GnRH release *in vitro* in the goldfish (108) and in the sea bream (110).

Neuropeptide-Y also regulates the HPG axis at the level of pituitary. *In vitro* treatment with NPY stimulates LH release from pituitary cells in the goldfish (107) and increases LHβ and GTHα, but not FSHβ mRNA levels in the tilapia pituitary (115). In addition, NPY fibers make close appositions on LH cells in the catfish pituitary (171).

These findings provide strong support for the stimulatory role of NPY in fish reproduction at the levels of hypothalamic GnRH and pituitary LH cells.

26RFamide (QRFP)

26RFamide is a 26-amino acid peptide, and was first isolated from the frog brain (121). In teleosts, 26RFa has been identified only in the goldfish (172). The 26RFa gene is highly expressed in the hypothalamus, and relatively less in the optic tectum-thalamus and in the testis (122). 26RFa and its mammalian homolog QRFP act as an orexigenic hormone in birds, mice (121, 172–174), and probably in fish (122).

The role of 26RFa has been implicated in the integration of metabolism and reproduction in vertebrates, including fish [see review in Ref. (89)]. In mammals, 26RFa stimulates LH and FSH release in rats *in vivo* and *in vitro* (pituitary culture) (175). In teleost, intraperitoneal injections of 26RFa significantly increase plasma LH levels in the goldfish (122). On the other hand, *in vitro* treatment with 26RFa shows no effects on LH release from pituitary cells. These facts indicate that 26RFa might act on the stimulation of the HPG axis through GnRH1 release in fish.

GALANIN (GAL)

GAL is a 29-amino acid peptide, expressed in the central nervous system and in the intestine. GAL stimulates feeding in the goldfish (123) and the doctor fish tench (124), indicating that GAL acts as an orexigenic hormone in fish as in mammals (176, 177).

Involvement of GAL in the control of HPG axis is evidenced in mammals. In rodents and humans, GAL neuronal fibers make close appositions with GnRH1 neurons (178–180), and GnRH neurons express a GAL receptor Gal-R1 in the rat (181). In fact, GAL stimulates *in vitro* GnRH release in rats (125). These data indicate that GAL is involved in the control of reproduction at the level of GnRH neurons.

In fish, there are no studies that demonstrated the role of GAL in the control of reproduction. However, close appositions of GAL fibers with gonadotropes in the proximal pars distalis (PPD) are seen in the sea bass (182). Similarly, fiber projections of GAL neurons in the PPD are observed in the rainbow trout (183), sea bream (184), and Senegalese sole (185), while no GAL fibers are observed in the pituitary of the Siberian sturgeon (170). Therefore, GAL might modulate the HPG axis at the pituitary level at least in some fish species.

GnRH2

As mentioned before, among the different forms of GnRH, neuronal fibers of GnRH2 (also known as chicken GnRH-II) are widely distributed in the vertebrate brain. In an insectivore, the musk shrew, GnRH2 stimulates sexual behavior and seduces food intake (186, 187), indicating that GnRH2 plays a role in connecting reproductive function and feeding regulation.

In fish species, the suppressive effect of GnRH2 on feeding has also been confirmed. Food consumption is significantly decreased by intracerebroventricular injections of GnRH2 but not GnRH3 in a dose dependent manner in the goldfish (102, 118) and the zebrafish (119).

GnRH2 also has effects on sexual behavior of fish. In the goldfish, GnRH2 stimulates reproductive behavior (188). Furthermore, there is a strong positive correlation between spawning behavior and GnRH2 gene expression (189), suggesting stimulatory role of GnRH2 in reproductive behavior. GnRH2 is also detected in the goldfish pituitary (190) and induces LH release *in vitro* (120). Positive correlation between the pituitary GnRH2 levels and gonadal development is also observed in the striped bass (51), suggesting that it also have a hypophysiotropic role in some fish species. In the grass puffer, the amount of GnRH2 mRNA is slightly higher in the post-spawning females compared to spawning female (191). Therefore, GnRH2 may have different physiological roles depending on the physiological conditions of the fish.

PITUITARY ADENYLATE CYCLASE-ACTIVATING POLYPEPTIDE (PACAP)

PACAP was first isolated from the rat hypothalamus (192). PACAP is an anorexigenic factor in various vertebrates, including rodents (193), chicks (194, 195), and fish (196). To date, the role of PACAP in feeding has been studied only in one fish species, the goldfish.

PACAP increases plasma LH levels *in vivo* in the goldfish (197). *In vitro* studies showed that the stimulatory effect of PACAP on LH release is exerted at the level of the pituitary (113, 114). PACAP also stimulates the levels of GTH subunit mRNAs and FSHβ mRNA in the pituitary of tilapia (115) and in the female blue gourami (116, 117), respectively. Dense projection of PACAP nerve terminals is seen in the pars distalis of the pituitary, where gonadotropes are localized, in the goldfish (198) and in the European eel (199). The expression of PACAP receptor in the pituitary is also observed in the goldfish (197). Therefore, PACAP stimulates GTH secretion in fish pituitary.

MELANIN-CONCENTRATING HORMONE (MCH)

MCH is a cyclic peptide, originally isolated from the pituitary of the chum salmon as a hormone involved in body color change (200). In the winter and barfin flounders, fasting stimulates hypothalamic expression of MCH (201, 202), suggesting that MCH acts as an orexigenic hormone as in mammals (203, 204). However, MCH acts as an anorexigenic hormone in the goldfish (105, 126, 205, 206). Therefore, like ghrelin, MCH acts as an orexigenic and anorexigenic neuropeptide depending on the fish species, although its orexigenic action in fish has to be confirmed.

In mammals, MCH modulates LH secretion in an estradiol-dependent manner [see a review in Ref. (207)]. The close appositions between MCH fibers and hypothalamic GnRH neurons (208, 209) and the expression of MCH receptors in GnRH neurons (209) suggest the direct action of MCH on GnRH neurons in mammals. MCH also acts at the pituitary level to modulate the release of LH (210). In teleosts, an *in vitro* study showed that salmon MCH stimulates the release of LH in a dose response manner from dispersed pituitary cells in the goldfish, suggesting a direct action of MCH on LH cells (127). Whether MCH acts on GnRH neurons in fish as in mammals remains unknown.

α-MELANOCYTE STIMULATING HORMONE (α-MSH)

α-MSH is one of melanocortins and derived from a precursor peptide encoded by the POMC gene (211). Among melanocortins and their receptors, α-MSH and melanocortin receptor 4 (MC4R) are involved in the control of food intake in vertebrates including fish. α-MSH or MC4R agonist inhibits food intake in the goldfish (128, 212) and in the rainbow trout (129), suggesting that the α-MSH/MC4R system play a role in the anorexigenic regulation of feeding in fish as in mammals.

Although the α-MSH/MC4R system is known to play a stimulatory role in reproduction at the level of GnRH neurons in mammals [see a review in Ref. (130)], available information is limited in teleost. Projection of α-MSH fibers in the PPD of the pituitary and differential expression of POMC gene between sexually inactive and active fish in the zebrafish suggests that some of POMC-derived products are involved in the stimulation of fish reproduction (213).

COCAINE- AND AMPHETAMINE-REGULATED TRANSCRIPT (CART)

CART is an anorexigenic neuropeptide originally isolated from the rat brain (160, 214). In fish, CART might also act as anorexigenic hormone in the goldfish (131), winter flounder (215), cod (216), channel catfish (217), zebrafish (218), and in the Atlantic salmon (219).

In mammals, CART is involved in the control of GnRH neurons. CART stimulates GnRH pulsatile release in rats (133, 134). The existence of close appositions between CART fibers and hypothalamic GnRH neurons in the Siberian hamster suggests the effect of CART on GnRH neuronal activity is a direct action (220).

In the catfish, the projections of CART fibers are observed in the PPD of the pituitary (221). CART is also expressed in LH cells of the catfish pituitary but only during sexual maturation period (222), suggesting its local function in the sexual maturation process. However, it should be noted that while similar expression of CART in LH cells is observed in the rat pituitary, CART inhibits the release of prolactin but not GTHs (223). Thus, the role of CART in the fish pituitary has to be examined.

CHOLECYSTOKININ (CCK)

CCK is found in the brain and in the gastrointestinal tract of various vertebrates. It has multiple biologically active forms, among which CCK-8 is the most abundant form in the brain (224). As in mammals, CCK has many physiological roles in fish, but functions primarily in the control of food intake as a satiety indicator (135).

In mammals, CCK decreases the pulse interval of GnRH release in goats (225). Furthermore, CCK implants into the POA, where GnRH neurons are located, increase the plasma levels of LH in rats (226). These data suggest that CCK acts at the levels of GnRH and stimulates reproduction. In fish, on the other hand, CCK seems to acts on the pituitary. An immunohistochemical study showed that CCK neurons innervate into the PPD of the pituitary and that CCK stimulates LH release *in vitro* in the goldfish (136).

NESFATIN-1

Nesfatin-1, a nucleobindin-2 (NUCB2) encoded unmodified peptide, was first characterized in rats (227), and was shown to have anorexigenic actions in the goldfish (137, 138).

The number of studies about the function of nesfatin-1 in the control of reproduction is still limited. However, recent studies showed that nesfatin-1 acts as an inhibitory signal in the control of fish reproduction. Although nesfatin-1 plays a stimulatory role in LH secretion in rats (228), an intraperitoneal injection of nesfatin-1 decreases plasma levels of LH in the goldfish (139). At the same time, nesfatin-1 down regulates expression of GnRH, LHβ, and FSHβ genes, suggesting that the inhibitory action of nesfatin-1 takes place at the levels of GnRH neurons. Whether nesfatin-1 also functions at the level of the pituitary remains unclear.

PERIPHERAL HORMONES INVOLVED IN FEEDING, METABOLISM, AND REPRODUCTION

LEPTIN

Leptin is primarily produced by adipocytes of the white adipose tissue (229), and secreted into the blood circulation in proportion to the mass of body fat. The change in plasma leptin levels is detected by the hypothalamus and thereby it acts as a peripheral factor that signals nutritional status to the CNS [see review by Crown et al. (230)]. In teleosts including the goldfish and the rainbow trout, leptin functions as a peripheral signal to inhibit food intake (140, 141, 231, 232) as in mammals (233).

In mammals, leptin stimulates the HPG axis by promoting the synthesis and release of GnRH from the hypothalamus, and LH and FSH from the pituitary (234–236). In teleosts, leptin also stimulates the reproductive axis. Leptin increases *in vitro* LH release from the pituitary culture in the sea bass (142) and the rainbow trout (143). However, it should be noted that the stimulating effect of leptin on LH release is observed only on the pituitary samples from the fish in maturational stages. Furthermore, leptin expression levels increase with the onset of sexual maturation in the Arctic char (237) and the Atlantic salmon (238). Therefore, the role of leptin in sexual maturation seems to be conserved among vertebrate species.

GHRELIN

As in mammals, ghrelin is highly expressed in the stomach and moderately in the brain (144, 239, 240), and is involved in appetite stimulation, energy balance, feeding, and metabolism [see reviews in Ref. (241, 242)]. Interestingly, the role of ghrelin in fish differs in different fish species. It acts as an orexigenic hormone in the goldfish (144–146) and probably in the sea bass (243) and the zebrafish (244). On the other hand, ghrelin acts as an anorexigenic hormone in the rainbow trout (147) and probably in the burbot (245, 246). The opposite effects of ghrelin on food intake can be explained by species-specific neural pathways mediating the effect of ghrelin (247). The variations in the role of ghrelin in feeding may reflect different regulatory mechanisms of feeding in different teleost species.

In fish species, ghrelin acts as a stimulatory factor in the reproduction, although ghrelin inhibits the HPG axis in mammals [reviewed by Tena-Sempere (248)]. Intracerebroventricular injection of ghrelin increases plasma LH levels in the goldfish (148), indicating its stimulatory action on the HPG axis. The increase of plasma LH levels is, however, small and slow compared to the increase of plasma GH levels. This suggests that the stimulatory effect of ghrelin on plasma LH levels is not through the action of ghrelin on hypothalamic GnRH. Actually, the highest levels of ghrelin receptor mRNA are observed in the sea bream and goldfish pituitary (249, 250). *In vitro* treatment with ghrelin stimulates LH release in the goldfish (148, 250) and in the common carp (149), while pituitary levels of mRNA for LHβ subunit is also increased. As no reports show fiber projections of hypothalamic ghrelin neurons into the pituitary in fish, ghrelin released from stomach/intestine might play a role in the LH secretion from the pituitary. Therefore, ghrelin might act as a stimulatory peripheral factor in reproduction at the level of pituitary, whereas its action on GnRH neurons is uncertain.

INTERACTIONS BETWEEN METABOLIC NEUROPEPTIDES AND THE REPRODUCTIVE SYSTEM IN THE CONTROL OF REPRODUCTION

As shown in the above section, many metabolic neuropeptides are involved in the control of reproduction at the level of hypothalamic GnRH neurons and at the level of pituitary gonadotropes (**Figure 1**). Among these metabolic neuropeptides, NPY and nesfatin-1 function as inhibitory factors on GnRH neurons, while orexin stimulates GnRH neurons. It is interesting that NPY and orexin, which possess orexigenic activity, act on the reproductive system in an opposite manner in the goldfish. It suggests that different metabolic neuropeptides might play a role in the control of reproduction under different physiological conditions. It should be noted, however, that the inhibitory role of orexin on spawning behavior and GnRH gene expression might be the result of orexin action on non-hypophysiotropic GnRH system. In the goldfish brain, hypophysiotropic GnRH type is expressed not only in the hypothalamic population but also in the olfactory bulb and midbrain populations (190). Therefore, the inhibitory effect of orexin on the HPG axis need to be confirmed although it is clear that orexin has suppressive role in some aspects of reproduction.

At the pituitary level, it is evident that many metabolic neuropeptides including NPY, MCH, GnRH2, PACAP, and CCK stimulate LH secretion. In addition, peripheral metabolic signals such as ghrelin and leptin also stimulate LH secretion at the pituitary. The fact that most central neuropeptides and peripheral metabolic signals regulate the reproductive system indicates fundamental interaction between energy balance and reproduction, which is evolutionarily conserved from fish to mammal. However, both orexigenic and anorexigenic metabolic signals act as stimulatory factors in the reproductive system in fish. In mammals, feeding and reproduction are two alternatives in general. Therefore, orexigenic factors inhibit reproduction and anorexigenic factors stimulate reproduction [see review in Ref. (251)]. In fish species, on the other hand, a central orexigenic neuropeptide NPY and a peripheral orexigenic peptide ghrelin inhibit LH secretion in the goldfish and other species (**Table 1**). This indicates that metabolic regulation of the reproductive system in teleost is different from that in mammals, at least in some species.

Fish species have a variety of feeding and reproductive behaviors. For example, most salmonids and the winter flounder undergo a period of fasting just before the spawning season as a part of their normal physiology (252), whereas the goldfish do not have such fasting period. The halt of food intake during final maturation might require the differential usage of metabolic signals in these species.

Recently, kisspeptin has been proposed as a mediator of metabolic signals in the mammalian reproductive system, in particular on GnRH neurons [see reviews in Ref. (89, 253)]. In mice (254) and in the sheep (255), kisspeptin neurons in the arcuate nucleus possess leptin receptors, suggesting direct action of leptin on kisspeptin neurons. Furthermore, kisspeptin neurons receive innervations from other neurons that express leptin receptor (255). These facts suggest that leptin controls GnRH neurons through kisspeptin neurons *via* direct and indirect actions. Furthermore, kisspeptin neurons receive fiber projections from NPY and POMC neurons in mammals (255, 256). Therefore kisspeptin neurons might play an important role in the integration of metabolic signals to control the reproductive system. In teleost, fasting induces a significant increase in *kiss2* mRNA levels in the hypothalamus, as well as an increase in LHβ and FSHβ mRNA levels in the pituitary in the Senegalese sole (*Solea senegalensis*) (257), suggesting negative correlation between energy balance and reproduction. However, to our knowledge, there is no information available regarding direct evidence of metabolic regulation of kisspeptin neurons in fish.

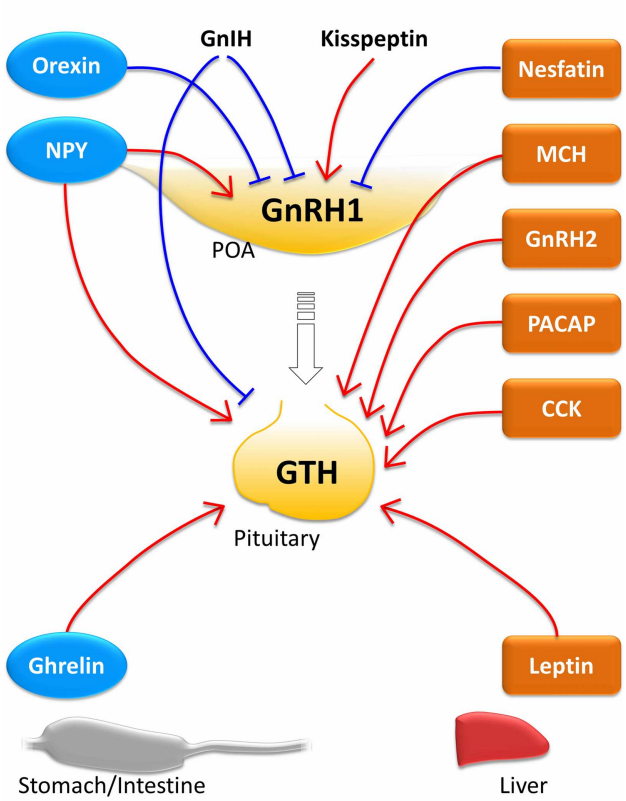

FIGURE 1 | Effects of central and peripheral metabolic hormones on the reproductive system. The hypothalamus–pituitary axis receives many stimulatory and inhibitory inputs from central metabolic neuropeptide neurons and peripheral metabolic signals to control the reproductive system according to the energy status. The hormones indicated with blue circles are orexigenic hormones, while those with orange squares are anorexigenic ones. Red lines indicate stimulatory action and blue lines indicate inhibitory action. Most anorexigenic hormones stimulate gonadotropin secretion at the pituitary level. In the goldfish, NPY and ghrelin, which act as orexigenic hormones, also stimulate the reproductive system at the brain and pituitary levels. It should be noted that this figure is drew primarily based on the information obtained from the goldfish. Function of each metabolic hormone might differ in different species [e.g., Ghrelin functions as an anorexigenic factor in the rainbow trout (147) and GnIH stimulates GTH subunit mRNA expression in the puffer fish (97) and the sockeye salmon (92).]. CCK, cholecystokinin; GnIH, gonadotropin-inhibitory hormone; GnRH, gonadotropin-releasing hormone; MCH, melanin-concentrating hormone; NPY, neuropeptide-Y; PACAP, pituitary adenylate cyclase-activating polypeptide.

In addition to its primary role in reproduction, GnIH stimulates food intake in chickens (258) and in rats (259, 260), suggesting its potential role to switch from reproduction to feeding. Close appositions of GnIH fibers with NPY, orexin, MCH, and POMC neurons in the sheep (261) indicate the involvement of several feeding regulatory pathways. However, there are no studies reporting metabolic regulation of GnIH neurons in vertebrates. On the other hand, GnIH is known to be regulated by stress, photoperiod, and gonadal steroids to suppress the reproductive system (89). Therefore, GnIH neurons might have a role in the modulation of feeding according to the environmental factors in mammals. Whether GnIH plays a similar role in teleosts requires more studies.

INTERACTIONS AMONG NEUROPEPTIDES TO CONTROL FEEDING

To monitor the amount of energy stock, central metabolic neuropeptide neurons receive peripheral signals including leptin and ghrelin. For example, in mammals leptin receptor is expressed in many metabolic neuropeptide neurons including orexin, NPY, GAL, MCH, POMC, CART, and CCK neurons [see reviews in Ref. (262, 263)]. In fish species, leptin also affects several central neuropeptide neurons. Administration of leptin reduces NPY mRNA levels in the goldfish (140), grass carp (264), and in the rainbow trout (141, 265). On the other hand, leptin increases the mRNA levels of CCK and POMC, which are anorexigenic neuropeptides, in the goldfish (140) and the rainbow trout (141, 265), respectively. A recent study showed that leptin receptor knockout medaka exhibit higher levels of NPY mRNA before and after feeding and lower levels of POMC mRNA levels after feeding together with increased food intake (266). Therefore leptin's anorexigenic effect might be mediated by these neuropeptides.

Double immunostaining revealed interactions among orexigenic/anorexigenic neuropeptide neurons in teleosts, in particular in the goldfish (**Figure 2**). Among anorexigenic neuropeptide neurons, MCH neuronal fibers project to α-MSH neurons (206) and α-MSH neuronal fibers project to CRH neurons (267). Furthermore, a study using antagonists against α-MSH receptor and CRH receptor showed that anorexigenic action of MCH is mediated by α-MSH and CRH (268). In addition, GnRH2 mediates anorexigenic effect of α-MSH and CRH (269). These results suggest that the MCH–α-MSH–CRH–GnRH2 pathway suppresses food intake in the goldfish, although it is not known whether CRH directly acts on GnRH2 neurons.

Among orexigenic neuropeptide neurons, NPY and orexin neurons make reciprocal connections in fish as in mammals. NPY neuronal fibers make close appositions with orexin neurons, whereas orexin neuronal fibers make close appositions with NPY neurons in the NPPv in the goldfish (270). Furthermore, co-injections of OXA and NPY result in food intake higher than that observed in fish treated with NPY alone (132). These results indicate that orexins and NPY induce orexigenic actions by mutual signaling pathways in the CNS in teleost. Probably the reciprocal interaction between NPY and orexin functions as a positive-feedback system to maintain food intake.

Moreover, the orexigenic and the anorexigenic circuits are also connected with each other. α-MSH neuronal fibers make close appositions with NPY neurons, whereas NPY neuronal fibers project to α-MSH neurons in the goldfish (271). In addition, MCH neuronal fibers make close appositions with NPY neurons (272). These inhibitory inputs between the orexigenic and anorexigenic neurons might function as an on/off switch to decide whether eat or not eat by activating only one of the two circuits.

Studies using antagonists against of the receptors for metabolic neuropeptides further provided possible interaction among central metabolic neuropeptides in the goldfish. For example, GAL mediates the orexigenic action of orexin, and orexin mediates the orexigenic action of GAL (123). GAL also mediates NPY's action on food intake and *vice versa* (123). Besides, orexin mediates central action of ghrelin in food intake and central ghrelin mediates the action of orexin (273). Furthermore, NPY mediates the orexigenic action of ghrelin (274). These results indicate complex neuronal interactions especially among central orexigenic neuropeptides. This complex neuronal network suggests that many central neuropeptide neurons function in a coordinated manner to regulate food intake. To fully understand the whole circuit that controls food intake, further information on the neuronal interaction among central metabolic neuropeptides have to be obtained.

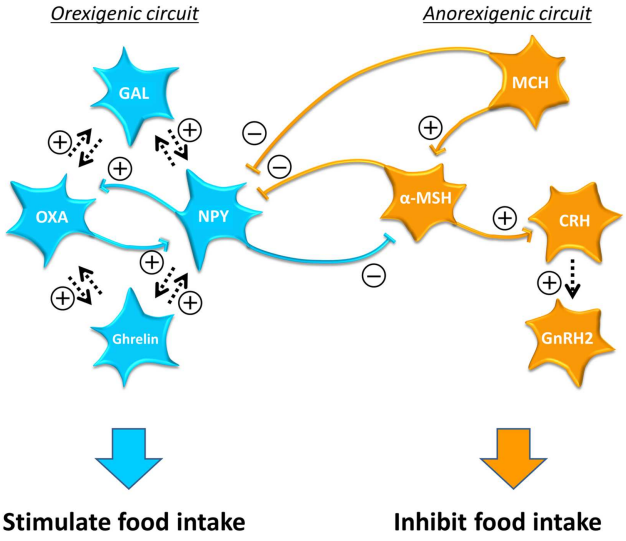

FIGURE 2 | Interaction between the orexigenic circuit and anorexigenic circuit and among neurons in each circuit in the goldfish. Hormones in the orexigenic circuit are shown in blue and those in the anorexigenic circuit are shown in orange. The orexigenic and anorexigenic circuits are connected each other via inhibitory neuronal fiber projections to form an on-off switch. Reciprocal interaction is observed especially between neurons in the orexigenic circuit. Arrows with dotted lines indicate the interactions that have not been confirmed whether direct or indirect. The complex interaction among metabolic neuropeptides suggests that a change in one metabolic signal can affect the reproductive system through the action via other metabolic neuropeptides. Note that in the goldfish ghrelin secreted in the brain also function as an orexigenic signal. α-MSH, alpha-melanocyte stimulating hormone; CRH, corticotropin-releasing hormone; GAL, galanin; GnRH, gonadotropin-releasing hormone; MCH, melanin-concentrating hormone; NPY, neuropeptide-Y; OXA, orexin A; PACAP, pituitary adenylate cyclase-activating polypeptide.

In addition to the evident neuronal interactions in the goldfish, more combinations of neuronal interactions were reported in other fish species. In the masu salmon (275) and the Siberian sturgeon (170), NPY and GAL neurons make reciprocal connections. In the medaka, orexin and MCH neurons send neuronal fibers to each other (156). In the barfin flounder, reciprocal connection between orexin and MCH neurons and between α-MSH and MCH neurons was reported (156). In the rainbow trout, CRH mediates the anorexigenic action of ghrelin (147). These facts suggest that the interaction among central metabolic neuropeptides is really complicated. Therefore, more fiber projection studies together with the localization of the neuropeptide receptors are necessary to understand proper relationships among these neuropeptides that consist of the regulatory circuits of food intake.

PERSPECTIVES

DIRECT INTERACTIONS AMONG CENTRAL METABOLIC NEUROPEPTIDES AND REPRODUCTIVE SYSTEM

Significant amount of information about the relationship among orexigenic and anorexigenic neuropeptides have been accumulated, particularly in the goldfish. However, the knowledge of direct interactions among these neurons is still not enough to draw a complete diagram of the neuronal circuit to control food intake and reproduction in fish. In particular, metabolic regulation of kisspeptin and GnRH neurons are still unknown, while it is suggested from mammalian studies. Further fiber projection studies using double immunostaining and localization of the neuropeptide receptors in certain neuronal cell bodies need to be performed.

SPECIES DIFFERENCES

There are many differences in the regulatory mechanism of food intake and reproduction not only between mammals and fish but also between fish species. The significant difference between fish species might be the result of the adaptation to a wide range of feeding habits and reproductive strategies. Therefore we have to be careful to combine data obtained from different species.

SEX AND MATURATIONAL STAGES

Several studies reported that the responses of the reproductive system to metabolic signals differ depending on the sex and the stage of sexual maturation. In fact, gonadal steroids modulate the effect of NPY on GnRH and LH release in the goldfish (276). Each study should use a particular sex and maturational stage to make comparison easy.

NUTRITIONAL CONDITIONS

Animals might change the metabolic control of reproduction according to the available energy stock. For example, short term food limitation attenuates sexual motivation, while remaining energy stock still maintains activity of the HPG axis. On the other hand, long term food limitation depletes the energy stock and stops the HPG axis to prioritize the energy supply to the survival. Thus, feeding conditions and the timing of experiment might be important to obtain comparable data.

ENDOCANNABINOID SYSTEM

The endocannabinoid system is involved in a variety of physiology including pain-sensation, mood, and memory. Importantly, both energy balance and reproduction are modulated by the endocannabinoid system. Endocannabinoids modulate several hypothalamic metabolic neuropeptides in mammals [see reviews in Ref. (277, 278)]. The endocannabinoid system also regulates food intake in fish (279, 280). In mammalian and non-mammalian vertebrates, the endocannabinoid system regulates hypothalamic GnRH neurons and pituitary LH cells directly and indirectly [see reviews in Ref. (281, 282)]. Interrelation among these systems might be an additional mechanism underlying the interaction between mood, stress, appetite, and reproduction.

CONCLUSION

In summary, the cellular and molecular basis for the integration of feeding and reproduction involves a complex interaction of the reproductive system with metabolic neuropeptides and peripheral fuels. The metabolic neuropeptides, particularly orexin, NPY, PACAP, MCH, nesfatin, GnRH2, and CCK play an important role in the reproduction by either regulating GnRH neurons in the hypothalamus or by stimulating gonadotropes in the pituitary. Peripheral metabolic signals such as ghrelin and leptin also act on the pituitary to stimulate LH secretion. It should be, however, noted that compared to mammals, fishes show a great variety of feeding and reproductive habits. The variations of metabolic control of reproduction in different teleost species may reflect different requirement of energy status for reproduction in different species. Compared to mammals, fish represent a vast phylogenetic group, which shows a significant level of diversity with regards to morphology, ecology, behavior, and genomes (283). Thus, species differences in the neuroendocrine control of reproduction have to be taken into consideration in teleosts. In addition, more detailed studies about the interconnections among metabolic neuropeptide neurons, effects of sexual maturation, and nutritional conditions will provide more precise figure of the metabolic control of reproduction. Furthermore, differential control of multiple GnRH neuronal population by the neuropeptides and metabolic signals should be examined to elucidate their roles in different aspects of metabolic control of reproduction.

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REFERENCES

1. Schneider JE. Energy balance and reproduction. *Physiol Behav* (2004) **81**(2):289–317. doi:10.1016/j.physbeh.2004.02.007
2. Fernandez-Fernandez R, Martini AC, Navarro VM, Castellano JM, Dieguez C, Aguilar E, et al. Novel signals for the integration of energy balance and reproduction. *Mol Cell Endocrinol* (2006) **254–5**:127–32. doi:10.1016/j.mce.2006.04.026
3. Castellano JM, Roa J, Luque RM, Dieguez C, Aguilar E, Pinilla L, et al. KiSS-1/kisspeptins and the metabolic control of reproduction: physiologic roles and putative physiopathological implications. *Peptides* (2009) **30**(1):139–45. doi:10.1016/j.peptides.2008.06.007
4. Hill JW, Elmquist JK, Elias CF. Hypothalamic pathways linking energy balance and reproduction. *Am J Physiol Endocrinol Metab* (2008) **294**(5):E827–32. doi:10.1152/ajpendo.00670.2007
5. Schneider JE, Buckley CA, Blum RM, Zhou D, Szymanski L, Day DE, et al. Metabolic signals, hormones and neuropeptides involved in control of

energy balance and reproductive success in hamsters. *Eur J Neurosci* (2002) **16**(3):377–9. doi:10.1046/j.1460-9568.2002.02118.x
6. Wade GN, Schneider JE, Li HY. Control of fertility by metabolic cues. *Am J Physiol* (1996) **270**(1 Pt 1):E1–19.
7. Volkoff H, Canosa LF, Unniappan S, Cerda-Reverter JM, Bernier NJ, Kelly SP, et al. Neuropeptides and the control of food intake in fish. *Gen Comp Endocrinol* (2005) **142**(1–2):3–19. doi:10.1016/j.ygcen.2004.11.001
8. Mircea CN, Lujan ME, Pierson RA. Metabolic fuel and clinical implications for female reproduction. *J Obstet Gynaecol Can* (2007) **29**(11):887–902.
9. Volkoff H, Xu M, MacDonald E, Hoskins L. Aspects of the hormonal regulation of appetite in fish with emphasis on goldfish, Atlantic cod and winter flounder: notes on actions and responses to nutritional, environmental and reproductive changes. *Comp Biochem Physiol A Mol Integr Physiol* (2009) **153**(1):8–12. doi:10.1016/j.cbpa.2008.12.001
10. Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B. Regulation of fish gonadotropins. *Int Rev Cytol* (2003) **225**:131–85. doi:10.1016/S0074-7696(05)25004-0
11. Zohar Y, Munoz-Cueto JA, Elizur A, Kah O. Neuroendocrinology of reproduction in teleost fish. *Gen Comp Endocrinol* (2010) **165**(3):438–55. doi:10.1016/j.ygcen.2009.04.017
12. Amoss M, Burgus R, Blackwell R, Vale W, Fellows R, Guillemin R. Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. *Biochem Biophys Res Commun* (1971) **44**(1):205–10. doi:10.1016/S0006-291X(71)80179-1
13. Matsuo H, Baba Y, Nair RM, Arimura A, Schally AV. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem Biophys Res Commun* (1971) **43**(6):1334–9. doi:10.1016/S0006-291X(71)80019-0
14. Breton B, Jalabert B, Billard R, Weil C. In vitro stimulation of the release of pituitary gonadotropic hormone by a hypothalamic factor in the carp *Cyprinus carpio* L. *C R Acad Sci Hebd Seances Acad Sci D* (1971) **273**(25):2591–4.
15. Sherwood N, Eiden L, Brownstein M, Spiess J, Rivier J, Vale W. Characterization of a teleost gonadotropin-releasing hormone. *Proc Natl Acad Sci U S A* (1983) **80**(9):2794–8. doi:10.1073/pnas.80.9.2794
16. Sherwood NM, Zoeller RT, Moore FL. Multiple forms of gonadotropin-releasing hormone in amphibian brains. *Gen Comp Endocrinol* (1986) **61**(2):313–22. doi:10.1016/0016-6480(86)90208-X
17. Miyamoto K, Hasegawa Y, Minegishi T, Nomura M, Takahashi Y, Igarashi M, et al. Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. *Biochem Biophys Res Commun* (1982) **107**(3):820–7. doi:10.1016/0006-291X(82)90596-4
18. Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H. Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. *Proc Natl Acad Sci U S A* (1984) **81**(12):3874–8. doi:10.1073/pnas.81.12.3874
19. Lovejoy DA, Stell WK, Sherwood NM. Partial characterization of four forms of immunoreactive gonadotropin-releasing hormone in the brain and terminal nerve of the spiny dogfish (Elasmobranchii; *Squalus acanthias*). *Regul Pept* (1992) **37**(1):39–48. doi:10.1016/0167-0115(92)90062-Y
20. Ngamvongchon S, Rivier JE, Sherwood NM. Structure-function studies of five natural, including catfish and dogfish, gonadotropin-releasing hormones and eight analogs on reproduction in Thai catfish (*Clarias macrocephalus*). *Regul Pept* (1992) **42**(1–2):63–73. doi:10.1016/0167-0115(92)90024-O
21. Sower SA, Chiang YC, Lovas S, Conlon JM. Primary structure and biological activity of a third gonadotropin-releasing hormone from lamprey brain. *Endocrinology* (1993) **132**(3):1125–31. doi:10.1210/endo.132.3.8440174
22. Jimenez-Linan M, Rubin BS, King JC. Examination of guinea pig luteinizing hormone-releasing hormone gene reveals a unique decapeptide and existence of two transcripts in the brain. *Endocrinology* (1997) **138**(10):4123–30. doi:10.1210/en.138.10.4123
23. Carolsfeld J, Powell JF, Park M, Fischer WH, Craig AG, Chang JP, et al. Primary structure and function of three gonadotropin-releasing hormones, including a novel form, from an ancient teleost, herring. *Endocrinology* (2000) **141**(2):505–12. doi:10.1210/endo.141.2.7300
24. Kavanaugh SI, Nozaki M, Sower SA. Origins of gonadotropin-releasing hormone (GnRH) in vertebrates: identification of a novel GnRH in a basal vertebrate, the sea lamprey. *Endocrinology* (2008) **149**(8):3860–9. doi:10.1210/en.2008-0184
25. Adams BA, Vickers ED, Warby C, Park M, Fischer WH, Grey Craig A, et al. Three forms of gonadotropin-releasing hormone, including a novel form, in a basal salmonid, *Coregonus clupeaformis*. *Biol Reprod* (2002) **67**(1):232–9. doi:10.1095/biolreprod67.1.232
26. Montaner AD, Mongiat L, Lux-Lantos VA, Park MK, Fischer WH, Craig AG, et al. Structure and biological activity of gonadotropin-releasing hormone isoforms isolated from rat and hamster brains. *Neuroendocrinology* (2001) **74**(3):202–12. doi:10.1159/000054687
27. Mohamed JS, Khan IA. Molecular cloning and differential expression of three GnRH mRNAs in discrete brain areas and lymphocytes in red drum. *J Endocrinol* (2006) **188**(3):407–16. doi:10.1677/joe.1.06423
28. Andersson E, Fjelldal PG, Klenke U, Vikingstad E, Taranger GL, Zohar Y, et al. Three forms of GnRH in the brain and pituitary of the turbot, *Scophthalmus maximus*: immunological characterization and seasonal variation. *Comp Biochem Physiol B Biochem Mol Biol* (2001) **129**(2–3):551–8. doi:10.1016/S1096-4959(01)00363-3
29. Okubo K, Amano M, Yoshiura Y, Suetake H, Aida K. A novel form of gonadotropin-releasing hormone in the medaka, *Oryzias latipes*. *Biochem Biophys Res Commun* (2000) **276**(1):298–303. doi:10.1006/bbrc.2000.3476
30. Sherwood NM, von Schalburg KR, Lescheid DW. Origin and evolution of GnRH in vertebrates and invertebrates. In: Parhar IS, Sakuma Y, editors. *GnRH Neurons: From Genes to Behavior*. Tokyo: Brain Shuppan (1997). p. 3–25.
31. Parhar IS. GnRH in tilapia: three genes, three origins and their roles. In: Parhar IS, Sakuma Y, editors. *GnRH Neurons: Gene to Behavior*. Tokyo: Brain Shuppan (1997). p. 99–122.
32. Parhar IS. Cell migration and evolutionary significance of GnRH subtypes. *Prog Brain Res* (2002) **141**:3–17. doi:10.1016/S0079-6123(02)41080-1
33. Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. *Endocr Rev* (2004) **25**(2):235–75. doi:10.1210/er.2003-0002
34. Karigo T, Oka Y. Neurobiological study of fish brains gives insights into the nature of gonadotropin-releasing hormone 1-3 neurons. *Front Endocrinol* (2013) **4**:177. doi:10.3389/fendo.2013.00177
35. Powell JF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, et al. Three forms of gonadotropin-releasing hormone characterized from brains of one species. *Proc Natl Acad Sci U S A* (1994) **91**(25):12081–5. doi:10.1073/pnas.91.25.12081
36. Okubo K, Nagahama Y. Structural and functional evolution of gonadotropin-releasing hormone in vertebrates. *Acta Physiol* (2008) **193**(1):3–15. doi:10.1111/j.1748-1716.2008.01832.x
37. White SA, Kasten TL, Bond CT, Adelman JP, Fernald RD. Three gonadotropin-releasing hormone genes in one organism suggest novel roles for an ancient peptide. *Proc Natl Acad Sci U S A* (1995) **92**(18):8363–7. doi:10.1073/pnas.92.18.8363
38. Gothilf Y, Munoz-Cueto JA, Sagrillo CA, Selmanoff M, Chen TT, Kah O, et al. Three forms of gonadotropin-releasing hormone in a perciform fish (*Sparus aurata*): complementary deoxyribonucleic acid characterization and brain localization. *Biol Reprod* (1996) **55**(3):636–45. doi:10.1095/biolreprod55.3.636
39. Gonzalez-Martinez D, Madigou T, Zmora N, Anglade I, Zanuy S, Zohar Y, et al. Differential expression of three different prepro-GnRH (gonadotropin-releasing hormone) messengers in the brain of the European sea bass (*Dicentrarchus labrax*). *J Comp Neurol* (2001) **429**(1):144–55. doi:10.1002/1096-9861(20000101)429:1<144::AID-CNE11>3.0.CO;2-B
40. Gonzalez-Martinez D, Zmora N, Mananos E, Saligaut D, Zanuy S, Zohar Y, et al. Immunohistochemical localization of three different prepro-GnRHs in the brain and pituitary of the European sea bass (*Dicentrarchus labrax*) using antibodies to the corresponding GnRH-associated peptides. *J Comp Neurol* (2002) **446**(2):95–113. doi:10.1002/cne.10190
41. Palevitch O, Kight K, Abraham E, Wray S, Zohar Y, Gothilf Y. Ontogeny of the GnRH systems in zebrafish brain: in situ hybridization and promoter-reporter expression analyses in intact animals. *Cell Tissue Res* (2007) **327**(2):313–22. doi:10.1007/s00441-006-0279-0
42. Vickers ED, Laberge F, Adams BA, Hara TJ, Sherwood NM. Cloning and localization of three forms of gonadotropin-releasing hormone, including the novel whitefish form, in a salmonid, *Coregonus clupeaformis*. *Biol Reprod* (2004) **70**(4):1136–46. doi:10.1095/biolreprod.103.023846
43. Pandolfi M, Munoz-Cueto JA, Lo Nostro FL, Downs JL, Paz DA, Maggese MC, et al. GnRH systems of *Cichlasoma dimerus* (*Perciformes*, *Cichlidae*) revisited: a localization study with antibodies and riboprobes to GnRH-associated peptides. *Cell Tissue Res* (2005) **321**(2):219–32. doi:10.1007/s00441-004-1055-7

44. Mohamed JS, Thomas P, Khan IA. Isolation, cloning, and expression of three prepro-GnRH mRNAs in Atlantic croaker brain and pituitary. *J Comp Neurol* (2005) **488**(4):384–95. doi:10.1002/cne.20596
45. Parhar IS, Iwata M. Gonadotropin releasing hormone (GnRH) neurons project to growth hormone and somatolactin cells in the steelhead trout. *Histochemistry* (1994) **102**(3):195–203. doi:10.1007/BF00268896
46. Breton B, Govoroun M, Mikolajczyk T. GTH I and GTH II secretion profiles during the reproductive cycle in female rainbow trout: relationship with pituitary responsiveness to GnRH-A stimulation. *Gen Comp Endocrinol* (1998) **111**(1):38–50. doi:10.1006/gcen.1998.7088
47. Mananos EL, Anglade I, Chyb J, Saligaut C, Breton B, Kah O. Involvement of gamma-aminobutyric acid in the control of GTH-1 and GTH-2 secretion in male and female rainbow trout. *Neuroendocrinology* (1999) **69**(4):269–80. doi:10.1159/000054428
48. Weil C, Carre F, Blaise O, Breton B, Le Bail PY. Differential effect of insulin-like growth factor I on in vitro gonadotropin (I and II) and growth hormone secretions in rainbow trout (*Oncorhynchus mykiss*) at different stages of the reproductive cycle. *Endocrinology* (1999) **140**(5):2054–62. doi:10.1210/endo.140.5.6747
49. Dickey JT, Swanson P. Effects of sex steroids on gonadotropin (FSH and LH) regulation in Coho salmon (*Oncorhynchus kisutch*). *J Mol Endocrinol* (1998) **21**(3):291–306. doi:10.1677/jme.0.0210291
50. Senthilkumaran B, Okuzawa K, Gen K, Ookura T, Kagawa H. Distribution and seasonal variations in levels of three native GnRHs in the brain and pituitary of perciform fish. *J Neuroendocrinol* (1999) **11**(3):181–6. doi:10.1046/j.1365-2826.1999.00304.x
51. Holland MC, Hassin S, Zohar Y. Seasonal fluctuations in pituitary levels of the three forms of gonadotropin-releasing hormone in striped bass, *Morone saxatilis* (*Teleostei*), during juvenile and pubertal development. *J Endocrinol* (2001) **169**(3):527–38. doi:10.1677/joe.0.1690527
52. Amano M, Okubo K, Yamanome T, Yamada H, Aida K, Yamamori K. Changes in brain GnRH mRNA and pituitary GnRH peptide during testicular maturation in barfin flounder. *Comp Biochem Physiol B Biochem Mol Biol* (2004) **138**(4):435–43. doi:10.1016/j.cbpc.2004.05.005
53. Amano M, Pham KX, Amiya N, Yamanome T, Yamamori K. Changes in brain seabream GnRH mRNA and pituitary seabream GnRH peptide levels during ovarian maturation in female barfin flounder. *Gen Comp Endocrinol* (2008) **158**(2):168–72. doi:10.1016/j.ygcen.2008.06.008
54. Okuzawa K, Gen K, Bruysters M, Bogerd J, Gothilf Y, Zohar Y, et al. Seasonal variation of the three native gonadotropin-releasing hormone messenger ribonucleic acids levels in the brain of female red seabream. *Gen Comp Endocrinol* (2003) **130**(3):324–32. doi:10.1016/S0016-6480(02)00629-9
55. Okubo K, Sakai F, Lau EL, Yoshizaki G, Takeuchi Y, Naruse K, et al. Forebrain gonadotropin-releasing hormone neuronal development: insights from transgenic medaka and the relevance to X-linked Kallmann syndrome. *Endocrinology* (2006) **147**(3):1076–84. doi:10.1210/en.2005-0468
56. Holland MC, Gothilf Y, Meiri I, King JA, Okuzawa K, Elizur A, et al. Levels of the native forms of GnRH in the pituitary of the gilthead seabream, *Sparus aurata*, at several characteristic stages of the gonadal cycle. *Gen Comp Endocrinol* (1998) **112**(3):394–405. doi:10.1006/gcen.1998.7138
57. Zandbergen MA, Kah O, Bogerd J, Peute J, Goos HJ. Expression and distribution of two gonadotropin-releasing hormones in the catfish brain. *Neuroendocrinology* (1995) **62**(6):571–8. doi:10.1159/000127052
58. Amano M, Oka Y, Yamanome T, Okuzawa K, Yamamori K. Three GnRH systems in the brain and pituitary of a pleuronectiform fish, the barfin flounder *Verasper moseri*. *Cell Tissue Res* (2002) **309**(2):323–9. doi:10.1007/s00441-002-0594-z
59. Lethimonier C, Madigou T, Munoz-Cueto JA, Lareyre JJ, Kah O. Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish. *Gen Comp Endocrinol* (2004) **135**(1):1–16. doi:10.1016/j.ygcen.2003.10.007
60. Yamamoto N, Oka Y, Kawashima S. Lesions of gonadotropin-releasing hormone-immunoreactive terminal nerve cells: effects on the reproductive behavior of male dwarf gouramis. *Neuroendocrinology* (1997) **65**(6):403–12. doi:10.1159/000127203
61. Kitahashi T, Sato A, Alok D, Kaeriyama M, Zohar Y, Yamauchi K, et al. Gonadotropin-releasing hormone analog and sex steroids shorten homing duration of sockeye salmon in Lake Shikotsu. *Zoolog Sci* (1998) **15**(5):767–71. doi:10.2108/zsj.15.767
62. Ogawa S, Akiyama G, Kato S, Soga T, Sakuma Y, Parhar IS. Immunoneutralization of gonadotropin-releasing hormone type-III suppresses male reproductive behavior of cichlids. *Neurosci Lett* (2006) **403**(3):201–5. doi:10.1016/j.neulet.2006.02.041
63. Umino O, Dowling JE. Dopamine release from interplexiform cells in the retina: effects of GnRH, FMRFamide, bicuculline, and enkephalin on horizontal cell activity. *J Neurosci* (1991) **11**(10):3034–46.
64. Walker SE, Stell WK. Gonadotropin-releasing hormone (GnRF), molluscan cardioexcitatory peptide (FMRFamide), enkephalin and related neuropeptides affect goldfish retinal ganglion cell activity. *Brain Res* (1986) **384**(2):262–73. doi:10.1016/0006-8993(86)91162-5
65. Eisthen HL, Delay RJ, Wirsig-Wiechmann CR, Dionne VE. Neuromodulatory effects of gonadotropin releasing hormone on olfactory receptor neurons. *J Neurosci* (2000) **20**(11):3947–55.
66. Saito D, Hasegawa Y, Urano A. Gonadotropin-releasing hormones modulate electrical activity of vasotocin and isotocin neurons in the brain of rainbow trout. *Neurosci Lett* (2003) **351**(2):107–10. doi:10.1016/j.neulet.2003.08.017
67. Kinoshita M, Kobayashi S, Urano A, Ito E. Neuromodulatory effects of gonadotropin-releasing hormone on retinotectal synaptic transmission in the optic tectum of rainbow trout. *Eur J Neurosci* (2007) **25**(2):480–4. doi:10.1111/j.1460-9568.2006.05294.x
68. Oka Y. Three types of gonadotrophin-releasing hormone neurones and steroid-sensitive sexually dimorphic kisspeptin neurones in teleosts. *J Neuroendocrinol* (2009) **21**(4):334–8. doi:10.1111/j.1365-2826.2009.01850.x
69. Amano M, Urano A, Aida K. Distribution and function of gonadotropin-releasing hormone (GnRH) in the teleost brain. *Zoolog Sci* (1997) **14**(1):1–11. doi:10.2108/zsj.14.1
70. Abraham E, Palevitch O, Gothilf Y, Zohar Y. The zebrafish as a model system for forebrain GnRH neuronal development. *Gen Comp Endocrinol* (2009) **164**(2–3):151–60. doi:10.1016/j.ygcen.2009.01.012
71. Parhar IS, Tosaki H, Sakuma Y, Kobayashi M. Sex differences in the brain of goldfish: gonadotropin-releasing hormone and vasotocinergic neurons. *Neuroscience* (2001) **104**(4):1099–110. doi:10.1016/S0306-4522(01)00153-1
72. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev* (2009) **30**(6):713–43. doi:10.1210/er.2009-0005
73. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The metastasis suppressor gene KiSS-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem* (2001) **276**(37):34631–6. doi:10.1074/jbc.M104847200
74. Kitahashi T, Ogawa S, Parhar IS. Cloning and expression of kiss2 in the zebrafish and medaka. *Endocrinology* (2009) **150**(2):821–31. doi:10.1210/en.2008-0940
75. Felip A, Zanuy S, Pineda R, Pinilla L, Carrillo M, Tena-Sempere M, et al. Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. *Mol Cell Endocrinol* (2009) **312**(1–2):61–71. doi:10.1016/j.mce.2008.11.017
76. Li S, Zhang Y, Liu Y, Huang X, Huang W, Lu D, et al. Structural and functional multiplicity of the kisspeptin/GPR54 system in goldfish (*Carassius auratus*). *J Endocrinol* (2009) **201**(3):407–18. doi:10.1677/joe-09-0016
77. Lee YR, Tsunekawa K, Moon MJ, Um HN, Hwang JI, Osugi T, et al. Molecular evolution of multiple forms of kisspeptins and GPR54 receptors in vertebrates. *Endocrinology* (2009) **150**(6):2837–46. doi:10.1210/en.2008-1679
78. Biran J, Ben-Dor S, Levavi-Sivan B. Molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates. *Biol Reprod* (2008) **79**(4):776–86. doi:10.1095/biolreprod.107.066266
79. Mechaly AS, Vinas J, Piferrer F. Identification of two isoforms of the Kisspeptin-1 receptor (kiss1r) generated by alternative splicing in a modern teleost, the Senegalese sole (*Solea senegalensis*). *Biol Reprod* (2009) **80**(1):60–9. doi:10.1095/biolreprod.108.072173
80. Shi Y, Zhang Y, Li S, Liu Q, Lu D, Liu M, et al. Molecular identification of the Kiss2/Kiss1ra system and its potential function during 17alpha-methyltestosterone-induced sex reversal in the orange-spotted grouper, *Epinephelus coioides*[1]. *Biol Reprod* (2010) **83**(1):63–74. doi:10.1095/biolreprod.109.080044
81. Shahjahan M, Motohashi E, Doi H, Ando H. Elevation of Kiss2 and its receptor gene expression in the brain and pituitary of grass puffer during the spawning season. *Gen Comp Endocrinol* (2010) **169**(1):48–57. doi:10.1016/j.ygcen.2010.07.008

82. Mechaly AS, Vinas J, Murphy C, Reith M, Piferrer F. Gene structure of the Kiss1 receptor-2 (Kiss1r-2) in the Atlantic halibut: insights into the evolution and regulation of Kiss1r genes. *Mol Cell Endocrinol* (2010) **317**(1–2):78–89. doi:10.1016/j.mce.2009.11.005
83. Yang B, Jiang Q, Chan T, Ko WKW, Wong AOL. Goldfish kisspeptin: molecular cloning, tissue distribution of transcript expression, and stimulatory effects on prolactin, growth hormone and luteinizing hormone secretion and gene expression via direct actions at the pituitary level. *Gen Comp Endocrinol* (2010) **165**(1):60–71. doi:10.1016/j.ygcen.2009.06.001
84. Kanda S, Akazome Y, Matsunaga T, Yamamoto N, Yamada S, Tsukamura H, et al. Identification of KiSS-1 product kisspeptin and steroid-sensitive sexually dimorphic kisspeptin neurons in medaka (*Oryzias latipes*). *Endocrinology* (2008) **149**(5):2467–76. doi:10.1210/en.2007-1503
85. Servili A, Le Page Y, Leprince J, Caraty A, Escobar S, Parhar IS, et al. Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. *Endocrinology* (2011) **152**(4):1527–40. doi:10.1210/en.2010-0948
86. Parhar IS, Ogawa S, Sakuma Y. Laser-captured single digoxigenin-labeled neurons of gonadotropin-releasing hormone types reveal a novel G protein-coupled receptor (Gpr54) during maturation in cichlid fish. *Endocrinology* (2004) **145**(8):3613–8. doi:10.1210/en.2004-0395
87. Ogawa S, Ng KW, Ramadasan PN, Nathan FM, Parhar IS. Habenular Kiss1 neurons modulate the serotonergic system in the brain of zebrafish. *Endocrinology* (2012) **153**(5):2398–407. doi:10.1210/en.2012-1062
88. Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, et al. A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem Biophys Res Commun* (2000) **275**(2):661–7. doi:10.1006/bbrc.2000.3350
89. Parhar I, Ogawa S, Kitahashi T. RFamide peptides as mediators in environmental control of GnRH neurons. *Prog Neurobiol* (2012) **98**(2):176–96. doi:10.1016/j.pneurobio.2012.05.011
90. Tsutsui K, Ubuka T, Bentley GE, Kriegsfeld LJ. Gonadotropin-inhibitory hormone (GnIH): discovery, progress and prospect. *Gen Comp Endocrinol* (2012) **177**(3):305–14. doi:10.1016/j.ygcen.2012.02.013
91. Sawada K, Ukena K, Satake H, Iwakoshi E, Minakata H, Tsutsui K. Novel fish hypothalamic neuropeptide. *Eur J Biochem* (2002) **269**(24):6000–8. doi:10.1046/j.1432-1033.2002.03351.x
92. Amano M, Moriyama S, Iigo M, Kitamura S, Amiya N, Yamamori K, et al. Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon. *J Endocrinol* (2006) **188**(3):417–23. doi:10.1677/joe.1.06494
93. Moussavi M, Wlasichuk M, Chang JP, Habibi HR. Seasonal effect of GnIH on gonadotrope functions in the pituitary of goldfish. *Mol Cell Endocrinol* (2012) **350**(1):53–60. doi:10.1016/j.mce.2011.11.020
94. Zhang Y, Li S, Liu Y, Lu D, Chen H, Huang X, et al. Structural diversity of the GnIH/GnIH receptor system in teleost: its involvement in early development and the negative control of LH release. *Peptides* (2010) **31**(6):1034–43. doi:10.1016/j.peptides.2010.03.003
95. Qi X, Zhou W, Li S, Lu D, Yi S, Xie R, et al. Evidences for the regulation of GnRH and GTH expression by GnIH in the goldfish, *Carassius auratus*. *Mol Cell Endocrinol* (2013) **366**(1):9–20. doi:10.1016/j.mce.2012.11.001
96. Qi X, Zhou W, Lu D, Wang Q, Zhang H, Li S, et al. Sexual dimorphism of steroidogenesis regulated by GnIH in the goldfish, *Carassius auratus*. *Biol Reprod* (2013) **88**(4):89. doi:10.1095/biolreprod.112.105114
97. Shahjahan M, Ikegami T, Osugi T, Ukena K, Doi H, Hattori A, et al. Synchronised expressions of LPXRFamide peptide and its receptor genes: seasonal, diurnal and circadian changes during spawning period in grass puffer. *J Neuroendocrinol* (2011) **23**(1):39–51. doi:10.1111/j.1365-2826.2010.02081.x
98. Ubuka T, Inoue K, Fukuda Y, Mizuno T, Ukena K, Kriegsfeld LJ, et al. Identification, expression, and physiological functions of Siberian hamster gonadotropin-inhibitory hormone. *Endocrinology* (2012) **153**(1):373–85. doi:10.1210/en.2011-1110
99. Umatani C, Abe H, Oka Y. Neuropeptide RFRP inhibits the pacemaker activity of terminal nerve GnRH neurons. *J Neurophysiol* (2013) **109**(9):2354–63. doi:10.1152/jn.00712.2012
100. Yokobori E, Kojima K, Azuma M, Kang KS, Maejima S, Uchiyama M, et al. Stimulatory effect of intracerebroventricular administration of orexin A on food intake in the zebrafish, *Danio rerio*. *Peptides* (2011) **32**(7):1357–62. doi:10.1016/j.peptides.2011.05.010
101. Facciolo RM, Crudo M, Giusi G, Alo R, Canonaco M. Light- and dark-dependent orexinergic neuronal signals promote neurodegenerative phenomena accounting for distinct behavioral responses in the teleost *Thalassoma pavo*. *J Neurosci Res* (2009) **87**(3):748–57. doi:10.1002/jnr.21886
102. Hoskins LJ, Xu M, Volkoff H. Interactions between gonadotropin-releasing hormone (GnRH) and orexin in the regulation of feeding and reproduction in goldfish (*Carassius auratus*). *Horm Behav* (2008) **54**(3):379–85. doi:10.1016/j.yhbeh.2008.04.011
103. Lopez-Patino MA, Guijarro AI, Isorna E, Delgado MJ, Alonso-Bedate M, de Pedro N. Neuropeptide Y has a stimulatory action on feeding behavior in goldfish (*Carassius auratus*). *Eur J Pharmacol* (1999) **377**(2–3):147–53. doi:10.1016/S0014-2999(99)00408-2
104. Aldegunde M, Mancebo M. Effects of neuropeptide Y on food intake and brain biogenic amines in the rainbow trout (*Oncorhynchus mykiss*). *Peptides* (2006) **27**(4):719–27. doi:10.1016/j.peptides.2005.09.014
105. Matsuda K. Recent advances in the regulation of feeding behavior by neuropeptides in fish. *Ann N Y Acad Sci* (2009) **1163**:241–50. doi:10.1111/j.1749-6632.2008.03619.x
106. Yokobori E, Azuma M, Nishiguchi R, Kang KS, Kamijo M, Uchiyama M, et al. Neuropeptide Y stimulates food intake in the zebrafish, *Danio rerio*. *J Neuroendocrinol* (2012) **24**(5):766–73. doi:10.1111/j.1365-2826.2012.02281.x
107. Peng C, Humphries S, Peter RE, Rivier JE, Blomqvist AG, Larhammar D. Actions of goldfish neuropeptide Y on the secretion of growth hormone and gonadotropin-II in female goldfish. *Gen Comp Endocrinol* (1993) **90**(3):306–17. doi:10.1006/gcen.1993.1086
108. Peng C, Chang JP, Yu KL, Wong AO, Van Goor F, Peter RE, et al. Neuropeptide-Y stimulates growth hormone and gonadotropin-II secretion in the goldfish pituitary: involvement of both presynaptic and pituitary cell actions. *Endocrinology* (1993) **132**(4):1820–9. doi:10.1210/endo.132.4.8462479
109. Breton B, Mikolajczyk T, Popek W, Bieniarz K, Epler P. Neuropeptide Y stimulates in vivo gonadotropin secretion in teleost fish. *Gen Comp Endocrinol* (1991) **84**(2):277–83. doi:10.1016/0016-6480(91)90050-G
110. Senthilkumaran B, Okuzawa K, Gen K, Kagawa H. Effects of serotonin, GABA and neuropeptide Y on seabream gonadotropin releasing hormone release in vitro from preoptic-anterior hypothalamus and pituitary of red seabream, *Pagrus major*. *J Neuroendocrinol* (2001) **13**(5):395–400. doi:10.1046/j.1365-2826.2001.00645.x
111. Cerda-Reverter JM, Sorbera LA, Carrillo M, Zanuy S. Energetic dependence of NPY-induced LH secretion in a teleost fish (*Dicentrarchus labrax*). *Am J Physiol* (1999) **277**(6 Pt 2):R1627–34.
112. Matsuda K, Maruyama K, Nakamachi T, Miura T, Uchiyama M, Shioda S. Inhibitory effects of pituitary adenylate cyclase-activating polypeptide (PACAP) and vasoactive intestinal peptide (VIP) on food intake in the goldfish, *Carassius auratus*. *Peptides* (2005) **26**(9):1611–6. doi:10.1016/j.peptides.2005.02.022
113. Sawisky GR, Chang JP. Intracellular calcium involvement in pituitary adenylate cyclase-activating polypeptide stimulation of growth hormone and gonadotrophin secretion in goldfish pituitary cells. *J Neuroendocrinol* (2005) **17**(6):353–71. doi:10.1111/j.1365-2826.2005.01312.x
114. Chang JP, Wirachowsky NR, Kwong P, Johnson JD. Pacap stimulation of gonadotropin-II secretion in goldfish pituitary cells: mechanisms of action and interaction with gonadotropin releasing hormone signalling. *J Neuroendocrinol* (2001) **13**(6):540–50. doi:10.1046/j.1365-2826.2001.00667.x
115. Yaron Z, Gur G, Melamed P, Rosenfeld H, Levavi-Sivan B, Elizur A. Regulation of gonadotropin subunit genes in tilapia. *Comp Biochem Physiol B Biochem Mol Biol* (2001) **129**(2–3):489–502. doi:10.1016/S1096-4959(01)00345-1
116. Levy G, Jackson K, Degani G. Association between pituitary adenylate cyclase-activating polypeptide and reproduction in the blue gourami. *Gen Comp Endocrinol* (2010) **166**(1):83–93. doi:10.1016/j.ygcen.2009.09.015
117. Levy G, Degani G. Involvement of GnRH, PACAP and PRP in the reproduction of blue gourami females (*Trichogaster trichopterus*). *J Mol Neurosci* (2012) **48**(3):603–16. doi:10.1007/s12031-012-9730-8
118. Matsuda K, Nakamura K, Shimakura S, Miura T, Kageyama H, Uchiyama M, et al. Inhibitory effect of chicken gonadotropin-releasing hormone II on food intake in the goldfish, *Carassius auratus*. *Horm Behav* (2008) **54**(1):83–9. doi:10.1016/j.yhbeh.2008.01.011

119. Nishiguchi R, Azuma M, Yokobori E, Uchiyama M, Matsuda K. Gonadotropin-releasing hormone 2 suppresses food intake in the zebrafish, *Danio rerio*. *Front Endocrinol* (2012) **3**:122. doi:10.3389/fendo.2012.00122
120. Chang JP, Freedman GL, de Leeuw R. Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. II. Extracellular calcium dependence and dopaminergic inhibition of gonadotropin responses. *Gen Comp Endocrinol* (1990) **77**(2):274–82. doi:10.1016/0016-6480(90)90311-9
121. Chartrel N, Dujardin C, Anouar Y, Leprince J, Decker A, Clerens S, et al. Identification of 26RFa, a hypothalamic neuropeptide of the RFamide peptide family with orexigenic activity. *Proc Natl Acad Sci U S A* (2003) **100**(25):15247–52. doi:10.1073/pnas.2434676100
122. Liu Y, Zhang Y, Li S, Huang W, Liu X, Lu D, et al. Molecular cloning and functional characterization of the first non-mammalian 26RFa/QRFP orthologue in goldfish, *Carassius auratus*. *Mol Cell Endocrinol* (2009) **303**(1–2):82–90. doi:10.1016/j.mce.2009.01.009
123. Volkoff H, Peter RE. Interactions between orexin A, NPY and galanin in the control of food intake of the goldfish, *Carassius auratus*. *Regul Pept* (2001) **101**(1–3):59–72. doi:10.1016/S0167-0115(01)00261-0
124. Guijarro AI, Delgado MJ, Pinillos ML, López-Patiño MA, Alonso-Bedate M, De Pedro N. Galanin and β-endorphin as feeding regulators in cyprinids: effect of temperature. *Aquac Res* (1999) **30**(7):483–9. doi:10.1046/j.1365-2109.1999.00360.x
125. Merchenthaler I, Lopez FJ, Negro-Vilar A. Colocalization of galanin and luteinizing hormone-releasing hormone in a subset of preoptic hypothalamic neurons: anatomical and functional correlates. *Proc Natl Acad Sci U S A* (1990) **87**(16):6326–30. doi:10.1073/pnas.87.16.6326
126. Matsuda K, Shimakura S, Maruyama K, Miura T, Uchiyama M, Kawauchi H, et al. Central administration of melanin-concentrating hormone (MCH) suppresses food intake, but not locomotor activity, in the goldfish, *Carassius auratus*. *Neurosci Lett* (2006) **399**(3):259–63. doi:10.1016/j.neulet.2006.02.005
127. Cerda-Reverter JM, Canosa LF, Peter RE. Regulation of the hypothalamic melanin-concentrating hormone neurons by sex steroids in the goldfish: possible role in the modulation of luteinizing hormone secretion. *Neuroendocrinology* (2006) **84**(6):364–77. doi:10.1159/000098334
128. Cerda-Reverter JM, Schioth HB, Peter RE. The central melanocortin system regulates food intake in goldfish. *Regul Pept* (2003) **115**(2):101–13. doi:10.1016/S0167-0115(03)00144-7
129. Schjolden J, Schioth HB, Larhammar D, Winberg S, Larson ET. Melanocortin peptides affect the motivation to feed in rainbow trout (*Oncorhynchus mykiss*). *Gen Comp Endocrinol* (2009) **160**(2):134–8. doi:10.1016/j.ygcen.2008.11.003
130. Roa J. Role of GnRH neurons and their neuronal afferents as key integrators between food intake regulatory signals and the control of reproduction. *Int J Endocrinol* (2013) **2013**:518046. doi:10.1155/2013/518046
131. Volkoff H, Peter RE. Effects of CART peptides on food consumption, feeding and associated behaviors in the goldfish, *Carassius auratus*: actions on neuropeptide Y- and orexin A-induced feeding. *Brain Res* (2000) **887**(1):125–33. doi:10.1016/S0006-8993(00)03001-8
132. Volkoff H, Peter RE. Characterization of two forms of cocaine- and amphetamine-regulated transcript (CART) peptide precursors in goldfish: molecular cloning and distribution, modulation of expression by nutritional status, and interactions with leptin. *Endocrinology* (2001) **142**(12):5076–88. doi:10.1210/en.142.12.5076
133. Lebrethon MC, Vandersmissen E, Gerard A, Parent AS, Bourguignon JP. Cocaine and amphetamine-regulated-transcript peptide mediation of leptin stimulatory effect on the rat gonadotropin-releasing hormone pulse generator in vitro. *J Neuroendocrinol* (2000) **12**(5):383–5. doi:10.1046/j.1365-2826.2000.00497.x
134. Parent AS, Lebrethon MC, Gerard A, Vandersmissen E, Bourguignon JP. Leptin effects on pulsatile gonadotropin releasing hormone secretion from the adult rat hypothalamus and interaction with cocaine and amphetamine regulated transcript peptide and neuropeptide Y. *Regul Pept* (2000) **92**(1–3):17–24. doi:10.1016/S0167-0115(00)00144-0
135. Himick BA, Peter RE. CCK/gastrin-like immunoreactivity in brain and gut, and CCK suppression of feeding in goldfish. *Am J Physiol* (1994) **267**(3 Pt 2):R841–51.
136. Himick BA, Golosinski AA, Jonsson AC, Peter RE. CCK/gastrin-like immunoreactivity in the goldfish pituitary: regulation of pituitary hormone secretion by CCK-like peptides in vitro. *Gen Comp Endocrinol* (1993) **92**(1):88–103. doi:10.1006/gcen.1993.1146
137. Gonzalez R, Kerbel B, Chun A, Unniappan S. Molecular, cellular and physiological evidences for the anorexigenic actions of nesfatin-1 in goldfish. *PLoS One* (2010) **5**(12):e15201. doi:10.1371/journal.pone.0015201
138. Kerbel B, Unniappan S. Nesfatin-1 suppresses energy intake, co-localises ghrelin in the brain and gut, and alters ghrelin, cholecystokinin and orexin mRNA expression in goldfish. *J Neuroendocrinol* (2012) **24**(2):366–77. doi:10.1111/j.1365-2826.2011.02246.x
139. Gonzalez R, Shepperd E, Thiruppugazh V, Lohan S, Grey CL, Chang JP, et al. Nesfatin-1 regulates the hypothalamo-pituitary-ovarian axis of fish. *Biol Reprod* (2012) **87**(4):84. doi:10.1095/biolreprod.112.099630
140. Volkoff H, Eykelbosh AJ, Peter RE. Role of leptin in the control of feeding of goldfish *Carassius auratus*: interactions with cholecystokinin, neuropeptide Y and orexin A, and modulation by fasting. *Brain Res* (2003) **972**(1–2):90–109. doi:10.1016/S0006-8993(03)02507-1
141. Murashita K, Uji S, Yamamoto T, Ronnestad I, Kurokawa T. Production of recombinant leptin and its effects on food intake in rainbow trout (*Oncorhynchus mykiss*). *Comp Biochem Physiol B Biochem Mol Biol* (2008) **150**(4):377–84. doi:10.1016/j.cbpb.2008.04.007
142. Peyon P, Zanuy S, Carrillo M. Action of leptin on in vitro luteinizing hormone release in the European sea bass (*Dicentrarchus labrax*). *Biol Reprod* (2001) **65**(5):1573–8. doi:10.1095/biolreprod65.5.1573
143. Weil C, Le Bail PY, Sabin N, Le Gac F. In vitro action of leptin on FSH and LH production in rainbow trout (*Onchorynchus mykiss*) at different stages of the sexual cycle. *Gen Comp Endocrinol* (2003) **130**(1):2–12. doi:10.1016/S0016-6480(02)00504-X
144. Unniappan S, Lin X, Cervini L, Rivier J, Kaiya H, Kangawa K, et al. Goldfish ghrelin: molecular characterization of the complementary deoxyribonucleic acid, partial gene structure and evidence for its stimulatory role in food intake. *Endocrinology* (2002) **143**(10):4143–6. doi:10.1210/en.2002-220644
145. Matsuda K, Miura T, Kaiya H, Maruyama K, Shimakura S, Uchiyama M, et al. Regulation of food intake by acyl and des-acyl ghrelins in the goldfish. *Peptides* (2006) **27**(9):2321–5. doi:10.1016/j.peptides.2006.03.028
146. Miura T, Maruyama K, Kaiya H, Miyazato M, Kangawa K, Uchiyama M, et al. Purification and properties of ghrelin from the intestine of the goldfish, *Carassius auratus*. *Peptides* (2009) **30**(4):758–65. doi:10.1016/j.peptides.2008.12.016
147. Jonsson E, Kaiya H, Bjornsson BT. Ghrelin decreases food intake in juvenile rainbow trout (*Oncorhynchus mykiss*) through the central anorexigenic corticotropin-releasing factor system. *Gen Comp Endocrinol* (2010) **166**(1):39–46. doi:10.1016/j.ygcen.2009.11.001
148. Unniappan S, Peter RE. In vitro and in vivo effects of ghrelin on luteinizing hormone and growth hormone release in goldfish. *Am J Physiol Regul Integr Comp Physiol* (2004) **286**(6):R1093–101. doi:10.1152/ajpregu.00669.2003
149. Sokolowska-Mikolajczyk M, Socha M, Szczerbik P, Epler P. The effects of ghrelin on the in vitro spontaneous and sGnRH-A stimulated luteinizing hormone (LH) release from the pituitary cells of common carp (*Cyprinus carpio* L.). *Comp Biochem Physiol A Mol Integr Physiol* (2009) **153**(4):386–90. doi:10.1016/j.cbpa.2009.03.012
150. Wong KK, Ng SY, Lee LT, Ng HK, Chow BK. Orexins and their receptors from fish to mammals: a comparative approach. *Gen Comp Endocrinol* (2011) **171**(2):124–30. doi:10.1016/j.ygcen.2011.01.001
151. Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. *Cell* (1998) **92**(4):573–85. doi:10.1016/S0092-8674(02)09256-5
152. Nakamachi T, Matsuda K, Maruyama K, Miura T, Uchiyama M, Funahashi H, et al. Regulation by orexin of feeding behaviour and locomotor activity in the goldfish. *J Neuroendocrinol* (2006) **18**(4):290–7. doi:10.1111/j.1365-2826.2006.01415.x
153. Sasson R, Dearth RK, White RS, Chappell PE, Mellon PL. Orexin A induces GnRH gene expression and secretion from GT1-7 hypothalamic GnRH neurons. *Neuroendocrinology* (2006) **84**(6):353–63. doi:10.1159/000098333
154. Russell SH, Small CJ, Kennedy AR, Stanley SA, Seth A, Murphy KG, et al. Orexin A interactions in the hypothalamo-pituitary gonadal axis. *Endocrinology* (2001) **142**(12):5294–302. doi:10.1210/endo.142.12.8558
155. Campbell RE, Grove KL, Smith MS. Gonadotropin-releasing hormone neurons coexpress orexin 1 receptor immunoreactivity and receive direct contacts

by orexin fibers. *Endocrinology* (2003) **144**(4):1542–8. doi:10.1210/en.2002-220958
156. Amiya N, Amano M, Oka Y, Iigo M, Takahashi A, Yamamori K. Immunohistochemical localization of orexin/hypocretin-like immunoreactive peptides and melanin-concentrating hormone in the brain and pituitary of medaka. *Neurosci Lett* (2007) **427**(1):16–21. doi:10.1016/j.neulet.2007.07.043
157. Suzuki H, Miyoshi Y, Yamamoto T. Orexin-A (hypocretin 1)-like immunoreactivity in growth hormone-containing cells of the Japanese seaperch (*Lateolabrax japonicus*) pituitary. *Gen Comp Endocrinol* (2007) **150**(2):205–11. doi:10.1016/j.ygcen.2006.08.008
158. Suzuki H, Matsumoto A, Yamamoto T. Orexin-B-like immunoreactivity localized in both luteinizing hormone- and thyroid-stimulating hormone-containing cells in the Nile tilapia (*Oreochromis niloticus*) pituitary. *Tissue Cell* (2009) **41**(1):75–8. doi:10.1016/j.tice.2008.06.001
159. Tatemoto K, Carlquist M, Mutt V. Neuropeptide Y – a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* (1982) **296**(5858):659–60. doi:10.1038/296659a0
160. Valassi E, Scacchi M, Cavagnini F. Neuroendocrine control of food intake. *Nutr Metab Cardiovasc Dis* (2008) **18**(2):158–68. doi:10.1016/j.numecd.2007.06.004
161. de Pedro N, Lopez-Patino MA, Guijarro AI, Pinillos ML, Delgado MJ, Alonso-Bedate M. NPY receptors and opioidergic system are involved in NPY-induced feeding in goldfish. *Peptides* (2000) **21**(10):1495–502. doi:10.1016/S0196-9781(00)00303-X
162. Narnaware YK, Peyon PP, Lin X, Peter RE. Regulation of food intake by neuropeptide Y in goldfish. *Am J Physiol Regul Integr Comp Physiol* (2000) **279**(3):R1025–34.
163. Narnaware YK, Peter RE. Effects of food deprivation and refeeding on neuropeptide Y (NPY) mRNA levels in goldfish. *Comp Biochem Physiol B Biochem Mol Biol* (2001) **129**(2–3):633–7. doi:10.1016/S1096-4959(01)00359-1
164. Narnaware YK, Peter RE. Neuropeptide Y stimulates food consumption through multiple receptors in goldfish. *Physiol Behav* (2001) **74**(1–2):185–90. doi:10.1016/S0031-9384(01)00556-X
165. Kamijo M, Kojima K, Maruyama K, Konno N, Motohashi E, Ikegami T, et al. Neuropeptide Y in tiger puffer (*Takifugu rubripes*): distribution, cloning, characterization, and mRNA expression responses to prandial condition. *Zoolog Sci* (2011) **28**(12):882–90. doi:10.2108/zsj.28.882
166. Wojcik-Gladysz A, Polkowska J. Neuropeptide Y – a neuromodulatory link between nutrition and reproduction at the central nervous system level. *Reprod Biol* (2006) **6**(Suppl 2):21–8.
167. Breton B, Motin A, Billard R, Kah O, Geoffre S, Precigoux G. Immunoreactive gonadotropin-releasing hormone-like material in the brain and the pituitary gland during the periovulatory period in the brown trout (*Salmo trutta* L.): relationships with the plasma and pituitary gonadotropin. *Gen Comp Endocrinol* (1986) **61**(1):109–19. doi:10.1016/0016-6480(86)90255-8
168. Danger JM, Breton B, Vallarino M, Fournier A, Pelletier G, Vaudry H. Neuropeptide-Y in the trout brain and pituitary: localization, characterization, and action on gonadotropin release. *Endocrinology* (1991) **128**(5):2360–8. doi:10.1210/endo-128-5-2360
169. Chiba A, Sohn YC, Honma Y. Distribution of neuropeptide Y and gonadotropin-releasing hormone immunoreactivities in the brain and hypophysis of the ayu, *Plecoglossus altivelis* (*Teleostei*). *Arch Histol Cytol* (1996) **59**(2):137–48. doi:10.1679/aohc.59.137
170. Amiya N, Amano M, Tabuchi A, Oka Y. Anatomical relations between neuropeptide Y, galanin, and gonadotropin-releasing hormone in the brain of chondrostean, the Siberian sturgeon *Acipenser baeri*. *Neurosci Lett* (2011) **503**(2):87–92. doi:10.1016/j.neulet.2011.08.008
171. Gaikwad A, Biju KC, Subhedar N. GnRH-LH secreting cells axis in the pituitary of the teleost *Clarias batrachus* responds to neuropeptide Y treatment: an immunocytochemical study. *Gen Comp Endocrinol* (2003) **131**(2):126–33. doi:10.1016/S0016-6480(02)00631-7
172. Ukena K, Tachibana T, Iwakoshi-Ukena E, Saito Y, Minakata H, Kawaguchi R, et al. Identification, localization, and function of a novel avian hypothalamic neuropeptide, 26RFa, and its cognate receptor, G protein-coupled receptor-103. *Endocrinology* (2010) **151**(5):2255–64. doi:10.1210/en.2009-1478
173. Primeaux SD. QRFP in female rats: effects on high fat food intake and hypothalamic gene expression across the estrous cycle. *Peptides* (2011) **32**(6):1270–5. doi:10.1016/j.peptides.2011.03.022
174. Takayasu S, Sakurai T, Iwasaki S, Teranishi H, Yamanaka A, Williams SC, et al. A neuropeptide ligand of the G protein-coupled receptor GPR103 regulates feeding, behavioral arousal, and blood pressure in mice. *Proc Natl Acad Sci U S A* (2006) **103**(19):7438–43. doi:10.1073/pnas.0602371103
175. Navarro VM, Fernandez-Fernandez R, Nogueiras R, Vigo E, Tovar S, Chartrel N, et al. Novel role of 26RFa, a hypothalamic RFamide orexigenic peptide, as putative regulator of the gonadotropic axis. *J Physiol* (2006) **573**(Pt 1):237–49. doi:10.1113/jphysiol.2006.106856
176. Gundlach AL. Galanin/GALP and galanin receptors: role in central control of feeding, body weight/obesity and reproduction? *Eur J Pharmacol* (2002) **440**(2–3):255–68. doi:10.1016/S0014-2999(02)01433-4
177. Vrontakis ME. Galanin: a biologically active peptide. *Curr Drug Targets CNS Neurol Disord* (2002) **1**(6):531–41. doi:10.2174/1568007023338914
178. Rajendren G, Li X. Galanin synaptic input to gonadotropin-releasing hormone perikarya in juvenile and adult female mice: implications for sexual maturity. *Brain Res Dev Brain Res* (2001) **131**(1–2):161–5. doi:10.1016/S0165-3806(01)00257-7
179. Merchenthaler I, Lopez FJ, Lennard DE, Negro-Vilar A. Sexual differences in the distribution of neurons coexpressing galanin and luteinizing hormone-releasing hormone in the rat brain. *Endocrinology* (1991) **129**(4):1977–86. doi:10.1210/endo-129-4-1977
180. Dudas B, Merchenthaler I. Bi-directional associations between galanin and luteinizing hormone-releasing hormone neuronal systems in the human diencephalon. *Neuroscience* (2004) **127**(3):695–707. doi:10.1016/j.neuroscience.2004.05.018
181. Mitchell V, Bouret S, Prevot V, Jennes L, Beauvillain JC. Evidence for expression of galanin receptor Gal-R1 mRNA in certain gonadotropin releasing hormone neurones of the rostral preoptic area. *J Neuroendocrinol* (1999) **11**(10):805–12. doi:10.1046/j.1365-2826.1999.00399.x
182. Batten TF, Moons L, Cambre M, Vandesande F. Anatomical distribution of galanin-like immunoreactivity in the brain and pituitary of teleost fishes. *Neurosci Lett* (1990) **111**(1–2):12–7. doi:10.1016/0304-3940(90)90336-8
183. Anglade I, Wang Y, Jensen J, Tramu G, Kah O, Conlon JM. Characterization of trout galanin and its distribution in trout brain and pituitary. *J Comp Neurol* (1994) **350**(1):63–74. doi:10.1002/cne.903500105
184. Power DM, Canario AV, Ingleton PM. Somatotropin release-inhibiting factor and galanin innervation in the hypothalamus and pituitary of seabream (*Sparus aurata*). *Gen Comp Endocrinol* (1996) **101**(3):264–74. doi:10.1006/gcen.1996.0029
185. Rodriguez-Gomez FJ, Rendon-Unceta MC, Sarasquete C, Munoz-Cueto JA. Localization of galanin-like immunoreactive structures in the brain of the Senegalese sole, *Solea senegalensis*. *Histochem J* (2000) **32**(2):123–31. doi:10.1023/A:1004074430973
186. Kauffman AS, Rissman EF. The evolutionarily conserved gonadotropin-releasing hormone II modifies food intake. *Endocrinology* (2004) **145**(2):686–91. doi:10.1210/en.2003-1150
187. Kauffman AS, Wills A, Millar RP, Rissman EF. Evidence that the type-2 gonadotrophin-releasing hormone (GnRH) receptor mediates the behavioural effects of GnRH-II on feeding and reproduction in musk shrews. *J Neuroendocrinol* (2005) **17**(8):489–97. doi:10.1111/j.1365-2826.2005.01334.x
188. Volkoff H, Peter RE. Actions of two forms of gonadotropin releasing hormone and a GnRH antagonist on spawning behavior of the goldfish *Carassius auratus*. *Gen Comp Endocrinol* (1999) **116**(3):347–55. doi:10.1006/gcen.1999.7377
189. Canosa LF, Stacey N, Peter RE. Changes in brain mRNA levels of gonadotropin-releasing hormone, pituitary adenylate cyclase activating polypeptide, and somatostatin during ovulatory luteinizing hormone and growth hormone surges in goldfish. *Am J Physiol Regul Integr Comp Physiol* (2008) **295**(6):R1815–21. doi:10.1152/ajpregu.00166.2008
190. Kim MH, Oka Y, Amano M, Kobayashi M, Okuzawa K, Hasegawa Y, et al. Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, *Carassius auratus*. *J Comp Neurol* (1995) **356**(1):72–82. doi:10.1002/cne.903560105
191. Shahjahan M, Hamabata T, Motohashi E, Doi H, Ando H. Differential expression of three types of gonadotropin-releasing hormone genes during the spawning season in grass puffer, *Takifugu niphobles*. *Gen Comp Endocrinol* (2010) **167**(1):153–63. doi:10.1016/j.ygcen.2010.01.018

192. Miyata A, Arimura A, Dahl RR, Minamino N, Uehara A, Jiang L, et al. Isolation of a novel 38 residue-hypothalamic polypeptide which stimulates adenylate cyclase in pituitary cells. *Biochem Biophys Res Commun* (1989) **164**(1):567–74. doi:10.1016/0006-291X(89)91757-9
193. Morley JE, Horowitz M, Morley PM, Flood JF. Pituitary adenylate cyclase activating polypeptide (PACAP) reduces food intake in mice. *Peptides* (1992) **13**(6):1133–5. doi:10.1016/0196-9781(92)90019-Y
194. Tachibana T, Saito S, Tomonaga S, Takagi T, Saito ES, Boswell T, et al. Intracerebroventricular injection of vasoactive intestinal peptide and pituitary adenylate cyclase-activating polypeptide inhibits feeding in chicks. *Neurosci Lett* (2003) **339**(3):203–6. doi:10.1016/S0304-3940(03)00017-X
195. Tachibana T, Tomonaga S, Oikawa D, Saito S, Takagi T, Saito ES, et al. Pituitary adenylate cyclase activating polypeptide and vasoactive intestinal peptide inhibit feeding in the chick brain by different mechanisms. *Neurosci Lett* (2003) **348**(1):25–8. doi:10.1016/S0304-3940(03)00646-3
196. Matsuda K, Kang KS, Sakashita A, Yahashi S, Vaudry H. Behavioral effect of neuropeptides related to feeding regulation in fish. *Ann N Y Acad Sci* (2011) **1220**:117–26. doi:10.1111/j.1749-6632.2010.05884.x
197. Wong AO, Li WS, Lee EK, Leung MY, Tse LY, Chow BK, et al. Pituitary adenylate cyclase activating polypeptide as a novel hypophysiotropic factor in fish. *Biochem Cell Biol* (2000) **78**(3):329–43. doi:10.1139/o00-055
198. Wong AO, Leung MY, Shea WL, Tse LY, Chang JP, Chow BK. Hypophysiotropic action of pituitary adenylate cyclase-activating polypeptide (PACAP) in the goldfish: immunohistochemical demonstration of PACAP in the pituitary, PACAP stimulation of growth hormone release from pituitary cells, and molecular cloning of pituitary type I PACAP receptor. *Endocrinology* (1998) **139**(8):3465–79. doi:10.1210/endo.139.8.6145
199. Montero M, Yon L, Rousseau K, Arimura A, Fournier A, Dufour S, et al. Distribution, characterization, and growth hormone-releasing activity of pituitary adenylate cyclase-activating polypeptide in the European eel, *Anguilla anguilla*. *Endocrinology* (1998) **139**(10):4300–10. doi:10.1210/endo.139.10.6239
200. Kawauchi H, Kawazoe I, Tsubokawa M, Kishida M, Baker BI. Characterization of melanin-concentrating hormone in chum salmon pituitaries. *Nature* (1983) **305**(5932):321–3. doi:10.1038/305321a0
201. Takahashi A, Tsuchiya K, Yamanome T, Amano M, Yasuda A, Yamamori K, et al. Possible involvement of melanin-concentrating hormone in food intake in a teleost fish, barfin flounder. *Peptides* (2004) **25**(10):1613–22. doi:10.1016/j.peptides.2004.02.022
202. Tuziak SM, Volkoff H. A preliminary investigation of the role of melanin-concentrating hormone (MCH) and its receptors in appetite regulation of winter flounder (*Pseudopleuronectes americanus*). *Mol Cell Endocrinol* (2012) **348**(1):281–96. doi:10.1016/j.mce.2011.09.015
203. Qu D, Ludwig DS, Gammeltoft S, Piper M, Pelleymounter MA, Cullen MJ, et al. A role for melanin-concentrating hormone in the central regulation of feeding behaviour. *Nature* (1996) **380**(6571):243–7. doi:10.1038/380243a0
204. Pissios P, Bradley RL, Maratos-Flier E. Expanding the scales: the multiple roles of MCH in regulating energy balance and other biological functions. *Endocr Rev* (2006) **27**(6):606–20. doi:10.1210/er.2006-0021
205. Matsuda K, Kojima K, Shimakura S, Takahashi A. Regulation of food intake by melanin-concentrating hormone in goldfish. *Peptides* (2009) **30**(11):2060–5. doi:10.1016/j.peptides.2009.02.015
206. Shimakura S, Kojima K, Nakamachi T, Kageyama H, Uchiyama M, Shioda S, et al. Neuronal interaction between melanin-concentrating hormone- and alpha-melanocyte-stimulating hormone-containing neurons in the goldfish hypothalamus. *Peptides* (2008) **29**(8):1432–40. doi:10.1016/j.peptides.2008.04.009
207. Naufahu J, Cunliffe AD, Murray JF. The roles of melanin-concentrating hormone in energy balance and reproductive function: are they connected? *Reproduction* (2013) **146**(5):R141–50. doi:10.1530/REP-12-0385
208. Smith MS, Grove KL. Integration of the regulation of reproductive function and energy balance: lactation as a model. *Front Neuroendocrinol* (2002) **23**(3):225–56. doi:10.1016/S0091-3022(02)00002-X
209. Williamson-Hughes PS, Grove KL, Smith MS. Melanin concentrating hormone (MCH): a novel neural pathway for regulation of GnRH neurons. *Brain Res* (2005) **1041**(2):117–24. doi:10.1016/j.brainres.2004.11.066
210. Chiocchio SR, Gallardo MG, Louzan P, Gutnisky V, Tramezzani JH. Melanin-concentrating hormone stimulates the release of luteinizing hormone-releasing hormone and gonadotropins in the female rat acting at both median eminence and pituitary levels. *Biol Reprod* (2001) **64**(5):1466–72. doi:10.1095/biolreprod64.5.1466
211. Schauer E, Trautinger F, Kock A, Schwarz A, Bhardwaj R, Simon M, et al. Proopiomelanocortin-derived peptides are synthesized and released by human keratinocytes. *J Clin Invest* (1994) **93**(5):2258–62. doi:10.1172/JCI117224
212. Cerda-Reverter JM, Ringholm A, Schioth HB, Peter RE. Molecular cloning, pharmacological characterization, and brain mapping of the melanocortin 4 receptor in the goldfish: involvement in the control of food intake. *Endocrinology* (2003) **144**(6):2336–49. doi:10.1210/en.2002-0213
213. Salbert G, Chauveau I, Bonnec G, Valotaire Y, Jego P. One of the two trout proopiomelanocortin messenger RNAs potentially encodes new peptides. *Mol Endocrinol* (1992) **6**(10):1605–13. doi:10.1210/mend.6.10.1448114
214. Kuhar MJ, Adams S, Dominguez G, Jaworski J, Balkan B. CART peptides. *Neuropeptides* (2002) **36**(1):1–8. doi:10.1054/npep.2002.0887
215. Sakata I, Nakamura K, Yamazaki M, Matsubara M, Hayashi Y, Kangawa K, et al. Ghrelin-producing cells exist as two types of cells, closed- and opened-type cells, in the rat gastrointestinal tract. *Peptides* (2002) **23**(3):531–6. doi:10.1016/S0196-9781(01)00633-7
216. Kehoe AS, Volkoff H. Cloning and characterization of neuropeptide Y (NPY) and cocaine and amphetamine regulated transcript (CART) in Atlantic cod (*Gadus morhua*). *Comp Biochem Physiol A Mol Integr Physiol* (2007) **146**(3):451–61. doi:10.1016/j.cbpa.2006.12.026
217. Kobayashi Y, Peterson BC, Waldbieser GC. Association of cocaine- and amphetamine-regulated transcript (CART) messenger RNA level, food intake, and growth in channel catfish. *Comp Biochem Physiol A Mol Integr Physiol* (2008) **151**(2):219–25. doi:10.1016/j.cbpa.2008.06.029
218. Nishio S, Gibert Y, Berekelya L, Bernard L, Brunet F, Guillot E, et al. Fasting induces CART down-regulation in the zebrafish nervous system in a cannabinoid receptor 1-dependent manner. *Mol Endocrinol* (2012) **26**(8):1316–26. doi:10.1210/me.2011-1180
219. Murashita K, Kurokawa T, Ebbesson LO, Stefansson SO, Ronnestad I. Characterization, tissue distribution, and regulation of agouti-related protein (AgRP), cocaine- and amphetamine-regulated transcript (CART) and neuropeptide Y (NPY) in Atlantic salmon (*Salmo salar*). *Gen Comp Endocrinol* (2009) **162**(2):160–71. doi:10.1016/j.ygcen.2009.03.015
220. Leslie RA, Sanders SJ, Anderson SI, Schuhler S, Horan TL, Ebling FJ. Appositions between cocaine and amphetamine-related transcript- and gonadotropin releasing hormone-immunoreactive neurons in the hypothalamus of the Siberian hamster. *Neurosci Lett* (2001) **314**(3):111–4. doi:10.1016/S0304-3940(01)02291-1
221. Singru PS, Mazumdar M, Sakharkar AJ, Lechan RM, Thim L, Clausen JT, et al. Immunohistochemical localization of cocaine- and amphetamine-regulated transcript peptide in the brain of the catfish, *Clarias batrachus* (Linn.). *J Comp Neurol* (2007) **502**(2):215–35. doi:10.1002/cne.21295
222. Barsagade VG, Mazumdar M, Singru PS, Thim L, Clausen JT, Subhedar N. Reproductive phase-related variations in cocaine- and amphetamine-regulated transcript (CART) in the olfactory system, forebrain, and pituitary of the female catfish, *Clarias batrachus* (Linn.). *J Comp Neurol* (2010) **518**(13):2503–24. doi:10.1002/cne.22349
223. Kuriyama G, Takekoshi S, Tojo K, Nakai Y, Kuhar MJ, Osamura RY. Cocaine- and amphetamine-regulated transcript peptide in the rat anterior pituitary gland is localized in gonadotrophs and suppresses prolactin secretion. *Endocrinology* (2004) **145**(5):2542–50. doi:10.1210/en.2003-0845
224. Moran TH, Kinzig KP. Gastrointestinal satiety signals II. Cholecystokinin. *Am J Physiol Gastrointest Liver Physiol* (2004) **286**(2):G183–8. doi:10.1152/ajpgi.00434.2003
225. Ichimaru T, Matsuyama S, Ohkura S, Mori Y, Okamura H. Central cholecystokinin-octapeptide accelerates the activity of the hypothalamic gonadotropin-releasing hormone pulse generator in goats. *J Neuroendocrinol* (2003) **15**(1):80–6. doi:10.1046/j.1365-2826.2003.00965.x
226. Kimura F, Hashimoto R, Kawakami M. The stimulatory effect of cholecystokinin implanted in the medial preoptic area on luteinizing hormone secretion in the ovariectomized estrogen-primed rat. *Endocrinol Jpn* (1983) **30**(3):305–9. doi:10.1507/endocrj1954.30.305
227. Oh IS, Shimizu H, Satoh T, Okada S, Adachi S, Inoue K, et al. Identification of nesfatin-1 as a satiety molecule in the hypothalamus. *Nature* (2006) **443**(7112):709–12. doi:10.1038/nature05162

228. Garcia-Galiano D, Navarro VM, Roa J, Ruiz-Pino F, Sanchez-Garrido MA, Pineda R, et al. The anorexigenic neuropeptide, nesfatin-1, is indispensable for normal puberty onset in the female rat. *J Neurosci* (2010) **30**(23):7783–92. doi:10.1523/JNEUROSCI.5828-09.2010
229. Zhang Y, Proenca R, Maffei M, Barone M, Leopold L, Friedman JM. Positional cloning of the mouse obese gene and its human homologue. *Nature* (1994) **372**(6505):425–32. doi:10.1038/372425a0
230. Crown A, Clifton DK, Steiner RA. Neuropeptide signaling in the integration of metabolism and reproduction. *Neuroendocrinology* (2007) **86**(3):175–82. doi:10.1159/000109095
231. de Pedro N, Martinez-Alvarez R, Delgado MJ. Acute and chronic leptin reduces food intake and body weight in goldfish (*Carassius auratus*). *J Endocrinol* (2006) **188**(3):513–20. doi:10.1677/joe.1.06349
232. Aguilar AJ, Conde-Sieira M, Polakof S, Miguez JM, Soengas JL. Central leptin treatment modulates brain glucosensing function and peripheral energy metabolism of rainbow trout. *Peptides* (2010) **31**(6):1044–54. doi:10.1016/j.peptides.2010.02.026
233. Maffei M, Halaas J, Ravussin E, Pratley RE, Lee GH, Zhang Y, et al. Leptin levels in human and rodent: measurement of plasma leptin and ob RNA in obese and weight-reduced subjects. *Nat Med* (1995) **1**(11):1155–61. doi:10.1038/nm1195-1155
234. Watanobe H. Leptin directly acts within the hypothalamus to stimulate gonadotropin-releasing hormone secretion in vivo in rats. *J Physiol* (2002) **545**(Pt 1):255–68. doi:10.1113/jphysiol.2002.023895
235. Smith GD, Jackson LM, Foster DL. Leptin regulation of reproductive function and fertility. *Theriogenology* (2002) **57**(1):73–86. doi:10.1016/S0093-691X(01)00658-6
236. Barb CR, Hausman GJ, Czaja K. Leptin: a metabolic signal affecting central regulation of reproduction in the pig. *Domest Anim Endocrinol* (2005) **29**(1):186–92. doi:10.1016/j.domaniend.2005.02.024
237. Froiland E, Murashita K, Jorgensen EH, Kurokawa T. Leptin and ghrelin in anadromous arctic charr: cloning and change in expressions during a seasonal feeding cycle. *Gen Comp Endocrinol* (2010) **165**(1):136–43. doi:10.1016/j.ygcen.2009.06.010
238. Trombley S, Schmitz M. Leptin in fish: possible role in sexual maturation in male Atlantic salmon. *Fish Physiol Biochem* (2013) **39**(1):103–6. doi:10.1007/s10695-012-9731-0
239. Kaiya H, Kojima M, Hosoda H, Riley LG, Hirano T, Grau EG, et al. Amidated fish ghrelin: purification, cDNA cloning in the Japanese eel and its biological activity. *J Endocrinol* (2003) **176**(3):415–23. doi:10.1677/joe.0.1760415
240. Kaiya H, Kojima M, Hosoda H, Moriyama S, Takahashi A, Kawauchi H, et al. Peptide purification, complementary deoxyribonucleic acid (DNA) and genomic DNA cloning, and functional characterization of ghrelin in rainbow trout. *Endocrinology* (2003) **144**(12):5215–26. doi:10.1210/en.2003-1085
241. Kaiya H, Miyazato M, Kangawa K, Peter RE, Unniappan S. Ghrelin: a multifunctional hormone in non-mammalian vertebrates. *Comp Biochem Physiol A Mol Integr Physiol* (2008) **149**(2):109–28. doi:10.1016/j.cbpa.2007.12.004
242. Kang KS, Yahashi S, Matsuda K. Central and peripheral effects of ghrelin on energy balance, food intake and lipid metabolism in teleost fish. *Peptides* (2011) **32**(11):2242–7. doi:10.1016/j.peptides.2011.05.006
243. Terova G, Rimoldi S, Bernardini G, Gornati R, Saroglia M. Sea bass ghrelin: molecular cloning and mRNA quantification during fasting and refeeding. *Gen Comp Endocrinol* (2008) **155**(2):341–51. doi:10.1016/j.ygcen.2007.05.028
244. Amole N, Unniappan S. Fasting induces preproghrelin mRNA expression in the brain and gut of zebrafish, *Danio rerio*. *Gen Comp Endocrinol* (2009) **161**(1):133–7. doi:10.1016/j.ygcen.2008.11.002
245. Jonsson E, Forsman A, Einarsdottir IE, Kaiya H, Ruohonen K, Bjornsson BT. Plasma ghrelin levels in rainbow trout in response to fasting, feeding and food composition, and effects of ghrelin on voluntary food intake. *Comp Biochem Physiol A Mol Integr Physiol* (2007) **147**(4):1116–24. doi:10.1016/j.cbpa.2007.03.024
246. Nieminen P, Mustonen AM, Hyvarinen H. Fasting reduces plasma leptin- and ghrelin-immunoreactive peptide concentrations of the burbot (*Lota lota*) at 2 degrees C but not at 10 degrees C. *Zoolog Sci* (2003) **20**(9):1109–15. doi:10.2108/zsj.20.1109
247. Jonsson E. The role of ghrelin in energy balance regulation in fish. *Gen Comp Endocrinol* (2013) **187**:79–85. doi:10.1016/j.ygcen.2013.03.013
248. Tena-Sempere M. Ghrelin as a pleotrophic modulator of gonadal function and reproduction. *Nat Clin Pract Endocrinol Metab* (2008) **4**(12):666–74. doi:10.1038/ncpendmet1003
249. Chan CB, Cheng CH. Identification and functional characterization of two alternatively spliced growth hormone secretagogue receptor transcripts from the pituitary of black seabream *Acanthopagrus schlegeli*. *Mol Cell Endocrinol* (2004) **214**(1–2):81–95. doi:10.1016/j.mce.2003.11.020
250. Grey CL, Grayfer L, Belosevic M, Chang JP. Ghrelin stimulation of gonadotropin (LH) release from goldfish pituitary cells: presence of the growth hormone secretagogue receptor (GHS-R1a) and involvement of voltage-sensitive Ca2+ channels. *Mol Cell Endocrinol* (2010) **317**(1–2):64–77. doi:10.1016/j.mce.2009.12.024
251. Schneider JE, Wise JD, Benton NA, Brozek JM, Keen-Rhinehart E. When do we eat? Ingestive behavior, survival, and reproductive success. *Horm Behav* (2013) **64**(4):702–28. doi:10.1016/j.yhbeh.2013.07.005
252. Milla S, Wang N, Mandiki SN, Kestemont P. Corticosteroids: friends or foes of teleost fish reproduction? *Comp Biochem Physiol A Mol Integr Physiol* (2009) **153**(3):242–51. doi:10.1016/j.cbpa.2009.02.027
253. Castellano JM, Bentsen AH, Mikkelsen JD, Tena-Sempere M. Kisspeptins: bridging energy homeostasis and reproduction. *Brain Res* (2010) **1364**:129–38. doi:10.1016/j.brainres.2010.08.057
254. Smith JT, Acohido BV, Clifton DK, Steiner RA. KiSS-1 neurones are direct targets for leptin in the ob/ob mouse. *J Neuroendocrinol* (2006) **18**(4):298–303. doi:10.1111/j.1365-2826.2006.01417.x
255. Backholer K, Smith JT, Rao A, Pereira A, Iqbal J, Ogawa S, et al. Kisspeptin cells in the ewe brain respond to leptin and communicate with neuropeptide Y and proopiomelanocortin cells. *Endocrinology* (2010) **151**(5):2233–43. doi:10.1210/en.2009-1190
256. Fu LY, van den Pol AN. Kisspeptin directly excites anorexigenic proopiomelanocortin neurons but inhibits orexigenic neuropeptide Y cells by an indirect synaptic mechanism. *J Neurosci* (2010) **30**(30):10205–19. doi:10.1523/JNEUROSCI.2098-10.2010
257. Mechaly AS, Vinas J, Piferrer F. Gene structure analysis of kisspeptin-2 (Kiss2) in the Senegalese sole (*Solea senegalensis*): characterization of two splice variants of Kiss2, and novel evidence for metabolic regulation of kisspeptin signaling in non-mammalian species. *Mol Cell Endocrinol* (2011) **339**(1–2):14–24. doi:10.1016/j.mce.2011.03.004
258. Tachibana T, Sato M, Takahashi H, Ukena K, Tsutsui K, Furuse M. Gonadotropin-inhibiting hormone stimulates feeding behavior in chicks. *Brain Res* (2005) **1050**(1–2):94–100. doi:10.1016/j.brainres.2005.05.035
259. Johnson MA, Tsutsui K, Fraley GS. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. *Horm Behav* (2007) **51**(1):171–80. doi:10.1016/j.yhbeh.2006.09.009
260. Murakami M, Matsuzaki T, Iwasa T, Yasui T, Irahara M, Osugi T, et al. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. *J Endocrinol* (2008) **199**(1):105–12. doi:10.1677/JOE-08-0197
261. Qi Y, Oldfield BJ, Clarke IJ. Projections of RFamide-related peptide-3 neurones in the ovine hypothalamus, with special reference to regions regulating energy balance and reproduction. *J Neuroendocrinol* (2009) **21**(8):690–7. doi:10.1111/j.1365-2826.2009.01886.x
262. Ahima RS, Saper CB, Flier JS, Elmquist JK. Leptin regulation of neuroendocrine systems. *Front Neuroendocrinol* (2000) **21**(3):263–307. doi:10.1006/frne.2000.0197
263. Williams G, Bing C, Cai XJ, Harrold JA, King PJ, Liu XH. The hypothalamus and the control of energy homeostasis: different circuits, different purposes. *Physiol Behav* (2001) **74**(4–5):683–701. doi:10.1016/S0031-9384(01)00612-6
264. Li GG, Liang XF, Xie Q, Li G, Yu Y, Lai K. Gene structure, recombinant expression and functional characterization of grass carp leptin. *Gen Comp Endocrinol* (2010) **166**(1):117–27. doi:10.1016/j.ygcen.2009.10.009
265. Aguilar AJ, Conde-Sieira M, Lopez-Patino MA, Miguez JM, Soengas JL. In vitro leptin treatment of rainbow trout hypothalamus and hindbrain affects glucosensing and gene expression of neuropeptides involved in food intake regulation. *Peptides* (2011) **32**(2):232–40. doi:10.1016/j.peptides.2010.11.007

266. Chisada S-I, Kurokawa T, Murashita K, Rønnestad I, Taniguchi Y, Toyoda A, et al. Leptin receptor-deficient (knockout) medaka, *Oryzias latipes*, show chronical up-regulated levels of orexigenic neuropeptides, elevated food intake and stage specific effects on growth and fat allocation. *Gen Comp Endocrinol* (2014) **195**:9–20. doi:10.1016/j.ygcen.2013.10.008
267. Matsuda K, Kojima K, Shimakura S, Wada K, Maruyama K, Uchiyama M, et al. Corticotropin-releasing hormone mediates alpha-melanocyte-stimulating hormone-induced anorexigenic action in goldfish. *Peptides* (2008) **29**(11):1930–6. doi:10.1016/j.peptides.2008.06.028
268. Shimakura S, Miura T, Maruyama K, Nakamachi T, Uchiyama M, Kageyama H, et al. Alpha-melanocyte-stimulating hormone mediates melanin-concentrating hormone-induced anorexigenic action in goldfish. *Horm Behav* (2008) **53**(2):323–8. doi:10.1016/j.yhbeh.2007.10.009
269. Kang KS, Shimizu K, Azuma M, Ui Y, Nakamura K, Uchiyama M, et al. Gonadotropin-releasing hormone II (GnRH II) mediates the anorexigenic actions of alpha-melanocyte-stimulating hormone (alpha-MSH) and corticotropin-releasing hormone (CRH) in goldfish. *Peptides* (2011) **32**(1):31–5. doi:10.1016/j.peptides.2010.10.013
270. Kojima K, Kamijo M, Kageyama H, Uchiyama M, Shioda S, Matsuda K. Neuronal relationship between orexin-A- and neuropeptide Y-induced orexigenic actions in goldfish. *Neuropeptides* (2009) **43**(2):63–71. doi:10.1016/j.npep.2009.01.004
271. Kojima K, Amiya N, Kamijo M, Kageyama H, Uchiyama M, Shioda S, et al. Relationship between alpha-melanocyte-stimulating hormone- and neuropeptide Y-containing neurons in the goldfish hypothalamus. *Gen Comp Endocrinol* (2010) **167**(3):366–72. doi:10.1016/j.ygcen.2009.12.004
272. Matsuda K, Kojima K, Shimakura S, Miura T, Uchiyama M, Shioda S, et al. Relationship between melanin-concentrating hormone- and neuropeptide Y-containing neurons in the goldfish hypothalamus. *Comp Biochem Physiol A Mol Integr Physiol* (2009) **153**(1):3–7. doi:10.1016/j.cbpa.2008.10.002
273. Miura T, Maruyama K, Shimakura S, Kaiya H, Uchiyama M, Kangawa K, et al. Regulation of food intake in the goldfish by interaction between ghrelin and orexin. *Peptides* (2007) **28**(6):1207–13. doi:10.1016/j.peptides.2007.03.023
274. Miura T, Maruyama K, Shimakura S, Kaiya H, Uchiyama M, Kangawa K, et al. Neuropeptide Y mediates ghrelin-induced feeding in the goldfish, *Carassius auratus*. *Neurosci Lett* (2006) **407**(3):279–83. doi:10.1016/j.neulet.2006.08.071
275. Amano M, Amiya N, Hiramatsu M, Tomioka T, Oka Y. Interaction between neuropeptide Y immunoreactive neurons and galanin immunoreactive neurons in the brain of the masu salmon, *Oncorhynchus masou*. *Neurosci Lett* (2009) **462**(1):33–8. doi:10.1016/j.neulet.2009.06.067
276. Peng C, Trudeau VL, Peter RE. Seasonal variation of neuropeptide Y actions on growth hormone and gonadotropin-II secretion in the goldfish: effects of sex steroids. *J Neuroendocrinol* (1993) **5**(3):273–80. doi:10.1111/j.1365-2826.1993.tb00483.x
277. Flores A, Maldonado R, Berrendero F. Cannabinoid-hypocretin cross-talk in the central nervous system: what we know so far. *Front Neurosci* (2013) **7**:256. doi:10.3389/fnins.2013.00256
278. Bermudez-Silva FJ, Cardinal P, Cota D. The role of the endocannabinoid system in the neuroendocrine regulation of energy balance. *J Psychopharmacol* (2012) **26**(1):114–24. doi:10.1177/0269881111408458
279. Piccinetti CC, Migliarini B, Petrosino S, Di Marzo V, Carnevali O. Anandamide and AM251, via water, modulate food intake at central and peripheral level in fish. *Gen Comp Endocrinol* (2010) **166**(2):259–67. doi:10.1016/j.ygcen.2009.09.017
280. Valenti M, Cottone E, Martinez R, De Pedro N, Rubio M, Viveros MP, et al. The endocannabinoid system in the brain of *Carassius auratus* and its possible role in the control of food intake. *J Neurochem* (2005) **95**(3):662–72. doi:10.1111/j.1471-4159.2005.03406.x
281. Cottone E, Pomatto V, Bovolin P. Role of the endocannabinoid system in the central regulation of nonmammalian vertebrate reproduction. *Int J Endocrinol* (2013) **2013**:941237. doi:10.1155/2013/941237
282. Battista N, Meccariello R, Cobellis G, Fasano S, Di Tommaso M, Pirazzi V, et al. The role of endocannabinoids in gonadal function and fertility along the evolutionary axis. *Mol Cell Endocrinol* (2012) **355**(1):1–14. doi:10.1016/j.mce.2012.01.014
283. Volff JN. Genome evolution and biodiversity in teleost fish. *Heredity* (2004) **94**(3):280–94. doi:10.1038/sj.hdy.6800635

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Pleiotropic activities of HGF/c-Met system in testicular physiology: paracrine and endocrine implications

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In the last decades, a growing body of evidence has been reported concerning the expression and functional role of hepatocyte growth factor (HGF) on different aspects of testicular physiology. This review has the aim to summarize what is currently known regarding this topic. From early embryonic development to adult age, HGF and its receptor c-Met appeared to be clearly detectable in the testis. These molecules acquire different distribution patterns and roles depending on the developmental stage or the post-natal age considered. HGF acts as a paracrine modulator of testicular functions promoting the epithelium–mesenchyme cross-talk as described even in other organs. Interestingly, it has been reported that testicular HGF acts even as an autocrine factor and that its receptor might be modulated by endocrine signals that change at puberty: HGF receptor expressed by Sertoli cells, in fact, is up-regulated by FSH administration. HGF is in turn able to modify endocrine state of the organism being able to increase testosterone secretion of both fetal and adult Leydig cells. Moreover, c-Met is expressed in mitotic and meiotic male germ cells as well as in spermatozoa. The distribution pattern of c-Met on sperm cell membrane changes in the caput and cauda epididymal sperms and HGF is able to maintain epididymal sperm motility *in vitro* suggesting a physiological role of this growth factor in the acquisition of sperm motility. Noteworthy changes in HGF concentration in seminal plasma have been reported in different andrological diseases. All together these data indicate that HGF has a role in the control of spermatogenesis and sperm quality either directly, acting on male germ cells, or indirectly acting on tubular and interstitial somatic cells of the testis.

Keywords: HGF, c-Met receptor, testis, male gonad development, testicular cell differentiation, sex hormones

THE HEPATOCYTE GROWTH FACTOR MACHINERY AND ITS BIOLOGICAL FUNCTIONS

The hepatocyte growth factor (HGF) is a pleiotropic cytokine originally purified as a potent mitogen for hepatocytes (1, 2) and subsequently identified as a "scatter factor" (3, 4). HGF is synthesized as an inactive single chain precursor that is cleaved to acquire the bioactive disulfite-linked heterodimeric form (2, 5). One of the most recognized activators of HGF precursor is the HGF activator protein (HGFA), which is a serine protease able to cleave immature HGF precursor to form a mature bioactive HGF (6). Interestingly, HGF activation may be provided also by active metalloproteinases (MMP2 and MMP9) and by plasminogen activator (PA). More recently, it has been discovered also an inhibitor of HGF activation (called HGF inhibitor or HAI) that is a serine protease inhibitor that blocks HGFA activation (7). The modulation of HGFA and HAI in the tissue microenvironment is able to maintain the correct HGF availability since HGF has been established as an important factor for tissue homeostasis (8).

c-Met is the unique HGF receptor and it is normally expressed by cells of epithelial origin whilst HGF expression has been mainly found restricted to cells of mesenchymal origin. c-Met receptor presents tyrosine-kinase activity and, upon HGF stimulation, this receptor triggers several transduction pathways responsible for its multiple biological responses including proliferation, motility, migration, morphogenesis, tubulogenesis, differentiation, and angiogenesis (8–10). In particular, c-Met activation by its ligand HGF triggers transphosphorylation of the catalytic tyrosines Tyr 1234 and Tyr 1235, which positively modulate its enzymatic activity. c-Met c-terminal tail contains tyrosines Tyr 1349 and Tyr 1356, which represent, when phosphorylated, the multifunctional docking site of the receptor. These two amino acidic residues are able to recruit several transducers and adaptors after c-Met activation, thus explaining the whole spectrum of pleiotropic biological activities exerted by HGF/c-Met system (11). These transducers interact with the intracellular multi-substrate docking site of c-Met either directly, such as GRB2, SHC, SRC, and the p85 regulatory subunit of phosphatidylinositol-3 kinase (PI3K), or indirectly through the scaffolding protein Gab1 such as PLC-γ (12–17).

c-Met knock-out mice have provided only partial information in the understanding of the role of HGF/c-Met system in the embryonic development of mammals since this animal model showed an embryonic lethal phenotype due to severe placental defects. However, some information was drawn by these knock-out animals indicating an essential role of this growth factor in gastrulation, angiogenesis, myoblast migration, and liver development (12, 18, 19). The study of HGF/c-Met system expression

during the mouse organogenesis has provided great insights in the understanding of HGF function. This system, in fact, has been found in several developing organs being HGF expressed in the mesenchyme and c-Met in the epithelial part of the developing tissue. On the basis of this preliminary observation, it has been demonstrated and well established that HGF/c-Met system mediates signal exchange between mesenchymal and epithelial cells in embryonic morphogenesis as well as in post-natal stroma–parenchyma cross-talk and tissue homeostasis (20). In addition, HGF has been demonstrated to exert unique developing capability as a morphogen of tubular structures and inducer of harmonic cell migratory activities (21–23). Even if HGF could be mainly identified as paracrine factor in the mesenchyme–epithelium cross-talk, it has been also found that this growth factor is actively delivered *via* blood vessels to injured organs allowing their repair and homeostasis (24, 25). Besides its action as a hormone, it has been demonstrated that HGF expression is regulated by blood hormones, neurotransmitters, and cytokines, such as GH (26), Norepinephrine (27), and systemic prostaglandin E (28). In addition, the discovery of HGF/c-Met system in the regulation of testis and ovary differentiation and physiology has given rise to an increasing amount of evidences that support the intriguing hypothesis of a cross-talk among gonadotropins, sex hormones, and HGF in both the female and male gonads (29, 30). This review aims to focus on what is known on the implications of HGF in the autocrine, paracrine, and endocrine regulation of testicular physiology.

ROLES OF HGF IN THE PHYSIOLOGY OF THE TESTIS

Hepatocyte growth factor/c-Met system has been found expressed and active during all the phases of pre-natal and post-natal testis development. The activities of the HGF machinery on the testicular tissue vary depending on the different phases of both pre-natal and post-natal ages: these ranges from the modulation of both steroidogenesis and apoptosis to guiding mitosis, morphogenesis, and differentiation. Overall, the emerging picture suggests HGF as one of the growth factors which cooperates at different levels to support male reproductive health and is deeply involved in the harmonic control of spermatogenetic process.

HGF/c-MET SYSTEM IN TESTIS EMBRYONIC DEVELOPMENT

As previously stated during pre-natal development, HGF/c-Met system is expressed and active in a wide variety of developing organs, such as the liver, lung, pancreas, intestine, and kidney. HGF transcripts were mainly found localized in the mesenchymal part of these organs whereas c-Met expression appeared mainly restricted to the epithelial portion (23), thereby indicating an important role for HGF in epithelial–mesenchymal interaction during embryonic morphogenesis. Moreover, it has been reported that HGF may induce mesenchymal to epithelial cell conversion (31). Testis develops from the collaboration and the cross-talk of intermediate mesoderm and celomic epithelium. In addition, its morphogenesis is characterized by a conversion of mesenchyme in epithelial cells (for instance Sertoli and Leydig cells) as well as by a tight cross-talk between its epithelial and mesenchymal cells. By this point of view, it may be intriguing but not surprising that HGF/c-Met system has been found during the entire period of testis embryonic development. What seems noteworthy is that the distribution patterns and functional roles of this molecular machinery change in the different morphogenetic phases of the testicular embryonic development.

Early testicular morphogenesis

Hepatocyte growth factor/c-Met expression has been reported starting from 11.5 dies post coitum (dpc) and continues through the entire period of pre-natal development. Actually, at 11.5 dpc only HGF is present in the celomic epithelium underlying male urogenital ridges whereas, at the same pre-natal age, c-Met is not already expressed in the gonads even if it is clearly detectable in the mesonephric mesenchyme (32, 33). One of the first specific features of male gonad development is represented by the mesonephric cell migration toward the developing testis; this cell migration begins at 11.5 dpc and was described up to 16.5 dpc in a sex-specific manner (34–37). Consistent with the observed HGF/c-Met distribution pattern in the early gonad, HGF has been established as one of the growth factors potentially involved in the chemo-attraction of mesonephric cells (32, 33, 38) thus collaborating with other growth factors such as FGF9, PDGF, and neurotrophins, to establish the male differentiation niche for somatic and germ cells (38–41) (**Figure 1**).

Testicular cord morphogenesis

Seminiferous cord formation is the critical morphogenetic event of testis development. To obtain a correct cord formation, the previously reported migration of mesenchymal cells from the mesonephros into the developing gonads is necessary (37, 42). However, this complex phenomenon requires the harmonic coordination of several biological processes such as proliferation, differentiation, and polarization of pre-Sertoli cells present in the "morphologically indifferent" gonad and their association with the primordial germ cells. The coordination of these events requires a tight epithelium–mesenchyme cross-talk that is guaranteed by the action of specific local growth factors (43–45) and HGF has been indicated as one of them. Testicular cords begin to organize at 12.5 dpc. At the same developmental age, c-Met starts to be expressed by the developing testis and seems to be confined in the testicular cords of this organ. As reported for other organs, at the same stage of development, HGF expression is present in the differentiating stroma and in differentiating myoid cells confirming the capability of HGF/c-Met system to mediate epithelium–mesenchyme exchange (32, 33). Using organ culture of indifferent male urogenital ridges, it has been demonstrated that HGF is able to mediate testis differentiation and testicular cord formation in *ex vivo* organ culture condition (32, 33, 38). Since this *ex vivo* differentiation is the result of the coordination of cell migration, cell proliferation, and tubulogenesis, using *in vitro* assays able to discriminate between these different phenomena, it has been established that HGF is able to trigger all these events, again confirming the multiple biological activities that are mediated by this molecular machinery (32, 33, 38) (**Figure 1**).

Fetal Leydig cell differentiation and endocrine implications

c-Met receptor continues to be expressed by testicular cord up to 15.5 dpc. It is interesting to notice that in the late part of pre-natal

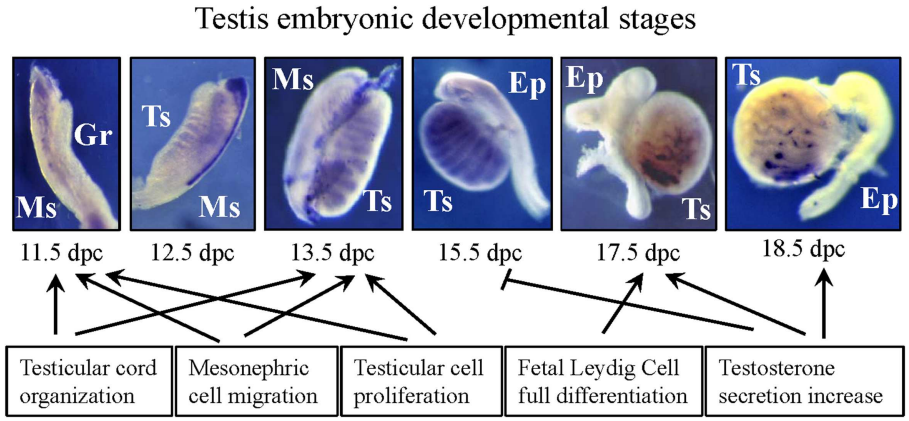

FIGURE 1 | Picture summarizing the main effects exerted by HGF during embryonic testis development. Purple signal in the developing urogenital ridges at different pre-natal stages indicates c-Met mRNA expression. Gr, genital ridge; Ts, testis; Ms, mesonephros; Ep, epididymis.

development (since 17.5 dpc to birth) c-Met distribution pattern drastically changes, being down-regulated in the testicular cords and up-regulated in the interstitial fetal Leydig cells (46). HGF is always present in the interstitial compartment, but, interestingly, it is not produced by mouse fetal Leydig cells. Thus HGF acts as a paracrine factor even on differentiating fetal Leydig cells reproducing in the interstitial compartment the epithelium–mesenchyme cross-talk present between interstitium and testicular cords in the embryonic stages before this pre-natal age. Actually, steroid-producing fetal Leydig cell lineage starts to be detectable in the testis early on 12.5 dpc but their differentiation process involves all the further stages of pre-natal development when they increase in number and gradually acquire the capability to modulate androgen secretion in response to local and endocrine cues (39, 47, 48). It is worth to highlight that when mesenchyme derived fetal Leydig cells start to acquire their fully differentiated phenotype in late embryogenesis (49), they start to express c-Met on their surface. The latter observation allowed to hypothesize a role for this growth factor in the modulation of testicular pre-natal endocrine function. In rodents, in fact, fetal masculinization and increase of plasma androgens occur before the hypothalamic–pituitary–gonadal axis to be functional (50) since LURKO mice (that lack the luteinizing hormone receptor) have normal androgen levels and testicular phenotype at birth (51). Thus there is a common agreement in the scientific community that states the differentiation of fetal Leydig cells is under the control of local growth factors and HGF seems to be one of them. In fact, in 17.5 and 18.5 dpc testicular organ culture, HGF is able to stimulate testosterone production (46) and fetal Leydig cell survival and full development (52). Noteworthy at 15.5 dpc, when c-Met is not detectable on fetal Leydig cells but is still expressed in seminiferous cords, HGF has been demonstrated not able to modify testosterone secretion in testicular organ culture. From these data HGF can be numbered besides PDGF, DHH, TGF-β, and IGF-I as one of the growth factors responsible of the normal pre-natal steroidogenesis (39, 48, 52–55).

This phenomenon may be relevant for the onset of the first cross-talk among gonad, hypothalamus, and pituitary gland, and potentially involved in the acquisition of secondary sex-specific features and brain testosterone imprinting (**Figure 1**).

HGF/c-MET SYSTEM IN THE PHYSIOLOGY OF POST-NATAL TESTIS

Even if the studies on pre-natal morphogenetic functions of HGF have been established using mouse models, the post-natal roles of HGF on testicular physiology have been determined mainly using rats since testicular cell isolation is better characterized for this animal model. Intriguingly, many parallel mechanisms exist comparing ovarian and testicular function and some of them, with particular attention to endocrine function, will be highlighted in this part of the review.

Sertoli cells

Correct Sertoli cell differentiation is crucial in order to maintain the local microenvironment necessary to sustain spermatogenetic process (56, 57). Several growth factors have been described as local modulators of Sertoli cell physiological niche and HGF can be numbered in this cohort since it has been demonstrated as a paracrine and autocrine modulator of Sertoli cell physiology. In the rat, c-Met mRNA and protein were detected in the post-natal testis starting from 10 dies post partum (dpp) even if they appear on Sertoli cells not before 25 dpp and their expression increases at 35 dpp. As previously reported in the embryonic testis, also in the post-natal testis the main source of HGF seems to be represented by the interstitial cells and by the myoid cells, reproducing the epithelium–mesenchyme cross-talk observable during the morphogenesis of the testis. Despite this observation, in the post-natal testis, HGF has often been demonstrated to be expressed also by the same cell lineages that express c-Met and Sertoli cells are not an exception. These data suggest that HGF levels could be finely regulated by different testicular cell types and that the action of this factor is not only paracrine but seems to be also autocrine and maybe endocrine. Interestingly, dissociated rat Sertoli cells as well as the Sertoli cell line SF7 cultured in the presence of HGF, are able to organize in tubular-like structures showing, even in the post-natal testis, the morphogenetic and tubulogenic ability of this growth factor (58, 59). This observation demonstrates that HGF

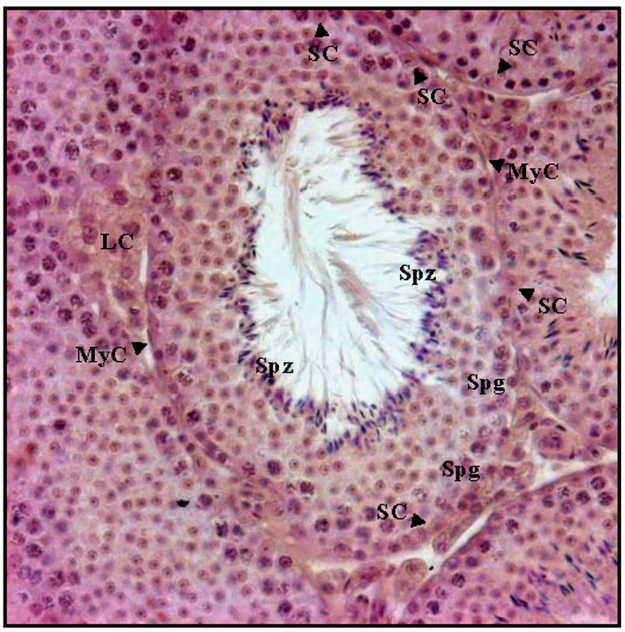

FIGURE 2 | Picture summarizing the main effects exerted by HGF on post-natal testis derived cells. A hematoxylin–eosin stained section of rodent adult testis is also reported. SC, Sertoli cells; LC, Leydig cells; MyC, myoid cells; Spg, spermatogonia; Spz, spermatozoa.

is able to guarantee the right cue for the maintenance of correct Sertoli cell polarization that is one of the parameters showing full differentiated Sertoli cell phenotype. These data together with the age of onset of Sertoli cell sensitivity to HGF have strongly suggested that this growth factor may be involved in testicular cord lumen and blood–testis barrier (BTB) formation. This particular topic will be fully expanded in a paragraph below.

Sertoli cells are one of the key somatic actors of endocrine hypothalamus–pituitary–gonadal axis cross-talk being able to produce and secrete estrogens in response to FSH and thus to regulate the spermatogenetic process. As HGF has been proposed as one of the factors able to modify Sertoli cell physiology and to maintain their differentiated phenotype, it is conceivable to hypothesize that this factor has a role in the modulation of gonad–pituitary cross-talk. It was suggested by Zachow and Uzumcu in 2007 (30), that there is an intriguing parallelism between male and female gonad that hypothesizes some endocrine implications of HGF in the physiology of both organs. It has been reported, also, that HGF can down-modulate ovarian steroidogenesis suppressing FSH-dependent 17β-estradiol production by directly impairing CYP19 enzyme (60, 61). It is well known that Sertoli cells represent the testicular counterpart of granulosa cells and are the testicular source of 17β-estradiol. Sertoli cells are capable to produce the greatest quantity of 17β-estradiol in the first 10–20 days of post-natal age (62). Interestingly, HGF together with c-Met is not present on Sertoli cells at post-natal day 10 (63, 64) whilst both the receptor and the ligand appear expressed by Sertoli cells since post-natal day 25 (64, 65), which is the time when 17β-estradiol production by Sertoli cells begins to be reduced (62). All together these observations allow to hypothesize that HGF could locally control *in vivo* Sertoli cell 17β-estradiol production in a similar way of what observed in granulosa cells. Consistent with these data, FSH has been demonstrated able to up-regulate c-Met expression in Sertoli cell cultures (65) whose activation in turn suppresses, following this hypothesis, Sertoli cells 17β-estradiol synthesis. The proposed mechanism, that deserves further investigations, suggests that HGF/c-Met system may be responsible for a local modulation of FSH-dependent estrogen production, modulating both enzyme activity and c-Met receptor availability (**Figure 2**).

Germ cells and spermatozoa

It has been reported by several groups that c-Met is expressed in both human and rodent male germ cells: this observation is interesting since it means the expression of this receptor is conserved at least among mammals (66–68). In rats, this receptor is always present during spermatogenetic process from spermatogonia to spermatozoa. HGF has been shown to control germ cell mitotic activity being able to significantly increase spermatogonial cell proliferation from 8 to 30 days old rat testis in *ex vivo* organ culture. This result on spermatogonia cells appears to be more relevant highlighting that the HGF activator inhibitor (HAI-2) is expressed exclusively in primary spermatocytes (69) strongly indicating that mitotic germ cells need HGF signal whereas at the beginning of meiotic process this proliferating cue needs to be inhibited in order to allow germ cell meiotic entry.

Germ cell apoptosis is finely controlled in the testis in order to guarantee the best selection of differentiating male gametes and HGF has been demonstrated to be involved even in the control of this biological process. This growth factor, in fact, has been demonstrated to act also as a survival factor for male germ cells

since it is capable to significantly decrease germ cell apoptosis (67). The lowest protective effect (35% reduction of apoptosis) was found in late prepubertal rats and this finding is probably due to the fact that the highest level of apoptosis has been described at this age (70). Alternatively, this observation could be due to the presence of a more complex network of pro-apoptotic and anti-apoptotic factors present in the older animals (**Figure 2**). This result on healthy animals is reinforced by Goda and coworkers who studied a rat experimental cryptorchid model (71). This pathology is characterized by spermatogenic failure and germ cell loss. The adenovirus-mediated HGF gene transfer in the testis of these animals induced over-expression of HGF and significantly decreased apoptotic germ cell number, restoring spermatogenesis and testicular weight.

It is well known that germ cell proliferation and survival are controlled by endocrine signals: for instance FSH stimulates spermatogonial proliferation both *in vivo* and *in vitro* (72–74). In addition, testosterone and FSH regulate germ cell apoptosis (75, 76) probably acting via somatic cells considering the absence on the germ cells of their respective receptors (77, 78). By this point of view, HGF may be considered one of the local cytokines already identified (57, 79–83) that collaborates with the endocrine signals to promote the correct male germ cell homeostasis.

There has not been a report of any effect of HGF on meiotic germ cells, even if they express c-Met receptor on their surface (67). However, several groups have reported that c-Met is expressed on the surface of rat (66), and human epididymal spermatozoa (68, 84) and that HGF is present in mouse (85), rat (66), and human (86) epididymis. It is fair to say that literature data on the role of HGF on epididymal spermatozoa are often controversial. The first finding that strongly suggested a role of HGF in epididymal sperm maturation was reported by Naz and coworkers in 1994 (85). This group showed a specific region distribution pattern of HGF in the mouse epididymal tract with the highest levels of the growth factor in the distal corpus and cauda, where sperms acquire their motility. In the same study, HGF was found able to slightly induce cell motility on immotile sperms. Noteworthy, also c-Met distribution pattern on sperm surface seems to change from testicular to caput and cauda epididymal spermatozoa in rats (66). In addition, the previously mentioned effect on sperm motility was, at least in part, confirmed on rat epididymal sperms in which HGF has a positive effect on the *in vitro* maintenance of epididymal sperm motility even if, actually, the factor was not able to significantly increase the percentage of motile cells (66). Interestingly, c-Met receptor on human sperm has been found activated indicating that the HGF/c-Met system is functionally active in epididymal spermatozoa (84). However, the same motogenic effect reported in rodents failed to be demonstrated on human sperms (87) and the role of this growth factor on human sperm physiology is still a matter of debate (**Figure 2**). It was suggested that modifications in HGF seminal plasma concentration could be related with different andrological diseases and male infertility, but actually as in this case conflicting data are reported in the literature (87–89). It is fair to notice that male infertility could be due to really different causes: it is likely that the reported literature controversy may depend on the difficulties in classification of andrological diseases considered eligible for this particular study by the different research groups. Further studies are needed to better clarify this point that deserves to be deeper investigated.

Blood–testis barrier

The described morphogenic and motogenic effect on cultured prenatal and post-natal Sertoli cells allowed to hypothesize that HGF may modulate junctional capability of this cell type. It is well known, in fact, that HGF is a "Scatter Factor" able to modulate junctional behavior of target cells. In adult mammalian testes, Sertoli cells form junctional complexes with neighboring Sertoli cells that have been described from a long time (90). These junctional complexes consist of tight junctions and testis specific cell to cell actin based anchoring junctions which are both involved in the formation of the BTB. BTB separates the seminiferous epithelium in two different niches: the basal compartment, that encloses mitotic spermatogonia, and the adluminal compartment that encloses male meiotic germ cells. BTB integrity is necessary to allow the correct spermatogenic process, but its structure is highly dynamic. Junctional complexes of BTB, in fact, are able to disassemble and reassemble to allow the passage of pre-leptotene spermatocytes across the barrier (90, 91). In the last years, multiple reports from different laboratories have indicated that BTB permeability and physiological dynamics are regulated by a complex interaction of bioactive molecules including gonadotropins, testosterone, TGF-β, TNF-α, and interleukin-1a (92–98). Since 2008, HGF must be included in this number of factors (58, 99). In pubertal and adult rats it has been reported, in fact, that HGF is involved in the disassembling of the polygonal structures formed by occludin around the Sertoli cells. Moreover, it was demonstrated a quantitative occludin decrease in the tubules cultured in the presence of HGF by means of both confocal microscopy and Western blot analysis. In addition, HGF modifies the position of the tight junctions: it is indicated by the shift in the position of the occludin within the tubule treated with the growth factor compared to the controls. These data indicate a role of HGF in the modification of Sertoli cells junctional behavior. Interestingly, in adult rats HGF is maximally expressed at stages VII–VIII of the cycle, when germ cells traverse the BTB, whereas its levels fall in the subsequent stages IX–XII and XIII–I (58). This observation gives rise to the intriguing hypothesis that HGF produced by Sertoli cells could autocrinally regulate BTB in a stage dependent manner. It is relevant to highlight that HGF mediated BTB dynamism may be potentially due not only to a direct motogenic effect of HGF on Sertoli cells but also to the capability of HGF to modify the seminiferous tubule microenvironment promoting the increase of TGF-β active fraction. This phenomenon could be ascribed, at least in part, to the uPA level increase mediated by HGF in seminiferous tubule cultures (58, 99) (**Figure 2**).

Myoid cells

Myoid cell lineage was the first isolated testicular cell type in which HGF/c-Met system has been discovered (63). Even if HGF has always been found expressed both in pre-natal and post-natal myoid cells (33, 63, 64), c-Met was detectable only in post-natal cells indicating a paracrine function during embryonic development and a paracrine/autocrine role in post-natal testis. Noteworthy, myoid cells isolated from prepubertal rat testis (10 and

20 days old rats) express c-Met receptor at high level whereas the expression level decreases in pubertal and adult myoid cells (35 and 60 days old rats). Consistent with these results, HGF is able to induce a faster cell spreading of prepubertal myoid cells but not able to modify this parameter on myoid cells isolated from pubertal animals (**Figure 2**) (64). Despite myoid cells were the first testicular lineage in which HGF/c-Met system has been described, the role exerted by this machinery in their physiology needs to be better investigated. Probably, one of the roles exerted by myoid cells is to provide a source of this factor both for Leydig and Sertoli cells.

Leydig cells

As well as mouse fetal Leydig cells, also Leydig cells isolated from pubertal rats expressed c-Met receptor (100). Physiological activity of HGF on pubertal rat Leydig cells presents some similarities with their fetal counterparts: in particular as HGF has been demonstrated to promote basal testosterone secretion and Leydig cell survival (100) both in Leydig cell primary culture and in *ex vivo* organ culture. Intriguingly, the steroid modulator activity is not confined in male gonad but was also described in theca cells, which are the Leydig cell ovarian counterparts. In rat theca cells, in fact, HGF suppressed LH-dependent androsterone secretion, while stimulated basal and LH-induced progesterone production (60). It is fair to highlight the parallelism between male and female gonad and to notice that steroid production modulation in response to local cues is quite relevant for gonad physiology since sex hormones are not only important for endocrine homeostasis via pituitary–gonadal axis cross-talk but also act as paracrine regulators of male and female gametogenesis. In addition, local increase of testosterone could be important for BTB dynamism that, as previously stated, was also directly modulated by HGF.

Besides its effect on testosterone secretion, it is worth mentioning that HGF exerts a broader effect on Leydig cell secretory activities: HGF administration to isolated rat Leydig cells was able to increase the secretion of active form of several proteases such as MMP2 and uPA (101). Interestingly, HGF administration was also able to increase active TGF-β. Viewed together these data suggest that increased amount of active TGF-β could not be a direct effect of HGF but a consequence of the HGF-dependent increased uPA and MMP2 activity (102–104).

Different types of molecules can be substrates of MMPs including growth factors, tyrosine-kinase receptors, extracellular matrix proteins, and cell adhesion molecules. HGF administration on isolated Leydig cells significantly reduces the amount of fibronectin indicating that this growth factor might modify interstitial extracellular matrix components and in turn changes significantly adhesive microenvironment and cytokine availability (**Figure 2**). It is fair to highlight that extracellular matrix homeostasis is necessary for spermatogenesis and that a thickened lamina propria has been reported associated with impaired spermatogenesis (105) and that HGF has demonstrated to have anti-fibrotic effect in the regulation of extracellular matrix composition even in other organs (8). All together, the presented data indicate a relevant role of HGF in the regulation of Leydig cell metabolic activities and in the composition of the interstitial tissue. The reported results strongly indicate that HGF/c-Met system is implicated in the local control of endocrine testicular machinery.

CONCLUDING REMARKS

Hepatocyte growth factor has been well established as a key regulator of the development and homeostasis of many organs. An increasing amount of evidences is demonstrating its important role in several aspects of pre-natal and post-natal testicular physiology. A huge job has been done but we must deal with a major one to figure out what might be the implications of this factor in the reproductive health of human beings. A better understanding of the molecular mechanisms carried out *in vivo* by this growth factor could be a useful prerequisite in order to address idiopathic andrological diseases.

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REFERENCES

1. Nakamura T, Nawa K, Ichihara A. Partial purification and characterization of hepatocyte growth factor from serum of hepatectomized rats. *Biochem Biophys Res Commun* (1984) **122**:1450–9. doi:10.1016/0006-291X(84)91253-1
2. Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, et al. Molecular cloning and expression of human hepatocyte growth factor. *Nature* (1989) **342**:440–3. doi:10.1038/342440a0
3. Stoker M, Gherardi E, Perryman M, Gray J. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* (1987) **327**:239–42. doi:10.1038/327239a0
4. Weidner KM, Hartmann G, Sachs M, Birchmeier W. Properties and functions of scatter factor/hepatocyte growth factor and its receptor c-Met. *Am J Respir Cell Mol Biol* (1993) **8**:229–37. doi:10.1165/ajrcmb/8.3.229
5. Miyazawa K, Tsubouchi H, Naka D, Takahashi K, Okigaki M, Arakaki N, et al. Molecular cloning and sequence analysis of cDNA for human hepatocyte growth factor. *Biochem Biophys Res Commun* (1989) **163**:967–73. doi:10.1016/0006-291X(89)92316-4
6. Miyazawa K, Shimomura T, Kitamura A, Kondo J, Morimoto Y, Kitamura N. Molecular cloning and sequence analysis of the cDNA for a human serine protease responsible for activation of hepatocyte growth factor. Structural similarity of the protease precursor to blood coagulation factor XII. *J Biol Chem* (1993) **268**:10024–8.
7. Kawaguchi T, Qin L, Shimomura T, Kondo J, Matsumoto K, Denda K, et al. Purification and cloning of hepatocyte growth factor activator inhibitor type 2, a Kunitz-type serine protease inhibitor. *J Biol Chem* (1997) **272**:27558–64. doi:10.1074/jbc.272.44.27558
8. Nakamura T, Mizuno S. The discovery of hepatocyte growth factor (HGF) and its significance for cell biology, life sciences and clinical medicine. *Proc Jpn Acad Ser B Phys Biol Sci* (2010) **86**:588–610. doi:10.2183/pjab.86.588
9. Kim ES, Salgia R. MET pathway as a therapeutic target. *J Thorac Oncol* (2009) **4**:444–7. doi:10.1097/JTO.0b013e31819d6f91
10. Trusolino L, Bertotti A, Comoglio PM. MET signalling: principles and functions in development, organ regeneration and cancer. *Nat Rev Mol Cell Biol* (2010) **11**:834–48. doi:10.1038/nrm3012
11. Organ SL, Tsao MS. An overview of the c-MET signaling pathway. *Ther Adv Med Oncol* (2011) **3**:S7–19. doi:10.1177/1758834011422556
12. Maina F, Casagranda F, Audero E, Simeone A, Comoglio PM, Klein R, et al. Uncoupling of Grb2 from the Met receptor in vivo reveals complex roles in muscle development. *Cell* (1996) **87**:531–42. doi:10.1016/S0092-8674(00)81372-0
13. Pelicci G, Giordano S, Zhen Z, Salcini AE, Lanfrancone L, Bardelli A, et al. The motogenic and mitogenic responses to HGF are amplified by the Shc adaptor protein. *Oncogene* (1995) **10**:1631–8.

14. Weidner KM, Di CS, Sachs M, Brinkmann V, Behrens J, Birchmeier W. Interaction between Gab1 and the c-Met receptor tyrosine kinase is responsible for epithelial morphogenesis. *Nature* (1996) **384**:173–6. doi:10.1038/384173a0
15. Furge KA, Zhang YW, Vande Woude GF. Met receptor tyrosine kinase: enhanced signaling through adapter proteins. *Oncogene* (2000) **19**:5582–9. doi:10.1038/sj.onc.1203859
16. Gual P, Giordano S, Williams TA, Rocchi S, Van OE, Comoglio PM. Sustained recruitment of phospholipase C-gamma to Gab1 is required for HGF-induced branching tubulogenesis. *Oncogene* (2000) **19**:1509–18. doi:10.1038/sj.onc.1203514
17. Gual P, Giordano S, Anguissola S, Parker PJ, Comoglio PM. Gab1 phosphorylation: a novel mechanism for negative regulation of HGF receptor signaling. *Oncogene* (2001) **20**:156–66. doi:10.1038/sj.onc.1204047
18. Bladt F, Riethmacher D, Isenmann S, Aguzzi A, Birchmeier C. Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* (1995) **376**:768–71. doi:10.1038/376768a0
19. Schmidt C, Bladt F, Goedecke S, Brinkmann V, Zschiesche W, Sharpe M, et al. Scatter factor/hepatocyte growth factor is essential for liver development. *Nature* (1995) **373**:699–702. doi:10.1038/373699a0
20. Matsumoto K, Nakamura T. Emerging multipotent aspects of hepatocyte growth factor. *J Biochem* (1996) **119**:591–600. doi:10.1093/oxfordjournals.jbchem.a021283
21. Ohmichi H, Koshimizu U, Matsumoto K, Nakamura T. Hepatocyte growth factor (HGF) acts as a mesenchyme-derived morphogenic factor during fetal lung development. *Development* (1998) **125**:1315–24.
22. Santos OF, Barros EJ, Yang XM, Matsumoto K, Nakamura T, Park M, et al. Involvement of hepatocyte growth factor in kidney development. *Dev Biol* (1994) **163**:525–9. doi:10.1006/dbio.1994.1169
23. Sonnenberg E, Meyer D, Weidner KM, Birchmeier C. Scatter factor/hepatocyte growth factor and its receptor, the c-met tyrosine kinase, can mediate a signal exchange between mesenchyme and epithelia during mouse development. *J Cell Biol* (1993) **123**:223–35. doi:10.1083/jcb.123.1.223
24. Matsumoto K, Nakamura T. Hepatocyte growth factor: molecular structure, roles in liver regeneration, and other biological functions. *Crit Rev Oncog* (1992) **3**:27–54.
25. Yanagita K, Nagaike M, Ishibashi H, Niho Y, Matsumoto K, Nakamura T. Lung may have an endocrine function producing hepatocyte growth factor in response to injury of distal organs. *Biochem Biophys Res Commun* (1992) **182**:802–9. doi:10.1016/0006-291X(92)91803-X
26. Ekberg S, Luther M, Nakamura T, Jansson JO. Growth hormone promotes early initiation of hepatocyte growth factor gene expression in the liver of hypophysectomized rats after partial hepatectomy. *J Endocrinol* (1992) **135**:59–67. doi:10.1677/joe.0.1350059
27. Broten J, Michalopoulos G, Petersen B, Cruise J. Adrenergic stimulation of hepatocyte growth factor expression. *Biochem Biophys Res Commun* (1999) **262**:76–9. doi:10.1006/bbrc.1999.1183
28. Matsumoto K, Okazaki H, Nakamura T. Novel function of prostaglandins as inducers of gene expression of HGF and putative mediators of tissue regeneration. *J Biochem* (1995) **117**:458–64. doi:10.1093/jb/117.2.458
29. Uzumcu M, Pan Z, Chu Y, Kuhn PE, Zachow R. Immunolocalization of the hepatocyte growth factor (HGF) system in the rat ovary and the anti-apoptotic effect of HGF in rat ovarian granulosa cells in vitro. *Reproduction* (2006) **132**:291–9. doi:10.1530/rep.1.00989
30. Zachow R, Uzumcu M. The hepatocyte growth factor system as a regulator of female and male gonadal function. *J Endocrinol* (2007) **195**:359–71. doi:10.1677/JOE-07-0466
31. Tsarfaty I, Rong S, Resau JH, Rulong S, da Silva PP, Vande Woude GF. The Met proto-oncogene mesenchymal to epithelial cell conversion. *Science* (1994) **263**:98–101. doi:10.1126/science.7505952
32. Ricci G, Catizone A, Innocenzi A, Galdieri M. Hepatocyte growth factor (HGF) receptor expression and role of HGF during embryonic mouse testis development. *Dev Biol* (1999) **216**:340–7. doi:10.1006/dbio.1999.9505
33. Ricci G, Catizone A, Galdieri M. Pleiotropic activity of hepatocyte growth factor during embryonic mouse testis development. *Mech Dev* (2002) **118**:19–28. doi:10.1016/S0925-4773(02)00247-2
34. Merchant-Larios H, Moreno-Mendoza N, Buehr M. The role of the mesonephros in cell differentiation and morphogenesis of the mouse fetal testis. *Int J Dev Biol* (1993) **37**:407–15.
35. Buehr M, Gu S, McLaren A. Mesonephric contribution to testis differentiation in the fetal mouse. *Development* (1993) **117**:273–81.
36. Martineau J, Nordqvist K, Tilmann C, Lovell-Badge R, Capel B. Male-specific cell migration into the developing gonad. *Curr Biol* (1997) **7**:958–68. doi:10.1016/S0960-9822(06)00415-5
37. Tilmann C, Capel B. Mesonephric cell migration induces testis cord formation and Sertoli cell differentiation in the mammalian gonad. *Development* (1999) **126**:2883–90.
38. Ricci G, Catizone A, Galdieri M. Embryonic mouse testis development: role of platelet derived growth factor (PDGF-BB). *J Cell Physiol* (2004) **200**:458–67. doi:10.1002/jcp.20035
39. Brennan J, Tilmann C, Capel B. Pdgfr-alpha mediates testis cord organization and fetal Leydig cell development in the XY gonad. *Genes Dev* (2003) **17**:800–10. doi:10.1101/gad.1052503
40. Colvin JS, Green RP, Schmahl J, Capel B, Ornitz DM. Male-to-female sex reversal in mice lacking fibroblast growth factor 9. *Cell* (2001) **104**:875–89. doi:10.1016/S0092-8674(01)00284-7
41. Cupp AS, Kim GH, Skinner MK. Expression and action of neurotropin-3 and nerve growth factor in embryonic and early postnatal rat testis development. *Biol Reprod* (2000) **63**:1617–28. doi:10.1095/biolreprod63.6.1617
42. Combes AN, Wilhelm D, Davidson T, Dejana E, Harley V, Sinclair A, et al. Endothelial cell migration directs testis cord formation. *Dev Biol* (2009) **326**:112–20. doi:10.1016/j.ydbio.2008.10.040
43. Cool J, Capel B. Mixed signals: development of the testis. *Semin Reprod Med* (2009) **27**:5–13. doi:10.1055/s-0028-1108005
44. McClelland K, Bowles J, Koopman P. Male sex determination: insights into molecular mechanisms. *Asian J Androl* (2012) **14**:164–71. doi:10.1038/aja.2011.169
45. Wilhelm D, Palmer S, Koopman P. Sex determination and gonadal development in mammals. *Physiol Rev* (2007) **87**:1–28. doi:10.1152/physrev.00009.2006
46. Ricci G, Catizone A, Galdieri M. Expression and functional role of hepatocyte growth factor and its receptor (c-met) during fetal mouse testis development. *J Endocrinol* (2006) **191**:559–70. doi:10.1677/joe.1.06879
47. Griswold SL, Behringer RR. Fetal Leydig cell origin and development. *Sex Dev* (2009) **3**:1–15. doi:10.1159/000200077
48. Yao HH, Whoriskey W, Capel B. Desert Hedgehog/Patched 1 signaling specifies fetal Leydig cell fate in testis organogenesis. *Genes Dev* (2002) **16**:1433–40. doi:10.1101/gad.981202
49. Caprio M, Fabbrini E, Ricci G, Basciani S, Gnessi L, Arizzi M, et al. Ontogenesis of leptin receptor in rat Leydig cells. *Biol Reprod* (2003) **68**:1199–207. doi:10.1095/biolreprod.102.007831
50. El-Gehani F, Zhang FP, Pakarinen P, Rannikko A, Huhtaniemi I. Gonadotropin-independent regulation of steroidogenesis in the fetal rat testis. *Biol Reprod* (1998) **58**:116–23. doi:10.1095/biolreprod58.1.116
51. Zhang FP, Poutanen M, Wilbertz J, Huhtaniemi I. Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice. *Mol Endocrinol* (2001) **15**:172–83. doi:10.1210/me.15.1.172
52. Ricci G, Guglielmo MC, Caruso M, Ferranti F, Canipari R, Galdieri M, et al. Hepatocyte growth factor is a mouse fetal Leydig cell terminal differentiation factor. *Biol Reprod* (2012) **87**:146. doi:10.1095/biolreprod.112.104638
53. Barsoum IB, Yao HH. Fetal Leydig cells: progenitor cell maintenance and differentiation. *J Androl* (2010) **31**:11–5. doi:10.2164/jandrol.109.008318
54. El-Gehani F, Tena-Sempere M, Huhtaniemi I. Evidence that pituitary adenylate cyclase-activating polypeptide is a potent regulator of fetal rat testicular steroidogenesis. *Biol Reprod* (2000) **63**:1482–9. doi:10.1095/biolreprod63.5.1482
55. Sarraj MA, Escalona RM, Umbers A, Chua HK, Small C, Griswold M, et al. Fetal testis dysgenesis and compromised Leydig cell function in Tgfbr3 (beta glycan) knockout mice. *Biol Reprod* (2010) **82**:153–62. doi:10.1095/biolreprod.109.078766
56. Griswold MD. The central role of Sertoli cells in spermatogenesis. *Semin Cell Dev Biol* (1998) **9**:411–6. doi:10.1006/scdb.1998.0203
57. Huleihel M, Lunenfeld E. Regulation of spermatogenesis by paracrine/autocrine testicular factors. *Asian J Androl* (2004) **6**:259–68.
58. Catizone A, Ricci G, Caruso M, Ferranti F, Canipari R, Galdieri M. Hepatocyte growth factor (HGF) regulates blood-testis barrier (BTB) in adult rats. *Mol Cell Endocrinol* (2012) **348**:135–46. doi:10.1016/j.mce.2011.07.050

59. van der WK, Hofmann MC. An in vitro tubule assay identifies HGF as a morphogen for the formation of seminiferous tubules in the postnatal mouse testis. *Exp Cell Res* (1999) **252**:175–85. doi:10.1006/excr.1999.4630
60. Zachow RJ, Weitsman SR, Magoffin DA. Hepatocyte growth factor regulates ovarian theca-interstitial cell differentiation and androgen production. *Endocrinology* (1997) **138**:691–7. doi:10.1210/en.138.2.691
61. Zachow RJ, Ramski BE, Lee H. Modulation of estrogen production and 17beta-hydroxysteroid dehydrogenase-type 1, cytochrome P450 aromatase, c-met, and protein kinase Balpha messenger ribonucleic acid content in rat ovarian granulosa cells by hepatocyte growth factor and follicle-stimulating hormone. *Biol Reprod* (2000) **62**:1851–7. doi:10.1095/biolreprod62.6.1851
62. Le MB, Jegou B. Paracrine control of immature Sertoli cells by adult germ cells, in the rat (an in vitro study). Cell-cell interactions within the testis. *Mol Cell Endocrinol* (1988) **58**:65–72. doi:10.1016/0303-7207(88)90054-8
63. Catizone A, Ricci G, Arista V, Innocenzi A, Galdieri M. Hepatocyte growth factor and c-MET are expressed in rat prepuberal testis. *Endocrinology* (1999) **140**:3106–13. doi:10.1210/en.140.7.3106
64. Catizone A, Ricci G, Galdieri M. Expression and functional role of hepatocyte growth factor receptor (C-MET) during postnatal rat testis development. *Endocrinology* (2001) **142**:1828–34. doi:10.1210/en.142.5.1828
65. Catizone A, Ricci G, Galdieri M. HGF and postnatal testis development. *Mol Cell Endocrinol* (2005) **241**:32–40. doi:10.1016/j.mce.2005.04.010
66. Catizone A, Ricci G, Galdieri M. Functional role of hepatocyte growth factor receptor during sperm maturation. *J Androl* (2002) **23**:911–8. doi:10.1002/j.1939-4640.2002.tb02349.x
67. Catizone A, Ricci G, Del BJ, Galdieri M. Hepatocyte growth factor modulates in vitro survival and proliferation of germ cells during postnatal testis development. *J Endocrinol* (2006) **189**:137–46. doi:10.1677/joe.1.06528
68. Depuydt CE, Zalata A, de Potter CR, van EJ, Comhaire FH. The receptor encoded by the human C-MET oncogene is expressed in testicular tissue and on human spermatozoa. *Mol Hum Reprod* (1996) **2**:2–8. doi:10.1093/molehr/2.1.2
69. Yamauchi M, Itoh H, Naganuma S, Koono M, Hasui Y, Osada Y, et al. Expression of hepatocyte growth factor activator inhibitor type 2 (HAI-2) in human testis: identification of a distinct transcription start site for the HAI-2 gene in testis. *Biol Chem* (2002) **383**:1953–7. doi:10.1515/BC.2002.220
70. Jahnukainen K, Chrysis D, Hou M, Parvinen M, Eksborg S, Soder O. Increased apoptosis occurring during the first wave of spermatogenesis is stage-specific and primarily affects midpachytene spermatocytes in the rat testis. *Biol Reprod* (2004) **70**:290–6. doi:10.1095/biolreprod.103.018390
71. Goda K, Fujisawa M, Shirakawa T, Dobashi M, Shiota G, Zhang ZJ, et al. Adenoviral-mediated HGF expression inhibits germ cell apoptosis in rats with cryptorchidism. *J Gene Med* (2004) **6**:869–76. doi:10.1002/jgm.585
72. Boitani C, Politi MG, Menna T. Spermatogonial cell proliferation in organ culture of immature rat testis. *Biol Reprod* (1993) **48**:761–7. doi:10.1095/biolreprod48.4.761
73. van Alphen MM, van de Kant HJ, de Rooij DG. Follicle-stimulating hormone stimulates spermatogenesis in the adult monkey. *Endocrinology* (1988) **123**:1449–55. doi:10.1210/endo-123-3-1449
74. Arslan M, Weinbauer GF, Schlatt S, Shahab M, Nieschlag E. FSH and testosterone, alone or in combination, initiate testicular growth and increase the number of spermatogonia and Sertoli cells in a juvenile non-human primate (*Macaca mulatta*). *J Endocrinol* (1993) **136**:235–43. doi:10.1677/joe.0.1360235
75. Henriksen K, Hakovirta H, Parvinen M. Testosterone inhibits and induces apoptosis in rat seminiferous tubules in a stage-specific manner: in situ quantification in squash preparations after administration of ethane dimethane sulfonate. *Endocrinology* (1995) **136**:3285–91. doi:10.1210/en.136.8.3285
76. Henriksen K, Kangasniemi M, Parvinen M, Kaipia A, Hakovirta H. In vitro, follicle-stimulating hormone prevents apoptosis and stimulates deoxyribonucleic acid synthesis in the rat seminiferous epithelium in a stage-specific fashion. *Endocrinology* (1996) **137**:2141–9. doi:10.1210/en.137.5.2141
77. de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, Wreford N. Spermatogenesis. *Hum Reprod* (1998) **13**(Suppl 1):1–8. doi:10.1093/humrep/13.suppl_1.1
78. Heckert LL, Griswold MD. Expression of follicle-stimulating hormone receptor mRNA in rat testes and Sertoli cells. *Mol Endocrinol* (1991) **5**:670–7. doi:10.1210/mend-5-5-670
79. Parvinen M, Kangasniemi M, Kaipia A, Mali P, Soder O, Pollanen P. Local regulation of seminiferous epithelium: the role of cytokines. *Bull Assoc Anat (Nancy)* (1991) **75**:163–5.
80. Pollanen P, Soder O, Parvinen M. Interleukin-1 alpha stimulation of spermatogonial proliferation in vivo. *Reprod Fertil Dev* (1989) **1**:85–7. doi:10.1071/RD9890085
81. Boitani C, Stefanini M, Fragale A, Morena AR. Activin stimulates Sertoli cell proliferation in a defined period of rat testis development. *Endocrinology* (1995) **136**:5438–44. doi:10.1210/en.136.12.5438
82. Kubota H, Avarbock MR, Brinster RL. Culture conditions and single growth factors affect fate determination of mouse spermatogonial stem cells. *Biol Reprod* (2004) **71**:722–31. doi:10.1095/biolreprod.104.029207
83. Puglisi R, Montanari M, Chiarella P, Stefanini M, Boitani C. Regulatory role of BMP2 and BMP7 in spermatogonia and Sertoli cell proliferation in the immature mouse. *Eur J Endocrinol* (2004) **151**:511–20. doi:10.1530/eje.0.1510511
84. Herness EA, Naz RK. Presence and tyrosine phosphorylation of c-met receptor in human sperm. *J Androl* (1999) **20**:640–7. doi:10.1002/j.1939-4640.1999.tb02566.x
85. Naz RK, Joseph A, Lee Y, Ahmad K, Bhargava MM. Expression of scatter factor/hepatocyte growth factor is regionally correlated with the initiation of sperm motility in murine male genital tract: is scatter factor/hepatocyte growth factor involved in initiation of sperm motility? *Mol Reprod Dev* (1994) **38**:431–9. doi:10.1002/mrd.1080380411
86. Depuydt CE, Zalata A, Falmagne JB, Bosmans E, Comhaire FH. Purification and characterization of hepatocyte growth factor (HGF) from human seminal plasma. *Int J Androl* (1997) **20**:306–14. doi:10.1046/j.1365-2605.1997.00074.x
87. Kitamura M, Matsumiya K, Nishimura K, Yamanaka M, Matsumoto K, Okuyama A. Effect of hepatocyte growth factor on sperm motility. *Am J Reprod Immunol* (2000) **44**:193–6. doi:10.1111/j.8755-8920.2000.440401.x
88. Depuydt CE, de Potter CR, Zalata A, Baekelandt E, Bosmans E, Comhaire FH. Levels of hepatocyte growth factor/scatter factor (HGF/SF) in seminal plasma of patients with andrological diseases. *J Androl* (1998) **19**:175–82. doi:10.1111/j.8755-8920.2000.440401.x
89. Wiltshire EJ, Flaherty SP, Couper RT. Hepatocyte growth factor in human semen and its association with semen parameters. *Hum Reprod* (2000) **15**:1525–8. doi:10.1093/humrep/15.7.1525
90. Dym M, Fawcett DW. The blood-testis barrier in the rat and the physiological compartmentation of the seminiferous epithelium. *Biol Reprod* (1970) **3**:308–26.
91. Russell L. Movement of spermatocytes from the basal to the adluminal compartment of the rat testis. *Am J Anat* (1977) **148**:313–28. doi:10.1002/aja.1001480303
92. Capaldo CT, Nusrat A. Cytokine regulation of tight junctions. *Biochim Biophys Acta* (2009) **1788**:864–71. doi:10.1016/j.bbamem.2008.08.027
93. Lie PP, Cheng CY, Mruk DD. Interleukin-1alpha is a regulator of the blood-testis barrier. *FASEB J* (2011) **25**:1244–53. doi:10.1096/fj.10-169995
94. Lui WY, Cheng CY. Regulation of cell junction dynamics by cytokines in the testis: a molecular and biochemical perspective. *Cytokine Growth Factor Rev* (2007) **18**:299–311. doi:10.1016/j.cytogfr.2007.04.009
95. McCabe MJ, Tarulli GA, Meachem SJ, Robertson DM, Smooker PM, Stanton PG. Gonadotropins regulate rat testicular tight junctions in vivo. *Endocrinology* (2010) **151**:2911–22. doi:10.1210/en.2009-1278
96. Siu MK, Lee WM, Cheng CY. The interplay of collagen IV, tumor necrosis factor-alpha, gelatinase B (matrix metalloprotease-9), and tissue inhibitor of metalloproteases-1 in the basal lamina regulates Sertoli cell-tight junction dynamics in the rat testis. *Endocrinology* (2003) **144**:371–87. doi:10.1210/en.2002-220786
97. Wong CH, Cheng CY. Mitogen-activated protein kinases, adherens junction dynamics, and spermatogenesis: a review of recent data. *Dev Biol* (2005) **286**:1–15. doi:10.1016/j.ydbio.2005.08.001
98. Yan HH, Cheng CY. Blood-testis barrier dynamics are regulated by an engagement/disengagement mechanism between tight and adherens junctions via peripheral adaptors. *Proc Natl Acad Sci U S A* (2005) **102**:11722–7. doi:10.1073/pnas.0503855102
99. Catizone A, Ricci G, Galdieri M. Hepatocyte growth factor modulates Sertoli-Sertoli tight junction dynamics. *J Cell Physiol* (2008) **216**:253–60. doi:10.1002/jcp.21400

100. Del Bravo J, Catizone A, Ricci G, Galdieri M. Hepatocyte growth factor-modulated rat Leydig cell functions. *J Androl* (2007) **28**:866–74. doi:10.2164/jandrol.107.002865
101. Catizone A, Ricci G, Tufano MA, Perfetto B, Canipari R, Galdieri M. Hepatocyte growth factor (HGF) modulates Leydig cell extracellular matrix components. *J Androl* (2010) **31**:306–13. doi:10.2164/jandrol.109.007658
102. Nunes I, Shapiro RL, Rifkin DB. Characterization of latent TGF-beta activation by murine peritoneal macrophages. *J Immunol* (1995) **155**: 1450–9.
103. Odekon LE, Blasi F, Rifkin DB. Requirement for receptor-bound urokinase in plasmin-dependent cellular conversion of latent TGF-beta to TGF-beta. *J Cell Physiol* (1994) **158**:398–407. doi:10.1002/jcp.1041580303
104. Yu Q, Stamenkovic I. Cell surface-localized matrix metalloproteinase-9 proteolytically activates TGF-beta and promotes tumor invasion and angiogenesis. *Genes Dev* (2000) **14**:163–76. doi:10.1101/gad.14.2.163
105. Volkmann J, Muller D, Feuerstacke C, Kliesch S, Bergmann M, Muhlfeld C, et al. Disturbed spermatogenesis associated with thickened lamina propria of seminiferous tubules is not caused by dedifferentiation of myofibroblasts. *Hum Reprod* (2011) **26**:1450–61. doi:10.1093/humrep/der077

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Endocannabinoids are involved in male vertebrate reproduction: regulatory mechanisms at central and gonadal level

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Endocannabinoids (eCBs) are natural lipids regulating a large array of physiological functions and behaviors in vertebrates. The eCB system is highly conserved in evolution and comprises several specific receptors (type-1 and type-2 cannabinoid receptors), their endogenous ligands (e.g., anandamide and 2-arachidonoylglycerol), and a number of biosynthetic and degradative enzymes. In the last few years, eCBs have been described as critical signals in the control of male and female reproduction at multiple levels: centrally, by targeting hypothalamic gonadotropin-releasing-hormone-secreting neurons and pituitary, and locally, with direct effects on the gonads. These functions are supported by the extensive localization of cannabinoid receptors and eCB metabolic enzymes at different levels of the hypothalamic–pituitary–gonadal axis in mammals, as well as bonyfish and amphibians. *In vivo* and *in vitro* studies indicate that eCBs centrally regulate gonadal functions by modulating the gonadotropin-releasing hormone–gonadotropin–steroid network through direct and indirect mechanisms. Several proofs of local eCB regulation have been found in the testis and male genital tracts, since eCBs control Sertoli and Leydig cells activity, germ cell progression, as well as the acquisition of sperm functions. A comparative approach usually is a key step in the study of physiological events leading to the building of a general model. Thus, in this review, we summarize the action of eCBs at different levels of the male reproductive axis, with special emphasis, where appropriate, on data from non-mammalian vertebrates.

Keywords: GnRH, hypothalamus, pituitary, spermatogenesis, chromatin remodeling, male fertility

INTRODUCTION

Since the discovery of Δ^9-tetrahydrocannabinol (THC) as the main psychoactive ingredient in marijuana, the subsequent cloning of cannabinoid receptors and the identification of their endogenous ligands [i.e., endocannabinoids (eCBs)], our understanding of the functions of the eCB system (ECS) has evolved considerably. It has become evident that most components of the mammalian ECS are highly conserved in evolution, pointing to a fundamental modulatory role in basic cellular and organismic functions (1, 2). Accordingly, the ECS is widely expressed in vertebrates, central and peripheral organs, and regulates a large array of physiological functions and behaviors.

The basic eCB signaling system consists of (1) at least two G-protein-coupled receptors, known as the cannabinoid type-1 and type-2 receptors (CB1 and CB2); (2) the endogenous ligands, of which anandamide (AEA) and 2-arachidonoylglycerol (2-AG) are the best characterized; and (3) synthetic and degradative enzymes and transporters that regulate eCB levels and action at receptors. CB1 receptors are abundant in the whole vertebrate central nervous system (CNS) and some peripheral tissues (3–5), whereas CB2 receptors are mostly expressed in peripheral tissues and immune cells, but they have recently been found also in the CNS (6–8). Research in mammals has provided evidence that eCBs can also bind to and activate type-1 transient receptor potential vanilloid (TRPV1) channels (9).

An enormous amount of information on the general properties of the ECS has accumulated over the last two decades [for general reviews on the ECS, see Ref. (10–14)]. In the past years, growing evidence has been accumulating to show the central role of the ECS in controlling vertebrate reproductive functions at both central and gonadal level (15). This review will summarize the action of eCBs at different levels of the reproductive axis, including data from non-mammalian vertebrates.

EFFECTS OF eCBs ON HYPOTHALAMIC–PITUITARY CONTROL OF REPRODUCTION

Reproductive functions are under neuroendocrine control and require a tight crosstalk between the hypothalamus, pituitary, and gonads. Gonadotropin-releasing-hormone (GnRH) is a key molecule in reproductive behavior and physiology. This neuropeptide

is synthesized by hypothalamic neurons mostly located, in mammals, in the preoptic area and in the arcuate nucleus. GnRH axons project to the median eminence, where pulsatile release of GnRH into the hypophysial portal circulation drives the synthesis and secretion of follicle-stimulating hormone (FSH) and luteinizing hormone (LH) from anterior pituitary gonadotropic cells. Circulating FSH and LH, in turn, stimulate gametogenesis and the synthesis and secretion of the gonadal steroid hormones, androgens, estrogens, and progesterone. Under various physiological and pathological conditions, hormonal and metabolic signals regulate GnRH neurons both directly or through upstream neuronal circuitries to influence the pattern of GnRH secretion. The emerging picture from studies in different vertebrate models is that eCBs can modulate both GnRH and gonadotropic cell function, in other words that eCBs can influence the regulation of reproduction at both hypothalamic and pituitary levels (16, 17).

There is general agreement on the inhibitory effect exerted by cannabinoids and eCBs on GnRH release. Early studies in rats demonstrated that the ECS influence gonadal androgens via effects on the hypothalamus and the anterior pituitary. THC, as well as eCBs, lowers not only circulating testosterone levels but also the levels of LH and FSH (18). Most of this negative effect appears to be exerted by inhibition of GnRH secretion into median eminence blood portal vessels (19, 20). Serum LH decreases in response to AEA administration in wild-type mice, whereas CB1 knockout mice ($Cb1^{-/-}$) are unresponsive to the treatment (21) and show low levels of GnRH and FSH-beta mRNA at hypothalamic and pituitary levels (22), demonstrating the pivotal role exerted by CB1 in the regulation of GnRH and godanotropins synthesis and/or release.

The above effects require CB1 expression in ventro-medial telencephalic and hypothalamic regions. Early localization studies in rodents detected a low abundance of CB1-immunoreactive axons (23) and a low expression level of *CB1* mRNA (24–26) in the rodent hypothalamus. However, more recent immunocytochemical studies (27) revealed a dense CB1-immunoreactive fiber network in the mouse hypothalamus. These data are consistent with studies in teleosts and amphibians, showing the expression of CB1-immunoreactive fibers and cell bodies in several hypothalamic regions of adult teleosts (*Carassius auratus* and *Pelvicachromis pulcher*) and anuran amphibians (*Xenopus laevis* and *Rana esculenta*) (4, 5, 28, 29), as well as in zebrafish and in embryos of *X. laevis* (30, 31). The expression of *CB1* appears to be regulated in the diencephalon during the annual sexual cycle in anuran amphibians (32). Interestingly, *CB1* fluctuations show an opposite trend compared to *GnRH-I* mRNA variations, suggesting that maximal GnRH release corresponds to minimal CB1 levels in the diencephalon. Both *GnRH-I* and *GnRH-II* expressions are inhibited in the frog diencephlaon by AEA administration, indicating that both molecular forms might be involved in the regulation of gonatropin discharge (33). Only few data so far indicate that CB2 and TRPV1 receptors might have a role in GnRH cell regulation. Profiling neurotransmitter receptor expression in mouse GnRH-secreting neurons revealed CB2 expression in diestrous adult females (34), and CB1/TRPV1 co-localization has been reported in mouse hypothalamic paraventricular nucleus (35).

An important question is whether eCBs exert their effect directly on GnRH neurons, or on neighboring cells that control GnRH release. Gammon et al. (36) demonstrated that immortalized GnRH neurons (GT1 cells) are both a source and target of eCBs; they produce and secrete 2-AG and AEA, are able to take up and degrade eCBs, and possess CB1 and CB2, whose activation leads to the inhibition of pulsatile GnRH release. Nevertheless, such observations have not been confirmed *in vivo* in mammals, although GnRH-secreting neurons are close to cannabinergic fibers in male mice (37) and few hypothalamic GnRH neurons seem to express CB1 receptors (36). Close proximity between CB1-expressing fibers and GnRH cells has been well documented in non-mammalian vertebrates. In *P. pulcher*, *C. auratus*, *Solea solea*, and *Danio rerio*, CB1-containing cell bodies and terminals codistribute with GnRHIII (also called *salmon* GnRH) cell bodies and fibers (38–40). Similarly, codistribution of CB1- and GnRH-I-immunoreactivity has been found in corresponding brain regions of *X. laevis* and *R. esculenta* (39, 41). Noteworthy, a subset of frog GnRH-I-immunoreactive neurons in the septum and preoptic area are also CB1 immunopositive (28), suggesting the existence of a CB1-mediated autocrine mechanism in the control of GnRH secretion, in addition to presynaptic mechanisms. Ultrastructural studies in mammals indicate that CB1-immunoreactive terminals establish symmetric as well as asymmetric synapses on GnRH neurons, suggesting that retrograde eCB signaling might influence GABAergic and glutamatergic synaptic transmission, respectively (27). It should be noted that most recent studies examining the effects of endogenous GABA release on GnRH neurons indicate that the predominant action is that of excitation (42). In line with this, Farkas et al. (37) provided electrophysiological and morphological evidence that retrograde eCB signaling reduces GABAergic excitatory drive onto GnRH neurons via activation of presynaptic CB1 receptors, and that the reduced $GABA_A$ receptor signaling in turn inhibits GnRH neuron firing activity. Besides the major afferent regulation exerted on GnRH neurons by GABAergic and glutamatergic inputs, available neuroanatomical literature describes afferent inputs by peptidergic and monoaminergic neuronal systems (43). However, whether the ECS interacts also with these systems has not been determined yet.

Besides the effect on GnRH cells, eCBs could also modulate the activity of other hypothalamic cell types involved in reproduction. Cells containing aromatase, the enzyme that catalyzes the transformation of androgen into estrogens, are localized in the hypothalamus and are deeply involved in sexual differentiation of the brain and activation of male sexual behavior. Aromatase and CB1 are expressed in close contiguity in the goldfish preoptic area and periventricular gray of hypothalamic inferior lobes (16), suggesting a possible CB1-mediated regulation of aromatase activity, at least in bony fish.

Several lines of evidence indicate that eCBs may control adenohypophyseal hormone secretion also acting directly at pituitary level. Both AEA and 2-AG have been detected in the anterior pituitary, suggesting local synthesis (44). In addition, CB1 has been localized in the anterior pituitary within the gonadotroph and lactotroph cells in adult male rats (45, 46), in humans (47), and in *X. laevis* (48). CB1 expression in pituitary depends on steroids,

since it is reduced in both orchidectomized male and estradiol-replaced OVX female rats (46). Recently, the presence of ECS has been demonstrated in mammalian pars tuberalis (49). This finding might be functionally significant also for GnRH release, since this pituitary region is a key station for the anterograde signaling toward the pars distalis.

EFFECTS OF eCBs AT GONADAL LEVEL

Beside the role exerted at hypothalamic level to control reproductive activity in both sexes, the discovery of eCBs in gonads and reproductive fluids – from seminal plasma in males to oviductal fluid and milk in females – (50–52) pointed out the importance of eCB signaling in the gonads. Gonads have the ability to synthesize eCBs which in turn exert differential effects activating both different types of receptors or tissue-/cell-specific receptor subtypes, the latter obtained by both alternative splicing or transcription sites (53–55). The content of eCBs is regulated by biosynthetic/hydrolyzing enzyme balance, and the appropriate "eCBs tone" *in loco*, is critical for spermatogenesis progression in male and follicle maturation in female, for sperm quality and the acquisition of sperm functions related to fertilization (motility and capacitation), for fertilization, early-embryo migration, implantation and placentation, for parturition onset and labor as well (15, 17, 56–63). Focusing on males, evidence of eCB direct action into the testis has been provided in most vertebrates [fish (8, 64), frogs (32, 57, 65–68), mammals (21, 69–74)), whereas an ECS has also been described in spermatozoa (SPZ) collected from sea urchin (75), amphibians (65), rodents (76–79), bull (80), boar (81), and human (82–85). A specific and significant association between the use of marijuana and the occurrence of non-seminomatous and mixed testicular germ cell tumors (TGCT) has been recently reported in humans (86–88); although a deep characterization of ECS has never been provided in TGCT patients yet, these data may suggest that the recreational and therapeutic use of cannabinoids may represent a risk factor for TGCT. In general, a relationship between the expression of cannabinoid receptors and the outcome of sex-steroid-dependent cancer has been documented, thus the imbalance in the ECS and its interaction with sex-steroid hormone homeostasis may promote cancer development, proliferation, and migration [for recent review, see Ref. (89)]. Defects in eCB signaling or eCB tone have recently been reported in rat treated with HU210 – a synthetic analog of THC – (90) as well as in clinical cases of male infertility in humans (85, 91). Consistently, genetic inactivation of the AEA-hydrolyzing enzyme, *Faah* (Fatty acid amide hydrolase) results in increased levels of AEA in the male reproductive system that negatively affect sperm motility and impair sperm fertilizing ability (92), whereas defects in the acquisition of sperm motility during the epididymal transit have been reported in $Cb1^{-/-}$ mice (76, 77). Thus, ECS is nowadays considered a potential therapeutic target in male infertility. ECS is widely expressed in testis in both germ and somatic cells, and a map of ECS localization in several species is provided in **Table 1**. The first intratesticular targets of eCBs to be identified were the Leydig cells (21, 93), consistent with the low basal testosterone production observed in both $Cb1^{-/-}$ mice and AEA-treated controls, providing evidence of mechanisms other than the AEA/THC-dependent downregulation exerted at hypothalamic/pituitary levels. The direct effect of Bhang (cannabis) on 3β-HSD, a well-known marker of Leydig cell activity, also confirmed this issue (79). The involvement of CB1 signaling in the control of Leydig cell activity is not restricted to steroid (both testosterone/estradiol) production (21, 22, 93), but also extends to Leydig cells ontogenesis. In fact, as reported by Cacciola et al. (72), CB1 expression in differentiating adult Leydig cells negatively correlates with cell division and the characterization of $Cb1^{-/-}$ mice phenotype revealed a 30% decrease in Leydig cells number (72), as well as low circulating estradiol level (22) [for recent review, see Ref. (94)].

In the germinal compartment, AEA reduces the spermatogenic output inducing the apoptosis of Sertoli cells (70) in a mechanism reversed by FSH-dependent activation of aromatase and by estradiol-dependent upregulation of *Faah* (71). Recent studies carried out by Grimaldi et al. (95) demonstrated that in mature Sertoli cells *Faah* gene is a direct target of estradiol whose promoter contains two proximal estrogen-responsive element (ERE) sequences named ERE2/3. *In vivo*, a mechanism involving the binding of ERβ to ERE 2/3 and the epigenetic modifications of *Faah* gene proximal promoter (demethylation of both DNA at CpG site and histone H3 at lysine 9) has been demonstrated (95); consistently *FAAH* silencing abolished estrogen protection against AEA-dependent apoptosis (95). Thus, AEA content finely toned by its hydrolyzing enzyme FAAH is a fundamental tool to prevent the apoptosis in Sertoli cells.

Beside the activity exerted on Sertoli cells, eCBs are critical for the progression of spermatogenesis from mitotic stages throughout the meiotic stages and spermiogenesis events. In such a context, the FAAH-dependent modulation of eCB tone and the cell-specific expression of CB1, CB2, and TRVP1 provide evidence of multiple, differential eCB-dependent signaling involved in the spermatogenetic events. In mouse, decreasing levels of 2-AG have been detected from spermatogonia (SPG) to spermatocytes (SPC) and spermatids (SPT), suggesting that 2-AG, through CB2 – the receptor highly expressed just in mitotic and meiotic stages, but retained in residual body during the spermiogenesis – may act as an autocrine/paracrine mediator during spermatogenesis (73). Conversely, the high expression of *Trpv1* observed in meiotic stages (73) and the massive germ cell depletion detected in *Trpv1* null mice (96) candidate TRPV1 as a controller of meiotic stages. Very recently, the involvement of both CB1 and TRPV1 in the opposite modulation of testicular GnRH signaling (15, 68, 97) – a master system involved in the control of both spermatogenesis progression and steroidogenetic activity – has been reported in the anuran amphibian, the frog *R. esculenta* (97), a seasonal breeder in which two GnRH molecular forms (GnRH-I and GnRH-II) and three GnRH receptors (GnRH-RI, -RII and -RIII) have been characterized in testis (68). In such a context, AEA might act as an autocrine/paracrine factor via CB1 and as an intracrine signal via TRPV1; thus, it might be hypothesized that AEA, through the activation of specific receptors, switches on/off testicular GnRH signaling, leading to germ cell progression (**Figure 1**).

However, in mammalian and non-mammalian vertebrates, CB1 activity is linked to the control of post-meiotic stages (32, 65, 69, 73). In particular, it has been suggested that ECS controls different steps of spermiogenesis that is the phase of spermatogenesis consisting in the differentiation of SPT in SPZ. In particular,

Table 1 | Localization of ECS components [both mRNA and protein (Prot)] in testicular somatic and germ cells.

Cell type	CB1	CB2	TRPV1	FAAH	NAPE-PLD	MAGL	DAGLα/β	Species	Reference
Leydig cells	mRNA Prot Prot	Prot		Prot	mRNA			*R. esculenta* *M. musculus* *R. norvegicus*	(68, 69, 72, 79)
Sertoli cells	mRNA Prot	mRNA/Prot	mRNA	mRNA/Prot	mRNA			*R. esculenta* *M. musculus* *R. norvegicus*	(68, 70–73)
ISPG	Prot mRNA/Prot	mRNA/Prot	mRNA	mRNA	mRNA	mRNA	mRNA	*R. esculenta* *M. musculus*	(65, 69, 73)
IISPG	mRNA/Prot mRNA/Prot	mRNA/Prot	mRNA	mRNA	mRNA mRNA	mRNA	mRNA	*R. esculenta* *M. musculus*	(65, 68, 69, 73)
ISCP	Prot mRNA/Prot	mRNA/Prot	mRNA	Prot mRNA	mRNA mRNA	mRNA	mRNA	*R. esculenta* *M. musculus*	(65, 68, 69, 73)
IISPC	Prot mRNA	mRNA/Prot	mRNA	Prot mRNA	mRNA	mRNA	mRNA	*R. esculenta* *M. musculus*	(65, 73)
SPT	mRNA/Prot mRNA Prot	mRNA/Prot	mRNA	Prot mRNA	mRNA	mRNA	mRNA	*R. esculenta* *M. musculus* *R. norvegicus*	(65, 68, 69, 72, 73)
SPZ	mRNA/Prot mRNA/Prot Prot Prot mRNA/Prot mRNA/Prot	mRNA/Prot Prot mRNA/Prot Prot	mRNA/Prot Prot Prot	Prot mRNA/Prot Prot Prot Prot	mRNA/Prot Prot	mRNA/Prot	mRNA/Prot	*R. esculenta* *M. musculus* *R. norvegicus* *S. scrofa* *B. taurus* *H. sapiens*	(65, 68, 72, 78, 81, 83, 84)

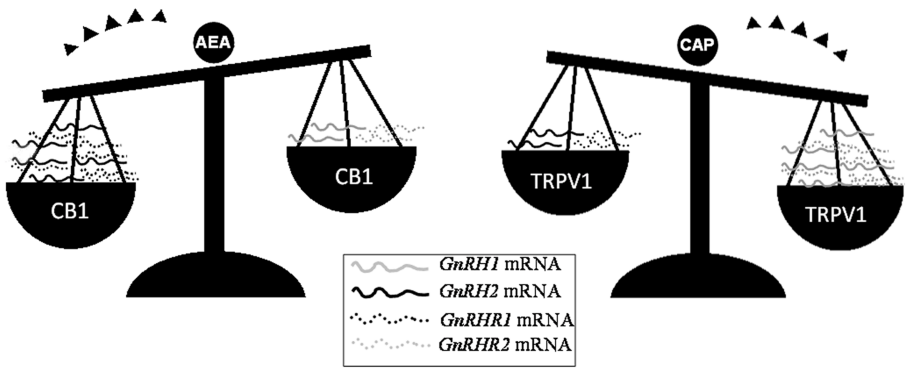

FIGURE 1 | Differential expression of GnRH system components after *in vitro* incubation of frog testis collected in post-reproductive periods with AEA or capsaicin (CAP), a selective agonist of TRPV1 receptor. Since intracellular AEA also bind TRPV1, the involvement of AEA in the modulation of testicular GnRH signaling may occur via the selective activation of different eCB receptors.

post-meiotic haploid round spermatids (rSPT) undergo biochemical and morphological changes becoming elongated cells (eSPT) and then SPZ. Sperm cells are differentially released from Sertoli cells by spermiation, a process characterized by species-specific features (65, 98). In mammals, SPZ undergo further transformations in the epididymis, which enables SPZ for fertilization (76, 77). These cellular modifications, and in particular some structural changes observed in SPT (i.e., acrosome development, nuclear shaping and chromatin condensation), seem to be related to ECS and in particular to CB1 activity.

A detailed immunolocalization of CB1 has been reported in rat SPT. CB1 appears in rSPT, around the nucleus, during acrosome development; the signal is retained in the head of elongating and condensing SPT, always close to the acrosome region, suggesting a role for CB1 in spermiogenesis, probably in chromatin packaging and in acrosome and/or cellular shape configuration

(57, 72, 81). In agreement, several data demonstrate that CB1 regulates acrosome reaction, chromatin condensation, and nuclear size of SPZ (82, 99). Recent observations demonstrate that CB1 is involved in chromatin remodeling of SPT. In fact, during spermiogenesis, as the nucleus elongates and assumes a specie-specific shape, the chromatin condenses. It is worth noting that chromatin condensation differentially occurs, depending on the species. In mammals, chromatin condensation starts in eSPT producing condensing and then condensed SPT, which are mature elongated cells with strongly packaged chromatin (100). Many events characterize these chromatin cyto-architecture changes (101). Early during spermiogenesis, it is possible to observe the expression and storage of specific proteins involved in condensation and in DNA integrity maintenance, such as transition proteins (TNPs) and protamines (PRMs) (102). Others events concern the following: (i) displacement and degradation of the nucleosome structure; (ii) histone replacement by TNPs and then by PRMs; (iii) transcriptional silencing; (iv) DNA repair; and finally, (v) repackaging of the protaminated chromatin into toroidal structures (103, 104) [for recent review, see Ref. (94)]. These events strongly preserve DNA by damage and are involved in mechanism related to sperm maturation. Indeed, it is well known that inefficient expression or activity of TNPs/PRMs deranges histone displacement and causes production of SPZ with histone retention, incomplete chromatin condensation, and DNA damage (74, 105, 106). In both humans and rodents, abnormal levels of sperm DNA damage are associated with lower conception, implantation, and fecundity rates, and with higher miscarriage probability (95, 107, 108). In this context, Chioccarelli et al. (74) showed that *Cb1* gene deletion negatively influences chromatin remodeling in SPT, by reducing either transition protein 2 (Tnp2) levels or histone displacement. Secondary effects, related to the inefficient histone displacement (i.e., histone retention, uncondensed chromatin, DNA damage, and nuclear size elongation) have been postulated (22, 74). In agreement, *in vivo* and *in vitro* experiments show that AEA is able to act locally and upregulate *Tnp2* mRNA levels through CB1, via PKC/PKA pathways (17, 74). Furthermore, in *caput* epididymis from $CB1^{-/-}$ mice, the percentage of SPZ retaining histones as well as the percentage of SPZ with uncondensed chromatin or with DNA damage, is higher as compared to normal mice. Interestingly, DNA damage increased during the epididymal transit, from *caput* to *cauda*, suggesting that CB1 preserve sperm DNA integrity of SPZ during epidydimal transit (74).

Recently, it has been demonstrated that estradiol, probably via stimulatory effects on FSH secretion and/or directly via paracrine actions within the testis, preserve chromatin condensation, and DNA integrity of SPZ, likely by promoting histone displacement in SPT (99). Indeed, it has been reported that $CB1^{-/-}$ male mice show low levels of circulating E_2, and when treated with 17β-estradiol, they rescue sperm chromatin quality by restoring histone content, chromatin packaging, DNA integrity, and nuclear length of SPZ (22, 99). These results corroborate the intriguing findings that the small nucleus of SPZ, containing chromatin that did not retain histones, appear fully condensed and able to preserve DNA from damage. On the contrary, the longer nucleus of SPZ, containing chromatin that retained histones, is uncondensed and unable to avoid DNA damage. The emerging exciting idea is that sperm nuclear dimensions can be a good marker for SPZ chromatin quality useful to select the SPZ qualitatively suitable for intracytoplasmic sperm injection (99).

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REFERENCES

1. Elphick MR, Egertová M. The phylogenetic distribution and evolutionary origins of endocannabinoid signalling. *Handb Exp Pharmacol* (2005) **168**:283–97. doi:10.1007/3-540-26573-2_9
2. Elphick MR. The evolution and comparative neurobiology of endocannabinoid signaling. *Philos Trans R Soc B Biol Sci* (2012) **367**:3201–15. doi:10.1098/rstb.2011.0394
3. Mackie K. Distribution of cannabinoid receptors in the central and peripheral nervous system. *Handb Exp Pharmacol* (2005) **168**:299–325. doi:10.1007/3-540-26573-2_10
4. Cottone E, Salio C, Conrath M, Franzoni MF. *Xenopus laevis* CB1 cannabinoid receptor: molecular cloning and mRNA distribution in the central nervous system. *J Comp Neurol* (2003) **464**:487–96. doi:10.1002/cne.10808
5. Cottone E, Forno S, Campantico E, Guastalla A, Viltono L, Mackie K, et al. Expression and distribution of CB1 cannabinoid receptors in the central nervous system of the African cichlid fish *Pelvicachromis pulcher*. *J Comp Neurol* (2005) **485**:293–303. doi:10.1002/cne.20502
6. Van Sickle MD, Duncan M, Kingsley PJ, Mouihate A, Urbani P, Mackie K, et al. Identification and functional characterization of brainstem cannabinoid CB2 receptors. *Science* (2005) **310**(5746):329–32. doi:10.1126/science.1115740
7. Atwood BK, Mackie K. CB2: a cannabinoid receptor with an identity crisis. *Br J Pharmacol* (2010) **160**:467–79. doi:10.1111/j.1476-5381.2010.00729.x
8. Cottone E, Pomatto V, Cerri F, Campantico E, Mackie K, Delpero M, et al. Cannabinoid receptors are widely expressed in goldfish: molecular cloning of a CB2-like receptor and evaluation of CB1 and CB2 mRNA expression profiles in different organs. *Fish Physiol Biochem* (2013) **39**:1287–96. doi:10.1007/s10695-013-9783-9
9. Di Marzo V, De Petrocellis L. Endocannabinoids as regulators of transient receptor potential (TRP) channels: a further opportunity to develop new endocannabinoid-based therapeutic drugs. *Curr Med Chem* (2010) **17**:1430–49. doi:10.2174/092986710790980078
10. Castillo PE, Younts TJ, Chávez AE, Hashimotodani Y. Endocannabinoid signaling and synaptic function. *Neuron* (2012) **76**(1):70–81. doi:10.1016/j.neuron.2012.09.020
11. Ahn K, McKinney MK, Cravatt BF. Enzymatic pathways that regulate endocannabinoid signaling in the nervous system. *Chem Rev* (2008) **108**:1687–707. doi:10.1021/cr0782067
12. Di Marzo V. The endocannabinoid system: its general strategy of action, tools for its pharmacological manipulation and potential therapeutic exploitation. *Pharmacol Res* (2009) **60**:77–84. doi:10.1016/j.phrs.2009.02.010
13. Pertwee RG, Howlett AC, Abood ME, Alexander SP, Di Marzo V, Elphick MR, et al. International union of basic and clinical pharmacology. LXXIX. Cannabinoid receptors and their ligands: beyond CB1 and CB2. *Pharmacol Rev* (2010) **62**:588–631. doi:10.1124/pr.110.003004
14. Piomelli D. The molecular logic of endocannabinoid signalling. *Nat Rev Neurosci* (2003) **4**:873–84. doi:10.1038/nrn1247
15. Meccariello R, Battista N, Bradshaw HB, Wang H. Updates in reproduction coming from the endocannabinoid system. *Int J Endocrinol* (2014) **2014**:412354. doi:10.1155/2014/412354
16. Cottone E, Pomatto V, Bovolin P. Role of the endocannabinoid system in the central regulation of nonmammalian vertebrate reproduction. *Int J Endocrinol* (2013) **2013**:941237. doi:10.1155/2013/941237
17. Battista N, Meccariello R, Cobellis G, Fasano S, Di Tommaso M, Piazzi V, et al. The role of endocannabinoids in gonadal function and fertility along

the evolutionary axis. *Mol Cell Endocrinol* (2012) **355**:1–14. doi:10.1016/j.mce.2012.01.014
18. Murphy LL, Steger RW, Smith MS, Bartke A. Effects of delta-9-tetrahydrocannabinol, cannabinol and cannabidiol, alone and in combinations, on luteinizing hormone and prolactin release and on hypothalamic neurotransmitters in the male rat. *Neuroendocrinology* (1990) **52**(4):316–21. doi:10.1159/000125604
19. Murphy LL, Muñoz RM, Adrian BA, Villanúa MA. Function of cannabinoid receptors in the neuroendocrine regulation of hormone secretion. *Neurobiol Dis* (1998) **5**:432–46. doi:10.1006/nbdi.1998.0224
20. Scorticati C, Fernandez-Solari J, De Laurentiis A, Mohn C, Prestifilippo JP, Lasaga M, et al. The inhibitory effect of anandamide on luteinizing hormone releasing hormone secretion is reversed by estrogen. *Proc Natl Acad Sci U S A* (2004) **32**:11891–6. doi:10.1073/pnas.0404366101
21. Wenger T, Ledent C, Csernus V, Gerendai I. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. *Biochem Biophys Res Commun* (2001) **284**:363–8. doi:10.1006/bbrc.2001.4977
22. Cacciola G, Chioccarelli T, Altucci L, Ledent C, Mason JI, Fasano S, et al. Low 17beta-estradiol levels in Cnr1 knock-out mice affect spermatid chromatin remodeling by interfering with chromatin reorganization. *Biol Reprod* (2013) **88**:152. doi:10.1095/biolreprod.112.105726
23. Tsou K, Brown S, Sanudo-Pena MC, Mackie K, Walker JM. Immunohistochemical distribution of cannabinoid CB1 receptors in the rat central nervous system. *Neuroscience* (1998) **83**:393–411. doi:10.1016/S0306-4522(97)00436-3
24. Cota D, Marsicano G, Tschöp M, Grübler Y, Flachskamm C, Schubert M, et al. The endogenous cannabinoid system affects energy balance via central orexigenic drive and peripheral lipogenesis. *J Clin Invest* (2003) **112**(3):423–31. doi:10.1172/JCI200317725
25. Marsicano G, Lutz B. Expression of the cannabinoid receptor CB1 in distinct neuronal subpopulations in the adult mouse forebrain. *Eur J Neurosci* (1999) **11**(12):4213–25. doi:10.1046/j.1460-9568.1999.00847.x
26. Matsuda LA, Bonner TI, Lolait SJ. Localization of cannabinoid receptor mRNA in rat brain. *J Comp Neurol* (1993) **327**:535–50. doi:10.1002/cne.903270406
27. Wittmann G, Deli L, Kalló I, Hrabovszky E, Watanabe M, Liposits Z, et al. Distribution of type 1 cannabinoid receptor (CB1)-immunoreactive axons in the mouse hypothalamus. *J Comp Neurol* (2007) **503**(2):270–9. doi:10.1002/cne.21383
28. Meccariello R, Franzoni MF, Chianese R, Cottone E, Scarpa D, Donna D, et al. Interplay between the endocannabinoid system and GnRH-I in the forebrain of the anuran amphibian *Rana esculenta*. *Endocrinology* (2008) **149**:2149–58. doi:10.1210/en.2007-1357
29. Valenti M, Cottone E, Martinez R, De Pedro N, Rubio M, Viveros MP, et al. The endocannabinoid system in the brain of *Carassius auratus* and its possible role in the control of food intake. *J Neurochem* (2005) **95**(3):662–72. doi:10.1111/j.1471-4159.2005.03406.x
30. Lam CS, Rastegar S, Strähle U. Distribution of cannabinoid receptor 1 in the CNS of zebrafish. *Neuroscience* (2006) **138**(1):83–95. doi:10.1016/j.neuroscience.2005.10.069
31. Migliarini B, Marucci G, Ghelfi F, Carnevali O. Endocannabinoid system in *Xenopus laevis* development: CB1 receptor dynamics. *FEBS Lett* (2006) **580**(8):1941–5. doi:10.1016/j.febslet.2006.02.057
32. Meccariello R, Chianese R, Cacciola G, Cobellis G, Pierantoni R, Fasano S. Type-1 cannabinoid receptor expression in the frog, *Rana esculenta*, tissues: a possible involvement in the regulation of testicular activity. *Mol Reprod Dev* (2006) **73**:551–8. doi:10.1002/mrd.20434
33. Chianese R, Ciaramella V, Fasano S, Pierantoni R, Meccariello R. Anandamide modulates the expression of *GnRH-II* and *GnRHRs* in frog, *Rana esculenta*, diencephalon. *Gen Comp Endocrinol* (2011) **173**:389–95. doi:10.1016/j.ygcen.2011.07.001
34. Todman MG, Han SK, Herbison AE. Profiling neurotransmitter receptor expression in mouse gonadotropin-releasing hormone neurons using green fluorescent protein-promoter transgenics and microarrays. *Neuroscience* (2005) **132**:703–12. doi:10.1016/j.neuroscience.2005.01.035
35. Cristino L, De Petrocellis L, Pryce G, Baker D, Guglielmotti V, Di Marzo V. Immunohistochemical localization of cannabinoid type 1 and vanilloid transient receptor potential vanilloid type 1 receptors in the mouse brain. *Neuroscience* (2006) **139**:1405–15. doi:10.1016/j.neuroscience.2006.02.074
36. Gammon CM, Freeman GM Jr, Xie W, Petersen SL, Wetsel WC. Regulation of gonadotropin-releasing hormone secretion by cannabinoids. *Endocrinology* (2005) **146**(10):4491–9. doi:10.1210/en.2004-1672
37. Farkas I, Kallo' I, Deli L, Vida B, Hrabovszky E, Fekete C, et al. Retrograde endocannabinoid signaling reduces GABA-ergic synaptic transmission to gonadotropin-releasing hormone neurons. *Endocrinology* (2010) **151**:5818–29. doi:10.1210/en.2010-0638
38. Cottone E, Campantico E, Guastalla A, Aramu S, Polzonetti-Magni A, Franzoni MF. Are the cannabinoids involved in fish reproduction? *Ann NY Acad Sci* (2005) **1040**:1–4. doi:10.1196/annals.1327.041
39. Cottone E, Guastalla A, Mackie K, Franzoni MF. Endocannabinoids affect the reproductive functions in teleosts and amphibians. *Mol Cell Endocrinol* (2008) **286**:S41–5. doi:10.1016/j.mce.2008.01.025
40. Cardinaletti G, Franzoni MF, Palermo FA, et al. Environmental and neuroendocrine control of fish reproduction. In: Garcia-Ayala A, Penalver JM, Chavez-Pozo E editors. *Recent Advances in Fish Reproduction Biology*. Kerala: Research Signpost (2010). p. 65–87.
41. Donna D, Cottone E, Aramu S, Campantico E, Guastalla A, Franzoni MF. Endocannabinoids and amphibian reproduction: an immunohistochemical study in the green frog. *Accad Sci Torino Atti Sci Fis* (2006) **140**:37–45.
42. Herbison AE, Moenter SM. Depolarising and hyperpolarising actions of GABA(A) receptor activation on gonadotrophin-releasing hormone neurones: towards an emerging consensus. *Neuroendocrinol* (2011) **23**(7):557–69. doi:10.1111/j.1365-2826.2011.02145.x
43. Hrabovszky E, Liposits Z. Afferent neuronal control of type-I gonadotropin releasing hormone neurons in the human. *Front Endocrinol* (2013) **4**:130. doi:10.3389/fendo.2013.00130
44. González S, Manzanares J, Berrendero F, Wenger T, Corchero J, Bisogno T, et al. Identification of endocannabinoids and cannabinoid CB(1) receptor mRNA in the pituitary gland. *Neuroendocrinology* (1999) **70**:137–45. doi:10.1159/000054468
45. Wenger T, Rettori V, Snyder GD, Dalterio S, McCann SM. Effects of delta-9-tetrahydrocannabinol on the hypothalamic-pituitary control of luteinizing hormone and follicle-stimulating hormone secretion in adult male rats. *Neuroendocrinology* (1987) **46**(6):488–93. doi:10.1159/000124870
46. González S, Mauriello-Romanazzi G, Berrendero F, Ramos JA, Franzoni MF, Fernández-Ruiz J. Decreased cannabinoid CB1 receptor mRNA levels and immunoreactivity in pituitary hyperplasia induced by prolonged exposure to estrogens. *Pituitary* (2000) **3**(4):221–6. doi:10.1023/A:1012874029689
47. Pagotto U, Marsicano G, Fezza F, Theodoropoulou M, Grübler Y, Stalla J, et al. Normal human pituitary gland and pituitary adenomas express cannabinoid receptor type 1 and synthesize endogenous cannabinoids: first evidence for a direct role of cannabinoids on hormone modulation at the human pituitary level. *J Clin Endocrinol Metab* (2001) **86**(6):2687–96. doi:10.1210/jc.86.6.2687
48. Cesa R, Guastalla A, Cottone E, Mackie K, Beltramo M, Franzoni MF. Relationships between CB1 cannabinoid receptors and pituitary endocrine cells in *Xenopus laevis*: an immunohistochemical study. *Gen Comp Endocrinol* (2002) **125**:17–24. doi:10.1006/gcen.2001.7720
49. Yasuo S, Koch M, Schmidt H, Ziebell S, Bojunga J, Geisslinger G, et al. An endocannabinoid system is localized to the hypophysial pars tuberalis of Syrian hamsters and responds to photoperiodic changes. *Cell Tissue Res* (2010) **340**:127–36. doi:10.1007/s00441-010-0930-7
50. Schuel H, Burkman LJ, Lippes J, Crickard K, Forester E, Piomelli D, et al. *N*-Acylethanolamines in human reproductive fluids. *Chem Phys Lipids* (2002) **121**:211–27. doi:10.1016/S0009-3084(02)00158-5
51. El-Talatini MR, Taylor AH, Konje JC. Fluctuation in anandamide levels from ovulation to early pregnancy in in-vitro fertilization-embryo transfer women, and its hormonal regulation. *Hum Reprod* (2009) **24**:1989–98. doi:10.1093/humrep/dep065
52. Gervasi MG, Marczylo TH, Lam PM, Rana S, Franchi AM, Konje JC, et al. Anandamide levels fluctuate in the bovine oviduct during the oestrous cycle. *PLoS One* (2013) **8**:e72521. doi:10.1371/journal.pone.0072521
53. Shire D, Carillon C, Kaghad M, Calandra B, Rinaldi-Carmona M, Le Fur G, et al. An ammino-terminal variant of the central cannabinoid receptor resulting from alternative splicing. *J Biol Chem* (1995) **270**:3726–31. doi:10.1074/jbc.270.8.3726
54. Ryberg E, Vu HK, Larsson N, Groblewski T, Hjorth S, Elebring T, et al. Identification and characterization of a novel splice variant of the human CB1 receptor. *FEBS Lett* (2005) **579**:259–64. doi:10.1016/j.febslet.2004.11.085

55. Liu QR, Pan CH, Hishimoto A, Li CY, Xi ZX, Llorente-Berzal A, et al. Species differences in cannabinoid receptor 2 (CNR2 gene): identification of novel human and rodent CB2 isoforms, differential tissue expression and regulation by cannabinoid receptor ligands. *Genes Brain Behav* (2009) **8**:519–30. doi:10.1111/j.1601-183X.2009.00498.x
56. Wang H, Dey SK, Maccarrone M. Jekyll and Hyde: two faces of cannabinoid signaling in male and female fertility. *Endocr Rev* (2006) **27**:427–48. doi:10.1210/er.2006-0006
57. Cacciola G, Chioccarelli T, Ricci G, Meccariello R, Fasano S, Pierantoni R, et al. The endocannabinoid system in vertebrate male reproduction: a comparative overview. *Mol Cell Endocrinol* (2008) **286**:S24–30. doi:10.1016/j.mce.2008.01.004
58. Pierantoni R, Cobellis G, Meccariello R, Cacciola G, Chianese R, Chioccarelli T, et al. CB1 activity in male reproduction: mammalian and nonmammalian animal models. *Vitam Horm* (2009) **81**:367–87. doi:10.1016/S0083-6729(09)81014-5
59. Pierantoni R, Cobellis G, Meccariello R, Cacciola G, Chianese R, Chioccarelli T, et al. Testicular gonadotropin-releasing hormone activity, progression of spermatogenesis, and sperm transport in vertebrates. *Ann N Y Acad Sci* (2009) **1163**:279–91. doi:10.1111/j.1749-6632.2008.03617.x
60. Acone G, Trabucco E, Colacurci N, Cobellis L, Mackie K, Meccariello R, et al. Low type I cannabinoid receptor levels characterize placental villous in labouring delivery. *Placenta* (2009) **30**:203–5. doi:10.1016/j.placenta.2008.11.018
61. Trabucco E, Acone G, Marenna A, Pierantoni R, Cacciola G, Chioccarelli T, et al. Endocannabinoid system in first trimester placenta: low FAAH and high CB1 expression characterize spontaneous miscarriage. *Placenta* (2009) **30**:516–22. doi:10.1016/j.placenta.2009.03.015
62. Cacciola G, Chianese R, Chioccarelli T, Ciaramella V, Fasano S, Pierantoni R, et al. Cannabinoids and reproduction: a lasting and intriguing history. *Pharmaceuticals* (2010) **3**:3275–323. doi:10.3390/ph3103275
63. Chan HW, McKirdy NC, Peiris HN, Rice GE, Mitchell MD. The role of endocannabinoids in pregnancy. *Reproduction* (2013) **146**:R101–9. doi:10.1530/REP-12-0508
64. Ruggeri B, Soverchia L, Mosconi G, Franzoni MF, Cottone E, Polzonetti-Magni AM. Changes of gonadal CB1 cannabinoid receptor mRNA in the gilthead seabream, Sparus aurata, during sex reversal. *Gen Comp Endocrinol* (2007) **150**:263–9. doi:10.1016/j.ygcen.2006.09.001
65. Cobellis G, Cacciola G, Scarpa D, Meccariello R, Chianese R, Franzoni MF, et al. Endocannabinoid system in frog and rodent testis: type-1 cannabinoid receptor and fatty acid amide hydrolase activity in male germ cells. *Biol Reprod* (2006) **75**:82–9. doi:10.1095/biolreprod.106.051730
66. Meccariello R, Chianese R, Cobellis G, Pierantoni R, Fasano S. Cloning of type 1 cannabinoid receptor in *Rana esculenta* reveals differences between genomic sequence and cDNA. *FEBS J* (2007) **274**:2909–20. doi:10.1111/j.1742-4658.2007.05824.x
67. Chianese R, Cobellis G, Pierantoni R, Fasano S, Meccariello R. Non-mammalian vertebrate models and the endocannabinoid system: relationships with gonadotropin-releasing hormone. *Mol Cell Endocrinol* (2008) **286**:S46–51. doi:10.1016/j.mce.2008.01.009
68. Chianese R, Ciaramella V, Scarpa D, Fasano S, Pierantoni R. Meccariello anandamide regulates the expression of GnRH1, GnRH2, and GnRH-Rs in frog testis. *Am J Physiol Endocrinol Metab* (2012) **303**:E475–87. doi:10.1152/ajpendo.00086.2012
69. Gye MC, Kang HH, Kang HJ. Expression of cannabinoid receptor 1 in mouse testes. *Arch Androl* (2005) **51**:247–55. doi:10.1080/014850190898845
70. Maccarrone M, Cecconi S, Rossi G, Battista N, Pauselli R, Finazzi-Agrò A. Anandamide activity and degradation are regulated by early postnatal aging and follicle-stimulating hormone in mouse Sertoli cells. *Endocrinology* (2003) **144**:20–8. doi:10.1210/en.2002-220544
71. Rossi G, Gasperi V, Paro R, Barsacchi D, Cecconi S, Maccarrone M. Follicle-stimulating hormone activates fatty acid amide hydrolase by protein kinase A and aromatase-dependent pathways in mouse primary Sertoli cells. *Endocrinology* (2007) **148**:1431–9. doi:10.1210/en.2006-0969
72. Cacciola G, Chioccarelli T, Mackie K, Meccariello R, Ledent C, Fasano S, et al. Expression of type-1 cannabinoid receptor during rat postnatal testicular development: possible involvement in adult leydig cell differentiation. *Biol Reprod* (2008) **79**:758–65. doi:10.1095/biolreprod.108.070128
73. Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T, et al. The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. *Proc Natl Acad Sci U S A* (2009) **106**:11131–6. doi:10.1073/pnas.0812789106
74. Chioccarelli T, Cacciola G, Altucci L, Lewis SE, Simon L, Ricci G, et al. Cannabinoid receptor 1 influences chromatin remodeling in mouse spermatids by affecting content of transition protein 2 mRNA and histone displacement. *Endocrinology* (2010) **151**:5017–29. doi:10.1210/en.2010-0133
75. Schuel H, Goldstein E, Mechoulam R, Zimmerman AM, Zimmerman S. Anandamide (arachidonylethanolamide), a brain cannabinoid receptor agonist, reduces sperm fertilizing capacity in sea urchins by inhibiting the acrosome reaction. *Proc Natl Acad Sci U S A* (1994) **91**:7678–82. doi:10.1073/pnas.91.16.7678
76. Ricci G, Cacciola G, Altucci L, Meccariello R, Pierantoni R, Fasano S, et al. Endocannabinoid control of sperm motility: the role of epididymus. *Gen Comp Endocrinol* (2007) **153**:320–2. doi:10.1016/j.ygcen.2007.02.003
77. Cobellis G, Ricci G, Cacciola G, Orlando P, Petrosino S, Cascio MG, et al. A gradient of 2-arachidonoylglycerol regulates mouse epididymal sperm cell start-up. *Biol Reprod* (2010) **82**:451–8. doi:10.1095/biolreprod.109.079210
78. Catanzaro G, Battista N, Rossi G, Di Tommaso M, Pucci M, Pirazzi V, et al. Effect of capacitation on the endocannabinoid system of mouse sperm. *Mol Cell Endocrinol* (2011) **343**:88–92. doi:10.1016/j.mce.2011.01.022
79. Banerjee A, Singh A, Srivastava P, Turner H, Krishna A. Effects of chronic bhang (cannabis) administration on the reproductive system of male mice. *Birth Defects Res B Dev Reprod Toxicol* (2011) **92**:195–205. doi:10.1002/bdrb.20295
80. Gervasi MG, Osycka-Salut C, Caballero J, Vazquez-Levin M, Pereyra E, Billi S, et al. Anandamide capacitates bull spermatozoa through CB1 and TRPV1 activation. *PLoS One* (2011) **6**:e16993. doi:10.1371/journal.pone.0016993
81. Maccarrone M, Barboni B, Paradisi A, Bernabò N, Gasperi V, Pistilli MG, et al. Characterization of the endocannabinoid system in boar spermatozoa and implications for sperm capacitation and acrosome reaction. *J Cell Sci* (2005) **118**:4393–404. doi:10.1242/jcs.02536
82. Schuel H, Burkman LJ, Lippes J, Crickard K, Mahony MC, Giuffrida A, et al. Evidence that anandamide-signalling regulates human sperm functions required for fertilization. *Mol Reprod Dev* (2002) **63**:376–87. doi:10.1002/mrd.90021
83. Rossato M, Ion Popa F, Ferigo M, Clari G, Foresta C. Human sperm express cannabinoid receptor Cb1, the activation of which inhibits motility, acrosome reaction, and mitochondrial function. *J Clin Endocrinol Metab* (2005) **90**:984–91. doi:10.1210/jc.2004-1287
84. Francavilla F, Battista N, Barbonetti A, Vassallo MR, Rapino C, Antonangelo C, et al. Characterization of the endocannabinoid system in human spermatozoa and involvement of transient receptor potential vanilloid 1 receptor in their fertilizing ability. *Endocrinology* (2009) **150**:4692–700. doi:10.1210/en.2009-0057
85. Amoako AA, Marczylo TH, Marczylo EL, Elson J, Willets JM, Taylor AH, et al. Anandamide modulates human sperm motility: implications for men with asthenozoospermia and oligoasthenoteratozoospermia. *Hum Reprod* (2013) **28**:2058–66. doi:10.1093/humrep/det232
86. Darling JR, Doody DR, Sun X, Trabert BL, Weiss NS, Chen C, et al. Association of marijuana use and the incidence of testicular germ cell tumors. *Cancer* (2009) **115**:1215–23. doi:10.1002/cncr.24159
87. Trabert B, Sigurdson AJ, Sweeney AM, Strom SS, McGlynn KA. Marijuana use and testicular germ cell tumors. *Cancer* (2011) **117**:848–53. doi:10.1002/cncr.25499
88. Lacson JC, Carroll JD, Tuazon E, Castelao EJ, Bernstein L, Cortessis VK. Population-based case-control study of recreational drug use and testis cancer risk confirms an association between marijuana use and nonseminoma risk. *Cancer* (2012) **118**:5374–83. doi:10.1002/cncr.27554
89. Ayakannu T, Taylor AH, Marczylo TH, Willets JM, Konje JC. The endocannabinoid system and sex steroid hormone-dependent cancers. *Int J Endocrinol* (2013) **2013**:259676. doi:10.1155/2013/259676
90. Lewis SE, Paro R, Borriello L, Simon L, Robinson L, Dincer Z, et al. Long-term use of HU210 adversely affects spermatogenesis in rats by modulating the endocannabinoid system. *Int J Androl* (2012) **35**:731–40. doi:10.1111/j.1365-2605.2012.01259.x
91. Lewis SE, Rapino C, Di Tommaso M, Pucci M, Battista N, Paro R, et al. Differences in the endocannabinoid system of sperm from fertile and infertile men. *PLoS One* (2012) **7**:e47704. doi:10.1371/journal.pone.0047704
92. Sun X, Wang H, Okabe M, Mackie K, Kingsley PJ, Marnett LJ, et al. Genetic loss of Faah compromises male fertility in mice. *Biol Reprod* (2009) **80**:235–42. doi:10.1095/biolreprod.108.072736

93. Chianese R, Ciaramella V, Fasano S, Pierantoni R, Meccariello R. Hypothalamus–pituitary axis: an obligatory target for endocannabinoids to inhibit steroidogenesis in frog testis. *Gen Comp Endocrinol* (2014). doi:10.1016/j.ygcen.2014.02.010
94. Cacciola G, Chioccarelli T, Fasano S, Pierantoni R, Cobellis G. Estrogens and spermiogenesis: new insights from type 1 cannabinoid receptor knockout mice. *Int J Endocrinol* (2013) **2013**:501350. doi:10.1155/2013/501350
95. Grimaldi P, Pucci M, Di Siena S, Di Giacomo D, Pirazzi V, Geremia R, et al. The *faah* gene is the first direct target of estrogen in the testis: role of histone demethylase LSD1. *Cell Mol Life Sci* (2012) **69**:4177–90. doi:10.1007/s00018-012-1074-6
96. Mizrak SC, van Dissel-Emiliani FM. Transient receptor potential vanilloid receptor-1 confers heat resistance to male germ cells. *Fertil Steril* (2008) **90**:1290–3. doi:10.1016/j.fertnstert.2007.10.081
97. Chianese R, Ciaramella V, Scarpa D, Fasano S, Pierantoni R, Meccariello R. Endocannabinoids and endovanilloids: a possible balance in the regulation of the testicular GnRH signalling. *Int J Endocrinol* (2013) **2013**:904748. doi:10.1155/2013/904748
98. Cobellis G, Lombardi M, Scarpa D, Izzo G, Fienga G, Meccariello R, et al. Fra-1 activity in the frog, *Rana esculenta*, testis. *Ann N Y Acad Sci* (2005) **1040**:264–8. doi:10.1196/annals.1327.039
99. Cacciola G, Chioccarelli T, Altucci L, Viaggiano A, Fasano S, Pierantoni R, et al. Nuclear size as estrogen-responsive chromatin quality parameter of mouse spermatozoa. *Gen Comp Endocrinol* (2013) **193**:201–9. doi:10.1016/j.ygcen.2013.07.018
100. Zhao M, Shirley CR, Hayashi S, Marcon L, Mohapatra B, Suganuma R, et al. Transition nuclear proteins are required for normal chromatin condensation and functional sperm development. *Genesis* (2004) **38**:200–13. doi:10.1002/gene.20019
101. Marcon L, Boissonneault G. Transient DNA strand breaks during mouse and human spermiogenesis new insights in stage specificity and link to chromatin remodeling. *Biol Reprod* (2004) **70**:910–8. doi:10.1095/biolreprod.103.022541
102. Oliva R, Dixon GH. Vertebrate protamine genes and histone-to-protamine replacement reaction. *Prog Nucleic Acid Res Mol Biol* (1991) **40**:25–94. doi:10.1016/S0079-6603(08)60839-9
103. Rousseaux S, Caron C, Govin J, Lestrat C, Faure AK, Khochbin S. Establishment of male-specific epigenetic information. *Gene* (2005) **345**:139–53. doi:10.1016/j.gene.2004.12.004
104. Oliva R, Castillo J. Proteomics and the genetics of sperm chromatin condensation. *Asian J Androl* (2011) **13**:24–30. doi:10.1038/aja.2010.65
105. Shirley CR, Hayashi S, Mounsey S, Yanagimachi R, Meistrich ML. Abnormalities and reduced reproductive potential of sperm from Tnp1- and Tnp2-null double mutant mice. *Biol Reprod* (2004) **71**:1220–9. doi:10.1095/biolreprod.104.029363
106. Zhao M, Shirley CR, Yu YE, Mohapatra B, Zhang Y, Unni E, et al. Targeted disruption of the transition protein 2 gene affects sperm chromatin structure and reduces fertility in mice. *Mol Cell Biol* (2001) **21**:7243–55. doi:10.1128/MCB.21.21.7243-7255.2001
107. Lewis SE, Agbaje IM. Using the alkaline comet assay in prognostic tests for male infertility and assisted reproductive technology outcomes. *Mutagenesis* (2008) **23**:163–70. doi:10.1093/mutage/gem052
108. Hogeveen KN, Sassone-Corsi P. Regulation of gene expression in postmeiotic male germ cells: CREM-signalling pathways and male fertility. *Hum Fertil* (2006) **9**:73–9. doi:10.1080/14647270500463400

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Roles of reactive oxygen species in the spermatogenesis regulation

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Spermatogenesis is a complex process of male germ cells proliferation and maturation from diploid spermatogonia, through meiosis, to mature haploid spermatozoa. The process involves dynamic interactions between the developing germ cells and their supporting Sertoli cells. The gonadal tissue, with abundance of highly unsaturated fatty acids, high rates of cell division, and variety of testis enzymes results very vulnerable to the overexpression of reactive oxygen species (ROS). In order to address this risk, testis has developed a sophisticated array of antioxidant systems comprising both enzymes and free radical scavengers. This chapter sets out the major pathways of testis generation, the metabolism of ROS, and highlights the transcriptional regulation by steroid receptors of antioxidant stress enzymes and their functional implications. It also deals with of the advantages of the system biology for an antioxidant under steroid control, the major selenoprotein expressed by germ cells in the testis, the phospholipid hydroperoxide glutathione peroxidase (PHGPx/GPx4) having multiple functions and representing the pivotal link between selenium, sperm quality, and species preservation.

Keywords: spermatogenesis, reactive oxygen species, antioxidants, selenium, healthy reproduction

INTRODUCTION

Spermatogenesis appears to be a fairly conserved process throughout the vertebrate series. The balance between spermatogonial stem cell self-renewal and differentiation in the adult testis grants cyclic waves of spermatogenesis and potential fertility. These replicative processes imply a highest rate of mitochondrial oxygen consumption and reactive oxygen species (ROS) generation. Enzyme complexes of the respiratory chain of the oxidative phosphorylation, localized on the crests of the mitochondria, as the xanthines, the nicotinamide adenine dinucleotide phosphate (NADPH) oxidase and cytochrome P450, represent a source for a variety of ROS. As known, ROS are free radicals and/or oxygen derivatives that include superoxide anion, hydrogen peroxide, hydroxyl radical, lipid hydroperoxides, peroxyl radicals, and peroxynitrite. They have a dual role in biological systems, both beneficial than harmful depending on their nature and concentration as well as location and length of exposure (1). In this mini-review, we focused our attention on the relevance of ROS role in the spermatogenesis.

REACTIVE OXYGEN SPECIES AND TESTIS MECHANISTIC ANTIOXIDATIVE AND REDOX DEFENSE

Reactive oxygen species are involved in all cell physiological processes. In testis, they may be beneficial or even indispensable in the complex process of male germ cells' proliferation and maturation, from diploid spermatogonia through meiosis to mature haploid spermatozoa (2). Conversely high doses, and/or inadequate removal of ROS caused by several mechanisms, i.e., ionizing radiation, bioactivation of xenobiotics, inflammatory processes, increased cellular metabolism, activation of oxidases, and oxygenases, can be very dangerous, modifying susceptible molecules including DNA, lipids, and proteins. In addition, testis as tissue, containing large quantities of highly unsaturated fatty acids (particularly 20:4 and 22:6), results vulnerable to ROS attach. The low oxygen tension that characterizes this tissue may be an important component of the self-defense mechanism from free radical-mediated damage during spermatogenesis and Leydig cell steroidogenesis (3); together with an elaborate array of antioxidant enzymes and free radical scavengers ensures that spermatogenic and steroidogenic functions of Leydig cells are not impacted by the overexpression of ROS. In order to have a better understanding of ROS testis' neutralization or limitation by the antioxidant systems, we summarize the major pathways of ROS generation and the mechanistic antioxidative defense in **Figure 1**. Superoxide radical can be generated by specialized enzymes, such as the xanthine or NADPH oxidases, or as a by-product of cellular metabolism, particularly the mitochondrial electron transport chain, and are converted to hydrogen peroxide by the superoxide dismutase (SOD). Hydrogen peroxide, present as superoxide radical and iron, forms a more reactive form, subsequently converted in lipid peroxide. Lipid peroxide is scavenged to H_2O by glutathione peroxidase (GPx) or glutathione-*S*-transferase (GST) (4). The SOD defense by Cu/Zn-SOD, Fe/Mn-SOD, and extracellular SOD, is generally achieved by catalase or peroxidases, such as the GPxs, which use reduced glutathione (GSH) as electron donor. Glutathione keeps cells in a reduced state, acting as electron donor for other antioxidative enzymes too, and as a source for the formation of conjugates with some harmful endogenous and xenobiotic compounds,

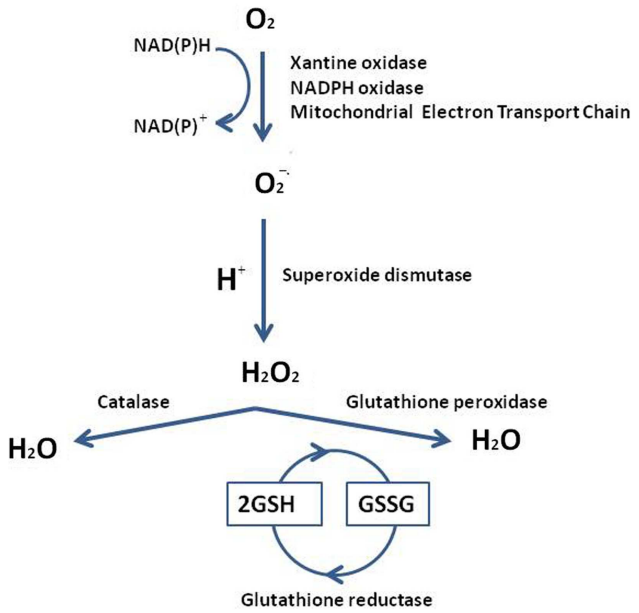

FIGURE 1 | Reactive oxygen species generation and the mechanistic antioxidative and redox defense. The testis overexpression of ROS accelerates a response by the superoxide dismutase (SOD), glutathione peroxidase (GPx), and the glutathione-*S*-transferase (GST). The resulting oxidation product is recycled by glutathione reductase (GR), which transforms the oxidized glutathione (GSSG) back to reduced form of glutathione (GSH) [from Ref. (13)].

via GST's catalysis. Levels of the reduced glutathione (GSH) are maintained via two ATP-consuming steps, involving c-glutamylcysteine synthetase (cGCS) and glutathione synthetase. The other option constitutes a recycling system involving glutathione reductase (GR): it reduces the oxidized glutathione (GSSG) back to GSH in an NADPH-dependent way. In the interaction of GSH with ROS, GSH serves as an electron donor. The resulting oxidation product, GSSG, is either recycled by GR via electron transfer from NADPH or pumped out of the cells. Thus, GR indirectly participates in the protection of cells against oxidative stress (5, 6). In addition to the major ROS processing enzymes, in testis small molecular weight antioxidant substances are present, protecting against oxidative damage. These factors include ions, as zinc and a wide variety of free radical scavengers, vitamins C or E, melatonin and cytochrome C (7).

REACTIVE OXYGEN SPECIES AND SPERMATOGENESIS TRANSCRIPTIONAL CONTROL

In vertebrates, the spermatogenesis is controlled by a complex network of endocrine, paracrine, and autocrine signals (8–10) Recent studies summarize different transcription factors, with a regulatory function, who modulate cellular and stage-specific gene expression. In particular, they can be subdivided in general transcription factors; nuclear receptors superfamily; transcription factors involved in testicular functions; testis-specific gene transcription, and transcriptional regulators of cell junction dynamics (11). As reported in **Figure 2** in response to the hypothalamic

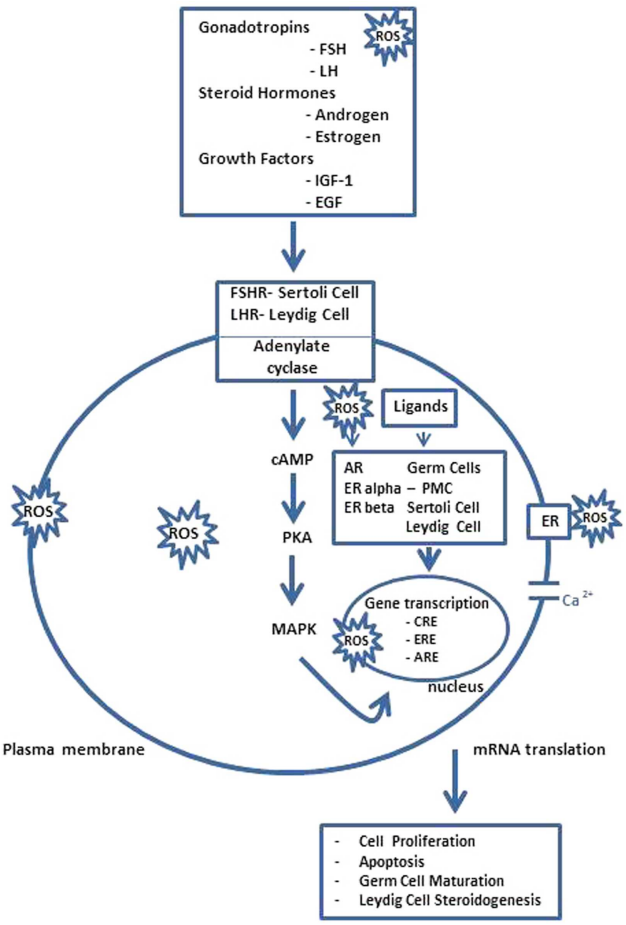

FIGURE 2 | Spermatogenesis ROS endocrine gene transcriptional regulation. FSH acts through its receptors in Sertoli cells (FSHR) to regulate the spermatogenesis and LH stimulates androgen production by Leydig cells after binding to LHR. However, steroid hormones, i.e., androgen and estrogen, and other agents that bind or prevent binding to steroid hormone receptors, which are present in Sertoli cells, germ cells, and Leydig cells also regulate testicular function as several growth factors, e.g., insulin like growth factor-1 (IGF-1) and epidermal growth factor (EGF), acting via their receptors possibly modulate AR and ERalpha and beta-mediated pathways. The pathway, mediated by adenosine monophosphate (cAMP), appears to be the primary intracellular signaling pathway in all testicular cells and stimulates the cAMP-dependent protein kinase (PKA). Thus, testicular function is disrupted by interactions between ROS and lipids, proteins, DNA, and several signaling pathways, some acting locally, e.g., AR and ER-mediated pathways, and others indirectly by modulating hypothalamus–pituitary function. Hormonal activation of transcriptional gene activity results in changes in cell differentiation and function. PMC, peritubular myoid cell; CRE, cAMP responsive elements; ARE, androgen-responsive elements; ERE, estrogen-responsive elements [modified from Ref. (15)].

gonadotropin hormone releasing (GnRH), the pituitary gland secretes two hormones, the luteinizing hormone (LH), and the follicle stimulating hormone (FSH), involved in the regulation of spermatogenesis, together with other important transcription factors (3). LH regulates the testosterone secretion by somatic Leydig cells located in the interstitium, between seminiferous

tubules; FSH acts in Sertoli cells by stimulating signaling, gene expression, and the secretion of peptides and other signaling molecules (12) In Sertoli cells, i.e., the cAMP response element binding protein (CREB) transcription factor, an important transducer of FSH signals. Transcription factors belonging to the CREB family are involved in the regulation of gene expression in response to a number of signaling pathways inducted by ROS overexpression (13). In rat testis, alternatively, the spliced variant CREB mRNAs are spermatogenic, cycle dependent, and expressed during development of the germ and Sertoli cells, indicating that the CREB isoforms may be the major players during spermatogenesis. The transcription factor cAMP response element modulator (CREM) is highly expressed in male germ cells and regulates the expression of several post-meiotic genes, such as the transition proteins and protamines, and it likely is the key regulator of gene expression during spermatogenesis. Targeted disruption of the CREM gene blocks the differentiation program in the first step of spermiogenesis. These findings indicate a crucial role of CREM in post-meiotic germ cells differentiation, linking the action of hormonal stimuli to direct regulation of spermatogenesis genes (14). Now, it is also clear that, not only testicular somatic cells (Leydig and Sertoli cells), but also germ cells express P450arom mRNA, which is translated in a biologically active enzyme involved in the production of estrogens. Therefore, the androgen/estrogen ratio is modified in germ cells, and if testosterone is involved in the regulation of testicular functions, estrogens are also necessary not only in the control of gonadotropins secretion but also in the modulation of the Leydig cells development and steroidogenesis, as well as in the development and/or maintenance of spermatogenesis and spermiogenesis in some mammalian species (15). However, the physiological linkage between different transcription factors and ROS overexpression showed regulation by the estrogen receptor of antioxidative stress enzymes (16), the molecular target genes of these transcription factors at different stages of the seminiferous epithelial cycle are largely unknown and this shall provide an unprecedented opportunity for further investigation in the field.

REACTIVE OXYGEN SPECIES AND SPECIE PRESERVATION

The maintenance of a high redox potential is a prerequisite to maintain the reproductive systems in a healthy state (17). Reproductive system needs ROS for reproduction, and minimizes the risk caused by ROS using antioxidative systems, such as SOD and GPx. When ROS levels exceed the scavenging capacity of the redox system, under such situations, can repair oxidized and damaged molecules using NADPH as an original electron source. In the context of defense against ROS, selenium as the glutathione (GSH) system plays key functions (18). Selenium has long been known to be necessary for the basal function of many systems of the male reproduction, also (19) is required for the synthesis of testosterone and the formation and development of the sperm (20); its deficiency affects testicular mass with damage to sperm motility, the sperm mid piece, and the shape of the sperm (21). In testis, however, most of the selenium, incorporated into proteins as selenocysteine, is associated to the enzyme phospholipid hydroperoxide GPx, PHGPx/GPx4 (22), member of the GPx named EC 1.11.1.12. PHGPx protects liposomes and biomembranes from peroxidative degradation and exhibits GPx activity on phosphatidylcholine hydroperoxides. It is, infact, able to react with hydroperoxides of fatty acids esterified in the phospholipids (23, 24); use protein thiol groups as donor substrates, to protect germ cell, by eliminating oxidative stress and reducing the levels of oxidized molecules. In rodents' testis, PHGPx is localized in the interstitial cells of Leydig, in the nucleus of round spermatids, at the level of the cytoplasm and in the mitochondrial capsule of spermatozoa (25). Here, it is present in three different isoforms: as a cytosolic, mitochondrial, and nuclear protein (26). Functional cis-regulatory elements are identified in the promoter region of nPHGPx (27), whose expression is mediated by the transcription factor CREM-t (28). In spermatids, it is abundantly expressed as active peroxidase and during final maturation, it is transformed into a structural protein enzymatically inactive; it surrounds the helix of mitochondria in the midpiece of the sperm. The nuclear isoform, in particular, is involved in the process of the chromatin condensation, which occurs in the final steps of spermatogenesis and requires the replacement of the majority of histones, with transition proteins and protamines, essential for the stabilization of DNA and condensation of spermatocytes. These changes in location suggest that the nPHGPx can play more than a role in spermatogenesis (29). PHGPx gene expression and activity are hormone dependent processes, and they are influenced by the levels of testosterone during spermatogenesis (30). Steroid hormones do not directly activate transcription and it has been documented that, *in vivo*, testosterone promote the expression only, as a consequence of the induction of spermatogenesis (30). The study of the mechanisms of gene transcription in testis (31), suggests a crucial role of this antioxidant in male fertility and its usefulness in the screening of a potential threat to the species' continuity (1, 32).

CONCLUDING REMARKS

The overall objective of our mini-review was to highlight the beneficial and detrimental role of ROS that comparatively determine and influence the cyclic waves of spermatogenesis and the species preservation.

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REFERENCES

1. Aitken RJ, Smith TB, Jobling MS, Baker MA, De Iuliis GN. Oxidative stress and male reproductive health. *Asian J Androl* (2014) **16**(1):31–8. doi:10.4103/1008-682X.122203
2. Shi Y, Buffenstein R, Pulliam DA, Van Remmen H. Comparative studies of oxidative stress and mitochondrial function in aging. *Integr Comp Biol* (2010) **50**:869–79. doi:10.1093/icb/icq079
3. Chen C, Ouyang WY, Grigura V, Zhou Q, Carnes K, Lim H, et al. ERM is required for transcriptional control of the spermatogonial stem cell niche. *Nature* (2005) **436**(7053):1030–4. doi:10.1038/nature03894
4. Aitken RJ, Jones KT, Robertson SA. Reactive oxygen species and sperm function – in sickness and in health. *J Androl* (2012) **33**(6):1096–106. doi:10.2164/jandrol.112.016535
5. Guerriero G, Di Finizio A, Ciarcia G. Oxidative defenses in the seabass, *Dicentrarchus labrax*. In: Dunn J, Swartz HM editors. *Advances in Experimental Medicine and Biology*. (Vol. 68), New York: Kluwer Academic/Plenum Plublisher (2003). p. 681–8.
6. Labuschagne CF, Brenkman AB. Current methods in quantifying ROS and oxidative damage in *Caenorhabditis elegans* and other model organism of aging. *Ageing Res Rev* (2013) **12**(4):918–30. doi:10.1016/j.arr.2013.09.003

7. Guerriero G, Ferro R, Russo GL, Ciarcia G. Vitamin E in early stages of sea bass (*Dicentrarchus labrax*) development. *Comp Biochem Physiol A Mol Integr Physiol* (2004) **138**(4):435–9. doi:10.1016/j.cbpb.2004.06.003
8. Huleihel M, Lunenfeld E. Regulation of spermatogenesis by paracrine/autocrine testicular factors. *Asian J Androl* (2004) **6**:259–68.
9. Cobellis G, Meccariello R, Pierantoni R, Fasano S. Intratesticular signals for progression of germ cell stages in vertebrates. *Gen Comp Endocrinol* (2003) **134**(3):220–8. doi:10.1016/S0016-6480(03)00281-8
10. Pierantoni R, Cobellis G, Meccariello R, Fasano S. Evolutionary aspects of cellular communication in the vertebrate hypothalamo-hypophysio-gonadal axis. *Int Rev Cytol* (2002) **218**:69–141. doi:10.1016/S0074-7696(02)18012-0
11. Lui W-Y, Cheng CY. Transcriptional regulation in spermatogenesis. In: Chang CY editor. *Advances in Experimental Medicine and Biology*. (Vol. 636), New York: Landes Bioscience and Springer Science + Business Media (2008). p. 115–32.
12. Grimes SR. Testis-specific transcriptional control. *Gene* (2004) **343**:11–22. doi:10.1016/j.gene.2004.08.021
13. Aitken RJ, Roman SD. Antioxidant systems and oxidative stress in the testes. *Oxid Med Cell Longev* (2008) **1**(1):15–24. doi:10.4161/oxim.1.1.6843
14. Ranawat P, Bansal MP. Modulatory effects of selenium on spermatogenesis: involvement of transcription factors CREB and CREM. *Am J Biomed Sci* (2010) **2**(4):329–41. doi:10.5099/aj100400329
15. Akingbemi BT. Estrogen regulation of testicular function. *Reprod Biol Endocrinol* (2005) **3**:51–64. doi:10.1186/1477-7827-3-51
16. Montano MM, Deng H, Liu M, Sun X, Singal R. Transcriptional regulation by the estrogen receptor of antioxidative stress enzymes and its functional implications. *Oncogene* (2004) **23**:2442–53. doi:10.1038/sj.onc.1207358
17. Fujii J, Iuchi Y, Okada F. Fundamental roles of reactive oxygen species and protective mechanisms in the female reproductive system. *Reprod Biol Endocrinol* (2005) **3**:43–53. doi:10.1186/1477-7827-3-43
18. Beckett GJ, Arthur JR. Selenium and endocrine systems. *J Endocrinol* (2005) **184**:455–65. doi:10.1677/joe.1.05971
19. Schreck CB. Stress and fish reproduction: the roles of allostasis and hormesis. *Gen Comp Endocrinol* (2010) **165**:549–56. doi:10.1016/j.ygcen.2009.07.004
20. Behne D, Hofer T, Von Berwordt-Wallrabe R, Elger W. Selenium in the testis of the rat: studies on its regulation and its importance for the organism. *J Nutr* (1982) **112**(9):1682–7.
21. Garrido N, Meseguer M, Carlos Simon C, Pellicer A, Remohi J. Pro-oxidative and anti-oxidative imbalance in human semen and its relation with male fertility. *Asian J Androl* (2004) **6**:59–65.
22. Ursini F, Maiorino M, Valente M, Ferri L, Gregolin C. Purification from pig liver of a protein which protects liposomes and biomembranes from peroxidative degradation and exhibits glutathione peroxidase activity on phosphatidylcholine hydroperoxides. *Biochim Biophys Acta* (1982) **710**: 197–211.
23. Hermesz E, Ferencz A. Identification of two phospholipid hydroperoxide glutathione peroxidase (gpx4) genes in common carp. *Comp Biochem Physiol C Toxicol Pharmacol* (2009) **150**(2):101–6. doi:10.1016/j.cbpc.2009.03.007
24. Saïd L, Banni M, Kerkeni A, Saïd K, Messaoudi I. Influence of combined treatment with zinc and selenium on cadmium induced testicular pathophysiology in rat. *Food Cheml Toxicol* (2010) **48**(10):2759–65. doi:10.1016/j.fct.2010.07.003
25. Nayernia K, Adham IM, Burkhardt-Göttges E, Neesen J, Rieche M, Wolf S, et al. Asthenozoospermia in mice with targeted deletion of the sperm mitochondrion-associated cysteine-rich protein (Smcp) gene. *Mol Cell Biol* (2002) **22**:3046–52. doi:10.1128/MCB.22.9.3046-3052.2002
26. Puglisi R, Tramer F, Carlomagno G, Gandini L, Panfili E, Stefanini M, et al. PHGPx in spermatogenesis: how many functions? *Contraception* (2005) **72**:291–3. doi:10.1016/j.contraception.2005.03.002
27. Borchert A, Wang CC, Ufer C, Schiebel H, Savaskan NE, Kuhn H. The role of phospholipid hydroperoxide glutathione peroxidase isoforms in murine embryogenesis. *J Biol Chem* (2006) **281**(28):19655–64. doi:10.1074/jbc.M601195200
28. Tramer F, Micali F, Sandri G, Bertoni A, Lenzi A, Gandini L, et al. Enzymatic and immunochemical evaluation of phospholipid hydroperoxide glutathione peroxidase (PHGPx) in testes and epididymal spermatozoa of rats of different ages. *Int J Androl* (2002) **25**:72–83. doi:10.1046/j.1365-2605.2002.00327.x
29. Boitani C, Puglisi R. Selenium, a key element in spermatogenesis and male fertility. In: Chang CY editor. *Advances in Experimental Medicine and Biology*. (Vol. 636), New York: Landes Bioscience and Springer Science + Business Media (2008). p. 65–73.
30. Maiorino M, Wissing JB, Brigelius-Flohe' R, Calabrese F, Roveri A, Steinert P, et al. Testosterone mediates expression of the selenoprotein PHGPx by induction of spermatogenesis and not by direct transcriptional gene activation. *FASEB J* (1998) **12**(13):1359–70.
31. Guthrie HD, Welch GR. Effects of reactive oxygen species on sperm function. *Theriogenology* (2012) **78**(8):1700–8. doi:10.1016/j.theriogenology.2012.05.002
32. Guerriero G, Ciarcia G. Biomarkers of stress and reproduction in fish. In: Reinecke M, Zaccone G, Kapoor BG editors. *Fish Endocrinology*. (Vol. 2), Enfield, NH: Sci Publ Inc (2006). p. 665–92.

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Spermatogenesis and cryptorchidism

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Cryptorchidism represents the most common endocrine disease in boys, with infertility more frequently observed in bilateral forms. It is also known that undescended testes, if untreated, lead to an increased risk of testicular tumors, usually seminomas, arising from mutant germ cells. In normal testes, germ cell development is an active process starting in the first months of life when the neonatal gonocytes transform into adult dark (AD) spermatogonia. These cells are now thought to be the stem cells useful to support spermatogenesis. Several researches suggest that AD spermatogonia form between 3 and 9 months of age. Not all the neonatal gonocytes transform into AD spermatogonia; indeed, the residual gonocytes undergo involution by apoptosis. In the undescended testes, these transformations are inhibited leading to a deficient pool of stem cells for post pubertal spermatogenesis. Early surgical intervention in infancy may allow the normal development of stem cells for spermatogenesis. Moreover, it is very interesting to note that intra-tubular carcinoma *in situ* in the second and third decades have enzymatic markers similar to neonatal gonocytes suggesting that these cells fail transformation into AD spermatogonia and likely generate testicular cancer (TC) in cryptorchid men. Orchidopexy between 6 and 12 months of age is recommended to maximize the future fertility potential and decrease the TC risk in adulthood.

Keywords: cryptorchidism, undescended testes, spermatogenesis, germ cells, testicular cancer, orchidopexy

INTRODUCTION

Undescended testis or cryptorchidism is the most common genital abnormality in boys. The prevalence of cryptorchidism in full-term newborns range between 1 and 3%, reaching 30% in prematures (1–3). The pathology is bilateral in about 20% of the cases. About 80% of undescended testes are palpable and 20% are non-palpable (3–5). Palpable undescended testes are located along the inguino-scrotal region. Non-palpable testes may fall into one of the following categories: intra-abdominal location, agenesis, intrauterine demise, or inguinal location caused by dysplasia or atrophy. It is important to differentiate the true cryptorchidism from the retractile testis, which is a normal finding and usually it does not require surgical treatment. Acquired cryptorchidism has been observed when the retractile testis ascent in the inguinal canal during the infancy (ascending testis).

The main risk factors for the cryptorchid testis are infertility and testicular cancer (TC).

The risk of infertility in adulthood is more significant in patients with bilateral undescended testes (6). Approximately 10% of the infertile men have a history of cryptorchidism and orchidopexy (7). Azoospermia is evident in 13% of unilateral cryptorchidism and increase to 89% in untreated bilateral cryptorchid patients (8), although boys with one undescended testis have a lower fertility rate, they have the same paternity rate as boys with bilateral descended testes. Boys with bilateral undescended testes have a lower fertility and a paternity rate (9). In some studies, patients with unilateral cryptorchidism had normal spermatogenesis, suggesting that additive detrimental factors may be responsible for impaired fertility. The studied mechanisms of the infertility in cryptorchidism are multiple (7). The hyperthermia, between 35 and 37°C rather than 33°C, evoked by the abnormal position of the testis may respond for the impaired spermatogenesis. Anatomical congenital anomalies associated to undescended testis as testis–epididymis disjunction or iatrogenic lesions of vas and testis during orchidopexy may also contribute to infertility. Retrospective studies in infertile patients with history of cryptorchidism have demonstrated an increased incidence of anti-sperm antibodies which is more evident in pubertal age (1, 8). Sinisi et al. showed that cryptorchidism may elicit an autoimmune response against sperm antigens in childhood independent of testis location and orchidopexy (1).

It is known that undescended testes, if untreated, lead to an increased risk of TC, usually seminomas (10), arising from mutant germ cells. TC is a solid neoplasm that has an incidence of 1% of all cancers in men and is the most common between 20 and 30 years of life (11, 12). Boys with an undescended testis have a 20-fold higher risk to develop a TC and about 10% of the cases of TC develop in men with a history of cryptorchidism (13).

In this review, we focus on the current knowledge about the abnormal germ cell development in the undescended testes and its possible relationship with the impaired spermatogenesis and TC in adulthood. In the second section of this review, we discuss the treatment of cryptorchidism and the possible role of the early orchidopexy in the prevention of both infertility and cancer.

GERM CELL DEVELOPMENT, INFERTILITY, AND TESTICULAR CANCER IN CRYPTORCHIDISM

The germ cell development and its modification in cryptorchidism have been recently matter of many researches (2, 14, 15).

Spermatogenesis is the process by which sperm cells are produced. In men, it starts at puberty, resulting from the increased levels of gonadotropins and testosterone. It is a complex process including sequential steps of mitosis, meiosis, and differentiation. In each of these steps, endocrine, paracrine, and autocrine factors are involved (16). Spermatogenesis takes place in the seminiferous tubule: here germ cells are organized from the base of the tubule to the lumen and progressively develop from spermatogonia to spermatids. In the last step, spermatids differentiate through morphological transformation into spermatozoa (spermiogenesis) (17) which are finally released from the Sertoli cells into the lumen of the seminiferous tubule (spermiation).

However, germ cell development is an active process. It starts during the first years after birth when neonatal gonocytes change into adult dark (AD) spermatogonia. These are stem cells and have a dark nucleus that specifically characterize them from the other germ cells. Therefore, AD spermatogonia do not directly take part to sperm production; nevertheless, they ensure a supply of stem cells for spermatogenesis. Indeed, AD spermatogonia replicate to produce adult pale (AP) spermatogonia, with light nuclei. These cells produce by mitosis the type B spermatogonia which further divide and differentiate into primary spermatocytes which are already evident in the testes of children 4 years of age (2, 18). Two sequential meiotic divisions and spermiogenesis lead to final development of round spermatids and spermatozoa, respectively (19).

Several data suggest that AD spermatogonia form between 3 and 9 months of age. This developmental cycle needs normal testicular hormones and the optimal scrotal temperature of 33°C (20, 21). The hormonal regulation of these changes is not fully understood, with evidence for a possible role of gonadotropins and androgens. Not all the neonatal gonocytes transform into AD spermatogonia. The remaining gonocytes undergo involution by apoptosis. Genetic aberrations and environmental conditions influence these processes.

The failure of transformation of gonocytes into AD spermatogonia may produce infertility in boys.

Hadziselimovic and Herzog (15) have demonstrated that the process of transformation of neonatal gonocytes into AD spermatogonia during the first year of life is crucial for male fertility. The inhibition of this process in undescended testis leads to a deficient pool of stem cells for post pubertal spermatogenesis and infertility. Moreover, in undescended testes, germ cells loss starts at 6 months of age. Testicular biopsies at time of orchidopexy confirmed the importance of AD spermatogonia for fertility in cryptorchid patients. Tasian and coworkers (22) observed greater germ cell depletion in abdominal testes compared with palpable testes and a progressive germ cell loss for each month the testes remain undescended.

It is very interesting to note that the intra-tubular carcinoma *in situ* (CIS) in the second and third decade has enzyme markers similar to neonatal gonocytes as placental alkaline phosphatase expression, suggesting that these cells, that fail to develop in AD spermatogonia at 3–9 months of age, are the origin of cancer in cryptorchid men (23). Studies have suggested that the precursor cells of testis cancer, testicular CIS, are similar to fetal gonocytes. A current hypothesis (2) is that, due to the high temperature anomaly of undescended testis, an abnormal apoptosis allows some gonocytes to persist and become CIS with progressive mutation and/or cellular unbalance, and eventually malignancy in adulthood. These abnormal gonocytes are kept in a defined environment "suspended animation" in the germ-line and, due to the accumulation of mutations, may undergo transformation becoming the source of the CIS (2, 21, 24).

The etiology accepted for germ cell carcinoma remains unknown, although disturbances in the microenvironment provided by the Sertoli and Leydig cells may play an important role. In fact, spermatogenesis is strictly controlled and depends on a succession of signals supplied from the local environment (11, 25, 26) and Leydig cells, next to their steroidogenic function, during development express the insulin-like-3 gene (INSL3), which is responsible for gubernaculum maturation and testicular descent (27). A specific association of mutations in INSL3 with cryptorchidism has been described but its possible role in TC development and infertility needs to be clarified (28).

Olesen et al. linked the development of TC not only with cryptorchidism but also with other urogenital anomalies such as hypospadia (29). In fact, epidemiological studies in males who presented fertility problems tend to lean toward an enhanced risk of testicular germ cell tumor (30). The development of TC is associated with many chromosomal abnormalities and this raises the problem for close monitoring of these patients. Kanetsky et al. (31) demonstrated common genetic variants associated to an increased risk of testicular germ cell cancer (TGCC) and found that seven markers at 12p22 within KITLG (c-KIT ligand) reached genome-wide significance. This gene has been involved in several aspects of primordial germ cell development, migration, and survival (32).

Concerning the development of the urogenital sinus and particularly the testis, the impacts of endocrine disruptors have been fairly well described on human and experimental models (33–35). This is especially true for hypospadia, cryptorchidism, and infertility; but the link with TGCC has to be explained. The unbalanced equilibrium between the estrogen and androgen levels *in utero* is hypothesized to influence the risk of TC. Thus, mutations in testosterone gene expression may change the level of testosterone *in vivo* and hypothetically the risk of developing TC (36).

As discussed before, hormonal regulation is very significant in the development of the germ-line. Beside the importance of fetal development, it seems that puberty should be an important moment, when hormone levels reach optimal concentrations for the secondary sex characters development. It has been shown that sperm agglutinating antibodies appear in young boys with cryptorchidism and they are more prevalent during puberty (1). This also coincides with the appearance of TGCC, as men affected are between 15 and 35 years old, suggesting that puberty and probably the increase in hormone concentrations should be central issues (37).

HORMONAL AND SURGICAL TREATMENT OF CRYPTORCHIDISM

The goals of treatment of cryptorchidism are mainly two: preserve fertility and reduce the risk of neoplastic disease. Last but not the least, treatment allows the testicular self-examination for an early diagnosis and detection of TC.

Hormonal treatment with human chorionic gonadotropin (hCG) or gonadotropin-releasing hormone (GnRH) may be initially administered for cryptorchidism because it should promote the testicular descent (38). The theoretical basis for its use is to stimulate the Leydig cells to produce testosterone, inducing inguinal–scrotal testicular descent. Potential harmful effects of hormonal treatment on the developing testes, including apoptosis, inflammation, and reduced number of germ cells are still under study. In addition, there are reports which suggest that the hormonal stimulation in infancy may be damaging to the testes (39). It has been observed, in hCG-treated rats, a poor differentiation of the seminiferous epithelium, with high Leydig cell evidence and increased inter-tubular eosinophilic material (40). These experimental data emphasize the possible negative outcome of hormone therapy on germ cell line and its main action on Leydig cells. The increased synthesis of vascular endothelial growth factor (VEGF), determined by hCG therapy also highlights the increased cell permeability causing interstitial edema. The role of VEGF on spermatogenesis is unclear. Several findings have revealed several inhibitory effects of VEGF on spermatogenesis (40, 41).

Considering the poor efficacy and the possible adverse effects of hormonal therapy, surgery must be preferred (42).

Orchidopexy is the cornerstone of cryptorchidism treatment. Inguinal operation is the standard approach for palpable testis. Laparoscopy is the gold standard technique for both diagnosis and treatment of non-palpable testes (3–5). Early surgical treatment may preserve fertility. Orchidopexy is commonly performed before 2 years of age and increasing research suggest that surgery before 1 year of age may permit the normal spermatogenesis by preventing degenerative changes of the testes and germ cell loss (22, 43). However, early orchidopexy does not guarantee normal fertility in adulthood. Hadziselimovic showed that despite orchidopexy before 6 months of age, up to 35% of boys will grow up to be infertile regardless of the normal total germ cell count on testicular biopsies performed at the time of orchidopexy (44). The current practice for the acquired cryptorchidism is to operate at diagnosis, by a scrotal approach, although the prognosis seems to be better than congenital cryptorchidism considering the normal development and apoptosis of the germinal cells during the first year of life.

Since the link between cryptorchidism and TC seems to be an abnormal development of the primary germ-line, any attempt to normalize this process, as early surgery, will permit a normal growth of germ cells, thereby avoiding cancer as well as oligospermia or azoospermia. However, it should be mentioned that some studies failed to demonstrate a correlation between the time of surgery and cancer risk (45). A systematic review and meta-analysis of the literature by an American group has concluded that prepubertal orchiopexy may decrease the risk of malignancy and that early surgical intervention is indicated in children with cryptorchidism leading to a better growth of the testis (46).

CONCLUSION

Cryptorchidism is a risk factor for infertility and TC in adulthood. To date, orchidopexy is recommended between 6 and 12 months of age. The aim of an early surgical intervention is to prevent the abnormal germ cell development and ultimately decrease the risk of infertility and malignancy in adulthood.

REFERENCES

1. Sinisi A, Pasquali D, Papparella A, Valente A, Orio F, Esposito D, et al. Antisperm antibodies in cryptorchidism before and after surgery. *J Urol* (1998) **160**:1834–7. doi:10.1016/S0022-5347(01)62428-5
2. Hutson JM. Undescended testis: the underlying mechanism and the effects on germ cells that cause infertility and cancer. *J Pediatr Surg* (2013) **48**:903–8. doi:10.1016/j.jpedsurg.2013.02.001
3. Papparella A, Romano M, Noviello C, Cobellis G, Nino F, Del Monaco C, et al. The value of laparoscopy in the management of non-palpable testis. *J Pediatr Urol* (2010) **6**:550–4. doi:10.1016/j.jpurol.2009.12.010
4. Papparella A, Parmeggiani P, Cobellis G, Mastroianni L, Stranieri G, Pappalepore N, et al. Laparoscopic management of nonpalpable testes: a multicenter study of the Italian society of videosurgery in infancy. *J Pediatr Surg* (2005) **40**:696–700. doi:10.1016/j.jpedsurg.2005.01.010
5. Papparella A, Zamparelli M, Cobellis G, Amici G, Saggiomo G, Parmeggiani P, et al. Laparoscopy for nonpalpable testis: is inguinal exploration always necessary when the cord structures enter the inguinal ring. *Pediatr Endosurg Innov Tech* (1999) **3**:29–33. doi:10.1089/pei.1999.3.29
6. Chung E, Brock GB. Cryptorchidism and its impact on male fertility: a state of art review of current literature. *Can Urol Assoc J* (2011) **5**(3):2010–4. doi:10.5489/cuaj.1010
7. Robin G, Boitrelle F, Marcelli F, Colin P, Leroy-Martin B, Mitchell V, et al. Cryptorchidism: from physiopathology to infertility. *Gynecol Obstet Fertil* (2010) **38**(10):588–99. doi:10.1016/j.gyobfe.2010.08.015
8. Urry RL, Carrel DT, Starr NT, Snow BW, Middleton RG. The incidence of antisperm antibodies in infertility patients with a history of cryptorchidism. *J Urol* (1994) **151**:381–3.
9. Tekgul S, Riedmiller H, Gerharz E, Hoebeke P, Kocvara R, Nijman R, et al. *The Cryptorchidism: Guidelines on Pediatric Urology*. Arnhem: European Association of Urology, European Society for Paediatric Urology (2009). p. 8–11.
10. Ferguson L, Agoulnik AI. Testicular cancer and cryptorchidism. *Front Endocrinol (Lausanne)* (2013) **4**:32. doi:10.3389/fendo.2013.00032
11. Vega A, Baptissart M, Caira F, Brugnon F, Lobaccaro JMA, Volle DH. Epigenetic: a molecular link between testicular cancer and environmental exposures. *Front Endocrinol (Lausanne)* (2012) **3**:150. doi:10.3389/fendo.2012.00150
12. Ziglioli F, Maestroni U, Dinale F, Ciuffreda M, Cortellini P. Carcinoma in situ (CIS) of the testis. *Acta Biomed* (2011) **82**(2):162–9.
13. Mannuel HD, Mitikiri N, Khan M, Hussain A. Testicular germ cell tumors: biology and clinical update. *Curr Opin Oncol* (2012) **24**:266–71. doi:10.1097/CCO.0b013e32835167fc
14. Hadziselimovic F, Hocht B, Herzog B, Buser MW. Infertility in cryptorchidism is linked to the stage of germ cell development at orchidopexy. *Horm Res* (2007) **68**(1):46–52. doi:10.1159/000100874
15. Hadziselimovic F, Herzog B. The importance of both an early orchidopexy and germ cell maturation for fertility. *Lancet* (2001) **358**:1156–7. doi:10.1016/S0140-6736(01)06274-2
16. Pierantoni R, Cobellis G, Meccariello R, Fasano S. Evolutionary aspects of cellular communication in the vertebrate hypothalamo-hypophysio-gonadal axis. *Int Rev Cytol* (2002) **218**:69–141. doi:10.1016/S0074-7696(02)18012-0
17. Cacciola G, Chioccarelli T, Fasano S, Pierantoni R, Cobellis G. Estrogens and spermiogenesis: new insight from type 1 cannabinoid receptor knockout mice. *Int J Endocrinol* (2013) **2013**:501350. doi:10.1155/2013/501350
18. Yuasa J, Ito H, Toyama Y, Yuasa S, Masai M. Postnatal development of the testis in Japanese children based on observations of undescended testes. *Int J Urol* (2001) **8**(9):490–4. doi:10.1046/j.1442-2042.2001.00357.x
19. de Kretser DM, Loveland KL, Meinhardt A, Simorangkir D, Wreford N. Spermatogenesis. *Hum Reprod* (1998) **13**(Suppl 1):1–8. doi:10.1093/humrep/13.suppl_1.1
20. Hadziselimovic F, Thommen L, Girard J. The significance of postnatal gonadotropin surge for testicular development in normal and cryptorchid testes. *J Urol* (1986) **136**:274–6.

21. Ong C, Hasthorpe S, Hutson JM. Germ cell development in the descended and cryptorchid testis and the effects of hormonal manipulation. *Pediatr Surg Int* (2005) **21**:240–54. doi:10.1007/s00383-005-1382-0
22. Tasian GE, Hittelman AB, Kim GE, Disandro MJ, Baskin LS. Age at orchiopexy and testis palpability predict germ and Leydig cell loss: clinical predictors of adverse histological features of cryptorchidism. *J Urol* (2009) **182**:704–9. doi:10.1016/j.juro.2009.04.032
23. Jørgensen N, Rajpert-De Meyts E, Graem N, Müller J, Giwercman A, Skakkebaek NE. Expression of immunohistochemical markers for testicular carcinoma in situ by normal human fetal germ cells. *Lab Invest* (1995) **72**(2):223.
24. Hutson JM, Balic A, Nation T, Southwell B. Cryptorchidism. *Semin Pediatr Surg* (2010) **19**:215–24. doi:10.1053/j.sempedsurg.2010.04.001
25. Skinner MK, Norton JN, Mullaney BP, Rosselli M, Whaley PD, Anthony CT. Cell interactions and the regulation of testis function. *Ann N Y Acad Sci* (1991) **637**:354–63. doi:10.1111/j.1749-6632.1991.tb27322.x
26. Verhoeven G. Local control systems within the testis. *Baillieres Clin Endocrinol Metab* (1992) **6**:313–33. doi:10.1016/S0950-351X(05)80152-1
27. Ivell R, Anand-Ivell R. Biological role and clinical significance of insulin-like peptide 3. *Curr Opin Endocrinol Diabetes Obes* (2011) **18**:210–6. doi:10.1097/MED.0b013e3283453fe6
28. Ferlin A, Bogatcheva NV, Gianesello L, Pepe A, Vinanzi C, Agoulnik AI, et al. Insulin-like factor 3 gene mutations in testicular dysgenesis syndrome: clinical and functional characterization. *Mol Hum Reprod* (2006) **12**:401–6. doi:10.1096/Molehr/gal043
29. Olesen IA, Sonne SB, Hoei-Hansen CE, Rajpert-De Meyts E, Skakkebaek NE. Environment, testicular dysgenesis and carcinoma in situ testis. *Best Pract Res Clin Endocrinol Metab* (2007) **21**:462–78. doi:10.1016/j.beem.2007.04.002
30. Burns WR, Sabanegh E, Dada R, Rein B, Agarwal A. Is male infertility a forerunner to cancer? *Int Braz J Urol* (2010) **36**:527–36. doi:10.1590/S1677-55382010000500002
31. Kanetsky PA, Mitra N, Vardhanabhuti S, Li M, Vaughn DJ, Letrero R, et al. Common variation in KITLG and at5q31.3 predisposes to testicular germ cell cancer. *Nat Genet* (2009) **41**:811–5. doi:10.1038/ng.393
32. Gu Y, Runyan C, Shoemaker A, Surani A, Wylie C. Steel factor controls primordial germ cell survival and motility from the time of their specification in the allantois, and provides a continuous niche throughout their migration. *Development* (2009) **136**:1295–303. doi:10.1242/dev.030619
33. Volle DH, Decourteix M, Garo E, McNeilly J, Fenichel P, Auwerx J, et al. The orphan nuclear receptor small heterodimer partner mediates male infertility induced by diethylstilbestrol in mice. *J Clin Invest* (2009) **119**:3752–64. doi:10.1172/JCI38521
34. Toppari J, Virtanen HE, Main KM, Skakkebaek NE. Cryptorchidism and hypospadias as a sign of testicular dysgenesis syndrome (TDS): environmental connection. *Birth Defects Res A Clin Mol Teratol* (2010) **88**(10):910–9. doi:10.1002/bdra.20707
35. Desdoits-Lethimonier C, Albert O, Le Bizec B, Perdu E, Zalko D, Courant F, et al. Human testis steroidogenesis is inhibited by phthalates. *Hum Reprod* (2012) **27**(5):1451–9. doi:10.1093/humrep/des069
36. Kristiansen W, Aschim EL, Andersen JM, Witczak O, Fosså SD, Haugen TB. Variations in testosterone pathway genes and susceptibility to testicular cancer in Norwegian men. *Int J Androl* (2012) **35**(6):819–27. doi:10.1111/j.1365-2605.2012.01297.x
37. Jacobsen GK, Henriques UV. A fetal testis with intratubular germ cell neoplasia (ITGCN). *Mod Pathol* (1992) **5**:547–9.
38. Papparella A, Coppola S, Nino F, Andrade Barrientos M, Gasparini N. Epidemiology and treatment of cryptorchidism and retractile testis. *Minerva Pediatr* (2013) **65**(1):77–82.
39. Cortes D, Thorup J, Visfeldt J. Hormonal treatment may harm the germ cells in 1 to 3-year-old boys with cryptorchidism. *J Urol* (2000) **163**:1290–2. doi:10.1016/S0022-5347(05)67763-4
40. Papparella A, Nino F, Noviello C, Romano M, Papparella S, Paciello O, et al. Morphologic changes due to human chorionic gonadotropin in the rat testis: role of vascular endothelial growth factor. *Open J Pediatr* (2013) **3**:85–91. doi:10.4236/ojped.2013.32016
41. Nalbandian A, Dettin L, Dym M, Ravindranath N. Expression of vascular endothelial growth factor receptors during male germ cell differentiation in the mouse. *Biol Reprod* (2003) **69**:985–94. doi:10.1095/biolreprod.102.013581
42. Ritzèn EM. Undescended testes: a consensus on management. *Eur J Endocrinol* (2008) **159**:S87–90. doi:10.1530/EJE-08-0181
43. Feyles F, Peiretti V, Mussa A, Manenti M, Canavese F, Cortese MG, et al. Improved sperm count and motility in young men surgically treated for cryptorchidism in the first year of life. *Eur J Pediatr Surg* (2013). doi:10.1055/s-0033-1349715
44. Hadziselimovic F. Early successful orchidopexy does not prevent from developing azoospermia. *Int Braz J Urol* (2007) **32**(5):570–3. doi:10.1590/S1677-55382006000500012
45. Hack WW, Sijstermans K, Van Der Voort-Doedens LM. Correction of cryptorchidism and testicular cancer. *N Engl J Med* (2007) **357**:825–7. doi:10.1056/NEJMc071510
46. Walsh TJ, Dall'Era MA, Croughan MS, Carroll PR, Turek PJ. Prepubertal orchiopexy for cryptorchidism may be associated with lower risk of testicular cancer. *J Urol* (2007) **178**(Pt 1):1440–6. doi:10.1016/j.juro.2007.05.166

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Intra-testicular signals regulate germ cell progression and production of qualitatively mature spermatozoa in vertebrates

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Spermatogenesis, a highly conserved process in vertebrates, is mainly under the hypothalamic–pituitary control, being regulated by the secretion of pituitary gonadotropins, follicle stimulating hormone, and luteinizing hormone, in response to stimulation exerted by gonadotropin releasing hormone from hypothalamic neurons. At testicular level, gonadotropins bind specific receptors located on the somatic cells regulating the production of steroids and factors necessary to ensure a correct spermatogenesis. Indeed, besides the endocrine route, a complex network of cell-to-cell communications regulates germ cell progression, and a combination of endocrine and intra-gonadal signals sustains the production of high quality mature spermatozoa. In this review, we focus on the recent advances in the area of the intra-gonadal signals supporting sperm development.

Keywords: testis, spermatogenesis, GnRH, kisspeptins, estrogens, sperm quality, spermatozoa

INTRODUCTION

In vertebrates, spermatogenesis is a hormonally controlled mechanism charged to produce gametes useful for reproduction. The production of high standard quality gametes is the main goal to preserve reproduction.

Spermatogenesis develops as a process consisting of mitotic, meiotic, and differentiation steps promoting germ cell progression from spermatogonia-to-spermatozoa (SPZ). In male, the hypothalamus–pituitary–gonadal axis supports germ cell progression, via gonadotropin releasing hormone (GnRH)–gonadotropin–steroid production and its activity is finely regulated by positive and negative feedbacks. Furthermore, a network of intra-gonadal factors, organized in a complex stage-specific multi-factorial net, is responsible for spermatogenesis control (1).

Using a comparative approach, this review summarizes the intriguing and sometimes conflicting information about the intra-testicular role played by GnRH, Kisspeptin, and estrogens in germ cell progression and production of high standard quality sperm.

GnRH, A HISTORICAL MODULATOR OF TESTIS PHYSIOLOGY

The GnRH, crucial player of the neural control of vertebrate reproduction, was originally isolated from the hypothalamus of pig and sheep (2). Basically, GnRH stimulates the synthesis and the discharge of pituitary gonadotropins [follicle stimulating hormone and luteinizing hormone (FSH and LH), respectively], which in turn induce both gametogenesis and the production of gonadal steroids. At present, 25 GnRH forms have been identified in protochordates and vertebrates (3, 4) and in many vertebrates three GnRH molecular forms have been identified: GnRH-1, GnRH-2, and GnRH-3 (formerly known as mammalian, chicken-II, and salmon GnRH, respectively) (3). GnRH action is mediated through high-affinity binding with the GnRH receptor (GnRH-R) (5, 6), a rhodopsin-like seven trans-membrane G protein-coupled receptor (GPCR). In vertebrates, GnRH-Rs exhibit a wide range of subtypes and alternate splicing derived forms (1, 3, 5–7). The presence of multiple forms of GnRHs and GnRH-Rs in the brain, with specific expression profiles, suggests the existence of different functional roles: in fact, GnRH-1 is considered the final regulator of the pituitary–gonadal axis; GnRH-2 is supposed to play a function for the control of sexual behavior, food intake, energy balance, stress, and many other environmental cues; GnRH-3, found only in the telencephalon of teleost fish, probably acts as neuro-modulator (1, 3, 8).

Extrahypothalamic synthesis and function of GnRHs and GnRH-Rs have been detected in many reproductive tissues in vertebrates, including human (gonads, prostate, endometrial tissue, oviduct, placenta), and in cancer cells (1, 5, 9–11).

GnRH plays several conserved roles in testis physiology, being the main paracrine modulator of the Leydig–Sertoli, Sertoli–germ cell, Sertoli–peritubular cell communications (1, 12). In this context, it drives steroidogenesis, germ cells progression, and acquisition of SPZ functions (1, 12–15).

The demonstration of a direct GnRH effect on testis has been provided in fish, frog, rodent, and human Leydig cells showing GnRH-specific high-affinity binding sites (1, 3, 15, 16). The finding of *GnRH* mRNA in Sertoli and spermatogenic cells in different species (17) suggests its involvement in paracrine Leydig–Sertoli cell communication (12). A similar pattern of expression has been confirmed in human (17), expressing two GnRH molecular forms and two GnRH-Rs (18, 19). However, the identification

of *GnRH-R2* antisense transcript in human testis (20) and the presence of frame-shift mutations and stop codons in human *GnRH-R2* (5) may indicate that these transcripts are not really functional.

The major reported effect of GnRH on vertebrate testis physiology concerns the modulation of steroidogenesis in *in vivo* and *in vitro* systems (1, 21–23). Interestingly, in elasmobranch and in dipnoi, this effect appears to be exerted trough the endocrine route (24, 25). Both GnRH-1 and GnRH-2 agonists have the ability to stimulate mouse pre-pubertal Leydig cell steroidogenesis, in a dose- and time-dependent manner, via transcriptional activation of 3β-hydroxy-steroid dehydrogenase (3β-HSD) (23). Accordingly, in human, the expression levels of *GnRH-1*, *GnRH-2*, *GnRH-Rs*, *cytochrome P450 side-chain cleavage* (*CYP11A1*), *3β-HSD type 2* enzyme, and the intra-testicular testosterone (T) levels are significantly increased in patients with spermatogenic failure (26). At molecular level, the transduction pathway involving the GnRH agonist-dependent activation of ERK1/2 has been reported (27). Interestingly, in mouse testis, GnRH-R activity well correlates with the increased steroidogenic activity observed during pubertal and adult stages and its decline parallels the decreased steroidogenic activity observed during the senescence (28). These expression profiles are consistent with the increasing expression of the gonadotropin inhibitory hormone (GnIH) during senescence, providing evidences of local interaction between GnRH and GnIH. The testicular localization of GnIH and its receptor GPR147, in both mammals and birds, opens new perspectives in the autocrine/paracrine control of testicular activity suggesting a possible interplay between GnRH and GnIH in order to modulate T secretion and spermatogenesis (29). Furthermore, GnRH activity in Leydig cells is not restricted to T production but is extended to the development of rat progenitor Leydig cells both *in vivo* and *in vitro* (30).

Several studies, carried out in cancer cell lines, demonstrated a direct anti-proliferative/apoptotic effect of GnRH and its synthetic agonists (31, 32). Accordingly, GnRH activity is a well-known modulator of germ cell apoptosis during the regression of fish gonad (33, 34). In rodents, GnRH agonists stimulate spermatogenic colony formation following spermatogonia (SPG) transplantation (35, 36) and induce SPG proliferation in damaged testis (37). In mollusk, a scallop GnRH-like peptide stimulates SPG cell division (38). In amphibian, a GnRH agonist induces G1-S transition of SPG cell cycle (39–43) whereas, in mouse, GnRH is expressed in gonocytes at birth (28). At molecular level, in the anuran amphibian *Rana esculenta*, SPG proliferation requires the cooperation between estradiol (E_2) and GnRH, in a mechanism involving the E_2-dependent transcriptional activation of *c-fos* (42) and a GnRH-mediated translocation of FOS protein from the SPG cytoplasm into the nucleus (43). Thus, GnRH activity may represent a key controller of proliferative/anti-proliferative events characteristic of testis renewal. Consistently, it has been found that GnRH induces proliferation of partially differentiated gonadotrope cells (44).

Lastly, the ability of GnRH agonists to induce spermiation (45) and the localization of GnRH and/or GnRH-Rs in spermatids (SPTs) and SPZ in mammalian and non-mammalian vertebrates (17, 28, 46, 47) suggest the involvement of GnRH signaling in SPZ functions and fertilization. Accordingly, GnRH antagonists inhibit, *in vivo* and *in vitro*, fertilization in rodents (14) whereas sperm binding to the human zona pellucida and calcium influx in response to GnRH and progesterone have been reported (13), providing evidence of functional role of GnRH-Rs in human SPZ.

The above indicated intra-testicular activity of GnRH has been described in detail in the frog *R. esculenta*, a species showing a complex GnRH system, deeply characterized at testicular level (46). In this seasonal breeder, two GnRH molecular forms (GnRH-1 and GnRH-2) and three receptor forms (GnRH-R1, -R2, -R3) (48) with specific expression pattern and localization in testis during the annual reproductive cycle (46) have been identified. *In situ* hybridization suggests a different role for *GnRH-1* and *GnRH-2*, as *GnRH-1* and *GnRH-R1* seem to be linked to germ cell progression and interstitial compartment activity, whereas *GnRH-2* and *GnRH-R2* seem to be linked to sperm function and release (46), confirming the hypothesis that each ligand might be involved in the modulation of specific processes. Interestingly, this functional portioning well correlates with the differential modulation of GnRH system counterparts exerted via the activation of endocannabinoid system, an evolutionarily conserved system deeply involved in central and local control of reproductive functions (49–52). At central level, in mammals, endocannabinoids interfere with GnRH production (53, 54) and signaling (55). In frog diencephalons, they modulate the expression of *GnRH-1*/*GnRH-2* (48, 56, 57) – both hypophysiotropic factors (1), *GnRH-R1* and *GnRH-R2* (48) (**Figure 1**). Furthermore, in frog testis, the endocannabinoid anandamide (AEA), via type 1 cannabinoid receptor (CB1) activation, modulates testicular GnRH activity at multiple levels and in a stage-dependent manner (46) (**Figure 2**). Interestingly, the activation of cannabinoid receptors other than CB1, such as the vanilloid transient receptor type 1 (TRPV1), differentially modulates the expression level of *GnRHs*/*GnRH-Rs*, but in an opposite manner as compared with CB1 (58). Thus, the transcriptional switch on/off of testicular GnRH system is finely toned through the activation of specific endocannabinoid receptors, providing evidence of a central role of this system in the local modulation of GnRH activity.

KISSPEPTINS, POSSIBLE PLAYERS IN TESTIS PHYSIOLOGY CURRENTLY UNDER INVESTIGATION

Kisspeptins are a novel class of neuro-peptides with a key position in the scenario of reproduction. They are encoded by the *kiss1* gene, originally discovered as a metastasis-suppressor gene in 1996 (59), and they are initially produced as an unstable 145-amino acid precursor peptide (kp145), then cleaved into shorter peptides (kp-54, kp14, kp-13, and kp-10). Interestingly, all kisspeptin shorter peptides are biologically active due to the binding to the "kiss" receptor GPR54 (60). The primary targets of kisspeptins are just the hypothalamic GnRH-secreting neurons (61) and, similarly to the deletion/mutation of *GnRH* or *GnRH-R* genes, target disruption of both *kiss1* and *GPR54* leads to hypogonadotropic hypogonadism and lack of sexual maturation (62, 63). Accordingly, the administration of kisspeptins accelerates the timing of puberty onset in fish (64–67) and mammals (68, 69), whereas circulating higher kisspeptin levels have been observed in clinical cases of precocious puberty in human (70, 71).

Several studies have been focused on the characterization of the kisspeptin-dependent signaling in the hypothalamus, with particular concern to the negative and the positive feedback action of sex steroids on *kiss1* gene expression in the arcuate and the anteroventral-preoptic nucleus, respectively [for review see Ref. (72)]. Therefore, in the last years, the idea that kisspeptin signaling is an essential guardian angel of reproduction, through the regulation of GnRH neurons, took place. These views strongly stride with evidences that genetic ablation of nearly all kisspeptin neurons does not impair reproduction, suggesting that possible compensatory mechanisms rescue reproduction (73). Probably, kisspeptin neurons and related products are in excess of what is really required to support reproductive functions. In this respect, male and female mice with a 95% reduction in *kiss1* transcript levels are normal and sub-fertile, respectively. This suggests that an overproduction of kisspeptin represents a failsafe to guarantee reproductive success (74).

A novel chapter of kisspeptin saga concerns the possible intragonadal action of these molecules. Kiss1 and/or GPR54 have been observed in several peripheral tissues, gonads included. In particular, the presence of both ligand and receptor has been observed in the human placenta (75) and testis (60, 75) whereas *GPR54* alone has been detected in mouse (76), rat (77), rhesus monkey (78, 79), and frog (80) testis. However, the functional mechanisms of kisspeptin/GPR54 system in gonads remain to be elucidated and several conflicting data concerning the direct involvement of kisspeptin activity in testis physiology emerged.

Long term kisspeptin-10 (kp-10) (81) and/or kp-54 (82) administration in maturing and adult rat testes gives rise to degenerative effects on spermatogenesis and suppresses the circulating levels of LH and T; no effects have been registered upon FSH levels. Specifically, germ cell number significantly decreases, many germ cells appear regressed, atrophied, and in necrosis; round and elongated SPTs show abnormal acrosome; intraepithelial vacuolization is visible, interstitial spaces are enlarged, and the germinal epithelium is irregularly shaped. Leydig cells frequently lose contacts with the seminiferous tubules and Sertoli–germ cell interaction is destroyed (81). A similar degenerative effect – caused by continuous administration of kp-10 – has also been discovered in rat seminal vesicles (83) and pre-pubertal prostate gland (84). Conversely, a physiological role of kisspeptins in testis has been completely excluded in mouse (85) and conflicting data concerning the localization of kiss1/GPR54 protein and mRNA recently emerged. The use of different antisera, strategies, and strains as well might be taken in account to explain these discrepancies and the missing overlapping in mRNA/protein detection described

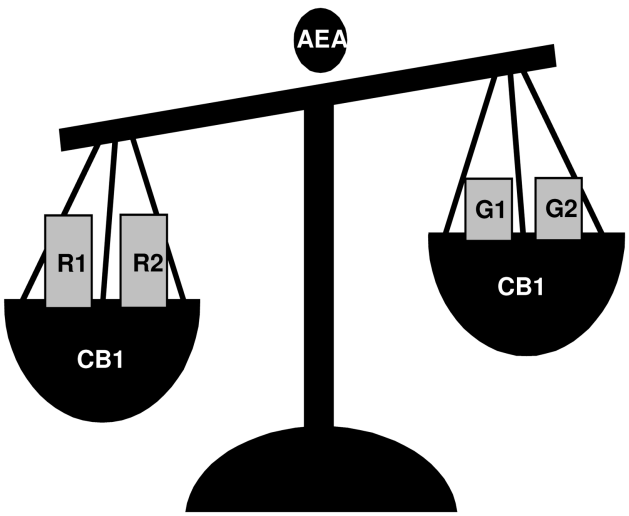

FIGURE 1 | A schematic view of the effects of AEA incubation on *GnRH-1* (G1), *GnRH-2* (G2), *GnRH-R1* (R1), and *GnRH-R2* (R2) expression in frog *R. esculenta* diencephalon. Animals were collected in June and testes were incubated *in vitro* for 1 h. Via CB1 activation, AEA treatment significantly increased the expression of *GnRH-R1* and *GnRH-R2* whereas it decreased the expression of both *GnRH-1* and *GnRH-2*; no effect on *GnRH-R3* was observed.

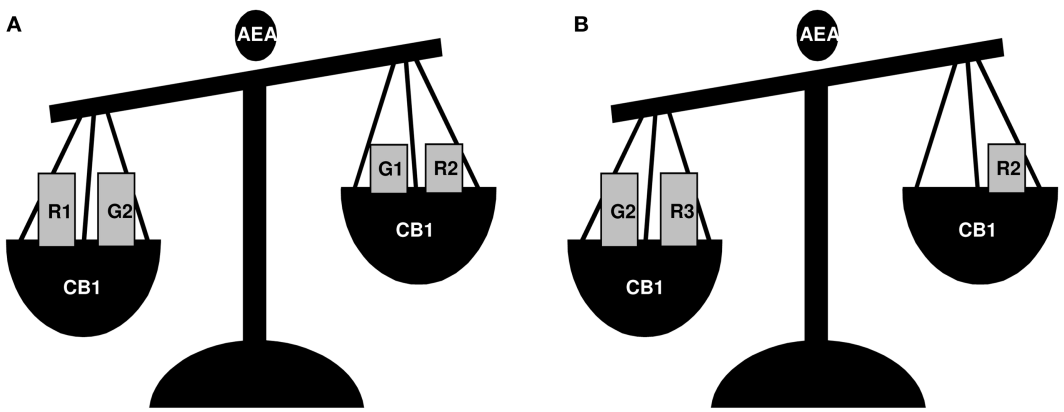

FIGURE 2 | A schematic view of the effects of AEA treatment on *GnRH-1* (G1), *GnRH-2* (G2), *GnRH-R1* (R1), *GnRH-R2* (R2), and *GnRH-R3* (R3) expression in frog *R. esculenta* testis. Animals were collected in June **(A)** and February **(B)** and testes were incubated *in vitro* for 1 h. In June, AEA treatment significantly increased the expression of *GnRH-R1* and *GnRH-2* whereas it decreased those of *GnRH-1* and *GnRH-R2*, and had no effect on *GnRH-R3*; in February, AEA treatment increased *GnRH-2* and *GnRH-R3* expression, decreased *GnRH-R2*, and had no effect on *GnRH-1* or *GnRH-R1*. In both periods, AEA-dependent effects occurred via CB1 activation.

so far. In fact, in transgenic mice with LacZ targeted to either *kiss1* or *GPR54* genes, *kiss1* and *GPR54* mRNA have been localized in mouse round SPTs, whereas kisspeptin protein has been shown in Leydig cells, with no staining in SPTs (85). Conversely, both GPR54 and kiss1 immunoreactivity has been provided in both Leydig and germ cells (primordial germ cells and elongating SPTs) with significant age-related variations (28). Studies conducted in Leydig cell line MA-10 – a cell line that expresses LH receptors and responds to human chorionic gonadotropin (hCG) stimulation, producing progesterone as major steroid hormone – confirm that these cells produced *GPR54* mRNA, but were unable to show any *kiss1* expression (85). Despite *GPR54* expression, from a functional point of view kp-10 does not exert any significant direct effects on steroid production in both MA-10 cell line or in physiological systems, such as mouse seminiferous tubule explants (85). However, evidences reported in other species examined so far, pointed out a possible role of kisspeptin system just in steroidogenetic activity. Although Leydig cells do not show any kisspeptin and/or GPR54 immuno-localization in rhesus monkey (78), intra-testicular action on steroidogenesis (79) has been demonstrated in monkeys treated with acyline, a GnRH-R antagonist (86), just to exclusively investigate kisspeptin activity without any influence of pituitary gonadotropic drive. In these clamped monkeys, kp-10 has a synergic effect with hCG to induce T production (79). Anyway, the real possible mechanism through which kisspeptin enhances T production in primates is not clear and may require additional paracrine routes involved in Leydig–Sertoli cell communications. In fact, in rhesus monkey kisspeptin immunoreactivity has been detected in spermatocytes (SPCs) and SPTs, whereas GPR54 has been localized in SPCs and Sertoli cells (78). Thus kisspeptin – produced by germ cells – might act in an autocrine/paracrine manner to control the progression of the spermatogenesis and/or to modulate Sertoli cells activity. It is noteworthy, however, that intravenous injection of the kisspeptin antagonist 234 (kp-234) (87) does not alter plasma T levels in adult rhesus monkey. Interestingly, Anjum and co-workers reported that kisspeptin expression – analyzed by slot blot analysis in Leydig cells of Parkes strain mice – significantly decreases from birth to pre-pubertal testis, increases during pubertal period, decreases in reproductive active mouse to further increase during the senescence. These expression profiles well correlate to GnIH expression and to the decreased steroidogenic activity observed during the senescence, providing evidence of a possible involvement of kisspeptin in the control of steroidogenesis in cooperation with testicular GnIH (28).

The detection of *kiss1* and *GPR54* mRNA in round/elongating SPTs (28, 78, 85) raises the possibility that autocrine or paracrine kisspeptin actions might be involved in spermiogenesis and in the acquisition of sperm functions, as recently demonstrated in human SPZ by Candenas and co-workers (88). This group immunolocalized kisspeptin and GPR54 in the post-acrosome region of the human SPZ head and in the equatorial segment of the tail, providing also evidence of some regulatory actions. In fact, 1 μM kp-13 increases $[Ca^{2+}]_i$ and induces a small, but significant change in sperm motility, leading to motility trajectories that characterize hyper-activated SPZ. Instead, the same treatment has no effect on acrosome reaction (88). Very recently, in mouse, GPR54 has been specifically localized in the acrosomal region of SPTs and mature SPZ whereas kisspeptin expression has been detected in the cumulus–oocyte complex and oviductal epithelium of ovarian and oviductal tissue (89). Since SPZ treatment with kp-234 decreases the *in vitro* fertilization rates, evidence emerged that kisspeptin modulates fertilization capability in mammals (89).

Interestingly, in sexually immature scombroid fish, kp-15 peripheral administration induced spermiation (67), accordingly to *GPR54* expression detected in the myoid peritubular cells in amphibians (80), indicating a possible involvement in sperm transport and release.

Compelling evidence about gonadal activity of kisspeptin system recently comes from a non-mammalian vertebrate, the anuran amphibian, the frog *R. esculenta*. In this seasonal breeder, germ cell progression is under the control of endocrine, environmental, and gonadal factors (90, 91), whereas spermatogenesis proceeds in cysts, typical formations consisting of Sertoli cells enveloping cluster of germ cells at a synchronous stage (91). During the frog annual sexual cycle, *GPR54* mRNA has been analyzed in testis, showing higher expression at the end of the winter stasis and during the breeding season (80). In these periods, in an E_2-dependent fashion, the recruitment of SPG and the onset of a new spermatogenetic wave take place (42, 91, 92). Consistently, in February, *GPR54* mRNA has been revealed in primary and secondary SPG by *in situ* hybridization (**Figure 3**) (80) accordingly to kisspeptin localization in primordial germ cells observed in mouse (28). In proliferating germ cells, a strong expression of *GPR54* mRNA has been found in interstitial compartment of frog testis all over the annual sexual cycle (**Figure 3**). Contrary to human, in frog post meiotic cells and SPZ do not express *GPR54* mRNA, but it is not excluded that the *GPR54* mRNA produced in SPG might be translated in later stages. Since E_2 is likely to be involved in various aspects of testicular activity such as steroidogenesis and primary SPG proliferation (42, 93–95), a possible relationship between E_2 and kisspeptin/GPR54 has been analyzed in frogs. In this respect, an E_2-dependent modulation of *GPR54* expression has been reported in testis. In addition, kp-10, *in vitro*, is able to modulate both *GPR54* and *ERα* expression at the end of the winter stasis (February) as well as during the breeding season (March) (80). Therefore, via kisspeptins/GPR54 activation, E_2 might regulate steroidogenic activity and SPG proliferation. This hypothesis is supported by the localization of *GPR54* mRNA that well correlates with the sites of E_2 action occurring in frog testis (90). Thus, the expression of *GPR54* inside the interstitium and in proliferating SPG, as well as its E_2-dependent expression, strongly support the hypothesis that kisspeptin might have a direct involvement in the onset of the spermatogenetic wave. Accordingly, subcutaneous administration of kp-15 accelerates spermatogenesis in the pre-pubertal teleost *Scomber japonicus* without any significant change in the expression of hypothalamic *GnRH-1* and pituitary *FSHβ* and *LHβ* subunits (66). In addition, kp-10 involvement in differentiation events has been further confirmed in the rhesus monkey derived stem cell line r366.4 (96).

It is evident that the several controversies regarding the "kisspeptin saga" make their history more intriguing with many "behind-the-scenes" yet to be written.

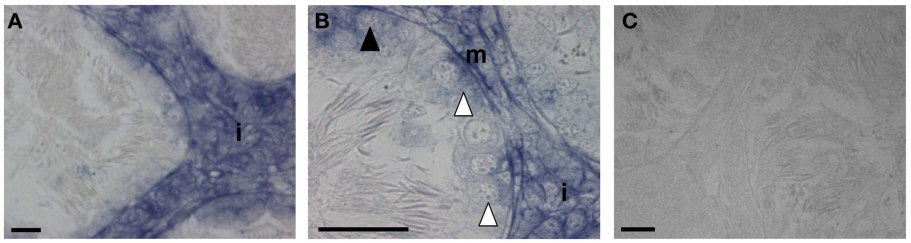

FIGURE 3 | Sections of *R. esculenta* testis, collected in February, analyzed by *in situ* hybridization for *GPR54*. *GPR54* mRNA was detected in the interstitial compartment **(A,B)**, in primary spermatogonia **(B)**, in secondary spermatogonia cysts **(B)** as well as in myoid peritubular cells **(B)**. The specificity of signals was tested through the reaction with a sense riboprobe **(C)**. i, Interstitial compartment; white arrow head, ISPG; dark arrow head, IISPG; m, myoid peritubular cells; scale bar: 20 μm.

ESTROGENS AND SPERM QUALITY

Traditionally, E_2 is stereotyped as the "female" and T as the "male" hormone. E_2 and T are instead present in both males and females, and in male the ratio between the two hormones controls reproduction via specific receptors (16). To date, nuclear (ERα and ERβ) and membrane-bound (GPR30) receptors, able to respond to E_2 via genomic and non-genomic pathways, respectively, have been identified [for review see Ref. (97, 98)].

Estrogens are synthesized via the irreversible transformation of androgens by the aromatase (P450arom; *Cyp19A1* is the related gene), an enzyme expressed in the endoplasmic reticulum of testicular cells. In male, E_2 is indeed primarily synthesized in the testis, which expresses also the specific receptors, ERα and ERβ (16). Recently, GPR30 has been studied in fish, rat, and human and localized in somatic (rat and human) and germ (fish and rat) cells (99–102).

P450arom and ERs expression has been studied in mammalian and non-mammalian testis and the specific mRNA and/or proteins localized in the interstitial (Leydig cells) and tubular (Sertoli and germ cells) compartments, depending on the species [for reviews see Ref. (1, 16, 97, 103)], demonstrating that both somatic and germ cells are able to produce E_2 that can act locally.

In vertebrates, E_2 acts at both central (hypothalamus and hypophysis) (55, 104) and local (testis, efferent ductules, and epididymis) (1, 105, 106) levels and studies in mammalian and non-mammalian species show that E_2 regulates proliferation (gonocytes, SPG, Leydig cells), apoptosis (pachytene SPC, Sertoli cells), and differentiation (SPTs) of germ and somatic cells, as well as it regulates spermiation, transport and motility of SPZ, epididymal sperm maturation, and scrotal testicular descent (42, 43, 80, 97, 107–116). Some of these functions are evolutionarily conserved from fish to mammals demonstrating that E_2 plays an important role in male reproduction physiology in vertebrates (1, 90, 117). Expression profiling of spermatogenesis in the rainbow trout identifies evolutionarily conserved genes involved in male gonadal maturation (118). Accordingly, E_2-responsive genes have been characterized in gonads enriched of SPG or in isolated germ cells: in both frog (42, 108) and fish (118, 119), some of these genes are associated to proliferation.

To date, although tissue and cell culture experiments show that E_2 may act on germ cells, its direct effect in *in vivo* systems has not yet been fully elucidated. However, data obtained in mouse, rat, and human models clearly show that E_2 is important to produce and sustain high standard quality mature SPZ. Two main observations suggest that E_2 is able to act locally into the testis: (1) germ cells express both P450arom and ER, in particular SPTs (120) produce E_2 that may act via specific receptors (121); (2) Sertoli cell barrier envelops the germinal epithelium, from SPCs to SPTs/SPZ, ensuring a specific micro-environment that allows a correct germ cell progression.

In mouse, P450arom activity is high in germ cells and in particular in SPTs, while is lowered in the interstitial compartment (120). Among germ cells, mainly SPTs and SPZ are responsive to inhibition/inactivation of P450arom and to low E_2 levels. Early studies, demonstrated that when rat (122, 123) or bonnet monkey (115) were treated with aromatase inhibitors, degeneration of round SPT and a massive decrease of elongated SPTs was found. Later, D'Souza showed that round SPT differentiation (steps 1–6) was largely dependent on E_2, whereas SPT elongation (steps 8–19) was androgen dependent (124). Indeed, high intra-testicular E_2 levels preserve round SPTs (steps 1–6) whereas T deficiency, induced by E_2, originate pyknotic bodies in elongated/condensed SPTs (steps 8–19) (124). Consistently, loss of E_2 in human testis promotes apoptosis of round SPTs with loss of elongated SPTs (125) and viable SPZ (126). Therefore, E_2 is now considered as a survival factor for SPTs and SPZ.

The bulk of information about the role of E_2 in germ cell differentiation, from SPT-to-SPZ, came from studies on mutant mice such as the hypogonadic (*hpg*), the *Cyp19A1* knock-out (ArKO), and the *Cb1* knock-out ($Cb1^{-/-}$) (55, 127, 128).

Due to a natural *GnRH* gene deletion, the *hpg* mice are functionally deficient in gonadotropins and sex steroids and show meiosis arrest at pachytene stage. Treatment with E_2 or ERα agonists restored meiosis in these animals which, in absence of T, produce haploid elongated SPTs (129). The E_2 treatment alone was as effective as FSH alone and the combination of both hormones did not produce a greater effect (130). Authors concluded that E_2 likely acts on *hpg* testis via a mechanism involving a weak neuroendocrine activation of FSH secretion (128–130).

The phenotype of ArKO mice and experimental analysis carried out using this mutant mice counteract with this conclusion. ArKO males (127) are initially fertile, but they develop progressive infertility between 4.5 months and 1 year. In the SPTs of these animals, multiple acrosome vesicles, irregularly scattered

over the nuclear surface, are observed (127) suggesting that acrosome biogenesis may be an E_2-dependent process. Accordingly, P450arom is at high levels in the Golgi complex of developing SPT (120). In ArKO mice, spermatogenesis is primarily arrested at early stages, with a decrease of round and elongated SPT numbers, without any detectable change of circulating FSH levels (127). Dietary phytoestrogens may partially prevent disruption of ArKO mice spermatogenesis, avoiding the decline of germ cell number. Interestingly, when young ArKO mice were exposed to a phytoestrogen free diet, the phenotype was severely disrupted as compared with mice under normal diet. This occurred in absence of a decreased gonadotropic stimulus, suggesting that the effects of dietary phytoestrogens are independent of changes concerning the pituitary–gonadal axis and they are probably related to direct activation of testicular ERs (131). In agreement with this conclusion, E_2 administration in irradiated rats suppressed serum LH, FSH, and T (both plasma and intratesticular) levels (132) and produced the recovery of spermatogenesis (133, 134) suggesting a gonadotropin-independent E_2 activity. However, gynecomastia and cardiovascular problems are secondary effects related to E_2 treatment and represent the major impediment to clinical application. Recently, it has been suggested that the phytoestrogen genistein may be a true substitute for E_2 (135).

Concerning the *Cb1*$^{-/-}$ mouse, it is a genetically modified animal model showing *Cb1*-gene deletion (136). This gene codifies CB1, which is broadly expressed in hypothalamus, pituitary, and testis (137, 138) of many vertebrates, from fish to mammals [for review see Ref. (52)]. CB1 is involved in GnRH and gonadotropin production (55–57, 139–141) at testicular level, it regulates both spermatogenesis (15, 46, 58, 137, 138, 142–145) and steroidogenesis (146, 147). Interestingly, *Cb1*$^{-/-}$ mice exhibit endocrine features in common with *hpg* and ArKO models: (1) down regulation of *GnRH* and *GnRH-R* mRNA, (2) low LH release and low expression of *FSH*β mRNA, (3) low T production, and (4) low E_2 plasma levels. Morphological and molecular analyses of epididymis and 3β-HSD, which are responsive to T, suggest that even low, T levels are enough (55). Unlike *hpg* and ArKO mice, *Cb1*$^{-/-}$ mutants are fertile; they show a quantitatively normal production of SPZ although, similarly to some fertile men, a consistent aliquot shows abnormalities (148, 149) that are mainly related to the motility and to chromatin quality (histone content, chromatin packaging, DNA integrity, and nuclear size, useful parameters to classify sperm chromatin quality). Therefore, *Cb1*$^{-/-}$ mice exhibit endocrine and phenotypic features, which are useful to extend the above studies about the role of E_2 in SPT differentiation and in the maintenance of sperm quality. Interestingly, when *Cb1*$^{-/-}$ mice were treated with E_2, all the abovementioned chromatin quality indices improved in SPZ (55, 150). Therefore, sperm chromatin quality appears to be responsive to E_2 treatment (151). Interestingly, *ER*α and *ER*β polymorphisms have been associated with semen quality (152). Accordingly, P450arom, either mRNA or protein, has been proposed as marker of sperm quality in men. Indeed, Carreau and co-workers reported that, in human ejaculated SPZ, the immotile sperm fraction showed low levels of P450arom, both mRNA and protein activity (30 and 50%, respectively), as compared with the motile sperm fraction (153–155). In addition, the same authors have recently reported that in SPZ from asthenospermic, teratospermic, and asthenoteratospermic patients, *P450arom* mRNA levels were progressively lower as compared with SPZ from control patients (156). The hypothesis that E_2 treatment improves motility by enhancing oxidative metabolism and the intracellular ATP concentrations in human sperm (157, 158) well fit with the observation that E_2 can regulate mitochondrial function in MCF7 cells by increasing nuclear respiratory factor-1 expression (159). However, in mouse, E_2 and phytoestrogens are able to improve capacitation as well as acrosome reaction and fertilizing capacity of SPZ (160), while natural and synthetic estrogens have stimulatory effect on boar sperm capacitation *in vitro* (161).

Results from mutant animal models, here reported, in combination with case reports concerning patients with few testicular germ cells or decreased sperm motility and number, have a common root: they are characterized by E_2 deficiency due to the mutation or low expression of *Cyp19A1* gene ((126, 162–164), suggesting that E_2 may have a instrumental role in quality sperm and its action is much more complex than previously predicted or suggested by ERα knock-out mice, which show impaired fluid re-adsorption within the efferent ducts as cause of sterility (105).

CLOSING REMARKS

In the last years, data provided by literature evidence that, besides endocrine route, intra-testicular paracrine and autocrine communications are fundamental to sustain spermatogenesis in order to gain high standard quality SPZ. New roles for stereotyped hypothalamic and female hormones – GnRH and E_2, respectively emerged, new potential modulators such as kisspeptins have been identified as well, but conflicting data reveal that several issues need to be further investigated. The modulators here reported – GnRH, kisspeptin, and estrogens – are critical for a successful spermatogenesis as clearly demonstrated by clinical cases of infertility in humans. However, several questions are still open. These different modulators strongly cooperate at hypothalamic level whereas, at testicular level, they control similar events (Leydig cell functions, proliferation/differentiation events, sperm functions); conversely, their possible local crosstalk is far away to be elucidated. Similarly, they may trigger, independently from each other, pathways controlling the same aspects that might represent two sides of the same coin. Both a comparative approach and the use of genetically modified experimental models may represent a successful tool to make giant strides in the building of general models, but to extricate this intriguing story, there is still much to be done.

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AUTHOR CONTRIBUTIONS

Rosaria Meccariello, conception and design of the work, interpretation of data, manuscript drafting, critical revision, final approval of the submitted version; Rosanna Chianese, acquisition, analysis,

interpretation of data for the work, manuscript drafting, final approval of the submitted version; Teresa Chioccarelli, acquisition, analysis, interpretation of data for the work, manuscript drafting, final approval of the submitted version; Vincenza Ciaramella, acquisition, analysis, interpretation of data for the work, manuscript drafting, final approval of the submitted version; Silvia Fasano, conception and design of the work, manuscript drafting, critical revision, final approval of the submitted version; Riccardo Pierantoni, conception and design of the work, manuscript drafting, critical revision, final approval of the submitted version; Gilda Cobellis, conception and design of the work, interpretation of data, manuscript drafting, critical revision, final approval of the submitted version.

REFERENCES

1. Pierantoni R, Cobellis G, Meccariello R, Fasano S. Evolutionary aspects of cellular communication in the vertebrate hypothalamo-hypophysio-gonadal axis. *Int Rev Cytol* (2002) **218**:69–141. doi:10.1016/S0074-7696(02)18012-0
2. Amoss M, Burgus R, Blackwell R, Vale W, Fellows R, Guillemin R. Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. *Biochem Biophys Res Commun* (1971) **44**:205–10. doi:10.1016/S0006-291X(71)80179-1
3. Kah O, Lethimonier C, Somoza G, Guilgur LG, Vaillant C, Lareyre JJ. GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective. *Gen Comp Endocrinol* (2007) **153**:346–64. doi:10.1016/j.ygcen.2007.01.030
4. Kavanaugh SI, Nozaki M, Sower SA. Origins of gonadotropin-releasing hormone (GnRH) in vertebrates: identification of a novel GnRH in a basal vertebrate, the sea lamprey. *Endocrinology* (2008) **149**:3860–9. doi:10.1210/en.2008-0184
5. Millar RP, Lu ZL, Pawson AJ, Flanagan CA, Morgan K, Maudsley SR. Gonadotropin-releasing hormone receptors. *Endocr Rev* (2004) **25**:235–75. doi:10.1210/er.2003-0002
6. Morgan K, Millar RP. Evolution of GnRH ligand precursors and GnRH receptors in protochordate and vertebrate species. *Gen Comp Endocrinol* (2004) **139**:191–7. doi:10.1016/j.ygcen.2004.09.015
7. Oh DY, Song JA, Moon JS, Moon MJ, Kim JI, Kim K, et al. Membrane-proximal region of the carboxyl terminus of the gonadotropin-releasing hormone receptor (GnRHR) confers differential signal transduction between mammalian and nonmammalian GnRHRs. *Mol Endocrinol* (2005) **19**:722–31. doi:10.1210/me.2004-0220
8. King JA, Millar RP. Evolutionary aspects of gonadotropin-releasing hormone and its receptor. *Cell Mol Neurobiol* (1995) **15**:5–23. doi:10.1007/BF02069556
9. Wu HM, Cheng JC, Wang HS, Huangm HY, MacCalman CD, Leung PC. Gonadotropin-releasing hormone type II induces apoptosis of human endometrial cancer cells by activating GADD45alpha. *Cancer Res* (2009) **69**:4202–8. doi:10.1158/0008-5472.CAN-08-4591
10. Aguilar-Rojas A, Huerta-Reyes M. Human gonadotropin-releasing hormone receptor-activated cellular functions and signaling pathways in extra-pituitary tissues and cancer cells. *Oncol Rep* (2009) **22**:981–90. doi:10.3892/or_00000525
11. Pawson AJ, Morgan K, Maudsley SR, Millar RP. Type II gonadotrophin-releasing hormone (GnRH-II) in reproductive biology. *Reproduction* (2003) **126**:271–8. doi:10.1530/rep.0.1260271
12. Sharpe RM. Paracrine control of the testis. *Clin Endocrinol Metab* (1986) **15**:185–207. doi:10.1016/S0300-595X(86)80049-4
13. Morales P, Pizzarro E, Kong M, Kerr B, Ceric F, Vigil P. Gonadotropin releasing hormone stimulated sperm binding to the human zona is mediated by a calcium influx. *Biol Reprod* (2000) **63**:635–42. doi:10.1095/biolreprod63.2.635
14. Morales P, Pasten C, Pizzarro E. Inhibition of *in vivo* and *in vivo* fertilization in rodents by gonadotropin-releasing hormone antagonist. *Biol Reprod* (2002) **67**:1360–5. doi:10.1095/biolreprod67.4.1360
15. Pierantoni R, Cobellis G, Meccariello R, Cacciola G, Chianese R, Chioccarelli T, et al. Testicular gonadotropin-releasing hormone activity, progression of spermatogenesis, and sperm transport in vertebrates. *Ann N Y Acad Sci* (2009) **1163**:279–91. doi:10.1111/j.1749-6632.2008.03617.x
16. Chianese R, Chioccarelli T, Cacciola G, Ciaramella V, Fasano S, Pierantoni R, et al. The contribution of lower vertebrate animal models in human reproduction research. *Gen Comp Endocrinol* (2011) **171**:17–27. doi:10.1016/j.ygcen.2010.12.011
17. Bahk JY, Hyun JS, Chung SH, Lee H, Kim MO, Lee BH, et al. Stage specific identification of the expression of GnRH mRNA and localization of the GnRH receptor in mature rat and adult human testis. *J Urol* (1995) **154**:1958–61. doi:10.1097/00005392-199511000-00105
18. White RB, Fernald RD. Genomic structure and expression sites of three gonadotropin-releasing hormone genes in one species. *Gen Comp Endocrinol* (1998) **112**:217–25. doi:10.1006/gcen.1998.7125
19. van Biljon W, Wykes S, Scherer S, Krawetz SA, Hapgood J. Type II gonadotropin-releasing hormone receptor transcripts in human sperm. *Biol Reprod* (2002) **67**:1741–9. doi:10.1095/biolreprod.101.002808
20. Millar R, Conklin D, Lofton-Day C, Hutchinson E, Troskie B, Illing N, et al. A novel human GnRH receptor homolog gene: abundant and wide tissue distribution of the antisense transcript. *J Endocrinol* (1999) **162**:117–26. doi:10.1677/joe.0.1620117
21. Pierantoni R, Fasano S, Di Matteo L, Minucci S, Varriale B, Chieffi G. Stimulatory effect of a GnRH agonist (buserelin) in *in vitro* and *in vivo* testosterone production by the frog (*Rana esculenta*) testis. *Mol Cell Endocrinol* (1984) **38**:215–9. doi:10.1016/0303-7207(84)90120-5
22. Ramakrishnappa N, Rajamahendran R, Lin YM, Leung PC. GnRH in non-hypothalamic reproductive tissues. *Anim Reprod Sci* (2005) **88**:95–113. doi:10.1016/j.anireprosci.2005.05.009
23. Lin YM, Liu MY, Poon SL, Leu SF, Huang BM. Gonadotropin-releasing hormone-I and -II stimulate steroidogenesis in prepubertal murine Leydig cells *in vitro*. *Asian J Androl* (2008) **10**:929–36. doi:10.1111/j.1745-7262.2008.00434.x
24. Fasano S, Pierantoni R, Minucci S, Di Matteo L, D'Antonio M, Chieffi G. Effects of intratesticular injections of estradiol and gonadotropin-releasing hormone (GnRHA, HOE 766) on plasma androgen levels in intact and hypophysectomized *Torpedo marmorata* and *Torpedo ocellata*. *Gen Comp Endocrinol* (1989) **75**:349–54. doi:10.1016/0016-6480(89)90169-X
25. King JA, Millar RP, Vallarino M, Pierantoni R. Localization and characterization of gonadotropin-releasing hormones in the brain, gonads, and plasma of a dipnoi (lungfish, *Protopterus annectens*). *Regul Pept* (1995) **57**:163–74. doi:10.1016/0167-0115(95)00025-7
26. Lin YM, Poon SL, Choi JH, Lin JS, Leung PC, Huang BM. Transcripts of testicular gonadotropin-releasing hormone, steroidogenic enzymes, and intratesticular testosterone levels in infertile men. *Fertil Steril* (2008) **90**:1761–8. doi:10.1016/j.fertnstert.2007.08.078
27. Yao B, Liu HY, Gu YC, Shi SS, Tao XQ, Li XJ, et al. Gonadotropin-releasing hormone positively regulates steroidogenesis via extracellular signal-regulated kinase in rat Leydig cells. *Asian J Androl* (2011) **13**:438–45. doi:10.1038/aja.2010.158
28. Anjum S, Krishna A, Sridaran R, Tsutsui K. Localization of gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin and GnRH receptor and their possible roles in testicular activities from birth to senescence in mice. *J Exp Zool A Ecol Genet Physiol* (2012) **317**:630–44. doi:10.1002/jez.1765
29. Ubuka T, Son YL, Tobari J, Narihiro M, Bentley GE, Kriegsfelf LJ, et al. Central and direct regulation of testicular activity by gonadotropin-inhibitory hormone and its receptor. *Front Endocrinol* (2014) **5**:8. doi:10.3389/fendo.2014.00008
30. Guo JJ, Ma X, Wang CQ, Ge YF, Lian QQ, Hardy DO, et al. Effect of luteinizing hormone and androgen on the development of rat progenitor Leydig cells *in vitro* and *in vivo*. *Asian J Androl* (2013) **15**:685–91. doi:10.1038/aja.2013.55
31. So WK, Cheng JC, Poon SL, Leung PC. Gonadotropin-releasing hormone and ovarian cancer: a functional and mechanistic overview. *FEBS J* (2008) **275**:5496–511. doi:10.1111/j.1742-4658.2008.06679.x
32. White CD, Stewart AJ, Lu ZL, Millar RP, Morgan K. Antiproliferative effects of GnRH agonists: prospects and problems for cancer therapy. *Neuroendocrinology* (2008) **88**:67–79. doi:10.1159/000119093
33. Andreu-Vieyra CV, Buret AG, Habibi HR. Gonadotropin-releasing hormone induction of apoptosis in the testes of goldfish (*Carassius auratus*). *Endocrinology* (2005) **146**:1588–96. doi:10.1210/en.2004-0818

34. Soverchia L, Carotti M, Andreu-Vieyra C, Mosconi G, Cannella N, Habibi H, et al. Role of gonadotropin-releasing hormone (GnRH) in the regulation of gonadal differentiation in the gilthead seabream (*Sparus aurata*). *Mol Reprod Dev* (2007) **74**:57–67. doi:10.1002/mrd.20484
35. Ogawa T, Dobrinski I, Avarbock MR, Brinster RL. Leuprolide, a gonadotropin-releasing hormone agonist, enhances colonization after spermatogonial transplantation into mouse testes. *Tissue Cell* (1998) **30**:583–8. doi:10.1016/S0040-8166(98)80039-6
36. Ogawa T, Dobrinski I, Brinster RL. Recipient preparation is critical for spermatogonial transplantation in the rat. *Tissue Cell* (1999) **31**:461–72. doi:10.1054/tice.1999.0060
37. Shuttlesworth GA, de Rooij DG, Huhtaniemi I, Reissmann T, Russel LD, Shetty G, et al. Enhancement of a spermatogonial proliferation and differentiation in irradiated rats by gonadotropin-releasing hormone antagonist administration. *Endocrinology* (2000) **141**:37–49. doi:10.1210/en.141.1.37
38. Treen N, Itoh N, Miura H, Kikuchi I, Ueda T, Takahashi KG, et al. Mollusc gonadotropin-releasing hormone directly regulates gonadal functions: a primitive endocrine system controlling reproduction. *Gen Comp Endocrinol* (2012) **176**:167–72. doi:10.1016/j.ygcen.2012.01.008
39. Minucci S, Di Matteo L, Pierantoni R, Varriale B, Rastogi RK, Chieffi G. In vivo and *in vitro* stimulatory effect of a gonadotropin-releasing hormone analog (HOE 766) on spermatogonial multiplication in the frog, *Rana esculenta*. Endocrinology (1986) **119**:731–6. doi:10.1210/endo-119-2-731
40. Minucci S, Fasano S, Pierantoni R. Induction of S-phase entry by a gonadotropin releasing hormone agonist (buserelin) in the frog, *Rana esculenta*, primary spermatogonia. *Comp Biochem Physiol C Pharmacol Toxicol Endocrinol* (1996) **113**:99–102. doi:10.1016/0742-8413(95)02046-2
41. Di Matteo L, Minucci S, Fasano S, Pierantoni R, Varriale B, Chieffi G. A gonadotropin-releasing hormone (GnRH) antagonist decreases androgen production and spermatogonial multiplication in frog (*Rana esculenta*): indirect evidence for the existence of GnRH or GnRH-like material receptors in the hypophysis and testis. *Endocrinology* (1988) **122**:62–7. doi:10.1210/endo-122-1-62
42. Cobellis G, Meccariello R, Fienga G, Pierantoni R, Fasano S. Cytoplasmic and nuclear Fos protein forms regulate resumption of spermatogenesis in the frog, *Rana esculenta*. *Endocrinology* (2002) **143**:163–70. doi:10.1210/en.143.1.163
43. Cobellis G, Meccariello R, Minucci S, Palmiero C, Pierantoni R, Fasano S. Cytoplasmic versus nuclear localization of Fos-related proteins in the frog, *Rana esculenta*, testis: *in vivo* and direct *in vitro* effect of a gonadotropin-releasing hormone agonist. *Biol Reprod* (2003) **68**:954–60. doi:10.1095/biolreprod.102.008938
44. Savulescu D, Feng J, Ping YS, Mai O, Boehm U, He B, et al. Gonadotropin-releasing hormone-regulated prohibitin mediates apoptosis of the gonadotrope cells. *Mol Endocrinol* (2013) **27**:1856–70. doi:10.1210/me.2013-1210
45. Minucci S, Di Matteo L, Chieffi Baccari G, Pierantoni R. A gonadotropin releasing hormone analog induces spermiation in intact and hypophysectomized frogs, *Rana esculenta*. *Experientia* (1989) **45**:1118–21. doi:10.1007/BF01950175
46. Chianese R, Ciaramella V, Scarpa D, Fasano S, Pierantoni R, Meccariello R. Anandamide regulates the expression of *GnRH1*, *GnRH2* and *GnRHRs* in frog testis. *Am J Physiol Endocrinol Metab* (2012) **303**:E475–87. doi:10.1152/ajpendo.00086.2012
47. Zerani M, Catone G, Quassinti L, Maccari E, Bramucci M, Gobbetti A, et al. *In vitro* effects of gonadotropin-releasing hormone (GnRH) on Leydig cells of adult alpaca (*Lama pacos*) testis: GnRH receptor immunolocalization, testosterone and prostaglandin synthesis, and cyclooxygenase activities. *Domest Anim Endocrinol* (2011) **40**:51–9. doi:10.1016/j.domaniend.2010.08.006
48. Chianese R, Ciaramella V, Fasano S, Pierantoni R, Meccariello R. Anandamide modulates the expression of GnRH-II and GnRHRs in frog, *Rana esculenta*, diencephalon. *Gen Comp Endocrinol* (2011) **173**:389–95. doi:10.1016/j.ygcen.2011.07.001
49. Cacciola G, Chianese R, Chioccarelli T, Ciaramella V, Fasano S, Pierantoni R, et al. Cannabinoid and reproduction: a lasting and intriguing history. *Pharmaceuticals* (2010) **3**:3275–323. doi:10.3390/ph3103275
50. Pierantoni R, Cobellis G, Meccariello R, Cacciola G, Chianese R, Chioccarelli T, et al. CB1 activity in male reproduction: mammalian and nonmammalian anima models. *Vitam Horm* (2009) **81**:367–87. doi:10.1016/S0083-6729(09)81014-5
51. Battista N, Meccariello R, Cobellis G, Fasano S, Di Tommaso M, Pirazzi V, et al. The role of endocannabinoids in gonadal function and fertility along the evolutionary axis. *Mol Cell Endocrinol* (2012) **355**:1–14. doi:10.1016/j.mce.2012.01.014
52. Meccariello R, Battista N, Bradshaw HB, Wang H. Updates in reproduction coming from the endocannabinoid system. *Int J Endocrinol* (2014) **2014**(412354):16. doi:10.1155/2014/412354
53. Scorticati C, Fernandez-Solari J, De Laurentiis A, Mohn C, Prestifilippo JP, Lasaga M, et al. The inhibitory effect of anandamide on luteinizing hormone-releasing hormone secretion is reversed by oestrogen. *Proc Natl Acad Sci U S A* (2004) **32**:11891–6. doi:10.1073/pnas.0404366101
54. Farkas I, Kallo I, Deli L, Vida B, Hrabovszky E, Fekete C, et al. Retrograde endocannabinoids signaling reduces GABAergic synaptic transmission to gonadotropin-releasing hormone neurons. *Endocrinology* (2010) **151**:5818–29. doi:10.1210/en.2010-0638
55. Cacciola G, Chioccarelli T, Altucci L, Ledent C, Mason JI, Fasano S, et al. Low 17beta-estradiol levels in CNR1 knock-out mice affect spermatid chromatin remodeling by interfering with chromatin reorganization. *Biol Reprod* (2013) **152**:1–12. doi:10.1095/biolreprod.112.105726
56. Meccariello R, Franzoni MF, Chianese R, Cottone E, Scarpa D, Donna D, et al. Interplay between the endocannabinoid system and *GnRH-I* in the forebrain oh the anuran amphibian *Rana esculenta*. *Endocrinology* (2008) **149**:2149–58. doi:10.1210/en.2007-1357
57. Chianese R, Cobellis G, Pierantoni R, Fasano S, Meccariello R. Non-mammalian vertebrate models and the endocannabinoid system: relationships with gonadotropin-releasing hormone. *Mol Cell Endocrinol* (2008) **286**:S46–51. doi:10.1016/j.mce.2008.01.009
58. Chianese R, Ciaramella V, Scarpa D, Fasano S, Pierantoni R, Meccariello R. Endocannabinoids and endovanilloids: a possible balance in the regulation of testicular GnRH signaling. *Int J Endocrinol* (2013) **2013**:904748. doi:10.1155/2013/904748
59. Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, et al. Kiss-1, a novel human malignant melanoma metastasis-suppressor gene. *J Natl Cancer Inst* (1996) **88**:1731–7. doi:10.1093/jnci/88.23.1731
60. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The metastasis suppressor gene Kiss-1 encodes kisspeptins, the natural ligands of the orphan G protein-coupled receptor GPR54. *J Biol Chem* (2001) **276**:34631–6. doi:10.1074/jbc.M104847200
61. Messager S, Chatzidaki EE, Ma D, Hendrick AG, Zahn D, Dixon J, et al. Kisspeptin directly stimulates gonadotropin releasing hormone release via G protein coupled receptor 54. *Proc Natl Acad Sci U S A* (2005) **102**:1761–6. doi:10.1073/pnas.0409330102
62. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Shagoury JK, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med* (2003) **349**:1614–27. doi:10.1056/NEJMoa035322
63. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U S A* (2003) **100**:10972–6. doi:10.1073/pnas.1834399100
64. Beck BH, Fuller SA, Peatman E, McEntire ME, Darwish A, Freeman DW. Chronic exogenous kisspeptin administration accelerates gonadal development in basses of the genus Morone. *Comp Biochem Physiol A Mol Integr Physiol* (2012) **162**:265–73. doi:10.1016/j.cbpa.2012.03.019
65. Nocillado JN, Zohar Y, Biran J, Levavi-Sivan B, Elizur A. Chronic kisspeptin administration stimulated gonadal development in pre-pubertal male yellowtail kingfish (*Seriola lalandi*; perciformes) during the breeding and non-breeding season. *Gen Comp Endocrinol* (2013) **191**:168–76. doi:10.1016/j.ygcen.2013.06.005
66. Selvaraj S, Ohga H, Nyuji M, Kitano H, Nagano N, Yamaguchi A, et al. Subcutaneous administration of Kiss1 pentadecapeptide accelerates spermatogenesis in prepubertal male chub mackerel (*Scomber japonicus*). *Comp Biochem Physiol A Mol Integr Physiol* (2013) **166**:228–36. doi:10.1016/j.cbpa.2013.06.007
67. Selvaraj S, Ohga H, Kitano H, Nyuji M, Yamaguchi A, Matsuyama M. Peripheral administration of Kiss1 pentadecapeptide induces gonadal development in sexually immature adult scombroid fish. *Zoolog Sci* (2013) **30**:446–54. doi:10.2108/zsj.30.446
68. Saito H, Sawada T, Yaegashi T, Goto Y, Jin J, Sawai K, et al. Kisspeptin-10 stimulates the release of luteinizing hormone and testosterone in pre- and post-pubertal male goats. *Anim Sci J* (2012) **83**:487–92. doi:10.1111/j.1740-0929.2011.00978.x

69. Kauffman AS. Coming of age in the kisspeptin era: sex differences, development, and puberty. *Mol Cell Endocrinol* (2010) **324**:51–63. doi:10.1016/j.mce.2010.01.017
70. Demirbilek H, Gonc EN, Ozon A, Alikasifoglu A, Kandemir N. Evaluation of serum kisspeptin levels in girls in the diagnosis of central precocious puberty and in the assessment of pubertal suppression. *J Pediatr Endocrinol Metab* (2012) **25**:313–6. doi:10.1515/jpem-2011-0445
71. Ratnasabapathy R, Dhillo WS. The effects of kisspeptin in human reproductive function therapeutic implications. *Curr Drug Targets* (2013) **14**:365–71. doi:10.2174/138945013804998981
72. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev* (2009) **30**:713–43. doi:10.1210/er.2009-0005
73. Mayer C, Boehm U. Female reproductive maturation in the absence of kisspeptin/GPR54 signaling. *Nat Neurosci* (2011) **14**:704–10. doi:10.1038/nn.2818
74. Popa SM, Moriyama RM, Caligioni CS, Yang JJ, Cho CM, Concepcion TL, et al. Redundancy in Kiss1 expression safeguards reproduction in the mouse. *Endocrinology* (2013) **154**:2784–94. doi:10.1210/en.2013-1222
75. Ohtaki T, Shintani Y, Honda S, Matsumoto H, Hori A, Kanehashi K, et al. Metastasis suppressor gene KiSS-1 encodes peptide ligand of a G-protein-coupled receptor. *Nature* (2001) **411**:613–7. doi:10.1038/35079135
76. Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, et al. The KiSS-1 receptor GPR54 is essential for the development of the murine reproductive system. *Biochem Biophys Res Commun* (2003) **312**:1357–63. doi:10.1016/j.bbrc.2003.11.066
77. Terao Y, Kumano S, Takatsu Y, Hattori M, Nishimura A, Ohtaki T, et al. Expression of KiSS-1, a metastasis suppressor gene, in trophoblast giant cells of the rat placenta. *Biochim Biophys Acta* (2004) **1678**:102–10. doi:10.1016/j.bbaexp.2004.02.005
78. Tariq AR, Shahab M, Clarke IJ, Pereira A, Smith JT, Khan SH, et al. Kiss1 and kiss1 receptor expression in the rhesus monkey testis: a possible local regulator of testicular function. *Cent Eur J Biol* (2013) **8**:968–74. doi:10.2478/s11535-013-0219-4
79. Irfan S, Ehmcke J, Wahab F, Shahab M, Schlatt S. Intratesticular action of kisspeptin in rhesus monkey (*Macaca mulatta*). *Andrologia* (2013). doi:10.1111/and.12121
80. Chianese R, Ciaramella V, Fasano S, Pierantoni R, Meccariello R. Kisspeptin receptor, GPR54, as a candidate for the regulation of testicular activity in the frog *Rana esculenta*. *Biol Reprod* (2013) **88**:73. doi:10.1095/biolreprod.112.103515
81. Ramzan F, Qureshi IZ. Intraperitoneal kisspeptin-10 administration induces dose-dependent degenerative changes in maturing rat testes. *Life Sci* (2011) **88**:246–56. doi:10.1016/j.lfs.2010.11.019
82. Thompson EL, Murphy KG, Patterson M, Bewick GA, Stamp GW, Curtis AE, et al. Chronic subcutaneous administration of kisspeptin-54 causes testicular degeneration in adult male rats. *Am J Physiol Endocrinol Metab* (2006) **291**:E1074–82. doi:10.1152/ajpendo.00040.2006
83. Ramzan F, Qureshi IZ, Ramzan M, Ramzan MH, Ramzan F. Immature rat seminal vesicles show histomorphological and ultrastructural alterations following treatment with kisspeptin-10. *Reprod Biol Endocrinol* (2012) **10**:18. doi:10.1186/1477-7827-10-18
84. Ramzan F, Qureshi IZ, Ramzan M, Ramzan MH, Ramzan F. Kisspeptin-10 induces dose dependent degeneration in prepubertal rat prostate gland. *Prostate* (2013) **73**:690–9. doi:10.1002/pros.22609
85. Mei H, Doran J, Kyle V, Yeo SH, Colledge WH. Does kisspeptin signalling have a role in the testes? *Front Endocrinol* (2013) **4**:198. doi:10.3389/fendo.2013.00198
86. Shahab M, Mastronardi C, Seminara SB, Crowley WF, Ojeda SR, Plant TM. Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates. *Proc Natl Acad Sci U S A* (2005) **102**:2129–34. doi:10.1073/pnas.0409822102
87. Roseweir AK, Kauffman AS, Smith JT, Guerriero KA, Morgan K, Pielecka-Fortuna J, et al. Discovery of potent kisspeptin antagonists delineate physiological mechanisms of gonadotropin regulation. *J Neurosci* (2009) **29**:3920–9. doi:10.1523/JNEUROSCI.5740-08.2009
88. Pinto FM, Cejudo-Román A, Ravina CG, Fernández-Sánchez M, Martín-Lozano D, Illanes M, et al. Characterization of the kisspeptin system in human spermatozoa. *Int J Androl* (2012) **35**:63–73. doi:10.1111/j.1365-2605.2011.01177.x
89. Hsu MC, Wang JY, Lee YJ, Jong DS, Tsui KH, Chiu CH. Kisspeptin modulates fertilization capability of mouse spermatozoa. *Reproduction* (2014). doi:10.1530/REP-13-0368
90. Pierantoni R, Cobellis G, Meccariello R, Palmiero C, Fienga G, Minucci S, et al. The amphibian testis as model to study germ cell progression during spermatogenesis. *Biochem Physiol B Biochem Mol Biol* (2002) **132**:131–9. doi:10.1016/S1096-4959(01)00543-7
91. Rastogi RK, Iela L, Saxena PK, Chieffi G. The control of spermatogenesis in the green frog, *Rana esculenta*. *J Exp Zool* (1976) **169**:151–66. doi:10.1002/jez.1401960203
92. Fasano S, Minucci S, Di Matteo L, D'Antonio M, Pierantoni R. Intratesticular feedback mechanisms in the regulation of steroid profiles in the frog, *Rana esculenta*. *Gen Comp Endocrinol* (1989) **75**:335–42. doi:10.1016/0016-6480(89)90167-6
93. Sirianni R, Chimento A, Ruggiero C, De Luca A, Lappano R, Andò S, et al. The novel estrogen receptor, G protein-coupled receptor 30, mediates the proliferative effects induced by 17beta-estradiol on mouse spermatogonial GC-1 cell line. *Endocrinology* (2008) **149**:5043–51. doi:10.1210/en.2007-1593
94. Chimento A, Sirianni R, Casaburi I, Pezzi V. Role of estrogen receptors (ERs) and G protein-coupled estrogen receptor (GPER) in regulation of hypothalamic-pituitary-testis axis and spermatogenesis. *Front Endocrinol* (2014) **5**:1. doi:10.3389/fendo.2014.00001
95. Lidke AK, Bannister S, Löwer AM, Apel DM, Podleschny M, Kollmann M, et al. 17β-Estradiol induces supernumerary primordial germ cells in embryos of the polychaete *Platynereis dumerilii*. *Gen Comp Endocrinol* (2014) **196**:52–61. doi:10.1016/j.ygcen.2013.11.017
96. Huma T, Ullah F, Hanif F, Rizak JD, Shahab M. Peripheral administration of kisspeptin antagonist does not alter basal plasma testosterone but decreases plasma adiponectin levels in adult male rhesus macaques. *Eur J Sci Res* (2013) **109**:668–77. doi:10.3906/biy-1401-53
97. Carreau S, Bouraima-Lelong H, Delalande C. Estrogens in male germ cells. *Spermatogenesis* (2011) **1**:90–4. doi:10.4161/spmg.1.2.16766
98. Maggiolini M, Picard D. The unfolding stories of GPR30, a new membrane-bound estrogen receptor. *J Endocrinol* (2010) **204**:105–14. doi:10.1677/JOE-09-0242
99. Liu X, Zhu P, Sham KW, Yuen JM, Xie C, Zhang Y, et al. Identification of a membrane estrogen receptor in zebrafish with homology to mammalian GPR30 and its high expression in early germ cells of the testis. *Biol Reprod* (2009) **80**:1253–61. doi:10.1095/biolreprod.108.070250
100. Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Andò S, et al. 17 Beta-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ER alpha. *Mol Cell Endocrinol* (2010) **320**:136–44. doi:10.1016/j.mce.2010.01.035
101. Alves MG, Socorro S, Silva J, Barros A, Sousa M, Cavaco JE, et al. In vitro cultured human Sertoli cells secrete high amounts of acetate that is stimulated by 17β-estradiol and suppressed by insulin deprivation. *Biochim Biophys Acta* (2012) **1823**:1389–94. doi:10.1016/j.bbamcr.2012.06.002
102. Rago V, Romeo F, Giordano F, Maggiolini M, Carpino A. Identification of the estrogen receptor GPER in neoplastic and non-neoplastic human testes. *Reprod Biol Endocrinol* (2011) **9**:135. doi:10.1186/1477-7827-9-135
103. O'Donnell L, Robertson KM, Jones ME, Simpson ER. Estrogen and spermatogenesis. *Endocr Rev* (2001) **22**:289–318. doi:10.1210/er.22.3.289
104. Shanbacker BD. Regulation of luteinizing hormone secretion in male sheep by endogenous estrogen. *Endocrinology* (1974) **115**:944–50. doi:10.1210/endo-115-3-944
105. Eddy EM, Washburn TF, Bunch DO, Goulding EH, Gladen BC, Lubahn DB, et al. Targeted disruption of the estrogen receptor gene in male mice causes alteration of spermatogenesis and infertility. *Endocrinology* (1996) **137**:4796–805. doi:10.1210/en.137.11.4796
106. Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, et al. A role for oestrogens in the male reproductive system. *Nature* (1997) **390**:509–12. doi:10.1038/37352
107. Miura T, Miura C, Ohta T, Nader MR, Todo T, Yamauchi K. Estradiol-17β stimulates the renewal of spermatogonial stem cells in males. *Biochem Biophys Res Commun* (1999) **264**:230–4. doi:10.1006/bbrc.1999.1494
108. Cobellis G, Pierantoni R, Minucci S, Pernas-Alonso R, Meccariello R, Fasano S. c-Fos activity in *Rana esculenta* testis: seasonal and estradiol-induced changes. *Endocrinology* (1999) **140**:3238–44. doi:10.1210/en.140.7.3238

109. Cobellis G, Lombardi M, Scarpa D, Izzo G, Fienga G, Meccariello R, et al. Fra-1 activity in the frog, *Rana esculenta*, testis. *Ann N Y Acad Sci* (2005) **1040**:264–8. doi:10.1196/annals.1327.039
110. Cobellis G, Lombardi M, Scarpa D, Izzo G, Fienga G, Meccariello R, et al. Fra1 activity in the frog, *Rana esculenta*, testis: a new potential role in sperm transport. *Biol Reprod* (2005) **72**:1101–8. doi:10.1095/biolreprod.104.036541
111. Cobellis G, Cacciola G, Chioccarelli T, Izzo G, Meccariello R, Pierantoni R, et al. Estrogen regulation of the male reproductive tract in the frog, *Rana esculenta*: a role in Fra-1 activation in peritubular myoid cells and in sperm release. *Gen Comp Endocrinol* (2008) **155**:838–46. doi:10.1016/j.ygcen.2007.10.004
112. Lucas TF, Pimenta MT, Pisolato R, Lazari MF, Porto CS. 17Beta-estradiol signaling and regulation of Sertoli cell function. *Spermatogenesis* (2011) **1**:318–24. doi:10.4161/spmg.1.4.18903
113. Ruwanpura SM, McLachlan RI, Meachem SJ. Hormonal regulation of male germ cell development. *J Endocrinol* (2010) **205**:117–31. doi:10.1677/JOE-10-0025
114. Shetty G, Krishnamurthy H, Krishnamurthy HN, Bhatnagar S, Moudgal RN. Effect of estrogen deprivation on the reproductive physiology of male and female primates. *J Steroid Biochem Mol Biol* (1997) **61**:157–66. doi:10.1016/S0960-0760(97)80008-8
115. Shetty G, Krishnamurthy H, Krishnamurthy HN, Bhatnagar AS, Moudgal NR. Effect of long-term treatment with aromatase inhibitor on testicular function of adult male bonnet monkeys (*M. radiata*). *Steroids* (1998) **63**:414–20. doi:10.1016/S0039-128X(98)00042-7
116. Staub C, Rauch M, Ferrière F, Trépos M, Dorval-Coiffec I, Saunders PT, et al. Expression of estrogen receptor ESR1 and its 46-kDa variant in the gubernaculum testis. *Biol Reprod* (2005) **73**:703–12. doi:10.1095/biolreprod.105.042796
117. Schulz RW, de França LR, Lareyre JJ, Le Gac F, Chiarini-Garcia H, Nobrega RH, et al. Spermatogenesis in fish. *Gen Comp Endocrinol* (2010) **165**:390–411. doi:10.1016/j.ygcen.2009.02.013
118. Rolland AD, Lareyre JJ, Goupil AS, Montfort J, Ricordel MJ, Esquerré D, et al. Expression profiling of rainbow trout testis development identifies evolutionary conserved genes involved in spermatogenesis. *BMC Genomics* (2009) **10**:546. doi:10.1186/1471-2164-10-546
119. Pinto PI, Teodósio HR, Galay-Burgos M, Power DM, Sweeney GE, Canário AV. Identification of estrogen-responsive genes in the testis of sea bream (*Sparus auratus*) using suppression subtractive hybridization. *Mol Reprod Dev* (2006) **73**:318–29. doi:10.1002/mrd.20402
120. Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Milette CF, et al. Germ cells of the mouse testis express P450 aromatase. *Endocrinology* (1993) **132**:1396–401. doi:10.1210/en.132.3.1396
121. Carreau S, Bouraima-Lelong H, Delalande C. Role of estrogens in spermatogenesis. *Front Biosci (Elite Ed)* (2012) **4**:1–11. doi:10.2741/356
122. Tsutsumi I, Toppari J, Campeau JD, Di Zerega GS. Reduction of fertility in the male rat by systemic treatment with follicle regulatory protein. *Fertil Steril* (1987) **47**:689–95.
123. Tsutsumi I, Fugimori K, Nakamura RM. Disruption of seminiferous epithelial function in the rat by ovarian protein. *Biol Reprod* (1987) **36**:451–61. doi:10.1095/biolreprod36.2.451
124. D'Souza R, Gill-Sharma MK, Pathak S, Kedia N, Kumar R, Balasinor N. Effect of high intratesticular estrogen on the seminiferous epithelium in adult male rats. *Mol Cell Endocrinol* (2005) **241**:41–8. doi:10.1016/j.mce.2005.04.011
125. Pentikäinen V, Erkkilä K, Suomalainen L, Parvinen M, Dunkel L. Estradiol acts as a germ cell survival factor in the human testis in vitro. *J Clin Endocrinol Metab* (2000) **85**:2057–67. doi:10.1210/jcem.85.5.6600
126. Lanfranco F, Zirilli L, Baldi M, Pignatti E, Corneli G, Ghigo E, et al. A novel mutation in the human aromatase gene: insights on the relationship among serum estradiol, longitudinal growth and bone mineral density in an adult man under estrogen replacement treatment. *Bone* (2008) **43**:628–35. doi:10.1016/j.bone.2008.05.011
127. Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, et al. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci U S A* (1999) **96**:7986–91. doi:10.1073/pnas.96.14.7986
128. Ebling FJ, Brooks AN, Cronin AS, Ford H, Kerr JB. Estrogenic induction of spermatogenesis in the hypogonadal mouse. *Endocrinology* (2000) **141**:2861–9. doi:10.1210/en.141.8.2861
129. Allan CM, Couse JF, Simanainen U, Spaliviero J, Jimenez M, Rodriguez K, et al. Estradiol induction of spermatogenesis is mediated via an estrogen receptor-falphag mechanism involving neuroendocrine activation of follicle-stimulating hormone secretion. *Endocrinology* (2010) **151**:2800–10. doi:10.1210/en.2009-1477
130. Baines H, Nwagwu MO, Hastie GR, Wiles RA, Mayhew TM, Ebling FJ. Effects of estradiol and FSH on maturation of the testis in the hypogonadal (hpg) mouse. *Reprod Biol Endocrinol* (2008) **6**:4. doi:10.1186/1477-7827-6-4
131. Robertson KM, O'Donnell L, Simpson ER, Jones ME. The phenotype of the aromatase knockout mouse reveals dietary phytoestrogens impact significantly on testis function. *Endocrinology* (2002) **143**:2913–21. doi:10.1210/en.143.8.2913
132. Shetty G, Weng CC, Bolden-Tiller OU, Huhtaniemi I, Handelsman DJ, Meistrich ML. Effects of medroxyprogesterone and estradiol on the recovery of spermatogenesis in irradiated rats. *Endocrinology* (2004) **145**:4461–9. doi:10.1210/en.2004-0440
133. Zhou W, Bolden-Tiller OU, Shao SH, Weng CC, Shetty G, AbuElhija M, et al. Estrogen-regulated genes in rat testes and their relationship to recovery of spermatogenesis after irradiation. *Biol Reprod* (2011) **85**:823–33. doi:10.1095/biolreprod.111.091611
134. Porter KL, Shetty G, Shuttlesworth GA, Weng CC, Huhtaniemi I, Pakarinen P, et al. Estrogen enhances recovery from radiation-induced spermatogonial arrest in rat testes. *J Androl* (2009) **30**:440–51. doi:10.2164/jandrol.108.006635
135. Chi H, Chun K, Son H, Kim J, Kim G, Roh S. Effect of genistein administration on the recovery of spermatogenesis in the busulfan-treated rat testis. *Clin Exp Reprod Med* (2013) **40**:60–6. doi:10.5653/cerm.2013.40.2.60
136. Ledent C, Valverde O, Cossu G, Petitet F, Aubert JF, Beslot F, et al. Unresponsiveness to cannabinoids and reduced addictive effects of opiates in CB1 receptor knockout mice. *Science* (1999) **283**:401–4. doi:10.1126/science.283.5400.401
137. Cobellis G, Cacciola G, Scarpa D, Meccariello R, Chianese R, Franzoni MF, et al. Endocannabinoid system in frog and rodent testis: type-1 cannabinoid receptor and fatty acid amide hydrolase activity in male germ cells. *Biol Reprod* (2006) **75**:82–9. doi:10.1095/biolreprod.106.051730
138. Cacciola G, Chioccarelli T, Ricci G, Meccariello R, Fasano S, Pierantoni R, et al. The endocannabinoid system in vertebrate male reproduction: a comparative overview. *Mol Cell Endocrinol* (2008) **286**:S24–30. doi:10.1016/j.mce.2008.01.004
139. Wenger T, Ledent C, Tramu G. The endogenous cannabinoid, anandamide, activates the hypothalamo-pituitary-adrenal axis in CB1 cannabinoid receptor knockout mice. *Neuroendocrinology* (2003) **78**:294–300. doi:10.1159/000074882
140. Maccarrone M, Wenger T. Effects of cannabinoids on hypothalamic and reproductive function. *Handb Exp Pharmacol* (2005) **168**:555–71. doi:10.1007/3-540-26573-2_18
141. Oláh M, Milloh H, Wenger T. The role of endocannabinoids in the regulation of luteinizing hormone and prolactin release. Differences between the effects of AEA and 2AG. *Mol Cell Endocrinol* (2008) **286**:S36–40. doi:10.1016/j.mce.2008.01.005
142. Grimaldi P, Orlando P, Di Siena S, Lolicato F, Petrosino S, Bisogno T, et al. The endocannabinoid system and pivotal role of the CB2 receptor in mouse spermatogenesis. *Proc Natl Acad Sci U S A* (2009) **106**:11131–6. doi:10.1073/pnas.0812789106
143. Fasano S, Meccariello R, Cobellis G, Chianese R, Cacciola G, Chioccarelli T, et al. The endocannabinoid system: an ancient signaling involved in the control of male fertility. *Ann N Y Acad Sci* (2009) **1163**:112–24. doi:10.1111/j.1749-6632.2009.04437.x
144. Cacciola G, Chioccarelli T, Mackie K, Meccariello R, Ledent C, Fasano S, et al. Expression of type-1 cannabinoid receptor during rat postnatal testicular development: possible involvement in adult Leydig cell differentiation. *Biol Reprod* (2008) **79**:758–65. doi:10.1095/biolreprod.108.070128
145. Meccariello R, Chianese R, Cacciola G, Cobellis G, Pierantoni R, Fasano S. Type-1 cannabinoid receptor expression in the frog, *Rana esculenta*, tissues: a possible involvement in the regulation of testicular activity. *Mol Reprod Dev* (2006) **73**:551–8. doi:10.1002/mrd.20434
146. Wenger T, Ledent C, Csernus V, Gerendai I. The central cannabinoid receptor inactivation suppresses endocrine reproductive functions. *Biochem Biophys Res Commun* (2001) **284**:363–8. doi:10.1006/bbrc.2001.4977
147. Chianese R, Ciaramella V, Fasano S, Pierantoni R, Meccariello R. Hypothalamus-pituitary axis: an obligatory target for endocannabinoids to

inhibit steroidogenesis in frog testis. *Gen Comp Endocrinol* (2014). doi:10.1016/j.ygcen.2014.02.010
148. Cobellis G, Ricci G, Cacciola G, Orlando P, Petrosino S, Cascio MG, et al. A gradient of 2-arachidonoylglycerol regulates mouse epididymal sperm cell start-up. *Biol Reprod* (2010) **82**:451–8. doi:10.1095/biolreprod.109.079210
149. Ricci G, Cacciola G, Altucci L, Meccariello R, Pierantoni R, Fasano S, et al. Endocannabinoid control of sperm motility: the role of epididymis. *Gen Comp Endocrinol* (2007) **153**:320–2. doi:10.1016/j.ygcen.2007.02.003
150. Cacciola G, Chioccarelli T, Altucci L, Viaggiano A, Fasano S, Pierantoni R, et al. Nuclear size as estrogen-responsive chromatin quality parameter of mouse spermatozoa. *Gen Comp Endocrinol* (2013) **193**:201–9. doi:10.1016/j.ygcen.2013.07.018
151. Cacciola G, Chioccarelli T, Fasano S, Pierantoni R, Cobellis G. Estrogens and spermiogenesis: new insights from type 1 cannabinoid receptor knockout mice. *Int J Endocrinol* (2013) **2013**:501350. doi:10.1155/2013/501350
152. Lazaros LA, Xita NV, Kaponis AI, Zikopoulos KA, Plachouras NI, Georgiou IA. Estrogen receptor alpha and beta polymorphisms are associated with semen quality. *J Androl* (2010) **31**:291–8. doi:10.2164/jandrol.109.007542
153. Lambard S, Galeraud-Denis I, Bouraïma H, Bourguiba S, Chocat A, Carreau S. Expression of aromatase in human ejaculated spermatozoa: a putative marker of motility. *Mol Hum Reprod* (2003) **9**:117–24. doi:10.1093/molehr/gag020
154. Lambard S, Galeraud-Denis I, Martin G, Levy R, Chocat A, Carreau S. Analysis and significance of mRNA in human ejaculated sperm from normozoospermic donors: relationship to sperm motility and capacitation. *Mol Hum Reprod* (2004) **10**:535–41. doi:10.1093/molehr/gah064
155. Lambard S, Galeraud-Denis I, Saunders PT, Carreau S. Human immature germ cells and ejaculated spermatozoa contain aromatase and oestrogen receptors. *J Mol Endocrinol* (2004) **32**:279–89. doi:10.1677/jme.0.0320279
156. Said L, Saad A, Carreau S. Differential expression of mRNA aromatase in ejaculated spermatozoa from infertile men in relation to either asthenozoospermia or teratozoospermia. *Andrologia* (2014) **46**:136–46. doi:10.1111/and.12058
157. Beck KJ, Herschel S, Hungershöfer R, Schwinger E. The effect of steroid hormones on motility and selective migration of X- and Y-bearing human spermatozoa. *Fertil Steril* (1976) **27**:407–12.
158. Idaomar M, Guerin JF, Lornage J, Czyba JC. Stimulation of motility and energy metabolism of spermatozoa from asthenozoospermic patients by 17 beta-estradiol. *Arch Androl* (1989) **22**:197–202. doi:10.3109/01485018908986772
159. Mattingly KA, Ivanova MM, Riggs KA, Wickramasinghe NS, Barch MJ, Klinge CM. Estradiol stimulates transcription of nuclear respiratory factor-1 and increases mitochondrial biogenesis. *Mol Endocrinol* (2008) **22**:609–22. doi:10.1210/me.2007-0029
160. Adeoya-Osiguwa SA, Markoulaki S, Pocock V, Milligan SR, Fraser LR. 17Beta-estradiol and environmental estrogens significantly affect mammalian sperm function. *Hum Reprod* (2003) **18**:100–7. doi:10.1093/humrep/deg037
161. Ded L, Dostalova P, Dorosh A, Dvorakova-Hortova K, Peknicova J. Effect of estrogens on boar sperm capacitation *in vitro*. *Reprod Biol Endocrinol* (2010) **8**:87. doi:10.1186/1477-7827-8-87
162. Carani C, Qin K, Simoni M, Faustini-Fustini M, Serpente S, Boyd J, et al. Effect of testosterone and estradiol in a man with aromatase deficiency. *N Engl J Med* (1997) **337**:91–5. doi:10.1056/NEJM199707103370204
163. Herrmann BL, Saller B, Janssen OE, Gocke P, Bockisch A, Sperling H, et al. Impact of estrogen replacement therapy in a male with congenital aromatase deficiency caused by a novel mutation in the CYP19 gene. *J Clin Endocrinol Metab* (2002) **87**:5476–84. doi:10.1210/jc.2002-020498
164. Maffei L, Murata Y, Rochira V, Tubert G, Aranda C, Vazquez M, et al. Dysmetabolic syndrome in a man with a novel mutation of the aromatase gene: effects of testosterone, alendronate, and estradiol treatment. *J Clin Endocrinol Metab* (2004) **89**:61–70. doi:10.1210/jc.2003-030313

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