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MODULATORS OF HYPOTHALAMIC-PITUITARY-GONADAL AXIS FOR THE CONTROL OF SPERMATOGENESIS AND SPERM QUALITY IN VERTEBRATES

Topic Editors
Rosaria Meccariello, Silvia Fasano,
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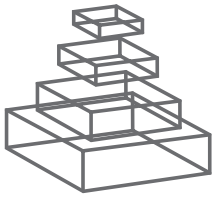

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MODULATORS OF HYPOTHALAMIC-PITUITARY-GONADAL AXIS FOR THE CONTROL OF SPERMATOGENESIS AND SPERM QUALITY IN VERTEBRATES

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Spermatogenesis is a process highly conserved throughout vertebrate species and is mainly under hypothalamic-pituitary control. It occurs in the testis in a stepwise fashion so that committed spermatogonia develop into spermatocytes and enter meiosis to produce round spermatids. These undergo a morphological transformation (spermiogenesis) into mature spermatids (i.e.: spermatozoa), which are differentially released from Sertoli cells (spermiation) depending on the species. In mammals, further transformations are necessary to form mature spermatozoa, suitable for fertilization. Gonadotropins, mainly responsive to gonadotropin-releasing hormone, control spermatogenesis through specific receptors located at the gonadal level. However, besides the endocrine route, the chemical mediators may also act locally in the gonad. Indeed, it is documented that testis physiology, including steroidogenesis and spermatogenesis, does not fully account for traditional endocrine control but an intragonadal network of autocrine and/or paracrine regulators also exists, whose activity, via cell-to-cell communication, regulates germ cell progression and development of qualitatively mature spermatozoa. Of note, a number of testicular modulators, such as gonadotropin releasing hormone, Kiss-peptin, endocannabinoids, has been early isolated in the brain and latest in the gonads. To fully understand precise mechanisms underlying the functional interaction of this intricate network, needless to say, it is crucially required to have detailed information about modulators and target cells.

Through synergy between the respective specializations of all the authors, this topic reviewed emerging knowledge about neuroendocrine and local mediators controlling germ cell progression and maturation.

Table of Contents

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EDITORIAL
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Modulators of hypothalamic–pituitary–gonadal axis for the control of spermatogenesis and sperm quality in vertebrates

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In both male and female, gametogenesis is regulated by hypothalamus–pituitary–gonadal axis (HPG) that corresponds to the hormonal axis, gonadotropin-releasing hormone (GnRH)–gonadotropins–steroids. Indeed, the main target of GnRH is the gonadotrope cells, located in the adenohypophysis. These, in turn, release two gonadotropin hormones, the follicle stimulating hormone (FSH) and the luteinizing hormone (LH), that through the main circulation reach gonads to regulate gametogenesis via the synthesis of steroid hormones. It is now accepted that further non-steroid factors support germ cell progression via intragonadal action (1).

The first evidence of relationships between pituitary and gonad came out in 1905 from a study on castrated animals, which showed hypertrophy of the pituitary gland (2). Later in 1910, Homans and co-workers (3) showed that the "experimental hypophysectomy" in prepubertal animals induced persistence of gonadal infantilism. Surprisingly, only in 1930 the reciprocal relationship between gonads and pituitary via feedbacks was elucidated (4). Later in 1954, the long feedback connecting the hypothalamus and the gonad was described (5), but only in the 1970s did the picture become complete through the description of the short- and ultrashort-feedback mechanisms. It was at the end of the 1970s that paracrine and autocrine communications were described as being carried out also by "classic" hormones (6). In particular, it was observed that chemical messengers acting through the bloodstream could be produced in multiple tissues, not necessarily including any of the traditional ductless glands. This observation led to the new definition of what constitutes a hormone by considering its function (ὁρμάω, to excite) rather than its source (ductless glands). A hormone may now be considered as a chemical messenger acting through endocrine (bloodstream), paracrine, and/or autocrine (local) routes. Furthermore, any chemical mediators, not only hormones, besides the endocrine route may also act locally in the gonad (7, 8).

In the testis, it has been demonstrated that a network of intragonadal endocrine, paracrine, and autocrine factors converge in a complex stage-specific multi-factorial control of spermatogenesis (6). Indeed, it has been documented that traditional endocrine control does not fully account for testis physiology, including steroidogenesis and spermatogenesis, and an intragonadal network of autocrine and/or paracrine regulators also exist, which regulates germ cell progression and development of qualitatively mature spermatozoa via cell-to-cell communication (9, 10).

The aim of this Research Topic is to give a comparative track on HPG axis activity for the control of spermatogenesis and quality sperm production. Through synergy between the respective specializations of all the authors, this Research Topic reviews the emerging knowledge about neuroendocrine and local mediators controlling progression and maturation of germ cells in male vertebrates.

The Research Topic firstly reports the description of a primitive HPG in hagfish, one of the only two extant members of the class of agnathans – the most primitive vertebrates known, living or extinct – providing evidence that there are neuroendocrine–pituitary hormones that share common structure and functional features compared to later evolved vertebrates (11). A complex set of neuronal network converges information concerning environmental, stressors, and metabolic cues onto the centers governing the reproductive axis. In this respect, the most recent discoveries in the central pathways integrating metabolism and reproduction in teleost fish have been reviewed here (12). However, the list of central and local modulators of HPG is growing up and currently comprises gonadotropin-inhibiting hormone, firstly identified in Japanese quail in 2000 (13) as an inhibitor of gonadotropin synthesis and release but subsequently identified in all vertebrates (14); classical female hormone such as estrogens that elicit their activity through genomic and non-genomic mechanisms (15); lastly endocannabinoids (16), a set of lipid mediators that share some of the effects with delta-9-tetrahydrocannabinol (Δ^9-THC), the active principle of marijuana plant, *Cannabis sativa*. The middle part of this Research Topic comprises a set of four review articles dedicated to the control of fetal and postnatal development of both Leydig and germ cells and to the intragonadal networks controlling the progression of the spermatogenesis (17–20); two original research articles point out the discussed involvement of new players such as kisspeptins in the local control of testis physiology (21) and the difficulties to reproduce the testicular environment *in vitro* to get a successful spermatogenesis (22). Lastly, in order

to gain the production of high quality sperm, the importance of antioxidant defenses (23), GnRH, kisspeptins, estradiol (24), and endocannabinoids (25) has been reported.

The last part of this Research Topic is focused on disease models such as Kallmann Syndrome (26), blindness (27), lysosomal storage disease (28), and cryptorchidism (29).

We hope that this contribution published in Frontiers in Endocrinology may represent a comprehensive guide in the plethora of data concerning the control of male reproductive activity and that readers might find new insights for the building of general models.

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Endocannabinoids as markers of sperm quality: hot spots

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Male reproductive health is under threat from a range of environmental and lifestyle assaults, including endocrine disrupters, toxic pollutants, and ionizing radiations, as well as lifestyle factors such as sexually transmitted infections, alcoholism, smoking, and anabolic steroid use. The latest potential hazard in our modern lifestyle is the use of plant-derived cannabinoids present in hashish and marijuana as recreational drugs, and more recently as therapeutic agents (1). In the last decade, a highly sophisticated endogenous cannabinoid system (ECS) has been discovered in mammals, where it regulates many physiological functions including human male reproduction (2–5). Here, I shall briefly discuss the activity of distinct ECS elements that can be useful to assess sperm function, and hence to potentially monitor sperm quality. Among others, these include the effect of type-1 cannabinoid receptor (CB_1) in regulating energy metabolism and motility of human sperm, and that of transient receptor potential vanilloid 1 (TRPV1) channels in controlling their fertilizing ability. Remarkably, both receptors share a common natural agonist, that is the endocannabinoid (eCB) *N*-arachidonoylethanolamine (anandamide, AEA); instead, another major eCB like 2-arachidonoylglycerol (2-AG) can activate CB_1, but is ineffective at TRPV1 receptors (6). The potential therapeutic exploitation of these ECS elements for the treatment of human infertility will be also addressed.

Human sperm express CB_1, and its activation by AEA affects motility and acrosome reaction (AR). Both processes require energy, and a major role for glycolysis in supplying ATP for sperm motility has been recognized. Recently, human sperm exposure to methanandamide, a non-hydrolyzable analog of AEA, has been shown to significantly decrease mitochondrial transmembrane potential without triggering any mitochondria-dependent apoptotic death, and such an effect was prevented by the CB_1 antagonist SR141716, but not by the CB_2 antagonist SR144528, nor by the TRPV1 antagonist iodoresiniferatoxin (7). Interestingly, in the presence of glucose human sperm exposure to methanandamide for up to 18 h failed to affect sperm motility, that instead was dramatically reduced by the same substance under glycolysis blockage; again, the latter effect was prevented by SR141716 (7). Overall, CB_1 activation induced a non-apoptotic decrease of mitochondrial potential, whose detrimental reflection on sperm motility could be revealed only when blocking glycolysis. These findings contribute to elucidate the relationship between CB_1, energetic metabolism and mitochondria, an issue that appears relevant well beyond sperm biology. Indeed, mitochondrial CB_1 activation has been recently reported to control energy metabolism in neurons (8), though the actual receptor localization on mitochondria remains controversial (9).

Another hot spot is the involvement of the AEA-binding TRPV1 receptor in human sperm fertilizing ability. Immunoreactivity for CB_1 has been localized in the post-acrosomal region and in the midpiece of human sperm, whereas for TRPV1 it was restricted to the post-acrosomal region (10). Capsazepine (CPZ), a selective antagonist of TRPV1, was shown to inhibit progesterone (P)-enhanced sperm/oocyte fusion, as evaluated by the hamster egg penetration test. This inhibition was due to a reduction of the P-induced AR rate above that of spontaneous AR, which was instead increased (10). Altogether, these data demonstrate that TRPV1 plays a key-role in the human sperm fertilizing ability, by impacting on its fusion with the oocyte membrane. In line with this, a marked decrease of the ability of TRPV1 to bind its ligands has been shown in infertile versus fertile sperm, again supporting a major role for this ion channel in sperm functionality (11). On this basis, one might speculate that the reduction of AEA causes infertile sperm to lose their quiescent state and with that, the ability to prevent premature capacitation. This could then precipitate a premature AR, rendering that sperm infertile because of a reduced ability to penetrate an oocyte *in vivo*, or in assisted conception such as in *in vitro* fertilization (IVF) protocols. This hypothesis has recently found grounds through a clinical study performed on men affected by asthenozoospermia and oligoasthenoteratozoospermia (12). Indeed, AEA levels in seminal plasma were found to be halved in patients with respect to normal subjects (∼0.08 versus ∼0.20 nM). Remarkably, these differences in AEA content in men with different pathological semen subtypes were associated with poor semen quality, such as decreased sperm count and abnormal sperm motility, as well as with alterations of CB_1 at transcriptional level (12). Therefore, evaluation of eCBs content in human sperm and/or in seminal plasma could be proposed as a novel diagnostic tool in reproductive medicine. In line with this, a marked reduction (down to ∼25%) of both AEA and 2-AG content in seminal plasma from infertile men has been recently documented (11). Instead, no significant alterations were found in sperm from infertile versus fertile men, neither for AEA nor for 2-AG (11). Collectively,

these data pinpoint eCBs (and AEA in particular) as new biomarkers to determine semen quality, thus opening new avenues for the treatment of infertility in humans.

Further points of interest in the regulation of sperm quality by ECS are related to the role of membrane properties and epigenetic control of chromatin activity.

Mammalian sperm become fertile after completing capacitation, a process associated with cholesterol loss and changes in the biophysical properties of the membranes, e.g., at the level of cholesterol-rich microdomains termed lipid rafts (13). Membrane raft dynamics prepares the sperm to undergo AR, and in addition it may have a role in sperm-egg membrane interaction (14). Interestingly, CB_1 and TRPV1 are affected by sperm membrane properties (15), and CB_1 signal transduction in enhanced by lipid raft disruption in different neuronal and immune cells (16). In addition, the AEA congener *N*-palmitoylethanolamine (PEA), that has been shown in the male reproductive tract, modulates plasma membrane polarity with an effect on Ca^{2+} influx during the capacitation process (17). Remarkably, PEA might also affect some physiological sperm kinematic parameters (like sperm motility), thus impacting on the development of hyperactivation during capacitation, ultimately leading to idiopathic infertility (18). Taken together, further investigations into the contribution of sperm membrane lipid composition to the control of eCB signaling, and hence to its relevance for sperm quality and fertilizing ability, hold promise for a better design of preventive and/or therapeutic strategies against infertility. In this context, it remains to be assessed whether (and to what extent) sperm functionality might be affected by accumulation of AEA and congeners in intracellular stores called adiposomes (or lipid droplets), that are present in sperm (19), and are important for eCB signaling in different cell types (20, 21).

The last hot spot that I would like to address concerns chromatin remodeling and epigenetic regulation of sperm functions. Because CB_1 activation plays a pivotal role in spermiogenesis (that is the developmental stage where DNA is remodeled), it has been recently hypothesized that regulation of the CB_1 gene (*Cnr1*) might also influence chromatin quality in sperm (22). By using *Cnr1* null mutant ($Cnr1^{-/-}$) mice, CB_1 activation was demonstrated to regulate indeed chromatin remodeling of spermatids, *via* either increasing the levels of the *Tnp2* gene (encoding for the transition protein 2, that stimulates DNA nick repair *in vitro*), or enhancing histone displacement. Comparative analysis of wild-type, $Cnr1^{+/-}$ and $Cnr1^{-/-}$ animals suggested the possible occurrence of haploinsufficiency for *Tnp2* turnover under CB_1 control, whereas histone displacement was disrupted in $Cnr1^{+/-}$ and $Cnr1^{-/-}$ mice to a lesser extent. Furthermore, flow cytometry analysis demonstrated that the genetic loss of *Cnr1* decreased sperm chromatin quality and was associated with sperm DNA fragmentation. Of note, this damage increased during epididymal transit, from caput to cauda (22). Collectively, these results demonstrate that the expression (and expectedly the activity) of CB_1 controls the physiological alterations of DNA packaging during spermiogenesis and epididymal transit, which might have major implications for male fertility, given the deleterious effects of sperm DNA damage (22). On a final note, it should be recalled that the epigenetic regulation of target genes by eCBs, and conversely that of ECS genes (in particular CB_1) by pathological conditions, are emerging as a major issue to understand the fine tuning of eCB signaling in human health and disease (23). Therefore, it can be anticipated that epigenetic studies on sperm quality and fertilizing capacity will open new avenues for preventing or curing (e.g., through a correct lifestyle) human infertility with innovative therapeutics.

In conclusion, distinct ECS elements like CB_1 and TRPV1, along with the endogenous levels of their common ligand AEA, hold the promise to represent useful diagnostic biomarkers and therapeutic targets of male fertility defects. It seems noteworthy that, while CB_1 has major effects also on female reproductive events (from oocyte development, to ovarian transport, and embryo implantation), apparently TRPV1 does not impact on female fertility (24), apart from generating hyperalgesia via primary sensory neurons during endometriosis (25). Therefore, the latter ion channel seems to represent an ideal target to specifically combat reproductive dysfunctions in males.

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Paracrine mechanisms involved in the control of early stages of mammalian spermatogenesis

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Within the testis, Sertoli-cell is the primary target of pituitary FSH. Several growth factors have been described to be produced specifically by Sertoli cells and modulate male germ cell development through paracrine mechanisms. Some have been shown to act directly on spermatogonia such as GDNF, which acts on self-renewal of spermatogonial stem cells (SSCs) while inhibiting their differentiation; BMP4, which has both a proliferative and differentiative effect on these cells, and KIT ligand (KL), which stimulates the KIT tyrosine-kinase receptor expressed by differentiating spermatogonia (but not by SSCs). KL not only controls the proliferative cycles of KIT-positive spermatogonia, but it also stimulates the expression of genes that are specific of the early phases of meiosis, whereas the expression of typical spermatogonial markers is down-regulated. On the contrary, FGF9 acts as a meiotic inhibiting substance both in fetal gonocytes and in post-natal spermatogonia through the induction of the RNA-binding protein NANOS2. Vitamin A, which is metabolized to Retinoic Acid in Sertoli cells, controls both SSCs differentiation through KIT induction and NANOS2 inhibition, and meiotic entry of differentiating spermatogonia through STRA8 upregulation.

Keywords: primordial germ cells, spermatogonial stem cells, spermatogenesis, meiosis, growth factors, paracrine control, signal transduction, gene expression

BRIEF INTRODUCTION: PARACRINE CONTROL OF FETAL MALE GERM CELL DEVELOPMENT

The control of the germ cell fate by paracrine factors secreted by the surrounding somatic environment already starts in the fetal life in the period of germ cell specification, independently from the influence of the hypothalamic-pituitary axis. Bone Morphogenetic Protein 4 (BMP4) has been shown to induce primordial germ cell (PGC) formation, to act as a PGC survival and localization factor within the allantois (1) and as a mitogen in *in vitro* cultured PGCs (2). During PGC specification in the extraembryonic mesoderm, SOX2 induction is required for the transcriptional regulation of KIT expression in PGCs (3). KIT is a tyrosine-kinase receptor, which is activated by KIT Ligand (KL), a growth factor expressed by the surrounding somatic environment. KL/KIT interaction is essential in the fetal period both during the specification of PGCs and for their proliferation and migration [(3–7), and references therein]. KIT expression is then down-regulated both in fetal oocytes undergoing meiosis and in gonocytes, which stop to proliferate after germ cell sex determination. Sertoli cells can prevent meiotic entry of gonocytes through the production of paracrine factors acting as meiotic inhibiting substances. The best characterized meiotic inhibiting substance produced by fetal Sertoli cells is Fibroblast Growth factor 9 (FGF9). FGF9 is a SRY/SOX9-dependent growth factor crucial for male sex differentiation acting on the somatic compartment of the fetal testis (8, 9). However, FGF9 also acts directly on male fetal gonocytes by upregulating levels of the RNA-binding protein NANOS2 (10, 11). NANOS2 prevents meiosis through the post-transcriptional regulation of key genes involved in the meiotic program (10, 12, 13). Recently, it has been shown that the meiosis-preventing activity of FGF9 in the fetal testis is mediated, at least in part, by NODAL, a member of the TGF-β family, and its partner Cripto (14–16).

In the same period in which FGF9 is expressed during testis determination, Sertoli cells produce an enzyme, CYP26B1, which degrades Retinoic Acid (RA) of mesonephric origin, in order to block Stimulated by Retinoic Acid 8 (STRA8) expression, and, as a consequence, to prevent premature gonocyte entry into meiosis (17–20). Although the identification of RA as the CYP26B1 substrate in the fetal testis (required for STRA8 induction and meiosis initiation in the fetal ovary) has been questioned (21), most of the available data in the literature support the role of RA as a master inducer of the mitotic-meiotic switch in germ cells (22). In line with this evidence is the finding that RA treatment down-regulates NANOS2 expression in fetal gonocytes (10).

PARACRINE CONTROL OF POST-NATAL MALE GERM CELL DEVELOPMENT

Pituitary gonadotropins, FSH, and LH, were originally identified for their essential role in ovarian function, as the stimulator of follicular activity and the inducer of follicular luteinization, respectively (23). Later on, it became clear that the same hormones play important roles also in testicular function, FSH being involved in the induction of spermatogenesis at puberty, and LH being the main inducer of androgen production (24). Spermatogenesis is a highly ordered differentiative process that occurs under FSH and androgen control. Sertoli cells, the only known targets for these hormones in the seminiferous tubules, mediate hormone action on spermatogenesis by controlling the germinal stem cell niche and by creating a suitable environment for the complex developmental events of germ cell proliferation and differentiation. Sertoli

cells directly orchestrate these complex events through both membrane intercellular communications and the production of growth factors and cytokines that act directly on the germ cell compartment. In the following paragraphs we will focus on the better characterized Sertoli-cell controlled paracrine mechanisms acting on the early stages of mammalian spermatogenesis, which are schematically summarized in **Figure 1**.

MAINTENANCE OF THE GERM STEM CELL NICHE

Spermatogonial stem cells (SSCs) are the direct descendants of fetal gonocytes. In the testis, SSCs are a subpopulation of undifferentiated spermatogonia residing in the basal layer of the seminiferous epithelium. Their mitotic expansion allows continuous production of germ cells committed to differentiation. One of the specific properties of SSCs and other undifferentiated spermatogonia that distinguishes them from differentiating spermatogonia is the expression of the Glial cell line-derived neurotrophic factor (GDNF)-family receptor α1 (GFRα1) and the c-Ret receptor tyrosine-kinase, which are both required for signaling in response to the Sertoli-cell-derived GDNF (25–28). GDNF has been shown to be essential for fate determination of SSCs, since in aging males heterozygotes for GDNF deletion, testes appear devoided of germ cells and show a phenotype similar to Sertoli-cell-only syndrome (25). Furthermore, overexpression of GDNF in mouse testes appeared to stimulate self-renewal of stem cells and block spermatogonial differentiation, inducing a seminomatous phenotype (25, 27). GDNF-induced activation of AKT and MEK signaling pathways in SSCs leads to increased generation of reactive oxygen species (ROS) generated by NAPDH oxidase 1, and apparently (contrary to their alleged detrimental role for spermatogenesis) ROS stimulate proliferation and self-renewal of SSCs through the activation of p38 and JNK MAPKs (29). Thus, GDNF is important for SSCs self-renewal, and, at the same time negatively controls their differentiation. This notion has been recently challenged by the finding that GFRα1-positive chained spermatogonia (A paired and A aligned) are more numerous than GFRα1-positive

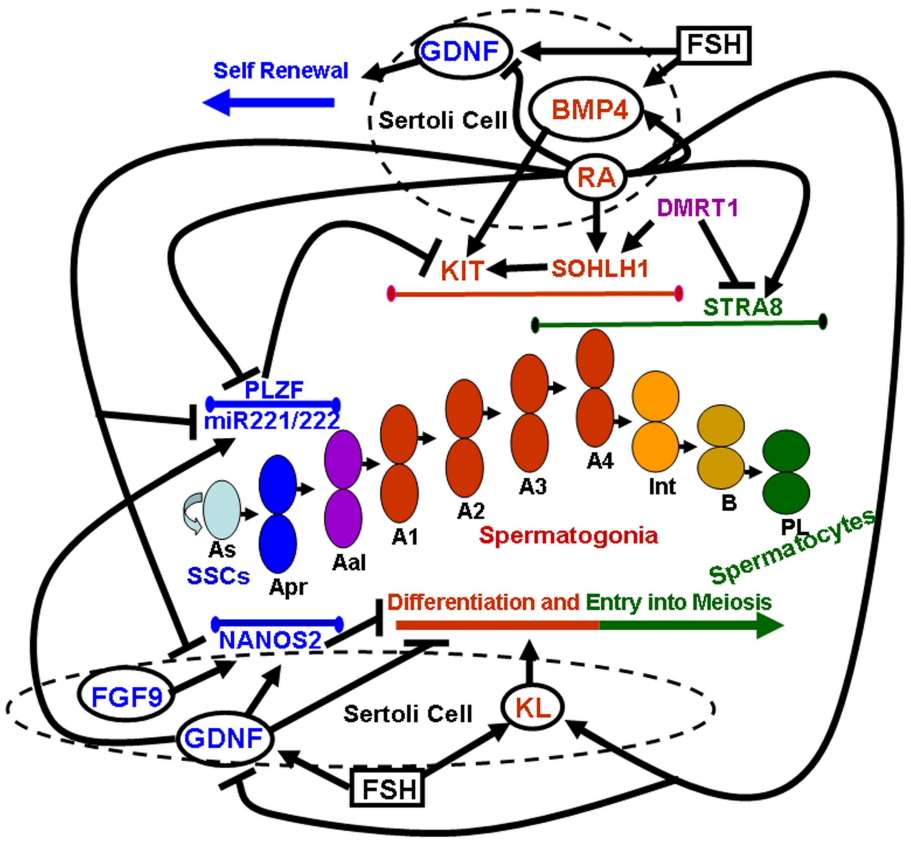

FIGURE 1 | Sertoli-cell controlled paracrine mechanisms acting on the early stages of mammalian spermatogenesis. Paracrine factors secreted by Sertoli cells (whose membrane is represented by dashed circles) are enclosed within solid line circles. Follicle stimulating hormone (FSH) is enclosed within a solid line square. Endogenous factors expressed by germ cells are represented by non-enclosed words. Blue colors refer to paracrine and endogenous factors that promote self-renewal of spermatogonial stem cells (SSCs) and inhibit spermatogonial differentiation and/or meiotic entry. Red colors refer to paracrine and endogenous factors that promote spermatogonial differentiation. Purple colors refer to endogenous factors which promote spermatogonial differentiation but at the same time inhibit meiotic entry. Green colors refer to endogenous factors that drive entry into meiosis. Lines delimited by small ellipsoids refer to the stage of expression of the germ cell endogenous factors involved in either self-renewal of SSCs and inhibition of differentiation (blue colors) or in differentiation (red colors) and meiotic entry (green colors). The succession of the various types of germ cells during the earliest stages of mouse spermatogenesis is represented in the center of the image: As, a single spermatogonia; Apr, a paired spermatogonia; Aal, a aligned spermatogonia; A1, A2, A3, A4, type A1–A4 spermatogonia; Int, intermediate spermatogonia; B, type B spermatogonia; PL, pre-leptotene spermatocytes.

A single spermatogonia, which are thought to represent the major SSCs reservoir in the mouse testis (30). However, GDNF signaling is essential to maintain NANOS2 expression in SSCs, and it has been proposed that this RNA-binding protein, besides its well-established role in preventing meiosis in fetal gonocytes, is also important to prevent spermatogonial differentiation in the post-natal testis (31). Overall, it is clear that GDNF mainly acts in positively regulating the proliferation of SSCs and maintenance of their undifferentiated state. Importantly, FSH and its second messenger cyclic AMP (cAMP) have been reported to stimulate GDNF expression in Sertoli cells (32, 33), which is instead down-regulated by RA treatment (33). These evidences suggest that that GDNF might be one of the paracrine factors that influences SSCs proliferation and population size under the control of the hypothalamic-pituitary axis.

CONTROL OF SPERMATOGONIAL DIFFERENTIATION

Undifferentiated SSCs (A single spermatogonia) have been described as single cells that are able both to renew themselves and to produce more differentiated A paired spermatogonia. The A paired cells then divide into A aligned spermatogonia that further differentiate into A1 spermatogonia (34). Appearance of A1 (differentiating) spermatogonia coincides with regain of the expression of KIT, encoding the receptor for KL (35–38). KIT mediates proliferation, survival, and differentiation in type A spermatogonia (33, 39–41). Upon KIT expression, spermatogonia become sensitive to KL produced by Sertoli cells (39, 42) and undergo a definite number of proliferative cycles, forming the A2-A4, intermediate, and B spermatogonia, before entering meiosis. The temporal appearance of KIT expression and of KL sensitivity in mouse spermatogonia, between 4 and 7 days postpartum (dpp) (33, 35, 36, 40), marks the switch from the A aligned spermatogonia to the A1-B differentiating cell types. Indeed, KIT is universally considered the most important marker that distinguishes differentiating spermatogonia from their undifferentiated precursors, including SSCs. Thus, paracrine factors in the testicular environment that stimulate KIT expression in mitotic germ cells play an essential role for the start of spermatogenesis at puberty. One of the paracrine signals involved in this event is BMP4, which is produced by Sertoli cells very early in the post-natal life, and whose expression is positively regulated by cAMP and RA (33, 43). Its receptor ALK3 and the SMAD5 transducer are expressed in undifferentiated spermatogonia, and *in vitro* treatment of these cells with BMP4 exerts both mitogenic and differentiative effects, inducing [^{3}H]thymidine incorporation and KIT expression both at the RNA and protein levels (43). As a result of the latter event, KIT-negative spermatogonia acquire sensitivity to KL (43). Since SSCs are able to renew themselves and at the same time to progress through differentiation (i.e., to the KIT-dependent stages of proliferation), BMP4 could be one of the factors that regulates such process. Alternatively, BMP4 could act on a subset of undifferentiated spermatogonia that have lost SSC features, i.e., that have entered the differentiative stage but are not yet KIT-positive. In agreement with the first possibility, BMP4 addition, on the opposite of GDNF, was shown to impair *in vitro* maintenance of mouse primary SSCs (44). Moreover, more recently BMP4 was shown to induce differentiation and KIT expression in a rat SSC cell line (45). In the adult testis, BMP4 has been reported to be produced by spermatogonia, but not by Sertoli cells (46), suggesting that it might work as a paracrine-autocrine factor modulating the establishment of the cycle of the seminiferous epithelium.

Another well-established paracrine factor involved in spermatogonial differentiation is the Vitamin A derivative RA. Mice kept on a diet deficient on vitamin A (VAD mice) or lacking vitamin A derivatives are sterile because the seminiferous tubules contain only undifferentiated KIT-negative spermatogonia, indicating a role of vitamin A in spermatogonia differentiation (38, 47). RA functions inside the nucleus recognizing two different classes of retinoid receptors. Both classes (RARs and RXRs) consist of three types of receptors, α, β, and γ, encoded by distinct genes and transduce RA signal by binding directly to RA-responsive elements. During post-natal development, each RAR is detected predominantly in a specific cell type of the seminiferous epithelium: RARα in Sertoli cells, RARβ in round spermatids and RARγ in type A spermatogonia (48). RARα conditional ablation in Sertoli cells showed germ cell apoptosis and seminiferous epithelium dysfunctions related to the disruption of Sertoli cells cyclical gene expression, which preceded testis degeneration (49). It has been reported that during the first, prepubertal, spermatogenic cycle RALDH-dependent synthesis of RA by Sertoli cells is indispensable to initiate differentiation of A aligned into A1 spermatogonia, and that this effect is mainly mediated by autocrine action of RA through RARα in the somatic compartment (50). However, RA (either the all-trans or the 9-cis Retinoic isomers) treatment *in vitro* exerts a direct effect on the differentiation of mitotic germ cell compartment by promoting KIT expression in undifferentiated spermatogonia (33, 51). This effect has been confirmed *in vivo* by the observation that targeted ablation of RARγ impairs the A aligned to A 1 transition in the course of some of the seminiferous epithelium cycles (52). Altogether these data indicate that RA favors spermatogonial differentiation through a direct action on spermatogonia and an indirect action mediated by changes in the expression pattern of paracrine factors such as KL, BMP4, and GDNF secreted by Sertoli cells (33).

Due to its importance for promoting expansion of differentiating spermatogonia, KIT expression in SSCs is subjected to a very tight transcriptional control. Promyelocytic Leukemia Zinc Finger (PLZF, also known as ZFP145, or ZBTB16) is a DNA sequence-specific transcriptional repressor that can exert local and long-range chromatin remodeling activity through the recruitment of DNA histone deacetylases and through the action of several nuclear corepressors (53). PLZF is specifically expressed in SSCs, and male PLZF knock-out (KO) mice show progressive spermatogonia depletion due to the deregulated expression of genes controlling the switch between self-renewal and differentiation (54–56). PLZF represses both endogenous KIT expression and expression of a reporter gene under the control of KIT regulatory elements (57). A discrete sequence of the KIT promoter, required for PLZF-mediated KIT transcriptional repression, was demonstrated to be bound by PLZF *in vitro* and also *in vivo*, by using chromatin immunoprecipitation (ChIP) of spermatogonia. Moreover, a 3-bp mutation in this PLZF binding site abolishes the responsiveness of the KIT promoter to PLZF repression In agreement with these findings, a significant increase in KIT expression

was found in the undifferentiated spermatogonia isolated from PLZF KO mice (57). Thus, one mechanism by which PLZF maintains the pool of SSCs is through a direct repression of KIT transcription, thus acting as a gatekeeper of spermatogonial differentiation. RA was shown to trigger downregulation of PLZF in SSCs (58), which might be part of the mechanisms which triggers up-regulation of KIT during spermatogonial differentiation.

Positive regulators of KIT transcription in spermatogonia are two b-Helix-Loop-Helix (HLH) transcription factors specifically expressed in germ cells, SOHLH1 (Spermatogenesis and Oogenesis HLH1), and SOHLH2. Both SOHLHs have been involved in the differentiation of spermatogonia and oocytes (59–64). In the male, deletion of each transcription factor leads to the disappearance of KIT-expressing spermatogonia in the prepuberal testis. An expression study of SOHLH1 and SOHLH2 during fetal and post-natal development showed a strong positive correlation between KIT and the two transcription factors in post-natal spermatogonia (65). SOHLH2 was found enriched mainly in undifferentiated spermatogonia, whereas SOHLH1 expression was maximal in KIT-dependent stages. Reporter gene expression driven by sequences contained within the KIT promoter and first intron was strongly up-regulated in transfection experiments overexpressing either SOHLH1 or SOHLH2, and co-transfection of both factors showed a cooperative effect (65). *In vivo*, co-immunoprecipitation results evidenced that the two proteins interact and overexpression of both factors increased endogenous KIT expression. Using ChIP analysis, SOHLH1 was found to occupy discrete bHLH binding site containing regions within the KIT promoter in spermatogonia chromatin (64, 65). Interestingly, expression of SOHLH1 was increased in post-natal mitotic germ cells by treatment with All-trans RA (65), which might be another mechanisms through which vitamin A derivatives triggers KIT up-regulation and spermatogonial differentiation. Using conditional gene targeting, it has been shown that loss of the Doublesex-related transcription factor DMRT1 in spermatogonia causes a precocious exit from the spermatogonial program and entry into meiosis (66). Apparently, DMRT1 acts in differentiating spermatogonia by restricting RA responsiveness, directly repressing transcription of the meiotic inducer STRA8, and activating transcription of SOHLH1, thereby preventing meiosis and promoting spermatogonial development (66). In agreement with the direct role played by SOHLH1 in regulating KIT transcription (65), a drastic reduction of KIT expression in spermatogonia was evident in testes from DMRT1 conditional KO mice (66).

Retinoic acid can up-regulate KIT expression in spermatogonia also at the post-transcriptional level, by interfering with the action of two X-linked microRNAs, miR-221 and miR-222 (67). Since miR-221/222 negatively regulate both KIT mRNA and KIT protein abundance in spermatogonia, impaired expression of these microRNAs in mouse undifferentiated spermatogonia induces transition from a KIT-negative to a KIT-positive state and loss of stem cell capacity to regenerate spermatogenesis. Undifferentiated spermatogonia overexpressing miR-221/222 were found to be resistant to RA-induced transition to a KIT-positive state and incapable of differentiation *in vivo* (67). Moreover, growth factors that promote maintenance of undifferentiated spermatogonia, such as GDNF, were found to up-regulate miR-221/222 expression. On the contrary, exposure to RA down-regulates miR-221/222 abundance (67). In conclusion, RA promotes progression of SSCs to differentiating spermatogonia through different mechanisms, all of which positively influence KIT expression: downregulation of PLZF and of miR-221/222, and up-regulation of SOHLH1.

CONTROL OF SPERMATOGONIAL EXPANSION

KIT ligand/KIT interaction is essential during post-natal stages of spermatogenesis for the expansion of the differentiating spermatogonia pool. KL, expressed by Sertoli cells, stimulates proliferation of differentiating type A1-A4 spermatogonia both by inducing their progression into the mitotic cell cycle and by reducing their apoptotic rate. This effect is exerted by the activated KIT tyrosine-kinase using as signal transducers both PI3K-AKT and MEK-ERK1/2 (39, 40, 68). The role of KIT/KL in the maintenance and proliferation of differentiating spermatogonia has been highlighted by a mouse genetic model with a point mutation of KIT that eliminates the PI3K docking site (Y719F) through a single bp change (69, 70). While PGC specification and proliferation in both sexes is not compromised during embryonic development, KIT(Y719F)/KIT(Y719F) males are sterile due to the lack of spermatogonia proliferation during the prepuberal period and an arrest of spermatogenesis at the pre-meiotic stages. The KIT/KL system is also an important mediator of the influence of hypothalamic-pituitary axis on the spermatogenic process. Indeed, the expression of the mRNA for KL is induced by FSH in prepuberal mouse Sertoli cells cultured *in vitro*, through an increase in cAMP levels (39, 42). The cAMP-dependent increase in KL expression in Sertoli cells is mainly due to direct activation of transcription from proximal promoter elements within the KL gene (71). Stage-dependent induction of KL mRNA expression by FSH has also been observed in the adult rat testis (72), and the maximal levels of KL mRNA induction are observed in stages of the seminiferous epithelium which show the maximal sensitivity to FSH stimulation, and in which type A spermatogonia are actively dividing. Interestingly, the soluble and membrane forms of KL, produced by alternative splicing, are differentially expressed during testis development. Sertoli cells from prepuberal mice mainly express the mRNA encoding for the transmembrane form, while the mRNA encoding for the soluble form is expressed at higher levels later, in coincidence with the beginning of the spermatogenic process, and the two transcripts are expressed at equivalent levels in the adult testis (39). Moreover, FSH and/or cAMP analogs, beside increasing KL mRNA levels, also modify the splicing pattern of the two isoforms in cultured mouse Sertoli cells in favor of the mRNA encoding for the soluble form (39). In agreement with these observations is the finding that the highest levels of the transmembrane form of KL are detected immunohistochemically in stages VII-VIII of the mouse seminiferous epithelium (73), which are the less sensitive to FSH stimulation in the adult testis (74). It has been hypothesized that the transmembrane form of KL could be physiologically relevant for the progression through the blood-testis barrier of mitotic germ cells entering the first meiotic prophase at stages VII-VIII (5). Moreover, even though at the onset of meiosis KIT expression in male germ cells ceases at both the RNA and protein levels (5), KL/KIT interaction, besides its well-established role in the expansion of differentiating type A spermatogonia, is

also important for entry into the meiotic program, i.e., the transition from type B spermatogonia to pre-leptotene spermatocytes, as discussed in the next paragraph.

CONTROL OF ENTRY INTO MEIOSIS

Retinoic acid acts in a bimodal mode to promote the spermatogenic process. Indeed, besides its important role in promoting progression of SSCs to differentiating spermatogonia through activation of KIT expression, RA also promotes expression of the meiotic inducer STRA8 in spermatogonia (33, 51). Besides RA of Sertoli-cell origin, it has been reported that also RA synthesized by pre-meiotic spermatocytes cell autonomously induces meiotic initiation through controlling the RAR-dependent expression of STRA8 in the same cells (50). Targeted ablation of STRA8 revealed a crucial role for this gene in the initial stages of the meiotic process in post-natal male germ cells, either in the transition from type B spermatogonia/pre-leptotene to leptotene spermatocytes (75), or in slightly later stages of the meiotic prophase, with mutant leptotene spermatocytes undergoing a premature mitotic-like chromosome condensation (76). The mechanisms through which STRA8 regulates the initial stages of meiosis in both sexes are currently unknown. However, the role played by STRA8 in male meiosis appears to be different from that played in the induction of the meiotic process in the fetal ovary, in which STRA8 ablation leads to an arrest of pre-meiotic DNA synthesis in pre-leptotene oocytes (18), whereas the last round of germ cell DNA synthesis appears not be affected in STRA8-deficient pre-leptotene spermatocytes (75, 76). RA was found to increase meiotic entry of mouse KIT-positive differentiating spermatogonia *in vitro*, as evaluated by both morphological and biochemical criteria (33). Increased expression of STRA8 and of early meiotic markers, such as DMC1, accompanied the morphological switch from spermatogonia to pre-leptotene and leptotene spermatocytes. RA treatment also increased STRA8 expression in *in vitro* cultured KIT-negative undifferentiated spermatogonia, which included SSCs, but this was not followed by induction of meiotic entry, suggesting that spermatogonial competence to enter meiosis is acquired only during the differentiative stages in which they undergo KIT-dependent mitotic divisions (33). Transcriptome analysis of *in vitro* cultured differentiating spermatogonia stimulated with recombinant KL revealed a pattern of RNA expression compatible with the qualitative changes of the cell cycle that occur during the subsequent cell divisions in type A and B spermatogonia, i.e., the progressive lengthening of the S phase and the shortening of the G2/M transition (41). Moreover, KL treatment was found to up-regulate in differentiating spermatogonia the expression of early meiotic genes, and to down-regulate at the same time typical spermatogonial markers, suggesting an important role for KL/KIT interaction in the transition from the mitotic to the meiotic cell cycle, and also an active role in the induction of meiotic differentiation (41). Indeed, morphological and biochemical analysis of *in vitro* cultured spermatogonia treated with KL revealed an induction of STRA8 and DMC1 expression and of meiotic entry, evaluated as a dramatic increase in the number of pre-leptotene and leptotene spermatocytes similar to the one induced by RA treatment (33). The effect of RA and KL on meiotic entry did not appear to be additive, implying that these factors converge on common signal transduction pathways to exert this effect. Indeed, similarly to KL, RA treatment induced KIT autophosphorylation, MEK-ERK1/2 and PI3K-AKT activation, and selective inhibitors of any of these pathways inhibited the biochemical and morphological signs of meiotic entry. Thus, together with genomic effects leading to increased expression of KIT in spermatogonia and of KL in Sertoli cells, RA also exerts rapid non-genomic effects in differentiating spermatogonia and converge with KL on common KIT-dependent signaling pathways for the induction of meiotic entry (33).

In order to ensure the homeostasis of the spermatogenic process, paracrine mechanisms, and endogenous effectors which negatively regulate spermatogonial differentiation and the onset of the meiotic process in post-natal spermatogenesis must coexist with positive inducers such as RA and KL. One of these paracrine mechanisms is analogous to the one operating to prevent meiosis onset in the fetal testis, and involves FGF9 expression in the somatic environment of the seminiferous epithelium and expression of the RNA-binding protein NANOS2 in pre-meiotic germ cells. In the post-natal testis, NANOS2 was found to be specifically expressed at both the RNA and protein level in KIT-negative undifferentiated spermatogonia, but not in KIT-positive differentiating spermatogonia, nor in meiotic or postmeiotic germ cells (10). FGF9 stimulation of *in vitro* cultured differentiating spermatogonia resulted in a dramatic induction of NANOS2 expression and inhibition of the morphological and biochemical signs of entry into meiosis, without apparent effects on the expression of STRA8, whereas RA treatment resulted in a deep inhibition in the levels of NANOS2 expression in undifferentiated spermatogonia, together with the previously described stimulation of STRA8 expression (10). Thus, together with playing an essential role in preventing meiosis of gonocytes in the male fetal testis, FGF9 acts as an inhibitor of meiotic differentiation through the upregulation of NANOS2 also in post-natal male mitotic germ cells.

FUTURE PERSPECTIVES

Obviously there must be also a paracrine influence of germ cells on Sertoli-cell production of factors involved in the local control of spermatogenesis, but, up to now, little information is available in the literature about these germ cell-generated signals. On the other hand, Sertoli cells are clearly the only mediators of the influence of the hypothalamic-pituitary axis on the spermatogenic process. FSH drives both Sertoli-cell secretion of GDNF, on one side, and of BMP4 and KL, on the other side. This actually fits with the double role exerted by the pituitary hormones, as inducers of spermatogenesis at puberty (through the local mediation of BMP4 and KL), but at the same time as essential for its maintenance and quantitative output (through GDNF stimulation of SSCs self-renewal). The factors which locally control the balance between GDNF vs. BMP4 and KL secretion by Sertoli cells in response to FSH might be germ cell-generated signals, and they must be the object of further studies.

Another puzzling observation is that FGF9 exerts opposite effects in KIT-positive differentiating spermatogonia with respect to those elicited by RA and KL signaling. Indeed, it is intriguing to notice that KL and FGF9 act on the same germ cell type stimulating receptor tyrosine-kinase activities (and thus presumably partially shared signal transduction pathways), yet they exert

opposite effects (differentiation and promotion of meiosis vs. prevention of meiotic entry). It will be very important to dissect the differences in intracellular signaling elicited in differentiating spermatogonia by these two antagonistic growth factors and the downstream cascade of events that lead to RA/KL-mediated induction of meiotic entry and FGF9-mediated inhibition of the same process. For instance, it will be interesting to characterize the subtypes of FGF receptors expressed in spermatogonia, and to investigate whether activation of NODAL signaling is involved in FGF9 action in post-natal male germ cells as it has been reported for male fetal gonocytes (14–16). Preliminary results from our laboratory indicate that both FGF9 and KL stimulate transient ERK1/2 activation in spermatogonia, but PI3K-dependent AKT activation is elicited by KL, but not by FGF9 (V. Tassinari, P. Rossi, and S. Dolci, unpublished results). This might be of particular importance, in light of the notion that in the mouse testis, as mentioned previously, a point mutation of KIT that eliminates the PI3K docking site cause a total block of the spermatogenic process between 8 and 10 dpp (69, 70), coinciding with of the onset of meiosis in the male germ cell line, and that PI3K inhibitors completely block induction of meiotic entry elicited *in vitro* by RA and/or KL treatment of differentiating spermatogonia (33).

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The endocannabinoid system and spermatogenesis

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Spermatogenesis is a complex process in which male germ cells undergo a mitotic phase followed by meiosis and by a morphogenetic process to form mature spermatozoa. Spermatogenesis is under the control of gonadotropins, steroid hormones and it is modulated by a complex network of autocrine and paracrine factors. These modulators ensure the correct progression of germ cell differentiation to form mature spermatozoa. Recently, it has been pointed out the relevance of endocannabinoids as critical modulators of male reproduction. Endocannabinoids are natural lipids able to bind to cannabinoid receptors and whose levels are regulated by specific biosynthetic and degradative enzymes. Together with their receptors and metabolic enzymes, they form the "endocannabinoid system" (ECS). In male reproductive tracts, they affect Sertoli cell activities, Leydig cell proliferation, germ cell differentiation, sperm motility, capacitation, and acrosome reaction. The ECS interferes with the pituitary-gonadal axis, and an intricate crosstalk between ECS and steroid hormones has been highlighted. This mini-review will focus on the involvement of the ECS in the control of spermatogenesis and on the interaction between ECS and steroid hormones.

Keywords: male germ cells, spermatogenesis, endocannabinoid system, sex hormones, cannabinoid

INTRODUCTION

Infertility affects 10–15% of couples, and it has been estimated that a male factor is responsible in approximately half of these cases. Male infertility is diagnosed with the analysis of several semen parameters, such as the number of total sperm, sperm motility, and percentage of sperm cells with a normal morphology. It is known that marijuana, the commonest recreational drug of abuse, has adverse effects on male reproductive physiology. Its use is associated with impotence, decreased testosterone plasma level, impairment of spermatogenesis, production of spermatozoa with abnormal morphology, reduction of sperm motility and viability and, more recently, with the occurrence of non-seminoma germ cell tumors (1). The identification of endogenous cannabinoids (ECBs) that mimic some effects of delta-9-THC, the active principle of *Cannabis sativa*, has opened new studies on the biological role of ECBs in male reproduction. In this mini-review we focused on the relevance of endocannabinoids and "endocannabinoid system" (ECS) in spermatogenesis and sperm functions, and on the interplay between ECS and sex hormone.

SPERMATOGENESIS

Spermatogenesis is a complex differentiative process starting from spermatogonial stem cells (SSCs), known as A-single (A_s). The A_s cells, similarly to other stem cells, have the capability to self-renew, producing daughter A_s cells, and to progress into "undifferentiated spermatogonia" known as A-paired (A_{pr}), and A-aligned (A_{al}) that represent committed cells. The A_{al} spermatogonia then differentiate into A1-4, intermediate (In) and B spermatogonia which undergo meiosis as pre-leptotene spermatocytes (2). Spermatocytes pass sequentially through leptotene, zygotene, pachytene, and diplotene phases of prophase I, and then quickly undergo two M-phase divisions, yielding haploid spermatids, that became spermatozoa through the morphogenetic process called spermiogenesis. Sperm released from the seminiferous epithelium into the tubule lumen are still immature and are not able to fertilize an egg. Sperm maturation occur in the epididymis. During spermatogenesis, germ cells, at each stage of differentiation, are in close contact with Sertoli cells which provide physical and metabolic support for their proliferation, meiosis, and successful progression into spermatozoa. Sertoli cells proliferate quickly during perinatal period and they switch to a mature, non-proliferative state, around the onset of puberty. Since only a limited number of germ cells can be supported by each Sertoli cell (3), in adult testis, the number of Sertoli cells will be a critical factor with obvious consequences on fertility.

Spermatogenesis continues throughout life and it is regulated by a complex assortment of hormones as well as numerous locally produced factors that include growth factors, cytokines, and chemokines, that act through autocrine and paracrine pathways. Sertoli cell-secreted growth factors are known to have direct effects mainly on spermatogonia: Gdnf acts on self-renewal of SSCs and inhibits their differentiation (4), Bmp4 has both a proliferative and differentiative effect on these cells (5), and Kit Ligand (KL), acts on the kit tyrosine-kinase receptor expressed by differentiating type A spermatogonia (6) stimulating their progression into the mitotic cell cycle and reducing apoptosis (7). The major hormonal control system of spermatogenesis is the hypothalamic-pituitary-gonadal axis, based essentially on the release of two gonadotropins, luteinizing hormone (LH) and follicle stimulating hormone (FSH), under the stimulation of hypothalamic GnRHs. Leydig and Sertoli cells, the somatic cells of the testis, are primary responders to circulating gonadotropin hormones and their failure to respond appropriately, results in male infertility (8). LH stimulates Leydig cells to synthesize testosterone (T) and FSH acts

on Sertoli cells stimulating their proliferation and expression of several trophic factors essential for spermatogenesis.

THE ENDOCANNABINOID SYSTEM

Endocannabinoids are lipid-signal molecules that are endogenous ligands for cannabinoid receptors, and together with enzymes responsible for their synthesis and degradation, they form the "ECS" (9). ECS is conserved from invertebrate to mammals and it assumes important role in physiological and pathological processes. The two best characterized endocannabinoids are *N*-arachidonoyl ethanolamine (AEA, anandamide) and 2 arachidonoyl glycerol (2-AG).

Endogenous cannabinoids bind to and activate their target receptors, causing several biological effects on different tissues. The main cannabinoid receptor targets type-1 (CB_1) and type-2 (CB_2) are seven trans-membrane G protein-coupled receptors (10). CB_1 is widely expressed in the nervous system mainly at the terminal ends of central and peripheral neurons, but it is also expressed in ovary, uterus, testis, vas deferens, and urinary bladder. CB_2 is mainly expressed in the cells of the immune system but it is also found in brainstem (11). ECBs are released from membrane phospholipid precursors by specific phospholipases, that are activated "on demand." AEA synthesis is catalyzed by an *N*-acylphosphatidylethanolamine-specific phospholipase D (NAPE-PLD) (12). Similarly, the formation of 2-AG involves a rapid hydrolysis of inositol phospholipids by a specific phospholipase C (PLC) to generate diacylglycerol (DAG), which is then converted into 2-AG by an sn-1-DAG lipase (DAGL) (13). As lipid molecules, ECBs diffuse passively through the membrane, but the presence of a membrane transporter, EMT, that acts by a facilitated diffusion mechanism, has been hypothesized (14, 15). More recently an anandamide transporter named FLAT, which facilitates its translocation into cells has been identified in neural cells (16). The biological effects of ECBs depend on their lifespan in the extracellular space, which is limited by a re-uptake by cells. Once inside the cells ECBs are hydrolyzed by two specific enzymes: the fatty acid amide hydrolase (FAAH) cleaves AEA into arachidonic acid and ethanolamine, and the monoacylglycerol lipase (MAGL) (17) transforms the 2-AG into arachidonic acid and glycerol (18).

AEA, but not 2-AG, behaves also as an endovanilloid binding to the type-1 vanilloid receptor (transient receptor potential vanilloid 1, TRPV1) at an intracellular site (19). TRPV1 is a six trans-membrane spanning non-selective cation channel, whose expression is found mainly in specialized sensory neurons that detect painful stimuli (20). However it is now established that TRPV1 is expressed also in non-neuronal cells, such as keratinocytes and epithelial and endothelial cells, where it could play a wide variety of physiological functions.

THE ENDOCANNABINOID SYSTEM AND SPERMATOGENESIS

ECS AND GERM CELLS

Following the discovery of ECS, many studies about its expression and function in male reproductive system have been carried out (21). The presence of components of ECS has been demonstrated in the testis, in the reproductive fluids and tracts, in different organisms from invertebrates to mammals. All the components of the ECS have been identified in mammalian germ cells, from spermatogonia to spermatozoa.

First evidence of an effect of cannabinoid in male reproduction comes from a study in sea urchin in which it was demonstrated that exogenous cannabinoid THC directly reduced the fertilizing capacity of sperm (22) through the inhibition of the acrosome reaction (23). Next, endogenous cannabinoid AEA was shown to induce the same effects of THC on sea urchin sperm (24).

Endocannabinoids have been identified in human seminal plasma (25), in the amphibian cloacal fluid (26) and in mouse epididymis (27) indicating a role in the control of sperm functions. Most of the *in vitro* studies reported an adverse effect of AEA on sperm function with inhibition of motility, capacitation and acrosome reaction, and indicated a pivotal role of CB_1 receptor in mediating AEA effects. In humans, AEA inhibits sperm motility by decreasing mitochondrial activity and this effect was blocked by the CB_1 receptor antagonist SR141716 (28). In boar (*Sus scropha*), a stable AEA analog, methanandamide, reduces sperm capacitation and inhibits acrosome reaction (29). Also in frog *Rana esculenta* AEA has been shown to inhibit sperm motility through CB_1 receptor (26).

It has been described a role of CB_1 in spermiogenesis, when elongated spermatids are remodeled to form mature spermatozoa with a change in the chromatin structure. Indeed, genetic inactivation of CB_1 causes an inefficient histone displacement, poor chromatin condensation, and DNA damage in sperm (30), indicating a role of ECS in spermatid differentiation.

Further interesting findings supporting a role of AEA and CB_1 receptor on sperm function arise from the gene knockout animal models. In the absence of CB_1 signaling, sperm acquire motility precociously and the percentage of motile spermatozoa recovered from the caput of epididymis was higher with respect to wild-type mice, suggesting a physiological inhibitory regulation of endocannabinoids on sperm motility in the epididymis (31). Genetic loss of FAAH results in increased levels of AEA in the reproductive system and impairment of sperm fertilizing ability (32). These results lead to hypothesize that an "adequate tone" of AEA and the expression of CB_1 receptor are critical in the formation of morphologically and functionally normal sperm. In support of this observation, it has been recently reported that, in rats, *in vivo* administration of HU210, a synthetic analog of THC and a potent agonist of CB receptors, causes a marked impairment of spermatogenesis with reduction in total sperm count and motility, and a deregulation of the ECS, confirming the *in vitro* observations and indicating that the use of exo-cannabinoids may influence adversely male fertility (33).

Another molecular target of AEA is the vanilloid receptor TRPV1 (34), expressed in sperm cells of mouse (35), boar (29), bull (36), and humans (37). Activation of TRPV1 receptor by AEA, seems to play a role in the stabilization of the plasma membranes in capacitated boar sperm, preventing spontaneous acrosome reaction (29). Therefore, AEA can bring different signals in sperm cells, depending on the target receptor (CB_1 or TRPV1) that is activated.

Besides AEA, also the endocannabinoid 2-AG has been reported to affect male reproduction. Using mouse male germ cell populations at different stage of differentiation we highlighted a pivotal role of 2-AG and CB_2 receptor in mouse spermatogenesis (35).

We demonstrated that mammalian male germ cells, from mitotic to haploid stage, have a complete ECS which is modulated during spermatogenesis. Spermatogonia possess higher level of 2-AG that decreases in spermatocytes and drastically drops in spermatids. This correlates to higher level of biosynthetic (DAGL) and lower level of degrading enzymes (MAGL) in spermatogonia with respect to spermatocytes and spermatids. On the contrary, AEA levels remain unchanged during spermatogenesis and probably are crucial to maintain, locally, an appropriate "anandamide tone" for a correct progression of spermatogenesis as seen for normal development of mouse embryos (38). Interestingly, activation of CB_2 receptor in spermatogonia promotes their progression into meiosis as revealed by an increased number of cells positive for the meiotic marker SCP3 and by the expression of premeiotic and early meiotic genes. Thus, during spermatogenesis an autocrine endocannabinoid regulation of mitotic germ cell differentiation might occur as proposed in **Figure 1**.

Endocannabinoid 2-AG has been also found to play a role in regulating the ability of spermatozoa to become motile during their transit in the epididymis. 2-AG levels are high in mouse spermatozoa isolated from the caput of the epididymis, where they do not move regularly, and decrease dramatically in spermatozoa isolated from the cauda, where they acquire vigorous motility, suggesting that, along the epididymis, the decrease of 2-AG levels from caput to cauda promotes start-up of spermatozoa (27). Finally mouse sperm capacitation has been found to be linked to an enhancement of the endogenous tone of both AEA and 2-AG (39), underlying the important role of ECS in regulating important step of spermatogenesis and sperm functions.

With the aim to investigate, in humans, a possible relationship between male reproductive dysfunction and deregulation of the ECS, recent studies have shown a marked reduction of AEA and 2-AG content in the seminal plasma of infertile patients. This reduction in sperm from infertile versus fertile men can be determined by either an increased ratio of degradation/biosynthesis, or by lower levels of CB_1 mRNA expression (40, 41), indicating that the ECBs signaling is involved in the preservation of normal human sperm function.

ECS AND TESTICULAR SOMATIC CELLS

Endocannabinoid system components are expressed also in somatic cells of mammalian testis. Sertoli cells possess the biochemical machinery to synthesize, transport, degrade, and bind both AEA (42) and 2-AG (43). Mouse Sertoli cells express a functional CB_2 receptor, an AEA membrane transporter and the AEA-degrading enzyme FAAH (42). AEA has been shown to have a pro-apoptotic effect on Sertoli cells, inducing DNA fragmentation. Lower level of AEA correlates with higher level of FAAH protein and with a decrease in Sertoli cell apoptosis, suggesting a protective and pro-survival role of FAAH in Sertoli cells. More interestingly, FAAH activity and expression is hormonally up-regulated in Sertoli cells by FSH and estrogen (43, 44).

Rat Leydig cells express CB_1 which is modulated during development and it negatively correlates to cell division. Immature Leydig cells in mitosis were negative for CB_1, while immature non-mitotic Leydig cells were positive, indicating a negative effect of CB_1 on Leydig cell proliferation and suggesting that their differentiation may depend on the ECS (45).

THE ENDOCANNABINOID SYSTEM AND SEX HORMONE

As described above, the ECS is widely distributed in testicular cells and it is an important regulator of spermatogenesis and sperm functions. Recently, many evidence indicate the existence of interplay between ECS and sex hormones, testosterone and estrogen, thus stressing the relevant role of ECS in regulating male reproduction. Testosterone is produced by Leydig cells under the stimulation of LH and it is essential for the occurrence of events like blood-testis-barrier formation, germ cells progression beyond meiosis, mature sperm release. Sertoli cells are the major cellular target for the testosterone signaling and the absence of testosterone or of the androgen receptor, results in the failure of spermatogenesis and infertility. Several studies on human males smoking cannabis, reported a decrease in plasma levels of testosterone, FSH, and LH and this effect was also evident in animal studies after acute and chronic administration of THC (46, 47). Decreased levels of testosterone correlate to an inhibitory effect of cannabinoids on male sexual behavior (48). Moreover *in vitro* studies on Leydig cells showed a decrease in testosterone secretion induced by THC (49). Similarly endogenous cannabinoid AEA suppresses LH and testosterone levels in wild-type, but not in CB_1 knockout mice (50), providing evidence that the ECS acts to suppress testosterone levels.

It is now well documented that, beside testosterone, also estrogens are important modulator of male reproduction (51). The

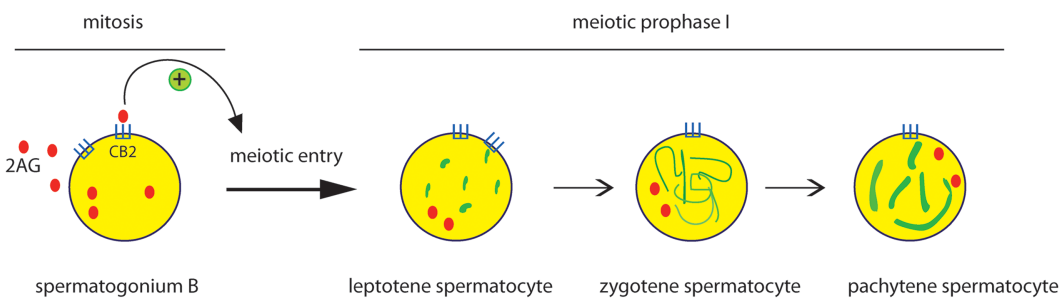

FIGURE 1 | Effect of CB_2 activation on the early steps of spermatogenic differentiation. Mitotic male germ cells express CB_2 receptor and high level of 2-AG. Activation of CB_2 receptor by 2-AG, through an autocrine pathway, promotes meiotic entry and progression of spermatogonia, as revealed by the meiotic organization of nuclear SCP3 (green) during the prophase I phases (leptotene, zygotene, pachytene).

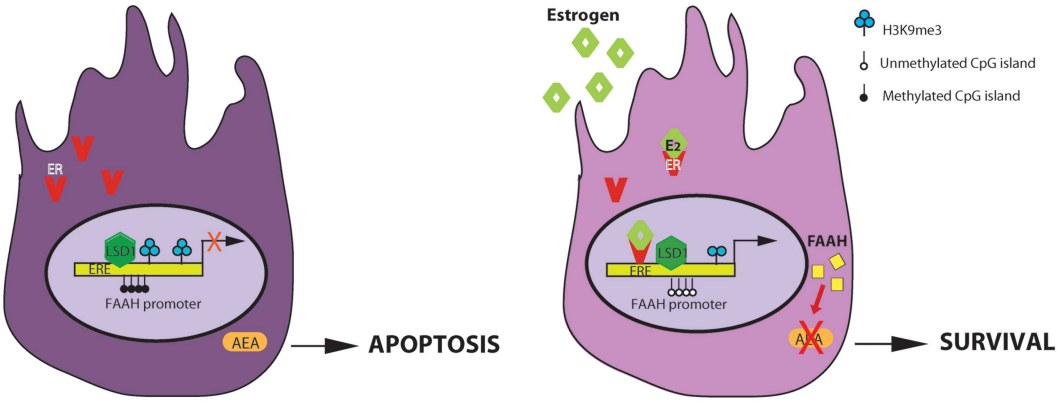

FIGURE 2 | Regulation of AEA-degrading enzyme FAAH expression by estrogen in Sertoli cells. E_2 regulates FAAH transcription by direct binding of estrogen receptor (ER) and epigenetic mechanisms including histone modification and DNA methylation. On the left: in the absence of estrogens, faah proximal promoter is methylated at DNA CpG sites and at lysine 9 of H3 histone and it is not competent for transcription. The final outcome is an increase AEA-induced apoptosis of Sertoli cells. On the right: estrogens activates the AEA-degrading enzyme FAAH transcription, through ER binding at ERE sites and reduction of DNA and H3K9me3 methylation. The direct/indirect interaction with histone demethylase LSD1, constitutively recruited at this site, is necessary for estrogen-induced transcription. The final outcome is a decrease of AEA-induced apoptosis of Sertoli cells (ERE, estrogen response element).

presence of estrogens in male reproductive tracts of numerous mammals has been reported (52). Aromatase is the enzyme that converts irreversibly androgen into estrogens and is expressed, in mammals, in all testicular cells except peritubular cells. The biological effects of estrogens are mediated by the estrogen receptors α (ERα) and β (ERβ), both expressed in mammalian testis. A role of estrogens in spermatogenesis is strongly supported by the observation that mice lacking estrogen receptors or aromatase are infertile and show impaired spermatogenesis in adulthood (53, 54).

Between all the components of ECS, the AEA-degrading enzyme *faah* gene has been demonstrated to be the only gene to be hormonally regulated in the testis. In Sertoli cells, FSH regulates FAAH expression and activity by triggering protein kinase A or aromatase-dependent pathway (42).

The PKA-dependent pathway enhances FAAH activity by inducing phosphorylation of other proteins that could activate the enzyme. On the other hand, the aromatase-dependent pathway, that leads to the conversion of testosterone into estrogens, induces FAAH expression at transcriptional level. Indeed we recently clarify the molecular mechanisms by which estrogens directly upregulate *faah* gene transcription (55). This involves direct binding of ER to the ERE sites in the *faah* promoter and the induction of epigenetic modifications in order to confer transcriptional competence.

As presented in **Figure 2**, in Sertoli cells, E_2 engages ER, which binds to ERE sites in the *faah* proximal promoter determining demethylation of both DNA, at CpG site, and histone H3, at lysine 9 (H3K9). The presence of histone demethylase LSD1, which is recruited at this site, ensures estrogens stimulation of *faah* transcription. LSD1 could interact with ligand-bound ER or with other different partners and activate gene transcription. The biological relevance of E_2-stimulation of FAAH expression consists in decreasing AEA levels in Sertoli cells and protect them against apoptosis induced by AEA. The pro-survival role of E_2 in Sertoli cells has a clear impact on spermatogenesis. In fact regulation of Sertoli cell apoptosis could be important to maintain their population size, and consequently, to sustain a normal spermatogenic output.

This is not the only example about the cross-talks between estrogens and ECS in the testis. Recent evidences reveal that estrogens affect spermiogenesis and regulate chromatin remodeling of germ cells (56). Indeed, in mice, genetic loss of CB_1 receptor causes a reduction in FSH and estrogen plasma levels and alteration in spermatid differentiation due to an inefficient histone displacement in the sperm. Estrogens treatment is able to rescue histone displacement suggesting a role in preserving chromatin condensation in spermatozoa.

CONCLUDING REMARKS

In this mini-review we highlighted the physiological role of ECS and its interplay with sex hormones, in male reproduction. A full comprehension of the molecular events regulated by ECS in the testis will allow to better define the "protective" role of this system in maintaining and ensuring the correct progression of spermatogenesis and the formation of mature and fertilizing sperm. Interfering with this system by exposure to exogenous cannabinoids, may alter the physiological function of ECS in male reproduction thus affecting male fertility.

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Does kisspeptin signaling have a role in the testes?

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Kisspeptins are a family of overlapping neuropeptides encoded by the *Kiss1* gene that regulate the mammalian reproductive axis by a central action in the hypothalamus to stimulate GnRH release. Kisspeptins and their receptor (GPR54 also called KISS1R) are also expressed in the testes but a functional role in this tissue has not been confirmed. We examined which cell types in the testes expressed kisspeptin and its receptor by staining for β-galactosidase activity using tissue from transgenic mice with *LacZ* targeted to either the *Kiss1* or the *Gpr54* genes. Expression of both genes appeared to be restricted to haploid spermatids and this was confirmed by a temporal expression analysis, which showed expression appearing with the first wave of haploid spermatid cells at puberty. We could not detect any kisspeptin protein in spermatids however, suggesting that the *Kiss1* mRNA may be translationally repressed. We tested whether kisspeptin could act on Leydig cells by examining the effects of kisspeptin on the immortalized Leydig cell line MA-10. Although MA-10 cells were shown to express *Gpr54* by RT-PCR, they did not respond to kisspeptin stimulation. We also tested whether kisspeptin could stimulate testosterone release by a direct action on the testes using explants of seminiferous tubules. The explants did not show any response to kisspeptin. The functional integrity of the MA-10 cells and the seminiferous tubule explants was confirmed by showing appropriate responses to the LH analog, human chorionic gonadotropin. These data suggest that kisspeptin signaling does not have a significant role in testes function in the mouse.

Keywords: kisspeptins, *Gpr54/Kiss1r*, testes, Leydig cells, testosterone secretion, spermatids

INTRODUCTION

Kisspeptins, encoded by the *Kiss1* gene, are an overlapping family of neuropeptides required for activation and maintenance of the mammalian reproductive axis [for review, see Ref. (1)]. Kisspeptins are encoded as a 145-amino-acid precursor protein in humans that is cleaved into shorter peptides (Kp54, Kp14, Kp13, and Kp10) that share a common RF-amide C-terminal decapeptide sequence. They all act as potent stimulators of GnRH release by signaling through the G-protein coupled receptor, GPR54 (also called KISS1R) expressed by GnRH neurons. Disruption of kisspeptin signaling causes hypogonadotropic hypogonadism in mice and humans (2–7). Mutant mice do not undergo sexual maturation at puberty and have low gonadotropic and sex steroid hormones levels caused by defective GnRH secretion from the hypothalamus. Conversely, activating mutations of *GPR54* cause precocious puberty in humans (8).

In addition to the role of kisspeptins in the central regulation of the reproductive axis, *Gpr54* expression has been detected in the testes of humans (9, 10), mice (3), rats (11), and frogs (12) raising the possibility that kisspeptins may also act at this location. Kisspeptins have been immunolocalized to Leydig cells in mice (13) and kisspeptin and GPR54 have been detected in human sperm, mainly localized to the head, neck, and the flagellum midpiece (14).

Although the expression profile of *Kiss1* and *Gpr54* suggests that kisspeptin signaling might have a role in the testes, very little has been done to test this hypothesis. The data to support a role for kisspeptin in the testes is largely circumstantial and based on discrepancies between the normally direct relationship of LH and testosterone levels. For example, in rats, chronic (13 days) subcutaneous administration of kisspeptin reduced testosterone secretion without a significant decrease in plasma LH (15). In Rhesus monkeys, continuous intravenous infusion of human kisspeptin over 4 days maintained plasma testosterone levels even after the LH stimulation levels had fallen (16). When circulating testosterone levels were expressed relative to LH levels, the [T]:[LH] ratios were significantly higher in the morning in the high dose kisspeptin treatment group compared to the vehicle group. This led to the suggestion that kisspeptins might augment the LH-induced secretion of testosterone. Support for this has come from kisspeptin administration in Rhesus monkeys pre-treated with acyline, a GnRH receptor antagonist, to allow the intratesticular actions of kisspeptin to be evaluated without the confounding effects of LH release from the pituitary (17). Kisspeptin administration significantly increased human chorionic gonadotropin (hCG)-stimulated testosterone levels in acycline treated monkeys compared to hCG treatment alone (17) suggesting that kisspeptin might enhance LH responses in Leydig cells.

To further investigate the possible function(s) of kisspeptin in the mouse testes, we used transgenic mice with *Kiss1* and *Gpr54* alleles targeted with a *LacZ* reporter gene to define the testicular cell expression profile of these genes. We also tested whether

kisspeptins can stimulate testosterone release from an immortalized mouse Leydig cell line and from primary testes explants in culture.

MATERIALS AND METHODS

MOUSE LINES AND MAINTENANCE

The 129S6/SvEv mutant mice with a targeted disruption of the *Gpr54* or *Kiss1* genes were generated as described previously (2, 5). All mice were maintained on a 12:12-h light-dark cycle (light on between 6:30 a.m. and 6:30 p.m.) with *ad libitum* access to food and water. Experimental procedures were performed under authority of a Home Office Project License and approved by a Local Ethics Committee.

MA-10 CELL CULTURE

The mouse Leydig tumor cell line MA-10 (18) was a generous gift from Dr. Mario Ascoli (University of Iowa, Iowa City, IA, USA). The MA-10 cells were maintained in RPMI-1640 medium (Sigma-Aldrich, Dorset, UK) containing 10% horse serum (Sigma-Aldrich, Dorset, UK) and 10% newborn calf serum (Sigma-Aldrich, Dorset, UK), and the cells were grown at 37°C in an humidified atmosphere of 5% CO_2. The growth medium was refreshed every 2 days to provide sufficient nutrition for cell growth.

RT-PCR GENE EXPRESSION ANALYSIS OF MA-10 CELLS

Total RNA was isolated from MA-10 cells using a NucleoSpin® RNA II kit (Cat No: 740955, MACHEREY-NAGEL GmbH & Co. KG) following the manufacturer's protocol. The time of the on-column DNA digestion was extended from 15 to 45 min to ensure complete removal of genomic DNA. The RNA was reverse transcribed into cDNA using SuperScript III Reverse Transcriptase (Cat No: 18080-044, Invitrogen, UK) following the protocol provided by the manufacturer. Standard PCR was performed as follows: the samples were denatured for 5 min at 95°C and amplified for 44 cycles (30 s at 93°C, 1 min at 60°C, and 2 min at 70°C). The primer sequences were: *Kiss1* (Forward: tgctgcttctctctgtgtcg; Reverse: gccgaaggagttccagttgta, 310 bp product), *Gpr54* (Forward: gccttcgcgctctacaacctgctg; Reverse: aaggcatagagcagcggattgagc, 367 bp product), *GnRH* (Forward: cggcattctactgctgactgt; Reverse: catcttcttctgcctggcttc, 229 bp product), β-actin (Forward: ctgtattcccctccatcgtg; Reverse: gggtcaggatacctctcttgc, 113 bp product). RNA without a reverse transcription step was used as a negative control for identification of genomic DNA contamination and cDNA from wild-type hypothalamus was used as a positive control for *Kiss1* amplification.

X-GAL STAINING OF TESTES SECTIONS

Testes were fixed in 1% paraformaldehyde/PBS overnight at 4°C, cryoprotected with 30% sucrose/PBS overnight at 4°C, and cryosectioned at 20 μm onto poly-lysine coated slides. Sections were air dried at room temperature, rehydrated in PBS and β-galactosidase activity detected using a *LacZ* staining solution [1 mM $MgCl_2$, 1 mg/ml X-gal (5-bromo-4-chloro-3-indolyl β-D-galactopyranoside), 5 mM potassium ferricyanide, 5 mM potassium ferrocyanide in PBS] at 37°C overnight, and counterstained with 1% Saffronin.

IMMUNOHISTOCHEMISTRY TO DETECT KISSPEPTIN EXPRESSION

Testes were fixed in 4% paraformaldehyde/Tris-buffered saline (TBS) for 5 h at room temperature and transferred to 30% sucrose/TBS overnight at 4°C. The testes were then cryosectioned at 15 μM, air dried at room temperature, rehydrated in TBS, and slide-mounted immunohistochemistry was performed to detect kisspeptin expression. Polyclonal antibody AC566 raised in rabbits against mouse Kp10 was a generous gift from Alain Caraty, Tours, France. Characterization and specificity of AC566 has been described previously (5, 19–21).

Sections were treated with 3% hydrogen peroxide for 15 min to quench endogenous peroxidase and then washed in TBS. To visualize kisspeptin expression, sections were incubated with the antibody at 1:2000 dilution for 8 h at room temperature. For secondary antibody labeling, sections were incubated with biotinylated goat anti-rabbit (1:100; Cat No: BA-1000, Vector Laboratories, Peterborough, UK) immunoglobulins at room temperature followed by incubation with Vector avidin-peroxidase (1:50; Cat No: PK-4000, Vector Laboratories, Peterborough, UK). Finally, the sections were rinsed and immunoreactivity was revealed with glucose-oxidase and nickel-enhanced diaminobenzidine hydrochloride (12.5 mg/ml). Sections were counterstain with hematoxylin, dehydrated in ethanol followed by Histoclear, and then coverslipped with DPX.

PROGESTERONE RELEASE EXPERIMENTS FROM MA-10 CELLS

The MA-10 cells were seeded at 2.5×10^5 cells/well (24-well plates) 24 h before the hormone treatment. The cells were treated with increasing concentrations of Kp10 (human Metastin 45–54) (1, 10, or 20 μM) (Cat No: M2816, Sigma-Aldrich, Dorset, UK) or Kp10 followed by hCG (0.012 IU/ml as the final concentration) (Cat No: CG5, Sigma, Saint Louis, MO, USA). PBS was added as a negative control. Each condition was tested in triplicate. After 4 h, the media was collected for progesterone measurement. After collection of media, the MA-10 cells were rinsed twice with PBS and lysed in 1× lysis buffer (reporter lysis buffer, Cat No: E397A, Promega, UK) by a freeze-thaw cycle. The lysate was briefly centrifuged and the protein content of the supernatant determined with a Bio-Rad Bradford Assay following the standard protocol.

PRIMARY CULTURE OF TESTES EXPLANTS

The testes from adult wild-type mice were cut into two pieces (approximately 40 mg/piece) without removing the tunica and each piece was cut and flattened to a 1-mm thickness on a Nylon membrane (Cat No: 1417240, Boehringer-Mannheim, Indianapolis, IN, USA) floating in phenol red-free DMEM medium (Cat No: 21063, GIBCO) (300 μl/well for 12-well plate) supplemented with 1× penicillin/streptomycin. The tissues were immediately treated with vehicle, Kp10 (1 μM), hCG (0.6 IU/ml), or a mixture of Kp10 (1 μM) and hCG (0.6 IU/ml), respectively. Each condition was tested in at least four repeat wells. The tissues were cultured at 37°C in an atmosphere of 5% CO_2 and the media collected at different time points. Fresh media was added at each time point after media collection.

HORMONE ASSAYS

Testosterone and progesterone were measured using ELISA kits (Cat No: EIA1559 and EIA1561 from DRG International, USA)

according to the manufacturer's instructions. The testosterone ELISA kit had a sensitivity of 0.083 ng/ml, an inter-assay variation of 6.7%, and an intra-assay variation of 3.3%. The progesterone ELISA kit had a sensitivity of 0.045 ng/ml, intra-assay variation of 7%, and inter-assay variation of 5%.

RESULTS

Gpr54 AND *KISS1* ARE EXPRESSED IN THE MOUSE TESTES

The *Gpr54* and *Kiss1* alleles in the transgenic mice have been tagged with a *LacZ* gene that allows their gene expression patterns to be visualized by staining for β-galactosidase activity. Staining was observed within seminiferous tubules from both *Kiss1*$^{+/-}$ (**Figure 1A**) and *Gpr54*$^{+/-}$ mice (data not shown) but not in wild-type mice (**Figure 1A**). Background staining was observed in the epididymis and the vas deferens of wild-type mice as the epithelial cells in these tissues express an endogenous galactosidase-like enzymatic activity. To define the cells in which *Kiss1* and *Gpr54* are expressed, cryosection of testes were stained for β-galactosidase activity. The staining in cryosections was localized to the region of the seminiferous tubules that contained round spermatids (arrowed in **Figures 1B,C**). The spermatids are easily recognized as they have smaller nuclei than spermatocytes and are four-times more abundant as they have just completed meiosis. Very faint β-galactosidase staining was also found in the Leydig cells in the *Gpr54*$^{+/-}$ mice (**Figure 1D**) but not in the *Kiss1*$^{+/-}$ mice (data not shown).

To confirm that the β-galactosidase expression was localized to spermatid cells, the time point at which expression was first observed during the first spermatogenic cycle was determined. Expression of *Kiss1* and *Gpr54* could not be observed prior to 3 weeks of age but staining was seen from 1 month of age which corresponds to the time at which the spermatids first appear in mice (**Figure 2**).

Kisspeptin protein expression in the mouse testes was visualized using a well characterized rabbit antiserum highly specific for mouse Kp10 (20). Strong immunoreactivity was found in Leydig cells with no staining in spermatids (**Figure 1E**). As a control for antibody specificity, testes sections from *Kiss1* mutant mice lacking kisspeptin protein were used and no immunoreactivity was observed (**Figure 1E**). This Leydig staining may be non-specific however, as no kisspeptin protein was detected in *Gpr54* mutant mice (data not shown).

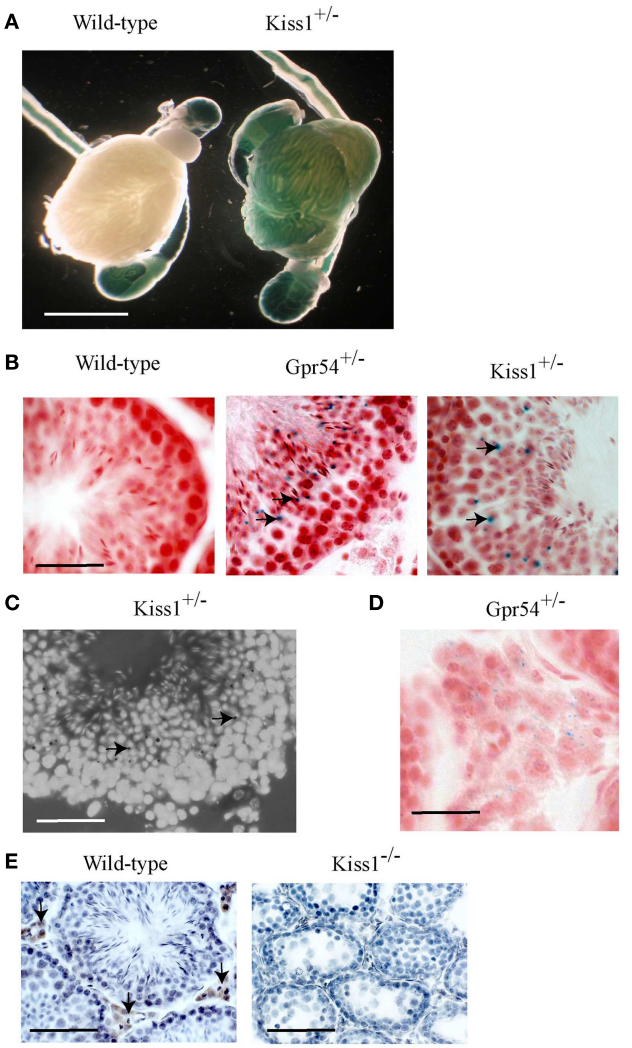

FIGURE 1 | *Kiss1* and *Gpr54* expression in the mouse testes. **(A)** Expression of *Kiss1* in seminiferous tubules of the testes visualized by X-gal staining (blue) for β-galactosidase activity. Note the non-specific staining in the epididymis and vas deferens of the wild-type testes. Scale bar = 5 mm. **(B)** Cryosections of testes from adult wild-type, *Gpr54*$^{+/-}$, and *Kiss1*$^{+/-}$ mice showing expression (arrowed) localized to spermatid cells of seminiferous tubules. Sections were stained for β-galactosidase activity (blue) and counterstained with Saffronin (red). Scale bar = 100 μm. **(C)** Testes cryosection from *Kiss1*$^{+/-}$ mice stained for β-galactosidase activity (black dots, arrowed) and counterstained with DAPI to visualize cell nuclei illustrating clearer expression in spermatid cells. Scale bar = 100 μm. **(D)** Low expression of *Gpr54* in Leydig cells visualized by X-gal staining. Scale bar = 50 μm. **(E)** Kisspeptin immunoreactivity localized to Leydig cell in wild-type mice (arrowed) but not *Kiss1* mutant mice. Scale bar = 200 μm.

Kp10 DOES NOT STIMULATE STEROIDOGENESIS IN THE LEYDIG CELL LINE, MA-10

To test whether Kp10 could stimulate testosterone release, the mouse Leydig cell line, MA-10, was used (18, 22). MA-10 cells, like normal Leydig cells, express LH receptors and respond to hCG stimulation. MA-10 cells have low expression and activity of P450c17 that is the enzyme that converts progesterone into 17-OH progesterone and finally into testosterone, thus MA-10 cells produce progesterone as the principle steroid hormone instead of testosterone (18, 22).

The MA-10 cells were examined for expression of *Kiss1* and *Gpr54* to determine whether they might be capable of responding to kisspeptins. There was a PCR product for *Gpr54* (**Figure 3A**), indicating that MA-10 cells endogenously express this gene but there was no detectable *Kiss1* expression in the MA-10 cells. The MA-10 cell also expressed GnRH and β-actin transcripts (**Figure 3A**). No products were observed when non-transcribed RNA was used as the template, indicating that the RNA was free of genomic DNA contamination. A *Kiss1* product was observed when hypothalamic cDNA was used from wild-type mice as a positive control.

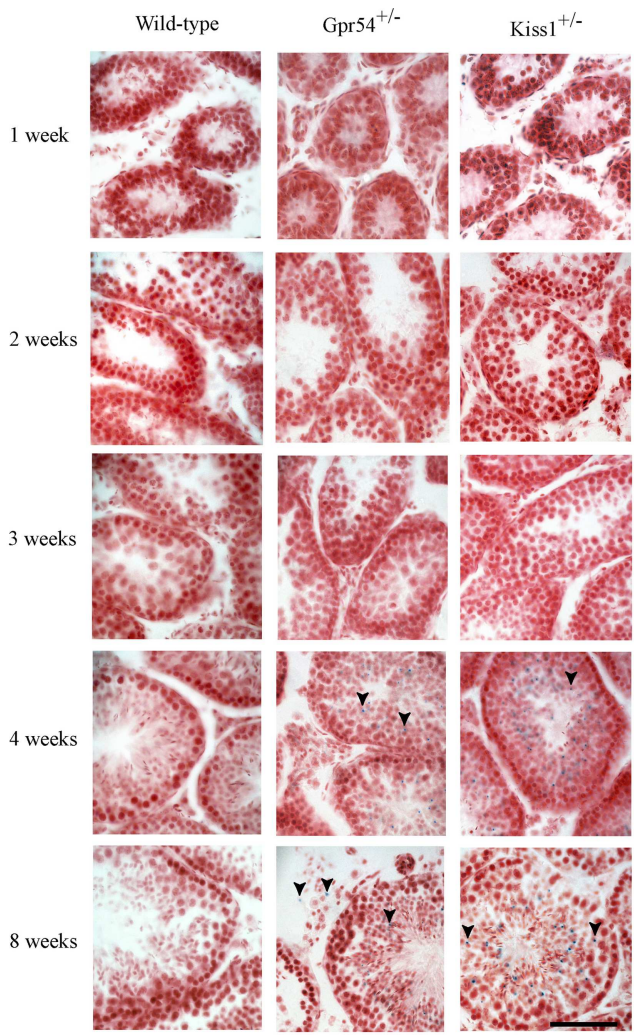

FIGURE 2 | Developmental time course of *Gpr54* and *Kiss1* expression in the mouse testes. Testes of wild-type (WT), *Kiss1*$^{+/-}$ and *Gpr54*$^{+/-}$ mice at different ages were cryosectioned, stained for β-galactosidase activity, and counterstained with Saffronin. Blue dots (arrowed) indicate expression of the *Kiss1* and *Gpr54* genes. All photographs are at the same magnification. Scale bar = 50 μm.

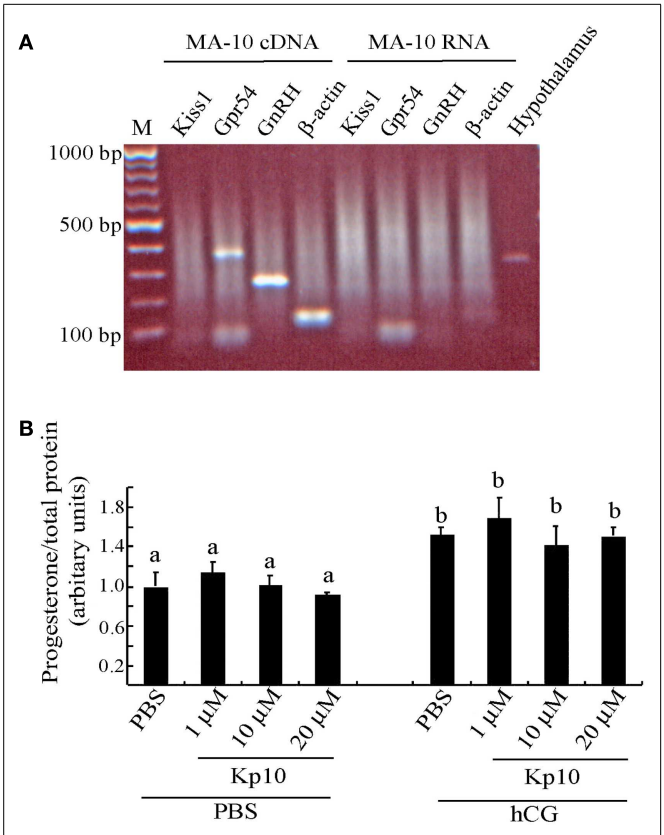

FIGURE 3 | The MA-10 Leydig cell line expresses *Gpr54* but do not respond to Kp10 treatment. **(A)** RT-PCR gene expression analysis in the MA-10 Leydig cell line. Expression of *Gpr54, GnRH*, and β-*actin* was detected in cDNA from the MA-10 cells but *Kiss1* expression was not detected. Hypothalamic cDNA was included as a positive control for *Kiss1* expression. **(B)** Effect of Kp10 on stimulating progesterone release from the MA-10 cell line. The MA-10 cells were stimulated with the hormones indicated and media collected after 4 h and tested for progesterone. Different letters (a and b) indicate statistically significant differences between groups ($P < 0.05$, one way ANOVA with a Tukey comparison post-test).

As the MA-10 cells expressed the kisspeptin receptor, they were tested to see whether Kp10 could stimulate progesterone release. The cells were divided into two experimental groups. The first group was treated with increasing concentrations of human Kp10 followed by PBS, and the second group was treated with Kp10 followed by hCG to examine possible synergistic effects. After 4 h, the media was assayed for progesterone, which was normalized to the protein content of the cell lysate to correct for variations in cell number. No significant difference in progesterone release was found between the vehicle (PBS) treatment and any of the three concentrations of Kp10 (**Figure 3B**), indicating that Kp10 cannot enhance progesterone release from the Leydig cell line even at a high concentration (20 μM). There was also no significant difference in progesterone release between the cells treated with hCG alone or those treated with hCG and Kp10 (**Figure 3B**), which suggests that Kp10 has no synergistic effect on progesterone release from MA-10 cells activated by hCG. However, there was a significant difference ($P < 0.05$) in progesterone release between the cells treated with PBS and those treated with hCG (**Figure 3B**), which indicates the functional responsiveness of the cells to hormonal stimulation.

Kp10 DOES NOT STIMULATE TESTOSTERONE RELEASE FROM TESTES TISSUE CULTURE EXPLANTS

To examine the possible action of Kp10 in a more physiological system, we tested whether Kp10 could stimulate testosterone release from primary explants of mouse testes. Pieces of adult wild-type mouse testes of similar weight (approximately 40 mg/piece) were treated with vehicle (PBS), Kp10 (1 μM), hCG (0.6 IU/ml), or a mixture of Kp10/hCG. Each condition was repeated with at least four samples. The media was collected at different time intervals for testosterone measurements. During the 0- to 4-h

time period, the testosterone released after hCG treatment was significantly higher than that with the vehicle (PBS) treatment (**Figure 4A**), indicating that the cultured testes maintained the ability to respond to hormone stimulation. However, there was no obvious stimulation of testosterone release after Kp10 treatment. Also, the testosterone released in the PBS and hCG groups was not significantly different at incubation times >4 h due to increased unstimulated testosterone release (**Figure 4A**). Therefore, a 4-h incubation time was used to test whether there was any synergy between Kp10 and hCG in stimulating testosterone release (**Figure 4B**). Once again, testosterone release after hCG treatment was significantly higher than after PBS treatment (**Figure 4B**). No difference in testosterone release was detected between the testes fragments cultured in PBS or Kp10. There was also no difference in testosterone release between testes treated with hCG only and testes treated with hCG and Kp10 together. These data indicate that Kp10 has no effect on testosterone release from adult mouse testes and it has no synergistic action on testosterone release stimulated by hCG.

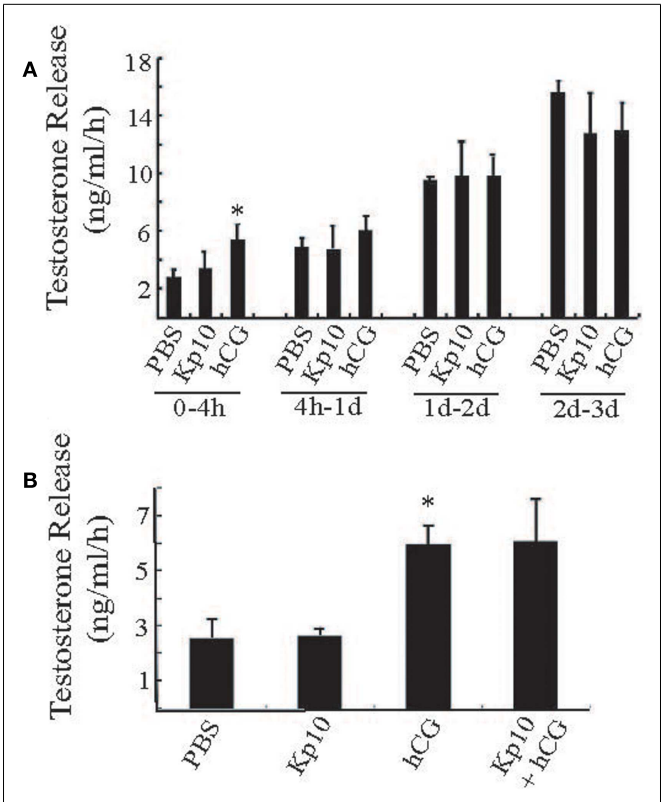

FIGURE 4 | Testing Kp10 stimulation of testosterone release from fresh testes explants. **(A)** Time course of testosterone release from explanted mouse testes tissue. Pieces of adult mouse testes were cultured in PBS, Kp10 (1 μM), or hCG (0.6 IU/ml), respectively. The media was collected at the time points indicated, and testosterone levels measured by an ELISA. **(B)** Evaluation of synergistic action of Kp10 on hCG-mediated testosterone release. Pieces of adult mouse testes were cultured in PBS, Kp10 (1 μM), hCG (0.6 IU/ml), or a mixture of Kp10 (1 μM) and hCG (0.6 IU/ml). The media was collected after 4 h incubation and testosterone levels measured by an ELISA. $n \geq 4$ for all columns. *Statistically significant difference ($P < 0.05$) between the hCG and the vehicle control group (PBS) (unpaired *t*-test with Welch's correction).

DISCUSSION

Kisspeptin neuropeptides are important central regulators of the mammalian reproductive axis with kisspeptin neurons acting upstream of GnRH neurons to stimulate GnRH release. In addition to this central role however, the expression profiles of *Kiss1* and *Gpr54* suggest that they may also have a function in peripheral tissues including the testes. We have shown using expression of a gene targeted *LacZ* reporter gene, that *Kiss1* and *Gpr54* are expressed by round spermatid cells in the mouse testes. Expression profiling during postnatal gonadal maturation confirmed this as the expression only started to emerge after 1 month of age, which is the time when the spermatids first appear. As both *Kiss1* and *Gpr54* were found to be expressed in spermatids, this raises the possibility that autocrine or paracrine kisspeptin signaling might be involved in spermiogenesis.

Round spermatid cells have just completed meiosis and will subsequently undergo the structural changes required to produce spermatozoa. During this structural remodeling, most of the cytoplasm is removed from the spermatids by the Sertoli cells, which will result in loss of β-galactosidase activity, which might explain why we do not observe staining in elongating spermatids and spermatozoa. Similarly, this cytoplasmic removal would remove any kisspeptin protein but GPR54 should be retained by virtue of its location in the plasma membrane. Indeed, GPR54 has been detected in the head region of human sperm and addition of kisspeptin can produce a modest rise in $[Ca^{2+}]_i$ and sperm motility (14).

The functional significance of *Kiss1* and *Gpr54* expression in spermatids and sperm is still not clear however. The infertility of the *Kiss1* and *Gpr54* mutant mice prevents performing functional tests with mutant sperm. It might be possible to initiate spermatogenesis in the mutant mice with pulsatile FSH and subcutaneous testosterone delivery. Although we have shown that *Kiss1* and *Gpr54* mutant mice can initiate a low level of spermatogenesis when given a chow diet containing phytoestrogens (23), the number of sperm that can be isolated from the vas deferens and epididymis is too small for functional studies. It is noteworthy, however, that several male patients with mutations in *GPR54* and hypogonadotropic hypogonadism have responded to exogenous hormone treatment and achieved fertility [for review, see Ref. (24)] suggesting that in humans, GPR54 function is not essential for sperm function.

There is an important caveat to this expression data however. Although the *LacZ* expression indicates that the *Kiss1* promoter is transcriptionally active in round spermatid cells, we could not detect kisspeptin immunoreactivity using a validated antibody capable of visualizing kisspeptin in the hypothalamus of mice (25). It is possible that the expression level of the kisspeptin protein is below the limits of detection and that X-gal staining for β-galactosidase activity is more sensitive. Alternatively, it is possible that *Kiss1* transcripts are not translated into protein in spermatid cells. Several gene transcripts encoding proteins required for late spermiogenesis are expressed in round spermatids and translationally repressed until the elongating spermatid stage (26). Translationally repressed mRNAs have unusually long poly(A) tails

of approximately 180 nt and translation is associated with shortening of these tails (27). The presence of long poly(A) tails on *Kiss1* transcripts, which are not subsequently shortened, might provide a mechanism for the proposed translational repression in spermatids.

We detected kisspeptin immunoreactivity in Leydig cells of wild-type mice similar to that reported by Anjum and colleagues (13). The specificity of this immunoreactivity was suggested by absence of staining in *Kiss1* mutant mice, which do not produce any kisspeptin protein (5). This notwithstanding, we believe that the kisspeptin immunoreactivity found in the Leydig cells may not be authentic for the following reasons. Firstly, we did not detect *Kiss1* promoter activity in Leydig cell by β-galactosidase staining in *Kiss1*$^{+/-}$ mice. Secondly, we did not detect *Kiss1* transcripts by RT-PCR in the immortalized Leydig cell line MA-10 although this may be a consequence of the cell immortalization process and the tendency for *Kiss1* expression to be suppressed during cell transformation and tumorigenesis. Finally, we failed to detect kisspeptin immunoreactivity in the Leydig cells of *Gpr54* mutant mice, which can produce kisspeptin protein. We believe that the staining pattern observed in the Leydig cells of the wild-type mice is an artifact perhaps associated the high levels of steroidogenesis in these cells, which does not occur in *Kiss1* or *Gpr54* mutant mice.

We also observed a very low level of β-galactosidase staining in Leydig cells from *Gpr54*$^{+/-}$ mice suggesting that these cells might express GPR54 protein. This was consistent with our detection of *Gpr54* transcripts in the immortalized mouse Leydig cell line MA-10. Unfortunately, there are no anti-GPR54 antibodies with sufficient specificity to confirm expression of the endogenous GPR54 protein in the Leydig cells.

If there was co-expression of GPR54 and kisspeptin in Leydig cells, this would allow local autocrine or paracrine action within the testes. Previously published work has suggested that kisspeptins are able to enhance testosterone release after LH stimulation (17). We therefore examined whether Kp10 was able to stimulate testosterone release from the MA-10 cell line as well as testes fragments in culture. We found no evidence that Kp10 could directly stimulate testosterone release or enhance the actions of LH. This is in contrast to the recent data that kisspeptin administration significantly increased hCG-stimulated testosterone levels in acyline treated Rhesus monkeys compared to the responses with hCG treatment alone (17). As the acyline inhibits endogenous LH secretion from the pituitary, these responses suggest a direct, synergistic action of kisspeptin on the testes. The reason for the difference from our data is not known, but apart from a species difference, it might be that the enhancement by kisspeptin requires a sub-threshold level of LH stimulation and the concentration of hCG that we used was too high. It would be informative to test Kp10 responses to a lower range of hCG treatments in the testes explants. It is noteworthy, however, that Huma and colleagues have found that an intravenous injection of the kisspeptin antagonist p234 does not alter plasma testosterone levels in adult Rhesus macaques (28) suggesting that any action of kisspeptin on the testes is small. This conclusion is consistent with the observation that fertility can be restored in *Gpr54* mutant mice by expression of a *Gpr54* transgene in GnRH neurons (29) indicating that GPR54 expression in the testes is also not essential for fertility in mice.

In summary, we have shown that the *Kiss1* and *Gpr54* are both expressed in round spermatid cells of the mouse testes and *Gpr54* is expressed by Leydig cells but we have not found any supporting data that kisspeptin signaling in the testes has a major role in spermatogenesis or testosterone secretion in the mouse.

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Hypothalamic-pituitary-gonadal endocrine system in the hagfish

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The hypothalamic-pituitary system is considered to be a seminal event that emerged prior to or during the differentiation of the ancestral agnathans (jawless vertebrates). Hagfishes as one of the only two extant members of the class of agnathans are considered the most primitive vertebrates known, living or extinct. Accordingly, studies on their reproduction are important for understanding the evolution and phylogenetic aspects of the vertebrate reproductive endocrine system. In gnathostomes (jawed vertebrates), the hormones of the hypothalamus and pituitary have been extensively studied and shown to have well-defined roles in the control of reproduction. In hagfish, it was thought that they did not have the same neuroendocrine control of reproduction as gnathostomes, since it was not clear whether the hagfish pituitary gland contained tropic hormones of any kind. This review highlights the recent findings of the hypothalamic-pituitary-gonadal endocrine system in the hagfish. In contrast to gnathostomes that have two gonadotropins (GTH: luteinizing hormone and follicle-stimulating hormone), only one pituitary GTH has been identified in the hagfish. Immunohistochemical and functional studies confirmed that this hagfish GTH was significantly correlated with the developmental stages of the gonads and showed the presence of a steroid (estradiol) feedback system at the hypothalamic-pituitary levels. Moreover, while the identity of hypothalamic gonadotropin-releasing hormone (GnRH) has not been determined, immunoreactive (ir) GnRH has been shown in the hagfish brain including seasonal changes of ir-GnRH corresponding to gonadal reproductive stages. In addition, a hagfish PQRFamide peptide was identified and shown to stimulate the expression of hagfish GTHβ mRNA in the hagfish pituitary. These findings provide evidence that there are neuroendocrine-pituitary hormones that share common structure and functional features compared to later evolved vertebrates.

Keywords: hagfish, agnathan, cyclostomes, HPG axis, pituitary gland, gonadotropin, GnRH, estradiol

INTRODUCTION

Reproduction in gnathostomes (jawed vertebrates) is controlled by a hierarchically organized endocrine system called the hypothalamic-pituitary-gonadal (HPG) axis (1). In spite of the diverged patterns of reproductive strategies and behaviors within this taxon, this endocrine network is remarkably conserved throughout gnathostomes. In response to hypothalamic gonadotropin-releasing hormone (GnRH), gonadotropins (GTHs) are secreted from the pituitary and stimulate the gonads, where they induce the synthesis and release of sex steroid hormones, which in turn elicit growth and maturation of the gonads (**Figure 1**).

The pituitary gland is present in all vertebrates from agnathans (jawless fishes) to mammals and consists of the same two principal elements, the neurohypophysis and adenohypophysis. The neurohypophysis develops from the floor of the diencephalon as an infundibular extension, whereas the adenohypophysis develops from the epithelium that comes in contact with this infundibulum. The enigma of the pituitary gland is that evolution of a composite organ with such a complex double developmental origin must have been associated with some functionally adaptive value. Yet demonstration of this adaptive value in the agnathans themselves remains elusive. Most surprising facts are that not only the pituitary gland but also all major adenohypophysial hormones such as GTHs, growth hormone (GH), prolactin, and adrenocorticotropin (ACTH) and their receptors are also considered to be vertebrate novelties (2). Thus, the hypothalamic-pituitary system, which is specific to vertebrates, is considered to be a seminal event that emerged prior to or during the differentiation of the ancestral agnathans. Such an evolutionary innovation is one of the key elements leading to physiological divergence, including reproduction, growth, metabolism, stress, and osmoregulation in subsequent evolution of gnathostomes.

Lampreys and hagfish are the only two extant representatives of agnathans. Paleontological analysis of extinct agnathans had suggested that lampreys were more closely related to gnathostomes than either group is to the hagfishes (3, 4). However, both recent molecular phylogenetic analyses (5–7) and developmental study on the craniofacial pattern of the hagfish (8) strongly support the monophyly of the cyclostomes (lampreys and hagfishes as closest relatives). Therefore, studies on reproduction of the cyclostomes are important for understanding the evolution of the HPG axis

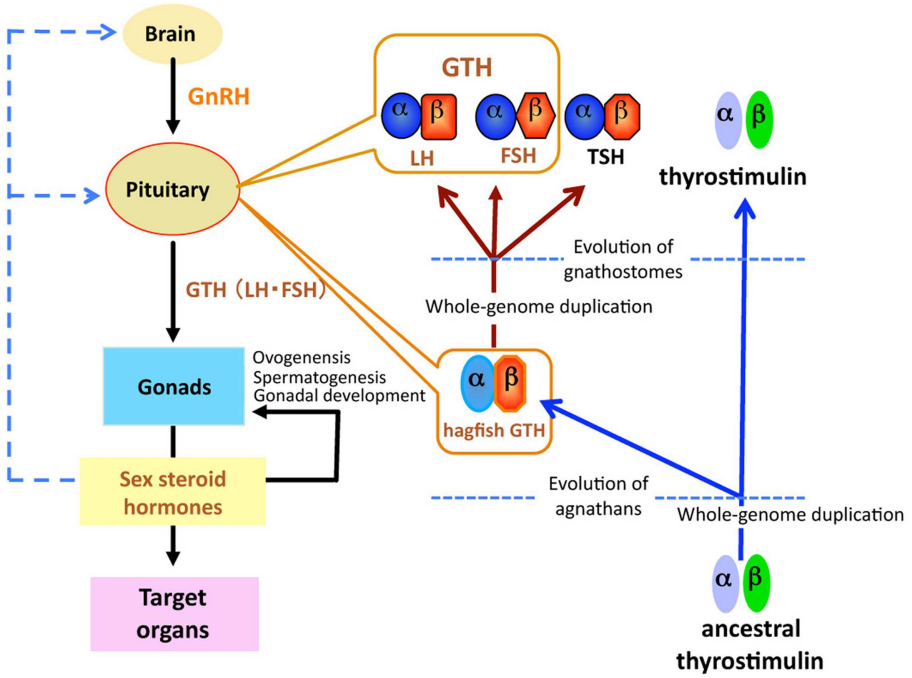

FIGURE 1 | Schematic diagram of the evolution of glycoprotein hormones in the hypothalamic-pituitary-gonadal axis. Ancestral thyrostimulin (α and β) existed before divergence of vertebrates. An ancestral thyrostimulin (α and β) diverged into GTH (α and β) and thyrostimulin (α and β) during the early phase of agnathan divergence. The GTH (α and β) formed a heterodimer in the pituitary and acted as the first adenohypophysial gonadotropic hormone during the evolution of agnathan species. This GTH dimer further diverged into three functional units of adenohypophysis, LH and FSH as two gonadotropins, and TSH as a thyrotropin, in the lineage to gnathostomes.

related to vertebrate reproduction. Findings from many molecular, biochemical, physiological, and morphological studies indicate that the HPG axis is present in the lamprey [for review, see Ref. (1)]. In contrast, endocrine regulation of reproduction in the hagfish is poorly understood [for reviews, see Ref. (9, 10)]. For example, until the recent identification of functional GTH in the brown hagfish, *Paramyxine atami* (11), it was not established whether the hagfish pituitary gland contains tropic hormones of any kind. Herein, this report summarizes the recent findings of the HPG endocrine system involved in reproduction in hagfish.

HAGFISH PITUITARY GLAND

The hagfish is considered the most primitive vertebrate known, living or extinct (3) (**Figure 2**). In addition to their primitive external body features, hagfish possess the most primitive hypothalamic-pituitary system among the vertebrates (12). The neurohypophysis is a flattened sac-like structure, whereas the adenohypophysis consists of a mass of clusters of cells embedded in connective tissue below the neurohypophysis (12, 13) (**Figures 3A,B**). The adenohypophysis and the neurohypophysis are completely separated by a layer of connective tissue, and there is no or little anatomical relationship between them (14, 15) (**Figure 3B**). In addition, there is no clear cytological differentiation between the pars distalis and the pars intermedia (12, 13) (**Figure 3B**). The question arises whether the simplicity of the hagfish pituitary gland is a primitive or a degenerate feature. For example, some authors have claimed that the pars intermedia seems to have been lost via a secondary degenerative process (13, 16). Moreover, until recent identification of a functional GTH in the hagfish pituitary (11), it had not been established whether the hagfish pituitary gland contained adenohypophysial hormones of any kind (9). Because of the simplicity and primitiveness of the pituitary morphology and equivocal data on the adenohypophysial hormones in the hagfish, many researchers had questioned whether there were any functional adenohypophysial hormones (9, 17). On the other hand, arginine vasotocin (AVT), as a single neurohypophysial hormone, was identified in the hagfish (18). In addition, the presence of GnRH has been suggested in the hagfish hypothalamus by both radioimmunoassay (RIA) and immunohistochemistry (19–22) (**Figure 4**). Thus, the hagfish appears to have neurohypophysial and hypothalamic hormones similar to those of other vertebrates.

At present, the adenohypophysis of the hagfish is the least understood of all the vertebrates. However, our immunohistochemical studies provided the first clear-cut evidence for the presence of GTH and ACTH in the hagfish (23–25). Although not conclusive, our data also suggested the presence of GH in the hagfish (23). In addition, these three adenohypophysial hormones were suggested to be the ancestral adenohypophysial hormones that have maintained their original functions throughout vertebrate evolution. On the other hand, the later derived hormones, such as prolactin and thyroid-stimulating hormone (TSH), may have contributed to the expansion of vertebrates into new environments, as suggested by Kawauchi et al. (26) and Kawauchi and Sower (27). Moreover, our study further revealed that GTH

cells, ACTH cells, and unidentified cells which were assumed to include both undifferentiated cells and GH cells, were packed together in the same cell cluster of the hagfish adenohypophysis, and thus each cluster appeared to serve as a separate functional unit (10, 24) (**Figures 3C–E**). If the absence of the pars intermedia is the most ancestral vertebrate pituitary gland, melanophore-stimulating hormone (MSH) activity seems to be gained secondarily together with the differentiation of the pars intermedia. Further studies are needed to clarify this possibility.

FIGURE 2 | Brown hagfish, *Paramyxine atami*.

GLYCOPROTEIN HORMONE FAMILY

Gonadotropins, in response to hypothalamic GnRH, are released from the pituitary and act on the gonads to regulate steroidogenesis and gametogenesis. Two GTHs, follicle-stimulating hormone (FSH) and luteinizing hormone (LH), together with TSH form a family of pituitary hormones (**Figure 1**). They are heterodimeric glycoproteins consisting of two subunits, an α-subunit and a unique β-subunit. These glycoprotein hormones (GPH) are believed to have evolved from a common ancestral molecule through duplication of β-subunit genes and subsequent divergence (27, 28). Two GTHs have been identified in all taxonomic groups of gnathostomes, including actinopterygians (29, 30), sarcopterygians (31), and chondrichthyans (32), but not in agnathans.

A single β-subunit of GTH was identified from the sea lamprey pituitary gland after extensive and exhaustive research for over 20 years (27, 33). However, the α-subunit of lamprey GTH is not found even in the lamprey genome (34). This is very strange fact, since a huge amount of physiological and morphological evidence has suggested the presence of GTH in the lamprey (33, 35–38). The lack of α-subunit of lamprey GTH makes difficulty to study the HPG axis in relation to GTH functions in the lamprey. The second form of β-subunit of pituitary GPHs as a candidate for TSHβ is not found in the lamprey genome (34), and thus the lamprey does not have TSH.

Recently, a fourth heterodimeric GPH has been discovered in the human genome and termed "thyrostimulin" due to its thyroid-stimulating activity (39). The thyrostimulin α-subunit, called glycoprotein α-subunit 2 (GPA2), is homologous but not identical to the common α-subunit (GPHα or GPA1). With the

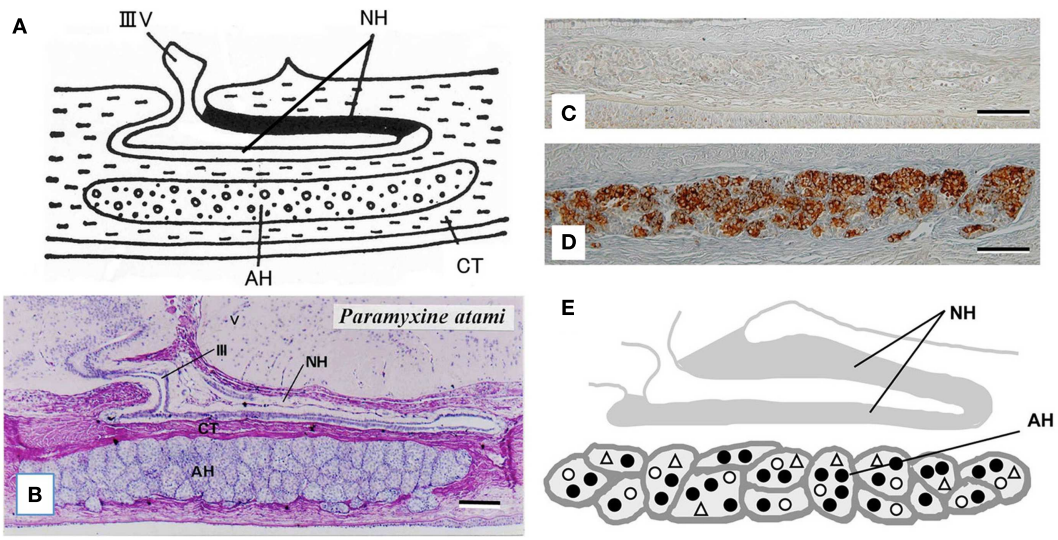

FIGURE 3 | (A) Diagrammatically sagittal section of the hagfish pituitary gland. Dark area of the neurohypophysis (NH) shows posterior part of the dorsal wall, where ir-GnRH nerve fibers and AVT nerve fibers are densely accumulated [for AVT, see Ref. (82)]. **(B)** Nearly mid-sagittal section of the pituitary gland of the brown hagfish, stained with hematoxylin and eosin. **(C,D)** GTHβ-like immunoreaction in the adenohypophysis of the juvenile **(C)** and sexually mature **(D)** brown hagfish stained with anti-hagfish GTHβ. Note that GTH-positive cells are almost absent in **(C)**, whereas they are abundant in **(D)**. **(E)**, Diagrammatically sagittal section of the hagfish pituitary gland showing the topographic distribution of adenohypophysial cells. Closed circle, GTH cell; open circle, ACTH cell; open triangle, undifferentiated cell and possible GH cell. AH, adenohypophysis; CT, connective tissue; IIIV, third ventricle. Scale bars: 100 μm.

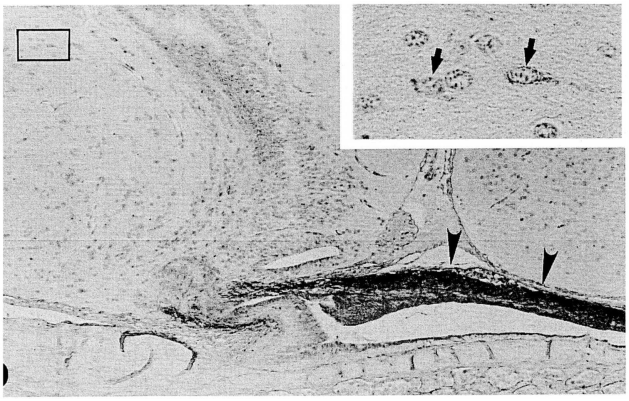

FIGURE 4 | A nearly mid-sagittal section through the neurohypophysis of the Atlantic hagfish, *Myxine glutinosa*, showing the accumulation of ir-GnRH in the dorsal wall of the neurohypophysis (arrowheads). This section was stained with anti-salmon GnRH. Inset, an enlargement of the rectangular area showing GnRH-positive neuronal cells. Arrows show GnRH-positive cell bodies. Scale bars: 100 μm; inset, 20 μm. From Oshima et al. (21).

discovery of GPA2 and glycoprotein β-subunit 5 (GPB5, thyrostimulin beta) homologs not only in other vertebrates but invertebrates including fly, nematode, and sea urchin (40, 41), it is proposed that ancestral glycoprotein existed before the divergence of vertebrates/invertebrates, and that later gene duplication events in vertebrates produced the thyrostimulin (GPA2 and GPB5) and GTH/TSH [GPHα and GPHβ (LHβ/FSHβ/TSHβ)] (40) (**Figure 1**). The basal lineage of chordates such as tunicates and amphioxus contains GPA2 and GPA5 in their genome but not GPHα or GPHβ (2, 42–45). Lamprey also has GPA2 and GPB5 genes in addition to the canonical GTHβ (1, 33, 34, 42). At present, no information is available as to the presence of GPA2/GPB5 in the hagfish.

IDENTIFICATION OF HAGFISH GTH

A single GPH, which comprises α- and β-subunits, was recently identified in the pituitary of the brown hagfish, *P. atami*, one of the Pacific hagfish (11) (**Figure 2**). Both subunits of GPH are produced in the same cells of the adenohypophysis, providing definitive evidence for the presence of a heterodimeric GPH in the hagfish. GPH increases at both the gene and protein levels corresponding to the reproductive stages of the hagfish (**Figures 3C,D**). Moreover, purified native GPH induces sex steroid release (estradiol-17β and testosterone) from cultured testis in a dose-dependent manner. From the phylogenetic analysis, the hagfish GPHα forms a clade with the gnathostome GPHαs. The hagfish GPHβ forms a clade with the TSHβs, however the bootstrap values are low and hagfishes evolved prior to the gnathostomes. The sea lamprey GTHβ also groups with the GPHβs but appears to be one of the outgroups of the LHβs. These results clearly show that the GPH identified in the hagfish acts as a functional gonadotropin, and hereafter it is referred as to GTH. Hagfish GTH is the earliest evolved pituitary GPH that has been identified in a basal vertebrate leading to the gnathostome GTH and TSH lineages.

FEEDBACK REGULATION OF HAGFISH GTH SYNTHESIS AND SECRETION

Gonadal steroid hormones and hypothalamic hormones play major roles in controlling the synthesis and release of LH and FSH in gnathostomes. Both positive and negative feedback effects of gonadal steroids have been demonstrated in teleosts, depending on modes of administration and reproductive stages of animals. In general, in sexually mature fish, sex steroids are considered to regulate gonadal maturation and recrudesce, whereas in juvenile fish, sex steroids are considered to regulate puberty. Thus, negative feedback effects of estradiol and testosterone are evident during the latter stages of gonadal development; specifically, it has been shown that gonadal removal increases LH secretion in salmon (46), goldfish (47), and African catfish (48). The observed increases in LH levels can be suppressed by treatment with estradiol, testosterone, or both. FSH is also controlled by steroid-dependent negative feedback loops in rainbow trout (49), salmon (50), and goldfish (51). The negative feedback effects of steroids may be mediated primarily at the levels of the hypothalamic GnRH neurons (52–54), because both *in vivo* and *in vitro* studies have shown that the expression of LHβ mRNA or FSHβ mRNA is often unchanged or increases following exposure to estradiol, testosterone, or both (49, 53, 55). However, in sexually immature teleosts, sex steroids appear to exert primarily a positive feedback effect that acts directly at the level of the pituitary and via effects on the GnRH system (55, 56). LH content and LH mRNA levels of the pituitary in juvenile fish increase in response to estrogens and aromatizable androgens (49, 57).

Estradiol treatment in the juvenile brown hagfish resulted in the marked accumulation of both immunoreactive (ir)-GTHα and ir-GTHβ in the pituitary (58). However, mRNA levels of GTHα and GTHβ in the pituitary were not, or only transiently, increased by the estradiol treatment (58). The latter results suggest that syntheses of both α- and β-subunits of GTH were not, or only transiently, affected by the estradiol treatment. Accordingly, the marked accumulation of both ir-GTH subunits could be attributed to the suppression of GTH secretion from the pituitary. From that study, the feedback effects of estradiol appeared to be inhibitory rather than stimulatory, and mediated by the possible suppression of the secretion of GTH from the pituitary in these juvenile hagfish. These conditions in juvenile hagfish resembled to those in adults, but not in juveniles, of teleosts (49, 53, 55). Such suppression of GTH secretion in the hagfish is probably regulated by the hypothalamic factors including GnRH, as mentioned below.

On the other hand, testosterone treatment in the juvenile brown hagfish had no effect on the staining intensities of the ir-GTHα and ir-GTHβ in the pituitary (58). Nevertheless, testosterone treatment resulted in the suppression of mRNA expressions of both GTHα and GTHβ in the pituitary (58). Therefore, testosterone probably acts to suppress both the synthesis and the secretion of GTH. This conclusion follows from the reasoning that if the secretion of GTH was not suppressed, the intensities of immunoreactions of both GTHα and GTHβ would have decreased due to decreased levels of mRNA expressions of both GTH subunits. Thus, it seems likely that estradiol and testosterone differ with regard to their roles in the regulation of synthesis and secretion of GTH in the pituitary of the hagfish.

PLASMA LEVELS OF SEX STEROID HORMONES IN THE HAGFISH

Only a few studies exist regarding sex steroid hormonal profiles in relation to gonadal function in hagfish. Matty et al. (17) reported that estradiol and testosterone were measurable in the plasma of *Eptatretus stouti* using RIA; however, the observed levels of these steroids were near the lower limit of RIA sensitivity. Schützinger et al. (59) found using a more sensitive RIA that plasma estradiol content increased in relation to the stages of ovarian development in female Atlantic hagfish, *Myxine glutinosa*. Powell et al. (60, 61) also reported using *in vitro* organ cultured ovaries that the number of females with large eggs increased following estradiol peaks in January in *M. glutinosa*. Thus, estrogen seems to be involved in the ovarian development.

Plasma concentrations of estradiol, testosterone, and progesterone were examined with respect to gonadal development, sexual differences, and possible function of atretic follicles in the brown hagfish, *P. atami*, using a time-resolved fluoroimmunoassay (62). In females, plasma estradiol levels showed a significant positive correlation with ovarian development, while plasma testosterone and progesterone levels were highest in non-vitellogenic adults (62). Thus, our data on plasma estradiol levels in female *P. atami* were consistent with the results of Schützinger et al. (59) and Powell et al. (60). In another study, Yu et al. (63) demonstrated that the synthesis of hepatic vitellogenin was inducible by estrogens, estradiol, and estrone, in *E. stouti*. Based on these results, estrogenic control of ovarian development and hepatic vitellogenesis seemed to have arisen early in vertebrate evolution.

In males, no clear relationships were observed between plasma estradiol or testosterone concentrations and testicular development, while plasma progesterone concentrations showed a significant inverse relationship with testicular development (62). However, in that study data on sexually mature males with high incidences of spermatids or spermatozoa were lacking, since they were very few in our populations (62). Therefore, it is still possible to consider that estradiol and testosterone are involved in the regulation of male reproduction in hagfish. In support of this possibility, it is reported that purified native hagfish GTH induced secretion of estradiol and testosterone from cultured hagfish testes (11). Moreover, intraperitoneal administration of these steroids in juvenile hagfish affected on the GTH functions as mentioned above.

On the other hand, in relation to our failure to correlate plasma concentrations of estradiol or testosterone to testicular development, recent studies in the lamprey have emphasized the importance of non-classical steroids, such as androstenedione and 15α-hydroxylated sex steroids (15α-hydroxytestosterone and 15α-hydroxyprogesterone) in serving as functional androgens (64–67). Indeed, evidence demonstrating testosterone functionality in lampreys was scarce [see Ref. (68)], while androstenedione was found in substantial amounts within the testicular tissue of lampreys, and plasma and tissue levels of the hormone increased significantly in prespermiating male sea lampreys after injection of GnRH (66). In addition, prespermiating males implanted with androstenedione reached maturation significantly faster and exhibited larger secondary sex characteristics than placebo or non-implanted males (66). A receptor for androstenedione was recently described by Bryan et al. (66). 15α-Hydroxylated steroids are also suggested to be involved in the regulation of lamprey reproduction (67). Since hagfish gonads also produce substantial amounts of unusual androgens, such as 6β-hydroxy testosterone and 5α-androstane-3β, 7α, 17β-triol, as well as androstenedione (69–71), some of these steroids may act as functional androgens in the hagfish.

HYPOTHALAMIC FACTORS REGULATING THE GONADOTROPIC FUNCTION OF HAGFISH

The synthesis and secretion of GnRH is the key neuroendocrine function in the hypothalamic regulation of the HPG axis. To date, two to three isoforms have been identified in representative species of all classes of gnathostomes and lampreys (1). GnRHs are also identified in tunicates (72), and several invertebrates belonging to lophotrochozoans [mollusk and annelid; (73, 74)], but not in the ecdysozoan lineages. On the other hand, adipokinetic hormone (AKH) has been identified as the ligand of the GnRH receptor of the insects, *Drosophila* and *Bombyx* (75). An AKH-GnRH-like neuropeptide has been identified in the nematode *C. elegans* (76). A comparative and phylogenetic approach shows that the ecdysozoan AKHs, lophotrochozoan GnRHs, and chordate GnRHs are structurally related and suggested that they all originate from a common ancestor (77).

In the hagfish, GnRH has not yet been identified, but previous chromatographic and immunohistochemical studies have suggested the presence of a GnRH-like molecule in the hypothalamic-neurohypophysial area (19, 20). Kavanaugh et al. (22) reported the seasonal changes in hypothalamic ir-GnRH contents in relation gonadal reproductive stages in the Atlantic hagfish (*M. glutinosa*). In *M. glutinosa*, a dense accumulation of GnRH-like immunoreaction was observed in the dorsal wall of the neurohypophysis with the use of antisera against chicken GnRH-II, salmon GnRH, lamprey GnRH-I, and lamprey GnRH-III (19, 21) (**Figure 4**). Neuronal cells containing ir-GnRH were found in the preoptic nucleus and the dorsal hypothalamic nucleus (20, 21). In another study, Osugi et al. (78) identified several PQRFamide peptides in the brain of the brown hagfish (*P. atami*). Based on *in situ* hybridization and immunohistochemistry, hagfish PQRFamide peptide precursor mRNA and its translated peptides were localized in the infundibular nucleus of the hypothalamus. Dense ir fibers were found in the infundibular nucleus and some of them were terminated on blood vessels within the infundibular nucleus. They further showed that one of the hagfish PQRFamide peptides significantly stimulated the expression of GTHβ mRNA in the cultured hagfish pituitary. The latter result clearly indicates that GTH functions of the hagfish pituitary are controlled by the hypothalamic factors.

Puzzling aspect of the hagfish hypothalamic-pituitary system is that there is no or little anatomical relationship between them. It is generally considered that the hypothalamic factors, such as GnRH, reach the adenohypophysis simply by diffusion (79, 80). However, the dorsal wall of the hagfish neurohypophysis, where ir-GnRH nerve fibers are terminated (**Figure 4**), is far from the adenohypophysis by the presence of the neurohypophysis itself. On the other hand, the blood vessels are richly distributed on the surface of the dorsal wall, and make the posterior hypophysial vascular

plexus (14, 15). Although most blood in the posterior hypophysial vascular plexus enter the posterior hypophysial vein of the anterior cardinal system, several small vessels proceed from the dorsal wall to the adenohypophysis in *Eptatretus burgeri* (15). These small vessels may contribute the regulation of the adenohypophysial functions. A pair of small blood vessels from the hypothalamus also enters the posterior hypophysial vascular plexus (14). Together with the fact that some PQRFamide neuronal fibers terminated on the blood vessels within the hypothalamus (78), further studies are needed to understand the hypothalamic-pituitary system of the hagfish.

CONCLUSION

Not only the pituitary gland but also all major adenohypophysial hormones and their receptors are considered to be vertebrate novelties. Since hagfish represent the most basal and primitive vertebrate that diverged over 550 millions years ago (81), they are of particular importance in understanding the evolution of the HPG axis related to vertebrate reproduction. Our data clearly show that the hagfish has a functional HPG axis similar to that of more advanced gnathostomes. It is strongly expected that the functional GTH found in hagfish pituitary helps to delineate the evolution of the complex HPG axis of reproduction in vertebrates. Furthermore, this HPG system likely evolved from an ancestral, pre-vertebrate exclusively neuroendocrine mechanism by gradual emergence of components of a new control level, the pituitary gland.

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Profiling, bioinformatic, and functional data on the developing olfactory/GnRH system reveal cellular and molecular pathways essential for this process and potentially relevant for the Kallmann syndrome

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During embryonic development, immature neurons in the olfactory epithelium (OE) extend axons through the nasal mesenchyme, to contact projection neurons in the olfactory bulb. Axon navigation is accompanied by migration of the GnRH+ neurons, which enter the anterior forebrain and home in the septo-hypothalamic area. This process can be interrupted at various points and lead to the onset of the Kallmann syndrome (KS), a disorder characterized by anosmia and central hypogonadotropic hypogonadism. Several genes has been identified in human and mice that cause KS or a KS-like phenotype. In mice a set of transcription factors appears to be required for olfactory connectivity and GnRH neuron migration; thus we explored the transcriptional network underlying this developmental process by profiling the OE and the adjacent mesenchyme at three embryonic ages. We also profiled the OE from embryos null for *Dlx5*, a homeogene that causes a KS-like phenotype when deleted. We identified 20 interesting genes belonging to the following categories: (1) transmembrane adhesion/receptor, (2) axon-glia interaction, (3) scaffold/adapter for signaling, (4) synaptic proteins. We tested some of them in zebrafish embryos: the depletion of five (of six) *Dlx5* targets affected axonal extension and targeting, while three (of three) affected GnRH neuron position and neurite organization. Thus, we confirmed the importance of cell–cell and cell-matrix interactions and identified new molecules needed for olfactory connection and GnRH neuron migration. Using available and newly generated data, we predicted/prioritized putative KS-disease genes, by building conserved co-expression networks with all known disease genes in human and mouse. The results show the overall validity of approaches based on high-throughput data and predictive bioinformatics to identify genes potentially relevant for the molecular pathogenesis of KS. A number of candidate will be discussed, that should be tested in future mutation screens.

Keywords: olfactory development, GnRH neuron, Kallmann syndrome, extracellular matrix, transcription profiling, disease gene prediction

INTRODUCTION

Central Hypogonadic Hypogonadism (CHH), is a heterogeneous genetic disorders characterized by absent or incomplete puberty, due to low circulating gonadotropins and sex steroids. Its mode of inheritance can be X-linked, autosomal dominant, or autosomal recessive, although unrelated sporadic cases occur more frequently (1). The disease is often associated with anosmia/hyposmia, in this case it is known as Kallmann Syndrome [KS, on-line Mendelian inheritance in man (OMIM) 308700], or with a normal sense of smell (normosmic CHH, or nCHH). These conditions are variably associated with non-reproductive phenotypes such as unilateral renal agenesis, skeletal abnormalities, midline malformations, or hearing loss. Neurological symptoms (including synkinesia of the hands, sensorineural deafness, eye-movement abnormalities, cerebellar ataxia, and gaze-evoked horizontal nystagmus) may also occur depending on the specific mode of inheritance (2).

Mutations affecting a large number of unrelated genes have been linked to the onset of KS/nCHH, currently including *Anosmin1* (*KAL1*), *Fibroblast Growth Factor Receptor-1* (*FGFR1*), *Fibroblast Growth Factor 8* (*FGF8*), *GnRH receptor* (*GNRH-R*), *Nasal Embryonic LHRH Factor (NELF)*, *Kisspeptin (KISS1)*; *Kisspeptin Receptor (KISS-R)/G-protein-Coupled Receptor 54 (GPR54)*, *Prokineticin-2 (PROK-2)*, *Prokineticin Receptor-2 (PROKR2)*, *Chromodomain Helicase DNA-binding Protein 7 (CHD7)*, *Neurokinin-B (TAC3)*, *Neurokinin-B Receptor (TAC3R)*, *Heparan Sulfate 6-O-SulphoTransferase 1 (HS6ST1)*, *SOX10*,

Semaphorin-3A (*SEMA3A*), and five novel genes, members of the "FGF8-synexpressome" (1–8). In addition, several mouse models of targeted gene disruption have been shown to exhibit a KS-like phenotype (6, 9–18).

Despite the number of genes mutated in KS/nCHH, the majority of patients (>60%) do not harbor mutations in known disease genes, thus it is expected that many additional disease loci remain to be identified. In addition, the mutations found in KS/nCHH patients, once thought to act alone, are now recognized as cooperating mutations, and in fact in some cases a bi-genic or oligo-genic origin of these disease has been reported, with specific genotype/phenotype correlations (19–22). These findings open questions on the actual prevalence of single and combined mutations, the functional cooperation between them, and the possibility to use these information for accurate prognostic evaluations.

Kallmann syndrome is righteously considered a developmental disease. During embryonic development the GnRH neurons originate in the primitive olfactory area, migrate along the extending axons from the olfactory epithelium (OE) and the vomero-nasal organ (VNO), reach the anterior-basal forebrain and home in the septo-hypothalamic region of the adult brain (23–28). The association of the olfactory axons with the immature GnRH neurons, hence their ability to migrate and reach the hypothalamus, is an ancient and highly conserved developmental process, justified by the fact that it is essential for puberty and reproduction, in addition to neuro-modulatory functions (27, 29, 30). Not surprisingly this process is governed by a large set of molecular cues. Several studies have identified specific signaling molecules and their cognate receptors, as well as adhesion molecules, axon-glia and axon-matrix molecules play a role in guiding the axons to the correct position and consent the penetration of the basement membrane and the brain parenchyma (31–40). For instance, the semaphorin co-receptor Nrp1 is expressed by extending axon and GnRH neurons, and mediates the guiding functions of Sema3a, expressed in the nasal mesenchyme (10, 11). FGF8 has been shown to act as survival factor for olfactory and migrating GnRH neurons, which express its receptor FGFR1 (41–43), and both genes are mutated in a subset of KS/nCHH patients. To further complicate the picture, a cell population on the surface of the OB interacts with incoming axons, GnRH neurons and the CNS, and provide key signals for basement membrane fenestration, hence axon connectivity (44).

Due to the close relationship of olfactory axon elongation/connectivity and GnRH migration that occurs during embryonic development, the GnRH neuronal migration is strictly dependent on the integrity and connectivity of the olfactory pathway (30). A premature termination or mislocalization of olfactory axons results in impaired odor perception and GnRH homing. Thus, defects in olfactory development and/or GnRH neuron migration are considered the main primary cause of KS. The genetic findings summarized above have revealed much about the abnormalities that can befall both the development of the olfactory sensory system and GnRH neuron ontogenesis, including their differentiation, migration, maturation, circuit formation, and senescence.

Experimentally, animal models with altered olfactory and GnRH development are becoming available, including mouse, Zebrafish, and Medaka. The zebrafish embryo is ideal for developmental genetic studies, and the depletion of *anosmin-1a* leads to altered olfactory development and a KS-like phenotype (45, 46). In mice, several mutant strains display a phenotype that closely resemble KS/nCHH, including mouse mutant for *Dlx5* (14, 16, 47), *Emx2* (18), *Klf7* (13), *FezF1* (17, 48), *Six1* (12), *Prok2* and its receptor *Prokr2* (6, 15), *Lhx2* (9), *Ebf2* (49), *Nrp1* and *Sema3a* (10, 11). Notably, 7 of these (*Dlx5*, *Emx2*, *Klf7*, *Six1*, *FezF1*, *Ebf2*, and *Lhx2*) code for unrelated transcription factors, thus it can be postulated the existence of transcription regulatory networks, yet to be uncovered, that sustain olfactory development and connectivity, consent migration of the GnRH neurons and may contribute to the onset of KS/nCHH when altered. Furthermore, it is increasingly recognized that biological processes are governed and regulated by regulatory modules and networks of molecular interaction, not limited to protein-coding genes, rather then simplistically by individual genes.

To advance in our knowledge on the molecular regulation of axon extension/connectivity and GnRH neuron migration, in the present study we adopted a strategy based on the generation of transcriptome-wide profile data, combined with bioinformatic analyses and meta-analyses. In addition to the normal olfactory tissue we have also included one of the mouse models of KS, i.e., the *Dlx5* null (14, 16, 47). We then used transgenic Zebrafishes to image the olfactory axons and the GnRH neurons, and use these to establish the function of Dlx5 targets for olfactory axon extension/contact and on GnRH neuron migration and neurite extension. The results confirm a role for *Dlx5* and *FGFR1*, and indicate *Lrrn1* and *Lingo2* as novel players for olfactory axon organization and for GnRH neuron migration. Finally, we applied a gene prediction algorithm based on conserved co-expression networks, on all known human and mouse KS-causing genes. We predict a set of best candidates for causing, con-causing, or modifying the KS/nCHH phenotype.

MATERIALS AND METHODS

MICE NULL FOR Dlx5

Mice with targeted disruption of *Dlx5* have been previously reported (50). The null allele, denominated $Dlx5^{lacZ}$, allows for detection of the *Dlx5*-expressing cells by staining for β-galactosidase (β-gal) expression. The olfactory phenotype has been previously characterized (14, 16, 47). To obtain the WT samples, only WT males and females were crossed. To obtain *Dlx5* mutant samples, $Dlx5^{+/-}$ (heterozygous) males and females were crossed; the progeny showed the expected Mendelian ratios of genotypes$^{+/+}$, $Dlx5^{+/-}$ and $Dlx5^{-/-}$. Pregnant females were sacrificed at the chosen embryonic age by cervical dislocation. The day of the vaginal plug was considered E0.5. All animal procedures were approved by the Ethical Committee of the University of Torino, and by the Italian Ministry of Health.

TISSUE COLLECTION FROM MOUSE EMBRYOS

Embryos were collected clean of extra-embryonic tissues (used for genotyping) by manual dissection, transferred in RNAse-free PBS, and further dissected to separate the head. This was then included in 3% low-melting agarose in PBS, let harden and sectioned by vibratome (250 μm). Sections were manually dissected

in cold PBS, with fine pins, to collect the OE or the VNO epithelia, or alternatively to collect the adjacent mesenchyme (Figure S1 in Supplementary Material). The excised tissues were individually collected in RNA-later (Ambion) and stored at −20°C until extraction. Following genotyping, samples of the same genotype were pooled. For the *Dlx5* mutant tissues, the entire epithelial lining of the nasal cavity was collected, since it was not possible to discriminate the OE vs. the respiratory epithelium.

RNA EXTRACTION, LABELING, AND HYBRIDIZATION ON MOUSE EXON-SPECIFIC ARRAYS

At least 15 embryos were used for each developmental age, the collected tissues were pooled in three independent biological samples, used to extract total RNA with the Trizol (Invitrogen).

After extraction, RNA samples were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies), the integrity of RNA molecules was assessed by capillary electrophoresis on a Agilent Bioanalyzer (Agilent), and found to have a RIN (RNA Integrity Number) value >5. One microgram of each total RNA sample (in triplicate) was processed using the Affymetrix platform's instruments, following the GeneChip Whole Transcript Sense Target Labeling procedure, according to instructions. Ribosomal RNA was depleted using the RiboMinus kit (Invitrogen), cDNA was synthesized with random primers coupled with the T7 Promoter sequence, using SuperScript II for first-strand synthesis, and DNA Polymerase I for second-strand synthesis. The cDNA was and used as template for IVT amplification, using T7 polymerase. The amplificated products were used to synthesize single-stranded cDNAs, with the incorporation of dUTP, the products were fragmented by uracil-DNA-glycosylase (UDG) and apurinic/apyrimidinic endonuclease-1 (APE 1) treatment. Finally, 5.5 μg of fragmented cDNA samples were biotinylated with terminal deoxynucleotidyl transferase and used to hybridize on GeneChip® Exon 1.0 ST Arrays (Affymetrix, Santa Clara, USA). The Chips were washed and stained with Streptavidin-phycoerithrin in the GeneChip Fluidic Station 450 and scanned with Affymetrix GeneChip® Scanner 3000 7G.

ANALYSIS OF MICROARRAY DATA

Quality control was performed using the Affymetrix Expression Console software[1]. All the experiments exhibited optimal quality controls and correctly clustered in the right sample groups; they were thus all included in the analysis. Normalization and probeset summarization steps were performed with RMA, within the OneChannelGUI package (51) included in Bioconductor (52), separately for each pairwise comparison including the six relevant arrays (three biological replicates per condition). Differentially expressed genes (DEG) for each pairwise comparison were obtained with Rank Products (53), adopting a 0.05 false discovery rate (adj. p-value $\leq$0.05).

SOFTWARES AND DATABASES

For preliminary Gene Ontology (G.O.) analyses we used DAVID[2] and KEGG[3]. For improved categorization and visualization, we used ClueGO (54). For the time course analysis we used default parameters. For the analysis of down-regulated DEGs in the *Dlx5*$^{-/-}$ samples we relaxed the analysis by using a cutoff of 0.001 on nominal enrichment p-value. For embryonic expression of RefSeq genes we used the two on-line *in situ* hybridization databases GenePaint[4] and Eurexpress[5]. For the position weight matrix (PWM) we used the JASPAR database. Tissue-specific conserved co-expression networks were obtained with the TS-CoExp Browser[6] (55).

We also used the following web resources: Ensembl Genome Browser[7], UCSC Genome Browser[8], RefSeq[9], Mouse Genome Informatix[10], OMIM[11].

GENOME-WIDE PREDICTION OF Dlx BINDING SITES AND PUTATIVE TARGET GENES

With the PWM of Dlx5 provided by JASPAR under accession PH0024.1 (56) Dlx5 sites were predicted by standard log-likelihood ratios, using as null model the nucleotide frequencies computed over the whole intergenic fraction of the mouse genome. We considered only those sites scoring 50% of the maximum possible score or better. We selected sites that are conserved in at least two (of eight vertebrate species). A site is defined as conserved with species S if it lies in a region of the mouse genome which is aligned with a region of the S genome and the aligned sequence in/S/is a site according to the same definition used for mouse sites. A ranked list of putative Dlx5 targets was obtained from the identified sites as described (57).

CONSERVED CO-EXPRESSION NETWORK, AND PREDICTION/PRIORITIZATION OF PUTATIVE DISEASE GENES

Tissue-specific conserved co-expression networks were obtained with the TS-CoExp Browser (see footnote text 6) (55, 58), based on 5188 human and 2310 mouse manually annotated microarray experiments. For disease prediction/prioritization we used a tool within the TS-CoExp Browser and the same approach based on conserved co-expression networks, but instead of using genes causing similar phenotypes, we used KS-disease genes as "reference" genes. These genes were selected based on documented mutations in KS patients (for human) or well described olfactory/GnRH embryonic phenotype recapitulating KS (mouse).

VALIDATION OF ARRAY DATA BY REAL-TIME qPCR

Tissue samples corresponding to WT and *Dlx5*$^{-/-}$ OE were collected from embryos at the age E12.5, transferred in RNA-later in individual tubes and stored at −20°C. The genotype was determined on extra-embryonic tissues. Samples were pooled according to the genotype, collected in Trizol (Invitrogen), and

[1] www.affymetrix.com
[2] http://david.abcc.ncifcrf.gov/
[3] http://www.genome.jp/kegg/pathway.html
[4] www.genepaint.org
[5] www.eurexpress.org
[6] http://www.mbcunito.it/cbu/ts-coexp
[7] http://www.ensembl.org/index.html
[8] http://genome.ucsc.edu
[9] http://www.ncbi.nlm.nih.gov/RefSeq
[10] http://www.informatics.jax.org/
[11] http://www.omim.org/

used to extract total RNA according to the instructions. For Real-Time qPCR, 250 ng of total RNA was reverse-transcribed at 42°C for 50 min in the presence of 500 ng/μl random hexamers, 10 mM of each dNTPs, RNasin and Improm Reverse Transcriptase (Promega). Relative cDNA abundance was determined using the AB7900 System and the GoTaq qPCR Master Mix (Promega). Specific cDNAs were amplified using primers and probes designed according the Universal Probe Library system (UPS, Roche). Experiments were repeated at least twice on independent samples, every point was done in triplicate, results were normalized to the level of *TATA-binding protein* (TBP) and *GAPDH* mRNAs. Data analysis was performed with ABI software, version 2.1 (Applied Biosystems) using the comparative Cq method, calculated with the formula of the DDCq. For each primer-pair, the melting curves of the amplified products revealed a single peak. Primer sequences are provided (Table S1 in Supplementary Material).

ZEBRAFISH STRAINS AND GENE KNOCK-DOWN IN EMBRYOS

The following two strains were used for visualization of the olfactory axons: *OMP^{2k}:gap-CFP^{rw034}* and *TRPC2$^{4.5k}$:gap-Venus^{rw037}* (59–61), and were obtained from Drs. Nobuhiko Miyasaka and Yoshihiro Yoshihara (RIKEN Brain Science Inst., Japan). The fish strain *GnRH3:GFP* (62–64) was obtained from Dr. Y. Zohar (University of Maryland Biotechnology Institute, Baltimore, USA) and Dr. Y. Gothilf (Life Sciences, Tel-Aviv University, Israel). Adult fishes were maintained, bred and genotyped according to standard procedures, kept under a 14 h-light and 10 h-dark photoperiod at 28°C. Allelic transmission followed the expected Mendelian ratios. Following fertilization, 1-cell zygotes were collected and maintained in the presence of 0.003% 1-phenyl-2-thiourea (PTU) to prevent formation of melanin.

To down-modulate specific genes, we injected antisense morpholino oligos (MO) into zebrafish oocytes (65, 66). MO were designed either to block splicing at a specific exon-intron junction (GeneTool oligo design), and consequently lead to present of aberrant transcripts and frame-shifted translation, or to anneal to the ATG start codon and inhibit translation initiation. For *z-dlx5a* we combined two MOs: one annealing with the exon1-intron1 splice junction and leading to a premature Stop codon upstream of the homeodomain; the other annealing with the Start codon. Sequences and properties of all the MO are in Table S2 in Supplementary Material. Zygotes were collected at one-cell stage and injected under stereological examination with 4 ng of MO, in presence of Phenol Red for subsequent selection. From 48 to 72 h post fertilization (hpf) embryos were fixed with 4% PFA at 4°C ON, washed in PBS, and embedded in 4% low-melting agarose, 0.1% Tween-20. The apical portion of the head was manually dissected from the rest of embryo. Confocal microscopy analysis was performed using a Leica TCS SP5 (Leica Microsystems). The OMP:CFP+ and the Trpc2:Venus+ (YFP+) axons were viewed in a frontal plane, while the GnRH3:GFP+ neurons were viewed in a ventral plane. Images were acquired as Z-stacks of 1 μm thick optical sections. Digital micrographs images were contrast balanced and color matched using Photoshop7 (Adobe), cropped, rotated, and assembled into figures with QuarkXpress (Pantone).

RESULTS

GENES DIFFERENTIALLY EXPRESSED DURING OLFACTORY DEVELOPMENT

We set forth to generate expression profiles of the OE at key stages of its development, comprising the time of axonal connection. We selected three developmental stages, i.e., the Olfactory Placode (OPL) at E11.5, the OE at E12.5, and either the OE or the VNO at E14.5. Mouse Affymetrix GeneChip® Exon 1.0 ST Arrays were used to analyze the gene expression profiles of the developing olfactory (neuro)epithelium (OE). Comparing the OE E12 vs. the OE at E11, with adj. p-value ≤ 0.05 and fold-change ≤ -0.9 or ≥ 0.9, we found 29 up-regulated and 62 down-regulated genes. Comparing the OE at E14 vs. the OPL E11 we found 358 up-regulated and 17 down-regulated genes. Comparing the VNO E14 vs. the OPL E11 we found 459 up-regulated and 21 down-regulated genes.

A fraction of the DEGs might derive from mesenchymal cells present in the epithelial samples; as a matter of fact, epithelial cells do not easily detach from the basement membrane and mesenchymal cells inevitably tend to remain attached. A survey of the embryonic expression territory of the modulated genes using the on-line expression databases and www.genepaint.org and www.eurexpress.org showed that about 10% of the DEGs was indeed expressed in the nasal mesenchyme adjacent to the OE, and not in the OE or VNO proper. Thus, we decided to estimate the extent of mesenchymal contamination in the OPL, OE, and VNO samples, by collecting pure mesenchymal tissue adjacent to the OPL, OE, and VNO, at the same embryonic ages, and use the RNA extracted from these to quantitatively determine the mRNA abundance of "epithelial only" (*FoxJ1*, *Fmo2*, and *Ehf*) and "mesenchymal only" (*Sp7* and *Lect1*) genes, by Real-Time qPCR. In the same experiment we compared the samples of the OE (mixed epithelium and mesenchyme) with "pure mesenchyme" samples at the same embryonic age. The results indicate that the abundance of a mesenchymal RNA in the OE samples is roughly 15% that of the pure mesenchyme samples, thus we assumed that the contribution of MES in the EPI samples is 15% (Figure S2 in Supplementary Material).

At the same time, using the GeneChip® Exon 1.0 ST Arrays and the same hybridization procedure and statistical analyses used before, we generated profiling data from the MES samples collected from wild-type embryos at E11.5, E12.5, and E14.5. At the age E14.5 the samples were collected adjacent to the OE or adjacent to the VNO, according to their anatomical position, and maintained separated. This effort was undertaken to: (1) explore the global changes of expression that underlie interaction between the OE and the MES, (2) carry out a subtraction step on the raw EPI data, to generate cleaner OE data.

By comparing the MES samples at E12 vs. E11 we found 118 up-regulated and 17 down-regulated genes; comparing the samples OE at E14 vs. OPL E11 we detected 284 up-regulated and 41 down-regulated genes, while comparing the VNO at E14 vs. OPL E11 we detected 293 up-regulated and 35 down-regulated genes (the non-annotated probes are not included). Then we subtracted the estimated expression of MES genes (15%) from the raw expression data, applying this general formula to all genes present

and expressed:

$$E_i^c(g) = E_i(g) - F \times M(g)$$

where $E_i(g)$ is the expression of gene g in the i-th replicate of the EPI dataset and $M(g)$ is the expression in the MES dataset, averaged over all replicates. F is the estimated mesenchymal fraction, equal to 0.15. Choosing F to be equal to 0.1 or 0.2 did not significantly alter the results. With this calculation we created a subtracted and corrected dataset with expression values more indicative of the sole EPI expression. Comparing the corrected EPI samples at E12 vs. E11 we found 9 up-regulated genes and 57 down-regulated; comparing the samples OE at E14 vs. OPL E11 we detected 250 up-regulated and 19 down-regulated genes, while comparing the VNO E14 vs. OPL E11 we detected 347 up-regulated genes and 14 down-regulated (the non-annotated probes and the OR genes are not counted). After the subtraction, a number of genes reached a "no expression" level. We assume that this is due mainly to the fact that their differential expression was relative to the MES. We examined how many of the genes that disappeared from the raw list are up-regulated in the MES samples, and detected highly significant enrichments ($p < 4e{-}12$).

The corrected lists of EPI DEGs up- and down-modulated in the OE E14 vs. OPL E11 are reported in Tables S3 and S4 in Supplementary Material, respectively, while the lists of DEGs up- and down-modulated in the VNO E14 vs. OP E11 are in Tables S5 and S6 in Supplementary Material, respectively. In the OE we find genes expected to be associated or to play a role in neuronal differentiation and/or olfactory development, such as *NeuroD*, *OMP*, *Peripherin*, *NCAM2*, *Claudins*, *Keratins*, and *Lhx2* (a gene causing a KS-like phenotype in the mouse) (9). In addition we find a set of olfactory receptor (OR) genes, as expected (Table S7 in Supplementary Material).

Next we carried out functional categorization analyses on the genes up-regulated in the OE, to identify enriched functional categories, using the Gene Ontology-based ClueGo tool (54, 67). Since this analysis could be biased by the OR genes, which are numerous (about 1000 in the mouse genome) and belong to a single category, we masked the OR genes. The results are shown in **Figure 1A**. From the comparisons OE 14 vs. OPL 11 we detect: regulation of epithelial cell proliferation, regulation of cell migration, regulation of extracellular matrix organization, and various categories of response to signals.

In the VNO, we find several genes expected to be associated or to play a role in VNO development, such as *NeuroD*, *OMP*, *Lhx2*, *Peripherin*, *Claudins*, *Keratins*, *EphA3*, *Neuropilin1*, *Laminin*β*3*, *Lhx2* (a Kallmann gene in the mouse), and *Dcx*. In addition we find several OR genes, as expected (Table S8 in Supplementary Material). We carried out functional categorization on the genes up-regulated in the VNO, after masking the differentially expressed OR genes, and detected the over-represented classes shown in **Figure 1B**. Focusing on the comparisons E14 vs.

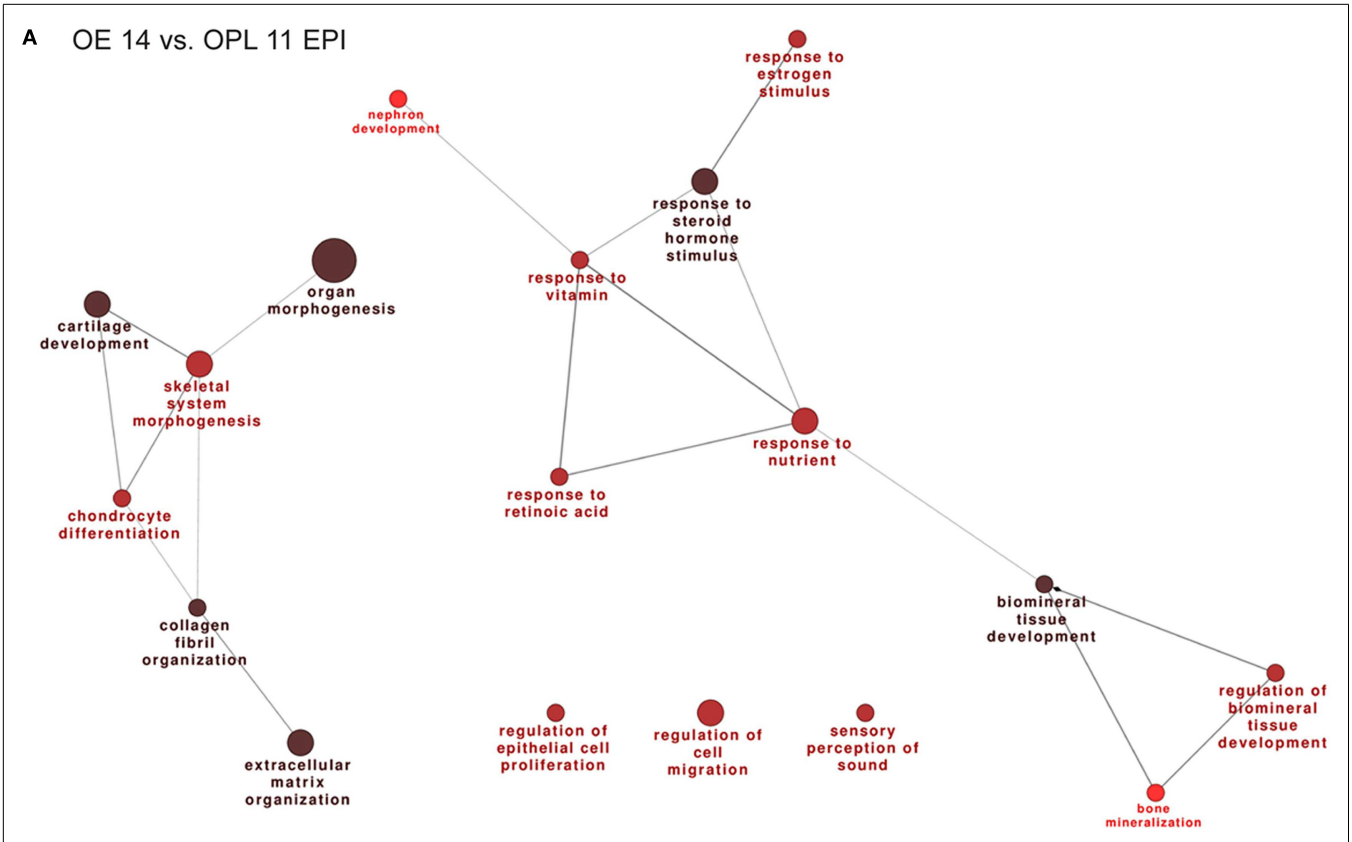


FIGURE 1 | Continued

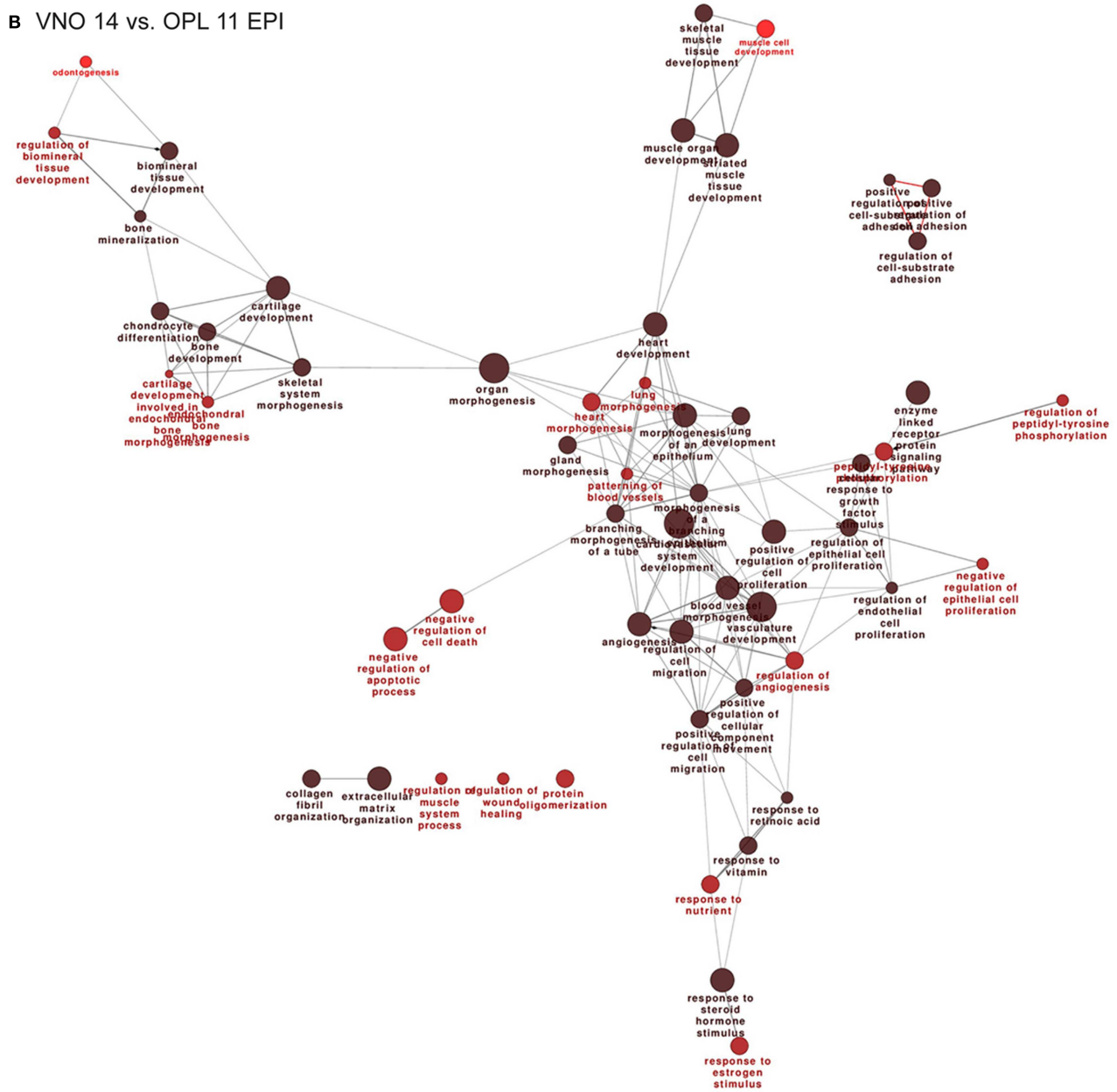


FIGURE 1 | Functional categorization of genes differentially expressed during normal OE and VNO development. Significantly over-represented functional categories of DEGs detected in the (corrected) EPI samples, comparing OE 14.5 vs. OPL 11.5 **(A)** or comparing the VNO 14.5 vs. OPL 11.5 **(B)** were organized them in networks using ClueGO. Only the results of the up-regulated DEGs are shown. Circles represent over-represented categories, lines indicate related categories.

E11, we detect: regulation of epithelial cell proliferation, regulation of cell migration, regulation of cell adhesion, gland and epithelium morphogenesis, cartilage development, bone development, extracellular matrix organization, and various categories of response to signals.

DEGs IN OLFACTORY-ASSOCIATED MES, DURING DEVELOPMENT

We then compared the profiles of the MES samples across the developmental ages E11.5–E12.5–E14.5. The full lists of up- and down-modulated DEGs relative to the OE are provided in Tables S9 and S10 in Supplementary Material, respectively, while the full lists of up- and down-regulated DEGs relative to the VNO are provided in Tables S11 and S12 in Supplementary Material, respectively. We recognized genes playing a role in cell–cell communication, signaling, matrix remodeling, etc. such as *Integrins*, *Contactins*, *Matrillins*, *Tenascin*, *Collagens*, *MMPs*, *Adams*, *Lectin Galactose Binding 9*, *Elastin*, *FGF7*, *FGF12*, *Sfrp2*, *Sfrp4*, *Sema3D*, *Sema3C*, *Nrp1*, *Wnt2*, *Bmp5*, *Follistatin*. We also found

some neuronal genes, likely due to a minimal presence of olfactory neuron in the MES sample and to the presence of migratory GnRH neurons in the E14 sample, minimal in the E11 sample. Functional categorization on these DEGs detected an enrichment of the following categories: extracellular matrix organization, cell-substrate adhesion, cartilage and bone development, organ morphogenesis, response to signals, and some neuronal categories (**Figures 2A,B**).

PROFILING OF THE Dlx5$^{-/-}$ VS. WILD-TYPE OE

The *Dlx5*$^{-/-}$ mutant mice represent a fully penetrant model of KS (14, 16, 47). Triplicates of the OE and VNO tissues were collected from WT and *Dlx5*$^{-/-}$ embryos at the age E12.5, total RNA was extracted and hybridized on the GeneChip® Exon 1.0 ST Arrays. Using the indicated statistical parameters (see Materials and Methods) we detected 121 down- and 25 up-regulated genes in the *Dlx5*$^{-/-}$ OE vs. the WT, not counting the non-annotated probes and the OR genes (**Figure 3A**; Table S13 in Supplementary Material). Again, the OR genes were removed (provided in Table S14 in Supplementary Material) prior to conducting functional categorization analysis by G.O. We detected: intermediate filament/cytoskeletal organization, endocrine system development, forebrain development, cell–cell signaling, and epithelial cell differentiation (**Figure 3B**).

We carried out a technical validation of the microarray results, by selecting 12 down- and 4 up-regulated DEGs and quantifying their expression on independently collected samples, by Real-Time qPCR. Of these DEGs, 11 down- and 2 up-regulated were confirmed (**Figure 3C**).

Next we verified whether the identified DEGs are expressed in the OE, in the adjacent mesenchyme, or in both, by consulting the on-line expression databases GenePaint and Eurexpress. We classified DEGs as either not expressed (−), expressed in the neurepithelium (NEp), expressed in the mesenchyme (Mes), expressed in the respiratory epithelium (Res) or ubiquitously expressed (Ub). We assumed that the OR were all expressed in NEp, and in any case they were excluded. Among the 72 down-modulated DEGs considered,

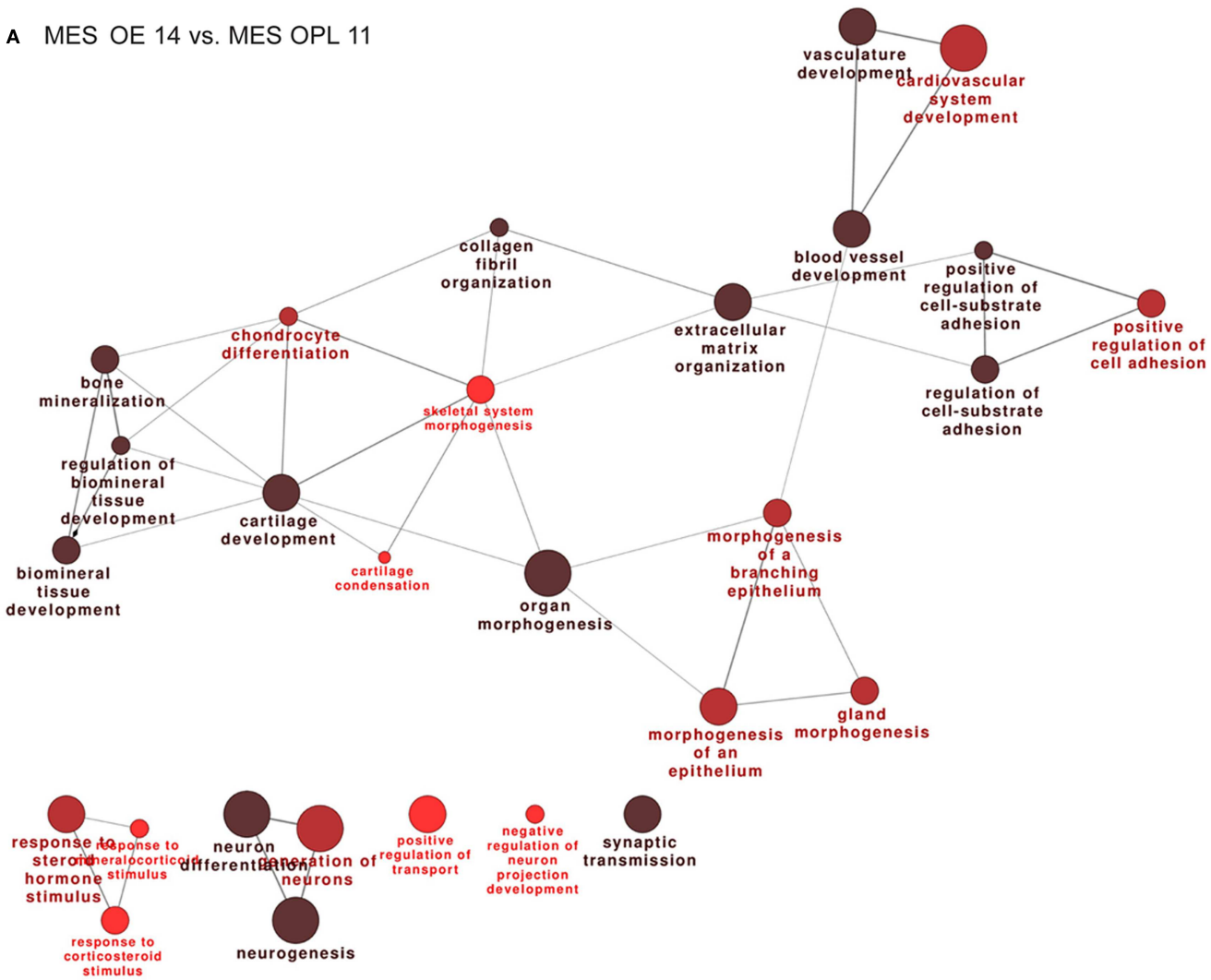


FIGURE 2 | Continued

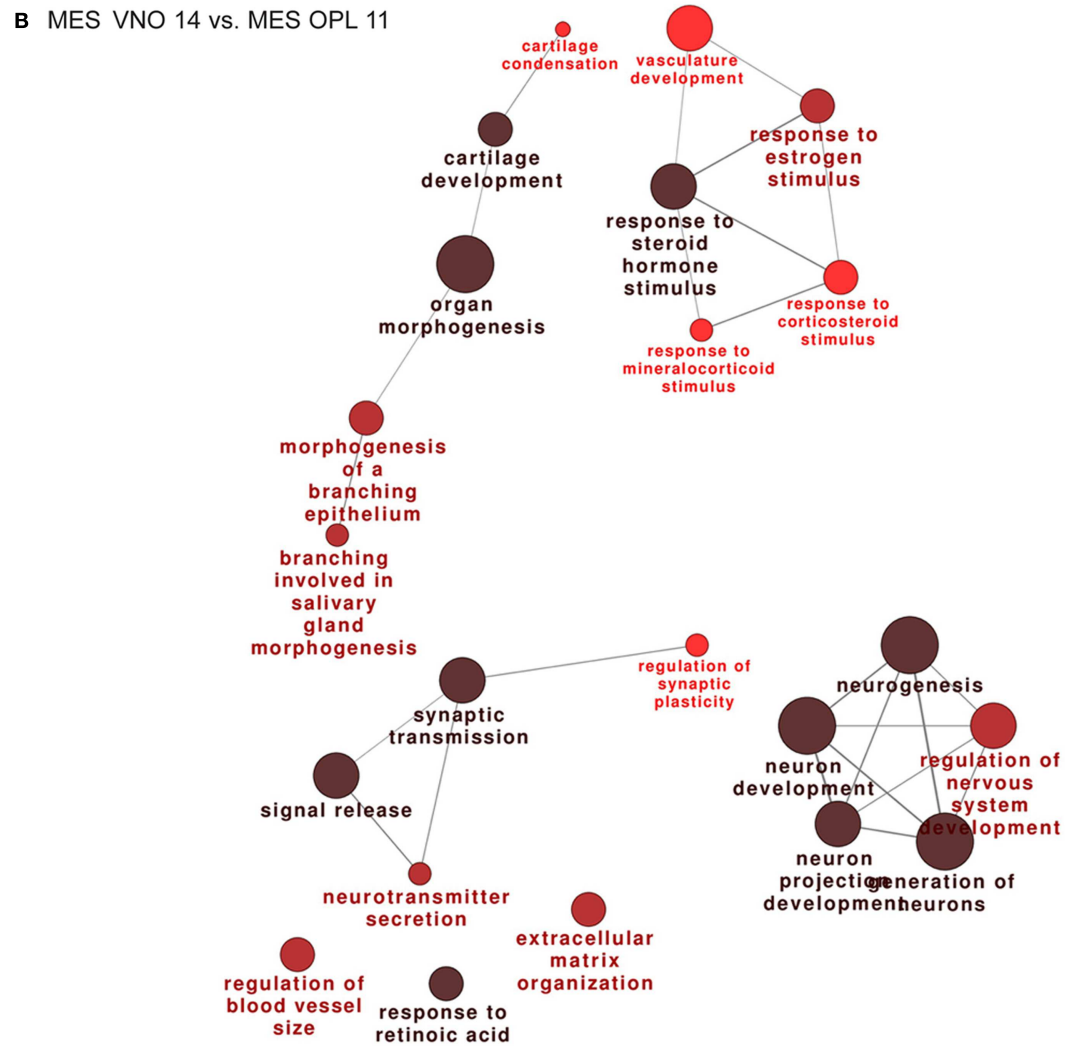


FIGURE 2 | Functional categorization of genes differentially expressed in the mesenchyme adjacent the OE and VNO, during development. Significantly over-represented functional categories of the DEGs detected in the mesenchymal tissue associated with the OE 14.5 vs. OPL 11.5 **(A)** or associated with the VNO 14.5 vs. OPL 11.5 **(B)** were organized in networks using ClueGO. Only the results of the up-regulated DEGs are shown.

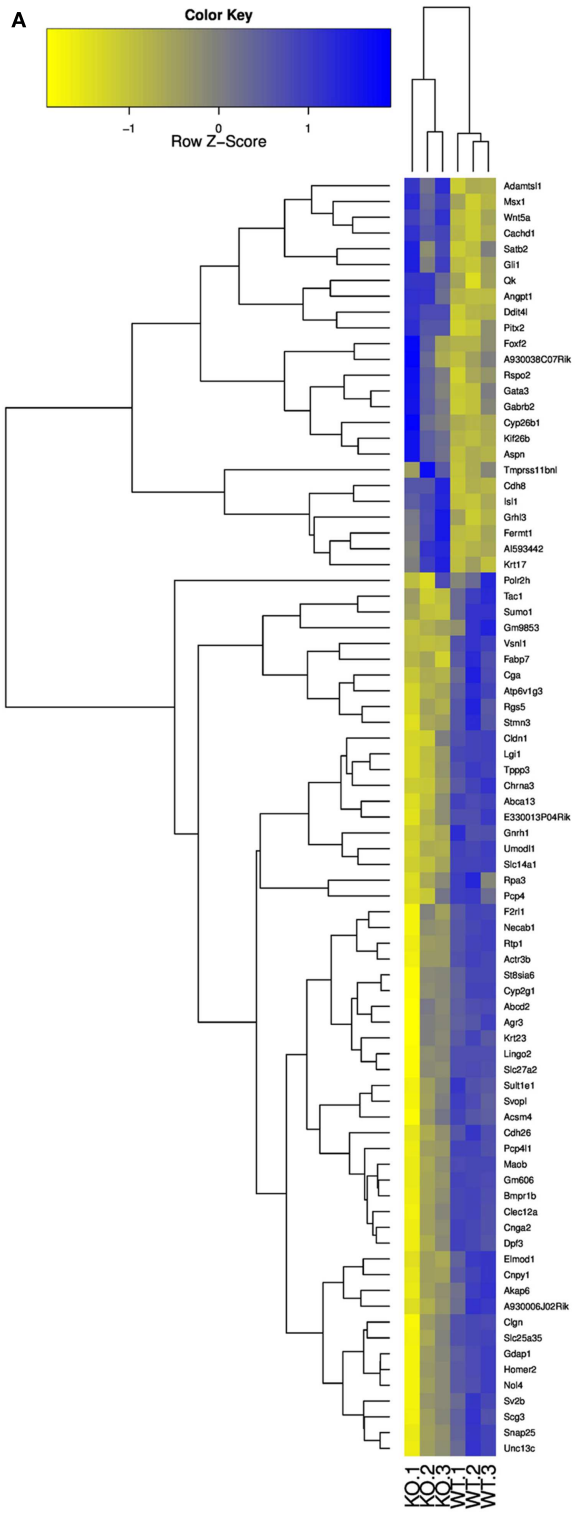
50 (69%) have a NEp expression, 14 (19%) are not expressed in the OE, 4 (5%) have a Mes expression, 3 (4%) have a Res expression, and 2 (3%) are ubiquitously expressed. Thus, conclude that the majority of down-modulated genes are expressed in the OE.

Then we examined whether the DEGs were differentially expressed also in other mouse tissues upon loss of *Dlx5*, specifically the inner ear and the pharyngeal arches (68, 69). No common DEG was found, indicating that Dlx5 targets are strongly tissue-specific. Next we examined whether the olfactory DEGs we detected were also differentially expressed in other published mouse models of KS, i.e., the *Klf7* and the *Emx2* mutants (70, 71). Three genes were found commonly differentially expressed the three models, namely *stathmin-like 3*, *synaptotagmin 1*, and *calmegin*, all expressed in the embryonic OE. Fifty genes were in common between Dlx5 and Emx2 datasets, seven were in common between *Emx2* and *Klf7*, and one was in common between Dlx5 and Klf7. However it should be noted that the profiles of the *Emx2* and *Klf7* mutants were obtained from the OB and not the OE.

The DEGs up-regulated in the absence of *Dlx5* are enriched in generic terms: biosynthesis, metabolic processes, morphogenesis. Of the 27 DEGs considered, 14 (52%) are expressed in the Mes, 6 (22%) are not expressed in the OE/VNO, 3 (11%) have a Res expression, 2 (7.5%) are expressed in the OE, and 2 (7.5%) are ubiquitously expressed. Thus, we conclude that most of the up-regulated DEGs are not expressed in the OE. Since the Dlx proteins are generally considered transcription activators (72, 73), the interest in these DEGs is low and they were not further considered.

GENOME-WIDE PREDICTION OF Dlx5 BINDING SITES AND TRANSCRIPTIONAL TARGETS

Using the consensus PWM for Dlx5 (56) (Figure S3 in Supplementary Material) we screened conserved regions of the vertebrate



B

Term	P value
GO:0045104~intermediate filament cytoskeleton	3 E-07
GO:0045103~intermediate filament-based process	6 E-07
GO:0045109~intermediate filament organization	8.1 E-05
GO:0007267~cell-cell signaling	7.7 E-04
GO:0019226~transmission of nerve impulse	8.4 E-04
GO:0021983~pituitary gland development	9.6 E-04
GO:0035270~endocrine system development	0.00142
GO:0007010~cytoskeleton organization	0.00163
GO:0021536~diencephalon development	0.00213
GO:0030900~forebrain development	0.00549
GO:0007268~synaptic transmission	0.00716
GO:0030855~epithelial cell differentiation	0.00968
GO:0048732~gland development	0.01083
GO:0031424~keratinization	0.01528

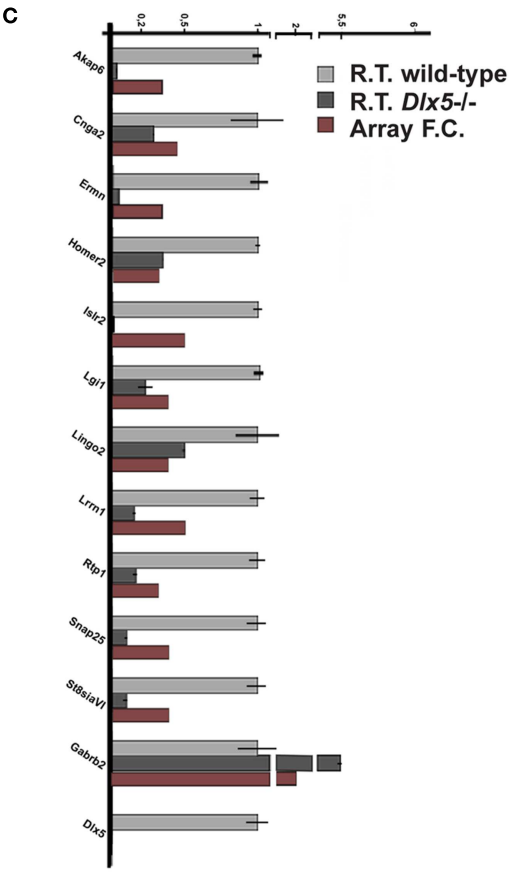


FIGURE 3 | Genes differentially expressed in the *Dlx5*$^{-/-}$ *OE* vs. wild-type OE. **(A)** Heatmap and cluster analyses on the MicroArray data, after elimination of non-annotated genes and of the OR genes. The triplicate *Dlx5*$^{-/-}$ (KO) and wild-type (WT) samples are indicated at the bottom. **(B)** Gene Ontology to detect over-represented functional categories. **(C)** Validation by Real-Time qPCR on selected down-regulated genes, done on independently collected samples. The abundance in the wild-type tissue is set = 1. For each gene, the fold-change determined by MicroArray hybridization is also reported.

genome and detected putative Dlx5 binding sites. We attributed to each site a score that reflects the number of species in which the site is conserved. We then associated the sites to an associated Refseq transcripts and found 3,426 RefSeq targets, corresponding to 2,683 unique Entrez-IDs [see Materials and Methods, and Ref. (57)]. The top scoring RefSeq are reported in Table S15 in Supplementary Material. We then categorized the predicted Dlx5 targets by ClueGO (54), and detected an enrichment categories such as neuronal differentiation, brain development, etc. as expected [there is ample literature on this; see Ref. (73)].

We then intersected the best predicted Dlx5 targets (having at least one binding site conserved in at least three mammalian species, and located <10 kb from the TSS) with the list of DEGs obtained comparing *Dlx5*$^{-/-}$ vs. WT OE, and we found that 16% of the down-regulated DEGs (19/121; $p = 0.0003$) were indeed predicted target of *Dlx5*, while 40% of up-regulated DEGs (9/21; $p = 0.00019$) were predicted targets. In both cases statistical significance was reached. This suggests that the prediction algorithm we have used is sensitive and sound. To restrict the number of candidate genes we intersected the profile datasets with: (a) embryonic expression databases, (b) conserved co-regulations, (c) predicted Dlx5 sites and target RefSeq, (d) data from published literature, in order to assign a score value to each DEG (**Tables 1A–E**). The expression of these putative *Dlx5* targets in the embryonic OE and nasal region, by *in situ* hybridization (see footnote text 4) is reported in Figure S5 in Supplementary Material. Some of the most functionally relevant genes are briefly described below:

Lrrn1 codes for a transmembrane protein related to Drosophila TRN/CAPS proteins, known play a role in neuromuscular target recognition, and to mediate interactions between incoming axons and the targets, possibly via homophilic adhesion. *Lrrn1* is expressed in the mouse embryonic OE.

Lingo2 (also known as *Lrrn6c*) codes for a transmembrane protein, expressed in the OE and in the ventricular region of the embryonic forebrain. Lingo proteins interact with the NOGO receptor and are able to modulate the NOGO pathway (74), however their precise functions are poorly known.

Lgi1 codes for a leucine-rich repeat secreted molecule of the SLIT family, involved in growth of neuronal processes on myelin substrates (75, 76).

St8siaVI is expressed by olfactory neurons and might be implicated in polysialylation the N-CAM to confer anti-adhesive properties to neuronal surfaces (77–79).

Homer2 codes for a protein present at post-synaptic density, likely to be involved in receptor clustering and trafficking, as well as calcium homeostasis (80). Recently, a role of Homer2 in tuning the activity of G protein-coupled receptors (such as ORs) has been reported (81). *Homer2* is expressed in the OE of the mouse embryo, however its function is unknown.

TESTING Dlx5, Dlx5 TARGETS, AND KS GENES IN ZEBRAFISH STRAINS: THE OLFACTORY AXONS

The development of olfactory system is well conserved during vertebrate evolution (27, 35, 82) and consists of two independent components: the main OE for detecting chemical compounds (odorants) and the VNO-accessory system for detecting pheromones. Fishes and primates lack a VNO organ and present only one olfactory organ, the OE (83). Within the OE of the fish, all ORNs project their axons to the OB – at different region in a mutually exclusive manner (60) – but display distinct properties with respect to their morphology, relative position in the OE, and molecular expression. The ciliated OSNs with long dendrites are situated in the deep layer of the OE, whereas microvillous ORNs with short dendrites are located in the superficial layer. The ciliated and microvillous ORNs are reported to express OR-type and V2R-type receptors, respectively (84, 85).

We opted to use *Danio rerio* (zebrafish) as a model to functionally examine *in vivo* the identified DEGs for their role in olfactory/GnRH development. We used two transgenic zebrafish strains expressing distinct fluorescent proteins in the fish olfactory neurons (59–61). In one strain the *CFP* reporter is expressed under the control of *OMP* promoter, which marks the majority of basal-layer ORN, projecting their axons to the dorsal OB. In the other strain, the *Venus* (YFP) reporter is expressed under the control of the *Trpc2* promoter, which marks a sub-population of apical-layer ORN, projecting to the ventro-lateral OB (scheme in Figure S4 in Supplementary Material) (60). The CFP+ and the Venus+ (YFP+) neurons are thought to correspond, respectively, to the OE and VNO receptors of the mammalian system. Since the reporter fluorescent proteins are efficiently translocated in the ORN axons, these two strains visualize the peripheral olfactory pathway.

We tested *z-fgfr1a*, the fish ortholog of mammalian *FGFR1*, to establish whether its depletion recapitulates the hallmarks of KS. Notably, mice hypomorphic for *FGF8* expression show distinctive signs of a KS phenotype, i.e., impaired migration of GnRH+ neurons and defects in olfactory development (41, 42). We injected *z-fgfr1a* MOs in 1-cell embryos of the *OMP:CFP* and the *Trpc2:Venus* strains, and 72 hpf we examined the number of fluorescent embryos, the organization of the OPL, the fasciculation, extension and glomeruli formation. In 61% (32/52) of the embryos we observed an altered morphogenesis of the OPL and an abnormal distribution of the CFP+ and the Venus+ neurons within the OLP (**Figure 4**); we defined this phenotype as "placode defect." In 30% (16/52) of cases we observed bundles of OE-type and VNO-type axons either overshooting past the OB or taking a misguided route (arrows in **Figure 4**). We also observed lack or impairment of connection with the OB, as indicated by the absence of typical glomerulus structures or their disorganized position at the OB (asterisks in **Figure 4**). We collectively defined these phenotypes as "connectivity and glomeruli defect." None of these phenotypes were seen in control embryos.

z-dlx5a is the fish ortholog of mammalian *Dlx5*, in fact the embryonic expression territory is similar (86), and its knockdown causes craniofacial and neuronal phenotypes resembling the *Dlx5*$^{-/-}$ phenotype in mice (87, 88). We depleted *z-dlx5a* in zebrafish embryos using a combination of two MO, and examined the organization of olfactory axons. Following MO injection, 72 hpf we recovered about 50% of CFP+ embryos (95% of the control injected) and about 72% of Venus+ embryos (78% of the controls). In 45% of cases (of 80 examined) we observed OPL defects, while in 54% of cases we observed OE-type and

Table 1 | Best *Dlx5* target gene, selected combining the profiling results with PWM-based site prediction and embryonic expression.

Gene title	Gene symbol	log2.FC.	Dlx site	Express	Score	Notes
(A) SURFACE RECEPTORS/ADHESION MOLECULES OR MODIFIERS						
Leucine-rich repeat and Ig domain containing 2	*Lingo2*	−1.3995539	+	N Ep	5	Structure similar to other Receptor Tyrosine Kinases, such as Trk. Associated to higher risk of tremor and Parkinson. Lingo1 is a component of the NOGO-66 receptor and may play a role in neurite outgrowth and oligodendrocyte differentiation
Leucine-rich repeat LGI family, member 1	*Lgi1*	−1.355411	+	N Ep	5	Secreted molecule of the SLIT family, promotes formation of stress fibers. Inhibits cell movement and invasion. Enhances growth of neuronal processes on myelin-based substrates. Its receptor forms complexes with Adam22
Leucine-rich repeat protein 1, neuronal	*Lrrn1*	−1.032383	+	N Ep	5	Transmembrane protein of unclear function. Regulates neurite growth
Ig superfamily containing leucine-rich repeat 2	*Islr2*	−0.9967503	+	Not/migr cell	4	Also known as Linx, could be a Receptor Tyrosine Kinase evolutionarily related to Trk receptor. Modulates axon extension and guidance
ST8 α-*N*-acetyl-neuraminide α-2,8-sialyltransferase VI	*St8siaVI*	−1.3472121	+	N Ep	4	Sialo-transferase expressed by neurons, essential for surface functions during neurite growth and neuronal migration
(B) SCAFFOLD INTRACELLULAR PROTEINS						
A kinase anchor protein 6	*Akap6*	−1.5186358	+	N Ep	5	Protein Kinase A-anchoring proteins. Serves as scaffold to bring together PKA and PDE and coordinate the timing and intracellular localization of cAMP signaling. Also binds to- and modulates-signaling through ERK, MAPK, and PP2A
Dual adaptor for phosphotyrosine and 3-phosphoinositides 1	*Dapp1*	−1.2094534		N Ep	4	Signaling adapter molecule, coordinates timing and location of signaling by PIP3 and PIP2 with that of ERK. Also binds F-actin and Rac
RIKEN cDNA 9330120H11 gene	*9330120H11Rik*	−1.1589186		N Ep	4	Also known as HOMER 2, present at post-synaptic density, involved in receptor clustering, trafficking, and in calcium homeostasis
(C) SYNAPTIC PROTEINS						
Synaptosomal-associated protein 25	*Snap25*	−1.3481758	+	N Ep	5	Controls membrane trafficking and fusion at the growth cone and at the synapse. Implicated in neuroblast migration and neuritogenesis during development. Forms complex with p140CAP which also binds to p130 CAS
γ-Aminobutyric acid (GABA) A receptor, subunit β 2	*Gabrb2*	1.0063619	+	N Ep	5	Receptor subunit for GABA. GABA-b receptors mediate signals inhibitory for olfactory axon elongation
Receptor transporter protein 1	*Rtp1*	−1.6548021		N Ep	4	Chaperon, required for the efficient translocation of OR molecules to the membrane. Interacts with the OR and with Homer
RIKEN cDNA 9330120H11 gene	*9330120H11Rik*	−1.1589186		N Ep	4	Also known as *Homer2*, present at post-synaptic density, involved in regulation of calcium fluxes and homeostasis

(Continued)

Table 1 | Continued

Gene title	Gene symbol	log2.FC.	Dlx site	Express	Score	Notes
(D) AXON-GLIA INTERACTION PROTEINS						
Fatty acid binding protein 7, brain	*Fabp7*	−1.9620307		N Ep-Gliale	4	Known as BLBP in human. Controls surface functions that are required for axon-Schwann cell interaction. May be involved in peripheral axon elongation and regeneration
Ermin, ERM-like protein	*Ermn*	−1.5033487		N Ep/Sust cell	4	Also known as Juxtanodin. Expressed in sustentacular cells, binds to F-actin and stabilizes the actin cytoskeleton. In the CNS promotes myelination
Ganglioside-induced differentiation-associated-protein 1	*Gdap1*	−1.1935825	+	N Ep/Res	4	Involved in the Charcot-Marie tooth disease, in particular those forms with axonal deficits. Cellular function unclear
UDP Galactosyltransferase 8A	*Ugt8a*	−1.1139671	+		4	Important for the biosynthesis of galacto-lipids and in myelin formation
(E) CALCIUM-REGULATION						
Cyclic nucleotide gated channel α 2	*Cnga2*	−1.129349	+	N Ep	5	Regulate axon extension and glomerular formation. KO mice have behavioral defects possibly linked to olfactory functions
Visinin-like 1	*Vsnl1*	−1.3860936	+	N Ep	4	Also known as GP2. Calcium-regulated guanylate cyclase transduction system. Play a role in adaptation. Inhibits the formation of cAMP. May affect dendrite and growth cone arborization

Genes are sub-divided in five general categories (A–E).

VNO-type axons targeting abnormal regions of the head near the OBs, often overshooting past the OB (arrows in **Figure 4**). We also observed impaired axon-OB connections, as judged by the absence of glomeruli-like bundles or their disorganized position (asterisks in **Figure 4**). None of these phenotypes were seen in non-injected or control MO-injected embryos. Thus, the depletion of *z-dlx5a* causes defects that recapitulates key aspects of the $Dlx5^{-/-}$ phenotype (14, 16, 47).

Next we focused on the putative Dlx5 targets *Lrrn1*, *Lingo2*, *Islr1*, *St8siaVI*, and *Homer2*, whose embryonic expression in the brain and olfactory system is reported in Figure S5 in Supplementary Material. We depleted z-*lrrn1* in 1-cell zygotes by MO injection. Of the injected embryos, only approximately 50% were recovered and positive for OMP:CFP (vs. 85% in the control injected), and in a majority of these (75% of a total of 62 examined) we observed a reduction of the CFP+ signal intensity. On the contrary, we recovered a not significantly different percentage of Venus+ embryos (71 vs. 78% in the control injected) and these occasionally (20%) showed a reduced YFP fluorescent signal (**Figure 5**). Twenty percent of *z-lrrn1* MO-injected embryos displayed placode defects, consisting in a reduced size, altered shape, and mispositioned neuron. Half of the *z-lrrn1* MO-injected embryos displayed an altered pattern of olfactory axon fasciculation and extension, with axons overshooting or taking an ectopic route (arrows) and reduced or absent glomeruli (asterisks). Thus, the depletion of *z-lrrn1* results in a delayed differentiation of the OMP+ type olfactory neurons and altered olfactory axons trajectory and connectivity, hallmarks of the phenotypes observed in $Dlx5^{-/-}$ mice and in *z-dlx5a* fish morphants.

Next we depleted *z-lingo2* in reporter zebrafish embryos. Injection of the anti-*z-lingo2* MO in 1-cell embryos caused minor OP defects, consisting in altered organization and shape, while axon trajectory and glomeruli formation appeared normal (**Figure 6**). Next we depleted *z-st8SiaVI* in zebrafish embryos. Injection of the anti-*z-st8siaVI* MO in 1-cell embryos resulted in a phenotype affecting axon extension, trajectory, and glomeruli formation (**Figure 6**). Next we depleted *Homer2* in the reporter fish embryos. Injection of the anti-*z-homer2* MO in 1-cell embryos resulted in defects of OP organization and axonal targeting, plus also resulted in a reduced expression of *Trpc2*, seen as reduced YFP fluorescent signal (**Figure 6**). This last result might indicated that *z-homer2* is involved in the differentiation of the VNO-type neurons, and its depletion may delay this process. Finally, the depletion of z-*islr1* yielded no appreciable phenotype affecting the olfactory pathway (data not shown). This gene is not expressed in the embryonic OE (Figure S5 in Supplementary Material).

TESTING Dlx5, Dlx5 TARGETS, AND KS GENES IN ZEBRAFISH STRAINS: THE GnRH NEURONS

To determine whether some of the DEGs that emerged from transcription profiling of *Dlx5* mutants had some function of GnRH neuronal migration and neurite organization, we used the

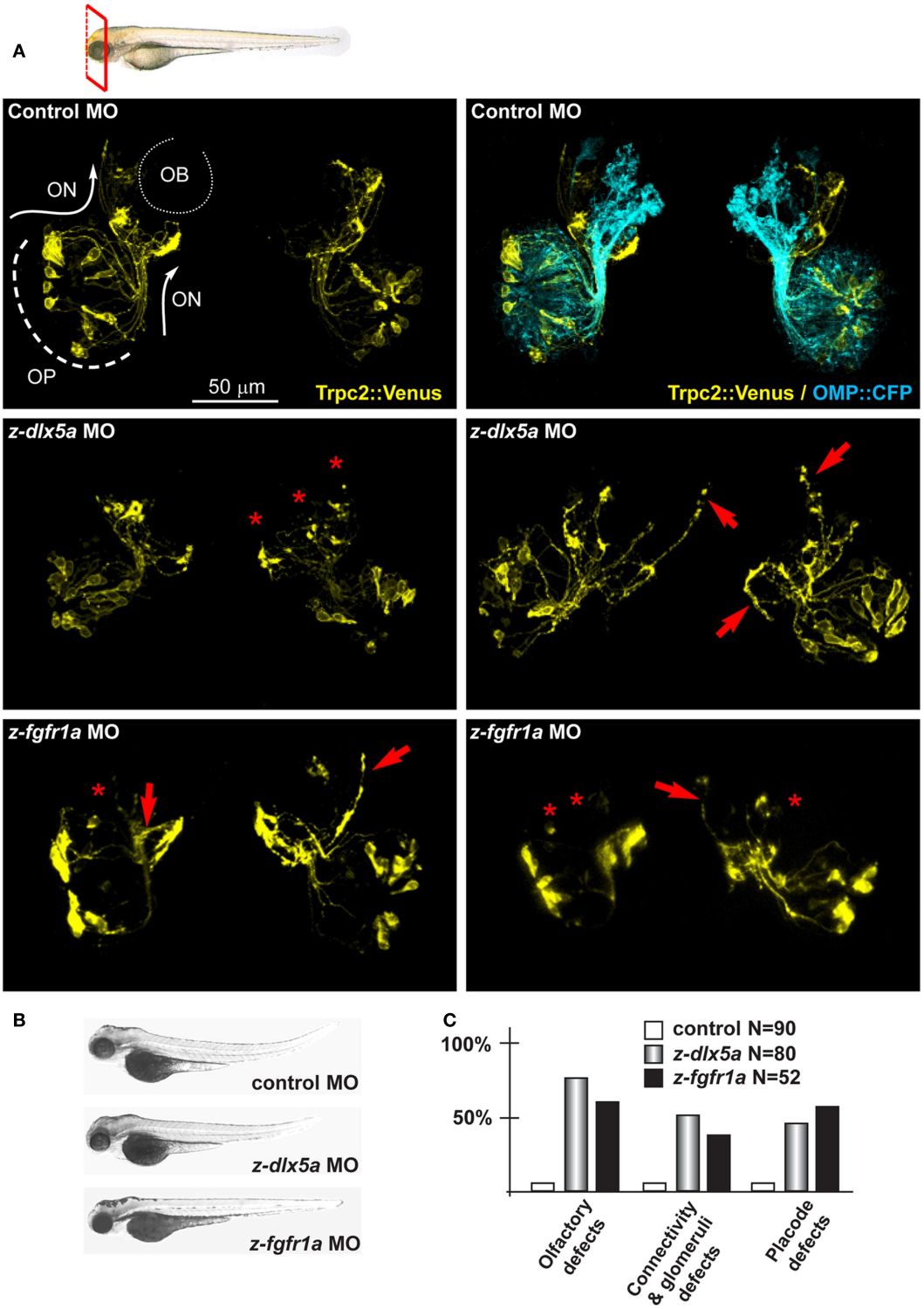


FIGURE 4 | Depletion of endogenous *z-dlx5a* and *z-fgfr1a* in zebrafish embryos, to image the olfactory axons. **(A)** Micrographs of *Trpc2:Venus* (YFP, yellow fluorescence) and *OMP:CFP* (cyan fluorescence) fish embryos injected with a control MO (top panels), injected with anti-*z-dlx5a* MO (middle panels) or injected with anti-*z-fgfr1a* MO (bottom panel). White arrows and lines indicate the normal axonal pathway in control embryos. Red asterisks indicate absence of glomeruli. Red arrows indicate altered axonal trajectories. **(B)** Whole-mount bright field micrographs of injected embryo, showing an overall normal embryonic morphology and growth rate in the injected embryos, compared to control injected ones. **(C)** Proportions of embryos showing either placode defects (OPL disorganization, altered neuron distribution), or connectivity/glomeruli defects (altered axon trajectory, altered fasciculation, reduced or absent glomeruli), or both, upon injection of control (open bars), anti-*z-dlx5a* (gray bars), or anti-*z-fgfr1a* (solid black bars) MOs.

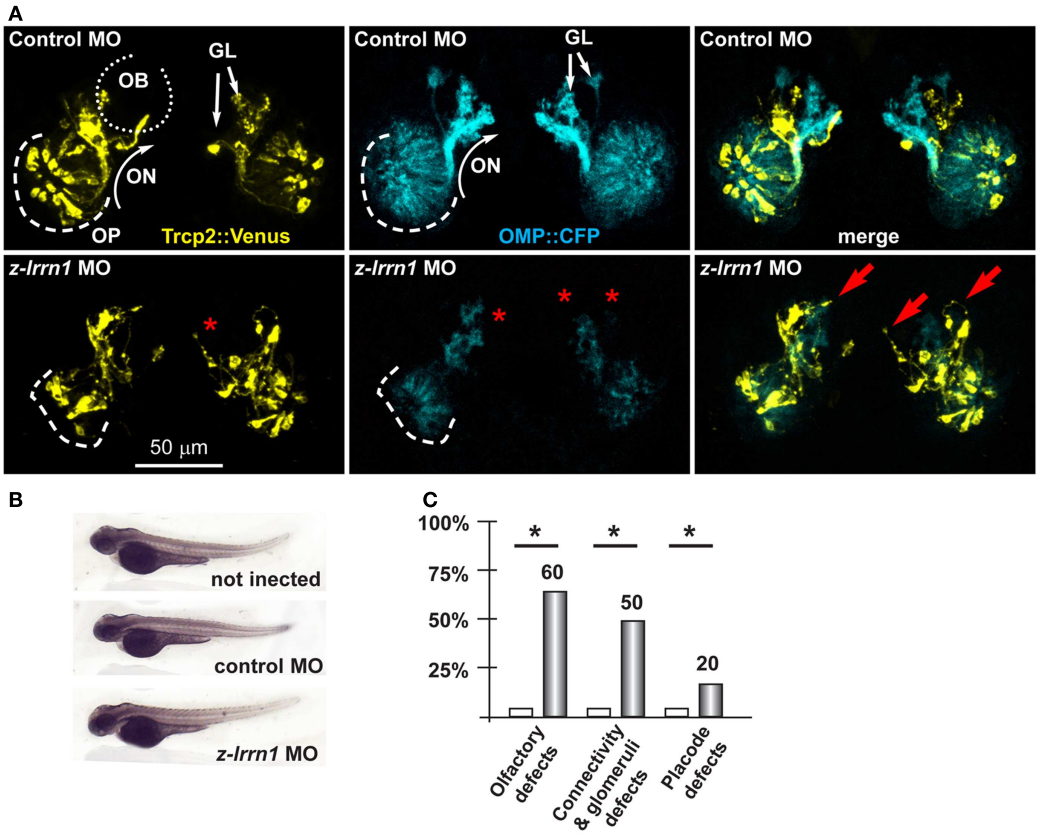


FIGURE 5 | Depletion of endogenous *z-lrrn1* in zebrafish embryos, to image the olfactory axons. **(A)** Micrographs of *Trpc2:Venus* (YFP, yellow fluorescence) and *OMP:CFP* (cyan fluorescence) zebrafish embryos injected with control (top panels) or with anti-*z-lrrn1* (bottom panels) MOs. The control MO did not cause any significant alteration. White arrows indicate the normal axonal pathway and glomeruli in the control embryos. Red asterisks indicate absence of glomeruli. Red arrows indicate altered axonal trajectories. **(B)** Whole-mount bright field micrographs of injected embryo, showing normal embryonic morphology and growth rate. **(C)** Proportions of embryos showing either OPL disorganization, or olfactory axon mistargeting, or both (last bars) upon injection of control (open bars) or anti-*z-lrrn1* (gray bars) MOs.

GnRH3:GFP transgenic zebrafish strain, previously reported (62–64). In these animals the GFP reporter is expressed under the transcription control of a fragment of the fish *GnRH3* promoter. The *GnRH3*-GFP+ neurons have been widely characterized, and they consist in a population of terminal nerve associated GnRH+ neurons, thought to represent the mammalian hypothalamic neurons with olfactory origin (27, 62–64, 89) (**Figures 7A,B**).

We depleted *z-dlx5a*, *z-fgfr1a/b*, and *z-lrrn1* in the *GnRH3:GFP* 1-cell zygotes, and examined the effect on the number, position, neurite organization, and commissure formation of the GFP3+ neurons associated to the terminal nerves. The depletion of *z-dlx5a* resulted in a reduced number of GFP+ neurons in 30% of cases, and in 70% of cases clearly appeared mispositioned (40 morphants examined) (**Figures 7C,D**; quantifications in **7G**). However, the depletion of *z-dlx5a* did not affect the ability of GFP+ axons to cross the midline at the anterior commissure. Thus, a reduction of *z-dlx5a* in the fish model recapitulates (some of) the GnRH phenotype observed in the mouse model (16, 47).

The depletion of *z-fgfr1a/b* resulted in a reduced number of GFP+ neurons in 80% of cases (a total of 40 morphants examined), and in 22% of cases these neurons were clearly mispositioned, and had shorter neurites (**Figure 7E**). In 35% of cases the GnRH+ neurites failed to properly cross the midline in the anterior commissure. This phenotype recapitulates that seen upon depletion of *z-kal1a/b* (45, 46), thus we conclude that, based on the results of two well-established KS/nCHH genes and one KS-causing gene in the mouse, the use of MO in the *GnRH3:GFP* strain is a valid approach to examine the KS phenotype *in vivo*, and assures that future analyses on this subject will be informative.

The depletion of *z-lrrn1* in the *GnRH3:GFP* fish embryo caused a reduction in the number of GFP+ neurons in 45% of cases, and in 65% of cases caused their misposition along the terminal nerve (40 morphants examined) (**Figure 7F**). We also observed a reduction of their neurite length, but little of no defect of the anterior commissure. Thus also one Dlx5 target is involved in the organization and the maturation of the olfactory/GnRH system.

BIOINFORMATIC PREDICTION/PRIORITIZATION OF NEW KALLMANN DISEASE GENES

A large set of genes has been found mutated, alone or in combination, in KS/nCHH patients, by classical mutation search approaches. However, with the exception of *KAL1* and *FGFR1*,

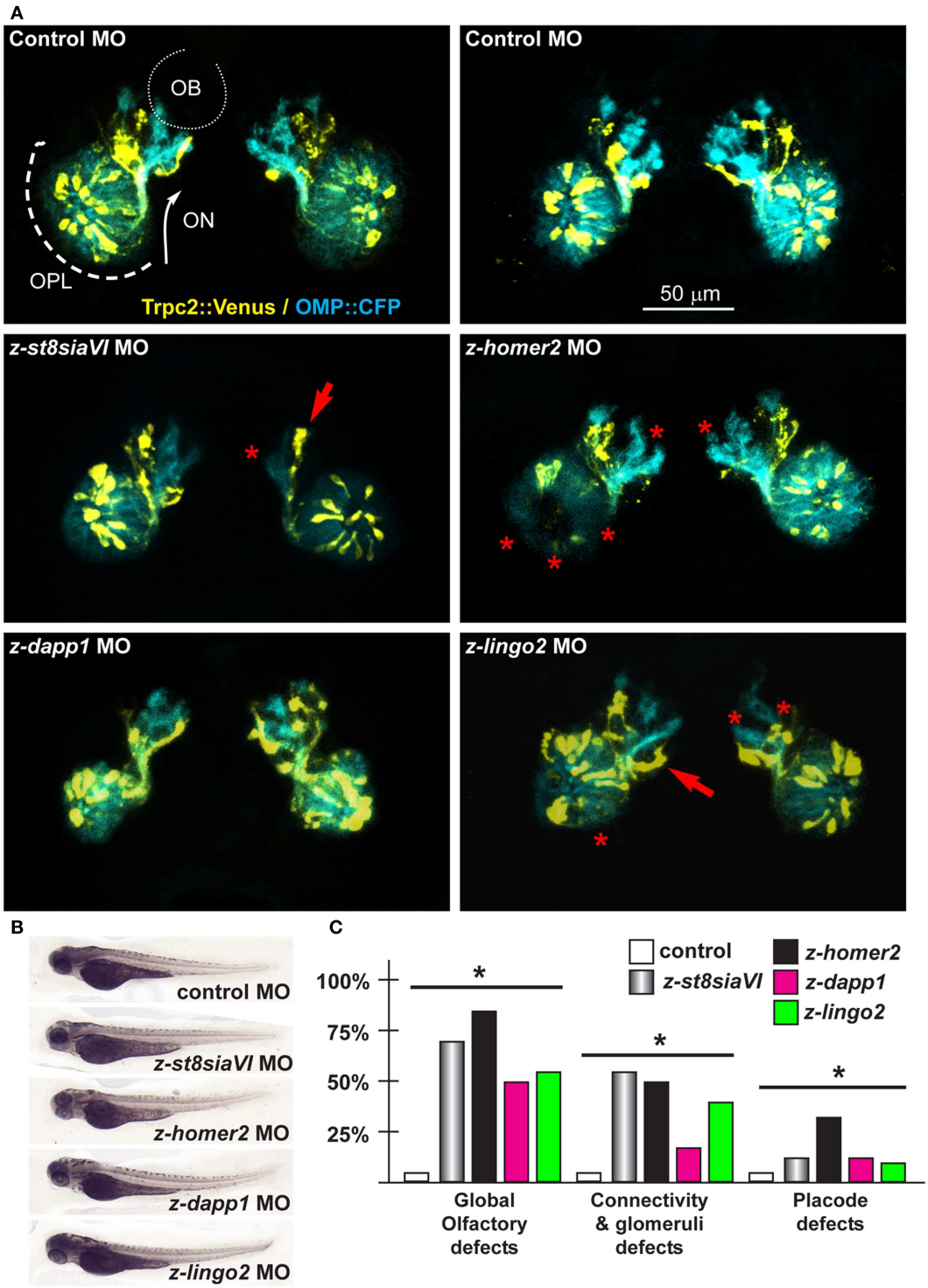


FIGURE 6 | Olfactory and VNO axons, upon depletion of endogenous *z-St8siaVI, z-lingo2,* and *z-homer2* in zebrafish embryos. **(A)** Micrographs of *Trpc2:Venus* (yellow fluorescence) and *OMP:CFP* (cyan fluorescence) embryos injected with control MO (top panels), or injected with anti-*z-st8siaVI*, anti-*z-homer2*, anti-*z-dapp1*, and anti-*z-lingo2* MOs, as indicated on top of each image. The control MO did not cause significant alterations. Arrows indicate altered axonal trajectory, asterisks indicate absence of glomeruli or altered OPL organization. Asterisks indicate the regions of reduced fluorescence intensity. **(B)** Whole-mount bright field micrographs of injected embryo, showing normal morphology and growth rate. **(C)** Proportions of embryos showing either placode defects, connectivity and glomeruli defects, or both, upon injection of the MOs indicated above (colored bars), compared to control MO (open bars). Asterisks indicate statistical significance.

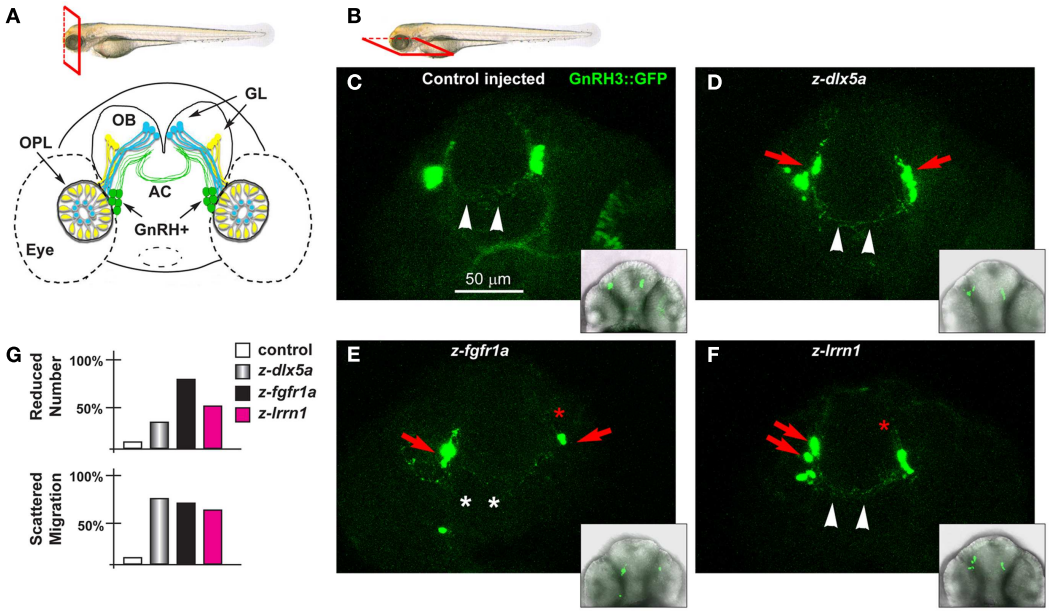


FIGURE 7 | The GnRH3 neurons upon depletion of endogenous *z-dlx5a, z-fgfr1a,* and *z-lrrn1* in zebrafish embryos. **(A)** Scheme showing the positions of the GnRH3:GFP+ neurons (green cells), relative to the OPL, the OB, and the olfactory nerves (yellow and blue), in a frontal view. The anterior commissure is shown at the basis of the OB. On top, a scheme illustrating the view plane (frontal) used in **(A)**. **(B)** Scheme illustrating the view plane (ventral) used for the fluorescent images in **(C–F)**. **(C–F)** Micrographs of *GnRH3:GFP* zebrafish embryos, at 60 hpf, injected at the 1-cell stage with either a control MO **(C)**, with anti-*z-dlx5a* MO **(D)**, with anti-*z-fgfr1a* MO **(E)**, or with anti-*z-lrrn1* MO **(F)**. Insets on the lower right of each panel is a low-magnification merged micrograph (bright field and GFP fluorescence) of the higher magnification one. Red asterisks indicate reduced number of cells, red arrowheads indicate scattering and delayed cell migration, white arrowheads indicate the anterior commissure, white asterisks indicate absence of anterior commissure. **(G)** Quantification of the observed phenotypes, as percent over the total number of GFP+ embryos examined with each MO.

each of them is mutated in a small fraction of the patients, and together account for no more than 40% of KS/nCHH cases. Five novel genes, functionally linked to FGF8, have been recently identified using predictive bioinformatics followed by mutation search in patients' DNAs (7). With the exception of some genes evidently linked (*Prok2* and *ProkR2*; *FGF8* and *FGFR1*; *GnRH* and *GnRH-R*) the remaining genes appear to be unrelated, or distantly related on a functional basis. We reasoned that relationships might exist between the KS-disease genes that are not obvious, or not easily detected, or that genes may have pleiotropic functions, not know as yet. Tools have been developed that search for such relationships in databases or newly generated data, and can be used to propose candidate disease genes (90).

HUMAN NETWORK

We compiled a list of genes known to cause KS, or KS and nCHH, excluding those causing only nCHH; the list included *FGFR1*, *FGF8*, *KAL1*, *PROK-2*, *PROKR2*, *CHD7*, *GnRH*, *GnRH-R*, *HS6ST1*, *TAC3*, *TACR3*, *SOX10 e SEMA3a*. We also included *FLRT3*, *IL17RD*, *FGF17*, *SPRY4*, *DUSP6*, members of the "FGF8 synexpression" group (7) and named all these "human reference genes." First we searched for the presence of the reference genes among the DEGs from the $Dlx5^{-/-}$ OE vs. WT, however none of them was found. Likewise, we searched for the presence of these genes among the DEGs from the time course of the normal OE and VNO. With the exception of *GnRH*, none of the other genes was found. Next, we positioned the "human reference genes" within the global conserved co-expression network, using TS-CoExp (with the exception of *KAL1/anosmin1* that lacks a mouse ortholog and for which the conservation criterium cannot be applied) (**Figure 8A**).

The network representation does not consent *per se* to derive relevant information. Instead, from the data we extracted those genes connected with at least six ($N = 2$), at least five ($N = 3$), at least four ($N = 10$), at least three ($N = 45$), at least two ($N = 317$), and at least one ($N = 1977$) reference genes. We then categorized these genes by G.O. and detected an enrichment of the following G.O. categories: phosphoproteins, kinase/transmembrane receptors, cell adhesion, cell junctions, regulation of cytoskeleton, cell migration/motility, neuronal projection. Among the most connected ones we did not find any gene causing KS in mice, but we identify *TRIM2*, *GATAD2A*, *SNRPN*, and *CDH2*. Being expressed in the embryonic OE (Figure S6 in Supplementary Material), these represent most interesting genes.

Next, we used the disease gene prediction tool of TS-CoExp to identify novel candidate KS genes: the "human input genes" were taken as reference to independently prioritize the following DEG lists: (a) $Dlx5^{-/-}$ OE vs. WT at E12; (b) EPI OE 14 vs. OPL E11 (WT); (c) EPI VNO 14 vs. OPL E11 (WT); (d) MES OE14 vs. OPL E11 (WT); (e) MES VNO 14 vs. OPL E11 (WT). From the DEG list (a) we found nine genes significantly associated with the KS phenotype, three of which (*RGS5*, *F2RL1*, and

DPF3) are expressed in the embryonic OE, while two (*GATA3* and *ADAMTS5*) are expressed in the olfactory mesenchyme (Figure S6 in Supplementary Material).

From the DEG lists (b) and (c) we found 21 and 73 genes respectively, significantly associated with the KS phenotype, 19 of which are present in both lists, and the majority of these are expressed in the nasal mesenchymal. Notably, the search predicted two genes known to cause KS in the mouse, namely *Ebf2* and *Nrp1*, confirming that our analysis is in principle correct. From the DEG lists (d) and (e) we found 47 and 189 genes respectively, significantly associated with the KS phenotype, 27 of which are present in both lists. Among these, 50% show expression in the embryonic OE (*ACAN*, *AKAP6*, *ATF5*, *KRT18*, *MYT1L*, *NDRG1*, *NRXN1*, *SYT1*, and *TPD52*) and 50% in the olfactory-associated mesenchyme (*ANXA1*, *DCN*, *FCGRT*, *PAPS2*, *PTRF*, *RUNX1*, *S100b*, and *TGM2*). Notably, the search predicted two genes known to cause KS in the mouse, namely *Ebf2* and *Nrp1*. Furthermore, we found genes such as *AKAP6*, *LINGO2*, *LGI1*, and *LGI2* that were found among the *Dlx5* targets in the OE, and *SEMA3C* and *MET*, known to play a role in axon guidance and cell migration, respectively.

MOUSE NETWORK

We applied the approach previously used to those genes causing a KS-like phenotype in mice; the list included *Dlx5*, *Emx2*, *Klf7*, *Fezf1*, *Six1*, *Prok2*, *ProkR2*, *Lhx2*, *Shep1*, *Ebf2*, *Nrp1*, and *Sema3a*. None of them was found in the DEG lists from the *Dlx5*$^{-/-}$ OE vs. wild-type, and none was found in the DEG list from the time course of the normal OE and VNO, with the exception of *Lhx2* and *GnRH*. We positioned the mouse reference genes within the conserved co-expression network (with the exception of *Fezf1* for which no result was obtained) (**Figure 8B**), and extracted lists of genes connected with at least five ($N = 1$), at least four ($N = 3$), at least three ($N = 33$), at least two ($N = 261$), and at least one ($N = 1850$) of them. These genes were then categorized by G.O. and we detected these over-represented terms: phosphoproteins, cell adhesion/cell junctions, neuronal projection, cell motility, cytoskeleton regulation, transcription regulation.

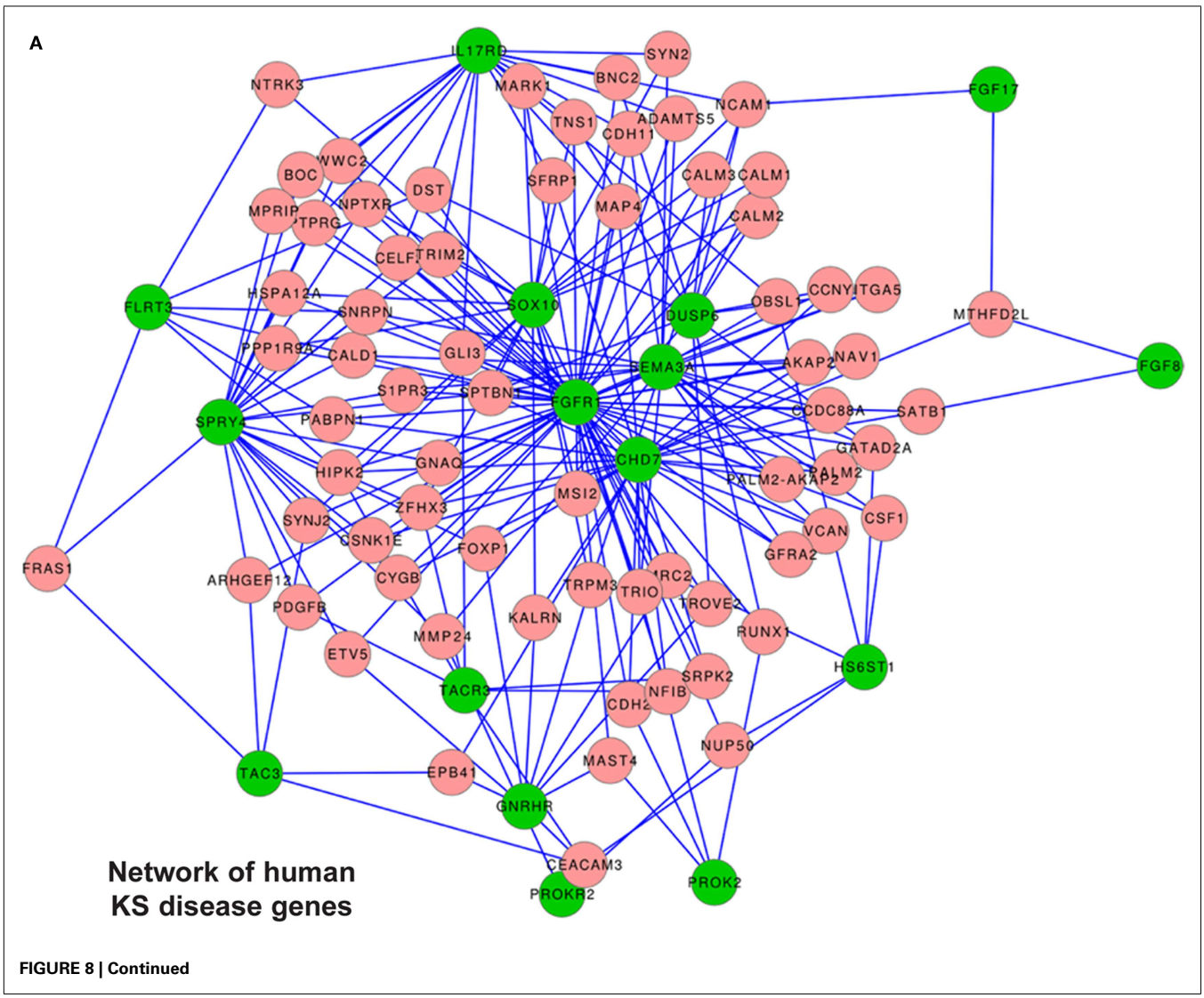


FIGURE 8 | Continued

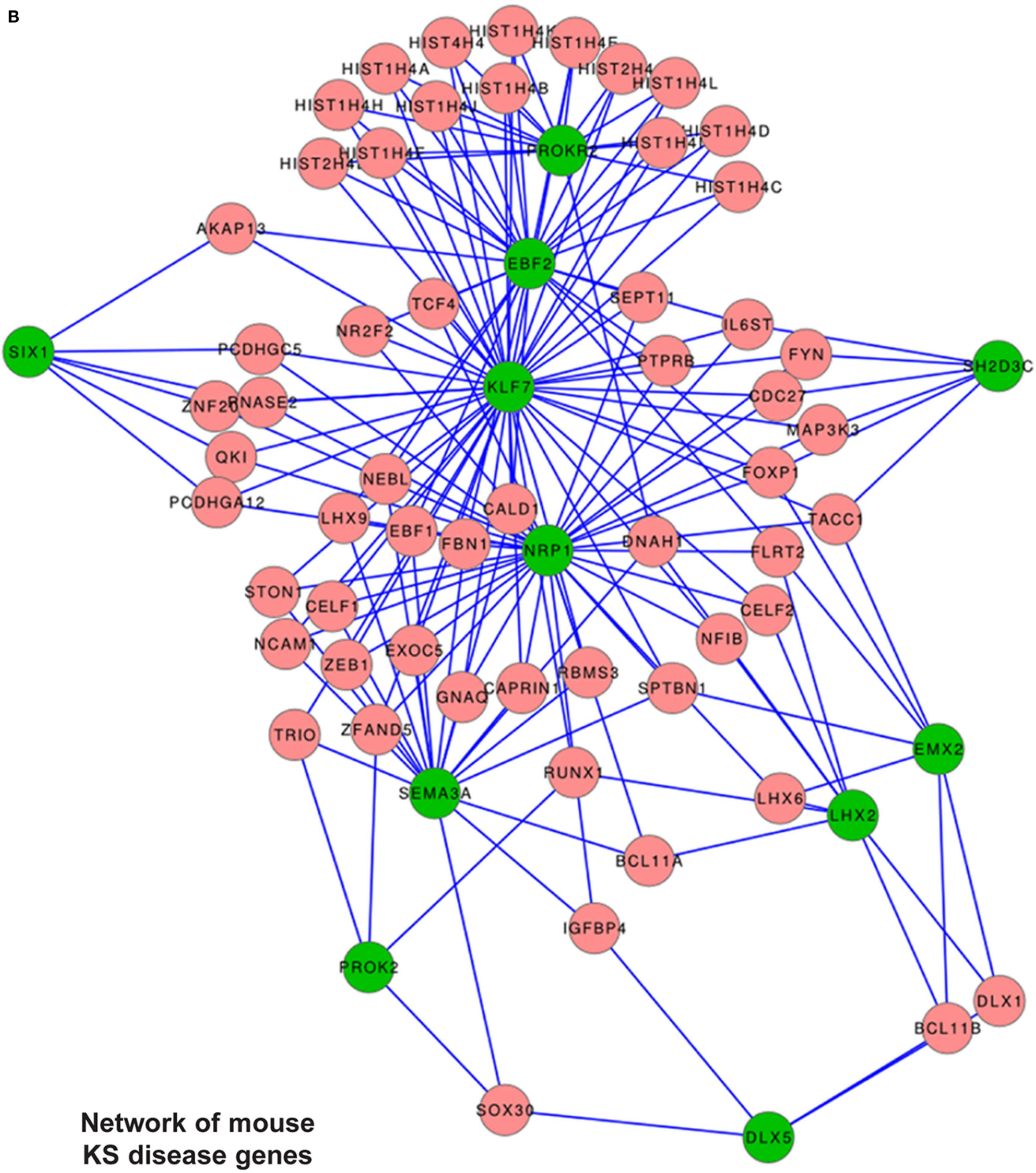


FIGURE 8 | Disease gene networks for KS. **(A)** Position of the human KS-causing genes within the global conserved co-expression network, as computed with the TS-CoExp algorithm. **(B)** Position of the genes causing a KS-like phenotype in mice, within the global conserved co-expression network. The lists of the input (human and mouse) "disease" genes used for these analyses are in the text. For simplicity, only the genes connected with at least three input genes are shown; the genes connected with "at least one" or "at least two" input genes are available upon request. Green circles represent the input genes, pink circles represent the connected genes, lines represent statistically significant co-regulations.

Next, for each of the four DEG list (from the profiling results, see above), we used the disease gene prediction tool in TS-CoExp to identify those genes most likely to be involved in KS, using the mouse reference genes as input. From the (a) list we found 13 genes, two of which (*Scn3B* and *Sv2B*) are expressed in the OE, while two (*Adamts5* and *Wnt5a*) are expressed in the olfactory mesenchyme (Figure S6 in Supplementary Material). From the (b) and (c) lists we found 19 and 65 genes respectively, 17 of which are present in both lists. Most are expressed in the nasal mesenchyme. Contrary to the human network, the mouse network did not predict any human KS gene. From the (d) and (e) lists we found 65 and 41 genes respectively, 17 of which are present in both lists and most of these are expressed in the nasal mesenchyme. Also in this case, we could not predicted any human KS genes. We found two genes: *Dcx* and *Lrrtm*, the first is relevant for migration of immature neurons, the second codes for a leucine-rich repeat protein similar to Lrrn1.

Finally, considering both the human and the mouse reference genes, five genes were found in at least three lists of genes associated with KS, namely *Dcn* (*Decorin*), *FGF7*, *Aspn*, *Ptfr*, and *Ntrk2*. Three of these, *Ntrk2*, *Dcn*, and *Ptfr* are clearly expressed in the juxta-OE and VNO mesenchyme, *Aspn* is ubiquitously expressed and the expression of *FGF7* is unclear. *FGF7* codes for a growth factor related to FGF22, the literature reports indications that it functions as pre-synaptic organizing molecule during hippocampal development (91, 92) and is needed for migration of enteric neurons (93). Its function in the developing OE is unknown. In conclusion, the most promising predicted KS genes are mesenchymally expressed. This is not surprising, considering that in all our profiling results, the prevalent categories are cell–cell and cell-matrix interactions, remodeling, signaling, etc. . .

Note: all gene lists, categorizations or tables not included in the manuscript or as supplementary material are available on request. All data are deposited at GEO repository, N° GSE52800.

DISCUSSION

Kallmann syndrome and nCHH are developmental/pediatric conditions phenotypically well characterized, however less well understood molecularly. Despite the number of genes found mutated in KS/nCHH patients, the majority of them still await a molecular definition. Thus, there is a strong basis to predict that many additional disease loci remain to be identified. Furthermore, the mutations found in KS patients, once thought to act alone, are now recognized as cooperating mutations, and the prevalent notion states that most KS/nCHH cases should be a bi-genic or oligogenic disease (21, 22). This raises hopes that a more exhaustive knowledge of cooperating genes and mutations, should consent a better prognosis and possibly personalized therapies.

Methods and algorithms have been proposed to identify/prioritize novel disease genes, based on (meta)-analyses of specific profiling data, co-expression networks, genome locations, functional categorizations, protein-protein interactions, etc. (90, 94). These methods have several advantages over direct whole exome sequencing of large panels of DNAs from KS/nCHH patients (95–97). In this study, on one side we have uncovered functional classes, possible networks, and individual genes involved in the olfactory/GnRH developmental, and validated some of them in the zebrafish model recapitulating the KS phenotype. On the other side we positioned known human genes causing KS and mouse models with a KS-like phenotype in gene-co-expression networks, in order to identify genes potentially relevant for the process and candidate KS-disease genes.

Embryonic development of the olfactory connection and the migration of immature GnRH neurons are anatomically and functionally linked. Since olfactory detection is a primary sensory system in most vertebrates, and sexual maturation/reproduction is essential for the species, it is not surprising the developmental process is highly conserved and is controlled by multiple – partially redundant – networks of molecular regulations. The high degree of conservation among vertebrates justifies the use of the zebrafish embryo for *in vivo* testing (98): not only its general anatomy has not greatly changed, but also the migration of GnRH neurons along the terminal nerve, in association with the VNO axons has been overall maintained (83).

We have generated profiling data, comparing the mouse normal OE and VNO at three developmental time-points, and comparing the normal vs. a mutant model characterized by a KS-like phenotype. The data have been used to identify novel gene categories involved in the development of the olfactory system, to identify Dlx5 target genes in the OE, and to intersect this wealth of information with data from other sources. As a further step, it might be useful to generate profiles from other models of KS in the mouse (i.e., *Prok2*, *Fezf1*, etc.) and intersect the results searching for common patterns and co-regulations. We have attempted this, however with little success, most likely because we specifically profiled the olfactory epithelia, while data form the *Emx2* and *Klf7* models were generated from the OB. Likewise, it would be useful to intersect our profiles from the *Dlx5* model with datasets from freshly dissociated embryonic GnRH neurons.

Categories that emerge from the "time course" profiles strongly implicate extracellular matrix remodeling, cell adhesion, and cell–cell signaling molecules. This is true both for the OE and VNO development, that after all appear more "similar" than "different." The profiles of the "pathologic" condition, i.e., the *Dlx5* knock-out model, indentified a number specific molecules in the categories of membrane receptors/adhesion molecules, axon-glia interaction molecules, but nothing specifically related to "axon elongation." This suggests that the cell-autonomous properties of the olfactory/VNO axons to establish connections, provided (directly or indirectly) by the transcription factor Dlx5, reflects cytoskeletal properties and cell surface events, mediated by receptors, scaffold proteins and cell adhesion (see below).

Since profile data may easily lead to false positives, functional validations are mandatory; we show that the zebrafish embryo can be effectively used either to examine the trajectory and of the olfactory axons, or the status of the GnRH3 neurons. We have functionally tested five genes for olfactory axons, and three genes for GnRH3 neurons, and the results clearly indicate that the chosen genes do affect axonal trajectory and GnRH3 migration. Previous works have shown that the depletion of *z-kal1a/kal1b* in the fish embryo also causes KS-like phenotypes (45, 46), thus the use of the reporter zebrafish strains we have adopted appears to be a valid approach in which to examine new KS-causing genes in human, or genes causing a KS-like phenotype when mutated in mice.

Work is continuing in this direction. The following interesting genes/categories emerge from the profile data.

THE LEUCINE-RICH REPEAT PROTEINS

We identified three leucine-rich repeat transmembrane protein genes among the *Dlx5*$^{-/-}$ targets, namely *Lrrn1*, *Lingo2*, and *Lgi1*. We functionally tested two of these using zebrafish embryos, and the results clearly show that these proteins participate in the development of the olfactory pathway. *Lrrn1* was also tested in GnRH3:GFP fish embryos, and the results show that it is required for correct GnRH neuron migration. Furthermore, *Lingo1*, *Lingo2*, and *Lgi* were prioritized with the human network, and *Lrrtm* (another member of this family) emerged from the mouse network.

Lrrn1 is a glycosylated single-pass transmembrane protein with 12 external leucine-rich repeats, a fibronectin domain, an immunoglobulin domain and short intracellular tails capable of mediating protein-protein interaction. Lrrn1 is closely related to drosophila tartan/capricious (trn/caps) proteins. Differential expression of trn/caps promotes an affinity difference and boundary formation between adjacent compartments in a number of contexts. The regulated embryonic expression and cellular location of these proteins suggest important roles during mouse development in the control of cell adhesion, movement, or signaling (99). Indeed, Lrrn1 has been identified as a positive and negative regulator of neurite growth (100). Lrrn1 appears to be a key regulator of the process of generating distinct cells at the midbrain-hindbrain boundary of the brain. In the chick embryo *Lrrn1* is dynamically expressed, the timing of its down-regulation correlates closely with the activation of signaling molecule expression at boundary regions. Cells over-expressing *Lrrn1* violate the boundary and this result in a loss of cell restriction at the midbrain-hindbrain boundary (101). Lrrn1 may regulate the subcellular localization of specific components of signaling or cell–cell recognition pathways in neuroepithelial cells (102).

Lingo2 is an exclusively neuronal transmembrane protein (103), containing 12 extracellular leucine-rich repeats, an immunoglobulin C2 domain and a short intracellular tail, and with a predicted structure similar to the Trk Receptor Tyrosine Kinases. In human Lingo2 been linked both to essential tremor and to Parkinson's disease (104). Interestingly, the combination of leucine-rich repeat and immunoglobulin-like domains is found in the domain architecture of the Trk neurotrophin receptor protein. In the mouse embryo *Lingo2* is expressed in a the olfactory neurepithelium and in various areas of the adult brain (99). Lingo1, another neuron-specific member of the same family, has been shown to be a component of the Nogo66 receptor/p75 signaling complex (74). This ternary complex confers responsiveness to oligodendrocyte myelin glycoprotein, as measured by RhoA activation. Such responsiveness is linked to the inhibition of axon regeneration of neurons in the adult brain, by myelin. Thus, Lingo proteins are likely to play a role in neurite outgrowth and oligodendrocyte differentiation.

Lgi1 is a leucine-rich repeat molecule, found to be down-regulated in the absence of Dlx5. This is a secreted molecule of the SLIT family, promotes formation of stress fibers, inhibits cell movement and invasion, and enhances growth of neuronal processes on myelin-based substrates (75, 76). At the moment we have no functional data on the possible role of Lgi1 in olfactory development, yet should be explored.

MULTI-ADAPTOR SCAFFOLD MOLECULES

Among the *Dlx5* targets we note the presence of a set of scaffold-adaptor proteins, including *Akap6*, *Dapp1* (also known as *BAM32*), and *Homer2*. Akap6 belongs to a class of protein kinase A-anchoring proteins, serving as scaffolds to cluster PKA and PDE and to coordinate the timing/intracellular localization of cAMP signaling. Akap proteins also bind to- and modulate-signaling through ERK, MAPK, and PP2A (105, 106). The potential importance of this class of molecules is suggested by the fact that *Akap6* (expressed in olfactory neurons) and *Akap2* (expressed in the mesenchyme) emerge as predicted/prioritized disease genes from the human network. *Akap6* is absent in the zebrafish genome and could not be tested.

Dapp1 codes for a signaling adapter molecule, much studied in B lymphocyte activation, in which it coordinates timing and location of signaling by PIP3 and PIP2 with that of ERK. Dapp1 also binds F-actin and Rac (107–109). *Dapp1* is not apparently expressed in the embryonic OE, nevertheless when *Dapp1* was depleted in the fish model a mild effect on axonal trajectory and OPL organization have been observed. It appears very likely that lipid signaling is involved in axonal trajectory and connectivity during olfactory development.

Homer2 is a post-synaptic scaffold molecule, involved in receptor clustering and modulating their downstream signaling. However, recently a role for Homer2 in tuning the activity of G-proteins coupled receptors (such as the ORs) by controlling calcium influxes has been demonstrated. We carried out functional experiments depleting Homer2 in zebrafish embryos: the results provide evidence for its involvement in olfactory axonal development. Considering the established importance of the OR for olfactory axon connectivity and guidance during embryonic development, much before their role in odor perception, an embryonic role of Homer2 can be envisioned, and our results with zebrafish clearly show this.

The p130CAS – Shep1 regulation

Mouse embryos null for *Shep1* show retarded OE differentiation, lack of primary axonal connections with the OB and retention of GnRH neurons in the nasal mesenchyme (110). These defects are accompanied by a reduced phosphorylation of the multi-adapter scaffold molecule p130CAS in the olfactory neurons and axons. Shep1 promotes Src-dependent phosphorylation of the multi-adapter molecule p130CAS, *in vitro* (111). These data implicate the phosphorylation of p130CAS in the establishment of olfactory contacts and in GnRH neuron migration, in line with previous studies suggesting that p130CAS is required for neurite outgrowth and axon guidance (112–114). p130CAS belongs to a family of multi-adaptor and scaffold molecules that spatially and temporally collect, integrate, and modulate signals coming from RTKs and adhesion receptors (115–117), undergoing changes in phosphorylation and interacting with a large set of effectors proteins. In light of the phenotype of *Shep1*$^{-/-}$ mice, the involvement of *p130CAS* in olfactory development and GnRH neuron migration

is a likely possibility to be explored. Since *p130CAS* null mice are embryonic lethal (118), this study will have to be pursued via conditional deletion of *p130CAS* in the olfactory system.

St8siaVI codes for a sialyl-transferase, expressed by olfactory neurons. The highly related St8siaII and St8siaIV proteins are required for polysialylation the N-CAM, confer to this neuronal surface molecule anti-adhesive properties and thereby promote neurite elongation and cell migration (77–79). Thus a role for this protein in OE development is conceivable, and supported by the presented data in fish embryos.

EphA3 codes for a receptor for the guidance molecules EphrinA3 and EphrinA5, which are expressed by VNO axons and have a preference for interacting with EphA expressing cells in the Accessory OB. Alterations of this pathway leads to abnormal topography, i.e., guidance defects, of the olfactory and VNO axons (119), indicating the EphrinA-EphA system is a positive guidance cue. *Dlx5* is co-expressed with *EphrinA3* and *EphrinA5* in the VNO, while *EphA3* is expressed in the mesenchyme near the VNO (Figure S7 in Supplementary Material). The link between Dlx5 and EphA signaling should be deeply explored.

GENES EMERGING FROM THE BIOINFORMATIC ANALYSES

A recent work has succeeded to use bioinformatics to prioritize candidate KS genes, focusing on the FGF8 co-expression and functional network (7). Inspired by this work, we opted for an unbiased approach that simultaneously searches for links between genes apparently unrelated. Limiting our search to co-regulations, we strongly introduce the notion of conservation, reasoning that the olfactory/GnRH development is highly conserved within vertebrates. Indeed, in our work we have attempted to use also mouse KS-disease genes to run the search. The advantage is the possibility to use all the disease genes, instead of focusing only on those logically related. An additional advantage of the present work derives from combining bioinformatic predictions, putative gene functions, phenotype descriptions, and information from the literature with "wet" profiling data *specifically* obtained from embryonic olfactory tissues.

The "human" network was able to predict few mouse KS gene (*Ebf2* and *Nrp1*), providing an evidence that the algorithm is effective. The outcome, both in terms of individual genes and the G.O. classes, assures that the pipeline works. The addition of protein-protein interaction data (when made available) or other data to carry out meta-analyses will certainly refine the results. On the contrary, it appears that the "mouse" gene network is little informative, i.e., less able to predict the human KS genes. This might be due to the fact that the definition of mouse input gene is based on accurate phenotypic analyses on the olfactory system, reported in the literature, that scientist don't routinely conduct (we may miss many other genes) or it is incomplete and does not examine olfactory axons but only hypothalamic GnRH neurons.

The predicted/prioritized genes emerging from our analyses may represent a novel set of KS-causing genes, or genes that might contribute when co-mutated with others. While the use of modern sequencing approach (WES) on KS patients' DNAs is the straightforward approach to define their role in the human disease, additional filters may be needed to further prioritize these genes, i.e., testing their function on GnRH3+ neurons fish embryos.

TRIM2 – tripartite motif containing 2, codes for an E3 ubiquitin-protein ligase that has been implicated in ubiquitination of neurofilament light chains. TRIM2 controls the dynamic of neuronal cytoskeleton, by which determines the specification of the choice of the axonal vs. dendritic projection in hippocampal neurons (120).

CDH2 – cadherin-2, also known as *N-cadherin*, codes for a well known calcium-dependent neuronal cell adhesion molecule that contributes to the formation of neural circuits by regulating growth cone migration and synapse formation. In the mammalian embryonic neocortex, radial migration is instructed by several signals that include homophilic interactions mediated by Cdh2 (121), and the fish embryo Cdh2 is involved in neuroblast migration within the hindbrain (122, 123). Chd2 function is required for guidance of afferent fibers of cranial sensory neurons (124) and regulates motor axon growth and branching, in fish embryos (125). During olfactory development, Cdh2 is expressed by receptor neurons and closely parallels expression of γ-catenin in neuronal axons (126), thus Cdh2 is positioned to underlie the formation of olfactory primary olfactory connections.

ADAMTS5 codes for a disintegrin-like and metallopeptidase extracellular protease, with thrombospondin-like motif. Adamts5 plays a role in the specification and patterning of progenitor cells in the lateral and medial ganglionic eminences (127). The proteolytic cleavage of astrocyte-derived proteoglycan, exerted by Adamts5, loosens the matrix environment and promotes neurite outgrowth (128). Being predicted by both the human and the mouse disease-gene networks, *Adamts5* appears to be a very interesting candidate.

RGS5 – regulator of G-protein signaling 5, codes for a protein that accelerates the inactivation of Gα-dependent signaling in various cells types. Down-regulation of *RGS5* induces GPCR-mediated signaling pathways and promotes migration of vascular and cancer cells (129, 130). A role of this protein in promoting the migration of GnRH neurons is possible, although RGS5 null mice don't show any obvious phenotype (131).

DPF3 – D4, zinc and *double PHD fingers, family 3*, codes for a component of the BAF chromatin remodeling protein, and acts a transcription co-activator in SWI/SNF complex-activation (132). DPF3 functions to activate transcription of the target genes *Pitx2* and *Jmjd1c* in association with the BAF complex, and binds histone H3 and H4 in an acetylation-dependent manner (133, 134). How this could be relevant for olfactory development, GnRH neuron migration and KS, is unclear.

FGF7 has been proposed to act as a pre-synaptic organizing molecule in the mammalian brain, and in particular during hippocampal development. Indeed FGF7-deficiency impairs inhibitory synapse formation, which results in mossy fiber sprouting and enhanced neurogenesis (91, 92). Neutralization of FGF7 inhibits pre-synaptic differentiation of mossy fibers at contact with granule cells, and inactivation of FGFR2 has similar effects (92). In neurons, FGFs and cell adhesion molecules stimulate neurite outgrowth via activation of FGF receptors. A role for FGF7 for the migration of enteric neuroblasts has been suggested from analyses of CAMs and FGFs expression in Hirschsprung Disease patients (93).

CONCLUSION

The molecular control over the ability of olfactory axons to contact the anterior forebrain, and/or the ability of GnRH neurons to efficiently migrate and home to the hypothalamus, entails numerous proteins of various functional classes, many of which appear to be directly and indirectly involved in matrix remodeling and signaling. Indeed, the data indicate that the navigation of OE and VNO axons is mostly governed by cell–cell and cell-matrix cues, rather than intrinsic properties of the axons. These include a set of scaffold molecules that, for their nature, are strong candidates for playing a key role in guiding axonal elongation-guidance and connectivity, as well as for GnRH neuron migration and homing. These molecules will be of great interest for developmental biologists.

Perturbations in the expression and sequence (mutations) of these molecules and in their associated gene networks may cause phenotypes similar to KS, a possibility that can be rapidly tested in zebrafish strains, and eventually in the mouse. Human geneticists should consider these molecules for mutation screens. This opens the possibility to test them in the mammalian model and to search for mutations in large collections of DNAs from KS/nCHH patients, hereditary, or sporadic, with the hope to find mutations, alone or in combination with mutations in known KS/nCHH genes.

Finally we show the validity of approaches based on high-throughput data generation and predictive bioinformatics to identify genes potentially relevant for specific developmental processes, and ultimately for disease. Indeed, we have uncover a set of molecules that might be candidate disease genes, to be tested in future mutation screens.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at http://www.frontiersin.org/Journal/10.3389/fendo.2013.00203/abstract

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New players in the infertility of a mouse model of lysosomal storage disease: the hypothalamus-pituitary-gonadal axis

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Mammalian spermatogenesis is a complex hormone-dependent developmental program where interactions between different cell types are finely regulated. Mouse models in which any of the sperm maturation steps are perturbed provide major insights into the molecular control of spermatogenesis. The Twitcher mouse is a model for the Krabbe disease, characterized by the deficiency of galactosylceramidase (GALC), a lysosomal enzyme that hydrolyzes the terminal galactose from galactosylceramide, a typical component of the myelin membrane. In addition, GALC catalyzes the hydrolysis of the terminal galactose from galactosyl-alkyl-acyl-glycerol, precursor of seminolipids, specifically expressed on the membrane of germ cells. Previous data reported by our group demonstrated that glycolipids play an important role in sperm maturation and differentiation. Moreover, we hypothesized that the severe impairment of the central nervous system that affects the Twitcher mouse could interfere with the hypothalamus-pituitary-gonadal axis function, contributing to infertility. To highlight this hypothesis we have determined, at molecular level, the potential variation in expression pattern of brain hormones involved in spermatogenesis regulation.

Keywords: spermatogenesis, Twitcher mouse, Krabbe disease, gene expression, hypothalamus-pituitary-gonadal axis

INTRODUCTION

Infertility is a major medical problem worldwide. Male infertility affects 1 in 25 men in the Western world and is the cause of considerable social and financial burden (1).

Spermatogenesis is a complex series of events which collectively involve the coordinated expression of about 2300 different genes (2, 3). Given the complex cellular and molecular interactions that are involved in spermatogenesis, the whole process cannot be modeled *in vitro*. However, mouse models provide an attractive alternative since the great majority of the genes and processes involved in sperm production are conserved between mice and men, thus making mice excellent models of human infertility (4, 5).

It is known that spermatogenesis in mammals requires the action of a complex assortment of peptides and hormones each of which plays an important role in the normal functioning of the seminiferous epithelium (6, 7). The gonadotropin-releasing hormone (GnRH), secreted from the hypothalamus, stimulates the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH). In turn, these two hormones regulate gametogenesis, hence the brain has a pivotal role in the control of spermatogenesis (8). LH stimulates the interstitial steroidogenic Leydig cells to produce testosterone, which has a local effect on interstitium and seminiferous tubules resulting in sperm production and maturation (9). FSH exerts its effect directly on the Sertoli cells whose direct contact with proliferating and differentiating germ cells within the seminiferous tubules makes them essential for providing both physical and nutritional support for spermatogenesis (10–12). Testosterone and estradiol, the latter converted through aromatase in the testis interstitium as well as in germ cells (13), are direct negative feedback modulators of GnRH, LH, and FSH (14).

Hence the maintenance of the proper crosstalk between the nervous system and the male gonads is mandatory for male fertility. This relationship becomes obvious if we take into account several unlinked autosomal mutations, which cause defects in both systems. Several studies on Lysosomal Storage Diseases (LSDs), genetic disorders caused by lysosomal enzyme deficiencies, demonstrate that lysosomal enzymes can elicit pleiotropic effects specifically on spermiogenesis (15, 16). In fact, in the knockout mice for the lysosomal enzymes sphingomyelinase α, H-hexosaminidase, or arylsulfatase A, both nervous and reproductive system are affected (17–19).

TWITCHER MOUSE SPERMATOGENESIS

New insights come also from the Twitcher mouse, a naturally occurring model of Krabbe disease, characterized by deficiency of galactosylceramidase (GALC) (20, 21). GALC is a lysosomal enzyme that hydrolyzes the terminal galactose from galactosylceramide, a typical component of the myelin membrane, and from galactosyl-alkyl-acyl-glycerol (GalAAG), precursor of seminolipids, glycolipids expressed on the membrane of germ cells

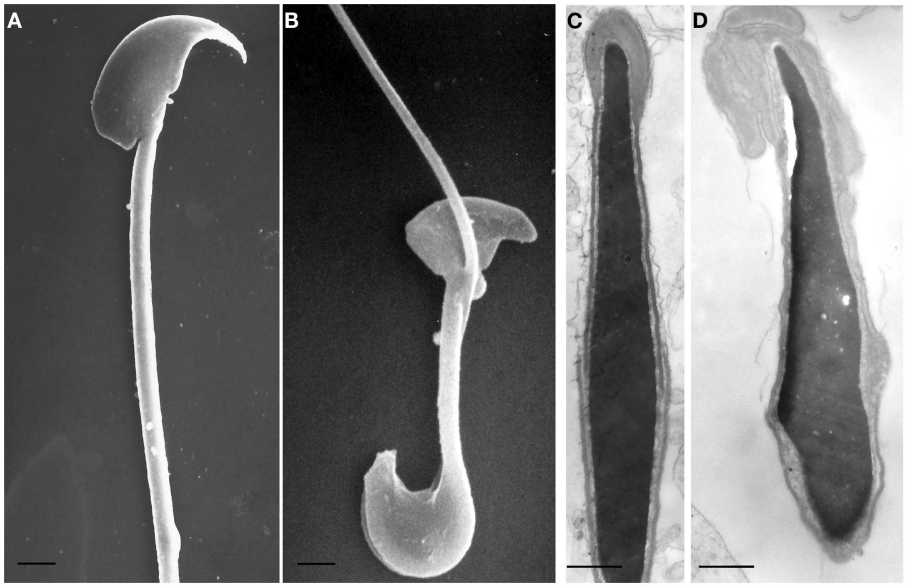

FIGURE 1 | Scanning (A,B) and transmission (C,D) electron microscopy micrographs of spermatozoa from wild type (A,C) and Twitcher (B,D) mouse, collected from vas deferens. In **(A)**, head and tail of control mouse sperm have a normal morphology: the crescent-like shape of the head and the acrosomal profile are evident and the flagellum is well developed. The sperm from Twitcher mouse shows the typical hairpin morphology **(B)**. At transmission electron microscopy level, sperm from control mouse show a normal structure of both acrosome and nucleus, with a well condensed chromatin **(C)**. By contrast, the acrosome of the Twitcher sperm is aberrant and detached from the nucleus, the plasma membrane is also enlarged and redundant; the nuclear profile is irregular and the chromatin appears granular and uncondensed **(D)**. [**(A,B)**: bars = 2 μm; **(C,D)**: bars = 0,25 μm]. Modified from Ref. (23).

(22). We have previously demonstrated that GALC deficiency causes metabolic and structural abnormalities in the spermatozoa of the Twitcher mouse as consequence of a significant accumulation of undegraded GalAAG and minor alterations in the concentration of seminolipids (23). In comparison with sperm obtained from wild type mice (**Figures 1A,C**), the spermatozoa of the Twitcher mouse recovered from the cauda epididymis or vas deferens (**Figures 1B,D**) reveal significant structural defects affecting both head and tail. Scanning electron microscopy analysis shows an altered shape of the sperm head (**Figure 1B**), which appears reduced in size and devoid of the acrosomal profile. Often the tail appears coiled at the level of the cytoplasmic droplet causing an incorrect development of the flagellum and its cytoskeletal structures (**Figure 1B**).

At ultrastructural level, the most severe alterations are detected in the acrosomal complex (**Figure 1D**): the inner acrosomal membrane is completely detached from the nucleus, the acrosome is swollen, redundant, and folded over. Furthermore, the plasma membrane is also enlarged and redundant. The nuclear profile is irregular and the chromatin appears granular and less compact than in control sperms (**Figure 1C**). These morphological abnormalities, the significant accumulation of undegraded GalAAG and the minor alterations in the concentration of seminolipids, previously reported in Twitcher mice by our group, demonstrated the pleiotropic effect of the *GALC* gene suggesting its importance in the development and function of the male reproductive system and indicating in its deficiency the cause of infertility of the Twitcher males.

It is known that hormones play a key role in controlling spermatogenesis and, moreover, that neurological impairment is often associated to infertility as demonstrated in several neurological mouse mutants. We have, therefore, hypothesized that an unbalanced hormonal profile, owing to severe brain degeneration, could contribute to male infertility in the Twitcher mouse.

At testicular level, the Leydig and Sertoli cells are the target of pituitary hormones, such as LH and FSH. The close interaction between germ cells and somatic cells, present in testis, was demonstrated to be essential for correct spermatozoa differentiation. Any alteration in their morphology/metabolism would result in the impairment of this relationship.

Among the testicular interstitial cells, Leydig cells are very important in testis development since they produce testosterone, a steroid hormone with a pivotal role in the regulation of spermatogenesis. To evaluate potential Leydig cells dysfunction, a careful morphological investigation of the tubular tissue of Twitcher mouse was performed in 35 days old mice, when the spermatogenetic process is already completed.

We observed, at light microscopy level, that Twitcher mouse tubules compared to age matched wild type were smaller in size and that the interstitial space was reduced allowing the tubular membranes to become adjacent (**Figures 2A,B**). These results indicate a loss not only of Leydig, but also of myoid cells.

At the ultrastructural level the Leydig cells of wild type mice were found in small clusters and most of them showed a normal ultrastructural pattern, with cell cytoplasm containing many lipid droplets (**Figure 2C**). Leydig cells from the Twitcher mice

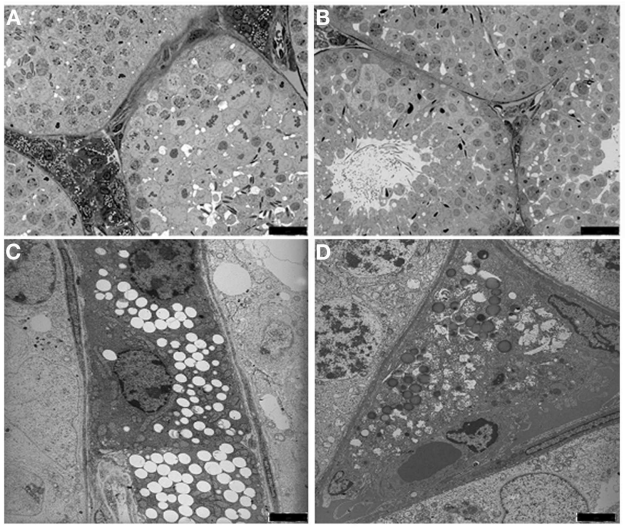

FIGURE 2 | Light microscopy (A,B) and transmission electron micrograph (C,D) of the interstitium from wild type (A,C) and Twitcher (B,D) testes. In the peritubular interstitium of wild type mice numerous Leydig cells are presents and filled with lipids droplets and lysosomes, whereas in Twitcher mice the interstitial cells are strongly reduced in number and appear partially degenerated [**(A,B)**: bars = 25 μm; **(C,D)**: bars = 2 μm].

appeared to be degenerated showing a significant decrease in the number of lipid droplets (**Figure 2D**). Based on the established correlation between the amount of testosterone and the number of lipid droplets (24, 25), a reduction of its synthesis in Leydig cells can be hypothesized.

HYPOTHALAMIC-PITUITARY-GONADAL AXIS

Gonadotropin-releasing hormone, secreted by hypothalamic neurons, is a key integrator between the neural and endocrine systems that stimulates the synthesis, storage, and secretion of gonadotropins by gonadotropic cells in the anterior pituitary. FSH and LH are the primary gonadotropins; in males, they stimulate testicular function through specific receptors (LH-R and FSH-R) expressed by Leydig and Sertoli cells, respectively. Thus, GnRH, FSH, and LH are the brain hormones that regulate testicular function and spermatogenesis. To establish if the hypothalamic-pituitary-gonadal axis is deregulated in the Twitcher mice, we have investigated by qRT-PCR the mRNA expression levels of genes encoding these hormones. The expression levels of the analyzed genes in wild type ($n = 6$) and Twitcher mice ($n = 6$) at PNDs 35, were normalized to the validated housekeeping gene eEF-2 (Eukaryotic elongation factor 2 kinase) (26) and referred to the wild type mouse (considered to be equal to 1).

Our results indicated that, at PND 35, GnRH expression is reduced by 70% in the Twitcher brain compared to wild type ($p < 0.01$). LH and FSH expression, were also significantly decreased (50 and 80% respectively, $p < 0.05$) in the Twitcher brain compared to aged matched wild type mouse.

Thus, gene expression analysis performed at brain level proved that hypothalamus and pituitary functions were affected.

CONCLUSION AND OPEN QUESTIONS

Since mammalian spermatogenesis is a complex hormone-dependent developmental program that ultimately give rise to spermatozoa, mouse models in which any of this step is perturbed have provided major insights into the molecular control of spermatogenesis.

The studies presented are the follow up of previous observations published by our group providing clues to the pleiotropic effect of the GALC gene and its importance in the development and function of the male reproductive system (23). The data that we have described demonstrate that the altered lipid metabolism, due to GALC deficiency, is not the only cause of male infertility. In addition, they support the hypothesis that the severe and progressive degeneration of the CNS affects the hypothalamus and hypophysis function, thus interfering with hypothalamus-pituitary-gonads axis. In fact, the GnRH produced by the hypothalamus mediates the secretion of the gonadotropin hormones FSH and LH by the hypophysis, that in turn regulate the testicular functions through their receptors (11, 27).

In conclusion, the data presented demonstrate that, in this mutant, the infertility may not be exclusively caused by the metabolic abnormalities in the sphingolipid pathway due to the GALC defect but, rather, to the severe involvement of the CNS that causes disruption of the hypothalamus-pituitary-gonadal axis.

Although further work is needed to fully clarify the complex interaction between brain and testis hormones, our data offer a new approach to study the spermatogenesis defects associated to CNS pathologies. Furthermore, the Twitcher mouse can be considered a model system for the study of hormone signaling orchestration between brain hormones with their testicular receptors.

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Opposite influence of light and blindness on pituitary–gonadal function

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Some environmental factors may influence the pituitary–gonadal function. Among these, light plays an important role in animals and in humans. The effect of light on the endocrine system is mediated by the pineal gland, through the modulation of melatonin secretion. In fact, melatonin secretion is stimulated by darkness and suppressed by light, thus its circadian rhythm peaks at night. Light plays a favorable action on the hypothalamic-pituitary axis likely inhibiting melatonin secretion, while the exogenous melatonin administration does not seem to impair the hormonal secretions of this axis. The basal and rhythmic pituitary–gonadal hormone secretions are regulated by a central clock gene and some independent clock genes in the peripheral tissues. Light is able to induce the expression of some of these genes, thus playing an important role in regulating the hormonal secretions of pituitary–gonadal axis and the sexual and reproductive function in animals and humans. The lack of light stimulus in blind subjects induces increase in plasma melatonin concentrations with a free-running rhythm of secretion, which impairs the hormonal secretions of pituitary–gonadal axis, causing disorders of reproductive processes in both sexes.

Keywords: light, blindness, clock genes, melatonin, pituitary–gonadal function

INTRODUCTION

Several endogenous and exogenous factors may influence endocrine secretions (1), including those of pituitary–gonadal axis (2). Among the exogenous environmental factors, light seems to play a pivotal role both in animals and in humans, especially as synchronizing agent of hormonal rhythmicity (3–5). Several structures are involved in the mechanism of transmission of light stimulus to the circadian timing system: a retinal component with photoreceptor and ganglion cells, a retino-hypothalamic tract (RHT) originating from these and projected to the suprachiasmatic nucleus (SCN), the circadian pacemaker, i.e., the SCN, efferent projections of SCN to a series of hypothalamic and thalamic nuclei (6). The major projections are to areas that themselves receive retinal input and project reciprocally to the SCN. Of particular importance are the projections of the SCN that reach the supraventricular zone and then the hypothalamus because they provide, among other functions, the neuroendocrine regulation and the pineal melatonin secretion, which plays an intermediate role between the environment and the endocrine system. Studies on the effects of light on the endocrine secretions in animals are usually performed by exposing them to different photoperiods or rendering them blind. In humans, blindness may be considered, despite unlucky, an experimental condition to study the effects of light on the hormonal secretions, but in this regard data are scarce and sometimes controversial. However, since light is one of the most important environmental factors, paying attention to its influence on the endocrine system may avoid misleading interpretation of individual hormonal data and may help prevent alterations in hormonal pattern and rhythmicity caused by variations of this environmental entraining-agent.

MOLECULAR ASPECTS

The recent identification of several clock genes in a number of organism, including mammals (7–14), seems to assign a pivotal role to the hypothalamus as pacemaker of pituitary–gonadal secretions. However, the findings of independent clocks in peripheral tissues (1, 9, 12–15) suggest a possible gonadal independent role in regulating the rhythmicity of gonadal steroids. In fact, recent findings support the assumption that some clock genes can influence fertility and testosterone (T) seasonality both in animals (16) and in humans (17). In particular, *Brain and muscle Arnt-like protein 1 (BMAL1)* and *Neuronal PAS domain protein 2 (NPAS2)* gene variants have been shown to influence fertility and seasonality in humans (17). Anyway, since light plays an important synchronizing role on the circadian rhythmicity, the alteration of photoperiod, or the lack of light stimulus, as occurring in blindness, may impair this rhythmicity (18). Consequently, the desynchronizing effect of altered light signal may influence circadian peripheral clocks in female and male reproductive tissues causing impairment of fertility (19) with disorders in estrus cycles, ovulation, sperm generation, implantation, and the progression of pregnancy (14).

In fact, light may act at molecular level inducing the expression of some immediate early genes in the SCN involved in entrainment of circadian clock (20, 21). These genes, activated by light, encode transcriptor factor proteins involved in molecular mechanism of resetting the circadian clock (20). Among these genes, are *c-fos* and *nur 77*, two of the early-response genes known to be induced in the SCN by light, and *egr-3*, a zinc-finger transcription factor, whose induction by light seems to be restricted to the ventral SCN, a structure involved in entrainment (22). Light also induces *Jun-B* messenger RNA expression and *AP-1* activity in the SCN (20).

Moreover, other mammalian genes involved in circadian regulation, like *mper 1* and *mper 2* have been shown to be expressed in SCN under light stimulus control (23). It has been demonstrated that light stimulus induces expression of *C-fos* gene in postnatal rat retinas (24). The earliest expression occurs between postnatal days 11 and 15 and is correlated to the genes coding for proteins involved in phototransduction, suggesting that it may play a role in the regulation of these genes in retinal cells during the light/dark cycle (24). This could in part explain the severe alteration of hormonal rhythmicity in born blinds. Further evidence that genes involved in clock regulation are reset by light has been given by studies in *Neurospora* (25). In particular, the *white collar-1 (wc-1)* and *white collar-2 (wc-2)*, both global regulators of photoresponses in *Neurospora*, encode DNA binding proteins containing PAS domains and acting as transcriptional activators, thus playing an essential role in the organization of circadian rhythmicity. Similarities between the PAS domain regions of molecules involved in light perception and circadian rhythmicity in several species suggest an evolutionary link between ancient photoreceptor protein and more recently described proteins required for circadian oscillation (25, 26).

ROLE OF PINEAL GLAND AND MELATONIN

The effects of environmental light on the hypothalamic–pituitary–gonadal axis are mediated by the pineal gland, through melatonin secretion (27, 28). Light stimulus from the environment reaches the retina; from here, through a RHT reaches the SCN, then the superior cervical ganglion, and finally the pineal gland, where it exerts an inhibiting effect on the pineal melatonin secretion. Instead, the darkness activates alpha1 and alpha2-adrenergic receptors in pineal gland, then it increases cyclic AMP and calcium concentration and activates arylalkylamine *N*-acetyltransferase, thus initiating the synthesis and release of melatonin, whose circadian rhythmicity is under control of an endogenous free-running pacemaker located in the SCN (29). As result of the opposite effect of light and darkness, melatonin rhythm normally peaks at night both in animals and in humans (29). Light exposure at night induces a parallel reduction in both plasma and salivary melatonin (30). A little amount of melatonin may be synthesized directly by retina: melatonin synthesis in cultured neural retinas of golden hamster exhibits a circadian rhythm entrained by light/dark cycles applied *in vitro*, whereas it shows a free-running rhythm when the culture is held on constant darkness (31). Several melatonin receptors have been found and cloned in animal and in humans. They belong to a superfamily of G-protein coupled receptors and mediate the physiological actions of melatonin with different specificity (29, 32–36). Among these, of particular importance are Mel 1a, isolated in brain, SCN, and pituitary, which is involved in circadian and reproductive processes (29, 32, 34); Mel 1b, isolated in retinas and brain, which is involved in retinas physiology regulation in some mammals (33); and Mel H9, isolated in pituitary, which is likely involved in genetically based neuroendocrine disorders (35).

Blindness affects melatonin secretion significantly. Blind patients show increased day-time melatonin levels or more complex changes in circadian rhythmicity (36–39). They exhibit a phase-advanced or a phase-delayed rhythm with respect to that of normal subjects. However, the exposure to bright light may suppress the high melatonin levels in some blind subjects with functional integrity of the RHT (40, 41). In fact, their melatonin secretion may be suppressed when their eyes are exposed to a bright light stimulus. Interestingly, these patients were less suffering for sleep alterations. The authors who studied these patients concluded that some blind people can have a functional integrity of RHT, allowing a melatonin suppression when exposed to light stimulus and consequently a sufficient sleep entrainment. Instead, blind patients with complete absence of bright input to the circadian system may represent a distinct form of blindness, associated with periodic insomnia correlated to abnormalities of melatonin rhythm, due to the persistent lack of synchronizing effect of light (40). In fact, changes in melatonin rhythmicity are more severe in patients with total blindness compared to those with only light perception (42). Interestingly, a reduced incidence of cancer has been observed in blind people (43). Even if other explanations have to be considered, the protective effect of high melatonin concentrations may not be excluded (43).

LIGHT, BLINDNESS, AND HYPOTHALAMIC–PITUITARY–GONADAL FUNCTION

Light influences favorably gonadal function in animals and this effect seems to be mediated by reduction of pineal melatonin production, whereas a reduction of photoperiod impairs this function through an activation of melatonin secretion (27, 28, 44). Sexual activity in animals is reduced during the months of the year with short day; this reduction is prevented by pinealectomy (28, 44). Moreover, increased melatonin levels and reduction of plasma luteinizing hormone (LH), follicle-stimulating hormone (FSH), prolactin (PRL), T levels, testis weight, spermatozoa production, and sexual activity have been documented in animals rendered blind or exposed to a short photoperiod (44–48). These effects are prevented by pinealectomy (28, 45). Seasonal variations in luminosity influence melatonin secretion and some functions correlated not only in animals (28) but also in humans. Women living in Finland, a region with a strong seasonal contrast in luminosity, showed increased melatonin and reduced gonadotropin secretion during dark season, with consequent reduction of conception rates (49). Seasonal variations of plasma LH and T concentrations have been demonstrated also in patients with primary and secondary hypogonadism, but with peak of values in season different from that of normal subjects (18). A possible negative feed-back mechanism between melatonin and hormones of pituitary–gonadal axis seems to be suggested by the presence of gonadotropin and gonadal steroid receptors in human pinealocytes (50) and conversely of melatonin receptors in human hypothalamus, pituitary, and in other tissues of gonadal tract (51). Other findings, instead, suggest that there is no classic feed-back between the pineal gland and the testes (52) and that administration of exogenous melatonin does not impair pituitary–gonadal hormone secretion in men (53); on the contrary it seems to amplify pulsatile LH secretion in women (54). However, this is in contrast with that occurring in patients with chronic endogenous melatonin increase that may show alterations of menstrual cycle in case of women (28, 55) and oligospermia or azoospermia in case of men (56).

Blindness can influence gonadal function in humans. Data on the age of puberty onset and fertility in blind women are

conflicting. Menarche in blind girls has been described as being advanced or delayed (57–59) and fertility in adult women as being normal or impaired (60, 61). Some blind adult patients showed a normal secretory rhythm of LH, FSH, and T in spite of impaired cortisol rhythm (62). However, in this study, the majority of patients had become blind from 14 years onward, an age in which mechanisms involved in pubertal development and gonadal function are quite completed. Instead, in a group of institutionalized blind boys, whose blindness was started in the first years of life, we found impaired basal and stimulated plasma levels of LH, FSH, PRL, and T (63). Since similar alterations had been described both in hypogonadotropic hypogonadism and in delayed puberty (64, 65), several years ago we studied the same hormonal pattern in a group of institutionalized adult blind males aged 20–29. They were divided in two subgroups: 14 with total blindness and 21 with only light perception, whose age of onset of impaired vision was reported by them as the first 5 years of life (36). Both subgroups showed increased plasma melatonin levels in comparison with a normal control group of sighted subjects, but normal LH, FSH, PRL, and T levels. However, the finding of a significant increase of FSH/LH ratio in both subgroups of blind patients versus the control group, could indicate a possible subclinical impairment of testicular function that however should be verified with studies of dynamic hormonal secretions and of seminal patterns, which the patients did not consent.

In conclusion, taking into account the data appeared in the literature and the results of our previous studies, light stimulus seems to influence favorably gonadal function both in animals and in humans, likely through inhibition of melatonin secretion. Instead, the lack or reduction of light stimulus in humans can induce:

– increased plasma melatonin concentrations;
– impairment of gonadotropins, PRL, and T secretion in prepubertal blind boys causing delayed puberty or more severe hypogonadism;
– impairment of pubertal development in young blind girls and of ovarian function and fertility in blind adult women.

These alterations seem to be more severe when the blindness occurs in the first years of life.

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Role of estrogen receptors and G protein-coupled estrogen receptor in regulation of hypothalamus–pituitary–testis axis and spermatogenesis

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Male reproductive function is under the control of both gonadotropins and androgens through a negative feedback loop that involves the hypothalamus, pituitary, and testis known as hypothalamus–pituitary–gonadal axis (HPG). Indeed, estrogens also play an important role in regulating HPG axis but the study on relative contribution to the inhibition of gonadotropins secretion exerted by the amount of estrogens produced within the hypothalamus and/or the pituitary or by the amount of circulating estrogens is still ongoing. Moreover, it is known that the maintenance of spermatogenesis is controlled by gonadotropins and testosterone, the effects of which are modulated by a complex network of locally produced factors, including estrogens. Physiological effects of estrogens are mediated by the classical nuclear estrogen receptor alpha and estrogen receptor beta, which mediate both genomic and rapid signaling events. In addition, estrogens induce rapid non-genomic responses through a membrane-associated G protein-coupled estrogen receptor (GPER). Ours and other studies reported that, in the testis, GPER is expressed in both normal germ cells and somatic cells and it is involved in mediating the estrogen action in spermatogenesis controlling proliferative and/or apoptotic events. Interestingly, GPER expression has been revealed also in the hypothalamus and pituitary. However, its role in mediating estrogen rapid actions in this context is under investigation. Recent studies indicate that GPER is involved in modulating gonadotropin-releasing hormone (GnRH) release as well as gonadotropins secretion. In this review, we will summarize the current knowledge concerning the role of estrogen/estrogen receptors molecular pathways in regulating GnRH, follicle-stimulating hormone, and luteinizing hormone release at the hypothalamic and pituitary levels in males as well as in controlling specific testicular functions such as spermatogenesis, focusing our attention mainly on estrogen signaling mediated by GPER.

Keywords: ESR1, ESR2, GPER, gonadotropins, HPG axis, spermatogenesis

INTRODUCTION

Male fertility and hence its reproductive potential is a result of a complex and intricate as a fine neuroendocrine control. Traditionally the adult male reproductive function was considered to be controlled by both gonadotropins and androgens through a negative feedback loop that involves the hypothalamus, pituitary, and testis known as the hypothalamus–pituitary–gonadal axis (HPG). As such, spermatogenesis is regulated by the pulsatile release of gonadotropin-releasing hormone (GnRH) from the arcuate nucleus of the hypothalamus, which stimulates the anterior pituitary to release follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (1). Accordingly, at the testicular level, LH stimulates the Leydig cells to produce testosterone, which has a local effect on the interstitium and seminiferous tubules and results in sperm production and maturation while FSH exerts its effect directly on the Sertoli cells that in turn promote and sustain spermatogenesis (1). Both GnRH and gonadotropin secretion could be modulated by testosterone and more surprisingly, estradiol (E2) acting on the hypothalamus or on the pituitary via a feedback regulating mechanisms (2). However, the specific role of each sex steroid in the regulation of gonadotropin negative feedback is still not completely clarified.

In males, the major source of circulating estrogens is the aromatization of androgens as a consequence of the action of the enzyme complex known as aromatase that is widely expressed in a number of male tissues including the testis and brain (3, 4).

Cellular effects of estrogens occur via classical estrogen receptor alpha (ESR1) and estrogen receptor beta (ESR2) located in the nucleus and cytoplasm of the target cells and belong to the nuclear receptor superfamily members that act as nuclear transcription factors, binding to estrogen response elements (EREs) within specific genes to alter their rate of transcription (5). However, it has become clear that estrogens also exert rapid, non-genomic effects by altering different signaling pathways both in central and nervous system peripheral tissues (6).

These "non-genomic effects" could be mediated by extranuclear estrogen receptors (ERs) or by non-classical membrane bound receptors such as G protein-coupled estrogen receptor also named GPR30/GPER that has been identified as a novel ER (7). Estradiol through GPER rapidly activates different pathways including the

stimulation of adenylyl cyclase, mobilization of intracellular calcium (Ca^{2+}) stores, and activation of mitogen-activated protein kinase (MAPK) and phosphoinositide 3-kinase (PI3K) signaling pathways (8, 9).

In this review, we will summarize the current knowledge concerning the role of estrogen/ERs signaling in regulating GnRH, FSH, and LH release at the hypothalamic and pituitary levels in males as well as in controlling specific testicular functions such as spermatogenesis, focusing our attention mainly on estrogen signaling mediated by GPER.

ROLE OF ESTROGEN AND ESTROGENS RECEPTORS IN GnRH, LH, AND FSH SECRETION IN MALES

ESTROGEN FUNCTIONS AT THE HYPOTHALAMIC LEVEL

Gonadotropins and gonadal steroids, being involved in the regulation of secondary sex characteristics, gametogenesis, cellular functions, and also behavior, are the main driving force for reproductive function. The hypothalamic GnRH neurons that control LH and FSH release from the pituitary represent the final common pathway for neuronally derived endogenous as well as exogenous stimuli (10). In both males and females, gonadal steroid hormones exert negative feedback regulation on HPG axis activity at both the hypothalamus and pituitary levels. In females, the feedback mechanism is more complex since estrogen and progesterone induce both negative and positive feedback responsible for generating the pre-ovulatory GnRH and LH surge (10). Thus, the neuroendocrine mechanism underlying the ovulatory LH and FSH surge, characteristic of the mature female reproductive system, is usually extinguished in males by neonatal androgen imprinting (10).

Several evidences indicate that testicular steroids, androgens, and estrogens could mediate the feedback actions on gonadotropin secretion interacting with their receptors, ERs or androgen receptors (ARs) that were found in the male hypothalamus (11). However, there is no clear consensus on the role of ER versus AR signaling in males (12, 13). Aromatization of testosterone to estradiol and reduction to 5α-dihydrotestosterone (DHT) is mandatory for normal male reproduction and occurs in peripheral (14) and central tissues (15, 16). Sharma and co-workers have demonstrated that aromatase inhibitor administration into the third cerebral ventricle of intact rams resulted in an increased frequency of LH pulses without affecting estradiol plasma concentrations (17). In addition, existence of these feedback actions is further clearly illustrated in a range of species by an increased secretion of the gonadotropins following castration (18–20). Accordingly, an increased LH secretion was found also in intact or castrated rams passively or actively immunized against estradiol (18). However, how testosterone and/or its primary metabolites act within the brain to suppress the synthesis and/or secretion of GnRH need more investigation.

In humans, androgen aromatization for normal gonadotropins feedback function (21) has been discovered by the use of testosterone or estradiol infusion in men affected by idiopathic hypothalamic hypogonadism (IHH). On the other hand, the authors did not record any change in LH and FSH secretion when pure androgen DHT was administered. These data indirectly suggest that the peripheral 5α-reduction of testosterone to DHT plays a minor role in the control of the secretion of gonadotropins (21). Thus, the inhibitory effect on gonadotropin secretion is mediated mainly by estradiol from endogenous conversion of testosterone rather than direct androgen action, at least in the pituitary gland (21). Indeed, other studies suggested that *in situ* aromatization of testosterone is required both at the hypothalamic and pituitary levels to insure a complete feedback mechanism of gonadotropins (22, 23). Moreover, the results coming from basal, GnRH-stimulated, and pulsatile evaluation of LH and FSH secretion in two aromatase-deficient men have provided direct evidence that circulating estrogens exert an inhibitory control in LH feedback at both the hypothalamic and pituitary levels (24).

It is universally accepted that estradiol actions were mediated by its interaction with ERs ESR1 and ESR2 that act as hormone-inducible transcription factors determining estrogen-dependent gene transactivation (1). Several studies, involving a range of species and both sexes, have demonstrated that GnRH neurons do not express ESR1 (25–27), even though a small number of GnRH neurons containing ESR1 were found in female rats (28). Indeed, accumulating evidence suggests that estrogen could act in GnRH neurons through ESR2. In fact, ESR2 immunoreactivity was detected first in rodents (29, 30) and later in humans (31). However, studies performed in *Esr1* knock-out mice suggest that in males, ESR1 is the predominant receptor involved in mediating estradiol suppression of GnRH content (12). Moreover, it was also demonstrated that in mouse LHRH neurons (29) ESR2 may mediate the rapid estradiol effects because mouse LHRH neurons expressed only ESR2, and the nuclear ER antagonist, ICI 182,780, suppressed the effect of estradiol on Ca^{2+} oscillations. However, in primate LHRH neurons, estradiol appears to cause its action through a different mechanism, because ICI 182,780 failed to block the estradiol-induced changes in Ca^{2+} oscillations and synchronization (32). This finding could be explained by the study of Noel and co-workers (33) suggesting a GPER involvement in the rapid action of estradiol in hypothalamic neurons. In fact these authors demonstrated that GPER is expressed in olfactory placode cultured cells and in a subset of LHRH neurons and that GPER gene knockdown in LHRH neurons completely abrogate both estradiol- and estrogen-dendrimer conjugate-induced changes in Ca^{2+} oscillations. Furthermore, using a selective specific GPER-agonist, they obtained changes in Ca^{2+} oscillations similar to those observed upon estradiol treatment confirming that estradiol rapid action appears to be mediated, at least partially, through GPER (33). However, further investigation is needed to better clarify what the specific target cells for estrogens action at the hypothalamic level are and what receptors are involved.

ESTROGEN FUNCTIONS AT THE PITUITARY LEVEL

In male vertebrates, LH and FSH plasma levels are largely regulated by GnRH and activins as stimulators and steroids and inhibins as inhibitors (34, 35). The negative feedback action of testicular androgens on serum LH and FSH was first demonstrated utilizing castrated animal models evidencing a substantial increase in LH and FSH levels that were prevented by the administration of physiological levels of testosterone (36). Later studies have pointed out the hypothalamus and pituitary as targets for such feedback. Although there are conflicting data concerning the effects of testosterone on GnRH synthesis and secretion, studies have

demonstrated that castration and steroid replacement alter levels of GnRH messenger RNA (mRNA) (37), processing of GnRH prohormone (38), hypothalamic GnRH contents (39), and patterns of pulsatile GnRH release (39, 40). Besides examining hypothalamic sites of action, a number of investigators have also examined feedback directly on the pituitary. Testosterone, DHT, or estradiol is able to suppress GnRH-stimulated LH secretion from pituitary cultures (41), whereas T treatments increase basal FSH secretion and intrapituitary FSH levels (42). Furthermore, molecular analyses of the promoter regions of the gonadotropin genes such as α-gonadotropin subunit (αGSU), FSHβ, and LHβ subunits (43) have revealed the presence of responsive elements through which AR or ER mediated the feedback effects exerted by testosterone or estradiol, respectively.

It is worth noting that estrogen responsiveness of the pituitary gland requires the presence of ERs, including the classical ESR1 and ESR2 (44). The ER expression and distribution patterns in pituitary glands have been studied in rats (45), sheep (46), and humans (47). The localization of ARs in the pituitary is also well-established since AR expression has been reported in the anterior pituitary gland of humans (48), rhesus monkeys, rats (49), Brazilian opossums (50), and mice (51).

Although these data support pituitary sites of steroid action, mainly in feedback regulation, it is unclear whether the effects of T are primarily mediated directly through the AR or indirectly via aromatization and activation of ERs. Experiments performed with a non-aromatizable androgen DHT has been demonstrated to suppress serum LH and basal levels of αGSU and LHβ mRNA in rats (52), confirming AR-mediated feedback. As such, antiandrogen flutamide induced up-regulates of LH serum concentrations (53). At the molecular levels it was also demonstrated that the enhancer elements of the αGSU gene is a target of AR-mediated suppression (43).

In addition, other studies have demonstrated that exogenous estradiol treatment (34) reduced LH and FSH concentrations and gonadotropin mRNAs content, while treatment with aromatase inhibitors determines an increase of LH serum levels (54). The roles of estrogens/ESR1 signaling are further supported clinically by the elevated serum FSH levels in an estrogen-resistant patient (55) as well as in aromatase-deficient humans (24). The unsolved debate focusing on what steroid receptor, AR and/or ESR1, is able to mediate negative feedback on serum gonadotropins is further complicated by the presence of ESR2 (56). Although ESR2 mRNA levels are very low in adult mouse pituitaries (57), there are studies, as already above mentioned, reporting that the hypothalamic nuclei of both rats and mice express ESR2 at both transcriptional and post-transcriptional levels (57, 58). Thus, it is reasonable to hypothesize that testicular steroids could modulate hypothalamic-pituitary activity directly through AR or indirectly through aromatization and activation of either ESR1 or ESR2 signaling pathways.

Estradiol effects in the pituitary gland occur mainly through genomic mechanisms (59) as evidenced in a mouse gonadotroph cell line (LβT2) where estradiol administration increased LHβ mRNA levels (60) due to the presence of EREs within the promoter region of LHβ gene (61). It is noteworthy that there is also experimental evidence for estrogen-independent ESR1 transcriptional activation in gonadotrope cells most probably through GnRH receptor and signaling via protein kinase C (PKC) and MAPK pathways (62). Recent studies indicate that GPER is involved in suppressing GnRH-stimulated LH release in primary pituitary cell culture derived from ovariectomized ewes (63). However, to date there are no studies showing GPER-mediated non-genomic signaling events in the male pituitary. Since GPER has been identified in the plasma membrane of a variety of target tissues, including anterior pituitary (64, 65), we can speculate that GPER could have a role in mediating the non-genomic effects of estradiol in the male pituitary.

ESTROGEN AND HPG AXIS IN MALES: LESSONS FROM ANIMAL MODELS

The development of knock-out or transgenic mice with targeted disruptions of ERs and/or aromatase has increased our understanding of estrogen function in reproduction (66).

Controversy aspect regarding the male hypothalamic and pituitary feedback regulation by steroids has been partially resolved by the observation of data coming from the castration and steroids replacement experiments in *Esr1* knock-out (ERKO) mouse (67) model. Lindzey and co-workers demonstrated that in males, ESR1 is the predominant receptor involved in mediating estradiol suppression of gonadotropin release and gonadotropin subunit mRNA expression (12). The role of an activated AR by testosterone is, of course, not secondary, as demonstrated by the ability of testosterone administration to suppress serum LH in ERKO male mice but its aromatization seems to produce a more functional inhibitory effect on the hypothalamic-pituitary feedback and this is also true for FSH production (12).

Other *in vivo* studies confirmed that estrogens have important roles in the regulation of spermatogenesis. The hypogonadal (*hpg*) mouse (68) that does not produce mature GnRH decapeptide due to a truncation in the GnRH gene is widely used as an animal model to investigate the endocrine regulation of spermatogenesis (69). *Hpg* mice are infertile because they do not produce gonadotropins and hence the testis failed to develop (70). By the *hpg* mice model it was demonstrated that treatment with LH stimulate steroidogenesis (71) and a combined treatment with FSH and androgens induce normal spermatogenesis (72, 73). More interestingly, later research demonstrated that chronic estradiol treatment of this animal model was able to restore spermatogenesis (69, 74, 75), via a mechanism involving a weak neuroendocrine activation of FSH secretion. These latter results raised the question about the site specific action of estrogen in *hpg* mouse model. Further studies based on traditional pharmacological approaches using selective ER agonists in engineered *hpg* animals knocked-out for ERs (*hpg*/ESR1 and *hpg*/ESR2) revealed that estradiol-mediated spermatogenesis takes place in *hpg* animals through the involvement of ESR1, but not ESR2, dependent mechanism responsible for the increase of FSH and testis (mainly Sertoli cells) function.

Spermatogenesis as a target for estrogen/ER signaling has been documented by the use of knock-out mice model for all three ERs (ESR1, ESR2, and GPER) as well as for the aromatase gene. *Esr1* KO animals have reduced fertility because of abnormal fluid reabsorption in the efferent ductules (76), whereas initially spermatogenesis, steroidogenesis, and fertility were found unaffected

in *Esr2* KO animals (66). However, all these *Esr2* mutants displayed alternative splicing transcripts that could compensate for the lack of full-length receptor isoform. An interesting study showed that a new *Esr2*$^{-/-}$ mutant mouse, in which exon 3 of *Esr2* was deleted by Cre/LoxP-mediated excision, completely avoiding any downstream transcripts, produced sterile males (77). The cause for the sterility of these male mice is still unknown, because their gonads and internal genital organs appear *histologically* normal and the mobility of their spermatozoa appears normal too (77). In aromatase knock-out (ArKO) mice the lack of estrogen production results in an alteration of a complex hormonal balance controlling meiosis progression, leading to a significant decrease in spermatocytes and round and elongated spermatids number associated with apoptotic features (78, 79). The more severe testicular phenotype observed in ArKO mice compared to ERKO mice (66) supports the hypothesis that an alternative receptor (i.e., GPER) and alternative pathways could be involved in mediating the effects of estrogen on spermatogenesis.

A study with *Gper* deficient mice (80) claimed that Gper was not involved in estrogenic responses of reproductive organs. However, even though male and female *Gper* KO mice were found fertile, it is noteworthy that the study did not show data on the spermatogenetic process, while a careful examination of estrogenic response was carried out only on the uterus and mammary glands.

A mouse model harboring a two amino-acid mutation of the DNA-binding domain (E207A, G208A) that precludes direct binding of ESR1 to an ERE has allowed discrimination between estrogen action through ERE versus non-ERE pathways (81). The loss of non-classical ESR1 signaling pathways is responsible for most of the reproductive tract defects observed in male ERKO mice (81). These data do not, however, distinguish between the various non-classical pathways (e.g., tethering versus membrane signaling) but support strongly the hypothesis that rapid estrogen signaling could play a crucial role in spermatogenesis.

An original study using estrogen non-responsive *Esr1* knock-in (ENERK1) mice, which have a point mutation in the LBD of *Esr1* that significantly reduces interaction with and response to endogenous estrogens, but does not affect activation of Esr1 by growth factors, showed that estrogen-dependent Esr1 signaling is required for germ cell viability (82).

New information on the role of ESR1 signaling in the regulation of chromatin remodeling during spermiogenesis were obtained from recent works on Type 1 Cannabinoid Receptor Knock-out Mice (*Cnr1*$^{-/-}$) model by Cacciola et al. (83, 84). The characterization of the reproductive *Cnr1*$^{-/-}$ Mice phenotype [reviewed in Ref. (85)] revealed that estrogen through its receptor is able to preserve chromatin condensation and DNA integrity of spermatozoa by promoting histone displacement in spermatids.

In summary, the studies *in vivo* support the findings that estrogen and its major receptor, ESR1, have important roles in the regulation of spermatogenesis, particularly with aging (86) and that this activity occurs through both rapid non-classical membrane-associated/growth factor receptors as well as classical transcriptional mediated pathways. Future studies are required to better understand the separation of these pathways and their potential interactions with other steroid receptors that coexist in the same cell types.

ESTROGEN AND ESTROGEN RECEPTORS IN SPERMATOGENESIS

Spermatogenesis, which takes place in the seminiferous epithelium, can be divided into three major steps: spermatogonia proliferation by mitosis, formation of preleptotene spermatocytes which then gives birth to round spermatids (RSs) via meiosis, and spermiogenesis that allows the maturation of spermatids into mature spermatozoa. This complex and coordinated process is regulated by numerous endocrine, paracrine, or autocrine factors (87, 88) including gonadotropins LH and FSH, androgens, and estrogens (86, 89, 90).

It is known that estrogen action mediated by its specific receptors, such as ESR1, ESR2, and GPER, has different localization and expression through the entire mammalian male reproductive tract (86, 91) with major differences between species, as well as between individuals belonging to the same species (86). In mouse testis, ESR1 was found in Leydig cells, in some peritubular myoid cells (92, 93), and in Sertoli cells (94), whereas ESR2 was found in Leydig cells, Sertoli cells, and some germ cells, particularly spermatocytes (92, 93). In the rat, ESR1 immunodetection was restricted to the Leydig cells (95), in immature rat Sertoli cells (94, 96), in the seminiferous compartment (97), and in purified germ cells (98, 99). Regarding ESR2, there is a general consensus concerning its localization in seminiferous tubules but conflicting data regarding its presence in germ cells (86, 100) although Bois and co-workers detected the presence of ESR2 in pachytene spermatocytes (PS) and RSs (101). The presence of ERs in testicular cells of humans is well documented (90, 102). The two types of ERs, 1 and 2, have been identified in isolated immature germ cells in men, the full-length protein ESR1 (66 kDa) and one isoform lacking the exon 1 (46 kDa). In mature spermatozoa, only the 46-kDa band was observed. For ESR2, two proteins that correspond to the long (60 kDa) and short (50 kDa) forms have been detected in germ cells (102). However, the presence of ESR1 and ESR2 in the human ejaculated spermatozoa has been demonstrated (90, 103).

Recently, ours and other studies have demonstrated the presence of a functional GPER in both normal (98, 99, 104, 105) and malignant testicular cell lines (106).

The important role of estrogens in spermatogonial cell proliferation has been evidenced by works of Chieffi et al. where the authors demonstrated at the molecular level the involvement of ERK/c-fos signaling (107, 108). Accordingly, studies with the mouse spermatogonial GC-1 cell line showed that estradiol rapidly activates EGFR/ERK/fos/cyclin D1 pathway through a functional cross-talk between GPER and ESR1 responsible for cell proliferation (104). Conversely, estradiol-mediated rapid ESR1 and/or GPER/EGFR/ERK/c-jun pathway activation in primary cultures of rat PS (98) and in GC-2 cells (105), an immortalized mouse pachytene spermatocyte-derived cell line, induces an apoptotic mechanism. In particular, in PS cells GPER activation is related to a reduction of cyclin A1 and B1 expression concomitantly with an increase of bax protein expression (98), while in GC-2 cells GPER signaling is associated with the phosphorylation of all MAPK family members initiating the intrinsic apoptotic pathway (105). Similarly, a functional cross-talk between ESR1 and GPER in mediating apoptotic effects was observed also in primary cultures of adult rat RSs (99). It is noteworthy that in this cellular context,

the contribution of ESR2 seems to be related to anti-apoptotic events (99).

G protein-coupled estrogen receptor expression and signaling was also investigated in cultured immature rat Sertoli cells (109, 110) where it has been observed that ERs are able to regulate gene expression involved in both cell proliferation and apoptosis. Indeed, ESR1 activated by its ligand rapidly induces EGFR/ERK1/2 and PI3K pathways that in turn increase cyclin D1 expression responsible for Sertoli cell proliferation (111). Interestingly, through the same molecular pathways the activation of GPER determines anti-apoptotic events by upregulating BCL2 and BCL2L2 proteins. Alternatively, the anti-apoptotic effects could be mediated by estradiol or G-1-GPER/EGFR/ERK1/2/pCREB dependent pathway driving a decrease of bax expression (111).

All these data evidenced that ERs and GPER through different molecular signaling may mediate estradiol action important for the function and maintenance of testicular cells where the complex balance between cellular maturation and cell death drive spermatogenesis and male (in)fertility.

Regarding GPER role in malignant testicular cell lines it has been shown that it is highly expressed in testicular germ cell cancer (TGCC) (112) as well as in Leydig and Sertoli cell tumors (113–115). However, also in this context, GPER activity appears to be cell type specific. In fact, in human testicular seminoma cell line, GPER activation is associated with increased cell proliferation (116), while in rat tumor, Leydig cell line is related to cell growth inhibition and apoptosis (106).

CONCLUDING REMARKS

The reproductive hormonal axis in males normally functions in a tightly regulated manner to produce concentrations of circulating steroids required for normal male sexual development, sexual function, and fertility. The testis has the ability to also produce significant amounts of estrogenic hormones and a regulated balance between androgens and estrogens seems to be essential for normal testicular physiology and reproduction acting both within the testis as well as in regulating HPG axis.

Studies discussed in this review have suggested that estradiol is the main hormone that provides negative feedback at the hypothalamic level, whereas the pituitary requires both estradiol and DHT for a complete negative feedback effect. However, further investigation is necessary to better understand how testosterone and/or its primary metabolites act within the brain to suppress the synthesis and/or secretion of GnRH. Accumulating evidence suggests that estrogen could act in the hypothalamus through rapid action mediated by ESR2, and at least partially, through GPER (33). However, it remains to establish: (i) the specific target cells (GnRH neurons, glia cells, etc.) for estrogen action at the hypothalamic level; (ii) the ER isoforms involved; (iii) the signal transduction activated by estrogen in the different cell types. An unsolved debate is focused on clarifying what steroid (DHT and/or E2) and consequently what steroid receptors (AR and/or ESR1, ESR2) are able to induce and mediate negative feedback at the pituitary level. Interesting studies using engineered *hpg* animals knocked-out for ERs (*hpg*/ESR1 and *hpg*/ESR2), revealed that estradiol-mediated spermatogenesis takes place in *hpg* animals through the involvement of ESR1, but not ESR2, which increases FSH release and testis (mainly Sertoli cells) functions. However, the debate on negative feedback at the pituitary level is further complicated by recent observations that GPER could be involved in suppressing GnRH-stimulated LH release in primary pituitary cell culture derived from ovariectomized ewes (63). However, to date, there are no studies showing GPER-mediated non-genomic signaling events in the male pituitary.

Another important finding is that estrogen plays a direct role in modulating spermatogenesis influencing, in a cell specific manner, germ cells proliferation, differentiation, as well as germ cell survival and apoptosis. The widespread presence of ESR1 and ESR2 in all testicular cells supports this finding and the discovery of GPER in the testis has opened new perspectives to better understand the rapid membrane pathways induced by estrogens. In fact, estrogenic activity in the testis as well as at the hypothalamic level appears to involve not only the classical genomic pathway, but also rapid membrane receptor initiated pathways. Studies discussed in this review indicate the ability of ERs to trigger rapid and converging pathways controlling proliferation (i.e., proliferation through ESR1 and GPER in spermatogonia or apoptosis through the same receptors in spermatids); or trigger, independently from each other, pathways controlling the same cell function (i.e., apoptosis through ESR1 and/or GPER in spermatocytes). Moreover, these studies support the hypothesis that in the testis, as in other tissues, estrogen effects are a result of the combination of different ER mediated activities, including the classic genomic as well as rapid actions at the membrane receptors via a functional cross-talk with growth factor receptors.

Another interesting aspect is that genomic and rapid pathways can work independently from each other but at same time cooperate to reach a common goal (i.e., in Sertoli cells E2-genomic action on cyclin D1 induces proliferation and estradiol rapid action through GPER activates anti-apoptotic signals).

Further studies are necessary to clarify the role of estrogen/ERs signaling in regulating GnRH, FSH, and LH release at the male hypothalamic and pituitary levels as well as in controlling spermatogenesis. Such studies could be helpful to better understand the impact of environmental endocrine disruptors' exposure, such as xenoestrogens, on male reproduction. In addition, more investigation is required to clarify the molecular mechanisms related to estrogen-dependent testicular tumorigenesis as well as to also provide a potential target for the development of a non-androgen male contraceptive.

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Insulin-like factor 3 and the HPG axis in the male

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The hypothalamic–pituitary–gonadal (HPG) axis comprises pulsatile GnRH from the hypothalamus impacting on the anterior pituitary to induce expression and release of both LH and FSH into the circulation. These in turn stimulate receptors on testicular Leydig and Sertoli cells, respectively, to promote steroidogenesis and spermatogenesis. Both Leydig and Sertoli cells exhibit negative feedback to the pituitary and/or hypothalamus via their products testosterone and inhibin B, respectively, thereby allowing tight regulation of the HPG axis. In particular, LH exerts both acute control on Leydig cells by influencing steroidogenic enzyme activity, as well as chronic control by impacting on Leydig cell differentiation and gene expression. Insulin-like peptide 3 (INSL3) represents an additional and different endpoint of the HPG axis. This Leydig cell hormone interacts with specific receptors, called RXFP2, on Leydig cells themselves to modulate steroidogenesis, and on male germ cells, probably to synergize with androgen-dependent Sertoli cell products to support spermatogenesis. Unlike testosterone, INSL3 is not acutely regulated by the HPG axis, but is a constitutive product of Leydig cells, which reflects their number and/or differentiation status and their ability therefore to produce various factors including steroids, together this is referred to as Leydig cell functional capacity. Because INSL3 is not subject to the acute episodic fluctuations inherent in the HPG axis itself, it serves as an excellent marker for Leydig cell differentiation and functional capacity, as in puberty, or in monitoring the treatment of hypogonadal patients, and at the same time buffering the HPG output.

Keywords: INSL3, RXFP2, Leydig cell, testosterone, puberty, hypothalamic hypogonadism

INTRODUCTION

Insulin-like factor 3 (INSL3) is a member of the peptide hormone family, which also includes insulin, IGF1 and IGF2, and relaxin, besides a small number of less well-known peptides (1, 2). There is insecurity about its precise structure *in vivo*. It has a very similar structure to insulin or relaxin, being made as a prepro-hormone, which after intracellular folding becomes post-translationally processed, to give rise to either an A–B heterodimeric peptide, like insulin, or possibly to an uncleaved B–C–A version, analogous to the IGFs. Why this is unclear is that both forms have been identified in the circulation of male mammals (3–5), and both forms are fully and equally bioactive (4). In the male mammal, the major site of INSL3 synthesis is the interstitial Leydig cells of both the fetal and the adult testis [Ref. (6); **Figure 1**]. There may be other sites of local synthesis in some peripheral tissues, but these do not contribute to the circulating levels of the hormone, which are exclusively derived from the testes, and could only have local autocrine or paracrine effects. Leydig cells are known for their production of androgenic steroids, of which testosterone (T), androstenedione (A4), and the derivative dihydrotestosterone (DHT) are the best characterized. However, besides contributing steroids to the circulation, Leydig cells also secrete large amounts of INSL3, giving rise to circulating concentrations of ca. 1 ng/ml in adult men (7–9), and higher levels in some other mammals (10, 11).

Thus, we need to reconsider the complexity of the hypothalamic–pituitary–gonadal (HPG) axis (**Figure 2**), since the gonads produce not only androgens, but also a major peptide hormone, INSL3. We still know very little about the functions attributable to INSL3, except that unlike testosterone there does not appear to be any negative feedback modulation of the hypothalamo-pituitary axis, although this has still not been very thoroughly investigated. Currently, INSL3 appears to have a systemic effect as well as both autocrine and paracrine effects within the testes themselves, in each case providing evidence for some kind of modulation of or by the classical HPG informational output, testosterone.

INSL3 IN THE MALE FETUS

Insulin-like factor 3 is a major product of fetal Leydig cells in all mammals so far investigated [reviewed in Ref. (6)], beginning its production shortly after sex determination and the expression of the key transcription factor SF-1 (steroidogenic factor-1). This represents about embryonic day 12 in the mouse, or week 11–12 of human pregnancy, effectively concurrent with the first detection of fetal androgens (12). In both the fetal testis as well as the adult testis, the production of INSL3 occurs only following a certain maturational differentiation of the Leydig cells. Whereas in the human fetus, as in the adults of all mammals, this differentiation appears to be dependent on the gonadotropin LH, but this is not the case for the mouse. In the fetal mouse,

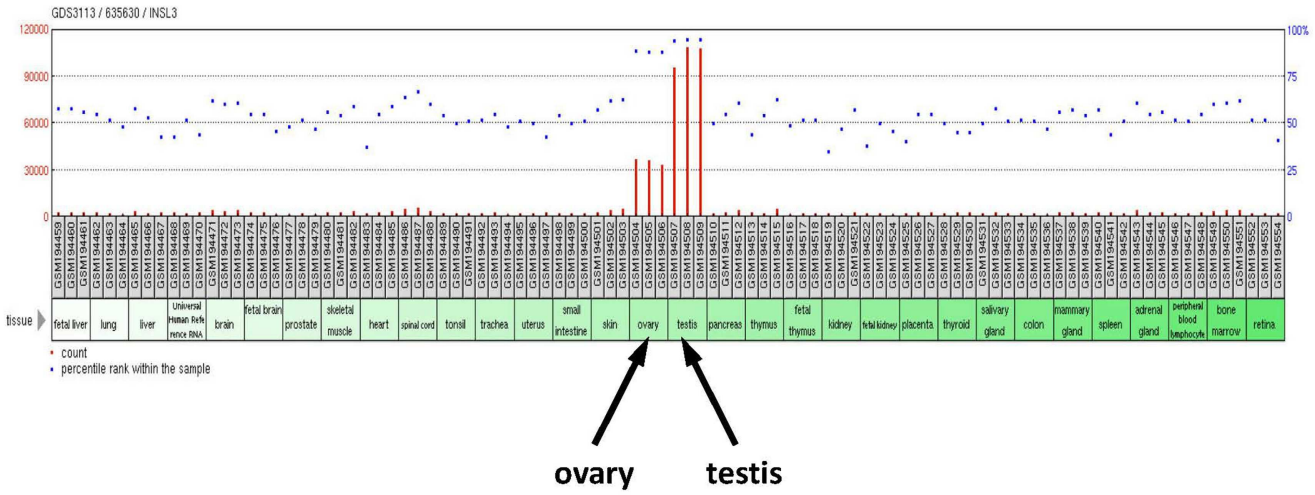

FIGURE 1 | Human tissue RNA profile based on Affymetrix microarrays (GEO profile database; GDS 3113/635630) probed for INSL3 gene expression. Significant INSL3 mRNA is only evident for testes and ovary samples. All tissues are represented in triplicate.

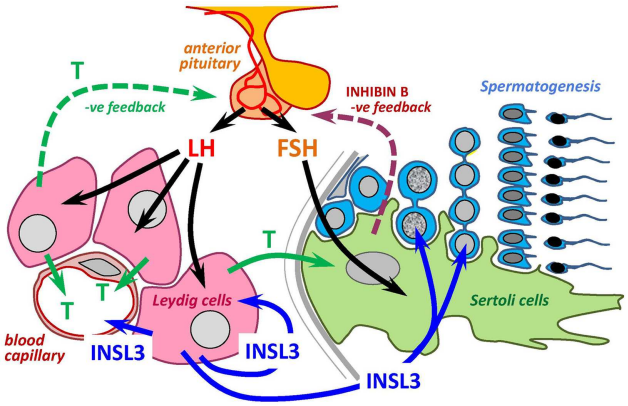

FIGURE 2 | INSL3 and HPG axis. Scheme to show the relationship between the INSL3/RXFP2 system and testosterone as endpoint effectors of the HPG axis within the testis. Arrows are directed only to cells where there are known to be specific cognate receptors.

Leydig cell differentiation is independent of LH production, but rather appears to be regulated by the adrenocorticotropic hormone ACTH (13), even though LH receptors may be present (14). A good illustration of this is the observation that INSL3 levels in fetal Leydig cells from hypogonadal (*hpg*, *gnrh*$^{-/-}$) mice are indistinguishable from those of wild type mice, even though LH levels are very low (15).

The main function of INSL3 in the male fetus is to induce the first, transabdominal phase of testicular descent, which ensues shortly after sex determination and concomitant with the first appearance of INSL3 or its mRNA in the fetus or in amniotic fluid (12). INSL3 acts on its unique receptor RXFP2 (relaxin family peptide receptor 2), which is a G-protein coupled receptor normally linked to G_s, activating adenylyl cyclase (1), and which in the male fetus is expressed by the cells of the gubernacular bulb. The gubernaculum is the ligament connecting the ventral aspect of the developing testis with the inguinal region. Activation of RXFP2 causes a thickening of the gubernacular bulb, which loses elasticity, and effectively retains the once perirenal testis in the inguinal region, at a time when other somatic development is causing the kidney and neighboring organs to grow away in an antero-dorsal direction. Although an active HPG axis is not essential for this process in mice, androgens act synergistically with INSL3 to achieve this important developmental step (16). Partly, it appears that androgens are required to induce the RXFP2 receptors (17, 18), and partly it seems that both androgens and INSL3 share very similar effector signaling pathways (19). INSL3 is not required for the subsequent inguino-scrotal migration of the testis, which appears to require only androgens, or at least an active HPG axis (20).

INSL3 AT PUBERTY AND IN THE ADULT

Following testicular descent at or after birth, the fetal Leydig cells mostly involute. Apart from the so-called "minipuberty" in humans at about 3 months of age, when Leydig cells appear to be transiently active again (21), the testes remain steroidogenically quiescent until puberty begins. The adult population of Leydig cells represent a completely separate lineage of cells from the fetal population, though presumably may share common Leydig stem cells with these. Adult-type Leydig cells differentiate during puberty in an LH-dependent manner, dependent both on the increasing production and pulse frequency of pituitary LH, as well as on the expression of full-length functional LH receptors by the immature Leydig cells. This latter feature is important to emphasize since early Leydig cell stages, at least in rodents, appear to express large amounts of non-functional truncated LH receptor gene transcripts (22–24).

During puberty, the HPG axis becomes hyperactivated, with large and more frequent pulses of LH causing the synthesis and secretion of large amounts of testosterone, which in turn feedback

on the pituitary and hypothalamus to regulate LH pulsatility (25). In rats, this is best illustrated less by changes in mean LH values, but rather by the range of LH concentration (**Figure 3**), which reflects the strong episodic secretion of LH during early puberty and becomes substantially reduced as puberty progresses (26). The average circulating testosterone levels follow a simple asymptotic curve as illustrated in **Figure 3**. This is the resultant both of chronic LH-dependent Leydig cell differentiation, causing long-term induction of appropriate steroidogenic genes, and acute androgen-dependent feedback mechanisms regulating acute LH pulse-dependent and consequent cAMP (PKA)-dependent regulation of steroidogenic enzyme activity. This is different for what happens to INSL3 (**Figure 3**). INSL3 production appears to follow the anatomical differentiation of Leydig cells consequent upon the massive pubertal LH pulsatility, and peaks at around day 40 in the rat, then subsequently declines to stabilize at a lower circulating concentration as the HPG axis attains its stable adult configuration, with the maximal testosterone output and negative feedback.

Cell culture studies using either MA10 mouse tumor or primary adult rat Leydig cells show that INSL3 is largely a constitutive secretory product of Leydig cells, and is not acutely regulated by cAMP or LH (hCG) in the short-term (hours), unlike steroidogenic enzyme activity (10, 28). However, if Leydig cells are subjected to differentiation processes, by being allowed to dedifferentiate in culture, or by collecting cells from immature testes, then LH or hCG have a markedly stimulatory effect on INSL3 production (**Figure 4**), because the gonadotropins can induce both Leydig cell proliferation and augment differentiation, and hence increase INSL3 production, which is a chronic (days) differentiation-dependent process. It should be noted that *in vivo* INSL3 is a biomarker for late Leydig cell differentiation (6). In **Figure 4**, immature Leydig cells prepared from rats at post-natal day 10 initially express no INSL3, as *in vivo*. Without additional gonadotropin, there is already some differentiation and INSL3 expression. However, with regular addition of hCG (as a surrogate for LH), these immature Leydig cells first proliferate until about day 8 of culture, equivalent to about day 18 *in vivo*, and then start to differentiate, with some cells also dying in culture, as reflected by the WST-1 assay (**Figure 4B**). Once differentiated, the Leydig cells cease further multiplication.

The difference between LH-dependent testosterone production and LH-dependent INSL3 production is well illustrated by **Figure 3**, because here we see that during puberty in rats, INSL3 first overshoots in response to the massive bursts of LH production (without feedback regulation), unlike testosterone which is acutely regulated at the level of enzyme activity. As androgen feedback leads progressively to a stabilization of the HPG axis (after day 60 in the rat) at a more moderate LH level (the "thermostat" model), and a correspondingly reduced level of Leydig cell metabolism (differentiation status), then so are the circulating INSL3 levels reduced to reflect that stable Leydig cell functional capacity. This situation is made a little more complex because not only do Leydig cells differentiate under chronic LH influence, but also immature Leydig cells can proliferate in an LH-dependent manner. What INSL3 as a constitutive biomarker is measuring is the sum of both differentiation status (individual cell maturity) and cell number, which together is captured by the term Leydig cell "functional capacity."

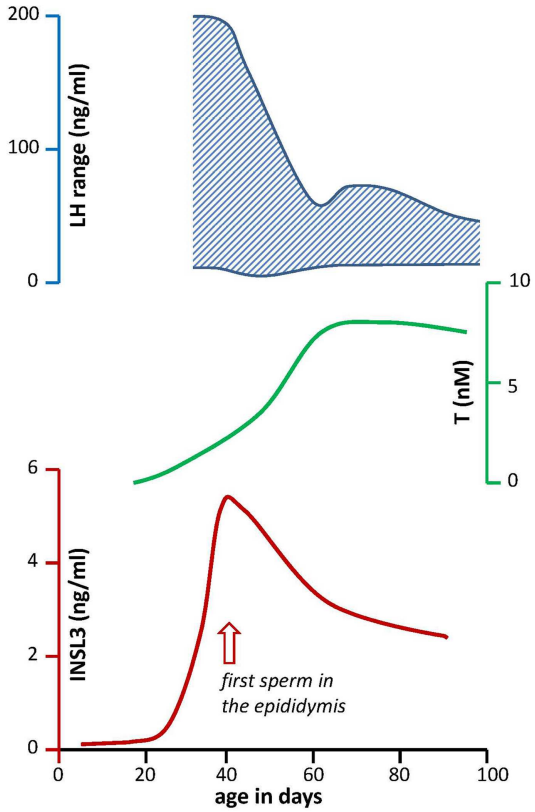

FIGURE 3 | Profiles through rat post-natal development for key circulating hormones of the HPG axis. LH (upper panel) is given as range to indicate the high degree of episodic secretion during early puberty, which is not represented in simple mean values (26). Testosterone (T; middle panel) concentrations are derived from Bartlett et al. (27) based on simple radioimmunassay. The profile for circulating INSL3 [lower panel; Ref. (10)] indicates the marked "overshoot" during early puberty, corresponding to the high LH variance (upper panel). Note that INSL3 values reduce to a stable lower concentration, concomitant with the asymptotic testosterone maximum, and the reduction in LH episodic fluctuation.

We have emphasized these important distinctions because the literature, particularly concerning INSL3 in hypothalamic hypogonadal men, is confusing [e.g., Ref. (29)]. Where such men are treated with hCG/LH for periods of less than a few days, there may be an acute increase in peripheral testosterone production, but there will be no change in circulating INSL3 (8). This is different where the hCG stimulus is chronic, for periods of weeks or months [e.g., Ref. (7, 29)]. The gonadotropin thereby induces the differentiation of the Leydig cells, thereby increasing their functional capacity, and concomitantly therefore increases also the levels of circulating INSL3. INSL3 is still being constitutively generated (in an acute sense) by those individual Leydig cells. Another example to illustrate this point is observed in uni-orchid men, who have one testis removed because of testicular cancer, but are otherwise healthy (9). Their Leydig cell functional capacity is obviously reduced compared to intact men, although those

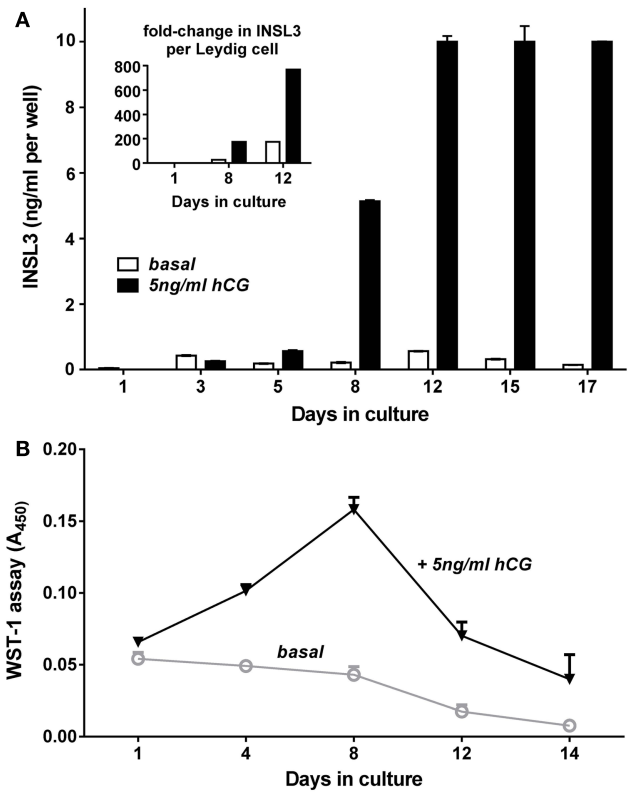

FIGURE 4 | Differentiation *in vitro* of post-natal day (PND) 10 rat Leydig cells in the absence (open bars) or presence (filled bars) of hCG. **(A)** Cells were purified from the abdominal testes of PND10 male Sprague Dawley rats by mechanical dispersion followed by unit sedimentation, then cultured in serum-free medium at 400,000 cells per well of 12-well plates at 37°C. Medium was changed every 2–3 days, with aliquots collected exactly 48 h after the last medium change, for measurement of INSL3 using rat INSL3-specific TRFIA (10). **(B)** Cells prepared as above were seeded in parallel at 30,000 cells per well into 96-well plates and subjected to the WST-1 (4-[3-(4-Iodophenyl)-2-(4-nitro-phenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate) assay to measure cell numbers, as described by the manufacturers (Roche Applied Science (Castle Hill, NSW, Australia). The inset in the upper panel indicates the fold-increase in INSL3 secretion calculated on a per cell basis for key times relative to basal expression on day 1, thus representing the differentiation of the individual Leydig cells, discrete from any effects on cell proliferation or cell death. This shows that while hCG has a marked effect on Leydig cell proliferation and/or survival, it is not essential for cell differentiation, though it does augment it. Animal experimentation was conducted under the terms of permit S-2010-102 of the Animal Ethics Committee, University of Adelaide.

individual Leydig cells will be metabolically highly stimulated. Whereas, as expected, compensatory feedback to the HPG axis has caused a significant increase in LH and an almost normalization of testosterone levels, circulating INSL3 concentration remains significantly reduced (9), and in fact there is an inverse relationship between circulating LH and INSL3 concentrations (9). This is because where the number of Leydig cells is limiting, the number of Leydig cells will be simply reflected by the INSL3 concentration which will be independent of LH. However, the more Leydig cells present, the less LH is required to maintain normal testosterone levels according to the "thermostat" model, and hence the inverse relationship.

A further example to illustrate this point is seen in aging men. When men become old, their circulating testosterone declines at approximately 6% per decade after the age of 40. However, this is continually being compensated by increasing LH, reflecting the continued acute feedback regulation via the HPG axis. For INSL3, produced by the same Leydig cells, the reduction is much greater (ca. 12% per decade) because this acute feedback compensation does not occur (9).

This concept of Leydig cell functional capacity is otherwise best captured only by the ratio of T/LH (30, 31), which of course, unlike a constitutive marker such as INSL3, is subject to the technical variation of being able to reliably measure both T and LH (32, 33). Another feature which reflects this notion of INSL3 as a constitutive biomarker is its technical consistency. We have measured INSL3 in repeated blood samples from young men and have found <10% variation over periods of several months (Anand-Ivell and Ivell, unpublished). Not only is it a technically more robust parameter to measure, but because it is constitutively measuring Leydig cell functional capacity, and is thus not subject to acute feedback fluctuations, as are testosterone and LH, it represents a valuable biomarker, particularly to follow treatments to remediate hypogonadism (29), or to map the progression of puberty (34).

ACTIONS OF INSL3 IN THE TESTIS

Besides the two known endocrine functions of INSL3, to induce the first transabdominal stage of testicular descent (35, 36), and to support bone metabolism and horn growth (37, 38), INSL3 appears to exert functions within the testis, thereby supplementing the conventional role of the HPG axis. The unique INSL3 receptor, RXFP2, has been identified at mRNA and at protein levels on both Leydig cells themselves (39), and also on germ cells within the seminiferous compartment (2, 39–41), but not on other testicular cell types.

Considering an autocrine/paracrine role within the interstitial compartment of the testis, it is important to recognize that under normal circumstances, the adult interstitial fluid will have constitutively high concentrations of INSL3 [in the rat, ca. 400 ng/ml; (10)], such that any surface RXFP2 receptors present are likely to be saturated and most likely desensitized [K_d <1 nM or <6 ng/ml; (1)]. Thus, any role for INSL3 in this compartment is likely to be relevant only in early puberty prior to the completion of Leydig cell differentiation, or similarly during early embryonic development for the fetal population of Leydig cells, or in equivalent disease states such as hypogonadism. In support of this, an interesting study by Pathirana and colleagues showed that INSL3 had a significant stimulatory effect upon Leydig cell steroidogenesis *in vitro*, but only where the cell density in culture was very low, and presumably endogenous INSL3 production was also low (42). Recent studies in the ovary using follicular theca cells, which are the female equivalent of Leydig cells, showed a similar stimulatory effect of INSL3 on theca cell steroidogenesis (18). This effect was absolutely dependent on RXFP2 expression, and could be reduced by transfecting cells with an RXFP2-specific siRNA (18). Thus, INSL3 appears to be part of a feed-forward mechanism buffering the production of steroids consequent upon LH stimulation, and

may have most impact during the first spermatogenic wave before Leydig cells have fully differentiated.

RXFP2 is also expressed by male germ cells (39, 40). In particular, the INSL3 receptor is found modestly expressed by spermatocytes, and to a greater amount on post-meiotic germ cells (39). Experiments in rats show that ca. 20 ng/ml of INSL3 can reach the seminiferous compartment across the blood–testis barrier by mechanisms, which are still unclear (10). This is sufficient to have a modulatory role on male germ cells. Several pieces of evidence support a survival factor/anti-apoptotic role for INSL3 in regard to germ cells, thus effectively abetting the role of FSH acting via Sertoli cells (**Figure 2**). First, in rats, it was shown that INSL3 was able to reduce the amount of germ cell death by apoptosis following GnRH antagonist treatment (40). Second, injection of an INSL3 antagonist into rat testes led to a significant reduction in testis weight (43), presumably resulting from germ cell death. Third, in men subjected to a steroidal contraceptive regimen to suppress the HPG axis, it was found that men retained most residual spermatogenesis when their circulating INSL3 levels were highest (44).

Taken together, these results strongly suggest that INSL3 is acting as an intratesticular autocrine/paracrine system to buffer the conventional output from the male HPG axis, thereby reducing unnecessary fluctuations induced by extrinsic influences (e.g., stress) or excessive pulsatility within the HPG axis, and modulating both LH and FSH actions.

INSL3 SYNERGY WITH ANDROGEN ACTION

Insulin-like factor 3 has been described as a "neohormone" (45, 46), i.e., as a hormone which has evolved specifically to address functions uniquely linked to the mammalian phenotype and evolution. One of the most obvious of these roles is the promotion of testicular descent and a scrotal testis. But also its role to promote horn and bone growth in the male (38) is closely linked to male reproductive behavior, another typical neohormone parameter (46). Inspection of the mechanisms of INSL3 action both as an endocrine, as well as a paracrine/autocrine hormone, indicates that INSL3 is mostly synergizing directly or indirectly with gonadotropin-induced androgen action, for example in bone and horn growth, in maturation of the male tract in the embryo, and in supporting germ cell survival within the seminiferous tubules. Also in the female, where INSL3 is not a highly expressed circulating hormone, it acts in concert with LH, FSH, and androstenedione to promote follicle growth and steroid production (18, 47). The precise molecular details of this synergy are not yet clear, although there is a good evidence to suggest that androgen receptor activation is required for RXFP2 expression (17, 18), and that, at least in the action of INSL3 on the gubernaculum, signaling pathways are induced very similar to those induced by androgen action (19).

INSL3 AND PATHOLOGY

Since INSL3 is part of a synergistic network modulating gonadotropin action, highly specific effects of INSL3 alteration are not to be expected. A complete loss of function of INSL3 or its receptor in mice or humans is associated with osteopenia/osteoporosis (37) and cryptorchidism (35, 36). Whilst a loss of INSL3 in the ovary appears to be linked to a reduction in antral follicle growth and maturation (48), no such gross aberration is evident for the adult testis, even when the receptor knockout is specifically targeted to the testis to avoid any repercussions caused by cryptorchidism (49). However, this latter study did not look at those phases of development such as puberty or during insult situations when the buffering or modulatory effect of INSL3 is likely to be most evident. A reduced INSL3 production by fetal Leydig cells appears to be instrumental in some aspects of the testicular dysgenesis syndrome induced by intra-uterine exposure to endocrine disrupting agents, such as phthalates in rats [reviewed in Ref. (12)]. It is also useful as a monitor to measure effects on Leydig cell development and functional capacity [reviewed in Ref. (6)], being less subject to random fluctuation than androgens. A recent observation resulting from a study of 1200 normal men in Australia also needs to be pursued. It was shown in this study that even young healthy men showed substantial variation (>4-fold) in their circulating levels of INSL3, presumably reflecting a very varied Leydig cell functional capacity (9). Whilst the absolute levels of this hormone are probably still sufficient to support normal physiology, it poses the question as to the causes of such variation, and the long-term impacts, for example, in terms of supporting gonadotropin-induced androgen action later in life. Leydig cell numbers once established in puberty do not appear to change substantially during the remainder of life, there being very little evidence for Leydig cell loss or proliferation in the adult (50). Whilst in the human it has been reported that there is a loss of Leydig cells in old age (51), only recognizably mature cells were counted here, excluding cells which may have dedifferentiated. Longitudinal studies are needed here to explore these aspects further.

CONCLUSION

Insulin-like factor 3 is an important new downstream effector of the HPG axis, which in the male, unlike androgens, does not appear to be subject to acute fluctuation, but through positive feed-forward mechanisms, rather acts to buffer the stimulus of LH (directly via Leydig cells) and of FSH (indirectly via Sertoli cells) on both steroidogenesis as well as germ cell production, respectively (**Figure 2**). Moreover, as a constitutive measure of Leydig cell functional capacity, it also acts as a kind of "memory" for historical insults which may during development, and possibly also in later life, have impacted on the final capacity of the testes to produce androgens.

AUTHOR CONTRIBUTIONS

Richard Ivell was responsible for the drafting of the manuscript. Ravinder Anand-Ivell was responsible for the overall conception of the manuscript and contributed substantially to the drafting, as well as carrying out a number of the experiments reported. Kee Heng carried out several experiments reported in this manuscript as part of her PhD thesis at the University of Adelaide. All authors have read and agree to the finally submitted text.

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Central and direct regulation of testicular activity by gonadotropin-inhibitory hormone and its receptor

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Gonadotropin-inhibitory hormone (GnIH) was first identified in Japanese quail to be an inhibitor of gonadotropin synthesis and release. GnIH peptides have since been identified in all vertebrates, and all share an LPXRFamide (X = L or Q) motif at their C-termini. The receptor for GnIH is the G protein-coupled receptor 147 (GPR147), which inhibits cAMP signaling. Cell bodies of GnIH neurons are located in the paraventricular nucleus (PVN) in birds and the dorsomedial hypothalamic area (DMH) in most mammals. GnIH neurons in the PVN or DMH project to the median eminence to control anterior pituitary function via GPR147 expressed in gonadotropes. Further, GnIH inhibits gonadotropin-releasing hormone (GnRH)-induced gonadotropin subunit gene transcription by inhibiting the adenylate cyclase/cAMP/PKA-dependent ERK pathway in an immortalized mouse gonadotrope cell line (LβT2 cells). GnIH neurons also project to GnRH neurons that express GPR147 in the preoptic area (POA) in birds and mammals. Accordingly, GnIH can inhibit gonadotropin synthesis and release by decreasing the activity of GnRH neurons as well as by directly inhibiting pituitary gonadotrope activity. GnIH and GPR147 can thus centrally suppress testosterone secretion and spermatogenesis by acting in the hypothalamic–pituitary–gonadal axis. GnIH and GPR147 are also expressed in the testis of birds and mammals, possibly acting in an autocrine/paracrine manner to suppress testosterone secretion and spermatogenesis. GnIH expression is also regulated by melatonin, stress, and social environment in birds and mammals. Accordingly, the GnIH–GPR147 system may play a role in transducing physical and social environmental information to regulate optimal testicular activity in birds and mammals. This review discusses central and direct inhibitory effects of GnIH and GPR147 on testosterone secretion and spermatogenesis in birds and mammals.

Keywords: gonadotropin-inhibitory hormone, GPR147, gonadotropins, testosterone, spermatogenesis, melatonin, stress, social environment

INTRODUCTION

Testicular activity is under the control of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which are synthesized in the anterior pituitary gland. LH and FSH are released into the circulation and activate their receptors expressed on Leydig cells and Sertoli cells, respectively, to stimulate testosterone secretion and spermatogenesis in the testis (1) (**Figure 1**). Spermatogenesis is a conserved process in vertebrate testis, where spermatogonia develop into spermatocytes that undergo meiosis to produce spermatids that enter spermiogenesis and undergo a morphological transformation into spermatozoa (2) (**Figure 1**). The process of germ cell development and maturation can be divided into two distinct patterns in vertebrates, one in anamniotes (fish and amphibia) and the other in amniotes (reptiles, birds, and mammals). In anamniotes, spermatogenesis occurs in spermatocysts, which for most species develop in seminiferous lobules. In amniotes, spermatogenesis occurs in seminiferous tubules that possess a permanent population of Sertoli cells, which support spermatogenesis and spermiogenesis, and spermatogonia, and act as a germ cell reservoir for succeeding bouts of spermatogenic activity (2) (**Figure 1**).

The hypothalamic decapeptide gonadotropin-releasing hormone (GnRH) is the primary factor that regulates gonadotropin secretion. GnRH is produced in the preoptic area (POA) and released at the median eminence to stimulate gonadotropin secretion from the pituitary (**Figure 1**). GnRH was first identified in mammals (6, 7) and subsequently in birds (8, 9) and other vertebrates. Testicular steroids and inhibin can modulate gonadotropin secretion by negative feedback. Although dopamine has been reported as an inhibitor of gonadotropin secretion in several fishes (10), no hypothalamic neuropeptide inhibitor of gonadotropin secretion was known in vertebrates. In 2000, a hypothalamic neuropeptide was shown to inhibit gonadotropin release from the cultured quail anterior pituitary gland and it was named gonadotropin-inhibitory hormone [GnIH; (11)] (**Figure 1**). GnIH was originally identified in birds (11) and subsequently in various vertebrates including mammals [for reviews, see Ref. (12–21)] (**Table 1**). Based on extensive studies on birds and mammals,

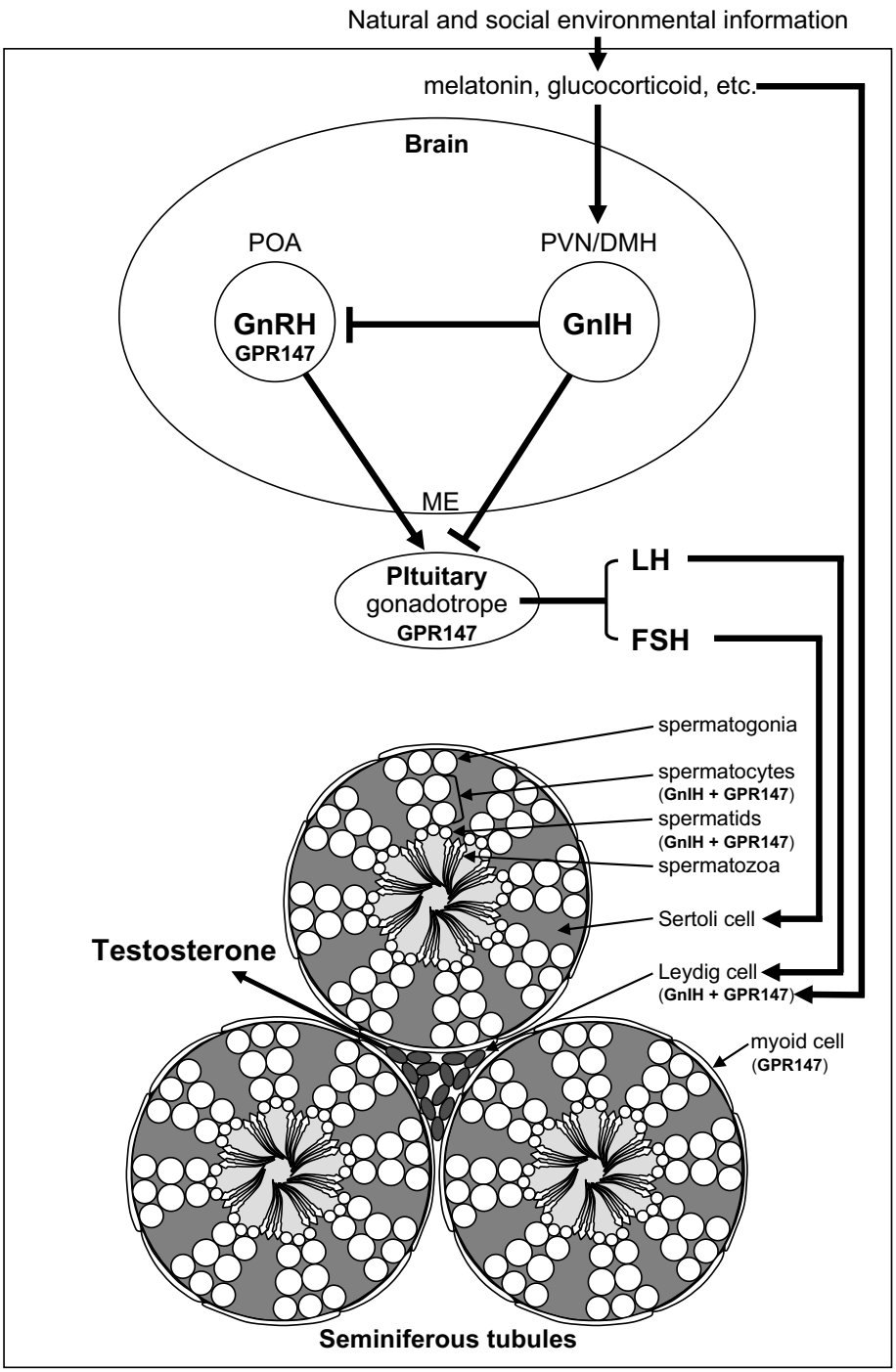

FIGURE 1 | Schematic model of central and direct actions of GnIH on testicular activity in birds and mammals. Neuronal cell bodies expressing gonadotropin-inhibitory hormone (GnIH) are located in the paraventricular nucleus (PVN) in birds and the dorsomedial hypothalamic area (DMH) in mammals. GnIH neurons in the PVN or DMH project to the median eminence (ME) to control anterior pituitary function via GnIH receptor (GPR147) expressed in gonadotropes. GnIH neurons also project to gonadotropin-releasing hormone (GnRH) neurons that express GPR147 in the preoptic area (POA) in birds and mammals. Accordingly, GnIH may inhibit gonadotropin [luteinizing hormone (LH) and follicle-stimulating hormone (FSH)] synthesis and release by decreasing the activity of GnRH neurons as well as directly inhibiting pituitary gonadotrope function. GnIH and/or GPR147 are also expressed in the testis of birds (3, 4) and mammals (5), possibly acting in an autocrine/paracrine manner to suppress testosterone secretion and spermatogenesis. GnIH and GPR147 can thus suppress testosterone secretion and spermatogenesis by acting at all levels of the hypothalamic–pituitary–testicular axis. GnIH expression is further regulated by melatonin, glucocorticoids, and the social environment in birds and mammals suggesting an important role in appropriate regulation of testicular activity seasonally, during times of stress and when interacting with conspecifics in birds and mammals.

Table 1 | Amino acid sequences of avian and mammalian GnIHs [LPXRFamide (X = L or Q) peptides].

	Animal	Name	Sequence	Reference
Birds	Quail	GnIH	SIKPSAY**LPLRFa**	Tsutsui et al. (11)
		GnIH-RP-1[a]	SLNFEEMKDWGSKNFMKVNTPT VNKVPNSVAN**LPLRFa**	Satake et al. (28)
		GnIH-RP-2	SSIQSLLN**LPQRFa**	Satake et al. (28)
	Chicken	GnIH[a]	SIRPSAY**LPLRFa**	Ikemoto et al. (29)
		GnIH-RP-1[a]	SLNFEEMKDWGSKNFLKVNTPT VNKVPNSVAN**LPLRFa**	Ikemoto et al. (29)
		GnIH-RP-2[a]	SSIQSLLN**LPQRFa**	Ikemoto et al. (29)
	Sparrow	GnIH[a]	SIKPFSN**LPLRFa**	Osugi et al. (30)
		GnIH-RP-1[a]	SLNFEEMEDWGSKDIIKMNPF TASKMPNSVAN**LPLRFa**	Osugi et al. (30)
		GnIH-RP-2[a]	SPLVKGSSQSLLN**LPQRFa**	Osugi et al. (30)
	Starling	GnIH	SIKPFAN**LPLRFa**	Ubuka et al. (31)
		GnIH-RP-1[a]	SLNFDEMEDWGSKDIIKMNPFT VSKMPNSVAN**LPLRFa**	Ubuka et al. (31)
		GnIH-RP-2[a]	GSSQSLLN**LPQRFa**	Ubuka et al. (31)
	Zebra finch	GnIH	SIKPFSN**LPLRFa**	Tobari et al. (32)
		GnIH-RP-1[a]	SLNFEEMEDWRSKDIIKMNPF AASKMPNSVAN**LPLRFa**	Tobari et al. (32)
		GnIH-RP-2[a]	SPLVKGSSQSLLN**LPQRFa**	Tobari et al. (32)
Mammals	Human	RFRP-1	MPHSFAN**LPLRFa**	Ubuka et al. (33)
		RFRP-3	VPN**LPQRFa**	Ubuka et al. (33)
	Macaque	RFRP-1[a]	MPHSVTN**LPLRFa**	Ubuka et al. (34)
		RFRP-3	SGRNMEVSLVRQVLN**LPQRFa**	Ubuka et al. (34)
	Bovine	RFRP-1	SLTFEEVKDWAPKIKMNKPV VNKMPPSAAN**LPLRFa**	Fukusumi et al. (35)
		RFRP-3	AMAHLPLRLGKNREDSLS RWVPN**LPQRFa**	Yoshida et al. (36)
	Ovine	RFRP-1[a]	SLTFEEVKDWGPKIKMNT PAVNKMPPSAAN**LPLRFa**	Clarke et al. (37)
		RFRP-3[a]	VPN**LPQRFa**	Clarke et al. (37)
	Rat	RFRP-1[a]	SVTFQELKDWGAKKDIKMS PAPANKVPHSAAN**LPLRFa**	Ukena et al. (38)
		RFRP-3	ANMEAGTMSHFPS**LPQRFa**	Ukena et al. (38)
	Hamster	RFRP-1	SPAPANKVPHSAAN**LPLRFa**	Ubuka et al. (39)
		RFRP-3	TLSRVPS**LPQRFa**	Ubuka et al. (39)

[a] *Putative peptides. The C-terminal LPXRFamide (X = L or Q) motifs are shown in bold.*

it appeared that GnIH can inhibit gonadotropin secretion by decreasing the activity of GnRH neurons as well as directly inhibiting pituitary gonadotropes [for reviews, see Ref. (12–21)]. GnIH and its receptor (GPR147) are also expressed in the gonads of birds (3, 4, 22, 23) and mammals (5, 24–26) including humans (27), possibly acting in an autocrine/paracrine manner (**Figure 1**). This review summarizes possible central and direct effects of GnIH and GPR147 on testosterone secretion and spermatogenesis in birds and mammals.

GnIH RECEPTOR AND CELL SIGNALING

Bonini et al. (40) have identified two G protein-coupled receptors (GPCRs) for neuropeptide FF (NPFF), which has a PQRFamide motif at its C-terminus, and named them as NPFF1 (identical to GPR147) and NPFF2 (identical to GPR74). Hinuma et al. (41) have reported a specific receptor for mammalian GnIH, RFamide-related peptide (RFRP), and named it OT7T022, which was identical to NPFF1 (GPR147). The binding affinities for GPR147 and GPR74 and the signal transduction pathway were examined, using various analogs of GnIHs (RFRPs) and NPFF. RFRPs showed a higher affinity for GPR147, whereas NPFF had potent agonistic activity for GPR74 (40, 42). Accordingly, GPR147 (NPFF1, OT7T022) was suggested to be the principal receptor for GnIH (RFRP). It was also shown that GnIHs (RFRPs) suppress cAMP production in Chinese hamster ovarian cells transfected with GPR147 cDNA, suggesting that GPR147 couples to $G_{\alpha i}$ protein (41).

Yin et al. (43) identified GnIH receptor (GPR147) in the quail diencephalon and characterized its binding activity. First, a cDNA encoding a putative *GPR147* was cloned using PCR primers

designed from the sequence of the receptor for RFRPs. The crude membrane fraction of COS-7 cells transfected with the putative *GPR147* cDNA specifically bound GnIH, GnIH-related peptides (-RPs), and RFRPs, which have an LPXRFamide (X = L or Q) motif at their C-termini, in a concentration-dependent manner (43). In contrast, C-terminal non-amidated GnIH failed to bind the receptor. Accordingly, the C-terminal LPXRFamide (X = L or Q) motif seems to be critical for its binding to GPR147 (43). It was suggested that there is no functional difference among GnIH and GnIH-RPs because GPR147 bound GnIH and GnIH-RPs with similar affinities (43). Further studies are required to investigate if GnIH and GnIH-RPs work additively or synergistically to achieve their effects on the target cells that express GnIH-R.

Ikemoto and Park (29) cloned *GnIH*, *GPR147*, and *GPR74* cDNAs in the chicken. *GPR147* cDNA was expressed only in the brain and pituitary, where GnIH may act directly on gonadotropes. On the other hand, *GPR74* cDNA was ubiquitously expressed in various tissue and organs where GnIH action is unknown. Quail GnIH and putative chicken GnIH inhibited $G_{\alpha i2}$ mRNA expression in COS-7 cells transiently transfected with chicken *GPR147* or *GPR74*. However, the effect of GnIHs on the inhibition of $G_{\alpha i2}$ mRNA expression in COS-7 cells was about 100-fold stronger in COS-7 cells transfected with *GPR147* than *GPR74* (29). These results further suggest that GPR147 is the principal receptor for GnIH in birds as in mammals.

To further investigate the intracellular signaling pathway responsible for the actions of GnIH and its possible interaction with GnRH, Son et al. (44) used a mouse gonadotrope cell line, LβT2. Using this cell line, this group established that mouse GnIHs (mRFRPs) effectively inhibit GnRH-induced cAMP signaling, indicating that mouse GnIHs (mRFRPs) function as inhibitors of adenylate cyclase (AC). They further showed that mouse GnIHs (mRFRPs) inhibit GnRH-stimulated ERK phosphorylation and gonadotropin subunit gene transcription. The results indicated that mouse GnIHs (mRFRPs) inhibit GnRH-induced gonadotropin subunit gene transcriptions by inhibiting AC/cAMP/PKA-dependent ERK activation in LβT2 cells (44).

Shimizu and Bédécarrats (45) showed that *GPR147* mRNA levels fluctuate in an opposite manner to GnRH-receptor-III, a pituitary specific form of GnRH receptor (GnRH-R), in the chicken (46, 47) according to reproductive stages. They demonstrated that the chicken GPR147 inhibits cAMP production, most likely by coupling to $G_{\alpha i}$. This inhibition significantly reduces GnRH-induced cAMP responsive element activation in a dose-dependent manner, and the ratio of GnRH/GnIH receptors was a significant modulatory factor. From these results they proposed that in avian species, sexual maturation is characterized by a change in GnIH/GnRH receptor ratio, changing pituitary sensitivity from GnIH inhibition of, to GnRH stimulation of, gonadotropin secretion (45).

SUPPRESSION OF TESTICULAR ACTIVITY BY GnIH INHIBITION OF GONADOTROPIN SECRETION

Gonadotropin-inhibitory hormone precursor mRNA was first localized by Southern blot analysis of the RT-PCR products in the quail brain. Within the samples from telencephalon, diencephalon, mesencephalon, and cerebellum, GnIH precursor mRNA was only expressed in the diencephalon (28). *In situ* hybridization for GnIH precursor mRNA showed that cells expressing *GnIH* mRNA are clustered in the paraventricular nucleus (PVN) in the hypothalamus (48). Immunohistochemistry using an antibody raised against avian GnIH has revealed that GnIH-ir neurons are clustered in the PVN in quail and other birds (11, 30–32, 49, 50) (**Figure 1**).

In mammals, GnIH (RFRP) precursor mRNA is expressed in the dorsomedial hypothalamic area (DMH) in mouse and hamster brains, as visualized by *in situ* hybridization (39, 51) (**Figure 1**). Mammalian GnIH (RFRP) precursor mRNA is expressed in the periventricular nucleus (PerVN), and in the area between the dorsomedial nucleus (DMN) and the ventromedial nucleus (VMN) of the hypothalamus in the rat brain (41, 52). *GnIH (RFRP)* mRNA expressing neuronal cell bodies are localized in the intermediate periventricular nucleus (IPe) of the hypothalamus in the macaque (34), and in the DMN and PVN in the sheep (37).

Immunohistochemical studies using light and confocal microscopy showed that GnIH (RFRP)-ir axon terminals are in close contact with GnRH neurons in birds (50), rodents (39, 51), monkeys (34), and humans (33) (**Figure 1**), suggesting direct inhibition of GnRH cells by GnIH. Ubuka et al. (31) investigated the interaction of GnIH neuronal fibers with GnRH neurons in the European starling brain. Birds possess at least two forms of GnRH in their brains. One form is GnRH1 which is thought to be released at the median eminence to stimulate the secretion of gonadotropins from the anterior pituitary (8, 9, 53–57). The second form of GnRH, GnRH2 (58, 59), is thought to influence reproductive behaviors in birds (60) and mammals (61, 62). Double-label immunocytochemistry showed GnIH axon terminals on GnRH1 and GnRH2 neurons in the songbird brain (31, 50, 63) suggesting regulation of both gonadotropin secretion and reproductive behavior. *In situ* hybridization of starling *GPR147* mRNA combined with GnRH immunocytochemistry further showed the expression of *GPR147* mRNA in GnRH1 and GnRH2 neurons (31). Similarly, in Siberian hamsters, double-label immunocytochemistry revealed GnIH axon terminals on GnRH neurons, with a subset of GnRH neurons expressing GPR147 (39). Using immunomagnetic purification of GnRH cells, single-cell nested RT-PCR, and *in situ* hybridization, Rizwan et al. (64) showed that 33% of GnRH neurons expressed GPR147, whereas GPR74 was not expressed in either population in mice.

Central administration of GnIH inhibits the release of gonadotropins in white-crowned sparrows (65), Syrian hamsters (51), rats (66), and Siberian hamsters (39) as does peripheral administration of GnIH (30, 51, 67). Direct application of mouse GnIH (RFRP-3) to GnRH cells in mouse brain slices decreased firing rate in a subpopulation of GnRH cells (68). GnIH (RFRP-3) also inhibited firing of kisspeptin-activated vGluT2 (vesicular glutamate transporter 2)-GnRH neurons as well as of kisspeptin-insensitive GnRH neurons (69). These findings suggest that GnIH may inhibit gonadotropin secretion by decreasing the activity of GnRH neurons in addition to directly regulating pituitary gonadotropes in birds and mammals (**Figure 1**). Importantly, the inhibitory action of GnIH (RFRP-1 and RFRP-3) was only observed in reproductively active long-day (LD) Siberian hamsters

that have high gonadotropin concentration, and GnIH (RFRP-1 and RFRP-3) increased basal gonadotropin concentration in reproductively inactive short-day (SD) hamsters (39).

Given the existence of GnIH-ir fibers at the median eminence in birds (11, 30, 31, 48, 50), much of the work to date has focused on the role of GnIH in pituitary gonadotrope regulation (**Figure 1**). As indicated previously, GnIH suppresses gonadotropin synthesis and/or release from cultured quail and chicken anterior pituitary gland (11, 70). In mammals, abundant GnIH (RFRP)-ir fibers are observed in the median eminence of sheep (37), macaque (34), hamsters (71), and humans (33). As in birds, mammalian GnIH (RFRP-3) inhibits gonadotropin synthesis and/or release from cultured pituitaries in sheep (72) and cattle (73). Peripheral administration of GnIH (RFRP-3) also inhibits gonadotropin release in sheep (37), rats (74), and cattle (73), suggesting actions on the pituitary. Finally, *GPR147* mRNA is expressed in gonadotropes in the human pituitary (33). Together, these findings suggest that GnIH and RFRP-3 act directly on the pituitary to inhibit gonadotropin secretion, at least in these avian and mammalian species (**Figure 1**).

Further evidence for a direct action of GnIH on the pituitary comes from a study by Sari et al. (72) where they investigated the effects of GnIH (RFRP-3) on the expression of gonadotropin β-subunit genes in ovine pituitary cells. GnRH or vehicle pulses were given to pituitary cells every 8 h for 24 h with and without GnIH (RFRP-3) treatment. GnIH (RFRP-3) reduced LH and FSH secretion stimulated by GnRH. GnIH (RFRP-3) also reduced GnRH-stimulated LHβ and FSHβ subunit gene expressions. Further, GnIH (RFRP-3) abolished GnRH-stimulated phosphorylation of ERK in the pituitary (72).

To establish whether or not GnIH is endogenously released into the anterior pituitary, Smith et al. (75) directly measured GnIH (RFRP-3) in hypophyseal portal blood in ewes during the non-breeding (anestrous) season and during the luteal and follicular phases of the estrous cycle in the breeding season. Pulsatile GnIH (RFRP-3) secretion was observed in the portal blood, with pulse amplitude and pulse frequency being higher during the non-breeding season. Additionally, the magnitude of the LH response to GnRH was reduced by GnIH (RFRP-3) administration in hypothalamo-pituitary-disconnected ewes, providing support for important functionality of this pathway. Together, these data provide convincing evidence that GnIH (RFRP-3) is secreted into portal blood to act on pituitary gonadotropes, reducing the action of GnRH in sheep (75).

To further establish the functional significance and mode of action of GnIH, Ubuka et al. (67) investigated the role of GnIH on gonadal development and maintenance in male quail. Continuous peripheral administration of GnIH to mature birds via osmotic pumps for 2 weeks decreased the expressions of gonadotropin *common* α and *LH*β subunit mRNAs in a dose-dependent manner. As expected, plasma LH and testosterone concentrations were also decreased dose dependently. Administration of GnIH to mature birds further induced testicular apoptosis, primarily observed in Sertoli cells, spermatogonia, and spermatocytes, and decreased spermatogenic activity in the testis, either through direct actions of GnIH at the level of the gonads (see below) or through decreased gonadotropin and testosterone concentrations. In immature birds, daily peripheral administration of GnIH for 2 weeks suppressed normal testicular growth and the rise in plasma testosterone concentrations. These results indicate that GnIH inhibits testicular development and maintenance either through decreased gonadotropin synthesis and release or via direct actions on the testes (67) (**Figure 1**).

GnIH AND GnIH RECEPTOR IN THE TESTIS

Vertebrate gonads are known to express many "neuropeptides." Bentley et al. (3) demonstrated the expression of GnIH and its receptor in the avian reproductive system, including the gonads and accessory reproductive organs of Passeriform and Galliform birds. Binding sites for GnIH were identified via receptor fluorography in the interstitial layer and seminiferous tubules of the testis. Immunocytochemistry detected GnIH in testicular interstitial cells and germ cells, and pseudostratified columnar epithelial cells in the epididymis. *In situ* hybridization for *GPR147* mRNA produced a strong reaction product in the germ cells and interstitium in the testes as well as pseudostratified columnar epithelial cells. The distribution of GnIH and its receptor suggested a potential for autocrine/paracrine regulation of testosterone production and germ cell differentiation and maturation in birds (3) (**Figure 1**).

To examine the functional significance of these findings, McGuire and Bentley (4) investigated the action of GnIH and GnIH receptor in the testis of house sparrow. GnIH precursor mRNA was expressed in the interstitium and *GPR147* mRNA was expressed in the interstitium and spermatocytes (**Figure 1**). GnIH significantly decreased the testosterone secretion from gonadotropin-stimulated testis cultures (4), suggesting that *GnIH* and *GPR147* are expressed in Leydig cells to reduce the effect of LH on testosterone secretion in an autocrine/paracrine manner (**Figure 1**).

To examine the generality of the findings in birds, Zhao et al. (5) examined GnIH (RFRP), GPR147, and GPR74 expression in the testes of Syrian hamsters. GnIH (RFRP) expression was observed in spermatocytes and in round to early elongated spermatids. GPR147 protein was observed in myoid cells in all stages of spermatogenesis, pachytene spermatocytes, maturation division spermatocytes, and in round and late elongated spermatids. GPR74 proteins only appeared in late elongated spermatids. As in birds, these findings suggest a possible autocrine and/or paracrine role for GnIH (RFRP) in Syrian hamster testis, potentially contributing to the differentiation of spermatids during spermiogenesis (5) (**Figure 1**).

Anjum et al. (76) investigated the changes in GnRH, GnIH, and GnRH-R in the testis from birth to senescence in mice. They found that increased staining of testicular GnRH-R coincided with increased steroidogenic activity during pubertal and adult stages, whereas decreased staining coincided with decreased steroidogenic activity during senescence, suggesting a putative role of GnRH during testicular pubertal development and senescence. The significant decline in GnRH-R during senescence was suggested to be due to a significant increase in GnIH synthesis during senescence. These observations provide new perspectives in the autocrine/paracrine control of testicular activity by GnRH and GnIH (76).

REGULATION OF GnIH GENE EXPRESSION

BY MELATONIN

Investigating the regulatory mechanisms of GnIH expression has important implications for understanding the physiological role of the GnIH system. Photoperiodic mammals regulate reproductive activities according to the annual cycle of changes in nocturnal secretion of melatonin (77). Despite the accepted dogma that birds do not use seasonal changes in melatonin secretion to time their reproductive effort (78, 79), there is some evidence that melatonin is involved in the regulation of several seasonal processes, including gonadal activity, gonadotropin secretion, and timing of egg-laying (80–83). Therefore, Ubuka et al. (84) investigated the action of melatonin on the expression of GnIH in quail, a highly photoperiodic bird species. Because the pineal gland and eyes are the major sources of melatonin in quail (85), Ubuka et al. (84) tested the effects of pinealectomy (Px) combined with orbital enucleation (Ex) (Px plus Ex) and melatonin administration on the expression of GnIH precursor mRNA and GnIH peptide. Px plus Ex decreased the expression of GnIH precursor mRNA and the content of mature GnIH peptide in the hypothalamus; melatonin administration caused a dose-dependent increase in GnIH precursor mRNA and GnIH peptide. Additionally, Mel_{1c} mRNA, a melatonin receptor subtype, was expressed in GnIH-ir neurons in the PVN. Melatonin receptor autoradiography further revealed the binding of melatonin in the PVN. The results suggested that melatonin acts directly on GnIH neurons through its receptor to induce expression of GnIH (84) (**Figure 1**). In agreement with this possibility, a later study showed that melatonin can stimulate GnIH release from the quail hypothalamus (86).

Opposite action of melatonin on the inhibition of GnIH (RFRP) expression was shown in Syrian and Siberian hamsters, both photoperiodic mammals (39, 87, 88). *GnIH (RFRP)* mRNA levels and the number of GnIH (RFRP)-ir cell bodies were reduced in sexually quiescent Syrian and Siberian hamsters acclimated to SD photoperiod, compared to sexually active animals maintained under LD photoperiod. The photoperiodic effects on GnIH (RFRP) expression were abolished in Px hamsters and injections of LD hamsters with melatonin reduced the expression of GnIH (RFRP) to SD levels (39, 87). There are also reports showing that the expression of GnIH (RFRP) is regulated by melatonin and season in sheep (89, 90) and rats (91). These results demonstrate that as in quail, GnIH (RFRP), expression is photoperiodically modulated via a melatonin-dependent process in mammals (**Figure 1**).

Given the localization of GnIH in gonadal tissue, McGuire et al. (23) investigated the possibility that melatonin affects sex steroid secretion and GnIH expression in the gonads of European starlings. Starling gonads expressed mRNAs for *GnIH*, *GPR147*, and melatonin receptors (Mel_{1b} and Mel_{1c}). *GnIH* and *GPR147* expression in the testes was relatively low during the breeding season. The expression levels of Mel_{1b} and Mel_{1c} were correlated with *GnIH* and *GPR147* expression, and melatonin up-regulated the expression of *GnIH* mRNA in starling gonads before the breeding season. GnIH and melatonin significantly decreased the testosterone secretion from gonadotropin-stimulated testes *in vitro* prior to, but not during, the breeding season. Thus, local inhibition of testosterone secretion appears to be regulated seasonally at the level of the testis by a mechanism involving melatonin and gonadal GnIH in birds (23) (**Figure 1**).

BY STRESS

Stress can lead to reproductive dysfunction across vertebrates (92). To explore whether or not stress might act to inhibit reproduction through the GnIH system, Calisi et al. (93) examined the effects of capture-handling stress on GnIH expression in male and female adult house sparrows. More GnIH-positive neurons were observed in fall birds versus those sampled in the spring, and GnIH-positive neurons were increased significantly by capture-handling stress in spring birds. These data imply that stress influences GnIH early during the breeding season, but not after birds have committed to reproduction (93) (**Figure 1**). McGuire et al. (94) tested the hypothesis that the gonads are directly influenced by stress hormones, showing that physiologically relevant concentrations of corticosterone can directly up-regulate GnIH expression and decrease the testosterone secretion from gonadotropin-stimulated testes prior to the breeding season (**Figure 1**). These findings suggest that, stress acts on both central and gonadal GnIH cell populations to inhibit reproductive function.

In agreement with the findings in house sparrows, Kirby et al. (95) showed that both acute and chronic immobilization stress lead to an up-regulation of the expression of GnIH (RFRP) in the DMH of adult male rats associated with the inhibition of downstream hypothalamic–pituitary–testicular activity. Adrenalectomy blocked the stress-induced increase in GnIH (RFRP) expression. Immunohistochemistry revealed that 53% of GnIH (RFRP) cells express receptors for glucocorticoids, suggesting that adrenal glucocorticoids act directly on GnIH (RFRP) cells to increase GnIH expression. Together, these data suggest that GnIH is an important integrator of stress-induced suppression of reproductive function (95) (**Figure 1**).

Son et al. investigated the mechanism by which glucocorticoids influence GnIH gene expression. As in sparrows and rats, *GR* mRNA was expressed in GnIH neurons in the PVN of quail suggesting direct modulation of GnIH in this species. Although acute corticosterone treatment had no effect on *GnIH* mRNA expression, chronic treatment with corticosterone increased *GnIH* mRNA expression in the quail diencephalon. Using a rat GnIH (RFRP)-expressing neuronal cell line, the authors confirmed the co-expression of *GR* mRNA and established that continuous corticosterone treatment increased *GnIH (RFRP)* mRNA expression. They further demonstrated that corticosterone directly regulates *GnIH* gene transcription by recruitment of GR to its promoter at the glucocorticoid responsive element (GRE) (You Lee Son, Takayoshi Ubuka, Narihiro Misato, Yujiro Fukuda, Itaru Hasunuma, Kazutoshi Yamamoto, and Kazuyoshi Tsutsui, unpublished observation) (**Figure 1**).

BY SOCIAL INTERACTION

To examine the impact of mating competition on GnIH, Calisi et al. (96) manipulated nesting opportunities for pairs of European starlings and examined brain *GnIH* mRNA and GnIH content as well as GnRH content. By limiting the number of nest boxes and thus the number of social pairing and nesting opportunities,

they observed that birds with nest boxes had significantly fewer numbers of GnIH-producing cells than those without nest boxes and this relationship reversed once eggs had been laid. On the other hand, GnRH content did not vary with nest box ownership. These data suggest that GnIH may serve as a modulator of reproductive function in response to social environment (96) (**Figure 1**).

It is known that the presence of a female bird as well as copulation rapidly decrease plasma testosterone concentrations in male quail (97, 98). Tobari et al. sought to explore the neurochemical mechanism translating social stimuli into reproductive physiology and behavior. They observed that visual presentation of a female quail decreased plasma LH and testosterone concentrations and this effect was likely to be caused by activation of GnIH neurons in the male quail hypothalamus (Yasuko Tobari, You Lee Son, Takayoshi Ubuka, Yoshihisa Hasegawa, Kazuyoshi Tsutsui, unpublished observation) (**Figure 1**). Together with the findings in starlings, these findings point to a prominent role for GnIH in mediating the impact of social stimuli on the reproductive axis.

SUMMARY

As described in the present review, GnIH, acting via GPR147, can suppress the testosterone secretion and spermatogenesis by acting at all levels of the hypothalamic–pituitary–gonadal axis of birds and mammals. GPR147 is expressed in GnRH cells, pituitary gonadotropes, and at the level of the testis and studies described herein at the organismal and cell culture levels provide functional evidence for control at each locus. Additionally, GnIH expression is regulated by melatonin, glucocorticoids, and the social environment. Together, these findings highlight a prominent role for GnIH–GPR147 in integrating physical and social environmental information to regulate reproductive activities appropriately in birds and mammals.

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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**).

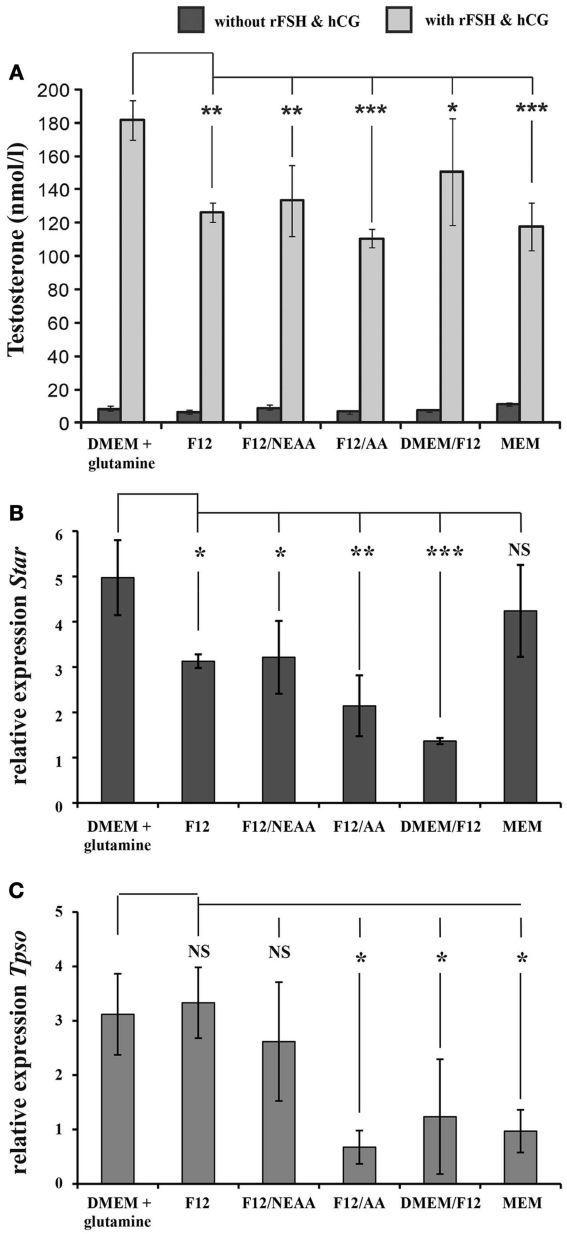

FIGURE 1 | The influence of various culture media on the capacity of the Leydig cells in three-dimensional cultures of rat testicular cells to produce testosterone and express steroidogenic genes. **(A)** On the *X*-axis are the different culture media employed [DMEM + glutamine (GL), F12, F12 + NEAA (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.

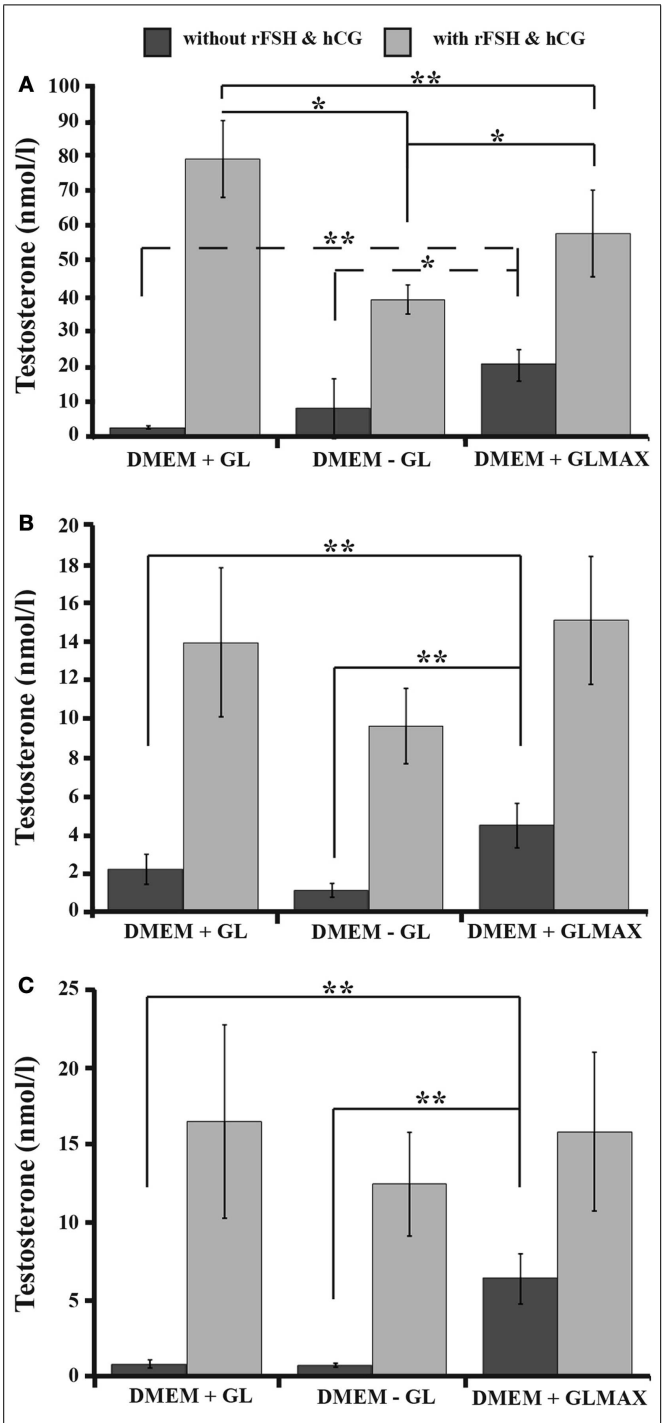

FIGURE 2 | The influence of different DMEM culture media on testosterone production by the Leydig cells in three-dimensional cultures of rat testicular cell. The cells were cultured for 14 days in DMEM + glutamine (GL), DMEM − glutamine (GL), or DMEM + Glutamax (GLMAX) (presented on the *X*-axis) either 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

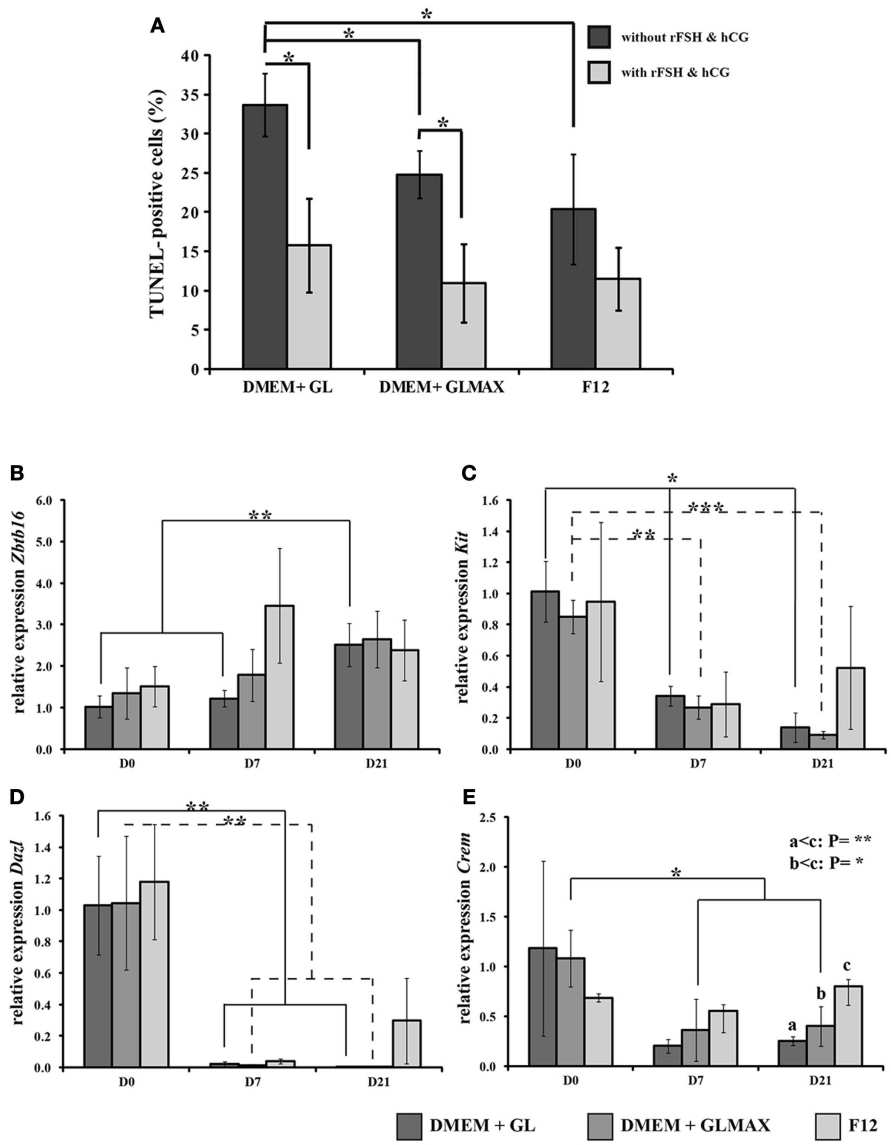

FIGURE 3 | The influence of different culture media on cell survival and expression of genes related to germ cell differentiation in a three-dimensional cultures of rat testicular cells. **(A)** The cells were cultured for 7 days in DMEM + glutamine (GL), DMEM + Glutamax (GLMAX), or F12 (presented on 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.

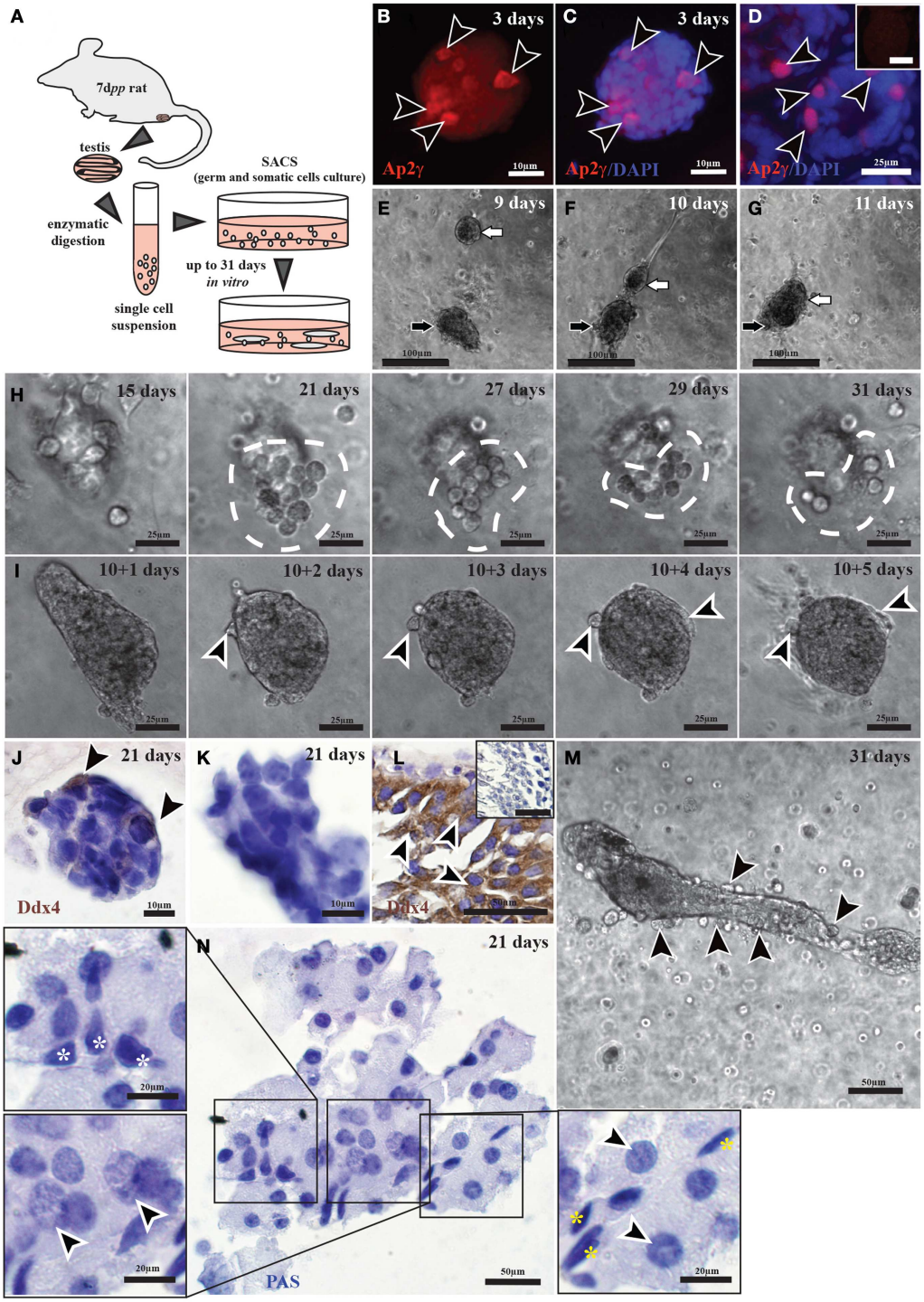

FIGURE 4 | Tubule formation by and germ cell differentiation of pre-pubertal rat testicular cells in three-dimensional cultures. **(A)** A schematic overview of the experimental conditions. **(B–D)** Immunofluorescent staining of undifferentiated spermatogonia (Ap-2γ) in cell colonies originated from 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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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.

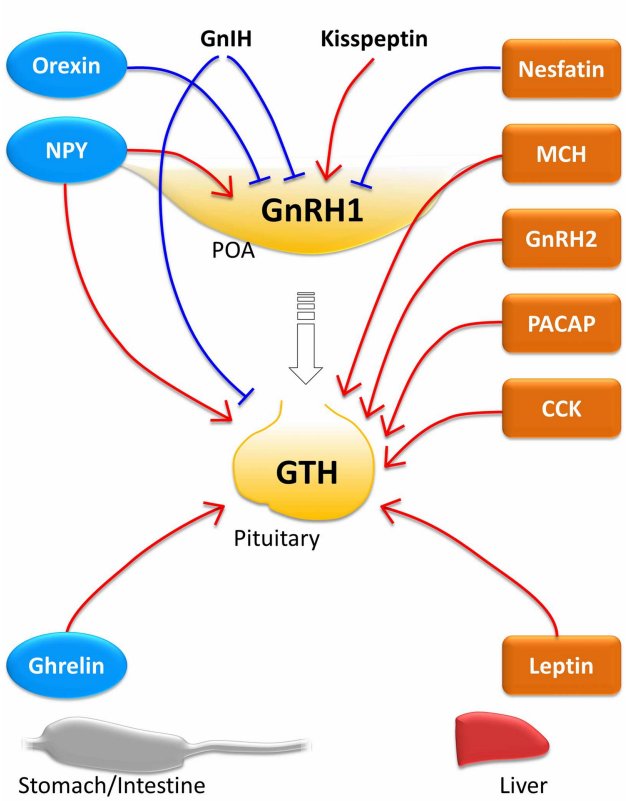

FIGURE 1 | Effects of central and peripheral metabolic hormones on the reproductive system. The hypothalamus–pituitary axis receives many stimulatory and inhibitory inputs from central metabolic neuropeptide neurons and peripheral metabolic signals to control the reproductive system according to the 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.

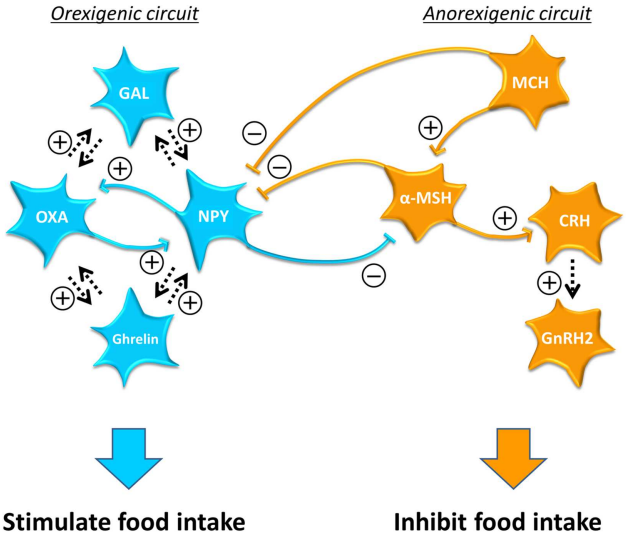

FIGURE 2 | Interaction between the orexigenic circuit and anorexigenic circuit and among neurons in each circuit in the goldfish. Hormones in the orexigenic circuit are shown in blue and those in the anorexigenic circuit are shown in orange. The orexigenic and anorexigenic circuits are connected each 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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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

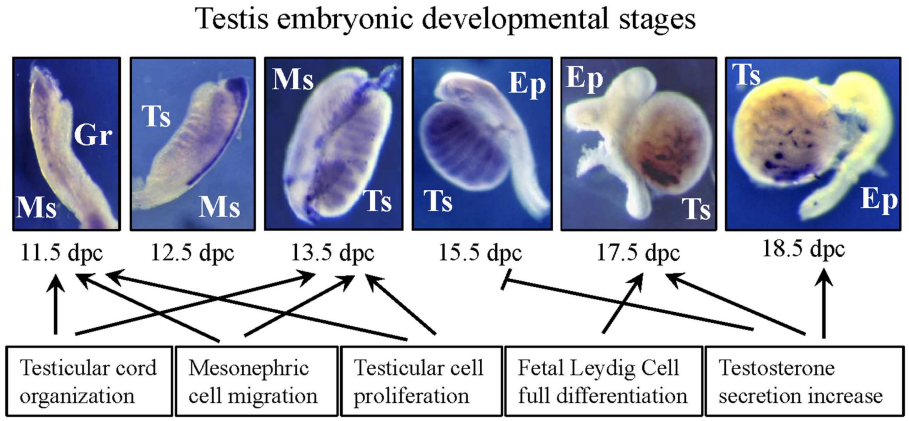

FIGURE 1 | Picture summarizing the main effects exerted by HGF during embryonic testis development. Purple signal in the developing urogenital ridges at different pre-natal stages indicates c-Met mRNA expression. Gr, genital ridge; Ts, testis; Ms, mesonephros; Ep, epididymis.

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

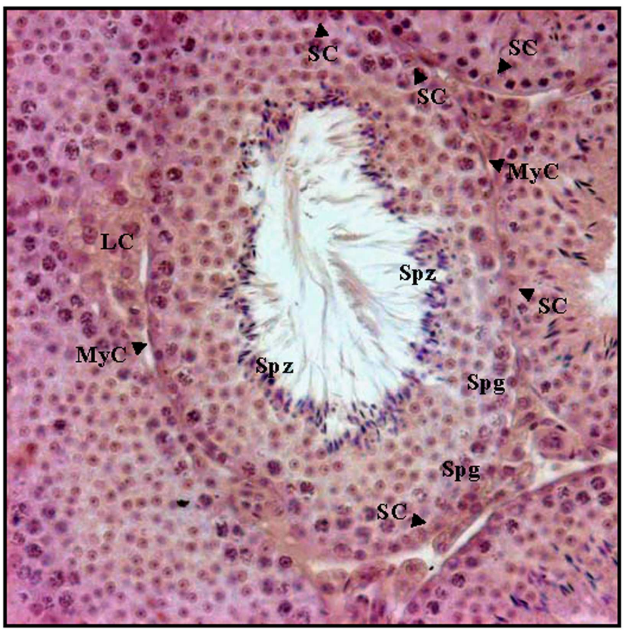

FIGURE 2 | Picture summarizing the main effects exerted by HGF on post-natal testis derived cells. A hematoxylin–eosin stained section of rodent adult testis is also reported. SC, Sertoli cells; LC, Leydig cells; MyC, myoid cells; Spg, spermatogonia; Spz, spermatozoa.

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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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)

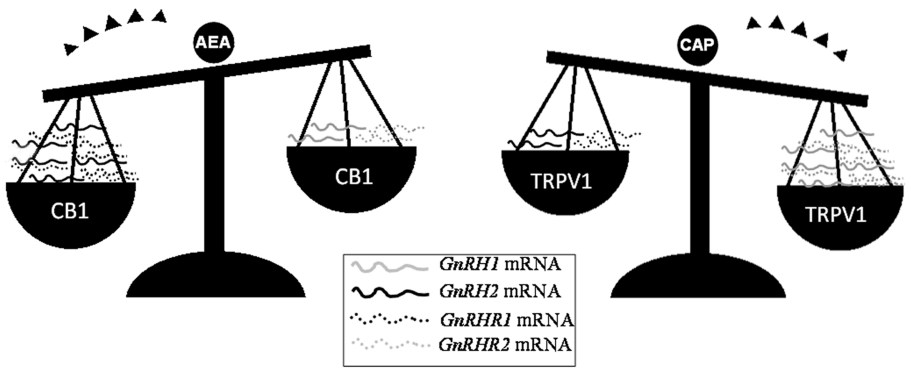

FIGURE 1 | Differential expression of GnRH system components after *in vitro* incubation of frog testis collected in post-reproductive periods with AEA or capsaicin (CAP), a selective agonist of TRPV1 receptor. Since intracellular AEA also bind TRPV1, the involvement of AEA in the modulation of testicular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

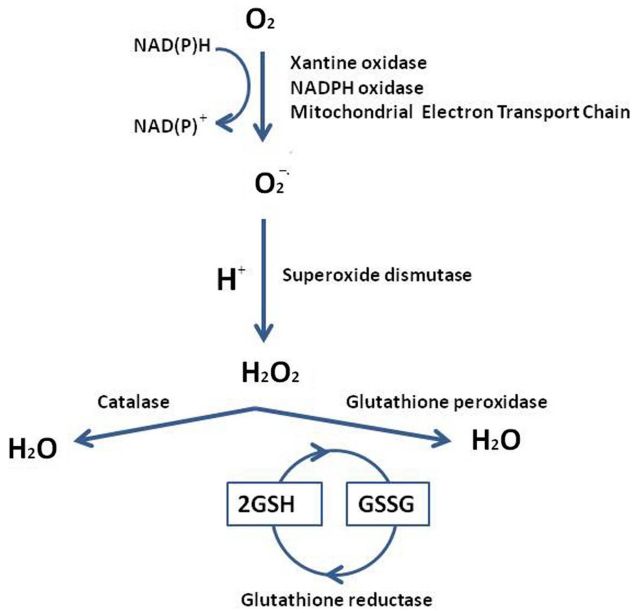

FIGURE 1 | Reactive oxygen species generation and the mechanistic antioxidative and redox defense. The testis overexpression of ROS accelerates a response by the superoxide dismutase (SOD), glutathione peroxidase (GPx), and the glutathione-*S*-transferase (GST). The resulting oxidation product is recycled 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

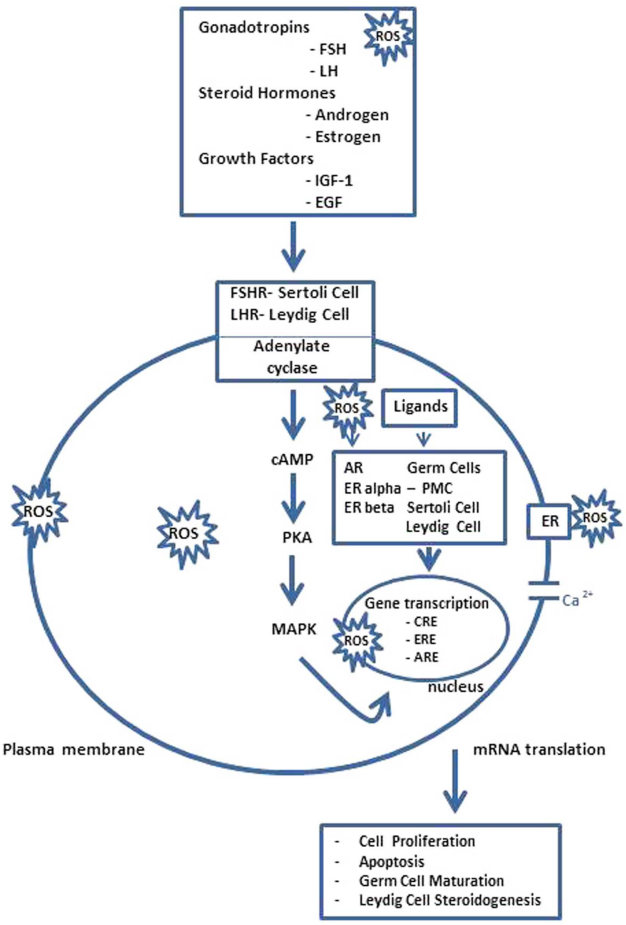

FIGURE 2 | Spermatogenesis ROS endocrine gene transcriptional regulation. FSH acts through its receptors in Sertoli cells (FSHR) to regulate the spermatogenesis and LH stimulates androgen production by Leydig cells after binding to LHR. However, steroid hormones, i.e., androgen and estrogen, and other 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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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.

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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

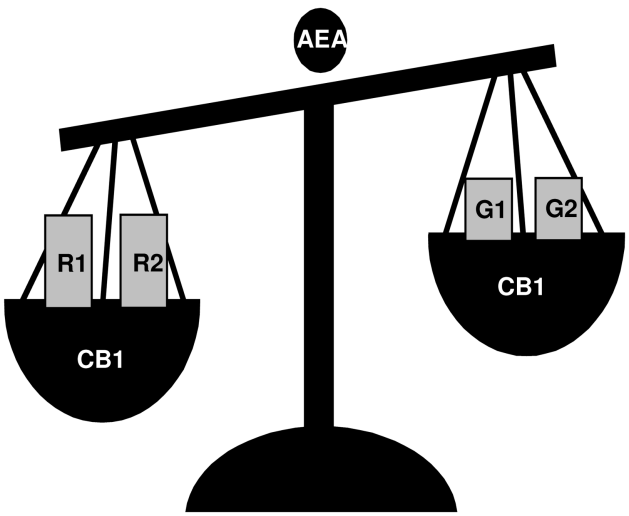

FIGURE 1 | A schematic view of the effects of AEA incubation on *GnRH-1* (G1), *GnRH-2* (G2), *GnRH-R1* (R1), and *GnRH-R2* (R2) expression in frog *R. esculenta* diencephalon. Animals were collected in June and testes were incubated *in vitro* for 1 h. Via CB1 activation, AEA treatment significantly 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.

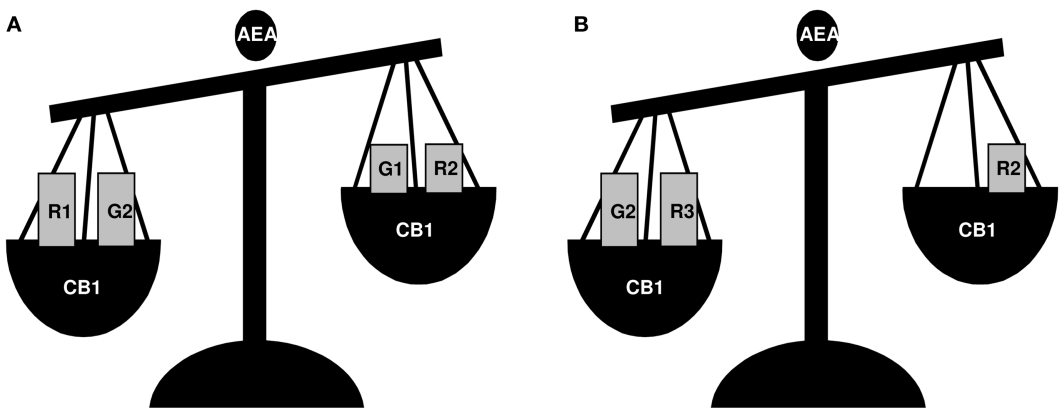

FIGURE 2 | A schematic view of the effects of AEA treatment on *GnRH-1* (G1), *GnRH-2* (G2), *GnRH-R1* (R1), *GnRH-R2* (R2), and *GnRH-R3* (R3) expression in frog *R. esculenta* testis. Animals were collected in June **(A)** and February **(B)** and testes were incubated *in vitro* for 1 h. In June, 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.

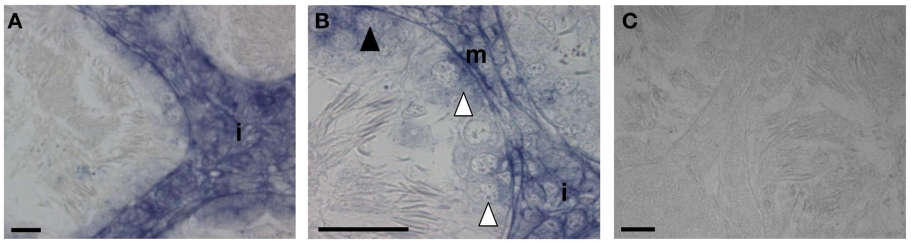

FIGURE 3 | Sections of *R. esculenta* testis, collected in February, analyzed by *in situ* hybridization for *GPR54*. *GPR54* mRNA was detected in the interstitial compartment **(A,B)**, in primary spermatogonia **(B)**, in secondary spermatogonia cysts **(B)** as well as in myoid peritubular cells **(B)**. 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.

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