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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.

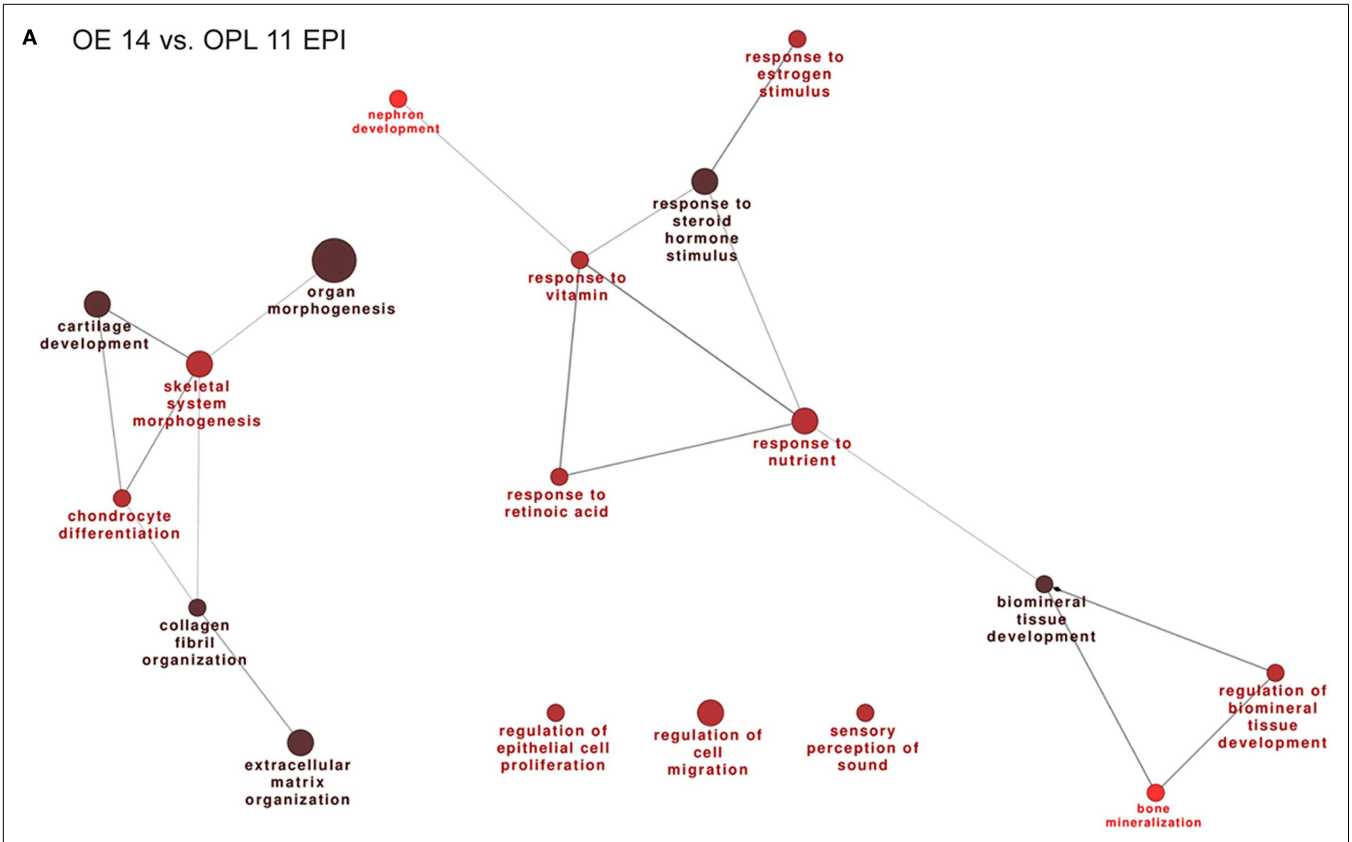


FIGURE 1 | Continued

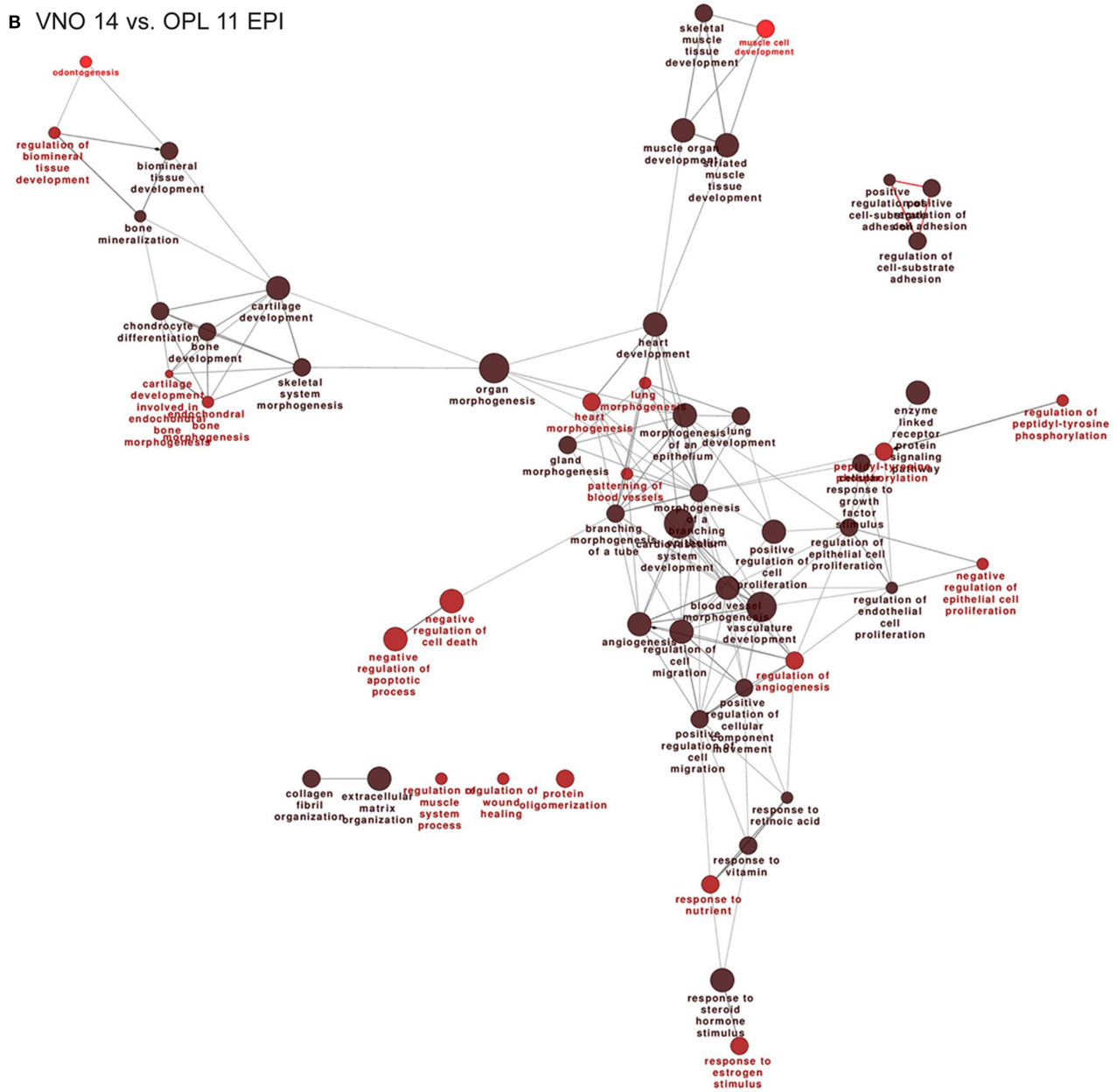


FIGURE 1 | Functional categorization of genes differentially expressed during normal OE and VNO development. Significantly over-represented functional categories of DEGs detected in the (corrected) EPI samples, comparing OE 14.5 vs. OPL 11.5 **(A)** or comparing the VNO 14.5 vs. OPL 11.5 **(B)** were 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,

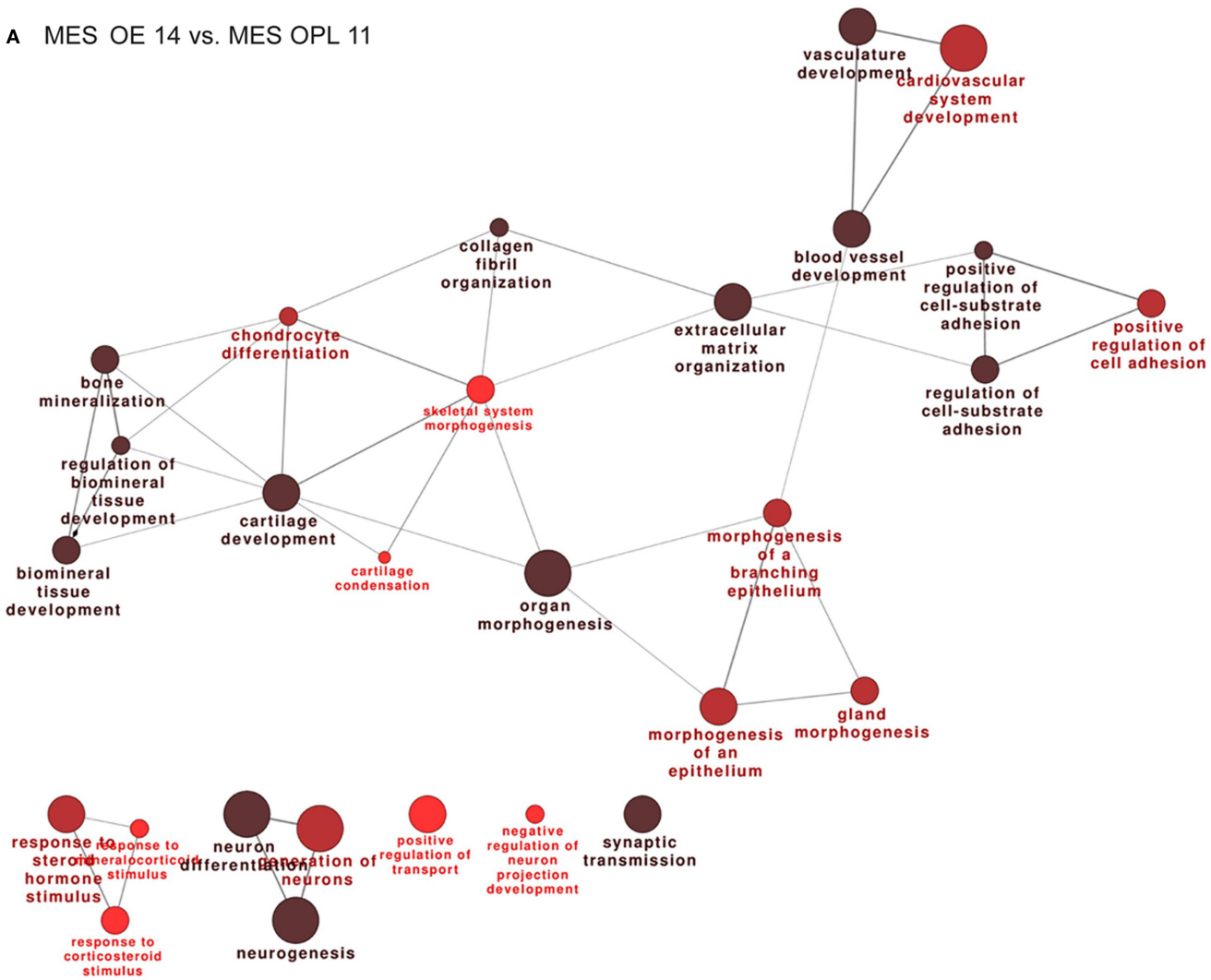


FIGURE 2 | Continued

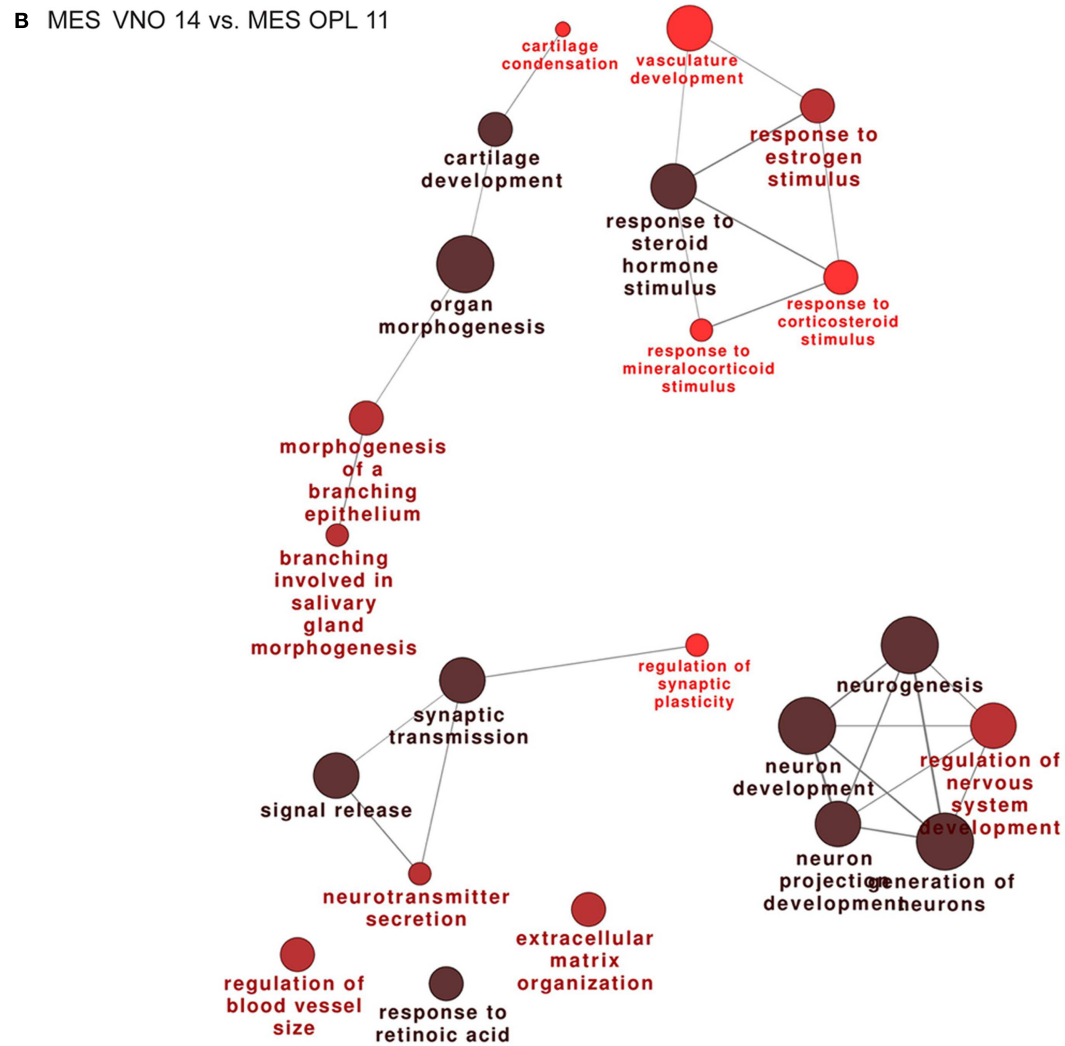


FIGURE 2 | Functional categorization of genes differentially expressed in the mesenchyme adjacent the OE and VNO, during development. Significantly over-represented functional categories of the DEGs detected in the mesenchymal tissue associated with the OE 14.5 vs. OPL 11.5 **(A)** or associated with 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.

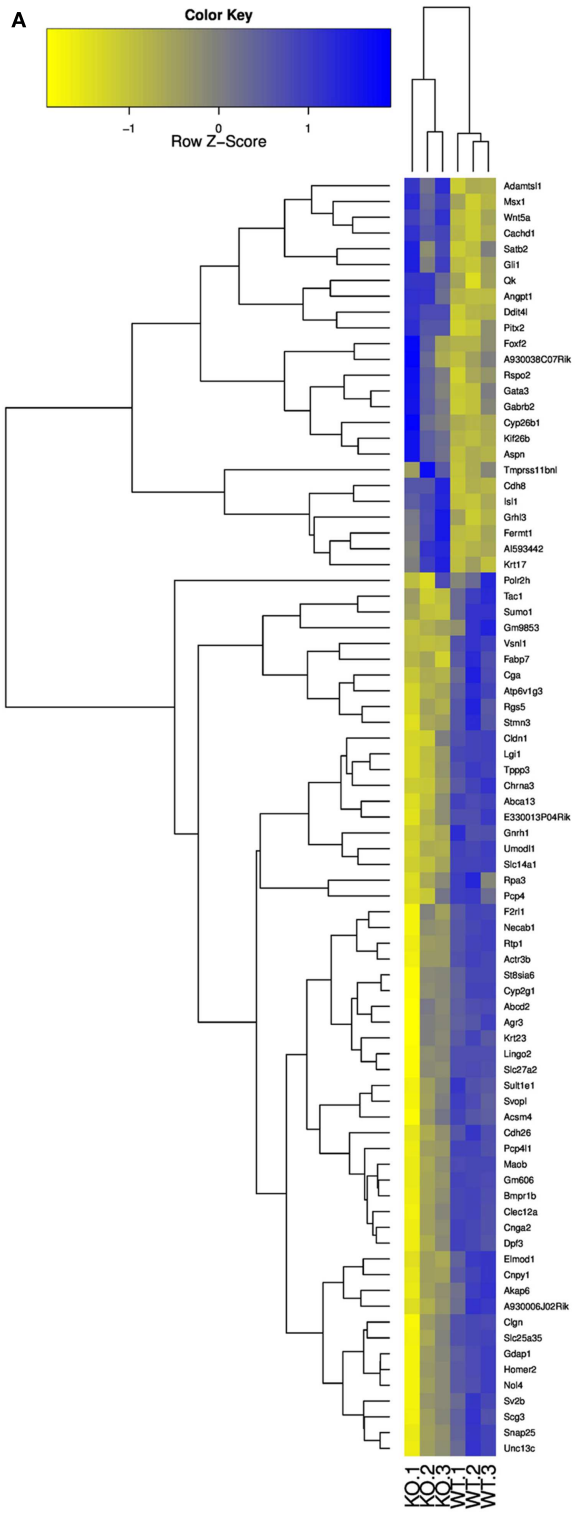
50 (69%) have a NEp expression, 14 (19%) are not expressed in the OE, 4 (5%) have a Mes expression, 3 (4%) have a Res expression, and 2 (3%) are ubiquitously expressed. Thus, conclude that the majority of down-modulated genes are expressed in the OE.

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

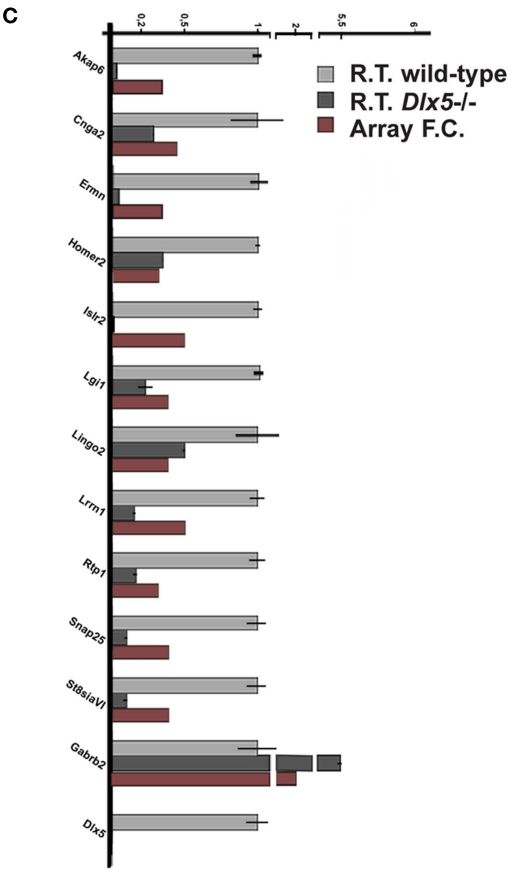


FIGURE 3 | Genes differentially expressed in the *Dlx5*$^{-/-}$ *OE* vs. wild-type OE. **(A)** Heatmap and cluster analyses on the MicroArray data, after elimination of non-annotated genes and of the OR genes. The triplicate *Dlx5*$^{-/-}$ (KO) and wild-type (WT) samples are indicated at the bottom. **(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

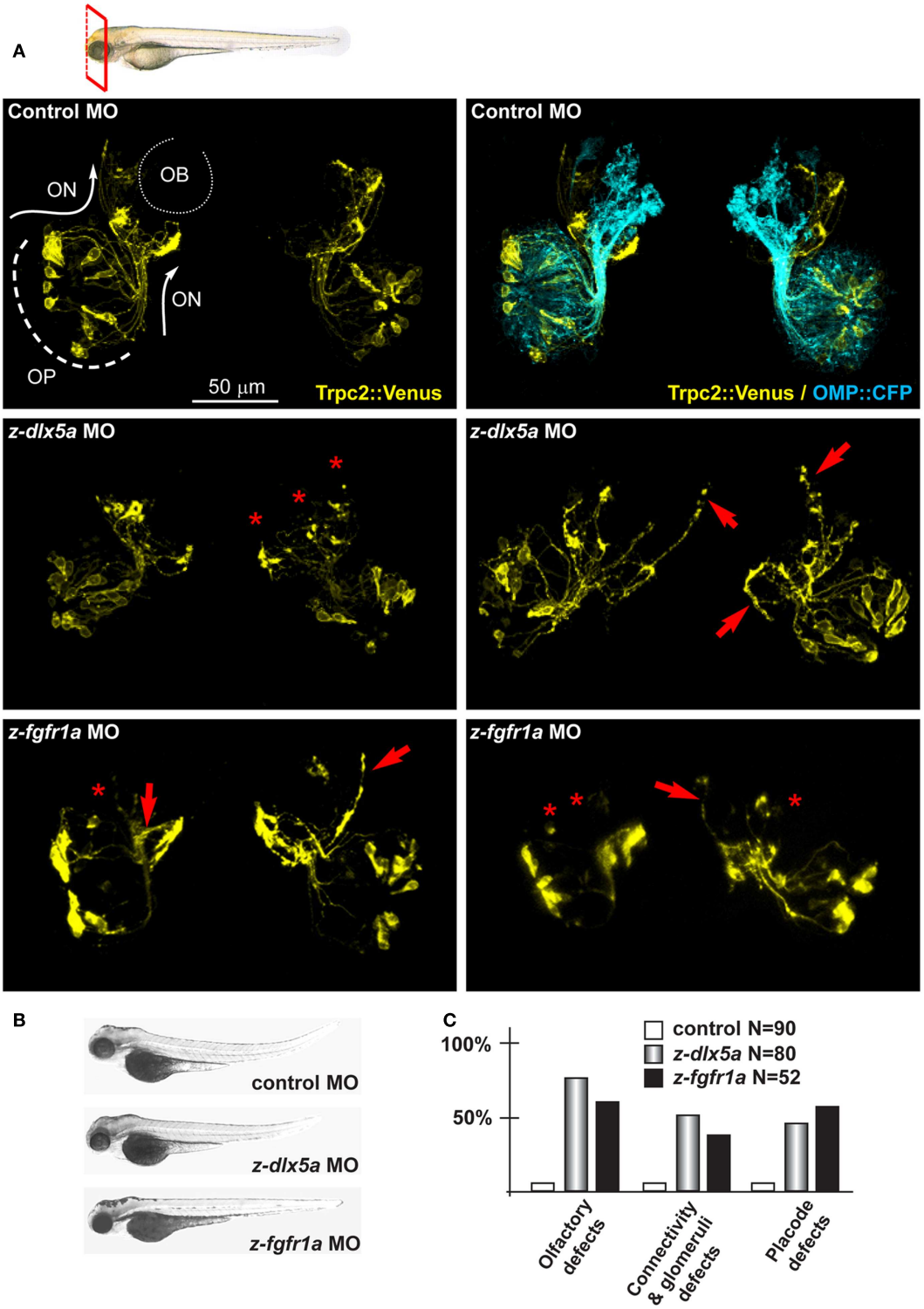


FIGURE 4 | Depletion of endogenous *z-dlx5a* and *z-fgfr1a* in zebrafish embryos, to image the olfactory axons. **(A)** Micrographs of *Trpc2:Venus* (YFP, yellow fluorescence) and *OMP:CFP* (cyan fluorescence) fish embryos injected with a control MO (top panels), injected with anti-*z-dlx5a* MO (middle 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.

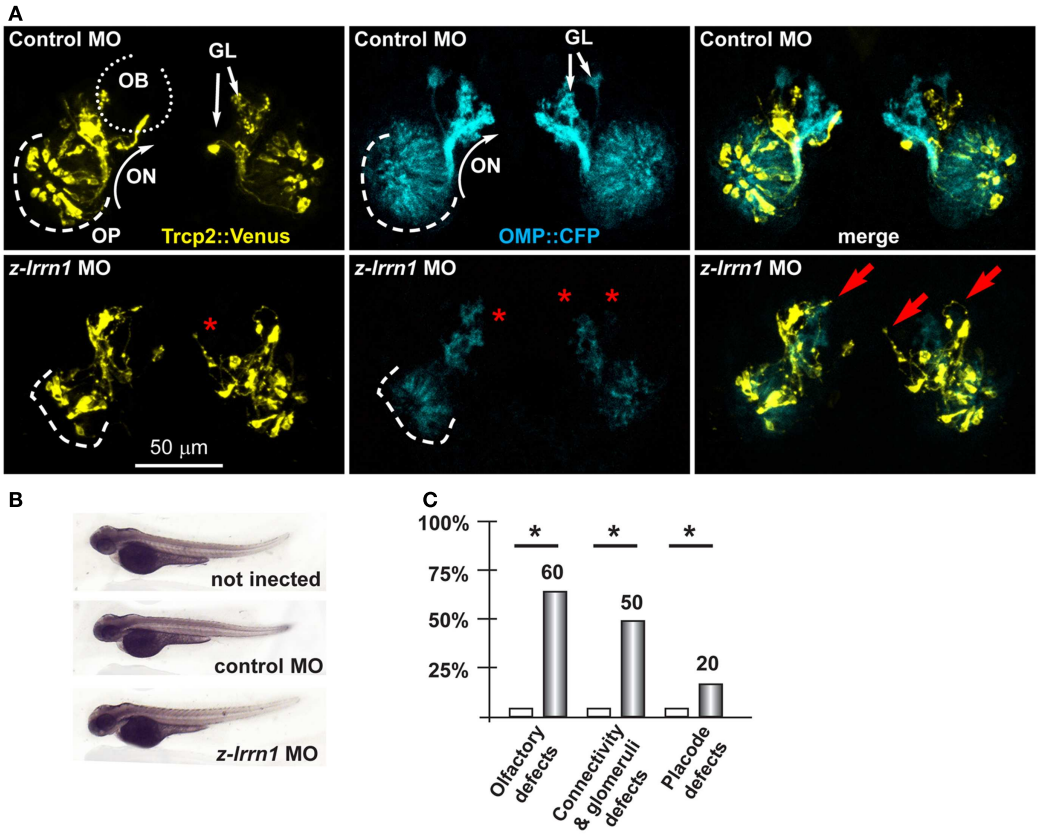


FIGURE 5 | Depletion of endogenous *z-lrrn1* in zebrafish embryos, to image the olfactory axons. **(A)** Micrographs of *Trpc2:Venus* (YFP, yellow fluorescence) and *OMP:CFP* (cyan fluorescence) zebrafish embryos injected with control (top panels) or with anti-*z-lrrn1* (bottom panels) MOs. The control 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*,

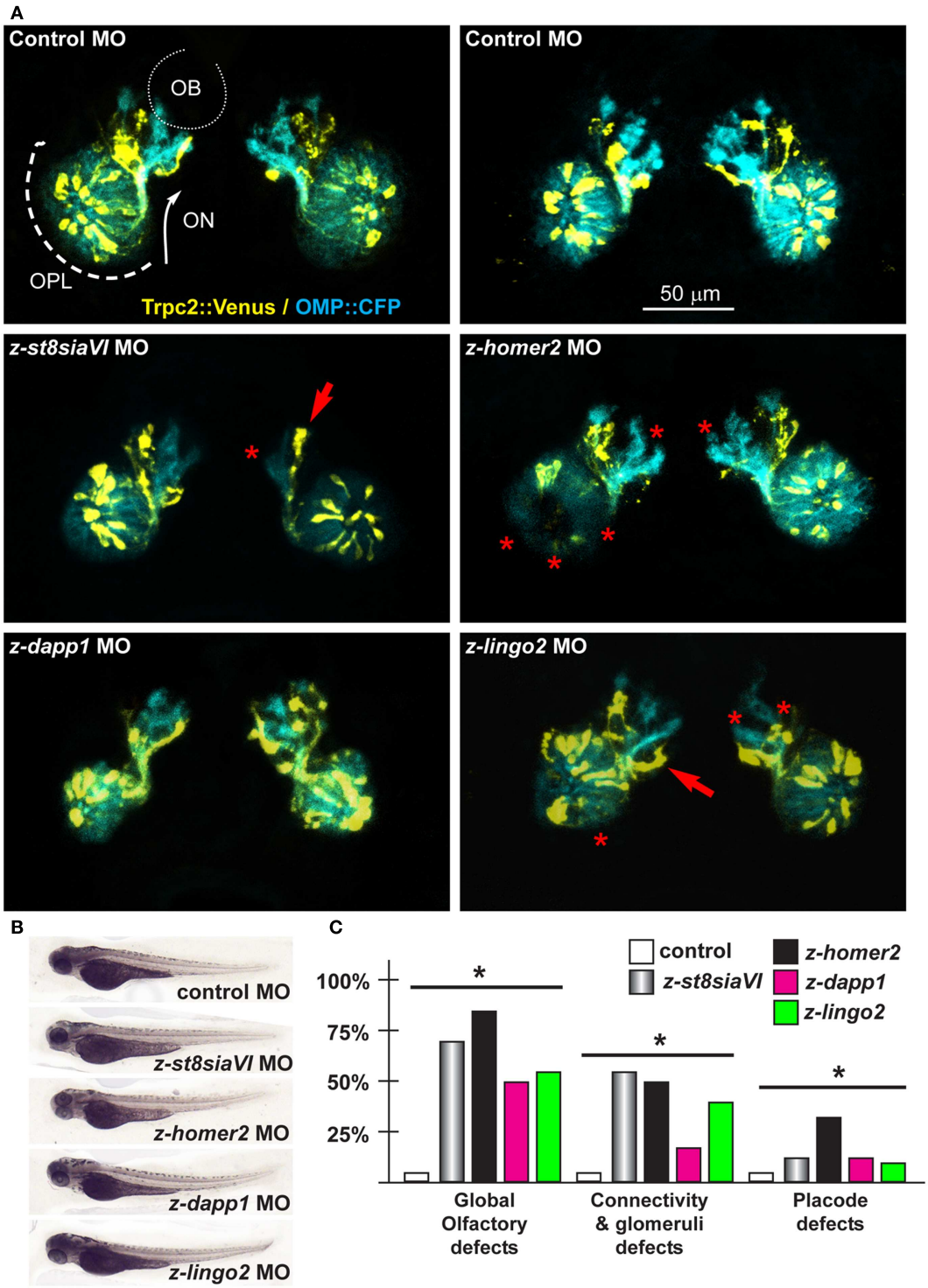


FIGURE 6 | Olfactory and VNO axons, upon depletion of endogenous *z-St8siaVI, z-lingo2,* and *z-homer2* in zebrafish embryos. **(A)** Micrographs of *Trpc2:Venus* (yellow fluorescence) and *OMP:CFP* (cyan fluorescence) embryos injected with control MO (top panels), or injected with anti-*z-st8siaVI*, 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.

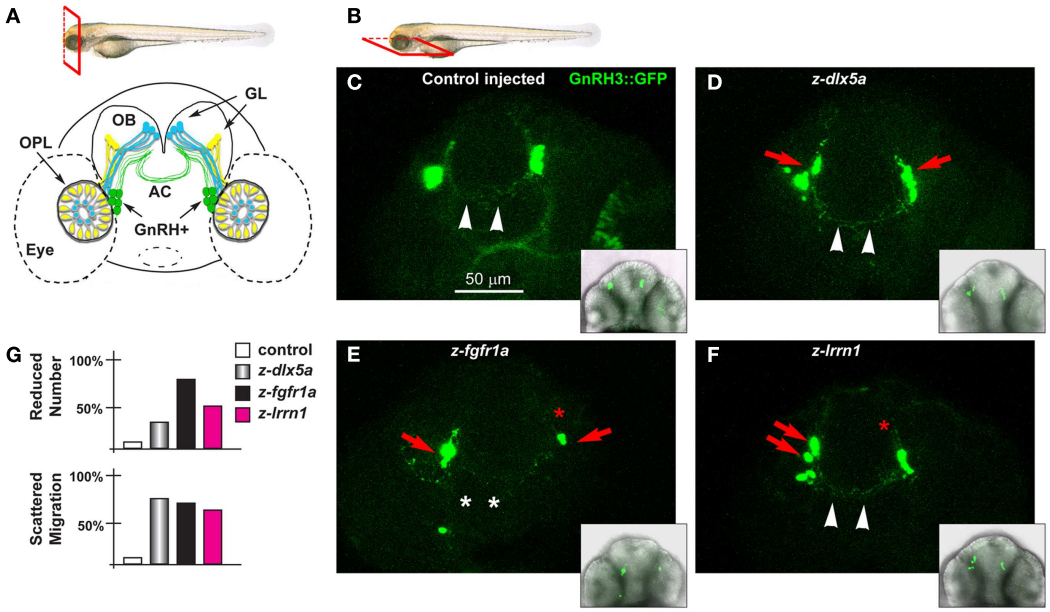


FIGURE 7 | The GnRH3 neurons upon depletion of endogenous *z-dlx5a, z-fgfr1a,* and *z-lrrn1* in zebrafish embryos. **(A)** Scheme showing the positions of the GnRH3:GFP+ neurons (green cells), relative to the OPL, the OB, and the olfactory nerves (yellow and blue), in a frontal view. The anterior commissure 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.

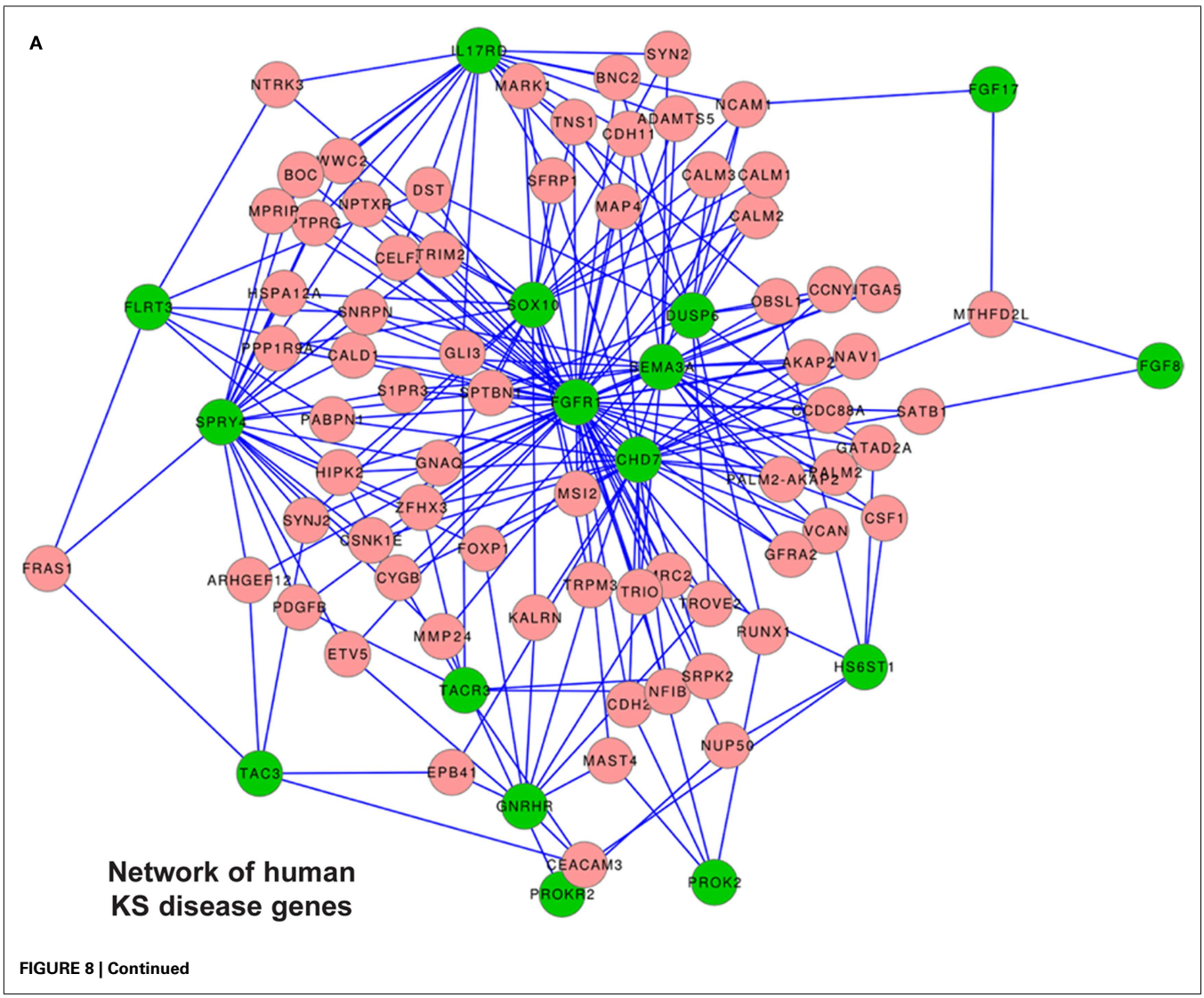


FIGURE 8 | Continued

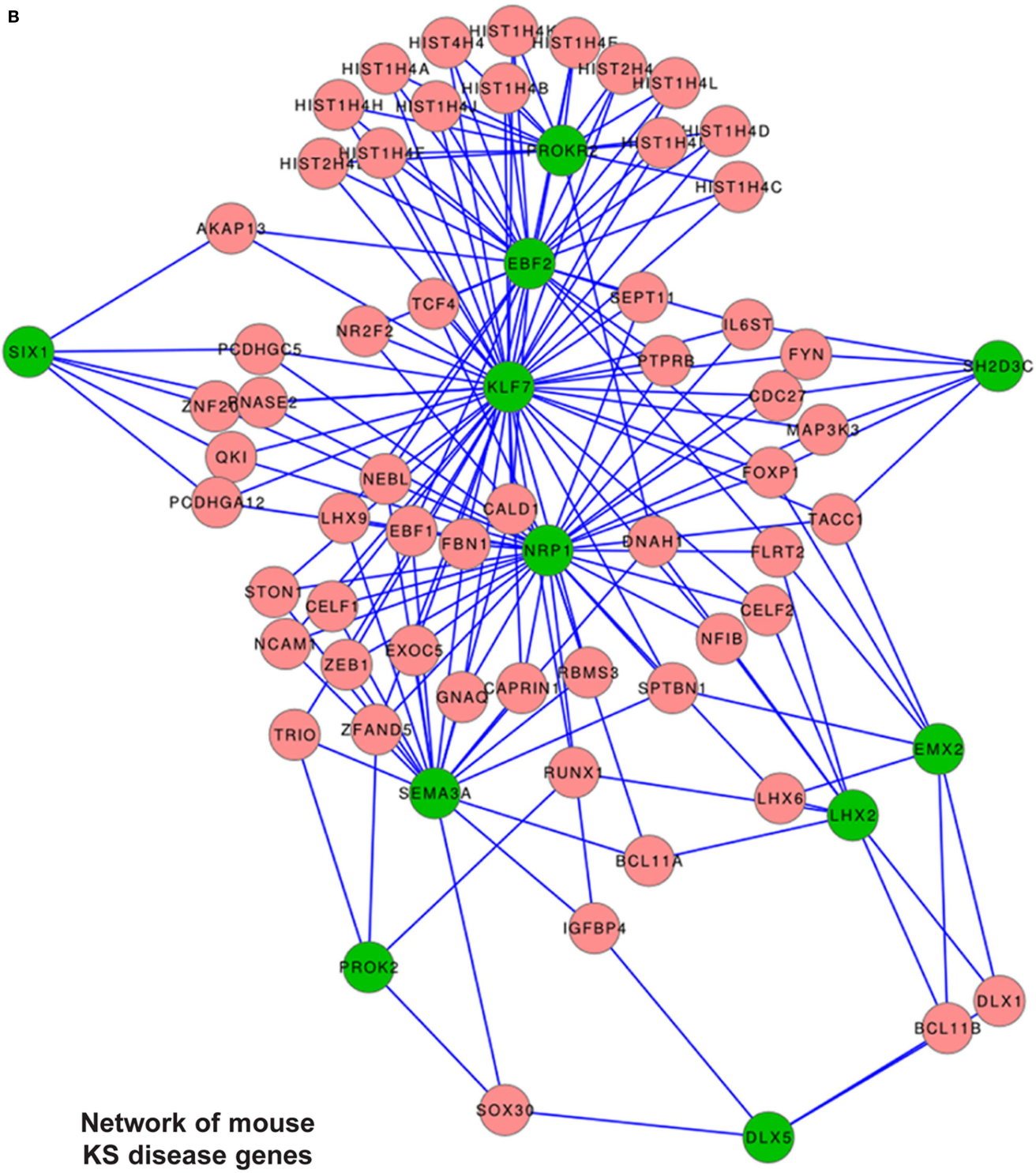


FIGURE 8 | Disease gene networks for KS. **(A)** Position of the human KS-causing genes within the global conserved co-expression network, as computed with the TS-CoExp algorithm. **(B)** Position of the genes causing a KS-like phenotype in mice, within the global conserved co-expression network. The 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

REFERENCES

1. Topaloglu AK, Kotan LD. Molecular causes of hypogonadotropic hypogonadism. *Curr Opin Obstet Gynecol* (2010) **22**:264–70. doi:10.1097/GCO.0b013e32833bb425
2. Hu Y, Tanriverdi F, MacColl GS, Bouloux PM. Kallmann's syndrome: molecular pathogenesis. *Int J Biochem Cell Biol* (2003) **35**:1157–62. doi:10.1016/S1357-2725(02)00395-3
3. Cadman SM, Kim SH, Hu Y, Gonzalez-Martinez D, Bouloux PM. Molecular pathogenesis of Kallmann's syndrome. *Horm Res* (2007) **67**:231–42. doi:10.1159/000098156
4. Cariboni A, Maggi R. Kallmann's syndrome, a neuronal migration defect. *Cell Mol Life Sci* (2006) **63**:2512–26. doi:10.1007/s00018-005-5604-3
5. Dode C, Hardelin JP. Kallmann syndrome. *Eur J Hum Genet* (2009) **17**:139–46. doi:10.1038/ejhg.2008.206
6. Hardelin JP, Dode C. The complex genetics of Kallmann syndrome: KAL1, FGFR1, FGF8, PROKR2, PROK2, et al. *Sex Dev* (2008) **2**:181–93. doi:10.1159/000152034
7. Miraoui H, Dwyer AA, Sykiotis GP, Plummer L, Chung W, Feng B, et al. Mutations in FGF17, IL17RD, DUSP6, SPRY4, and FLRT3 are identified in individuals with congenital hypogonadotropic hypogonadism. *Am J Hum Genet* (2013) **92**:725–43. doi:10.1016/j.ajhg.2013.04.008
8. Semple RK, Topaloglu AK. The recent genetics of hypogonadotrophic hypogonadism – novel insights and new questions. *Clin Endocrinol (Oxf)* (2010) **72**:427–35. doi:10.1111/j.1365-2265.2009.03687.x
9. Berghard A, Hagglund AC, Bohm S, Carlsson L. Lhx2-dependent specification of olfactory sensory neurons is required for successful integration of olfactory, vomeronasal, and GnRH neurons. *FASEB J* (2012) **26**:3464–72. doi:10.1096/fj.12-206193
10. Cariboni A, Davidson K, Rakic S, Maggi R, Parnavelas JG, Ruhrberg C. Defective gonadotropin-releasing hormone neuron migration in mice lacking SEMA3A signalling through NRP1 and NRP2: implications for the aetiology of hypogonadotropic hypogonadism. *Hum Mol Genet* (2011) **20**:336–44. doi:10.1093/hmg/ddq468
11. Hanchate NK, Giacobini P, Lhuillier P, Parkash J, Espy C, Fouveaut C, et al. SEMA3A, a gene involved in axonal pathfinding, is mutated in patients with Kallmann syndrome. *PLoS Genet* (2012) **8**:e1002896. doi:10.1371/journal.pgen.1002896
12. Ikeda K, Ookawara S, Sato S, Ando Z, Kageyama R, Kawakami K. Six1 is essential for early neurogenesis in the development of olfactory epithelium. *Dev Biol* (2007) **311**:53–68. doi:10.1016/j.ydbio.2007.08.020
13. Laub F, Dragomir C, Ramirez F. Mice without transcription factor KLF7 provide new insight into olfactory bulb development. *Brain Res* (2006) **1103**:108–13. doi:10.1016/j.brainres.2006.05.065
14. Long JE, Garel S, Depew MJ, Tobet S, Rubenstein JL. DLX5 regulates development of peripheral and central components of the olfactory system. *J Neurosci* (2003) **23**:568–78.
15. Matsumoto S, Yamazaki C, Masumoto KH, Nagano M, Naito M, Soga T, et al. Abnormal development of the olfactory bulb and reproductive system in mice lacking prokineticin receptor PKR2. *Proc Natl Acad Sci U S A* (2006) **103**:4140–5. doi:10.1073/pnas.0508881103
16. Merlo GR, Mantero S, Zaghetto AA, Peretto P, Paina S, Gozzo M. The role of Dlx homeogenes in early development of the olfactory pathway. *J Mol Histol* (2007) **38**:612–23.
17. Shimizu T, Hibi M. Formation and patterning of the forebrain and olfactory system by zinc-finger genes Fezf1 and Fezf2. *Dev Growth Differ* (2009) **51**:221–31. doi:10.1111/j.1440-169X.2009.01088.x
18. Yoshida M, Suda Y, Matsuo I, Miyamoto N, Takeda N, Kuratani S, et al. Emx1 and Emx2 functions in development of dorsal telencephalon. *Development* (1997) **124**:101–11.
19. Abel BS, Shaw ND, Brown JM, Adams JM, Alati T, Martin KA, et al. Responsiveness to a physiological regimen of GnRH therapy and relation to genotype in women with isolated hypogonadotropic hypogonadism. *J Clin Endocrinol Metab* (2013) **98**:E206–16. doi:10.1210/jc.2012-3294
20. Costa-Barbosa FA, Balasubramanian R, Keefe KW, Shaw ND, Al-Tassan N, Plummer L, et al. Prioritizing genetic testing in patients with Kallmann syndrome using clinical phenotypes. *J Clin Endocrinol Metab* (2013) **98**:E943–53. doi:10.1210/jc.2012-4116
21. Pitteloud N, Quinton R, Pearce S, Raivio T, Acierno J, Dwyer A, et al. Digenic mutations account for variable phenotypes in idiopathic hypogonadotropic hypogonadism. *J Clin Invest* (2007) **117**:457–63. doi:10.1172/JCI29884
22. Sykiotis GP, Plummer L, Hughes VA, Au M, Durrani S, Nayak-Young S, et al. Oligogenic basis of isolated gonadotropin-releasing hormone deficiency. *Proc Natl Acad Sci U S A* (2010) **107**:15140–4. doi:10.1073/pnas.1009622107
23. Astic L, Pellier-Monnin V, Godinot F. Spatio-temporal patterns of ensheathing cell differentiation in the rat olfactory system during development. *Neuroscience* (1998) **84**:295–307. doi:10.1016/S0306-4522(97)00496-X

24. Cariboni A, Maggi R, Parnavelas JG. From nose to fertility: the long migratory journey of gonadotropin-releasing hormone neurons. *Trends Neurosci* (2007) **30**:638–44. doi:10.1016/j.tins.2007.09.002
25. Forni PE, Taylor-Burds C, Melvin VS, Williams T, Wray S. Neural crest and ectodermal cells intermix in the nasal placode to give rise to GnRH-1 neurons, sensory neurons, and olfactory ensheathing cells. *J Neurosci* (2011) **31**:6915–27. doi:10.1523/JNEUROSCI.6087-10.2011
26. Tarozzo G, Peretto P, Fasolo A. Cell migration from the olfactory placode and the ontogeny of the neuroendocrine compartments. *Zoolog Sci* (1995) **12**:367–83. doi:10.2108/zsj.12.367
27. Whitlock KE, Illing N, Brideau NJ, Smith KM, Twomey S. Development of GnRH cells: setting the stage for puberty. *Mol Cell Endocrinol* (2006) **254–5**:39–50. doi:10.1016/j.mce.2006.04.038
28. Wray S, Grant P, Gainer H. Evidence that cells expressing luteinizing hormone-releasing hormone mRNA in the mouse are derived from progenitor cells in the olfactory placode. *Proc Natl Acad Sci U S A* (1989) **86**:8132–6. doi:10.1073/pnas.86.20.8132
29. Roa J. Role of GnRH neurons and their neuronal afferents as key integrators between food intake regulatory signals and the control of reproduction. *Int J Endocrinol* (2013) **2013**:518046.
30. Wierman ME, Kiseljak-Vassiliades K, Tobet S. Gonadotropin-releasing hormone (GnRH) neuron migration: initiation, maintenance and cessation as critical steps to ensure normal reproductive function. *Front Neuroendocrinol* (2011) **32**:43–52. doi:10.1016/j.yfrne.2010.07.005
31. Bailey MS, Puche AC, Shipley MT. Development of the olfactory bulb: evidence for glia-neuron interactions in glomerular formation. *J Comp Neurol* (1999) **415**:423–48. doi:10.1002/(SICI)1096-9861(19991227)415:4<423::AID-CNE2>3.3.CO;2-7
32. Bhasin N, Maynard TM, Gallagher PA, LaMantia AS. Mesenchymal/epithelial regulation of retinoic acid signaling in the olfactory placode. *Dev Biol* (2003) **261**:82–98. doi:10.1016/S0012-1606(03)00295-1
33. Cho JH, Prince JE, Cloutier JF. Axon guidance events in the wiring of the mammalian olfactory system. *Mol Neurobiol* (2009) **39**:1–9. doi:10.1007/s12035-008-8047-7
34. de Castro F. Wiring olfaction: the cellular and molecular mechanisms that guide the development of synaptic connections from the nose to the cortex. *Front Neurosci* (2009) **3**:52. doi:10.3389/neuro.22.004.2009
35. Franceschini I, Desroziers E, Caraty A, Duittoz A. The intimate relationship of gonadotropin-releasing hormone neurons with the polysialylated neural cell adhesion molecule revisited across development and adult plasticity. *Eur J Neurosci* (2010) **32**:2031–41. doi:10.1111/j.1460-9568.2010.07517.x
36. Julliard AK, Hartmann DJ. Spatiotemporal patterns of expression of extracellular matrix molecules in the developing and adult rat olfactory system. *Neuroscience* (1998) **84**:1135–50. doi:10.1016/S0306-4522(97)00544-7
37. LaMantia AS, Bhasin N, Rhodes K, Heemskerk J. Mesenchymal/epithelial induction mediates olfactory pathway formation. *Neuron* (2000) **28**:411–25. doi:10.1016/S0896-6273(00)00121-5
38. Nedelec S, Dubacq C, Trembleau A. Morphological and molecular features of the mammalian olfactory sensory neuron axons: what makes these axons so special? *J Neurocytol* (2005) **34**:49–64. doi:10.1007/s11068-005-5047-7
39. St John JA, Clarris HJ, Key B. Multiple axon guidance cues establish the olfactory topographic map: how do these cues interact? *Int J Dev Biol* (2002) **46**:639–47.
40. Tsim TY, Wong EY, Leung MS, Wong CC. Expression of axon guidance molecules and their related genes during development and sexual differentiation of the olfactory bulb in rats. *Neuroscience* (2004) **123**:951–65. doi:10.1016/j.neuroscience.2003.10.024
41. Chung WC, Moyle SS, Tsai PS. Fibroblast growth factor 8 signaling through fibroblast growth factor receptor 1 is required for the emergence of gonadotropin-releasing hormone neurons. *Endocrinology* (2008) **149**:4997–5003. doi:10.1210/en.2007-1634
42. Falardeau J, Chung WC, Beenken A, Raivio T, Plummer L, Sidis Y, et al. Decreased FGF8 signaling causes deficiency of gonadotropin-releasing hormone in humans and mice. *J Clin Invest* (2008) **118**:2822–31. doi:10.1172/JCI34538
43. Kim SH, Hu Y, Cadman S, Bouloux P. Diversity in fibroblast growth factor receptor 1 regulation: learning from the investigation of Kallmann syndrome. *J Neuroendocrinol* (2008) **20**:141–63. doi:10.1111/j.1365-2826.2007.01627.x
44. Zaghetto AA, Paina S, Mantero S, Platonova N, Peretto P, Bovetti S, et al. Activation of the Wnt-beta catenin pathway in a cell population on the surface of the forebrain is essential for the establishment of olfactory axon connections. *J Neurosci* (2007) **27**:9757–68. doi:10.1523/JNEUROSCI.0763-07.2007
45. Whitlock KE, Smith KM, Kim H, Harden MV. A role for foxd3 and sox10 in the differentiation of gonadotropin-releasing hormone (GnRH) cells in the zebrafish *Danio rerio*. *Development* (2005) **132**:5491–502. doi:10.1242/dev.02158
46. Yanicostas C, Herbomel E, Dipietromaria A, Soussi-Yanicostas N. Anosmin-1a is required for fasciculation and terminal targeting of olfactory sensory neuron axons in the zebrafish olfactory system. *Mol Cell Endocrinol* (2009) **312**:53–60. doi:10.1016/j.mce.2009.04.017
47. Levi G, Puche AC, Mantero S, Barbieri O, Trombino S, Paleari L, et al. The Dlx5 homeodomain gene is essential for olfactory development and connectivity in the mouse. *Mol Cell Neurosci* (2003) **22**:530–43. doi:10.1016/S1044-7431(02)00041-6
48. Hirata T, Nakazawa M, Yoshihara S, Miyachi H, Kitamura K, Yoshihara Y, et al. Zinc-finger gene Fez in the olfactory sensory neurons regulates development of the olfactory bulb non-cell-autonomously. *Development* (2006) **133**:1433–43. doi:10.1242/dev.02329
49. Corradi A, Croci L, Broccoli V, Zecchini S, Previtali S, Wurst W, et al. Hypogonadotropic hypogonadism and peripheral neuropathy in Ebf2-null mice. *Development* (2003) **130**:401–10. doi:10.1242/dev.00215
50. Acampora D, Merlo GR, Paleari L, Zerega B, Postiglione MP, Mantero S, et al. Craniofacial, vestibular and bone defects in mice lacking the Distal-less-related gene Dlx5. *Development* (1999) **126**:3795–809.
51. Sanges R, Cordero F, Calogero RA. oneChannelGUI: a graphical interface to bioconductor tools, designed for life scientists who are not familiar with R language. *Bioinformatics* (2007) **23**:3406–8. doi:10.1093/bioinformatics/btm469
52. Gentleman RC, Carey VJ, Bates DM, Bolstad B, Dettling M, Dudoit S, et al. Bioconductor: open software development for computational biology and bioinformatics. *Genome Biol* (2004) **5**:R80. doi:10.1186/gb-2004-5-10-r80
53. Breitling R, Armengaud P, Amtmann A, Herzyk P. Rank products: a simple, yet powerful, new method to detect differentially regulated genes in replicated microarray experiments. *FEBS Lett* (2004) **573**:83–92. doi:10.1016/j.febslet.2004.07.055
54. Bindea G, Mlecnik B, Hackl H, Charoentong P, Tosolini M, Kirilovsky A, et al. ClueGO: a cytoscape plug-in to decipher functionally grouped gene ontology and pathway annotation networks. *Bioinformatics* (2009) **25**:1091–3. doi:10.1093/bioinformatics/btp101
55. Piro RM, Ala U, Molineris I, Grassi E, Bracco C, Perego GP, et al. An atlas of tissue-specific conserved coexpression for functional annotation and disease gene prediction. *Eur J Hum Genet* (2011) **19**:1173–80. doi:10.1038/ejhg.2011.96
56. Portales-Casamar E, Thongjuea S, Kwon AT, Arenillas D, Zhao X, Valen E, et al. JASPAR 2010: the greatly expanded open-access database of transcription factor binding profiles. *Nucleic Acids Res* (2010) **38**:D105–10. doi:10.1093/nar/gkp950
57. Vieux-Rochas M, Bouhali K, Mantero S, Garaffo G, Provero P, Astigiano S, et al. BMP-mediated functional cooperation between Dlx5;Dlx6 and Msx1;Msx2 during mammalian limb development. *PLoS One* (2013) **8**:e51700. doi:10.1371/journal.pone.0051700
58. Ala U, Piro RM, Grassi E, Damasco C, Silengo L, Oti M, et al. Prediction of human disease genes by human-mouse conserved coexpression analysis. *PLoS Comput Biol* (2008) **4**:e1000043. doi:10.1371/journal.pcbi.1000043
59. Miyasaka N, Sato Y, Yeo SY, Hutson LD, Chien CB, Okamoto H, et al. Robo2 is required for establishment of a precise glomerular map in the zebrafish olfactory system. *Development* (2005) **132**:1283–93. doi:10.1242/dev.01698
60. Sato Y, Miyasaka N, Yoshihara Y. Mutually exclusive glomerular innervation by two distinct types of olfactory sensory neurons revealed in transgenic zebrafish. *J Neurosci* (2005) **25**:4889–97. doi:10.1523/JNEUROSCI.0679-05.2005
61. Yoshida T, Ito A, Matsuda N, Mishina M. Regulation by protein kinase A switching of axonal pathfinding of zebrafish olfactory sensory neurons through the olfactory placode-olfactory bulb boundary. *J Neurosci* (2002) **22**:4964–72.

62. Abraham E, Palevitch O, Gothilf Y, Zohar Y. The zebrafish as a model system for forebrain GnRH neuronal development. *Gen Comp Endocrinol* (2009) **164**:151–60. doi:10.1016/j.ygcen.2009.01.012
63. Abraham E, Palevitch O, Gothilf Y, Zohar Y. Targeted gonadotropin-releasing hormone-3 neuron ablation in zebrafish: effects on neurogenesis, neuronal migration, and reproduction. *Endocrinology* (2010) **151**:332–40. doi:10.1210/en.2009-0548
64. Abraham E, Palevitch O, Ijiri S, Du SJ, Gothilf Y, Zohar Y. Early development of forebrain gonadotrophin-releasing hormone (GnRH) neurones and the role of GnRH as an autocrine migration factor. *J Neuroendocrinol* (2008) **20**:394–405. doi:10.1111/j.1365-2826.2008.01654.x
65. Flynt AS, Li N, Thatcher EJ, Solnica-Krezel L, Patton JG. Zebrafish miR-214 modulates Hedgehog signaling to specify muscle cell fate. *Nat Genet* (2007) **39**:259–63. doi:10.1038/ng1953
66. Kloosterman WP, Plasterk RH. The diverse functions of microRNAs in animal development and disease. *Dev Cell* (2006) **11**:441–50. doi:10.1016/j.devcel.2006.09.009
67. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene ontology: tool for the unification of biology. The gene ontology consortium. *Nat Genet* (2000) **25**:25–9. doi:10.1038/75556
68. Jeong J, Li X, McEvilly RJ, Rosenfeld MG, Lufkin T, Rubenstein JL. Dlx genes pattern mammalian jaw primordium by regulating both lower jaw-specific and upper jaw-specific genetic programs. *Development* (2008) **135**:2905–16. doi:10.1242/dev.019778
69. Sajan SA, Rubenstein JL, Warchol ME, Lovett M. Identification of direct downstream targets of Dlx5 during early inner ear development. *Hum Mol Genet* (2011) **20**:1262–73. doi:10.1093/hmg/ddq567
70. Kajimura D, Dragomir C, Ramirez F, Laub F. Identification of genes regulated by transcription factor KLF7 in differentiating olfactory sensory neurons. *Gene* (2007) **388**:34–42. doi:10.1016/j.gene.2006.09.027
71. McIntyre JC, Bose SC, Stromberg AJ, McClintock TS. Emx2 stimulates odorant receptor gene expression. *Chem Senses* (2008) **33**:825–37. doi:10.1093/chemse/bjn061
72. Paina S, Garzotto D, DeMarchis S, Marino M, Moiana A, Conti L, et al. Wnt5a is a transcriptional target of Dlx homeogenes and promotes differentiation of interneuron progenitors *in vitro* and *in vivo*. *J Neurosci* (2011) **31**:2675–87. doi:10.1523/JNEUROSCI.3110-10.2011
73. Panganiban G, Rubenstein JL. Developmental functions of the Distal-less/Dlx homeobox genes. *Development* (2002) **129**:4371–86.
74. Mi S, Lee X, Shao Z, Thill G, Ji B, Relton J, et al. LINGO-1 is a component of the Nogo-66 receptor/p75 signaling complex. *Nat Neurosci* (2004) **7**:221–8. doi:10.1038/nn1188
75. Kunapuli P, Lo K, Hawthorn L, Cowell JK. Reexpression of LGI1 in glioma cells results in dysregulation of genes implicated in the canonical axon guidance pathway. *Genomics* (2010) **95**:93–100. doi:10.1016/j.ygeno.2009.10.001
76. Thomas R, Favell K, Morante-Redolat J, Pool M, Kent C, Wright M, et al. LGI1 is a Nogo receptor 1 ligand that antagonizes myelin-based growth inhibition. *J Neurosci* (2010) **30**:6607–12. doi:10.1523/JNEUROSCI.5147-09.2010
77. Jungnickel J, Bramer C, Bronzlik P, Lipokatic-Takacs E, Weinhold B, Gerardy-Schahn R, et al. Level and localization of polysialic acid is critical for early peripheral nerve regeneration. *Mol Cell Neurosci* (2009) **40**:374–81. doi:10.1016/j.mcn.2008.12.003
78. Nacher J, Guirado R, Varea E, Alonso-Llosa G, Rockle I, Hildebrandt H. Divergent impact of the polysialyltransferases ST8SiaII and ST8SiaIV on polysialic acid expression in immature neurons and interneurons of the adult cerebral cortex. *Neuroscience* (2010) **167**:825–37. doi:10.1016/j.neuroscience.2010.02.067
79. Rieger S, Volkmann K, Koster RW. Polysialyltransferase expression is linked to neuronal migration in the developing and adult zebrafish. *Dev Dyn* (2008) **237**:276–85. doi:10.1002/dvdy.21410
80. Worley PF, Zeng W, Huang G, Kim JY, Shin DM, Kim MS, et al. Homer proteins in Ca2+ signaling by excitable and non-excitable cells. *Cell Calcium* (2007) **42**:363–71. doi:10.1016/j.ceca.2007.05.007
81. Shin DM, Dehoff M, Luo X, Kang SH, Tu J, Nayak SK, et al. Homer 2 tunes G protein-coupled receptors stimulus intensity by regulating RGS proteins and PLCbeta GAP activities. *J Cell Biol* (2003) **162**:293–303. doi:10.1083/jcb.200210109
82. Miyasaka N, Sato Y, Yoshihara Y. Axon guidance of olfactory sensory neurons in zebrafish. *Chem Senses* (2005) **30**(Suppl 1):i92–3. doi:10.1093/chemse/bjh129
83. Niimura Y, Nei M. Evolutionary dynamics of olfactory and other chemosensory receptor genes in vertebrates. *J Hum Genet* (2006) **51**:505–17. doi:10.1007/s10038-006-0391-8
84. Hansen A, Anderson KT, Finger TE. Differential distribution of olfactory receptor neurons in goldfish: structural and molecular correlates. *J Comp Neurol* (2004) **477**:347–59. doi:10.1002/cne.20202
85. Hansen A, Rolen SH, Anderson K, Morita Y, Caprio J, Finger TE. Correlation between olfactory receptor cell type and function in the channel catfish. *J Neurosci* (2003) **23**:9328–39.
86. Quint E, Zerucha T, Ekker M. Differential expression of orthologous Dlx genes in zebrafish and mice: implications for the evolution of the Dlx homeobox gene family. *J Exp Zool* (2000) **288**:235–41. doi:10.1002/1097-010X(20001015)288:3<235::AID-JEZ4>3.0.CO;2-J
87. Ellies DL, Langille RM, Martin CC, Akimenko MA, Ekker M. Specific craniofacial cartilage dysmorphogenesis coincides with a loss of dlx gene expression in retinoic acid-treated zebrafish embryos. *Mech Dev* (1997) **61**:23–36. doi:10.1016/S0925-4773(96)00616-8
88. MacDonald RB, Debiais-Thibaud M, Talbot JC, Ekker M. The relationship between dlx and gad1 expression indicates highly conserved genetic pathways in the zebrafish forebrain. *Dev Dyn* (2010) **239**:2298–306. doi:10.1002/dvdy.22365
89. Wang X, Huang L, Li Y, Li X, Li P, Ray J, et al. Characterization of GFP-tagged GnRH-containing terminalis neurons in transgenic zebrafish. *J Cell Physiol* (2010) **226**:608–15. doi:10.1002/jcp.22369
90. Piro RM, Di Cunto F. Computational approaches to disease-gene prediction: rationale, classification and successes. *FEBS J* (2012) **279**:678–96. doi:10.1111/j.1742-4658.2012.08471.x
91. Lee CH, Javed D, Althaus AL, Parent JM, Umemori H. Neurogenesis is enhanced and mossy fiber sprouting arises in FGF7-deficient mice during development. *Mol Cell Neurosci* (2012) **51**:61–7. doi:10.1016/j.mcn.2012.07.010
92. Umemori H, Linhoff MW, Ornitz DM, Sanes JR. FGF22 and its close relatives are presynaptic organizing molecules in the mammalian brain. *Cell* (2004) **118**:257–70. doi:10.1016/j.cell.2004.06.025
93. Yoneda A, Wang Y, O'Briain DS, Puri P. Cell-adhesion molecules and fibroblast growth factor signalling in Hirschsprung's disease. *Pediatr Surg Int* (2001) **17**:299–303. doi:10.1007/s003830100598
94. Masoudi-Nejad A, Meshkin A, Haji-Eghrari B, Bidkhori G. Candidate gene prioritization. *Mol Genet Genomics* (2012) **287**:679–98. doi:10.1007/s00438-012-0710-z
95. Bromberg Y. Building a genome analysis pipeline to predict disease risk and prevent disease. *J Mol Biol* (2013) **425**:3993–4005. doi:10.1016/j.jmb.2013.07.038
96. Matullo G, Di Gaetano C, Guarrera S. Next generation sequencing and rare genetic variants: from human population studies to medical genetics. *Environ Mol Mutagen* (2013) **54**:518–32. doi:10.1002/em.21799
97. Wang Z, Liu X, Yang BZ, Gelernter J. The role and challenges of exome sequencing in studies of human diseases. *Front Genet* (2013) **4**:160.
98. Lohr H, Hammerschmidt M. Zebrafish in endocrine systems: recent advances and implications for human disease. *Annu Rev Physiol* (2011) **73**:183–211. doi:10.1146/annurev-physiol-012110-142320
99. Haines BP, Rigby PW. Expression of the Lingo/LERN gene family during mouse embryogenesis. *Gene Expr Patterns* (2008) **8**:79–86. doi:10.1016/j.modgep.2007.10.003
100. Buchser WJ, Slepak TI, Gutierrez-Arenas O, Bixby JL, Lemmon VP. Kinase/phosphatase overexpression reveals pathways regulating hippocampal neuron morphology. *Mol Syst Biol* (2010) **6**:391. doi:10.1038/msb.2010.52
101. Tossell K, Andreae LC, Cudmore C, Lang E, Muthukrishnan U, Lumsden A, et al. Lrrn1 is required for formation of the midbrain-hindbrain boundary and organiser through regulation of affinity differences between midbrain and hindbrain cells in chick. *Dev Biol* (2011) **352**:341–52. doi:10.1016/j.ydbio.2011.02.002
102. Andreae LC, Peukert D, Lumsden A, Gilthorpe JD. Analysis of Lrrn1 expression and its relationship to neuromeric boundaries during chick neural development. *Neural Dev* (2007) **2**:22. doi:10.1186/1749-8104-2-22

103. Homma S, Shimada T, Hikake T, Yaginuma H. Expression pattern of LRR and Ig domain-containing protein (LRRIG protein) in the early mouse embryo. *Gene Expr Patterns* (2009) **9**:1–26. doi:10.1016/j.gep.2008.09.004
104. Vilarino-Guell C, Wider C, Ross OA, Jasinska-Myga B, Kachergus J, Cobb SA, et al. LINGO1 and LINGO2 variants are associated with essential tremor and Parkinson disease. *Neurogenetics* (2010) **11**:401–8. doi:10.1007/s10048-010-0241-x
105. Dodge-Kafka KL, Bauman A, Kapiloff MS. A-kinase anchoring proteins as the basis for cAMP signaling. *Handb Exp Pharmacol* (2008) **186**:3–14. doi:10.1007/978-3-540-72843-6_1
106. Wong W, Scott JD. AKAP signalling complexes: focal points in space and time. *Nat Rev Mol Cell Biol* (2004) **5**:959–70. doi:10.1038/nrm1527
107. Allam A, Niiro H, Clark EA, Marshall AJ. The adaptor protein Bam32 regulates Rac1 activation and actin remodeling through a phosphorylation-dependent mechanism. *J Biol Chem* (2004) **279**:39775–82. doi:10.1074/jbc.M403367200
108. Sommers CL, Gurson JM, Surana R, Barda-Saad M, Lee J, Kishor A, et al. Bam32: a novel mediator of Erk activation in T cells. *Int Immunol* (2008) **20**:811–8. doi:10.1093/intimm/dxn039
109. Zhang TT, Li H, Cheung SM, Costantini JL, Hou S, Al-Alwan M, et al. Phosphoinositide 3-kinase-regulated adapters in lymphocyte activation. *Immunol Rev* (2009) **232**:255–72. doi:10.1111/j.1600-065X.2009.00838.x
110. Wang L, Vervoort V, Wallez Y, Core N, Cremer H, Pasquale EB. The SRC homology 2 domain protein Shep1 plays an important role in the penetration of olfactory sensory axons into the forebrain. *J Neurosci* (2010) **30**:13201–10. doi:10.1523/JNEUROSCI.3289-10.2010
111. Roselli S, Wallez Y, Wang L, Vervoort V, Pasquale EB. The SH2 domain protein Shep1 regulates the *in vivo* signaling function of the scaffolding protein Cas. *Cell Signal* (2010) **22**:1745–52. doi:10.1016/j.cellsig.2010.06.015
112. Huang J, Sakai R, Furuichi T. The docking protein Cas links tyrosine phosphorylation signaling to elongation of cerebellar granule cell axons. *Mol Biol Cell* (2006) **17**:3187–96. doi:10.1091/mbc.E05-12-1122
113. Liu G, Li W, Gao X, Li X, Jurgensen C, Park HT, et al. p130CAS is required for netrin signaling and commissural axon guidance. *J Neurosci* (2007) **27**:957–68. doi:10.1523/JNEUROSCI.4616-06.2007
114. Yang LT, Alexandropoulos K, Sap J. c-SRC mediates neurite outgrowth through recruitment of Crk to the scaffolding protein Sin/Efs without altering the kinetics of ERK activation. *J Biol Chem* (2002) **277**:17406–14. doi:10.1074/jbc.M111902200
115. Cabodi S, del Pilar Camacho-Leal M, Di Stefano P, Defilippi P. Integrin signalling adaptors: not only figurants in the cancer story. *Nat Rev Cancer* (2010) **10**:858–70. doi:10.1038/nrc2967
116. Defilippi P, Di Stefano P, Cabodi S. p130Cas: a versatile scaffold in signaling networks. *Trends Cell Biol* (2006) **16**:257–63. doi:10.1016/j.tcb.2006.03.003
117. Tikhmyanova N, Little JL, Golemis EA. CAS proteins in normal and pathological cell growth control. *Cell Mol Life Sci* (2010) **67**:1025–48. doi:10.1007/s00018-009-0213-1
118. Honda H, Oda H, Nakamoto T, Honda Z, Sakai R, Suzuki T, et al. Cardiovascular anomaly, impaired actin bundling and resistance to Src-induced transformation in mice lacking p130Cas. *Nat Genet* (1998) **19**:361–5. doi:10.1038/1246
119. Knoll B, Zarbalis K, Wurst W, Drescher U. A role for the EphA family in the topographic targeting of vomeronasal axons. *Development* (2001) **128**:895–906.
120. Khazaei MR, Bunk EC, Hillje AL, Jahn HM, Riegler EM, Knoblich JA, et al. The E3-ubiquitin ligase TRIM2 regulates neuronal polarization. *J Neurochem* (2011) **117**:29–37. doi:10.1111/j.1471-4159.2010.06971.x
121. Gil-Sanz C, Franco SJ, Martinez-Garay I, Espinosa A, Harkins-Perry S, Muller U. Cajal-Retzius cells instruct neuronal migration by coincidence signaling between secreted and contact-dependent guidance cues. *Neuron* (2012) **79**:461–77. doi:10.1016/j.neuron.2013.06.040
122. Stockinger P, Maitre JL, Heisenberg CP. Defective neuroepithelial cell cohesion affects tangential branchiomotor neuron migration in the zebrafish neural tube. *Development* (2011) **138**:4673–83. doi:10.1242/dev.071233
123. Wanner SJ, Prince VE. Axon tracts guide zebrafish facial branchiomotor neuron migration through the hindbrain. *Development* (2013) **140**:906–15. doi:10.1242/dev.087148
124. LaMora A, Voigt MM. Cranial sensory ganglia neurons require intrinsic N-cadherin function for guidance of afferent fibers to their final targets. *Neuroscience* (2009) **159**:1175–84. doi:10.1016/j.neuroscience.2009.01.049
125. Bruses JL. N-cadherin regulates primary motor axon growth and branching during zebrafish embryonic development. *J Comp Neurol* (2011) **519**:1797–815. doi:10.1002/cne.22602
126. Akins MR, Benson DL, Greer CA. Cadherin expression in the developing mouse olfactory system. *J Comp Neurol* (2007) **501**:483–97. doi:10.1002/cne.21270
127. Tucker ES, Segall S, Gopalakrishna D, Wu Y, Vernon M, Polleux F, et al. Molecular specification and patterning of progenitor cells in the lateral and medial ganglionic eminences. *J Neurosci* (2008) **28**:9504–18. doi:10.1523/JNEUROSCI.2341-08.2008
128. Hamel MG, Ajmo JM, Leonardo CC, Zuo F, Sandy JD, Gottschall PE. Multimodal signaling by the ADAMTSs (a disintegrin and metalloproteinase with thrombospondin motifs) promotes neurite extension. *Exp Neurol* (2008) **210**:428–40. doi:10.1016/j.expneurol.2007.11.014
129. Gunaje JJ, Bahrami AJ, Schwartz SM, Daum G, Mahoney WM Jr. PDGF-dependent regulation of regulator of G protein signaling-5 expression and vascular smooth muscle cell functionality. *Am J Physiol Cell Physiol* (2011) **301**:C478–89. doi:10.1152/ajpcell.00348.2010
130. Hu M, Chen X, Zhang J, Wang D, Fang X, Wang X, et al. Over-expression of regulator of G protein signaling 5 promotes tumor metastasis by inducing epithelial-mesenchymal transition in hepatocellular carcinoma cells. *J Surg Oncol* (2013) **108**:192–6. doi:10.1002/jso.23367
131. Nisancioglu MH, Mahoney WM Jr, Kimmel DD, Schwartz SM, Betsholtz C, Genove G. Generation and characterization of rgs5 mutant mice. *Mol Cell Biol* (2008) **28**:2324–31. doi:10.1128/MCB.01252-07
132. Ishizaka A, Mizutani T, Kobayashi K, Tando T, Sakurai K, Fujiwara T, et al. Double plant homeodomain (PHD) finger proteins DPF-3a and -3b are required as transcriptional co-activators in SWI/SNF complex-dependent activation of NF-kappaB RelA/p50 heterodimer. *J Biol Chem* (2012) **287**:11924–33. doi:10.1074/jbc.M111.322792
133. Lange M, Kaynak B, Forster UB, Tonjes M, Fischer JJ, Grimm C, et al. Regulation of muscle development by DPF3, a novel histone acetylation and methylation reader of the BAF chromatin remodeling complex. *Genes Dev* (2008) **22**:2370–84. doi:10.1101/gad.471408
134. Zeng L, Zhang Q, Li S, Plotnikov AN, Walsh MJ, Zhou MM. Mechanism and regulation of acetylated histone binding by the tandem PHD finger of DPF3b. *Nature* (2010) **466**:258–62. doi:10.1038/nature09139

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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

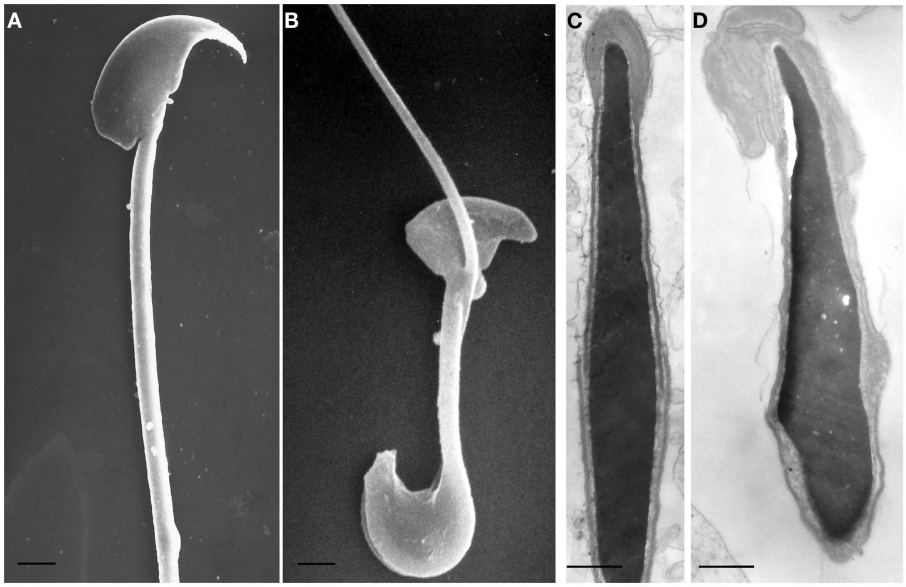

FIGURE 1 | Scanning (A,B) and transmission (C,D) electron microscopy micrographs of spermatozoa from wild type (A,C) and Twitcher (B,D) mouse, collected from vas deferens. In **(A)**, head and tail of control mouse sperm have a normal morphology: the crescent-like shape of the head and the acrosomal 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

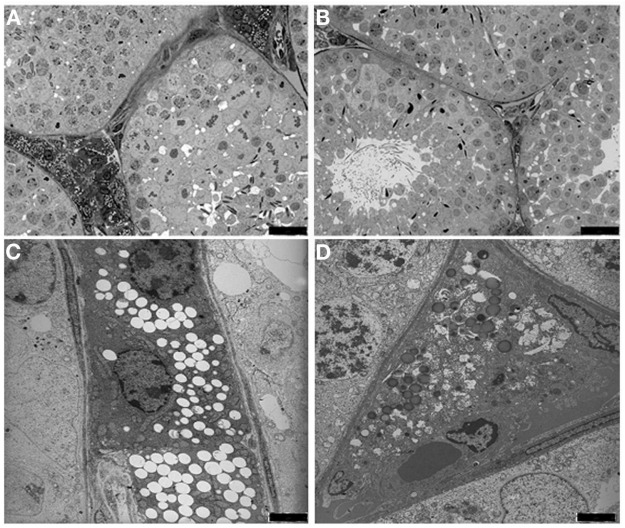

FIGURE 2 | Light microscopy (A,B) and transmission electron micrograph (C,D) of the interstitium from wild type (A,C) and Twitcher (B,D) testes. In the peritubular interstitium of wild type mice numerous Leydig cells are presents and filled with lipids droplets and lysosomes, whereas in Twitcher mice 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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REFERENCES

1. Rouchou B. Consequences of infertility in developing countries. *Perspect Public Health* (2013) **133**(3):174–9. doi:10.1177/1757913912472415
2. Schultz N, Hamra FK, Garbers DL. A multitude of genes expressed solely in meiotic or postmeiotic spermatogenic cells offers a myriad of contraceptive targets. *Proc Natl Acad Sci U S A* (2003) **100**(21):12201–6. doi:10.1073/pnas.1635054100
3. Ashrafzadeh A, Karsani SA, Nathan S. Mammalian sperm fertility related proteins. *Int J Med Sci* (2003) **10**(12):1649–57. doi:10.7150/ijms.6395
4. Jamsai D, O'Bryan MK. Mouse models in male fertility research. *Asian J Androl* (2011) **13**(1):139–51. doi:10.1038/aja.2010.101
5. Michaelis M, Langhammer M, Hoeflich A, Reinsch N, Schoen J, Weitzel JM. Initial characterization of an outbreed mouse model for male factor (in)fertility. *Andrology* (2013) **11**:772–8. doi:10.1111/j.2047-2927.2013.00108.x
6. Ruwanpura SM, McLachlan RI, Meachem SJ. Hormonal regulation of male germ cell development. *J Endocrinol* (2010) **205**(2):117–31. doi:10.1677/JOE-10-0025
7. Cheng CY, Wong EW, Yan HH, Mruk DD. Regulation of spermatogenesis in the microenvironment of the seminiferous epithelium: new insights and advances. *Mol Cell Endocrinol* (2010) **315**(1-2):49–56. doi:10.1016/j.mce.2009.08.004
8. Plant TM. Gonadal regulation of hypothalamic gonadotropin-releasing hormone release in primates. *Endocr Rev* (1986) **7**(1):75–88. doi:10.1210/edrv-7-1-75
9. Mendis-Handagama SM. Luteinizing hormone on Leydig cell structure and function. *Histol Histopathol* (1997) **12**(3):869–82.
10. Griswold M, McLean D. The Sertoli cell. In: Neill J, editor. *Knobil and Neill's Physiology of Reproduction.* (Vol. 1), San Diego: Elsevier (2006). p. 949–75.

11. Petersen C, Solder O. The Sertoli cell: a hormonal target and 'super' nurse for germ cells that determines testicular size. *Horm Res* (2006) **66**:153–61. doi:10.1159/000094142
12. Simoni M, Weinbauer GF, Gromoll J, Nieschlag E. Role of FSH in male gonadal function. *Ann Endocrinol* (1999) **60**(2):102–6.
13. Nitta H, Bunick D, Hess RA, Janulis L, Newton SC, Millette CF, et al. Germ cells of the mouse testis express P450 aromatase. *Endocrinology* (1993) **132**(3):1396–401. doi:10.1210/en.132.3.1396
14. de Kretser DM, Phillips DJ. Mechanisms of protein feedback on gonadotropin secretion. *J Reprod Immunol* (1998) **39**:1–12. doi:10.1016/S0165-0378(98)00025-4
15. Veeramachaneni DN, Smith MO, Ellinwood NM. Deficiency of fucosidase results in acrosomal dysgenesis and impaired sperm maturation. *J Androl* (1998) **19**:444–9.
16. Fan J, Akabane H, Graham SN, Richardson LL, Zhu GZ. Sperm defects in mice lacking a functional Niemann-Pick C1 protein. *Mol Reprod Dev* (2006) **73**:1284–91. doi:10.1002/mrd.20559
17. Butler A, He X, Gordon RE, Wu HS, Gatt S, Schuchman EH. Reproductive pathology and sperm physiology in acid sphingomyelinase-deficient mice. *Am J Pathol* (2002) **161**:1061–75. doi:10.1016/S0002-9440(10)64267-8
18. Trasler J, Saberi F, Somani IH, Adamali HI, Huang JQ, Fortunato SR, et al. Characterization of the testis and epididymis in mouse models of human Tay Sachs and Sandhoff diseases and partial determination of accumulated gangliosides. *Endocrinology* (1998) **139**:3280–8. doi:10.1210/en.139.7.3280
19. Xu H, Kongmanas K, Kadunganattil S, Smith CE, Rupar T, Goto-Inoue N, et al. Arylsulfatase A deficiency causes seminolipid accumulation and a lysosomal storage disorder in Sertoli cells. *J Lipid Res* (2011) **52**:2187–97. doi:10.1194/jlr.M019661
20. Kobayashi T, Yamanaka T, Jacobs JM, Teixera F, Suzuki K. The twitcher mouse: an enzymatically authentic model of human globoid cell leukodystrophy (Krabbe disease). *Brain Res* (1980) **202**:479–83. doi:10.1016/0006-8993(80)90159-6
21. Wenger DA, Suzuki K, Suzuki Y, Suzuki K. Galactosylceramide lipidosis: globoid cell leukodystrophy (Krabbe disease). In: Scriver CR, Beaudet AL, Sly WS, Valle D, editors. *Metabolic and Molecular Basis of Inherited Disease.* New York: McGraw-Hill (2001). p. 3669–94.
22. Ishizuka I. Chemistry and functional distribution of sulfoglycolipids. *Prog Lipid Res* (1997) **36**:245–319. doi:10.1016/S0163-7827(97)00011-8
23. Luddi A, Strazza M, Carbone M, Moretti E, Costantino-Ceccarini E. Galactosylceramidase deficiency causes sperm abnormalities in the mouse model of globoid cell leukodystrophy. *Exp Cell Res* (2005) **304**(1):59–68. doi:10.1016/j.yexcr.2004.10.034
24. Chigurupati S, Son TG, Hyun DH, Lathia JD, Mughal MR, Savell J, et al. Lifelong running reduces oxidative stress and degenerative changes in the testes of mice. *J Endocrinol* (2008) **199**(2):333–41. doi:10.1677/JOE-08-0306
25. Li WR, Chen L, Chang ZJ, Xin H, Liu T, Zhang JQ, et al. Autophagic deficiency is related to steroidogenic decline in aged rat Leydig cells. *Asian J Androl* (2011) **13**:881–8. doi:10.1038/aja.2011.85
26. Kouame KE, Nishida Y, Cadrin-Girard JF, Yoshioka M, St-Amand J. Housekeeping and tissue-specific genes in mouse tissues. *BMC Genomics* (2007) **8**:127. doi:10.1186/1471-2164-8-127
27. Nieschlag E, Behre HM, editors. Andrology. In: *Male Reproductive Health and Dysfunction.* 2nd ed. Berlin: Springer-Verlag (2001).

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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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REFERENCES

1. Patton DF, Mistlbergero RE. Circadian adaptations to meal timing: neuroendocrine mechanisms. *Front Neurosci* (2013) **7**:185. doi:10.3389/fnins.2013.00185
2. Maruska KP, Femald RD. Social regulation of gene expression in the hypothalamic-pituitary–gonadal axis. *Physiology (Bethesda)* (2011) **26**:412–23. doi:10.1152/physiol.00032.2011
3. Lewy AJ, Sack RL, Latham JM. Melatonin and the acute suppressant effect of light may help to regulate circadian rhythms in humans. In: Arendt J, Pevet P, editors. *Advances in Pineal Research*. London: Libbey (1991). p. 285–93.
4. Czeisler CA. The effect of light on the human circadian pace-maker. *Ciba Found Symp* (1995) **183**:254–90.
5. Bellastella A, Pisano G, Iorio S, Pasquali D, Orio F, Venditto T, et al. Endocrine secretions under abnormal light/dark cycles and in the blind. *Horm Res* (1998) **49**:153–7. doi:10.1159/000023163
6. Welsh DK, Takahashi JS, Kay SA. Suprachiasmatic nucleus: cell autonomy and network properties. *Annu Rev Physiol* (2010) **72**:551–79. doi:10.1146/annurev-physiol-021909-135919
7. Dunlap JC. Molecular bases for circadian clocks. *Cell* (1999) **96**:271–90. doi:10.1016/S0092-8674(00)80566-8
8. Schibler U, Sassone-Corsi P. A web of circadian pace-makers. *Cell* (2002) **111**:919–22. doi:10.1016/S0092-8674(02)01225-4
9. Doi M, Hirayama J, Sassone-Corsi P. Circadian regulator CLOCK is a histone acetyltransferase. *Cell* (2006) **125**:497–508. doi:10.1016/j.cell.2006.03.033
10. Hyrayama J, Sahar S, Grimaldi B, Tamaru T, Takamatsu K, Nakahata Y, et al. CLOCK mediated acetylation of BMAL1 controls circadian function. *Nature* (2007) **450**:1086–90. doi:10.1038/nature06394
11. Yan J, Wang H, Liu Y, Shao C. Analysis of gene regulatory networks in the mammalian circadian rhythm. *PLoS Comput Biol* (2008) **4**:e1000193. doi:10.1371/journal.pcbi.1000193
12. Dibner C, Schibler U, Albrecht U. The mammalian circadian timing system: organization and coordination of central and peripheral clocks. *Annu Rev Physiol* (2010) **72**:517–49. doi:10.1146/annurev-physiol-021909-135821
13. Sassone-Corsi P. Commentary: the year in circadian rhythms. *Mol Endocrinol* (2010) **24**:2081–7. doi:10.1210/me.2010-0359
14. Kennaway DJ, Boden MJ, Varcos TJ. Circadian rhythms and fertility. *Mol Cell Endocrinol* (2012) **349**:56–61. doi:10.1016/j.mce.2011.08.013
15. Wongchitrat P, Felder-Schmitbuhi MP, Govitrapong P, Phansuvan-Pujito P, Simonneaux V. A noradrenergic sensitive endogenous clock is present in the rat pineal gland. *Neuroendocrinology* (2011) **94**:75–83. doi:10.1159/000327430
16. Alvarez JD, Hansen A, Ord T, Bebas P, Chappell PE, Gielbultowicz JM, et al. The circadian clock protein BMAL1 is necessary for fertility and proper testosterone production in mice. *J Biol Rhythms* (2008) **23**:26–36. doi:10.1177/0748730407311254
17. Kovanen L, Saarkoski ST, Aromaa A, Lonnqvist J, Partonen T. ARNTL (BMAL1) and NPAS2 gene variants contribute to fertility and seasonality. *PLoS One* (2010) **5**:e10007. doi:10.1371/journal.pone.0010007
18. Bellastella G, Pane E, Iorio S, De Bellis A, Sinisi AA. Seasonal variations of plasma gonadotropin, prolactin, and testosterone levels in primary and secondary hypogonadism: evidence for an independent testicular role. *J Endocrinol Invest* (2013) **36**(5):339–42. doi:10.3275/8620
19. Sellix MT. Clocks underneath: the role of peripheral clocks in the timing of female reproductive physiology. *Front Endocrinol (Lausanne)* (2013) **4**:91. doi:10.3389/fendo.2013.00091
20. Kornhauser JM, Nelson DE, Mayo KE, Takahashi JS. Regulation of Jun-B messenger RNA and AP-1 activity by light and a circadian clock. *Science* (1992) **255**:1581–4. doi:10.1126/science.1549784
21. Kornhauser JM, Mayo KE, Takahashi JS. Light, immediate-early genes and circadian rhythms. *Behav Genet* (1996) **26**:221–40. doi:10.1007/BF02359382
22. Morris ME, Viswanathan N, Kuhlman S, Davis FC, Weitz CJ. A screen for genes induced in the suprachiasmatic nucleus by light. *Science* (1998) **279**:1544–7. doi:10.1126/science.279.5356.1544
23. Albrecht U, Sun ZS, Eichele G, Lee CC. A differential response of two putative mammalian circadian regulators, mper 1 and mper 2, to light. *Cell* (1997) **91**:1055–64. doi:10.1016/S0092-8674(00)80495-X
24. Ohki K, Yoshida K, Harada T, Takamura M, Matsuda H, Imaki J. C-fos gene expression in postnatal rat retinas with light/dark cycle. *Vision Res* (1996) **36**:1883–6. doi:10.1016/0042-6989(95)00284-7
25. Crosthwaite SK, Dunlap JC, Loros JJ. Neurospora wc-1 and wc-2 transcription, photoresponses and the origins of circadian rhythmicity. *Science* (1997) **276**:763–9. doi:10.1126/science.276.5313.763
26. Rastogi A, Kumary Y, Rami S, Kumar V. Neural correlates of migration: activation of hypothalamic clock(s) in and out of migratory state in the black-headed bunting (*Emberiza melanocephala*). *PLoS One* (2013) **8**(10):e70065. doi:10.1371/journal.pone.0070065
27. Reiter RJ. The pineal gland: an intermediary between the environment and the endocrine system. *Psychoneuroendocrinology* (1983) **8**:31–40. doi:10.1016/0306-4530(83)90039-2
28. Reiter RJ. Melatonin and human reproduction. *Ann Med* (1988) **30**:103–8. doi:10.3109/07853899808999391
29. Brzezinski A. Melatonin in human. *N Engl J Med* (1997) **16**:186–95.

30. McIntyre IM, Noman TR, Burrows GD, Armstrong SM. Melatonin rhythm in human plasma and saliva. *J Pineal Res* (1987) **4**:177–82. doi:10.1111/j.1600-079X.1987.tb00854.x
31. Tosini G, Menaker M. Circadian rhythm in cultured mammalian retina. *Science* (1996) **272**:419–21. doi:10.1126/science.272.5260.419
32. Weaver DR, Stehle JH, Stopa EG, Reppert SM. Melatonin receptors in human hypothalamus and pituitary: implications for circadian and reproductive responses to melatonin. *J Clin Endocrinol Metab* (1993) **76**:295–301. doi:10.1210/jc.76.2.295
33. Reppert SM. Melatonin receptors: molecular biology of a new family of G protein-coupled receptors. *J Biol Rhythms* (1997) **12**:528–31. doi:10.1177/074873049701200606
34. Sugden D, Pickering H, Teh MT, Garratt PJ. Melatonin receptor pharmacology: toward subtype specificity. *Biol Cell* (1998) **89**:531–7. doi:10.1016/S0248-4900(98)80009-9
35. Gubits AK, Reppert SM. Assignment of the melatonin-related receptor to human chromosome X (GPR50) and mouse chromosome X. *Genomics* (1999) **55**:248–51. doi:10.1006/geno.1998.5661
36. Bellastella A, Amato G, Bizzarro A, Carella C, Criscuolo T, Iorio S, et al. Light, blindness and endocrine secretions. *J Endocrinol Invest* (1999) **22**:874–85.
37. Lewy AJ, Newsome DA. Different types of melatonin circadian secretory rhythm in some blind subjects. *J Clin Endocrinol Metab* (1983) **56**:1103–7. doi:10.1210/jcem-56-6-1103
38. Bellastella A, Sinisi AA, Criscuolo T, De Bellis A, Carella C, Iorio S, et al. Melatonin and pituitary-thyroid axis status in blind adults: a possible resetting after puberty. *Clin Endocrinol* (1995) **43**:707–11. doi:10.1111/j.1365-2265.1995.tb00539.x
39. Sack RL, Lewy AJ, Blood ML, Keith LD, Nakagawa H. Circadian rhythm abnormalities in totally blind people: incidence and clinical significance. *J Clin Endocrinol Metab* (1992) **75**:127–34. doi:10.1210/jc.75.1.127
40. Czeisler CA, Shanahan TL, Klerman EB, Martens H, Brotman DJ, Emens JS, et al. Suppression of melatonin secretion in some blind patients by exposure to bright light. *N Engl J Med* (1995) **332**:6–11. doi:10.1056/NEJM199501053320102
41. Hatonen T, Laasko ML, Heiskala H, Alila-Johanson A, Sainio K, Santavuori P. Bright light suppresses melatonin in blind patients with neuronal ceroid-lipofuscinoses. *Neurology* (1998) **50**:1445–50. doi:10.1212/WNL.50.5.1445
42. Lockley SW, Skene DJ, Arendt J, Tabandeh H, Bird AC, Defrance R. Relationship between melatonin rhythms and visual loss in the blind. *J Clin Endocrinol Metab* (1997) **82**:3763–70. doi:10.1210/jc.82.11.3763
43. Feychting M, Osterlund B, Ahlbom A. Reduced cancer incidence among the blind. *Epidemiology* (1998) **9**:490–4. doi:10.1097/00001648-199809000-00004
44. Reiter RJ. Pineal melatonin: cell biology of its synthesis and of its physiological interactions. *Endocr Rev* (1991) **12**:151–80. doi:10.1210/edrv-12-2-151
45. Gala RR, Haisenleder DJ, Pieper DR. Influence of blinding, olfactory bulbectomy and pinealectomy on plasma prolactin levels in the neonatally androgenized rat. *Neuroendocrinology* (1983) **37**:9–12. doi:10.1159/000123509
46. Vanecek J, Illnerova H. Effect of short and long photoperiod on pineal acetyltransferase rhythm and on growth of testes and brown adipose tissue in developing rats. *Neuroendocrinology* (1985) **41**:186–91. doi:10.1159/000124176
47. Arendt J. The pineal gland: basic physiology and clinical implications. In: De Groot LJ, editor. *Endocrinology* (Vol. 1), Philadelphia: WB Saunders (1995). p. 432–44.
48. Olatunji-Bello II, Sofola OA. Effects of continuous light and darkness exposures on the pituitary-gonadal axis and thyroid activity in male rats. *African J Biomed Res* (2001) **4**:119–22. doi:10.4314/ajbr.v4i3.53888
49. Kauppila A, Kivela A, Pakarinen A, Vakkuri O. Inverse seasonal relationship between melatonin and ovarian activity in humans in a region with a strong seasonal contrast in luminosity. *J Clin Endocrinol Metab* (1993) **76**:295–301.
50. Luboshitzki R, Dharan M, Goldman D, Hiss Y, Herer P, Lavie P. Immunohistochemical localization of gonadotropin and gonadal steroid receptors in human pineal glands. *J Clin Endocrinol Metab* (1997) **82**:977–81. doi:10.1210/jc.82.3.977
51. Shang-Mian Y, Niles PL, Younglai V. Melatonin receptors on human granulose cell membranes. *J Clin Endocrinol Metab* (1995) **80**:1747–9. doi:10.1210/jc.80.5.1747
52. Ozata M, Bulur M, Bingol N, Behyan Z, Corakci A, Bolu E, et al. Daytime plasma melatonin levels in male hypogonadism. *J Clin Endocrinol Metab* (1996) **81**:18777–81. doi:10.1210/jc.81.5.1877
53. Luboshitzy R, Levi M, Shen-Orr Z, Blummenfeld Z, Herer P, Lavie P. Long-term melatonin administration does not alter pituitary-gonadal hormone secretion in normal men. *Hum Reprod* (2000) **15**:60–5. doi:10.1093/humrep/15.1.60
54. Cagnacci A, Elliot JA, Yen SS. Amplification of pulsatile LH secretion by exogenous melatonin in women. *J Clin Endocrinol Metab* (1991) **73**:210–2. doi:10.1210/jcem-73-1-210
55. Reiter RJ. Pineal function in the human: implication for the reproductive physiology. *J Obstet Gynecol* (1986) **6**(Suppl 2):77–81. doi:10.3109/01443618609081730
56. Karasek M, Pawilikowski M, Nowakowska-Jankiewicz B, Kolodziej-Maciejewska H, Zielieniewski J, Cieslak D, et al. Circadian variations in plasma melatonin, FSH, LH and prolactin and testosterone levels in infertile men. *J Pineal Res* (1990) **9**:149–57. doi:10.1111/j.1600-079X.1990.tb00703.x
57. Maege K, Basinski J, Quarrington B, Stancer HC. Blindness and menarche. *Life Sci* (1970) **9**:7. doi:10.1016/0024-3205(70)90003-2
58. Thomas JB, Pizzarello DJ. Blindness, biologic rhythms and menarche. *Obstet Gynecol* (1967) **30**:507.
59. Zacharias L, Wurtman RJ. Blindness: its relation to age of menarche. *Science* (1964) **144**:1154. doi:10.1126/science.144.3622.1154
60. Elden CA. Sterility of blind women. *Jpn J Fertil Steril* (1971) **16**:48–50.
61. Lehrer S. Fertility of blind women. *Fertil Steril* (1982) **38**:751–2.
62. Bodenheimer S, Winter JD, Faiman C. Diurnal rhythms of serum gonadotropins, testosterone, estradiol and cortisol in blind men. *J Clin Endocrinol Metab* (1973) **36**:472–5. doi:10.1210/jcem-37-3-472
63. Bellastella A, Criscuolo T, Sinisi AA, Iorio S, Mazzuca A, Parlato F, et al. Influence of blindness on plasma luteinizing hormone, follicle-stimulating hormone, prolactin and testosterone levels in prepubertal boys. *J Clin Endocrinol Metab* (1987) **64**:862–4. doi:10.1210/jcem-64-4-862
64. Spitz IM, Hirsch HJ, Trestian S. The prolactin response to thyrotropin-releasing hormone differentiates isolated gonadotropin deficiency from delayed puberty. *N Engl J Med* (1983) **308**:575–9. doi:10.1056/NEJM198303103081007
65. Moshang T Jr, Marxs BS, Cara JF, Snyder PJ. The prolactin response to thyrotropin-releasing hormone does not distinguish teenaged males with hypogonatropic hypogonadism from those with constitutional delay of growth and development. *J Clin Endocrinol Metab* (1985) **61**:1211–3. doi:10.1210/jcem-61-6-1211

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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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REFERENCES

1. Akingbemi BT. Estrogen regulation of testicular function. *Reprod Biol Endocrinol* (2005) **3**:51. doi:10.1186/1477-7827-3-51
2. Tilbrook AJ, Clarke IJ. Negative feedback regulation of the secretion and actions of gonadotropin-releasing hormone in males. *Biol Reprod* (2001) **64**(3):735–42. doi:10.1095/biolreprod64.3.735
3. Simpson ER, Mahendroo MS, Means GD, Kilgore MW, Hinshelwood MM, Graham-Lorence S, et al. Aromatase cytochrome P450, the enzyme responsible for estrogen biosynthesis. *Endocr Rev* (1994) **15**(3):342–55. doi:10.1210/er.15.3.342
4. Boon WC, Chow JD, Simpson ER. The multiple roles of estrogens and the enzyme aromatase. *Prog Brain Res* (2010) **181**:209–32. doi:10.1016/S0079-6123(08)81012-6

5. Mangelsdorf DJ, Thummel C, Beato M, Herrlich P, Schutz G, Umesono K, et al. The nuclear receptor superfamily: the second decade. *Cell* (1995) **83**(6):835–9. doi:10.1016/0092-8674(95)90199-X
6. Kelly MJ, Wagner EJ. Estrogen modulation of G-protein-coupled receptors. *Trends Endocrinol Metab* (1999) **10**(9):369–74. doi:10.1016/S1043-2760(99)00190-3
7. Prossnitz ER, Maggiolini M. Mechanisms of estrogen signaling and gene expression via GPR30. *Mol Cell Endocrinol* (2009) **308**(1–2):32–8. doi:10.1016/j.mce.2009.03.026
8. Lappano R, De Marco P, De Francesco EM, Chimento A, Pezzi V, Maggiolini M. Cross-talk between GPER and growth factor signaling. *J Steroid Biochem Mol Biol* (2013) **137**:50–6. doi:10.1016/j.jsbmb.2013.03.005
9. Prossnitz ER, Arterburn JB, Smith HO, Oprea TI, Sklar LA, Hathaway HJ. Estrogen signaling through the transmembrane G protein-coupled receptor GPR30. *Annu Rev Physiol* (2008) **70**:165–90. doi:10.1146/annurev.physiol.70.113006.100518
10. Herbison AE. Physiology of the GnRH neuronal network. In: Neill JD, editor. *Knobil and Neill's Physiology of Reproduction*, 3rd edn. San Diego: Academic Press (2006). p. 1415–82.
11. Scott CJ, Tilbrook AJ, Rawson JA, Clarke IJ. Gonadal steroid receptors in the regulation of GnRH secretion in farm animals. *Anim Reprod Sci* (2000) **60-61**:313–26. doi:10.1016/S0378-4320(00)00103-2
12. Lindzey J, Wetsel WC, Couse JF, Stoker T, Cooper R, Korach KS. Effects of castration and chronic steroid treatments on hypothalamic gonadotropin-releasing hormone content and pituitary gonadotropins in male wild-type and estrogen receptor-alpha knockout mice. *Endocrinology* (1998) **139**(10):4092–101. doi:10.1210/en.139.10.4092
13. Wersinger SR, Haisenleder DJ, Lubahn DB, Rissman EF. Steroid feedback on gonadotropin release and pituitary gonadotropin subunit mRNA in mice lacking a functional estrogen receptor alpha. *Endocrine* (1999) **11**(2):137–43. doi:10.1385/ENDO:11:2:137
14. Hileman SM, Lubbers LS, Kuehl DE, Schaeffer DJ, Rhodes L, Jackson GL. Effect of inhibiting 5 alpha-reductase activity on the ability of testosterone to inhibit luteinizing hormone release in male sheep. *Biol Reprod* (1994) **50**(6):1244–50. doi:10.1095/biolreprod50.6.1244
15. Naftolin F, Ryan KJ, Davies IJ, Petro Z, Kuhn M. The formation and metabolism of estrogens in brain tissues. *Adv Biosci* (1975) **15**:105–21.
16. Selmanoff MK, Brodkin LD, Weiner RI, Siiteri PK. Aromatization and 5alpha-reduction of androgens in discrete hypothalamic and limbic regions of the male and female rat. *Endocrinology* (1977) **101**(3):841–8. doi:10.1210/endo-101-3-841
17. Sharma TP, Blache D, Blackberry MA, Martin GB. Role of peripheral and central aromatization in the control of gonadotrophin secretion in the male sheep. *Reprod Fertil Dev* (1999) **11**(4–5):293–302. doi:10.1071/RD99084
18. Tilbrook AJ, Clarke IJ. Negative feedback regulation of the secretion and actions of GnRH in male ruminants. *J Reprod Fertil Suppl* (1995) **49**:297–306.
19. Plant TM. Effects of orchidectomy and testosterone replacement treatment on pulsatile luteinizing hormone secretion in the adult rhesus monkey (*Macaca mulatta*). *Endocrinology* (1982) **110**(6):1905–13. doi:10.1210/endo-110-6-1905
20. Damassa DA, Kobashigawa D, Smith ER, Davidson JM. Negative feedback control of LH by testosterone: a quantitative study in male rats. *Endocrinology* (1976) **99**(3):736–42. doi:10.1210/endo-99-3-736
21. Bagatell CJ, Dahl KD, Bremner WJ. The direct pituitary effect of testosterone to inhibit gonadotropin secretion in men is partially mediated by aromatization to estradiol. *J Androl* (1994) **15**(1):15–21.
22. Schnorr JA, Bray MJ, Veldhuis JD. Aromatization mediates testosterone's short-term feedback restraint of 24-hour endogenously driven and acute exogenous gonadotropin-releasing hormone-stimulated luteinizing hormone and follicle-stimulating hormone secretion in young men. *J Clin Endocrinol Metab* (2001) **86**(6):2600–6. doi:10.1210/jc.86.6.2600
23. Veldhuis JD, Dufau ML. Estradiol modulates the pulsatile secretion of biologically active luteinizing hormone in man. *J Clin Invest* (1987) **80**(3):631–8. doi:10.1172/JCI113115
24. Rochira V, Zirilli L, Genazzani AD, Balestrieri A, Aranda C, Fabre B, et al. Hypothalamic-pituitary-gonadal axis in two men with aromatase deficiency: evidence that circulating estrogens are required at the hypothalamic level for the integrity of gonadotropin negative feedback. *Eur J Endocrinol* (2006) **155**(4):513–22. doi:10.1530/eje.1.02254
25. Herbison AE, Robinson JE, Skinner DC. Distribution of estrogen receptor-immunoreactive cells in the preoptic area of the ewe: co-localization with glutamic acid decarboxylase but not luteinizing hormone-releasing hormone. *Neuroendocrinology* (1993) **57**(4):751–9. doi:10.1159/000126433
26. Shivers BD, Harlan RE, Morrell JI, Pfaff DW. Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. *Nature* (1983) **304**(5924):345–7. doi:10.1038/304345a0
27. Laflamme N, Nappi RE, Drolet G, Labrie C, Rivest S. Expression and neuropeptidergic characterization of estrogen receptors (ERalpha and ERbeta) throughout the rat brain: anatomical evidence of distinct roles of each subtype. *J Neurobiol* (1998) **36**(3):357–78. doi:10.1002/(SICI)1097-4695(19980905)36:3<357::AID-NEU5>3.0.CO;2-V
28. Butler JA, Sjoberg M, Coen CW. Evidence for oestrogen receptor alpha-immunoreactivity in gonadotrophin-releasing hormone-expressing neurones. *J Neuroendocrinol* (1999) **11**(5):331–5. doi:10.1046/j.1365-2826.1999.00347.x
29. Temple JL, Laing E, Sunder A, Wray S. Direct action of estradiol on gonadotropin-releasing hormone-1 neuronal activity via a transcription-dependent mechanism. *J Neurosci* (2004) **24**(28):6326–33. doi:10.1523/JNEUROSCI.1006-04.2004
30. Hrabovszky E, Steinhauser A, Barabas K, Shughrue PJ, Petersen SL, Merchenthaler I, et al. Estrogen receptor-beta immunoreactivity in luteinizing hormone-releasing hormone neurons of the rat brain. *Endocrinology* (2001) **142**(7):3261–4. doi:10.1210/en.142.7.3261
31. Hrabovszky E, Kallo I, Szlavik N, Keller E, Merchenthaler I, Liposits Z. Gonadotropin-releasing hormone neurons express estrogen receptor-beta. *J Clin Endocrinol Metab* (2007) **92**(7):2827–30. doi:10.1210/jc.2006-2819
32. Abe H, Keen KL, Terasawa E. Rapid action of estrogens on intracellular calcium oscillations in primate luteinizing hormone-releasing hormone-1 neurons. *Endocrinology* (2008) **149**(3):1155–62. doi:10.1210/en.2007-0942
33. Noel SD, Keen KL, Baumann DI, Filardo EJ, Terasawa E. Involvement of G protein-coupled receptor 30 (GPR30) in rapid action of estrogen in primate LHRH neurons. *Mol Endocrinol* (2009) **23**(3):349–59. doi:10.1210/me.2008-0299
34. Gharib SD, Wierman ME, Shupnik MA, Chin WW. Molecular biology of the pituitary gonadotropins. *Endocr Rev* (1990) **11**(1):177–99. doi:10.1210/edrv-11-1-177
35. Gregory SJ, Kaiser UB. Regulation of gonadotropins by inhibin and activin. *Semin Reprod Med* (2004) **22**(3):253–67. doi:10.1055/s-2004-831901
36. Ramirez VD, McCann SM. Inhibitory effect of testosterone on luteinizing hormone secretion in immature and adult rats. *Endocrinology* (1965) **76**:412–7. doi:10.1210/endo-76-3-412
37. Selmanoff M, Shu C, Petersen SL, Barraclough CA, Zoeller RT. Single cell levels of hypothalamic messenger ribonucleic acid encoding luteinizing hormone-releasing hormone in intact, castrated, and hyperprolactinemic male rats. *Endocrinology* (1991) **128**(1):459–66. doi:10.1210/endo-128-1-459
38. Roselli CE, Kelly MJ, Ronnekleiv OK. Testosterone regulates progonadotropin-releasing hormone levels in the preoptic area and basal hypothalamus of the male rat. *Endocrinology* (1990) **126**(2):1080–6. doi:10.1210/endo-126-2-1080
39. Giri M, Kaufman JM. Effects of long-term orchidectomy on in vitro pulsatile gonadotropin-releasing hormone release from the medial basal hypothalamus of the adult guinea pig. *Endocrinology* (1994) **134**(4):1621–6. doi:10.1210/en.134.4.1621
40. Levine JE, Duffy MT. Simultaneous measurement of luteinizing hormone (LH)-releasing hormone, LH, and follicle-stimulating hormone release in intact and short-term castrate rats. *Endocrinology* (1988) **122**(5):2211–21. doi:10.1210/endo-122-5-2211
41. Frawley LS, Neill JD. Biphasic effects of estrogen on gonadotropin-releasing hormone-induced luteinizing hormone release in monolayer cultures of rat and monkey pituitary cells. *Endocrinology* (1984) **114**(2):659–63. doi:10.1210/endo-114-2-659
42. Kennedy J, Chappel S. Direct pituitary effects of testosterone and luteinizing hormone-releasing hormone upon follicle-stimulating hormone: analysis by radioimmuno- and radioreceptor assay. *Endocrinology* (1985) **116**(2):741–8. doi:10.1210/endo-116-2-741
43. Heckert LL, Wilson EM, Nilson JH. Transcriptional repression of the alpha-subunit gene by androgen receptor occurs independently of DNA binding but requires the DNA-binding and ligand-binding domains of the receptor. *Mol Endocrinol* (1997) **11**(10):1497–506. doi:10.1210/me.11.10.1497

44. Demay F, Tiffoche C, Thieulant ML. Sex- and cell-specific expression of an estrogen receptor isoform in the pituitary gland. *Neuroendocrinology* (1996) **63**(6):522–9. doi:10.1159/000127081
45. Mitchner NA, Garlick C, Ben-Jonathan N. Cellular distribution and gene regulation of estrogen receptors alpha and beta in the rat pituitary gland. *Endocrinology* (1998) **139**(9):3976–83. doi:10.1210/en.139.9.3976
46. Sheng C, McNeilly AS, Brooks AN. Immunohistochemical distribution of oestrogen receptor and luteinizing hormone B subunit in the ovine pituitary gland during foetal development. *J Neuroendocrinol* (1998) **10**(9):713–8. doi:10.1046/j.1365-2826.1998.00255.x
47. Brandenberger AW, Tee MK, Lee JY, Chao V, Jaffe RB. Tissue distribution of estrogen receptors alpha (ER-alpha) and beta (ER-beta) mRNA in the midgestational human fetus. *J Clin Endocrinol Metab* (1997) **82**(10):3509–12. doi:10.1210/jc.82.10.3509
48. Kimura N, Mizokami A, Oonuma T, Sasano H, Nagura H. Immunocytochemical localization of androgen receptor with polyclonal antibody in paraffin-embedded human tissues. *J Histochem Cytochem* (1993) **41**(5):671–8. doi:10.1177/41.5.8468448
49. Okada Y, Fujii Y, Moore JP Jr, Winters SJ. Androgen receptors in gonadotrophs in pituitary cultures from adult male monkeys and rats. *Endocrinology* (2003) **144**(1):267–73. doi:10.1210/en.2002-220770
50. Iqbal J, Swanson JJ, Prins GS, Jacobson CD. Androgen receptor-like immunoreactivity in the Brazilian opossum brain and pituitary: distribution and effects of castration and testosterone replacement in the adult male. *Brain Res* (1995) **703**(1–2):1–18. doi:10.1016/0165-3806(96)83481-X
51. Crocoll A, Zhu CC, Cato AC, Blum M. Expression of androgen receptor mRNA during mouse embryogenesis. *Mech Dev* (1998) **72**(1–2):175–8. doi:10.1016/S0925-4773(98)00007-0
52. Wierman ME, Gharib SD, LaRovere JM, Badger TM, Chin WW. Selective failure of androgens to regulate follicle stimulating hormone beta messenger ribonucleic acid levels in the male rat. *Mol Endocrinol* (1988) **2**(6):492–8. doi:10.1210/mend-2-6-492
53. Urban RJ, Davis MR, Rogol AD, Johnson ML, Veldhuis JD. Acute androgen receptor blockade increases luteinizing hormone secretory activity in men. *J Clin Endocrinol Metab* (1988) **67**(6):1149–55. doi:10.1210/jcem-67-6-1149
54. Juniewicz PE, Oesterling JE, Walters JR, Steele RE, Niswender GD, Coffey DS, et al. Aromatase inhibition in the dog. I. Effect on serum LH, serum testosterone concentrations, testicular secretions and spermatogenesis. *J Urol* (1988) **139**(4):827–31.
55. Smith EP, Boyd J, Frank GR, Takahashi H, Cohen RM, Specker B, et al. Estrogen resistance caused by a mutation in the estrogen-receptor gene in a man. *N Engl J Med* (1994) **331**(16):1056–61. doi:10.1056/NEJM199410203311604
56. Tremblay GB, Tremblay A, Copeland NG, Gilbert DJ, Jenkins NA, Labrie F, et al. Cloning, chromosomal localization, and functional analysis of the murine estrogen receptor beta. *Mol Endocrinol* (1997) **11**(3):353–65. doi:10.1210/me.11.3.353
57. Couse JF, Lindzey J, Grandien K, Gustafsson JA, Korach KS. Tissue distribution and quantitative analysis of estrogen receptor-alpha (ERalpha) and estrogen receptor-beta (ERbeta) messenger ribonucleic acid in the wild-type and ERalpha-knockout mouse. *Endocrinology* (1997) **138**(11):4613–21. doi:10.1210/en.138.11.4613
58. Shughrue PJ, Komm B, Merchenthaler I. The distribution of estrogen receptor-beta mRNA in the rat hypothalamus. *Steroids* (1996) **61**(12):678–81. doi:10.1016/S0039-128X(96)00222-X
59. Hewitt SC, Korach KS. Oestrogen receptor knockout mice: roles for oestrogen receptors alpha and beta in reproductive tissues. *Reproduction* (2003) **125**(2):143–9. doi:10.1530/rep.0.1250143
60. Nicol L, McNeilly JR, Stridsberg M, Crawford JL, McNeilly AS. Influence of steroids and GnRH on biosynthesis and secretion of secretogranin II and chromogranin A in relation to LH release in LbetaT2 gonadotroph cells. *J Endocrinol* (2002) **174**(3):473–83. doi:10.1677/joe.0.1740473
61. Shupnik MA, Rosenzweig BA. Identification of an estrogen-responsive element in the rat LH beta gene. DNA-estrogen receptor interactions and functional analysis. *J Biol Chem* (1991) **266**(26):17084–91.
62. Demay F, De Monti M, Tiffoche C, Vaillant C, Thieulant ML. Steroid-independent activation of ER by GnRH in gonadotrope pituitary cells. *Endocrinology* (2001) **142**(8):3340–7. doi:10.1210/en.142.8.3340
63. Rudolf FO, Kadokawa H. Expression of estradiol receptor, GPR30, in bovine anterior pituitary and effects of GPR30 agonist on GnRH-induced LH secretion. *Anim Reprod Sci* (2013) **139**(1–4):9–17. doi:10.1016/j.anireprosci.2013.04.003
64. Brailoiu E, Dun SL, Brailoiu GC, Mizuo K, Sklar LA, Oprea TI, et al. Distribution and characterization of estrogen receptor G protein-coupled receptor 30 in the rat central nervous system. *J Endocrinol* (2007) **193**(2):311–21. doi:10.1677/JOE-07-0017
65. Hazell GG, Yao ST, Roper JA, Prossnitz ER, O'Carroll AM, Lolait SJ. Localisation of GPR30, a novel G protein-coupled oestrogen receptor, suggests multiple functions in rodent brain and peripheral tissues. *J Endocrinol* (2009) **202**(2):223–36. doi:10.1677/JOE-09-0066
66. Hewitt SC, Harrell JC, Korach KS. Lessons in estrogen biology from knockout and transgenic animals. *Annu Rev Physiol* (2005) **67**:285–308. doi:10.1146/annurev.physiol.67.040403.115914
67. Lubahn DB, Moyer JS, Golding TS, Couse JF, Korach KS, Smithies O. Alteration of reproductive function but not prenatal sexual development after insertional disruption of the mouse estrogen receptor gene. *Proc Natl Acad Sci U S A* (1993) **90**(23):11162–6. doi:10.1073/pnas.90.23.11162
68. Cattanach BM, Iddon CA, Charlton HM, Chiappa SA, Fink G. Gonadotrophin-releasing hormone deficiency in a mutant mouse with hypogonadism. *Nature* (1977) **269**(5626):338–40. doi:10.1038/269338a0
69. Ebling FJ, Brooks AN, Cronin AS, Ford H, Kerr JB. Estrogenic induction of spermatogenesis in the hypogonadal mouse. *Endocrinology* (2000) **141**(8):2861–9. doi:10.1210/en.141.8.2861
70. Myers M, Ebling FJ, Nwagwu M, Boulton R, Wadhwa K, Stewart J, et al. Atypical development of Sertoli cells and impairment of spermatogenesis in the hypogonadal (hpg) mouse. *J Anat* (2005) **207**(6):797–811. doi:10.1111/j.1469-7580.2005.00493.x
71. Scott IS, Charlton HM, Cox BS, Grocock CA, Sheffield JW, O'Shaughnessy PJ. Effect of LH injections on testicular steroidogenesis, cholesterol side-chain cleavage P450 mRNA content and Leydig cell morphology in hypogonadal mice. *J Endocrinol* (1990) **125**(1):131–8. doi:10.1677/joe.0.1250131
72. Singh J, O'Neill C, Handelsman DJ. Induction of spermatogenesis by androgens in gonadotropin-deficient (hpg) mice. *Endocrinology* (1995) **136**(12):5311–21. doi:10.1210/en.136.12.5311
73. Singh J, Handelsman DJ. The effects of recombinant FSH on testosterone-induced spermatogenesis in gonadotrophin-deficient (hpg) mice. *J Androl* (1996) **17**(4):382–93.
74. Baines H, Nwagwu MO, Furneaux EC, Stewart J, Kerr JB, Mayhew TM, et al. Estrogenic induction of spermatogenesis in the hypogonadal (hpg) mouse: role of androgens. *Reproduction* (2005) **130**(5):643–54. doi:10.1530/rep.1.00693
75. Nwagwu MO, Baines H, Kerr JB, Ebling FJ. Neonatal androgenization of hypogonadal (hpg) male mice does not abolish estradiol-induced FSH production and spermatogenesis. *Reprod Biol Endocrinol* (2005) **3**:48. doi:10.1186/1477-7827-3-48
76. Hess RA, Bunick D, Lee KH, Bahr J, Taylor JA, Korach KS, et al. A role for oestrogens in the male reproductive system. *Nature* (1997) **390**(6659):509–12. doi:10.1038/37352
77. Antal MC, Krust A, Chambon P, Mark M. Sterility and absence of histopathological defects in nonreproductive organs of a mouse ERbeta-null mutant. *Proc Natl Acad Sci U S A* (2008) **105**(7):2433–8. doi:10.1073/pnas.0712029105
78. Robertson KM, O'Donnell L, Jones ME, Meachem SJ, Boon WC, Fisher CR, et al. Impairment of spermatogenesis in mice lacking a functional aromatase (cyp 19) gene. *Proc Natl Acad Sci U S A* (1999) **96**(14):7986–91. doi:10.1073/pnas.96.14.7986
79. Robertson KM, O'Donnell L, Simpson ER, Jones ME. The phenotype of the aromatase knockout mouse reveals dietary phytoestrogens impact significantly on testis function. *Endocrinology* (2002) **143**(8):2913–21. doi:10.1210/en.143.8.2913
80. Otto C, Fuchs I, Kauselmann G, Kern H, Zevnik B, Andreasen P, et al. GPR30 does not mediate estrogenic responses in reproductive organs in mice. *Biol Reprod* (2009) **80**(1):34–41. doi:10.1095/biolreprod.108.071175
81. Weiss J, Bernhardt ML, Laronda MM, Hurley LA, Glidewell-Kenney C, Pillai S, et al. Estrogen actions in the male reproductive system involve estrogen response element-independent pathways. *Endocrinology* (2008) **149**(12):6198–206. doi:10.1210/en.2008-0122

82. Sinkevicius KW, Woloszyn K, Laine M, Jackson KS, Greene GL, Woodruff TK, et al. Characterization of the ovarian and reproductive abnormalities in prepubertal and adult estrogen non-responsive estrogen receptor alpha knock-in (ENERKI) mice. *Steroids* (2009) **74**(12):913–9. doi:10.1016/j.steroids.2009.06.012
83. Cacciola G, Chioccarelli T, Altucci L, Ledent C, Mason JI, Fasano S, et al. Low 17beta-estradiol levels in CNR1 knock-out mice affect spermatid chromatin remodeling by interfering with chromatin reorganization. *Biol Reprod* (2013) **88**(6):152. doi:10.1095/biolreprod.112.105726
84. Cacciola G, Chioccarelli T, Altucci L, Viggiano A, Fasano S, Pierantoni R, et al. Nuclear size as estrogen-responsive chromatin quality parameter of mouse spermatozoa. *Gen Comp Endocrinol* (2013) **193**:201–9. doi:10.1016/j.ygcen.2013.07.018
85. Cacciola G, Chioccarelli T, Fasano S, Pierantoni R, Cobellis G. Estrogens and spermiogenesis: new insights from type 1 cannabinoid receptor knockout mice. *Int J Endocrinol* (2013) **2013**:501350. doi:10.1155/2013/501350
86. Carreau S, Hess RA. Oestrogens and spermatogenesis. *Philos Trans R Soc Lond B Biol Sci* (2010) **365**(1546):1517–35. doi:10.1098/rstb.2009.0235
87. Franca LR, Parreira GG, Gates RJ, Russell LD. Hormonal regulation of spermatogenesis in the hypophysectomized rat: quantitation of germ-cell population and effect of elimination of residual testosterone after long-term hypophysectomy. *J Androl* (1998) **19**(3):335–40 discussion 41–2.
88. Handelsman DJ. Hormonal regulation of spermatogenesis: insights from constructing genetic models. *Reprod Fertil Dev* (2011) **23**(4):507–19. doi:10.1071/RD10308
89. O'Donnell L, Robertson KM, Jones ME, Simpson ER. Estrogen and spermatogenesis. *Endocr Rev* (2001) **22**(3):289–318. doi:10.1210/er.22.3.289
90. Carreau S, Wolczynski S, Galeraud-Denis I. Aromatase, oestrogens and human male reproduction. *Philos Trans R Soc Lond B Biol Sci* (2010) **365**(1546):1571–9. doi:10.1098/rstb.2009.0113
91. Rochira V, Granata AR, Madeo B, Zirilli L, Rossi G, Carani C. Estrogens in males: what have we learned in the last 10 years? *Asian J Androl* (2005) **7**(1):3–20. doi:10.1111/j.1745-7262.2005.00018.x
92. Zhou Q, Nie R, Prins GS, Saunders PT, Katzenellenbogen BS, Hess RA. Localization of androgen and estrogen receptors in adult male mouse reproductive tract. *J Androl* (2002) **23**(6):870–81. doi:10.1002/j.1939-4640.2002.tb02345.x
93. Kotula-Balak M, Gancarczyk M, Sadowska J, Bilinskai B. The expression of aromatase, estrogen receptor alpha and estrogen receptor beta in mouse Leydig cells in vitro that derived from cryptorchid males. *Eur J Histochem* (2005) **49**(1):59–62.
94. Lucas TF, Siu ER, Esteves CA, Monteiro HP, Oliveira CA, Porto CS, et al. 17Beta-estradiol induces the translocation of the estrogen receptors ESR1 and ESR2 to the cell membrane, MAPK3/1 phosphorylation and proliferation of cultured immature rat Sertoli cells. *Biol Reprod* (2008) **78**(1):101–14. doi:10.1095/biolreprod.107.063909
95. Fisher JS, Millar MR, Majdic G, Saunders PT, Fraser HM, Sharpe RM. Immunolocalisation of oestrogen receptor-alpha within the testis and excurrent ducts of the rat and marmoset monkey from perinatal life to adulthood. *J Endocrinol* (1997) **153**(3):485–95. doi:10.1677/joe.0.1530485
96. van Pelt AM, de Rooij DG, van der Burg B, van der Saag PT, Gustafsson JA, Kuiper GG. Ontogeny of estrogen receptor-beta expression in rat testis. *Endocrinology* (1999) **140**(1):478–83. doi:10.1210/en.140.1.478
97. Pelletier G, El-Alfy M. Immunocytochemical localization of estrogen receptors alpha and beta in the human reproductive organs. *J Clin Endocrinol Metab* (2000) **85**(12):4835–40. doi:10.1210/jc.85.12.4835
98. Chimento A, Sirianni R, Delalande C, Silandre D, Bois C, Ando S, et al. 17 Beta-estradiol activates rapid signaling pathways involved in rat pachytene spermatocytes apoptosis through GPR30 and ER alpha. *Mol Cell Endocrinol* (2010) **320**(1–2):136–44. doi:10.1016/j.mce.2010.01.035
99. Chimento A, Sirianni R, Zolea F, Bois C, Delalande C, Ando S, et al. Gper and ESRs are expressed in rat round spermatids and mediate oestrogen-dependent rapid pathways modulating expression of cyclin B1 and Bax. *Int J Androl* (2011) **34**(5 Pt 1):420–9. doi:10.1111/j.1365-2605.2010.01100.x
100. Yamada-Mouri N, Hirata S, Kato J. Existence and expression of the untranslated first exon of aromatase mRNA in the rat brain. *J Steroid Biochem Mol Biol* (1996) **58**(2):163–6. doi:10.1016/0960-0760(96)00022-2
101. Bois C, Delalande C, Nurmio M, Parvinen M, Zanatta L, Toppari J, et al. Age- and cell-related gene expression of aromatase and estrogen receptors in the rat testis. *J Mol Endocrinol* (2010) **45**(3):147–59. doi:10.1677/JME-10-0041
102. Lambard S, Galeraud-Denis I, Saunders PT, Carreau S. Human immature germ cells and ejaculated spermatozoa contain aromatase and oestrogen receptors. *J Mol Endocrinol* (2004) **32**(1):279–89. doi:10.1677/jme.0.0320279
103. Aquila S, Sisci D, Gentile M, Middea E, Siciliano L, Ando S. Human ejaculated spermatozoa contain active P450 aromatase. *J Clin Endocrinol Metab* (2002) **87**(7):3385–90. doi:10.1210/jc.87.7.3385
104. Sirianni R, Chimento A, Ruggiero C, De Luca A, Lappano R, Ando S, et al. The novel estrogen receptor, G protein-coupled receptor 30, mediates the proliferative effects induced by 17beta-estradiol on mouse spermatogonial GC-1 cell line. *Endocrinology* (2008) **149**(10):5043–51. doi:10.1210/en.2007-1593
105. Chimento A, Sirianni R, Casaburi I, Ruggiero C, Maggiolini M, Ando S, et al. 17Beta-estradiol activates GPER- and ESR1-dependent pathways inducing apoptosis in GC-2 cells, a mouse spermatocyte-derived cell line. *Mol Cell Endocrinol* (2012) **355**(1):49–59. doi:10.1016/j.mce.2012.01.017
106. Chimento A, Casaburi I, Bartucci M, Patrizii M, Dattilo R, Avena P, et al. Selective GPER activation decreases proliferation and activates apoptosis in tumor Leydig cells. *Cell Death Dis* (2013) **4**:e747. doi:10.1038/cddis.2013.275
107. Chieffi P, Colucci-D'Amato GL, Staibano S, Franco R, Tramontano D. Estradiol-induced mitogen-activated protein kinase (extracellular signal-regulated kinase 1 and 2) activity in the frog (*Rana esculenta*) testis. *J Endocrinol* (2000) **167**(1):77–84. doi:10.1677/joe.0.1670077
108. Chieffi P, Colucci D'Amato L, Guarino F, Salvatore G, Angelini F. 17 Beta-estradiol induces spermatogonial proliferation through mitogen-activated protein kinase (extracellular signal-regulated kinase 1/2) activity in the lizard (*Podarcis s. sicula*). *Mol Reprod Dev* (2002) **61**(2):218–25. doi:10.1002/mrd.1151
109. Lucas TF, Royer C, Siu ER, Lazari MF, Porto CS. Expression and signaling of G protein-coupled estrogen receptor 1 (GPER) in rat Sertoli cells. *Biol Reprod* (2010) **83**(2):307–17. doi:10.1095/biolreprod.110.084160
110. Lucas TF, Pimenta MT, Pisolato R, Lazari MF, Porto CS. 17Beta-estradiol signaling and regulation of Sertoli cell function. *Spermatogenesis* (2011) **1**(4):318–24. doi:10.4161/spmg.1.4.18903
111. Royer C, Lucas TF, Lazari MF, Porto CS. 17Beta-estradiol signaling and regulation of proliferation and apoptosis of rat Sertoli cells. *Biol Reprod* (2012) **86**(4):108. doi:10.1095/biolreprod.111.096891
112. Franco R, Boscia F, Gigantino V, Marra L, Esposito F, Ferrara D, et al. GPR30 is overexpressed in post-puberal testicular germ cell tumors. *Cancer Biol Ther* (2011) **11**(6):609–13. doi:10.4161/cbt.11.6.14672
113. Rago V, Romeo F, Giordano F, Maggiolini M, Carpino A. Identification of the estrogen receptor GPER in neoplastic and non-neoplastic human testes. *Reprod Biol Endocrinol* (2011) **9**:135. doi:10.1186/1477-7827-9-135
114. Rago V, Romeo F, Giordano F, Ferraro A, Ando S, Carpino A. Identification of ERbeta1 and ERbeta2 in human seminoma, in embryonal carcinoma and in their adjacent intratubular germ cell neoplasia. *Reprod Biol Endocrinol* (2009) **7**:56. doi:10.1186/1477-7827-7-56
115. Carpino A, Rago V, Pezzi V, Carani C, Ando S. Detection of aromatase and estrogen receptors (ERalpha, ERbeta1, ERbeta2) in human Leydig cell tumor. *Eur J Endocrinol* (2007) **157**(2):239–44. doi:10.1530/EJE-07-0029
116. Chevalier N, Bouskine A, Fenichel P. Role of GPER/GPR30 in tumoral testicular germ cells proliferation. *Cancer Biol Ther* (2011) **12**(1):2–3. doi:10.4161/cbt.12.1.15726

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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,

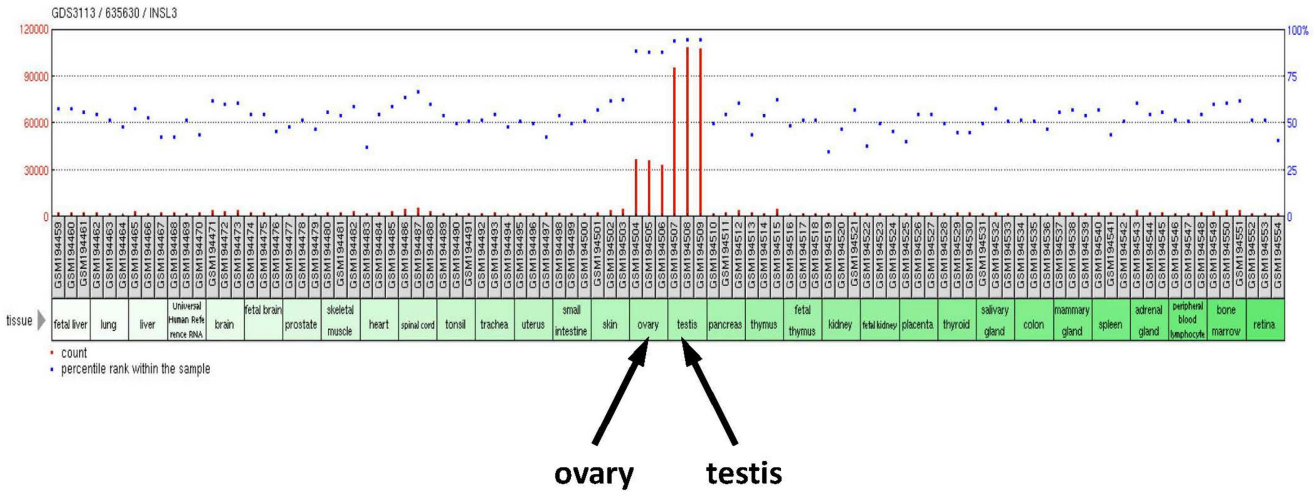

FIGURE 1 | Human tissue RNA profile based on Affymetrix microarrays (GEO profile database; GDS 3113/635630) probed for INSL3 gene expression. Significant INSL3 mRNA is only evident for testes and ovary samples. All tissues are represented in triplicate.

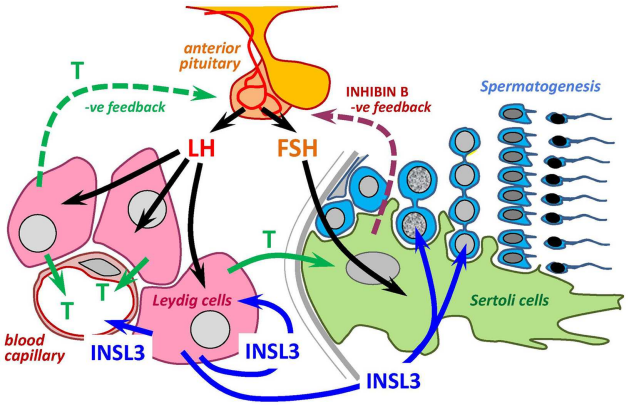

FIGURE 2 | INSL3 and HPG axis. Scheme to show the relationship between the INSL3/RXFP2 system and testosterone as endpoint effectors of the HPG axis within the testis. Arrows are directed only to cells where there are known to be specific cognate receptors.

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."

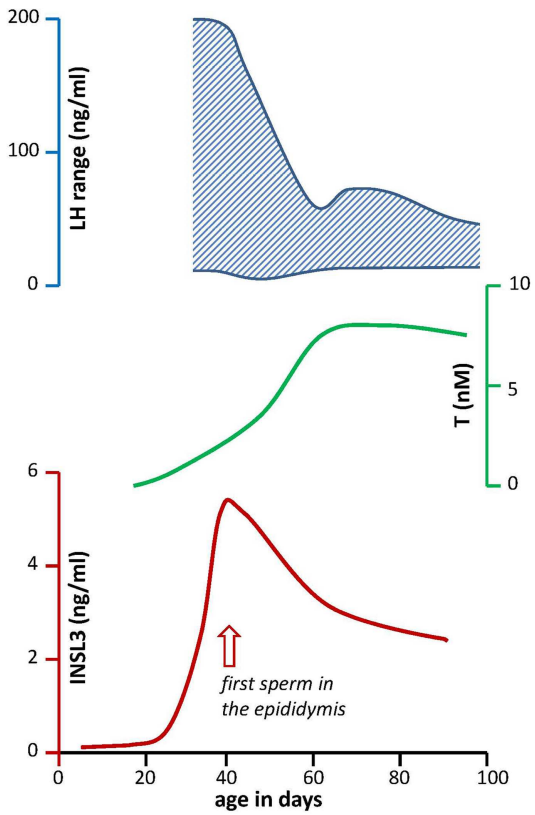

FIGURE 3 | Profiles through rat post-natal development for key circulating hormones of the HPG axis. LH (upper panel) is given as range to indicate the high degree of episodic secretion during early puberty, which is not represented in simple mean values (26). Testosterone (T; middle panel) concentrations 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

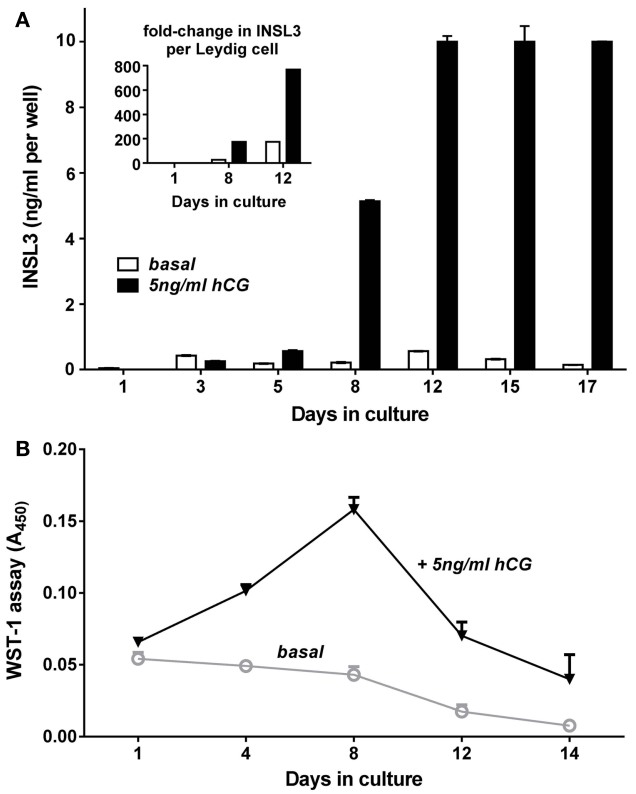

FIGURE 4 | Differentiation *in vitro* of post-natal day (PND) 10 rat Leydig cells in the absence (open bars) or presence (filled bars) of hCG. **(A)** Cells were purified from the abdominal testes of PND10 male Sprague Dawley rats by mechanical dispersion followed by unit sedimentation, then cultured 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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REFERENCES

1. Bathgate RA, Hsueh AJ, Ivell R, Sanborn BM, Sherwood OD, Summers RJ. International Union of Pharmacology. Recommendations for the nomenclature of receptors for relaxin family peptides. *Pharmacol Rev* (2006) **58**:7–31. doi:10.1124/pr.58.1.9
2. Ivell R, Kotula-Balak M, Glynn D, Heng K, Anand-Ivell R. Relaxin family peptides in the male reproductive system – a critical appraisal. *Mol Hum Reprod* (2011) **17**:71–84. doi:10.1093/molehr/gaq086
3. Bullesbach EE, Schwabe C. The primary structure and disulfide links of the bovine relaxin-like factor (RLF). *Biochemistry* (2002) **41**:274–81. doi:10.1021/bi0117302
4. Minagawa I, Fukuda M, Ishige H, Kohriki H, Shibata M, Park EY, et al. Relaxin-like factor (RLF)insulin-like peptide 3 (INSL3) is secreted from testicular Leydig cells as a monomeric protein comprising three domains B-C-A with full biological activity in boars. *Biochem J* (2012) **441**:265–73. doi:10.1042/BJ20111107
5. Siqin, Minagawa I, Okuno M, Yamada K, Sugawara Y, Nagura Y, et al. The active form of goat insulin-like peptide 3 (INSL3) is a single-chain structure comprising three domains B-C-A, constitutively expressed and secreted by testicular Leydig cells. *Biol Chem* (2013) **394**:1181–94. doi:10.1515/hsz-2012-0357
6. Ivell R, Wade JD, Anand-Ivell R. Insulin-like factor 3 (INSL3) as a biomarker of Leydig cell functional capacity. *Biol Reprod* (2013) **88**:147. doi:10.1095/biolreprod.113.108969
7. Foresta C, Bettella A, Vinanzi C, Dabrilli P, Meriggiola MC, Garolla A, et al. A novel circulating hormone of testis origin in humans. *J Clin Endocrinol Metab* (2004) **89**:5952–8. doi:10.1210/jc.2004-0575
8. Bay K, Hartung S, Ivell R, Schumacher M, Jurgensen D, Jorgensen N, et al. Insulin-like factor 3 serum levels in 135 normal men and 85 men with testicular disorders: relationship to the luteinizing hormone-testosterone axis. *J Clin Endocrinol Metab* (2005) **90**:3410–8. doi:10.1210/jc.2004-2257
9. Anand-Ivell RJK, Wohlgemuth J, Haren MT, Hope PJ, Hatzinikolas G, Wittert G, et al. Peripheral INSL3 concentrations decline with age in a large population of Australian men. *Int J Androl* (2006) **29**:618–26. doi:10.1111/j.1365-2605.2006.00714.x
10. Anand-Ivell R, Heng K, Hafen B, Setchell B, Ivell R. Dynamics of INSL3 peptide expression in the rodent testis. *Biol Reprod* (2009) **81**:480–7. doi:10.1095/biolreprod.109.077552
11. Kawate N, Ohnari A, Pathirana IN, Büllesbach EE, Takahashi M, Inaba T, et al. Changes in plasma concentrations of insulin-like peptide 3 and testosterone from birth to pubertal age in beef bulls. *Theriogenology* (2011) **76**:1632–8. doi:10.1016/j.theriogenology.2011.07.011
12. Anand-Ivell R, Ivell R. INSL3 as a monitor of endocrine disruption. *Reproduction* (2013). doi:10.1530/REP-13-0486.
13. O'Shaugnessy PJ, Fleming LM, Jackson G, Hochgeschwender U, Reed P, Baker PJ. Adrenocorticotropic hormone directly stimulates testosterone production by the fetal and neonatal mouse testis. *Endocrinology* (2003) **144**:3279–84. doi:10.1210/en.2003-0277
14. O'Shaugnessy PJ, Baker P, Sohnius U, Haavisto AM, Charlton HM, Huhtaniemi I. Fetal development of Leydig cell activity in the mouse is independent of pituitary gonadotroph function. *Endocrinology* (1998) **139**:1141–6. doi:10.1210/en.139.3.1141
15. Balvers M, Spiess AN, Domagalski R, Hunt N, Kilic E, Mukhopadhyay AK, et al. Relaxin-like factor expression as a marker of differentiation in the mouse testis and ovary. *Endocrinology* (1998) **139**:2960–70. doi:10.1210/endo.139.6.6046
16. Kubota Y, Temelcos C, Bathgate RA, Smith KJ, Scott D, Zhao C, et al. The role of insulin 3, testosterone, Müllerian inhibiting substance and relaxin in rat gubernacular growth. *Mol Hum Reprod* (2002) **8**:900–5. doi:10.1093/molehr/8.10.900
17. Yuan FP, Li X, Lin J, Schwabe C, Büllesbach EE, Rao CV, et al. The role of RXFP2 in mediating androgen-induced inguinoscrotal testis descent in LH receptor knockout mice. *Reproduction* (2010) **139**:759–69. doi:10.1530/REP-09-0518
18. Glister C, Satchell L, Bathgate RA, Wade JD, Dai Y, Ivell R, et al. A functional link between bone morphogenetic protein and insulin-like peptide 3 signaling in modulating ovarian androgen production. *Proc Natl Acad Sci U S A* (2013) **110**:E1426–35. doi:10.1073/pnas.1222216110
19. Barthold JS, Wang Y, Robbins A, Pike J, McDowell E, Johnson KJ, et al. Transcriptome analysis of the dihydrotestosterone-exposed fetal rat gubernaculum identifies common androgen and insulin-like 3 targets. *Biol Reprod* (2013) **89**:143. doi:10.1095/biolreprod.113.112953
20. Hutson JM, Southwell BR, Li R, Lie G, Ismail K, Harisis G, et al. The regulation of testicular descent and the effects of cryptorchidism. *Endocr Rev* (2013) **34**:725–52. doi:10.1210/er.2012-1089
21. Hadziselimovic F, Zivkovic D, Bica DT, Emmons LR. The importance of mini-puberty for fertility in cryptorchidism. *J Urol* (2005) **174**:1536–9. doi:10.1097/01.ju.0000181506.97839.b0
22. Veldhuizen Tsoerkan MB, Ivell R, Teerds K. Human chorionic gonadotrophin (hCG) induced changes in luteinizing hormone/hCG receptor messenger ribonucleic acid transcript levels in the testis of adult, hypophysectomized, ethane dimethyl sulphonate treated rats. *Mol Cell Endocrinol* (1994) **105**:37–44. doi:10.1016/0303-7207(94)90033-7
23. Zhang FP, Hämäläinen T, Kaipia A, Pakarinen P, Huhtaniemi I. Ontogeny of luteinizing hormone receptor gene expression in the rat testis. *Endocrinology* (1994) **134**:2206–13. doi:10.1210/en.134.5.2206
24. Tena-Sempere M, Zhang FP, Huhtaniemi I. Persistent expression of a truncated form of the luteinizing hormone receptor messenger ribonucleic acid in the rat testis after selective Leydig cell destruction by ethylene dimethane sulfonate. *Endocrinology* (1994) **135**:1018–24. doi:10.1210/endo.135.3.8070344
25. Wennink JM, Delemarre-van de Waal HA, Schoemaker R, Schomaker H, Schoemaker J. Luteinizing hormone and follicle stimulating hormone secretion patterns in boys throughout puberty measured using highly sensitive immunoradiometric assays. *Clin Endocrinol* (1989) **31**:551–64. doi:10.1111/j.1365-2265.1989.tb01279.x
26. Sharpe RM, Doogan DG, Cooper I. Intratesticular factors and testosterone secretion: the role of luteinizing hormone in relation to changes during puberty and experimental cryptorchidism. *Endocrinology* (1986) **119**:2089–96. doi:10.1210/endo-119-5-2089
27. Bartlett JMS, Charlton HM, Robinson ICAF, Nieschlag E. Pubertal development and testicular function in the male growth hormone-deficient rat. *J Endocrinol* (1990) **126**:193–201. doi:10.1677/joe.0.1260193
28. Sadeghian H, Anand-Ivell R, Balvers M, Relan V, Ivell R. Constitutive regulation of the INSL3 gene in rat Leydig cells. *Mol Cell Endocrinol* (2005) **124**:10–20. doi:10.1016/j.mce.2005.03.017
29. Trabado S, Maione L, Bry-Gauillard H, Affres H, Salenave S, Sarfati J, et al. Insulin-like peptide 3 (INSL3) in men with congenital hypogonadotropic hypogonadism/Kallmann syndrome and effects of different modalities of hormonal treatment: a single-center study of 281 patients. *J Clin Endocrinol Metab* (2013). doi:10.1210/jc.2013-2288.
30. Andersson AM, Jorgensen N, Frydelund-Larsen L, Rajpert-De Meyts E, Skakkebaek NE. Impaired Leydig cell function in infertile men: a study of 357 idiopathic infertile men and 318 proven fertile controls. *J Clin Endocrinol Metab* (2006) **89**:3161–7. doi:10.1210/jc.2003-031786
31. De Kretser DM. Editorial: is spermatogenic damage associated with Leydig cell dysfunction? *J Clin Endocrinol Metab* (2006) **89**:3158–60. doi:10.1210/jc.2004-0741
32. Diver MJ. Analytical and physiological factors affecting the interpretation of serum testosterone concentration in men. *Ann Clin Biochem* (2006) **43**:3–12. doi:10.1258/000456306775141803
33. Ivell R, Anand-Ivell R. The biology of Insulin-like Factor 3 (INSL3) in human reproduction. *Hum Reprod Update* (2009) **15**:463–76. doi:10.1093/humupd/dmp011
34. Johansen ML, Anand-Ivell R, Mouritsen A, Hagen CP, Mieritz MG, Soeborg T, et al. Serum levels of insulin-like factor 3, anti-Mullerian hormone, inhibin B and testosterone during pubertal transition in healthy boys: a longitudinal study. *Reproduction* (in press).
35. Nef S, Parada LF. Cryptorchidism in mice mutant for Insl3. *Nat Genet* (1999) **22**:295–9. doi:10.1038/10364
36. Zimmermann S, Steding G, Emmen JM, Brinkmann AO, Nayernia K, Holstein AF, et al. Targeted disruption of the Insl3 gene causes bilateral cryptorchidism. *Mol Endocrinol* (1999) **13**:681–91. doi:10.1210/me.13.5.681
37. Ferlin A, Pepe A, Gianesello L, Garolla A, Feng S, Giannini S, et al. Mutations in the insulin-like factor 3 receptor are associated with osteoporosis. *J Bone Miner Res* (2008) **23**:683–93. doi:10.1359/jbmr.080204
38. Johnston SE, Gratten J, Berenos C, Pilkinmgton JG, Clutton-Brock TH, Pemberton JM, et al. Life history trade-offs at a single locus maintain sexually selected genetic variation. *Nature* (2013) **502**:93–5. doi:10.1038/nature12489
39. Anand-Ivell RJK, Relan V, Balvers M, Fritsch M, Bathgate RAD, Ivell R. Expression of the Insulin-like peptide 3 (INSL3) hormone-receptor (LGR8)

system in the testis. *Biol Reprod* (2006) **74**:945–53. doi:10.1095/biolreprod.105.048165
40. Kawamura K, Kumagai J, Sudo S, Chun S-Y, Pisarska M, Morita H, et al. Paracrine regulation of oocyte maturation and male germ cell survival. *Proc Natl Acad Sci U S A* (2004) **101**:7323–8. doi:10.1073/pnas.0307061101
41. Filonzi M, Cardoso LC, Pimenta MT, Queiroz DB, Avellar MC, Porto CS, et al. Relaxin family peptide receptors Rxfp1 and Rxfp2: mapping of the mRNA and protein distribution in the reproductive tract of the male rat. *Reprod Biol Endocrinol* (2007) **5**:29. doi:10.1186/1477-7827-5-29
42. Pathirana IN, Kawate N, Büllesbach EE, Takahashi M, Hatoya S, Inaba T, et al. Insulin-like peptide 3 stimulates testosterone secretion in mouse Leydig cells via cAMP pathway. *Regul Pept* (2012) **178**:102–6. doi:10.1016/j.regpep.2012.07.003
43. Del Borgo MP, Hughes RA, Bathgate RA, Lin F, Kawamura K, Wade JD. Analogs of insulin-like peptide (INSL3) B-chain are LGR8 antagonists *in vitro* and *in vivo*. *J Biol Chem* (2006) **281**:13068–74. doi:10.1074/jbc.M600472200
44. Amory JK, Page ST, Anawalt BD, Coviello AD, Matsumoto AM, Bremner WJ. Elevated end-of-treatment serum INSL3 is associated with failure to completely suppress spermatogenesis in men receiving male hormone contraception. *J Androl* (2007) **28**:548–54. doi:10.2164/jandrol.106.002345
45. Ivell R, Bathgate RAD. Hypothesis: neohormone systems as exciting targets for drug development. *Trends Endocrinol Metab* (2006) **17**:123. doi:10.1016/j.tem.2006.03.004
46. Anand-Ivell R, Dai Y, Ivell R. Neohormones as biomarkers of reproductive health. *Fertil Steril* (2013) **99**:1153–60. doi:10.1016/j.fertnstert.2012.12.023
47. Xue K, Kim JY, Liu JY, Tsang BK. Insulin-like 3 induced rat preantral follicular growth is mediated by growth differentiation factor 9. *Endocrinology* (2014) **155**:156–67. doi:10.1210/en.2013-1491
48. Spanel-Borowski K, Schäfer I, Zimmermann S, Engel W, Adham IM. Increase in final stages of follicular atresia and premature decay of corpora lutea in Insl3-deficient mice. *Mol Reprod Dev* (2001) **58**:281–6. doi:10.1002/1098-2795(200103)58:3<281::AID-MRD6>3.0.CO;2-0
49. Huang Z, Rivas B, Agoulnik AI. Insulin-like 3 signaling is important for testicular descent but dispensable for spermatogenesis and germ cell survival in adult mice. *Biol Reprod* (2012) **87**:143. doi:10.1095/biolreprod.112.103382
50. Chen H, Hardy MP, Huhtaniemi I, Zirkin BR. Age-related decreased Leydig cell testosterone production in the brown Norway rat. *J Androl* (1994) **15**:551–7.
51. Neaves WB, Johnson L, Porter JC, Parker CR, Petty CS. Leydig cell numbers, daily sperm production and serum gonadotropin levels in aging men. *J Clin Endocrinol Metab* (1984) **59**:756–63. doi:10.1210/jcem-59-4-756

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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,

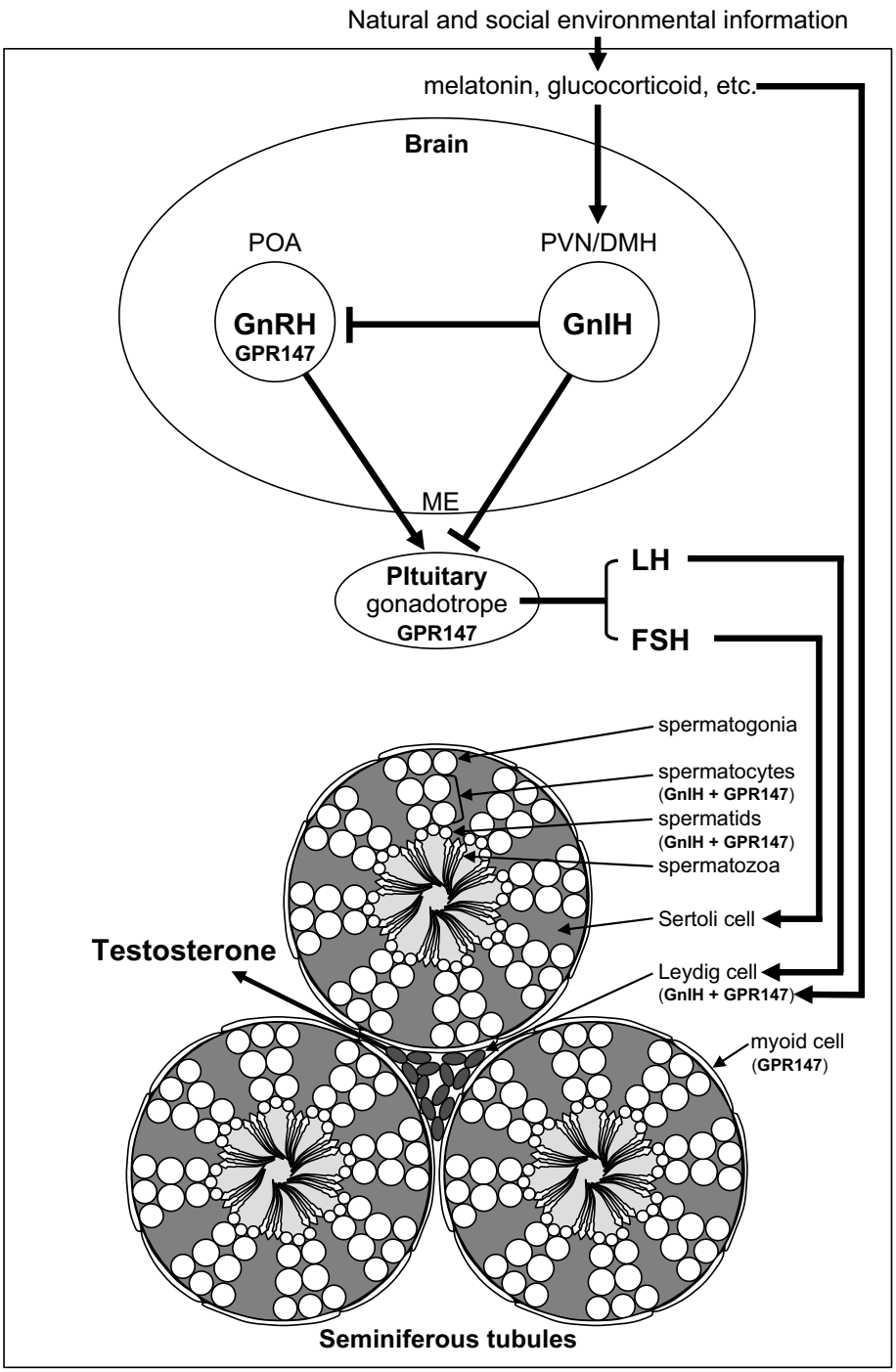

FIGURE 1 | Schematic model of central and direct actions of GnIH on testicular activity in birds and mammals. Neuronal cell bodies expressing gonadotropin-inhibitory hormone (GnIH) are located in the paraventricular nucleus (PVN) in birds and the dorsomedial hypothalamic area (DMH) in mammals. GnIH neurons 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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REFERENCES

1. Shalet SM. Normal testicular function and spermatogenesis. *Pediatr Blood Cancer* (2009) **53**:285–8. doi:10.1002/pbc.22000
2. Pudney J. Spermatogenesis in nonmammalian vertebrates. *Microsc Res Tech* (1995) **32**:459–97. doi:10.1002/jemt.1070320602
3. Bentley GE, Ubuka T, McGuire NL, Chowdhury VS, Morita Y, Yano T, et al. Gonadotropin-inhibitory hormone and its receptor in the avian reproductive system. *Gen Comp Endocrinol* (2008) **156**:34–43. doi:10.1016/j.ygcen.2007.10.003
4. McGuire NL, Bentley GE. A functional neuropeptide system in vertebrate gonads: gonadotropin-inhibitory hormone and its receptor in testes of field-caught house sparrow (*Passer domesticus*). *Gen Comp Endocrinol* (2010) **166**:565–72. doi:10.1016/j.ygcen.2010.01.010
5. Zhao S, Zhu E, Yang C, Bentley GE, Tsutsui K, Kriegsfeld LJ. RFamide-related peptide and messenger ribonucleic acid expression in mammalian testis: association with the spermatogenic cycle. *Endocrinology* (2010) **151**:617–27. doi:10.1210/en.2009-0978
6. Matsuo H, Baba Y, Nair RM, Arimura A, Schally AV. Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. *Biochem Biophys Res Commun* (1971) **43**:1334–9. doi:10.1016/S0006-291X(71)80019-0
7. Burgus R, Butcher M, Amoss M, Ling N, Monahan M, Rivier J, et al. Primary structure of the ovine hypothalamic luteinizing hormone-releasing factor (LRF). *Proc Natl Acad Sci U S A* (1972) **69**:278–82. doi:10.1073/pnas.69.1.278
8. King JA, Millar RP. Structure of chicken hypothalamic luteinizing hormone-releasing hormone. I. Structural determination on partially purified material. *J Biol Chem* (1982) **257**:10722–8.
9. Miyamoto K, Hasegawa Y, Minegishi T, Nomura M, Takahashi Y, Igarashi M, et al. Isolation and characterization of chicken hypothalamic luteinizing hormone-releasing hormone. *Biochem Biophys Res Commun* (1982) **107**:820–7. doi:10.1016/0006-291X(82)90596-4
10. Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O. Neuroendocrinology of reproduction in teleost fish. *Gen Comp Endocrinol* (2010) **165**:438–55. doi:10.1016/j.ygcen.2009.04.017
11. Tsutsui K, Saigoh E, Ukena K, Teranishi H, Fujisawa Y, Kikuchi M, et al. A novel avian hypothalamic peptide inhibiting gonadotropin release. *Biochem Biophys Res Commun* (2000) **275**:661–7. doi:10.1006/bbrc.2000.3350
12. Bentley GE, Tsutsui K, Kriegsfeld LJ. Recent studies of gonadotropin-inhibitory hormone (GnIH) in the mammalian hypothalamus, pituitary and gonads. *Brain Res* (2010) **1364**:62–71. doi:10.1016/j.brainres.2010.10.001
13. Kriegsfeld LJ, Gibson EM, Williams WP III, Zhao S, Mason AO, Bentley GE, et al. The roles of RFamide-related peptide-3 in mammalian reproductive function and behaviour. *J Neuroendocrinol* (2010) **22**:692–700. doi:10.1111/j.1365-2826.2010.02031.x
14. Tsutsui K. Review: a new key neurohormone controlling reproduction, gonadotropin-inhibitory hormone (GnIH): biosynthesis, mode of action and functional significance. *Prog Neurobiol* (2009) **88**:76–88. doi:10.1016/j.pneurobio.2009.02.003
15. Tsutsui K, Bentley GE, Bedecarrats G, Osugi T, Ubuka T, Kriegsfeld LJ. Review: gonadotropin-inhibitory hormone (GnIH) and its control of central and peripheral reproductive function. *Front Neuroendocrinol* (2010) **31**:284–95. doi:10.1016/j.yfrne.2010.03.001
16. Tsutsui K, Bentley GE, Kriegsfeld LJ, Osugi T, Seong JY, Vaudry H. Review: discovery and evolutionary history of gonadotrophin-inhibitory hormone and kisspeptin: new key neuropeptides controlling reproduction. *J Neuroendocrinol* (2010) **22**:716–27. doi:10.1111/j.1365-2826.2010.02018.x
17. Tsutsui K, Ubuka T, Bentley GE, Kriegsfeld LJ. Review: gonadotropin-inhibitory hormone (GnIH): discovery, progress and prospect. *Gen Comp Endocrinol* (2012) **177**:305–14. doi:10.1016/j.ygcen.2012.02.013
18. Tsutsui K, Ubuka T, Bentley GE, Kriegsfeld LJ. Review: regulatory mechanisms of gonadotropin-inhibitory hormone (GnIH) synthesis and release in photoperiodic animals. *Front Neurosci* (2013) **7**:60. doi:10.3389/fnins.2013.00060
19. Tsutsui K, Ubuka T. Gonadotropin-inhibitory Hormone. In: Kastin AJ, Vaudry H, editors. *Handbook of Biologically Active Peptides. Section on Brain Peptides.* London: Academic Press (2012). p. 802–11.
20. Ubuka T, Son YL, Tobari Y, Tsutsui K. Gonadotropin-inhibitory hormone action in the brain and pituitary. *Front Endocrinol (Lausanne)* (2012) **3**:148. doi:10.3389/fendo.2012.00148
21. Ubuka T, Son YL, Bentley GE, Millar RP, Tsutsui K. Gonadotropin-inhibitory hormone (GnIH), GnIH receptor and cell signaling. *Gen Comp Endocrinol* (2013) **190**:10–7. doi:10.1016/j.ygcen.2013.02.030
22. Maddineni SR, Ocón-Grove OM, Krzysik-Walker SM, Hendricks GL III, Ramachandran R. Gonadotropin-inhibitory hormone (GnIH) receptor gene is expressed in the chicken ovary: potential role of GnIH in follicular maturation. *Reproduction* (2008) **135**:267–74. doi:10.1530/REP-07-0369
23. McGuire NL, Kangas K, Bentley GE. Effects of melatonin on peripheral reproductive function: regulation of testicular GnIH and testosterone. *Endocrinology* (2011) **152**:3461–70. doi:10.1210/en.2011-1053
24. Singh P, Krishna A, Sridaran R, Tsutsui K. Immunohistochemical localization of GnRH and RFamide-related peptide-3 in the ovaries of mice during the estrous cycle. *J Mol Histol* (2011) **42**:371–81. doi:10.1007/s10735-011-9340-8
25. Singh P, Krishna A, Tsutsui K. Effects of gonadotropin-inhibitory hormone on folliculogenesis and steroidogenesis of cyclic mice. *Fertil Steril* (2011) **95**:1397–404. doi:10.1016/j.fertnstert.2010.03.052
26. Li X, Su J, Lei Z, Zhao Y, Jin M, Fang R, et al. Gonadotropin-inhibitory hormone (GnIH) and its receptor in the female pig: cDNA cloning, expression in

tissues and expression pattern in the reproductive axis during the estrous cycle. *Peptides* (2012) **36**:176–85. doi:10.1016/j.peptides.2012.05.008
27. Oishi H, Klausen C, Bentley GE, Osugi T, Tsutsui K, Gilks CB, et al. The human gonadotropin-inhibitory hormone ortholog RFamide-related peptide-3 suppresses gonadotropin-induced progesterone production in human granulosa cells. *Endocrinology* (2012) **153**:3435–45. doi:10.1210/en.2012-1066
28. Satake H, Hisada M, Kawada T, Minakata H, Ukena K, Tsutsui K. Characterization of a cDNA encoding a novel avian hypothalamic neuropeptide exerting an inhibitory effect on gonadotropin release. *Biochem J* (2001) **354**:379–85. doi:10.1042/0264-6021:3540379
29. Ikemoto T, Park MK. Chicken RFamide-related peptide (GnIH) and two distinct receptor subtypes: identification, molecular characterization, and evolutionary considerations. *J Reprod Dev* (2005) **51**:359–77. doi:10.1262/jrd.16087
30. Osugi T, Ukena K, Bentley GE, O'Brien S, Moore IT, Wingfield JC, et al. Gonadotropin-inhibitory hormone in Gambel's white-crowned sparrow (*Zonotrichia leucophrys gambelii*): cDNA identification, transcript localization and functional effects in laboratory and field experiments. *J Endocrinol* (2004) **182**:33–42. doi:10.1677/joe.0.1820033
31. Ubuka T, Kim S, Huang YC, Reid J, Jiang J, Osugi T, et al. Gonadotropin-inhibitory hormone neurons interact directly with gonadotropin-releasing hormone-I and -II neurons in European starling brain. *Endocrinology* (2008) **149**:268–78. doi:10.1210/en.2007-0983
32. Tobari Y, Iijima N, Tsunekawa K, Osugi T, Okanoya K, Tsutsui K, et al. Identification of gonadotropin-inhibitory hormone in the zebra finch (*Taeniopygia guttata*): peptide isolation, cDNA cloning and brain distribution. *Peptides* (2010) **31**:816–26. doi:10.1016/j.peptides.2010.01.015
33. Ubuka T, Morgan K, Pawson AJ, Osugi T, Chowdhury VS, Minakata H, et al. Identification of human GnIH homologs, RFRP-1 and RFRP-3, and the cognate receptor, GPR147 in the human hypothalamic pituitary axis. *PLoS One* (2009) **4**:e8400. doi:10.1371/journal.pone.0008400
34. Ubuka T, Lai H, Kitani M, Suzuuchi A, Pham V, Cadigan PA, et al. Gonadotropin-inhibitory hormone identification, cDNA cloning, and distribution in rhesus macaque brain. *J Comp Neurol* (2009) **517**:841–55. doi:10.1002/cne.22191
35. Fukusumi S, Habata Y, Yoshida H, Iijima N, Kawamata Y, Hosoya M, et al. Characteristics and distribution of endogenous RFamide-related peptide-1. *Biochim Biophys Acta* (2001) **1540**:221–32. doi:10.1016/S0167-4889(01)00135-5
36. Yoshida H, Habata Y, Hosoya M, Kawamata Y, Kitada C, Hinuma S. Molecular properties of endogenous RFamide-related peptide-3 and its interaction with receptors. *Biochim Biophys Acta* (2003) **1593**:151–7. doi:10.1016/S0167-4889(02)00389-0
37. Clarke IJ, Sari IP, Qi Y, Smith JT, Parkington HC, Ubuka T, et al. Potent action of RFamide-related peptide-3 on pituitary gonadotropes indicative of a hypophysiotropic role in the negative regulation of gonadotropin secretion. *Endocrinology* (2008) **149**:5811–21. doi:10.1210/en.2008-0575
38. Ukena K, Iwakoshi E, Minakata H, Tsutsui K. A novel rat hypothalamic RFamide-related peptide identified by immunoaffinity chromatography and mass spectrometry. *FEBS Lett* (2002) **512**:255–8. doi:10.1016/S0014-5793(02)02275-5
39. Ubuka T, Inoue K, Fukuda Y, Mizuno T, Ukena K, Kriegsfeld LJ, et al. Identification, expression, and physiological functions of Siberian hamster gonadotropin-inhibitory hormone. *Endocrinology* (2012) **153**:373–85. doi:10.1210/en.2011-1110
40. Bonini JA, Jones KA, Adham N, Forray C, Artymyshyn R, Durkin MM, et al. Identification and characterization of two G protein-coupled receptors for neuropeptide FF. *J Biol Chem* (2000) **275**:39324–31. doi:10.1074/jbc.M004385200
41. Hinuma S, Shintani Y, Fukusumi S, Iijima N, Matsumoto Y, Hosoya M, et al. New neuropeptides containing carboxy-terminal RFamide and their receptor in mammals. *Nat Cell Biol* (2000) **2**:703–8. doi:10.1038/35036326
42. Liu Q, Guan XM, Martin WJ, McDonald TP, Clements MK, Jiang Q, et al. Identification and characterization of novel mammalian neuropeptide FF-like peptides that attenuate morphine-induced antinociception. *J Biol Chem* (2001) **276**:36961–9. doi:10.1074/jbc.M105308200
43. Yin H, Ukena K, Ubuka T, Tsutsui K. A novel G protein-coupled receptor for gonadotropin-inhibitory hormone in the Japanese quail (*Coturnix japonica*): identification, expression and binding activity. *J Endocrinol* (2005) **184**:257–66. doi:10.1677/joe.1.05926
44. Son YL, Ubuka T, Millar RP, Kanasaki H, Tsutsui K. Gonadotropin-inhibitory hormone inhibits GnRH-induced gonadotropin subunit gene transcriptions by inhibiting AC/cAMP/PKA-dependent ERK pathway in LβT2 cells. *Endocrinology* (2012) **153**:2332–43. doi:10.1210/en.2011-1904
45. Shimizu M, Bédécarrats GY. Activation of the chicken gonadotropin-inhibitory hormone receptor reduces gonadotropin releasing hormone receptor signaling. *Gen Comp Endocrinol* (2010) **167**:331–7. doi:10.1016/j.ygcen.2010.03.029
46. Joseph NT, Morgan K, Sellar R, McBride D, Millar RP, Dunn IC. The chicken type III GnRH receptor homologue is predominantly expressed in the pituitary, and exhibits similar ligand selectivity to the type I receptor. *J Endocrinol* (2009) **202**:179–90. doi:10.1677/JOE-08-0544
47. Shimizu M, Bédécarrats GY. Identification of a novel pituitary-specific chicken gonadotropin-releasing hormone receptor and its splice variants. *Biol Reprod* (2006) **75**:800–8. doi:10.1095/biolreprod.105.050252
48. Ukena K, Ubuka T, Tsutsui K. Distribution of a novel avian gonadotropin-inhibitory hormone in the quail brain. *Cell Tissue Res* (2003) **312**:73–9. doi:10.1007/s00441-003-0700-x
49. Ubuka T, Ueno M, Ukena K, Tsutsui K. Developmental changes in gonadotropin-inhibitory hormone in the Japanese quail (*Coturnix japonica*) hypothalamo-hypophysial system. *J Endocrinol* (2003) **178**:311–8. doi:10.1677/joe.0.1780311
50. Bentley GE, Perfito N, Ukena K, Tsutsui K, Wingfield JC. Gonadotropin-inhibitory peptide in song sparrows (*Melospiza melodia*) in different reproductive conditions, and in house sparrows (*Passer domesticus*) relative to chicken-gonadotropin-releasing hormone. *J Neuroendocrinol* (2003) **15**:794–802. doi:10.1046/j.1365-2826.2003.01062.x
51. Kriegsfeld LJ, Mei DF, Bentley GE, Ubuka T, Mason AO, Inoue K, et al. Identification and characterization of a gonadotropin-inhibitory system in the brains of mammals. *Proc Natl Acad Sci U S A* (2006) **103**:2410–5. doi:10.1073/pnas.0511003103
52. Legagneux K, Bernard-Franchi G, Poncet F, La Roche A, Colard C, Fellmann D, et al. Distribution and genesis of the RFRP-producing neurons in the rat brain: comparison with melanin-concentrating hormone- and hypocretin-containing neurons. *Neuropeptides* (2009) **43**:13–9. doi:10.1016/j.npep.2008.11.001
53. Sharp PJ, Talbot RT, Main GM, Dunn IC, Fraser HM, Huskisson NS. Physiological roles of chicken LHRH-I and -II in the control of gonadotrophin release in the domestic chicken. *J Endocrinol* (1990) **124**:291–9. doi:10.1677/joe.0.1240291
54. Ubuka T, Bentley GE. Identification, localization, and regulation of passerine GnRH-I messenger RNA. *J Endocrinol* (2009) **201**:81–7. doi:10.1677/JOE-08-0508
55. Ubuka T, Bentley GE. Neuroendocrine control of reproduction in birds. In: Norris DO, Lopez KH, editors. *Hormones and Reproduction of Vertebrates-Vol. 4: Birds.* London: Academic Press (2011). p. 1–25.
56. Ubuka T, Cadigan PA, Wang A, Liu J, Bentley GE. Identification of European starling GnRH-I precursor mRNA and its seasonal regulation. *Gen Comp Endocrinol* (2009) **162**:301–6. doi:10.1016/j.ygcen.2009.04.001
57. Ubuka T, Bentley GE, Tsutsui K. Neuroendocrine regulation of gonadotropin secretion in seasonally breeding birds. *Front Neurosci* (2013) **7**:38. doi:10.3389/fnins.2013.00038
58. Miyamoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H. Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. *Proc Natl Acad Sci U S A* (1984) **81**:3874–8. doi:10.1073/pnas.81.12.3874
59. Millar RP. GnRH II and type II GnRH receptors. *Trends Endocrinol Metab* (2003) **14**:35–43. doi:10.1016/S1043-2760(02)00016-4
60. Maney DL, Richardson RD, Wingfield JC. Central administration of chicken gonadotropin-releasing hormone-II enhances courtship behavior in a female sparrow. *Horm Behav* (1997) **32**:11–8. doi:10.1006/hbeh.1997.1399
61. Temple JL, Millar RP, Rissman EF. An evolutionarily conserved form of gonadotropin-releasing hormone coordinates energy and reproductive behavior. *Endocrinology* (2003) **144**:13–9. doi:10.1210/en.2002-220883
62. Barnett DK, Bunnell TM, Millar RP, Abbott DH. Gonadotropin-releasing hormone II stimulates female sexual behavior in marmoset monkeys. *Endocrinology* (2006) **147**:615–23. doi:10.1210/en.2005-0662

63. Ubuka T, Mukai M, Wolfe J, Beverly R, Clegg S, Wang A, et al. RNA interference of gonadotropin-inhibitory hormone gene induces arousal in songbirds. *PLoS One* (2012) **7**:e30202. doi:10.1371/journal.pone.0030202
64. Rizwan MZ, Poling MC, Corr M, Cornes PA, Augustine RA, Quennell JH, et al. RFamide-related peptide-3 receptor gene expression in GnRH and kisspeptin neurons and GnRH-dependent mechanism of action. *Endocrinology* (2012) **153**:3770–9. doi:10.1210/en.2012-1133
65. Bentley GE, Jensen JP, Kaur GJ, Wacker DW, Tsutsui K, Wingfield JC. Rapid inhibition of female sexual behavior by gonadotropin-inhibitory hormone (GnIH). *Horm Behav* (2006) **49**:550–5. doi:10.1016/j.yhbeh.2005.12.005
66. Johnson MA, Tsutsui K, Fraley GS. Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat. *Horm Behav* (2007) **51**:171–80. doi:10.1016/j.yhbeh.2006.09.009
67. Ubuka T, Ukena K, Sharp PJ, Bentley GE, Tsutsui K. Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail. *Endocrinology* (2006) **147**:1187–94. doi:10.1210/en.2005-1178
68. Ducret E, Anderson GM, Herbison AE. RFamide-related peptide-3, a mammalian gonadotropin-inhibitory hormone ortholog, regulates gonadotropin-releasing hormone neuron firing in the mouse. *Endocrinology* (2009) **150**:2799–804. doi:10.1210/en.2008-1623
69. Wu M, Dumalska I, Morozova E, van den Pol AN, Alreja M. Gonadotropin inhibitory hormone inhibits basal forebrain vGluT2-gonadotropin-releasing hormone neurons via a direct postsynaptic mechanism. *J Physiol* (2009) **587**:1401–11. doi:10.1113/jphysiol.2008.166447
70. Ciccone NA, Dunn IC, Boswell T, Tsutsui K, Ubuka T, Ukena K, et al. Gonadotrophin inhibitory hormone depresses gonadotrophin alpha and follicle-stimulating hormone beta subunit expression in the pituitary of the domestic chicken. *J Neuroendocrinol* (2004) **16**:999–1006. doi:10.1111/j.1365-2826.2005.01260.x
71. Gibson EM, Humber SA, Jain S, Williams WP III, Zhao S, Bentley GE, et al. Alterations in RFamide-related peptide expression are coordinated with the preovulatory luteinizing hormone surge. *Endocrinology* (2008) **149**:4958–69. doi:10.1210/en.2008-0316
72. Sari IP, Rao A, Smith JT, Tilbrook AJ, Clarke IJ. Effect of RF-amide-related peptide-3 on luteinizing hormone and follicle-stimulating hormone synthesis and secretion in ovine pituitary gonadotropes. *Endocrinology* (2009) **150**:5549–56. doi:10.1210/en.2009-0775
73. Kadokawa H, Shibata M, Tanaka Y, Kojima T, Matsumoto K, Oshima K, et al. Bovine C-terminal octapeptide of RFamide-related peptide-3 suppresses luteinizing hormone (LH) secretion from the pituitary as well as pulsatile LH secretion in bovines. *Domest Anim Endocrinol* (2009) **36**:219–24. doi:10.1016/j.domaniend.2009.02.001
74. Murakami M, Matsuzaki T, Iwasa T, Yasui T, Irahara M, Osugi T, et al. Hypophysiotropic role of RFamide-related peptide-3 in the inhibition of LH secretion in female rats. *J Endocrinol* (2008) **199**:105–12. doi:10.1677/JOE-08-0197
75. Smith JT, Young IR, Veldhuis JD, Clarke IJ. Gonadotropin-inhibitory hormone (GnIH) secretion into the ovine hypophyseal portal system. *Endocrinology* (2012) **153**:3368–75. doi:10.1210/en.2012-1088
76. Anjum S, Krishna A, Sridaran R, Tsutsui K. Localization of gonadotropin-releasing hormone (GnRH), gonadotropin-inhibitory hormone (GnIH), kisspeptin and GnRH receptor and their possible roles in testicular activities from birth to senescence in mice. *J Exp Zool A Ecol Genet Physiol* (2012) **317**:630–44. doi:10.1002/jez.1765
77. Bronson FH. *Mammalian Reproductive Biology*. Chicago: University of Chicago Press (1990).
78. Wilson FE. Neither retinal nor pineal photoreceptors mediate photoperiodic control of seasonal reproduction in American tree sparrows (*Spizella arborea*). *J Exp Zool* (1991) **259**:117–27. doi:10.1002/jez.1402590114
79. Juss TS, Meddle SL, Servant RS, King VM. Melatonin and photoperiodic time measurement in Japanese quail (*Coturnix coturnix japonica*). *Proc Biol Sci* (1993) **254**:21–8. doi:10.1098/rspb.1993.0121
80. Ohta M, Kadota C, Konishi H. A role of melatonin in the initial stage of photoperiodism in the Japanese quail. *Biol Reprod* (1989) **40**:935–41. doi:10.1095/biolreprod40.5.935
81. Guyomarc'h C, Lumineau S, Vivien-Roels B, Richard J, Deregnaucourt S. Effect of melatonin supplementation on the sexual development in European quail (*Coturnix coturnix*). *Behav Processes* (2001) **53**:121–30. doi:10.1016/S0376-6357(01)00133-4
82. Rozenboim I, Aharony T, Yahav S. The effect of melatonin administration on circulating plasma luteinizing hormone concentration in castrated White Leghorn roosters. *Poult Sci* (2002) **81**:1354–9.
83. Greives TJ, Kingma SA, Beltrami G, Hau M. Melatonin delays clutch initiation in a wild songbird. *Biol Lett* (2012) **8**:330–2. doi:10.1098/rsbl.2011.1100
84. Ubuka T, Bentley GE, Ukena K, Wingfield JC, Tsutsui K. Melatonin induces the expression of gonadotropin-inhibitory hormone in the avian brain. *Proc Natl Acad Sci U S A* (2005) **102**:3052–7. doi:10.1073/pnas.0403840102
85. Underwood H, Binkley S, Siopes T, Mosher K. Melatonin rhythms in the eyes, pineal bodies, and blood of Japanese quail (*Coturnix coturnix japonica*). *Gen Comp Endocrinol* (1984) **56**:70–81. doi:10.1016/0016-6480(84)90063-7
86. Chowdhury VS, Yamamoto K, Ubuka T, Bentley GE, Hattori A, Tsutsui K. Melatonin stimulates the release of gonadotropin-inhibitory hormone by the avian hypothalamus. *Endocrinology* (2010) **151**:271–80. doi:10.1210/en.2009-0908
87. Revel FG, Saboureau M, Pévet P, Simonneaux V, Mikkelsen JD. RFamide-related peptide gene is a melatonin-driven photoperiodic gene. *Endocrinology* (2008) **149**:902–12. doi:10.1210/en.2007-0848
88. Mason AO, Duffy S, Zhao S, Ubuka T, Bentley GE, Tsutsui K, et al. Photoperiod and reproductive condition are associated with changes in RFamide-related peptide (RFRP) expression in Syrian hamsters (*Mesocricetus auratus*). *J Biol Rhythms* (2010) **25**:176–85. doi:10.1177/0748730410368821
89. Dardente H, Birnie M, Lincoln GA, Hazlerigg DG. RFamide-related peptide and its cognate receptor in the sheep: cDNA cloning, mRNA distribution in the hypothalamus and the effect of photoperiod. *J Neuroendocrinol* (2008) **20**:1252–9. doi:10.1111/j.1365-2826.2008.01784.x
90. Smith JT, Coolen LM, Kriegsfeld LJ, Sari IP, Jaafarzadehshirazi MR, Maltby M, et al. Variation in kisspeptin and RFamide-related peptide (RFRP) expression and terminal connections to gonadotropin-releasing hormone neurons in the brain: a novel medium for seasonal breeding in the sheep. *Endocrinology* (2008) **149**:5770–82. doi:10.1210/en.2008-0581
91. Gingerich S, Wang X, Lee PK, Dhillon SS, Chalmers JA, Koletar MM, et al. The generation of an array of clonal, immortalized cell models from the rat hypothalamus: analysis of melatonin effects on kisspeptin and gonadotropin-inhibitory hormone neurons. *Neuroscience* (2009) **162**:1134–40. doi:10.1016/j.neuroscience.2009.05.026
92. Chand D, Lovejoy DA. Stress and reproduction: controversies and challenges. *Gen Comp Endocrinol* (2011) **171**:253–7. doi:10.1016/j.ygcen.2011.02.022
93. Calisi RM, Rizzo NO, Bentley GE. Seasonal differences in hypothalamic EGR-1 and GnIH expression following capture-handling stress in house sparrows (*Passer domesticus*). *Gen Comp Endocrinol* (2008) **157**:283–7. doi:10.1016/j.ygcen.2008.05.010
94. McGuire NL, Koh A, Bentley GE. The direct response of the gonads to cues of stress in a temperate songbird species is season-dependent. *PeerJ* (2013) **1**:e139. doi:10.7717/peerj.139
95. Kirby ED, Geraghty AC, Ubuka T, Bentley GE, Kaufer D. Stress increases putative gonadotropin inhibitory hormone and decreases luteinizing hormone in male rats. *Proc Natl Acad Sci U S A* (2009) **106**:11324–9. doi:10.1073/pnas.0901176106
96. Calisi RM, Díaz-Muñoz SL, Wingfield JC, Bentley GE. Social and breeding status are associated with the expression of GnIH. *Genes Brain Behav* (2011) **10**:557–64. doi:10.1111/j.1601-183X.2011.00693.x
97. Delville Y, Sulon J, Hendrick JC, Balthazart J. Effect of the presence of females on the pituitary-testicular activity in male Japanese quail (*Coturnix coturnix japonica*). *Gen Comp Endocrinol* (1984) **55**:295–305. doi:10.1016/0016-6480(84)90115-1
98. Cornil CA, Stevenson TJ, Ball GF. Are rapid changes in gonadal testosterone release involved in the fast modulation of brain estrogen effects? *Gen Comp Endocrinol* (2009) **163**:298–305. doi:10.1016/j.ygcen.2009.04.029

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