# PLASTICITY IN THE VERTEBRATE PITUITARY, INCLUDING REGULATORY MECHANISMS

EDITED BY: Finn-Arne Weltzien, Karine Rizzoti and Romain Fontaine
PUBLISHED IN: Frontiers in Endocrinology

frontiers Research Topics

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ISSN 1664-8714
ISBN 978-2-88971-040-9
DOI 10.3389/978-2-88971-040-9

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# PLASTICITY IN THE VERTEBRATE PITUITARY, INCLUDING REGULATORY MECHANISMS

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**Citation:** Weltzien, F.-A., Rizzoti, K., Fontaine, R., eds. (2021). Plasticity in the Vertebrate Pituitary, Including Regulatory Mechanisms. Lausanne: Frontiers Media SA. doi: 10.3389/978-2-88971-040-9

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frontiers in Endocrinology

EDITORIAL
published: 18 May 2021
doi: 10.3389/fendo.2021.698916

# Editorial: Plasticity in the Vertebrate Pituitary, Including Regulatory Mechanisms

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**Keywords: pituitary, plasticity, adenohypophysis, hormones, stem cells, proliferation, transdifferentiation, regulation**

**Editorial on the Research Topic**

**Plasticity in the Vertebrate Pituitary, Including Regulatory Mechanisms**

## INTRODUCTION

In all vertebrates, the pituitary is involved in the control of many important and complex physiological processes such as growth, metabolism, homeostasis, reproduction, metamorphosis, and stress response. Located below the hypothalamus, the pituitary is divided into two main parts: the neurohypophysis (pars nervosa) and the adenohypophysis (pars distalis and pars intermedia). The latter, also named anterior pituitary (AP), is composed of up to 8 different hormone-producing cell types, highly specialized as they each mainly produce one type of hormone (1).

The adenohypophysis is characterized by its high plasticity, which is required to adapt hormone production to demand according to the environmental experience and life cycle of the animal. Pituitary plasticity occurs at three different levels (1): i) At the cellular level, cells can change activity in response to different signals by regulating receptor expression and by controlling hormone production and release; ii) At the structural level, cells can migrate and send projections that allows formation of homotypic and heterotypic networks that may influence the secretory behavior of the cells; iii) and finally at the population level, as endocrine cell numbers can be modulated. The latter encompasses differentiation of progenitor cells but also proliferation and changes of endocrine phenotype of specific hormone-producing cells which modify the cell type composition in the pituitary as a whole. Research questions such as when in the life cycle or under which environmental conditions does this plasticity play a role? What are the mechanisms allowing it? How are these mechanisms regulated? have been investigated for a long time but more intensely during the last decade due to the development of more powerful methodological tools.

On this basis, we conceived and coordinated the publication of a Research Topic on the "Plasticity in the Vertebrate Pituitary, Including Regulatory Mechanisms." This e-book presents the collection of three original research articles and 8 reviews, covering the different mechanisms involved in the three levels of plasticity described above, the way they are regulated, and the role they play in the life cycle of an animal, across vertebrates.

OPEN ACCESS

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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *22 April 2021*
***Accepted:*** *03 May 2021*
***Published:*** *18 May 2021*

***Citation:***
*Fontaine R, Rizzoti K and Weltzien F-A (2021) Editorial: Plasticity in the Vertebrate Pituitary, Including Regulatory Mechanisms. Front. Endocrinol. 12:698916. doi: 10.3389/fendo.2021.698916*

## MECHANISMS FOR PITUITARY PLASTICITY

One original research article and five reviews discuss the different mechanisms involved in anterior pituitary plasticity.

First, an original research article from Rojo-Ruiz et al. explores the cellular level of plasticity by investigating the contribution of endoplasmatic reticulum (ER) calcium release following stimulation by two important hypothalamic signals (GnRH and TRH) in transgenic mice expressing an ubiquitous calcium sensor. The authors report that while GnRH and TRH induce robust decreases of ER calcium concentration and concomitant rises of cytosolic calcium concentration, depolarization with potassium triggers a rise of cytosolic calcium concentration alone, indicating that the calcium-induced calcium-release through ryanodine receptors is not present in AP cells. A mini review from Clay et al. also explores the plasticity at cellular level, focusing on gonadotropes. It discusses the functional and organizational plasticity, including changes in sensitivity to GnRH and in cellular morphology, and their critical role for the generation of the LH surge leading to ovulation.

Second, the mini review from Childs et al. discusses the presence and potential role of multihormonal cells and the functional plasticity in somatotropes and gonadotropes. The review from Laporte et al. extensively discusses the potential role for stem cells during physiological and pathological remodeling of pituitary endocrine populations, from maturation of the post-natal organ to tumoral development. Along with the mini review from Camilletti et al., it also provides an interesting overview of the major advances in the development of protocols to generate specific pituitary hormone-producing cell types from stem cells, and discuss their application in pituitary diseases.

Finally, the review from Santiago-Andres et al. focuses on the plasticity at the structural level and discusses the establishment and role of pituitary cell networks throughout vertebrate evolution. As most endocrine cell populations have been shown to form homo- and heterotypic networks in several vertebrate groups, this review highlights the way by which pituitary networks serve to optimize generation of hormone pulses in vertebrates.

## REGULATION OF PITUITARY PLASTICITY

One original article and two reviews discuss the neuroendocrine and endocrine regulation of pituitary cells. Fleming et al. present an original research article on thyrotropes, characterizing the regulation of the two *tshb* genes that derived from the teleost-specific whole genome duplication (2) and are produced in two different cell types (3). Measuring their transcript levels in primary pituitary cell cultures, the authors report a differential regulation of the expression of the two *tshb* paralogs by CRH, somatostatin, TRH, thyroid hormones, cortisol and insulin-like growth factor 1. The review from Fontaine et al. focuses on the regulation of gonadotropes in fishes by internal factors, analyzing the extensive literature on the effect of sex steroids feedback mechanisms. It provides information on the complex effects of the different sex steroids which depend on their nature (androgens versus estrogens), on the species, sex of the animal and its physiological state. It discusses the three different levels of plasticity and discriminates direct effects on the gonadotropes from indirect effects at the brain and pituitary levels. In contrast, the second review from Vélez and Unniappan focuses on somatotropes and provides an overview and update on GH regulators, focusing on the main endogenous and synthetic factors, taking a comparative point of view within vertebrates.

One original article and one review discuss the environmental control of pituitary plasticity. The original research article from Ma et al. investigates seasonal changes in circulating LH and GH concentrations *in vivo* in female goldfish, and the sensitivity of transcript levels for several genes involved in reproduction and growth. The authors demonstrate the seasonality of LH, but not GH, circulating levels and the seasonally dependent effects of GnIH, GnRH and T3 on circulating levels of LH and GH. The authors also report seasonal patterns in basal and GnRH- and/or GnIH-induced transcript levels for ERα, ERβI, FSHR, aromatase, TRαI, TRβ, IGF-I, and VTG in the liver and ovary. The review by Ciani et al. summarizes the scarce literature on the regulation of anterior pituitary endocrine cell types by environmental factors through melatonin signaling in two vertebrate groups, mammals and fishes. It reveals the growing interest for the role of thyrotropes in the seasonal neuroendocrine regulation of the gonadotrope axis. Also distinguishing direct from indirect effects of melatonin, the comparison between these two vertebrate groups reveals similarities in several regulatory mechanisms.

In conclusion, this Research Topic offers an exhaustive update on the different aspects of pituitary plasticity, highlighting novels paradigms in primary research manuscripts, and synthetizing current knowledge in review articles. We hope that this compendium will remain useful to researchers interested in vertebrate neuroendocrinology.

## AUTHOR CONTRIBUTIONS

RF wrote the manuscript. KR and F-AW edited the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was funded by the University of Life Sciences and in part by the Wellcome Trust [grant number FC001107].

## ACKNOWLEDGMENTS

We want to thank the authors, reviewers, and editorial staff, whose contributions were immeasurable to the success of this Research Topic and the resulting e-book.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 07 April 2020
doi: 10.3389/fendo.2020.00175

# Seasonal Related Multifactorial Control of Pituitary Gonadotropin and Growth Hormone in Female Goldfish: Influences of Neuropeptides and Thyroid Hormone

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Female reproduction is under multifactorial control of brain-pituitary-peripheral origin. The present study provides information on seasonal changes in circulating LH and GH concentrations, as well as transcript levels for a number of genes involved in the regulation of reproduction and growth in female goldfish. We also provide information on the effects of treatments with GnRH and/or GnIH, and their interaction with T3, at three stages of gonadal recrudescence. Maximum basal concentration of LH was observed at late recrudescence (Spring) while no seasonal changes in basal serum GH levels was detected. Serum LH and GH levels were stimulated by GnRH as expected, depending on the season. GnIH stimulated basal GH concentrations in gonadally regressed fish. GnIH inhibitory action on GnRH-induced LH response was observed in late, but not in mid recrudescence. T3 actions on basal and GnRH- or GnIH-induced GH secretion were generally inhibitory, depending on season. Administration of T3 attenuated GnRH-induced LH responses in mid and late stages of gonadal recrudescence, and the presence of GnIH abolished inhibitory actions of T3 in fish at mid recrudescence. Our results also demonstrated seasonal patterns in basal and GnRH- and/or GnIH-induced transcript levels for ERα, ERβI, FSHR, aromatase, TRαI, TRβ, IGF-I, and Vtg in the liver and ovary. However, there were no clear correlations between changes in transcript levels and circulating levels of LH and GH. The results support the hypothesis that GnRH, GnIH, and T3 are contributing factors in complex reciprocal control of reproduction and growth in goldfish.

**Keywords: Gonadotropin-inhibitory hormone (GnIH), Gonadotropin-releasing hormone (GnRH), growth, reproduction, thyroid hormone, female, seasonality**

OPEN ACCESS

***Edited by:***
*Finn-Arne Weltzien, Norwegian University of Life Sciences, Norway*

***Reviewed by:***
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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *09 January 2020*
***Accepted:*** *11 March 2020*
***Published:*** *07 April 2020*

***Citation:***
*Ma Y, Ladisa C, Chang JP and Habibi HR (2020) Seasonal Related Multifactorial Control of Pituitary Gonadotropin and Growth Hormone in Female Goldfish: Influences of Neuropeptides and Thyroid Hormone. Front. Endocrinol. 11:175. doi: 10.3389/fendo.2020.00175*

## INTRODUCTION

In most seasonal reproducing oviparous species including fish, reproduction and growth cycles are usually not in-phase with one another because of the significant energy allocation needed to sustain each of these processes. The shift between reproduction and growth phase is associated with changes in the neuroendocrine control by hormones of brain-pituitary-peripheral axis, as well as the accompanying alterations in metabolism. Gonadotropin-releasing hormone (GnRH)

is an important neuroendocrine regulator of reproduction because of its ability to stimulate the release and gene expression of pituitary gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). Gonadotropins in turn promote gamete production and hormone production in the ovary and testis. Growth and metabolism are regulated by pituitary growth hormone (GH). GH production and secretion is stimulated by several neurohormones including GnRH (1–4). There are a number of vertebrate GnRH molecular forms and all vertebrates, including mammals, express multiple isoforms of GnRH (5). All vertebrates studied express GnRH2 in different parts of the brain, including the mid brain, and GnRH2 has been suggested to act as a neuromodulator of behavior, food intake, and energy balance rather than as a hypophysiotropic factor (6–8). GnRH3 is only found in teleosts and in species where the predominant vertebrate preoptic hypothalamic GnRH1 form is absent, GnRH3 acts as the main pituitary regulator (5). In goldfish, chicken (c)GnRHII (GnRH2) and salmon (s)GnRH (GnRH3) are the native isoforms (9). There is evidence that both GnRH2 and GnRH3 have hypophysiotropic functions in goldfish and both forms stimulate LH and GH production (2, 4, 5, 10).

While the importance of GnRH in the neuroendocrine regulation of reproduction in vertebrates including fish is well-accepted, there is clear evidence that GnRH is also a key factor in the control of somatotrope activity in fish. In goldfish, GnRH isoforms have been shown to directly stimulate GH release and synthesis from the pituitary (2, 11–16). GnRH binding sites have been observed in somatotrope cells in the pituitary of various fish species including goldfish, cichlids, and pejerry (*Odontesthes bonariensis*) (17–19). GnRH stimulatory actions on GH release has also been demonstrated in other fish species like tilapia (*Oreochromis niloticus*) (20), common carp (*Cyprinus carpio*) (21), and masu salmon (*Oncorhynchus masou*) (22). However, GnRH treatment was found to be without effect on GH production in turbot (*Scophthalmus maximus*) (23), eel (*Anguilla anguilla*) (24), and catfish (*Clarias gariepinus*) (25), indicating that species differences exist even among teleosts.

Multifactorial control of reproduction and growth also involves gonadotropin inhibitory hormone (GnIH). GnIH was first discovered in the Japanese quail (*Coturnix japonica*) as part of the RF-amide family of proteins and GnIH was found to inhibit FSH and LH production and secretion (26). The presence of multiple forms of GnIH and GnIH-related peptide transcripts have been demonstrated in each of a number of species (27). Goldfish have three GnIH genes, although only GnIH-3 (GnIH: SGTGLSATLPQRF-NH2) is expressed in the hypothalamus (28). In mammals and birds species studied, GnIH was found to inhibit gonadotrope functions (29–31). In fish and amphibians, GnIH effects are more complex and appear to be species specific (32). In cinnamon clownfish (*Amphiprion melanopus*), treatment with GnIH inhibited expression of gonadotropin α subunit as well as LHβ, and FSHβ subunits (33). In the cichlid fish (*Cichlasoma dimerus*), GnIH subtypes exert different actions; cdGnIH1 inhibited FSHβ and LH β expression, but cdGnIH2 stimulated FSHβ expression (34). In goldfish, GnIH exerts both stimulatory and inhibitory actions on gonadotropin and GH production, depending on the season (14, 35, 36). Treatment with GnIH inhibited both release and synthesis of LH during early recrudescence but not in late recrudescence goldfish (36). In grass puffer (*Takifugu alboplumbeus*), transcript levels for GnIH, FSH, and LH were found to be higher during spawning period, both *in vitro* and *in vivo* (37). GnIH also increased GH mRNA levels in grass puffer primary pituitary cell cultures (38) and GH release in cichlid *C. dimerus* (34). Similarly, GnIH stimulated GH release in mammals (39, 40). In static incubation of primary cultures of goldfish pituitary cells, GnIH stimulated GH release and mRNA expression in cells from goldfish in late recrudescence; however, GnIH reduced GH mRNA expression in cells obtained from early and mid recrudescence stage fish (14). *In vivo* application of GnIH to goldfish in early, mid, and late stages of recrudescence decreased serum GH concentration but increased pituitary GH mRNA levels (14). Overall, these studies have demonstrated GnIH has both stimulatory and inhibitory effects on LH and GH response that are species specific and seasonally related. To further add to the complexity of GnIH effects, many of these studies utilized mixed sex groups. The complete picture of GnIH regulation of somatotropes and gonadotropes in females remains to be clarified.

In addition to GnRH and GnIH, thyroid hormones (T3 and T4) also play important roles in the control of growth and reproduction. Thyroid hormones are also important factors involved in metamorphosis in amphibians and certain fish species (41–43). Thyroid hormones are known to work in concert with GH to increase growth and metabolism (44–47). Concomitant treatments of T4 and GH in Ames dwarf mice can increase body mass and growth to levels similar to regular non-dwarf mice (48). In contrast, thyroid hormones have both inhibitory and stimulatory effects on somatotrope functions in teleost species. For example, thyroid hormones directly inhibited GH release and synthesis in eel pituitary (49) but *in vivo* T3 treatments increased GH mRNA expression in rainbow trout (*Oncorhynchus mykiss*) (50) and had no effects in goldfish (51). A number of studies have demonstrated actions of thyroid hormone on reproduction in both female and male (52–56). In murrel, *Channa gachua*, and a carp, *Catla catla*, as well as Ranid frogs, GnRH variants increased plasma T4 levels (57, 58), although no changes in T3 levels were observed in the goldfish following GnRH treatment (59). Thyroid hormone levels in goldfish were found to undergo seasonal changes, increasing to maximum levels during the growth phase, and reaching a nadir during spawning (60). While injection with T3 reduced circulating estradiol (E2) level in male, it had no effect in female goldfish (51). T3 treatment increased vitellogenin (Vtg) mRNA levels in the liver of goldfish by increasing expression of estrogen receptor (ER)α mRNA levels at mid stages of gonadal recrudescence (61). In isolated rainbow trout ovarian follicles, T3 treatments enhanced gonadotropin-induced E2 secretion (53). In male fish, thyroid hormones significantly alter spermatogenesis (56). In zebrafish, T3 treatment was shown to stimulate Sertoli cell and spermatogonia type-A proliferation in testis (62).

The aim of the present study is to investigate the hypothesis that GnRH, GnIH, and T3 are players in the multifactorial regulation of growth and reproduction in female goldfish. To this end, the influence of GnRH and/or GnIH injections on

serum LH and GH levels, as well as transcript levels of several liver and ovarian genes important for growth and reproduction were monitored at three distinct ovarian recrudescence phases. Transcripts monitored include liver expression of ERs, Vtg, insulin-like growth factor (IGF-I), and thyroid hormone receptors (TRs); and ovarian expression of ERs, FSH receptor (FSHR), and aromatase. The effects of T3 injection on GnRH-elicited and GnIH-induced serum LH and GH responses were also examined to gain insight into the possible influences of thyroid hormones.

# MATERIALS AND METHODS

## Animals

Common goldfish (*Carassius auratus*) were purchased from Aquatic Imports (Calgary, AB, Canada; goldfish were imported from fish farm exposed to natural daylight and temperature cycles in Pennsylvania, USA). A total of 360 fish (20 fish per treatment group) was used per season representing different gonadal stages: regressed stage (July-August), mid recrudescence (December–January), late recrudescence (March–April). Average body weight of fish in regressed and mid recrudescence stage were 60 g, average weight for late recrudescence fish was 22 g, all fish were at least 1 year old and post pubertal. The difference in fish size was due to the supplier (Aquatic Imports, Calgary, AB, Canada) not able to provide the large number of fish needed for experiments at the time. Fish were housed in a flow-through system with daylight cycles and temperatures adjusted to match conditions in their previous environment to preserve seasonality and allowed to acclimate for 4–7 days prior to use (**Table 1**). Goldfish were fed once a day to satiation 2 h after lights on, with commercial flake diet (Nutrafin, Baie d'Urfé, QC, Canada). Buffered tricane methanesulfonate solution (MS-222, 160 mg/l, Sigma Aldrich St Louis, Missouri, USA) was used to anesthetize the animals prior to injection treatments, as well as to euthanize the animals after 24 h of treatment. Gonadal recrudescence stages were determined from visual inspection of goldfish ovaries after euthanization. Gonadal regressed stage is characterized by small ovaries and lack of developed follicles (July–August). Ovaries in mid recrudescence contained follicles that continue to increase in size for maximum levels of vitellogenesis (December-January). In late recrudescence, the ovaries are fully mature and have visible oocytes that are ready for ovulation (March–April). Sample of ovaries, liver, and blood from female fish were used for this study and separated based on the above criteria for gonadal recrudescence stage. All animal protocols were approved by the university's animal care committee and in accordance with the Canadian Council on Animal Care's guidelines.

**TABLE 1 |** Temperature and daylight cycles for goldfish housing in the three seasonal ovarian reproductive stages.

| Month | Gonadal phase | Water temperature (°C) | Daylight (h) |
|---|---|---|---|
| December–January | Mid recrudescence | 14 | 12 |
| March–April | Late recrudescence | 16 | 14 |
| June–August | Regressed | 19 | 14 |

*Lights were turned on at 9 a.m. every day and turned off at 9 or 11 p.m. These conditions are appropriate for preservation of gonadal stage of fish and similar to those in their natural environment in Pennsylvania (USA).*

**TABLE 2 |** Factorial design of hormone combination treatments using sGnRH (GnRH) and goldfish GnIH (GnIH).

| Group | 0 h | 12 h |
|---|---|---|
| 1 | PBS | GnRH |
| 2 | GnIH | GnIH |
| 3 | GnIH | GnRH |
| 4 | GnIH | GnIH & GnRH |
| 5 | PBS | PBS |
| 6 | PBS | GnIH |
| 7 | GnRH | GnRH |
| 8 | GnIH & GnRH | GnRH |
| 9 | GnIH & GnRH | GnIH & GnRH |

*Intraperitoneal injections of GnRH (100 ng/g fish, per gram of fish wet weight) and/or GnIH (50 ng/g fish) occurred at 0 h (9 a.m.) and 12 h (9 p.m.), followed by samples collection at 24 h. Phosphate buffered saline (PBS) was used as sham injections, double sham injections (PBS + PBS; first injection + second injection) were used as controls. T3 experimental groups had the addition of T3 at a dose of 1 ng/g of fish in both 0 and 12 h injections.*

## Injection Treatments

Hormone treatments were given at 0 and 12 h (9 a.m. and 9 p.m.) as intraperitoneal (ip) injections of 100 μL; fish were sacrificed and samples collected at 24 h after the first injection (9 a.m. the following day). The time course of treatments were chosen based on previous studies demonstrating significant effects of GnRH and gonadal steroids at 12 and 24 h following treatments (2, 13, 14, 35, 36, 63–65). GnRH and GnIH treatments were administered using a factorial design (**Table 2**) and injected with or without the addition of T3 to the hormone mixture. Mixtures of hormones were administered as a single injection (100 μL) for combined hormone treatments. Double injection method was utilized as a previous study has shown GnRH induced a potentiated response when compared to single injection GnRH in stimulating LH release (66). GnRH (sGnRH: Pyr-HWSYGWLPG-NH2) was purchased from Bachem (Torrance, California, USA). Goldfish GnIH (GnIH: SGTGLSATLPQRF-NH2) was made by the University of Calgary Peptide Services (Calgary, AB, Canada). 3,5,3′ tri-iodothyronine (T3) was bought from Sigma Aldrich (Sigma Aldrich St Louis, Missouri, USA) and dissolved in 0.2 M NaOH then serially diluted using Ultrapure water (Sigma Aldrich St Louis, Missouri, USA) to a concentration of 10 ng/ μL to create a stock T3 solution. All hormones were further diluted with phosphate buffered saline (PBS) prior to injection. Sham injections of PBS were used as controls (PBS+PBS; first injection + second injection). Doses of hormones per fish were adjusted based on the dosages per gram of average weight of fish in each season. Doses of hormone injected were chosen based on previous studies in our lab: 100 ng GnRH/g of fish (wet body weight), 50 ng GnIH/g of fish, and 1 ng T3/g of fish (14, 54, 55). At 24 h, samples of blood, liver and gonads were collected and separated by sex. Only

**TABLE 3 |** Primers and annealing temperatures used for mRNA transcript quantification in real time QPCR analysis of liver and ovarian tissues.

| Gene | Accession numbers | Primer | Sequence (5′-3′) | Annealing temperature (°C) |
|---|---|---|---|---|
| β-actin | AB039726 | Forward | CCTCCATTGTTGGCAGACC | 57 |
| | | Reverse | CCTCTCTTGCTTTGAGCCTC | |
| GAPDH | KT985226.1 | Forward | TGATGCTGGTGCCCTGTATG TAGT | 57 |
| | | Reverse | TGTCCTGGTTGACTCCCATC ACAA | |
| Vtg | DQ641252 | Forward | GAAGTGCGCATGGTGGCTT GTATT | 55 |
| | | Reverse | AGCTGCCATATCAGGAGCA GTGAT | |
| IGF-I | GU583648.1 | Forward | CAGGGGCATTGGTGTGA | 57.1 |
| | | Reverse | GCAGCGTGTCTACAAGC | |
| ERα | AY055725.1 | Forward | GAGGAAGAGTAGCAGCACT G | 55 |
| | | Reverse | GGCTGTGTTTCTGTCGTGAG | |
| ERβI | AF061269.1 | Forward | GGCAGGATGAGAACAAGTG G | 55 |
| | | Reverse | GTAAATCTCGGGTGGCTCTG | |
| TRαI | AY973629.1 | Forward | AGCCTGCCATGCCAGCC | 55 |
| | | Reverse | CCTCCTGATCCTCGAAGACC | |
| TRβ | AY973630.1 | Forward | GAGGAGCAGCAGAAGACGG | 55 |
| | | Reverse | GTTGCCTTGGGCGTTTGTGG | |
| FSHR | HM347775.1 | Forward | CGTCCACAATCCTACCTTCG | 56 |
| | | Reverse | TGAGAAACGGTGATTAGCGG | |
| Cyp19a1 (aromatase) | AB009336.1 | Forward | TTGTGCGGGTTTGGATCAAT GGTG | 58 |
| | | Reverse | TTCCGATACACTGCAGACCC AGTT | |

*All primers were designed and validated using Primer3 online software (Whitehead Institute of Biomedical Research, Cambridge, MA, USA).*

samples from female fish were used for the current study. Serum samples were isolated from blood for radioimmunoassays for GH and LH using well-established protocols (12, 67). All samples were stored at −80°C until various assays were performed. Serum FSH was not measured because of the lack of a goldfish FSH radioimmunoassay.

## RNA Extraction and QPCR

Total RNA in samples of ovarian and liver tissue were extracted using Trizol Reagent (Invitrogen, Burlington, ON, Canada) in accordance with manufacturer's instructions. Total RNA quantity and purity was determined by Nanodrop spectrophotometer (Thermo Scientific, Waltham, MA, USA). This was followed by DNase digestion (DNase I, Thermo Scientific, Waltham, MA, USA) and cDNA synthesis (High Capacity Multiscribe cDNA kit, Invitrogen, Burlington, ON, Canada).

Liver and ovarian samples were analyzed with real time quantitative (Q)PCR using previously validated primer sets (**Table 3**; **Table S1**) (14, 36, 51, 54, 68). GAPDH and β-actin were used as internal controls for ovary and gonads, respectively, based on their stability of expression between treatment groups. All PCR reactions were run in triplicates using SsoFast Eva Green Supermix (BioRad, Mississauga, ON, Canada). The QPCR thermal cycling steps commenced with an initial denaturing step at 95°C for 2 min which was followed by 36 cycle repeats of 95°C for 15 s, 55–60°C appropriate annealing temperature (**Table 3**) for 15 s, and a final 72°C extension for 1 min. After QPCR amplification, melt curves for each plate were run to ensure only one product was amplified.

## Statistical Analysis

A maximum of $n = 12$ randomly selected serum samples were processed for RIAs and a maximum of $n = 9$ samples were used for QPCR. Basal circulating levels of GH and LH in the reproductive seasons were measured in the appropriate control groups, reported as actual concentrations (means ± SEM, ng/ml), and analyzed by one-way ANOVA followed by with Tukey's *post-hoc* honestly significant (HSD) multiple comparison tests. In particular to examine the influence of ip injected T3, hormone levels of experimental treatment groups were further normalized to the averaged values of the control group having the highest hormone concentration among the three seasonal reproductive stages, reported as a percentage value, and analyzed with two-way ANOVA followed by Bonferroni's multiple comparisons tests.

Basal QPCR results were normalized to the appropriate housekeeping gene (β-actin or GAPDH) using the using the ΔΔCq method. Housekeeping genes were chosen based on stability of expression between treatment and control groups, and lowest variation (SD) in Ct values. Based on this criteria GAPDH was chosen for the ovaries and β-actin for the liver. QPCR data of experimental groups were further normalized with respect to the averaged value of the control group with highest basal transcript levels among the three reproductive stages examined. All QPCR results were analyzed by the Kruskal-Wallis test followed by Dunn's multiple comparison test. Kruskal-Wallis test was chosen because some of the data sets did not follow normal distribution. Prism 7 was used for these statistical tests (GraphPad Software Inc., La Jolla, CA, USA). Differences are considered significant when $P < 0.05$.

# RESULTS

## Basal Levels of Serum LH and GH

Fish ovaries were visually inspected for gonadal recrudescence status of the goldfish (**Figure 1A**). The gonadal regressed season starts following ovulation when post-ovulatory ovaries are characterized by their small size and the absence of developed follicles (July–August). Early recrudescence begins when follicular cells multiply and increase in size for higher steroidogenic activity needed for vitellogenin production (September-October). Mid recrudescence ovaries are characterized by follicles that continue to increase in size and this season corresponds to a period of maximum levels of vitellogenesis for yolk production (December–January). Fully matured ovaries with visible oocytes ready for ovulation are present during late recrudescence (March–April). We investigated female goldfish during three stages

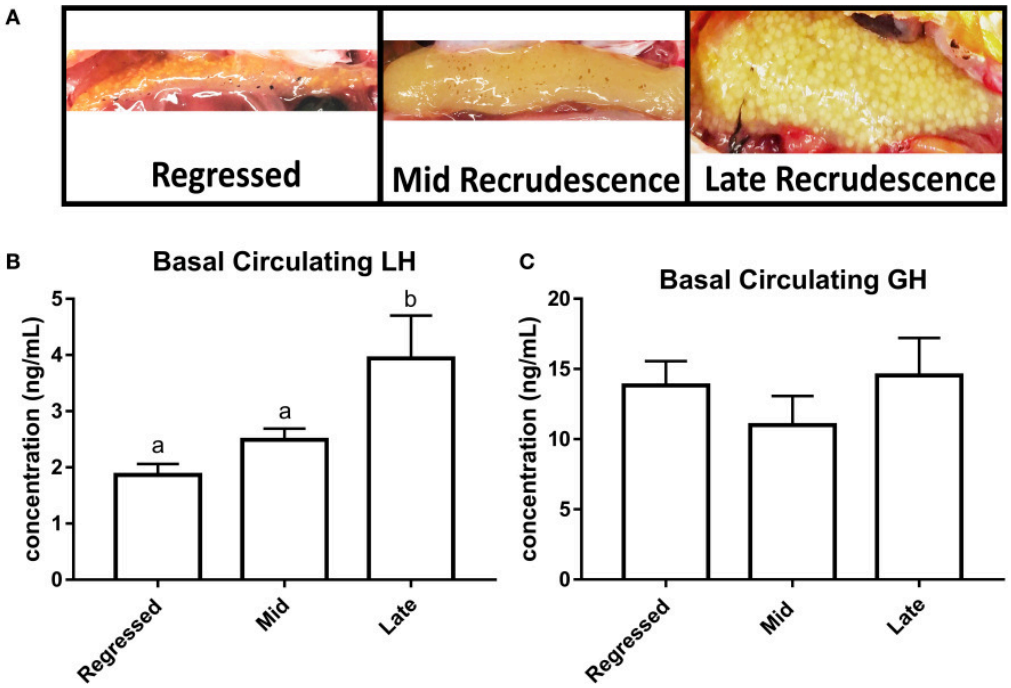


**FIGURE 1 |** Photographs of ovaries representing different gonadal stages **(A)**. Circulating basal serum levels of LH **(B)** and GH **(C)** in female goldfish at three seasonal reproductive stages (mean ± SEM, $n = 4$–12). Basal hormone levels were taken from control groups (PBS + PBS). Groups with significant differences between one another do not share the same letter of the alphabet (ANOVA followed by Tukey's multiple comparisons test, $p < 0.05$). Where ANOVA did not reveal the presence of significant differences between any of the groups within the whole data set, no identifier is included.

of recrudescence: regressed, mid, and late recrudescence. Significantly higher levels of LH were observed in serum of fish during late recrudescence compared to regressed and mid recrudescent stages (**Figure 1B**). Basal circulating GH levels did not significantly change between seasons (**Figure 1C**).

## Control of Serum LH and GH Levels by GnRH

The effects of the pre-optic hypothalamic goldfish GnRH form (sGnRH) (69), applied either as a single or double ip injection, were examined in the present study. Previous studies have shown that two ip injections of GnRH applied 12-h apart were generally more effective than a single GnRH injection in elevating circulating LH levels in goldfish (66), and thus both single and double injection protocols were used in the present study. Two-way ANOVA revealed the presence of GnRH effects on serum LH and GH levels among some of the reproductive stages examined (**Figure 2A**). Specifically, double injection with GnRH (GnRH + GnRH) did not affect serum LH levels in regressed fish but significantly elevated serum LH concentrations in fish at mid and late recrudescence by ∼70% relative to controls (PBS + PBS; **Figure 2A**). Increases in serum GH levels following single GnRH injection treatment (PBS + GnRH) were observed in regressed phase and late recrudescence as expected, and no significant changes were observed in mid recrudescence (**Figure 2C**).

## GnIH Effects on Basal and GnRH-Induced Serum LH and GH Levels

Treatment of GnIH was administered following a similar protocol as GnRH alone and combined with GnRH. Although two-way ANOVA suggested the presence of an overall GnIH alone treatment effects in mid recrudescence, treatment with GnIH alone did not significantly alter basal serum LH levels relative to controls during regressed, mid, or late gonadal recrudescence stages (**Figure 2B**). The combination of GnIH + GnRH treatment resulted in significant elevations in serum LH levels in sexually regressed fish when single injection of GnRH alone had no effects (**Figures 2A,E**). On the other hand treatment of GnIH tended to blunt GnRH-stimulated LH responses in late stages of recrudescence but did not block GnRH-induced responses in mid stage of recrudescence (**Figure 2E**). These observations are consistent with the presence of overall combination GnIH and GnRH treatment effects on serum LH at all three ovarian recrudescence stage as revealed by the two-way ANOVA.

Double injection of GnIH exerted significant stimulatory actions on circulating GH levels in fish at regressed stage but not at mid recrudescence (**Figure 2D**); these observations were consistent with the presence of an overall GnIH influence on serum GH levels at regressed but not late recrudescence. GnIH alone treatments at late stages of recrudescence were also without effect on serum GH levels relative to controls, although an overall influence of GnIH was revealed by two-way ANOVA

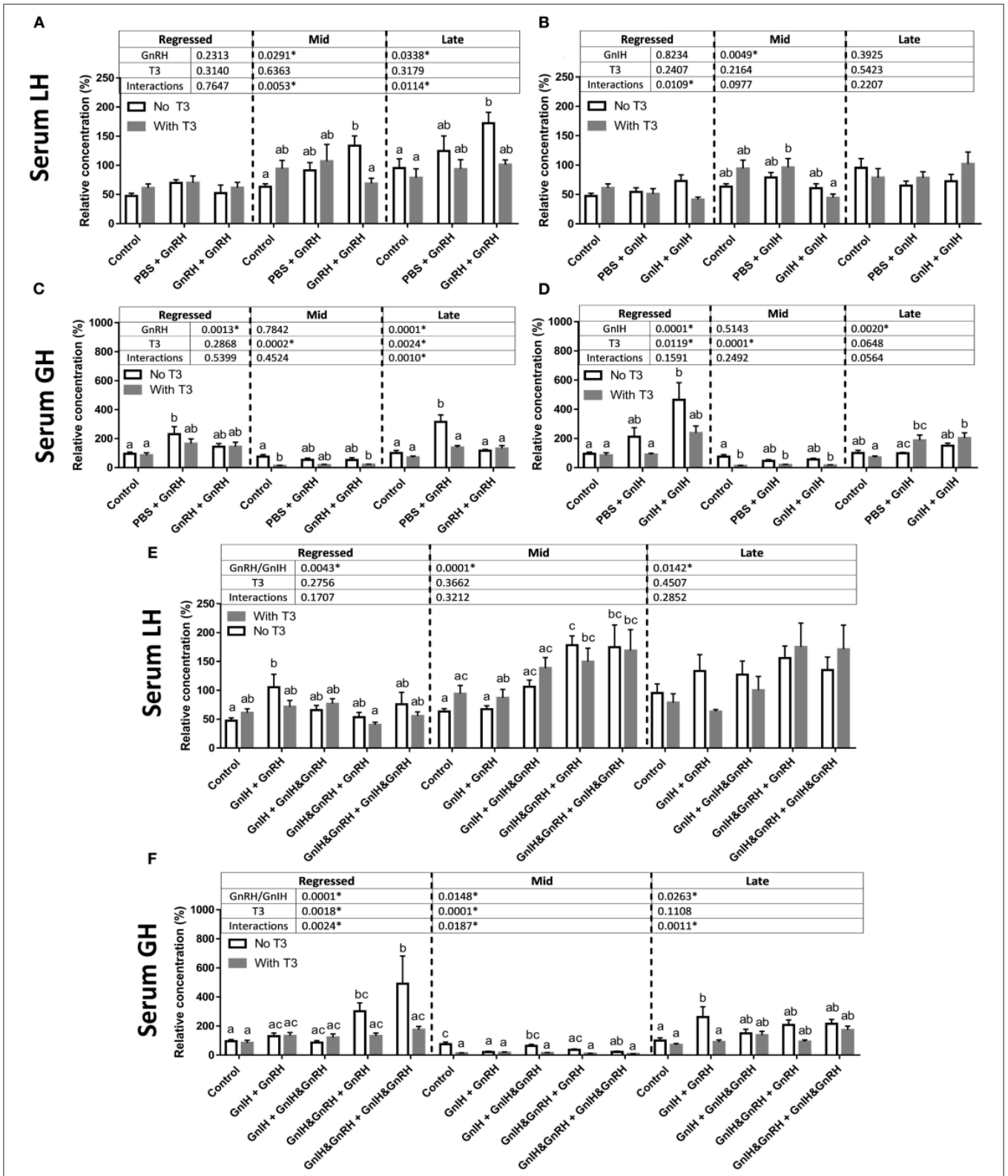


**FIGURE 2 |** Serum LH level changes in response to GnRH **(A)**, GnIH **(B)**, GnRH and GnIH **(E)** treatments. Serum GH changes in response to GnRH **(C)**, GnIH **(D)**, GnRH and GnIH **(F)** treatments. GnRH- and/or GnIH-induced changes in serum levels in female goldfish in the absence (open bar) and in the presence of T3 (gray bar) at three seasonal reproductive stages (mean ± SEM; regressed $n = 4–12$, mid recrudescence $n = 4–12$, and late recrudescence $n = 4–9$). Treatments are denoted as

*(Continued)*

**FIGURE 2 |** 0 + 12 h injection. Hormone values are presented as a percentage of the averaged concentrations of to control group (PBS + PBS injected) in late recrudescence. Controls of each respective season were placed at the start of each set of hormone treatments for visual comparison. Within each season, groups identified by different letters are significantly different from one another (two-way ANOVA followed by Bonferroni's multiple comparison test, $p < 0.05$). Vertical dashed line indicates groups within a seasonal gonadal stage that were statistically compared for significance. Groups were not tested for significance between seasons. In seasons where significant differences between groups are absent, identifiers are not included. *P*-values from two-way ANOVA are presented in tables above bars, and asterisks in tables indicate where factors (two factors analyzed in two-way ANOVA: GnRH and/or GnIH treatment, and the addition of T3) had significant effects, $p < 0.05$.

at this reproductive stage (**Figure 2D**). Significant effects on combination GnRH and GnIH treatments on serum GH levels were indicated by two-way ANOVA for all three reproductive seasons examined (**Figure 2F**). GnRH co-injection did not alter GnIH-induced GH responses in the regressed fish (**Figure 2F**). In mid stages of recrudescence, GnIH + GnRH and GnIH & GnRH + GnIH & GnRH treatments exerted inhibitory effects on serum GH levels but other combination treatments had no effects (**Figure 2F**). In late stages of recrudescence, GnIH + GnRH treatment increased serum GH to levels similar to those induced by a single GnRH injection while other combination treatments of GnIH and GnRH generally did not alter serum GH levels (**Figure 2F**).

## Effects of T3 on Basal, and GnRH- and/or GnIH-Induced Changes in LH and GH Levels

Two-way ANOVA identified significant interactions of T3 with GnRH at mid and late recrudescence, as well as interactions with GnIH at regressed state, but no significant overall effects of T3 alone on serum LH nor significant T3 interactions with combination GnRH and GnIH treatments (**Figures 2A,B,E**). Accordingly, treatment of T3 had no effects on basal LH release during any season but lowered the LH responses to double GnRH injections during mid and late stages of recrudescence (**Figure 2A**). Combination treatments with T3 and GnIH did not influence circulating LH levels (**Figure 2B**). T3 treatments with GnRH in the presence of GnIH did not alter GnRH-induced serum LH increases in mid recrudescent fish (**Figure 2E**). No significant effects were observed in these triple combination hormone treatment groups during regressed and late stage of gonadal recrudescence relative to controls (**Figure 2E**). Interestingly, the GnIH + GnRH-induced LH response was reduced by T3 injection to a level not different from controls in sexually regressed fish (**Figure 2E**).

The presence of significant overall T3 effects on serum GH were revealed at all reproductive seasons by two-way ANOVA, and in particular, interactions of T3 were identified with GnRH at late recrudescence, and with combination GnRH and GnIH treatments at all three ovarian stages (**Figures 2C,D,F**). T3 treatment significantly lowered basal serum GH levels during mid stages of recrudescence (**Figure 2D**) and reduced GnIH-induced GH response in regressed gonadal stage to levels not different to controls (**Figure 2F**). T3 also inhibited GnRH-induced GH release in late stages of recrudescence but not in regressed gonadal stage (**Figure 2C**). In addition, T3 reduced the GH responses to combination GnIH and GnRH treatments in regressed and late recrudescence stages (regressed: GnIH & GnRH + GnRH and GnIH & GnRH + GnIH & GnRH; late recrudescence: GnIH+GnRH; **Figure 2F**).

## Transcripts in Liver and Ovaries

Transcript levels for several genes important in reproduction and growth were measured with QPCR in liver and ovaries. The results for all transcript levels in liver and ovaries demonstrate clear seasonal pattern (**Figure 3**).

### ERα

ERα mRNA levels in the liver were significantly higher in mid recrudescent fish compared to fish in late recrudescence (**Figure 3D**). Similar to the changes in liver, ERα mRNA levels in the ovaries were significantly higher in mid stages of recrudescence but were lowest in regressed state fish (**Figure 3G**). Except for double GnRH injection treatment, changes in ERα mRNA levels in the liver did not correlate well with treatment-induced serum LH levels. GnRH + GnRH. Double GnRH injection treatment elevated ERα transcript levels in liver during late stages of gonadal recrudescence whereas single GnRH injection reduced the level of this transcript at regressed state (**Figure 4A**). In contrast, elevations in liver ERα mRNA levels were observed following double GnIH injections in regressed gonadal stage, GnIH & GnRH + GnIH & GnRH treatments in regressed and late recrudescence stages, as well as GnIH+GnRH treatments in late recrudescence (**Figure 4A**). Double GnRH injection treatment increased ERα transcript levels in ovaries during late stages of recrudescence and regressed gonadal stage (**Figure 5A**); these changes corresponded to the GnRH-induced increases in serum LH concentrations. However, GnIH treatments reduced ovary ERα mRNA levels during mid stages of recrudescence (**Figure 5A**). Treatment with GnIH+GnRH or GnIH+GnIH & GnRH increased levels of ERα mRNA level in ovaries during regressed gonadal stage (**Figure 5A**). GnIH+GnRH treatment reduced the levels of ERα mRNA in ovaries at mid recrudescence, but increased their ovarian expression during late recrudescence (**Figure 5A**).

### ERβI

There was a clear seasonal pattern in expression of ERβI mRNA in liver and ovaries of female goldfish with maximum levels seen in mid recrudescence, and lowest levels seen in liver of late recrudescence fish and ovaries of regressed stage fish (**Figures 3E,H**). ERβI mRNA levels in the liver of females showed no correlation with responses in LH concentrations elicited by hormone treatments. Single GnRH injection treatments reduced liver ERβI mRNA levels in sexually regressed fish, but increased these transcript levels in late recrudescence (**Figure 4B**). All GnRH alone treatments

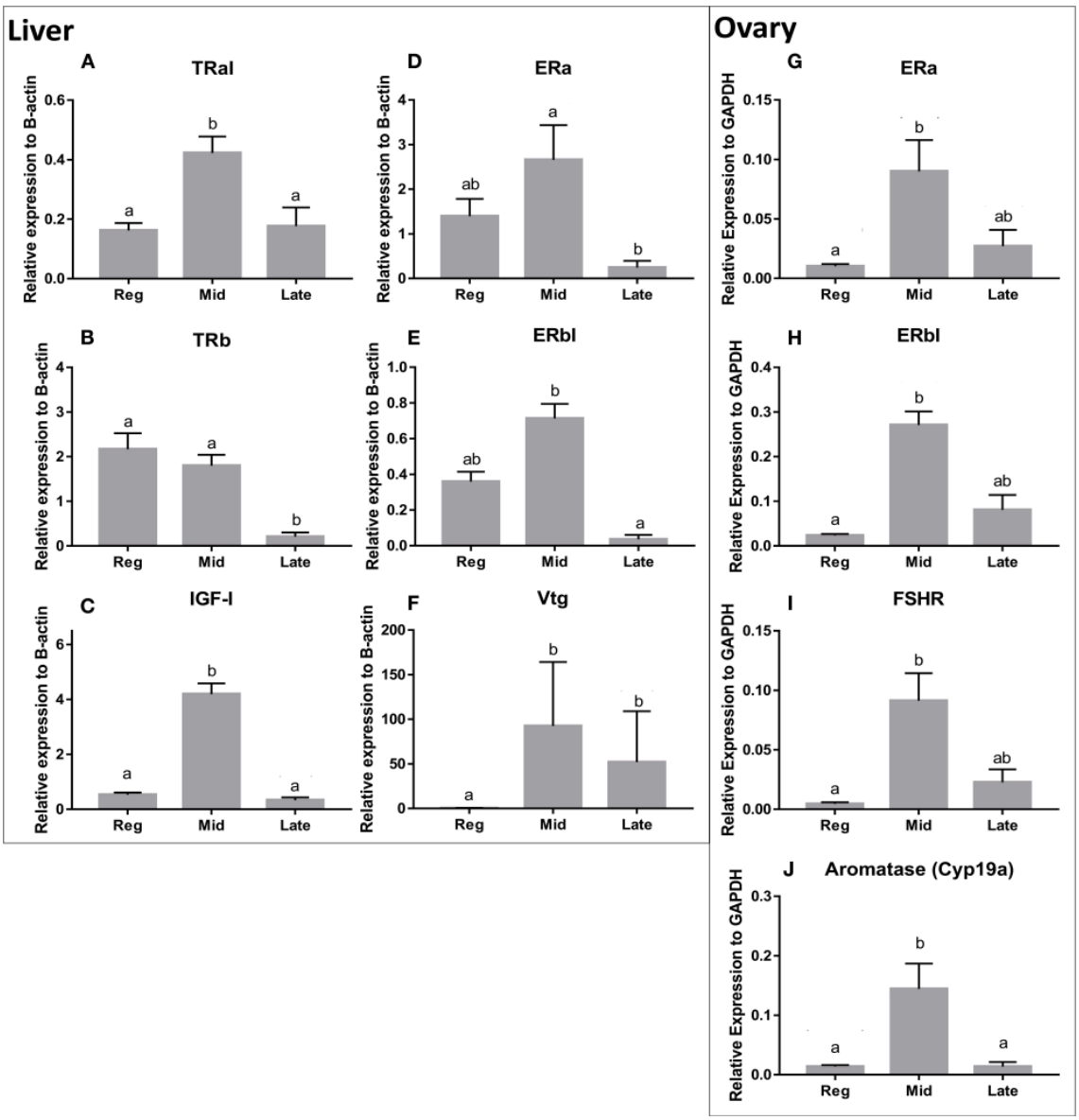


**FIGURE 3 |** Basal transcript levels of TRαI **(A)**, TRβ **(B)**, IGF-I **(C)**, ERα **(D)**, ERβ1 **(E)**, and vitellogenin **(F)** in the liver, and ERα **(G)**, ERβ1 **(H)**, FSHR **(I)**, and aromatase Cyp19a **(J)** in the ovaries of female goldfish in three stages of the reproductive season (regressed, mid, and late recrudescent). Expression was measured using QPCR and normalized against β-actin (liver) or GAPDH (gonads). Values are mean ± SEM ($n = 4$–9). Groups identified by different letters are significantly different from one another (Kruskal-Wallis test followed by Dunn's multiple comparisons test, $p < 0.05$).

in mid recrudescence lowered ERβI mRNA levels in liver (**Figure 4B**). Double GnIH injection treatment lowered liver ERβI mRNA levels during regressed and mid recrudescence stages and single GnIH injection similarly exerted inhibitory influences in mid recrudescence (**Figure 4B**). On the other hand, GnIH treatments increased ERβI mRNA levels in liver during late recrudescence (**Figure 4B**). Combined treatments with GnIH + GnRH and GnIH + GnIH & GnRH reduced ERβI mRNA levels in liver during regressed stage (**Figure 4B**). GnIH+GnRH combination treatment elevated ERβI mRNA levels in liver during late recrudescence stage (**Figure 4B**). In ovaries, ERβI mRNA levels partially correlated with changes in serum LH concentrations in late stages of gonadal recrudescence (**Figure 5B**). Single and double GnRH injection treatments increased ERβI mRNA levels in ovaries in regressed stage; double GnRH injection treatments also increased these levels in late recrudescence stage, but reduced these levels in mid recrudescence (**Figure 5B**). Double GnIH injection treatments increased ovarian ERβI mRNA levels in regressed phase fish, but both double and single GnIH injection treatments decreased ERβI mRNA levels in ovaries of fish at mid recrudescence (**Figure 5B**). GnIH+GnRH treatment reduced ERβI mRNA

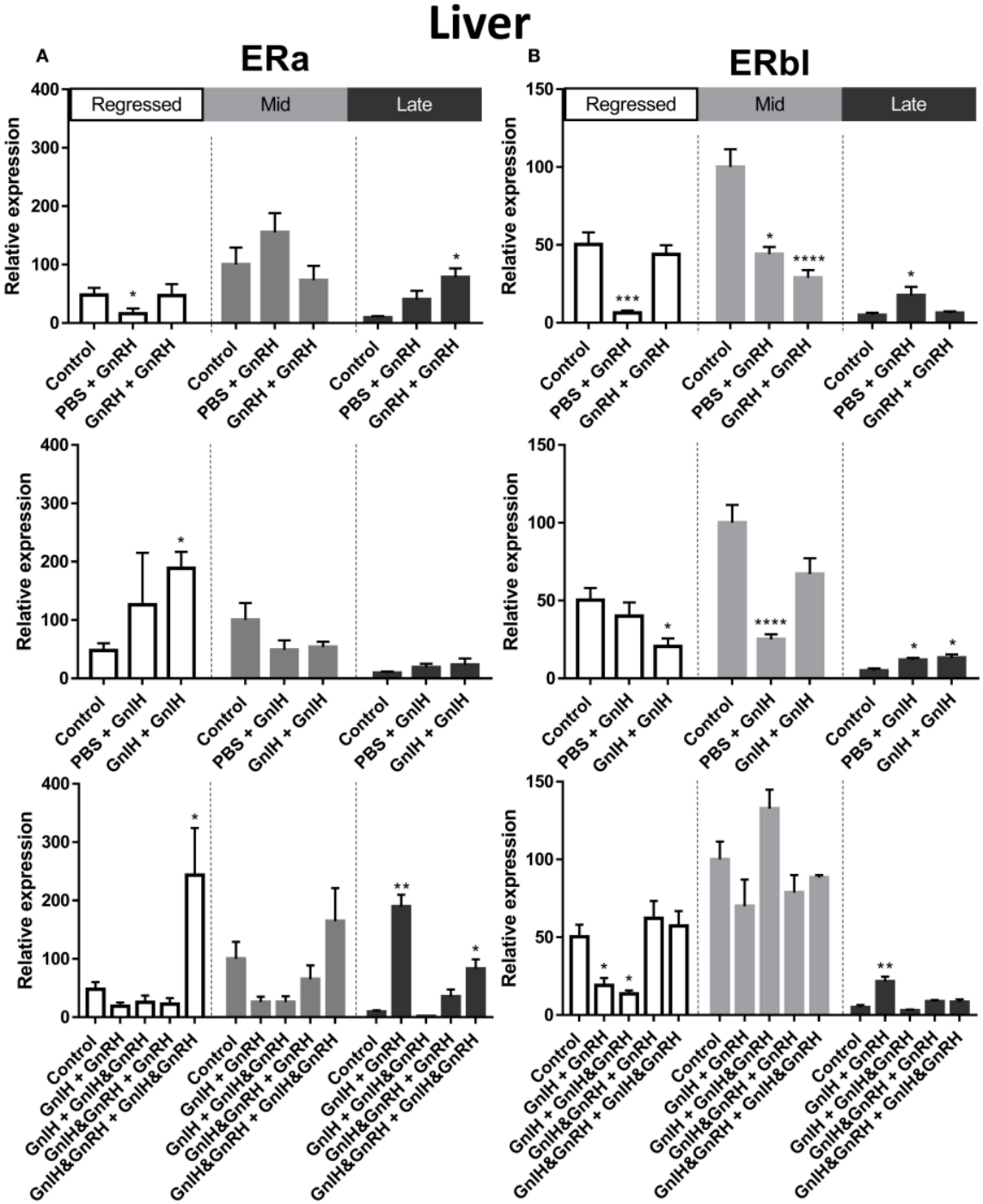


**FIGURE 4 |** Effects of GnRH and/or GnRH treatments on estrogen receptors ERα **(A)** and ERβ1 **(B)** mRNA levels in the liver of female goldfish at three seasonal reproductive stages (regressed, $n = 4$–9; mid recrudescence, $n = 4$–9; and late recrudescence $n = 4$–9). Treatments are denoted as 0 + 12 h injection. Levels of mRNA expression were detected using QPCR and normalized relative to β-actin. Results presented (mean ± SEM) are expressed as a percentage of the averaged relative expression levels in controls (PBS + PBS) at mid recrudescence. Each seasonal control group was placed in front of each set of hormone treatments for visual comparison. Asterisks indicate significance differences from controls (Kruskal-Wallis test followed by Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$).

levels in ovary during mid recrudescence, but no effects were seen in combination GnIH and GnRH treatment groups in other seasons (**Figure 5B**).

## Vtg

Liver Vtg mRNA levels were significantly higher in mid and late recrudescence compared to regressed stage (**Figure 3F**). There

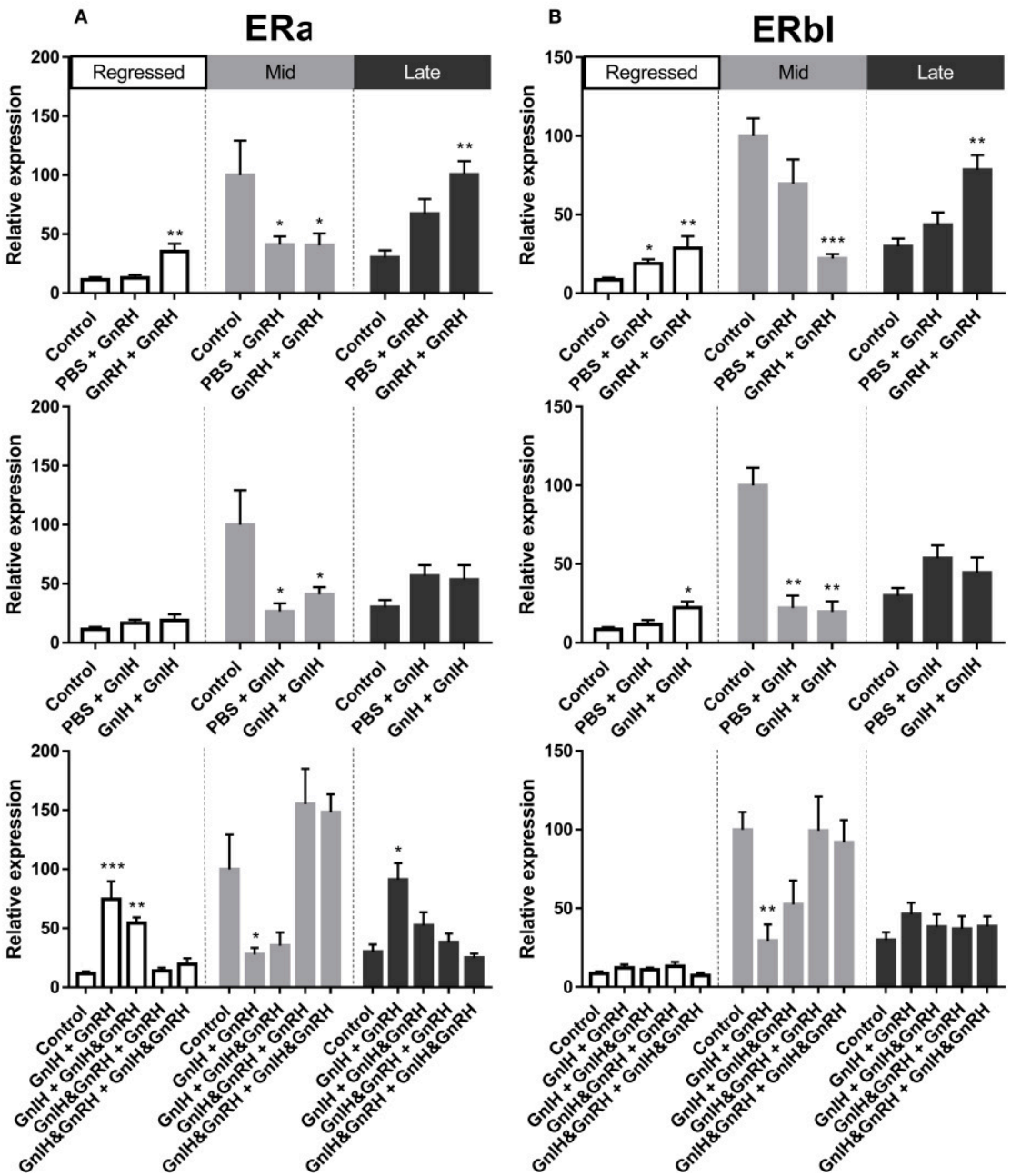


**FIGURE 5 |** Effects of GnRH and/or GnIH on estrogen receptors ERα **(A)** and ERβ1 **(B)** mRNA levels in the ovary of female goldfish at three seasonal reproductive stages (regressed, $n = 4$–9; mid recrudescence, $n = 4$–9; and late recrudescence $n = 4$–9). Treatments are denoted as 0 + 12 h injection. Levels of mRNA expression were detected using QPCR and normalized relative to GAPDH. Results presented (mean ± SEM) are expressed as a percentage of the averaged relative expression levels in control (PBS + PBS) group at mid recrudescence. Each seasonal control group was placed in front of each set of hormone treatments for visual comparison. Asterisks indicate significance differences from controls (Kruskal-Wallis test followed by Dunn multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

was a correlation between basal Vtg mRNA levels and changes in basal circulating LH levels in late recrudescence. Both single and double GnRH injection treatments increased Vtg mRNA levels in regressed stage females but single GnRH injection and GnIH + GnIH & GnRH treatments lowered these levels in mid recrudescence stage (**Figure 6**). Treatments with GnRH did not elicit any significant changes in Vtg mRNA levels in the liver of females during late recrudescence (**Figure 6**). In regressed

gonadal stage, double GnIH injection elevated Vtg mRNA levels in liver while GnIH + GnRH treatment was similarly stimulatory at late recrudescence (**Figure 6**). Other concomitant treatments with GnRH and GnIH were without effect on the liver Vtg mRNA level in the three seasons (**Figure 6**).

### IGF-I

In female liver, IGF-I mRNA levels were significantly higher in mid recrudescence (**Figure 3C**). A partial correlation was observed between changes in IGF-I mRNA level and serum GH concentration in response to hormone treatments during late stages of gonadal recrudescence. Single GnRH injection treatment reduced IGF-I mRNA levels in liver during regressed and mid recrudescence gonadal stage, but elevated IGF-I mRNA levels during late recrudescence (**Figure 7**). Single and double injection of GnIH increased IGF-I mRNA levels in liver at regressed stage, and single injection similarly increased mRNA levels in late recrudescent; on the other hand, treatment with double GnIH injection decreased IGF-I mRNA levels in liver during mid recrudescence (**Figure 7**). No significant effects were observed in combination treatment during mid recrudescence stage (**Figure 7**), but GnIH + GnRH treatment increased IGF-I mRNA levels in liver during regressed and late recrudescence stages (**Figure 7**).

### TRs

Thyroid hormones are important factors in reproduction and growth [for review see: (55)]. In female liver, TRαI transcript levels were observed to be significantly higher during mid recrudescent than in livers of regressed and mid recrudescent fish (**Figure 3A**). No clear correlation was observed between changes in TRαI mRNA levels and LH or GH secretion following hormone treatments. Double injections of GnRH decreased TRαI mRNA levels during mid and late stages of gonadal recrudescence in liver (**Figure 8A**). Treatments of GnIH alone, applied either as a single or double injection, reduced TRαI mRNA levels during mid recrudescence (**Figure 8A**). In late stages of recrudescence, GnIH + GnRH treatment increased TRαI mRNA levels (**Figure 8A**). In regressed stage fish, single GnRH injection treatment lowered TRαI mRNA levels and combination treatment with GnIH did not affect GnRH-induced responses in GnIH + GnRH and GnIH + GnIH & GnRH treatment groups (**Figure 8A**).

The seasonal expression pattern for liver TRβ mRNA was observed to be different from that of TRαI, with significantly higher levels in the regressed and mid recrudescence stages than at late recrudescence (**Figure 3B**). Similarly to TRαI, TRβ mRNA levels did not clearly correspond to changes in serum LH or GH levels elicited by hormone treatments. Treatments with GnRH alone reduced TRβ mRNA levels in liver of females during regressed stage, but a single GnRH injection increased TRβ mRNA levels during late recrudescence (**Figure 8B**). Double injections of GnIH reduced TRβ mRNA level in the liver in regressed gonadal stage females, but increased these transcript levels in late recrudescence (**Figure 8B**). In late recrudescence, treatments with GnIH + GnRH or GnIH & GnRH + GnRH increased TRβ mRNA levels (**Figure 8B**). All

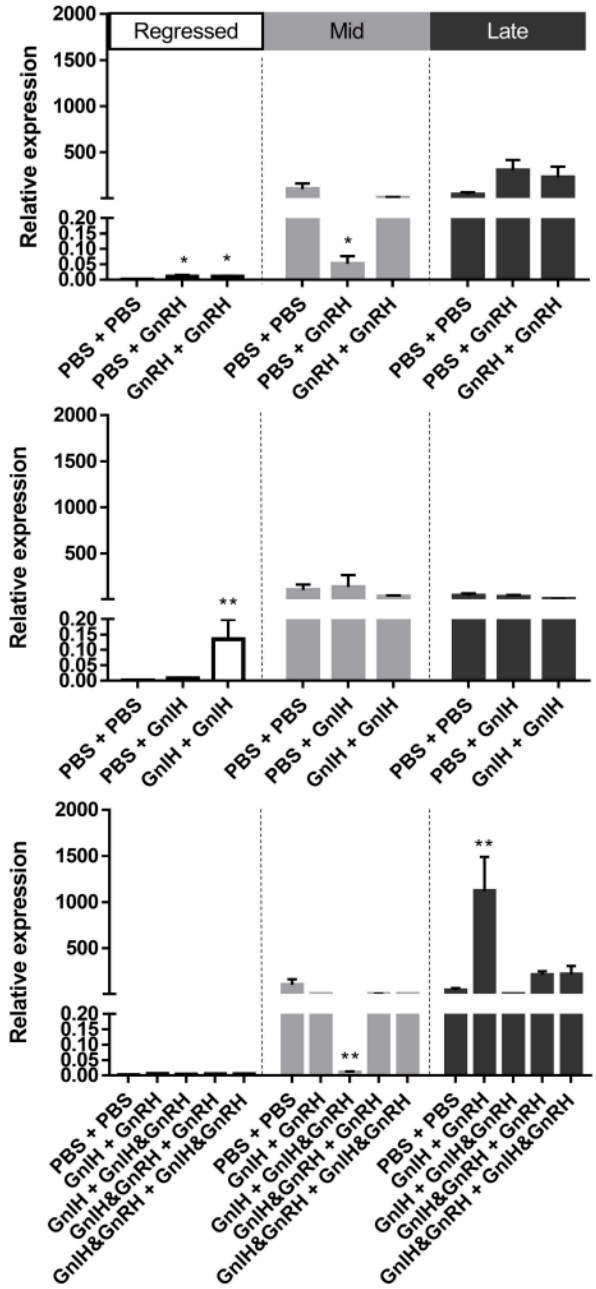


**FIGURE 6 |** Effects of GnRH and/or GnIH on Vtg mRNA levels in the liver of female goldfish at three seasonal reproductive stages (regressed, $n = 4$–9; mid recrudescence, $n = 4$–9; and late recrudescence $n = 4$–9). Treatments are denoted as 0 + 12 h injection. Levels of mRNA expression were detected using QPCR and normalized relative to β-actin. Results presented (mean ± SEM) are expressed as a percentage of the averaged relative expression levels in control (PBS + PBS) group at mid recrudescence. Each seasonal control group was placed in front of each set of hormone treatments for visual comparison. Asterisks indicate significance differences from controls (Kruskal-Wallis test followed by Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$).

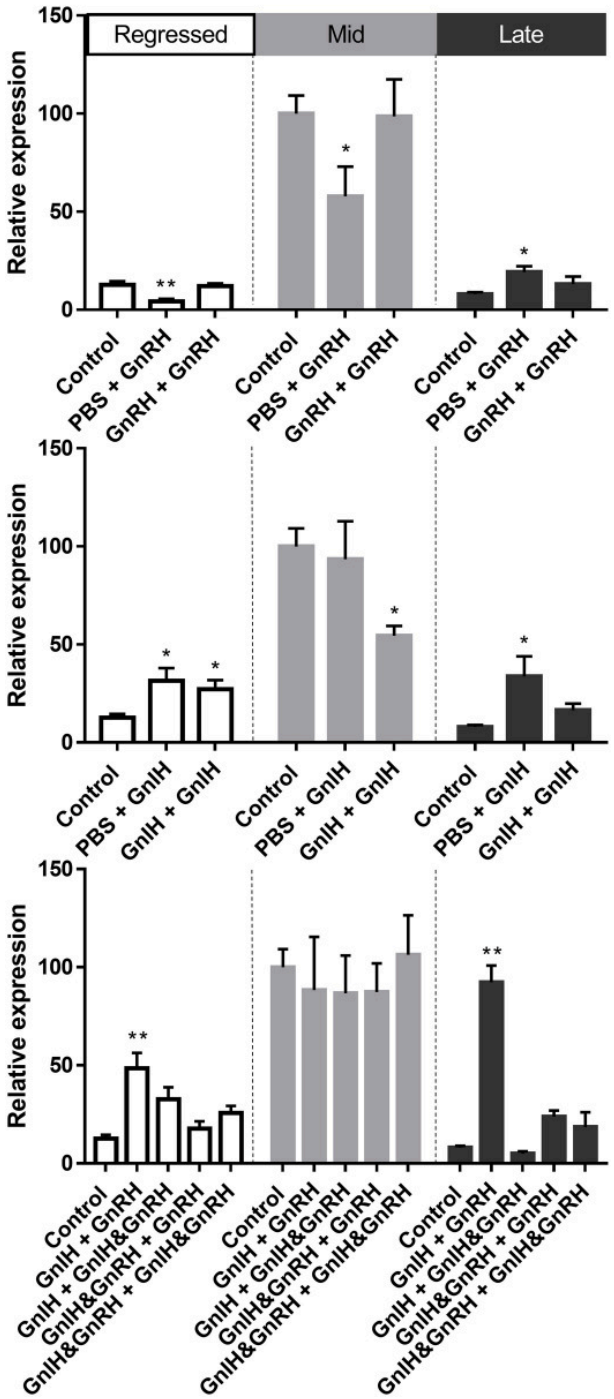


**FIGURE 7 |** Effects of GnRH and/or GnIH on IGF-I mRNA levels in the liver of female goldfish at three seasonal reproductive stages (regressed, $n = 4$–9; mid recrudescence, $n = 4$–9; and late recrudescence $n = 4$–9). Treatments are denoted as 0 + 12 h injection. Levels of mRNA expression were detected using QPCR and normalized relative to β-actin. Results presented (mean ± SEM) are expressed as a percentage of the averaged relative expression levels in control (PBS + PBS) group at mid recrudescence. Each seasonal control group was placed in front of each set of hormone treatments for visual comparison. Asterisks indicate significance differences from controls (Kruskal-Wallis test followed by Dunn multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$).

*(Continued)*

combination treatment groups in regressed gonadal stage fish reduced TRβ mRNA levels in liver, except treatment with GnIH & GnRH + GnIH & GnRH which had no effect on TRβ mRNA levels (**Figure 8B**). No significant changes were observed on TRβ transcript levels in mid stages of recrudescence with any treatment (**Figure 8B**).

## Aromatase

Gonadal aromatase (Cyp19a) is crucial for the aromatization of testosterones into estrogens (70). In the ovaries, relatively higher aromatase mRNA levels were observed in mid recrudescence compared to regressed and late recrudescence stages (**Figure 3J**). Partial correlation was observed between aromatase transcript expression and circulating LH concentrations following hormone treatments at late recrudescence stage (**Figure 9**). Treatments with either GnRH or GnIH alone reduced aromatase mRNA levels in ovaries during mid recrudescence and were without effect in regressed stage (**Figure 9**). Treatments with GnIH + GnRH or GnIH + GnIH & GnRH also lowered levels of aromatase mRNA in mid recrudescent ovaries, but not in regressed stage (**Figure 9**). At late stages of gonadal recrudescence, double GnRH injection treatment increase aromatase mRNA levels but no other treatments had any effects (**Figure 9**).

## FSHR

FSHR is a crucial facilitator of FSH actions on oocyte maturation and steroidogenesis (70). FSHR mRNA levels in ovary were highest during mid stages of recrudescence compared to regressed and late gonadal stages (**Figure 3I**). We observed partial correlation between FSHR mRNA levels and serum LH levels following GnRH treatments during late recrudescence phase. Treatments with either double or single injection of GnRH alone increased FSHR mRNA levels in ovaries of fish in late stages of recrudescence (**Figure 10**). In mid recrudescence, double injections of GnRH decreased ovarian FSHR mRNA levels, whereas treatment with a single injection of GnRH increased FSHR mRNA levels in regressed ovaries (**Figure 10**). Treatments with GnIH reduced FSHR mRNA levels in the ovary of mid recrudescent fish, while single GnIH injection treatments exerted a stimulatory influence at late recrudescence (**Figure 10**). Increased FSHR mRNA level was observed following combination GnIH + GnRH and GnIH & GnRH + GnIH & GnRH treatments in late recrudescence, but decreased transcript level was observed following GnIH + GnRH and GnIH + GnIH & GnRH treatments in mid recrudescence (**Figure 10**). Ovarian expression of the other gonadotropin receptor, LH receptor, was not quantified because of primer problems.

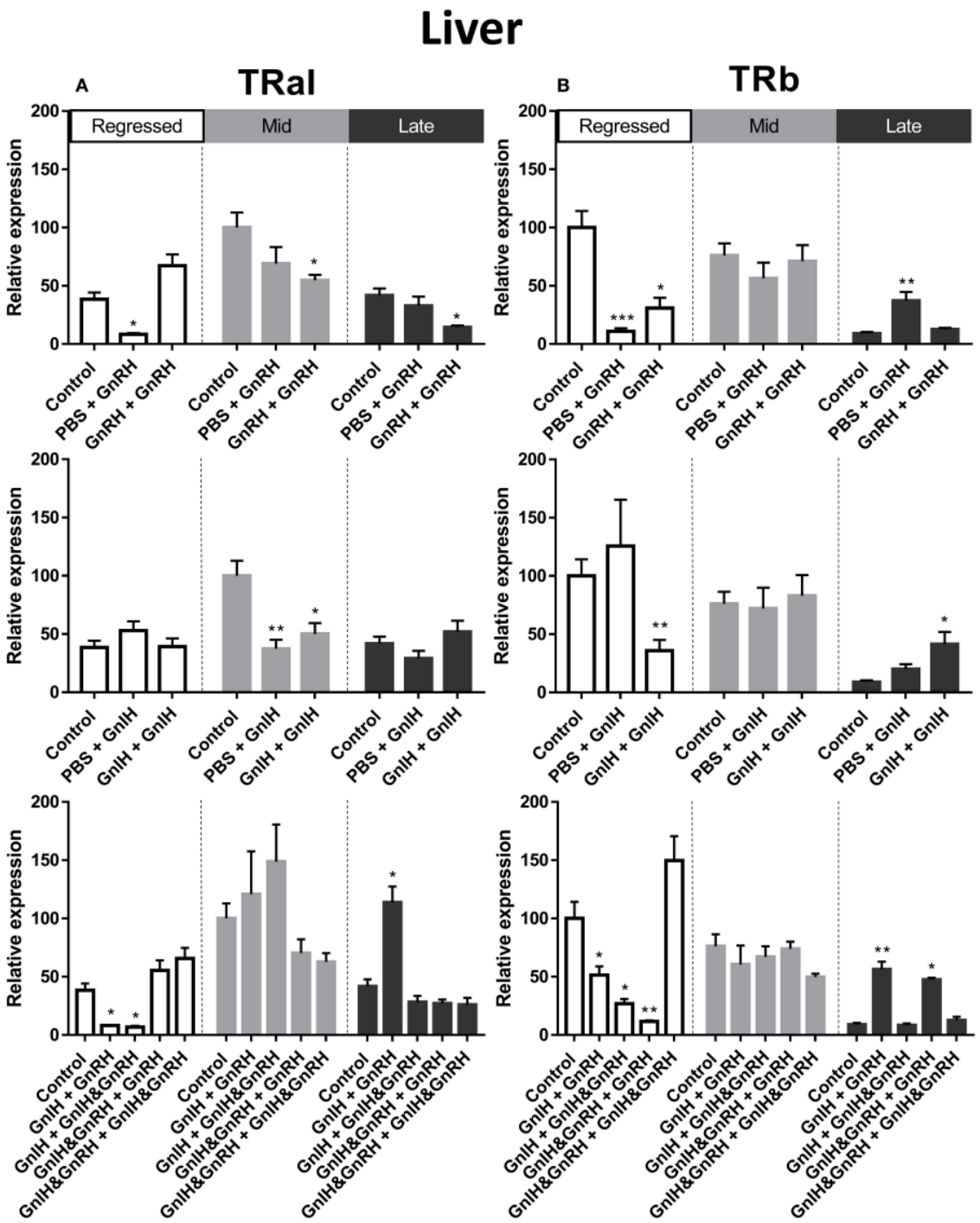


**FIGURE 8 |** Effects of GnRH and GnIH on thyroid hormone receptors TRαI **(A)** andTRβ **(B)** mRNA levels in the liver of female goldfish at three seasonal reproductive stages (regressed, $n = 4$–9; mid recrudescence, $n = 4$–9; and late recrudescence $n = 4$–9). Treatments are denoted as 0 + 12 h injection. Levels of mRNA expression were detected using QPCR and normalized relative to β-actin. Results presented (mean ± SEM) are expressed as a percentage of the averaged relative expression levels in control (PBS+PBS) group at mid recrudescence. Each seasonal control group was placed in front of each set of hormone treatments for visual comparison. Asterisks indicate significance differences from controls (Kruskal-Wallis test followed by Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p = 0.001$).

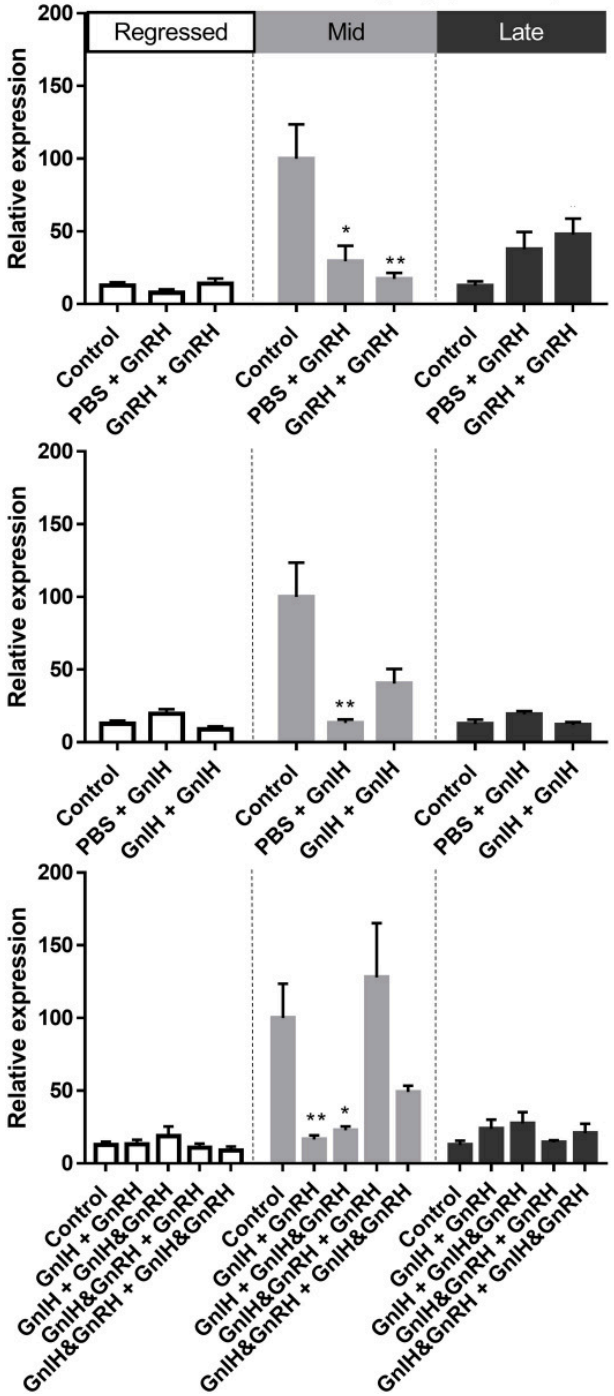


**FIGURE 9 |** Effects of GnRH and/or GnIH on gonadal aromatase (Cyp19a) mRNA levels in the ovary of female goldfish at three seasonal reproductive stages (regressed, $n = 4$–9; mid recrudescence, $n = 4$–9; and late recrudescence $n = 4$–9). Treatments are denoted as 0 + 12 h injection. Levels of mRNA expression were detected using QPCR and normalized relative to

*(Continued)*

**FIGURE 9 |** GAPDH. Results presented (mean ± SEM) are expressed as a percentage of the averaged relative expression levels in control (PBS + PBS) group at mid recrudescence. Each seasonal control group was placed in front of each set of hormone treatments for visual comparison. Asterisks indicate significance differences from controls (Kruskal-Wallis test followed by Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$).

## DISCUSSION

The present study provided insight into the neuroendocrine control of seasonal reproduction and growth in female goldfish. A seasonal pattern of change in basal serum LH concentrations in female goldfish was observed. The observed maximum circulating LH concentrations at late stages of recrudescence occurred in fish containing fully matured oocytes, and period of lower growth rates. This is consistent with a previous study in goldfish demonstrating maximum gonadosomatic index (GSI) in females during spring, lowest GSI during regressed phase, and increasing levels during mid recrudescence (71). In the present study, smaller ovary and follicle size was observed at regressed stage, increasing at mid recrudescence, and reaching a maximum size at late recrudescence, which also had higher circulating levels of LH. Marchant and Peter found higher rates of somatic and linear growth corresponding to the lowest GSI in female goldfish (71). In the present study, differences in basal circulating GH concentration in different reproductive seasons were not statistically significantly. In male fish, however, highest circulating levels of GH was observed at the regressed stage, compared to those at mid and late recrudescence (72). It should be noted that GH, as well as LH, contribute to stimulation of Vtg production, and thus ovarian maturation, in goldfish (73).

Treatment with GnRH had a stimulatory effect on serum LH levels at mid and late recrudescence stage, while GnRH-induced GH response was observed at regressed and late stages of recrudescence (summarized in **Table S2**). Previous studies demonstrated seasonally dependent changes and increased GnRH-induced LH release during late stages of gonadal recrudescence (36). GnRH has also been shown to increase LHβ subunit mRNA levels (2, 13). In the present study, serum GH concentrations were increased by both GnRH and GnIH injections at the regressed phase. In late recrudescence, however, only GnRH was stimulatory on circulating GH levels in female goldfish. Our results are consistent with a previous study demonstrating increases in serum GH levels and stimulation of GH mRNA expression during early and late gonadal recrudescence by both sGnRH and cGnRHII (14). A number of other studies also demonstrated GnRH stimulation of GH production in goldfish (11, 12, 15, 16, 21, 74), and other teleost species (20–22, 75). Thus, GnRH is an important factor in regulating GH and LH production and release in fish. The time course for both GnRH and GnIH were chosen based on previous studies (2, 13, 14, 35, 36, 63, 76). At 12 and 24 h following injection, GnRH significantly effects both GH and LH production as shown previously (2). GnRH-induced change in gonadotropins, in turn, effects production of gonadal steroids

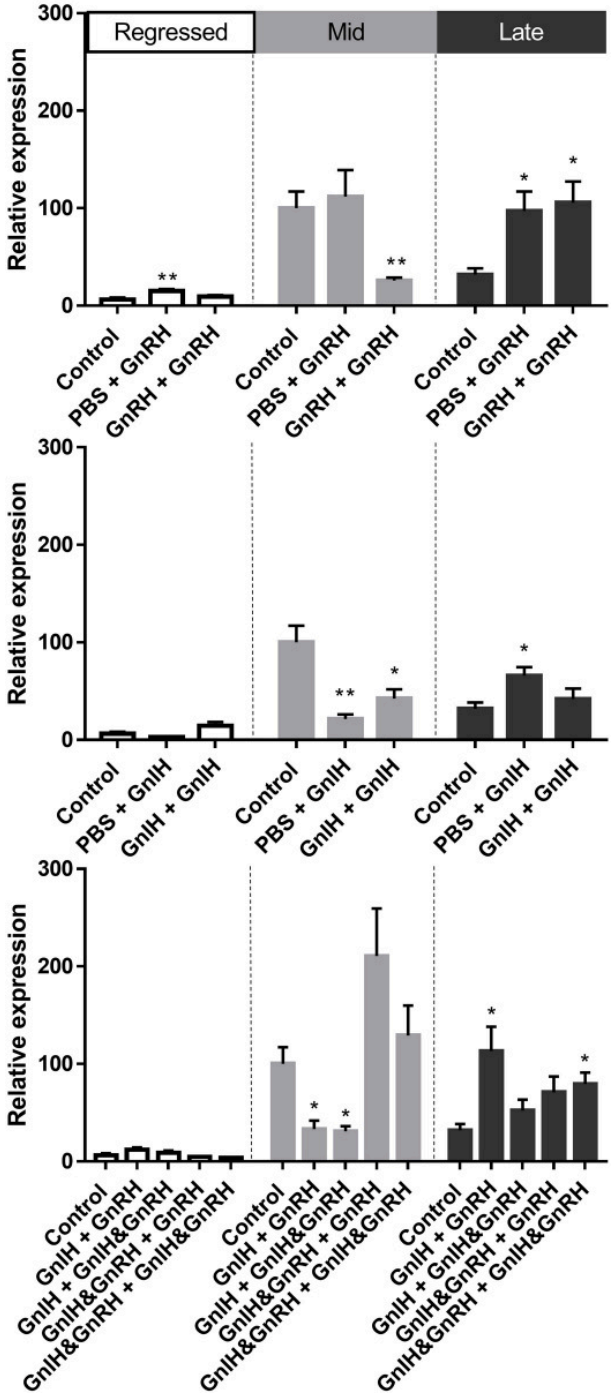


**FIGURE 10 |** Effects of GnRH and GnIH on follicle stimulating hormone receptor (FSHR) mRNA levels in the ovary of female goldfish at three seasonal reproductive stages (regressed, $n = 4$–9; mid recrudescence, $n = 4$–9; and late recrudescence $n = 4$–9). Treatments are denoted as 0 + 12 h injection. Levels of mRNA expression were detected using QPCR and normalized

*(Continued)*

**FIGURE 10 |** relative to GAPDH. Results presented (mean ± SEM) are expressed as a percentage of the averaged relative expression levels in control group at mid recrudescence. Each seasonal control group was placed in front of each set of hormone treatments for visual comparison. Asterisks indicate significance differences from controls (Kruskal-Wallis test followed by Dunn's multiple comparisons test, $^{*}p < 0.05$, $^{**}p < 0.01$).

which can have feedback effect on the brain and pituitary within the time frames used in the present study (64, 65). Therefore, 12 and 24 h timepoints were chosen based on previous studies. It is possible that more information may be obtained by investigating effects of hormones at earlier time points and it may be a limitation of this study. However, adding more time points would limit our ability to investigate multifactorial control of pituitary function, using a factorial design.

To investigate the seasonal differences and interactions between GnRH and GnIH, we combined the two hormones following a factorial design. In goldfish, GnIH has been shown to both stimulate and inhibit LH release and synthesis in the pituitary, depending on the stages of gonadal maturation (35, 36). Various combinations of GnRH and GnIH in cell perifusion experiments resulted in different effects on GH and LH release (14, 36). The present results demonstrate stimulation of basal GH release by GnIH during the somatic growth phase of female goldfish, and GnIH inhibition of GnRH-stimulated GH secretion during late gonadal recrudescence but not in sexually regressed stage (summarized in **Tables S3**, **S4**). These findings are consistent with a previous study demonstrating direct action of GnIH on GH release *in vitro* (14). GnIH stimulation of GH release and production has been demonstrated in a variety of other vertebrate species including the grass puffer (*Takifugu alboplumbeus*) (38), cichlid fish (*Cichlasoma dimerus*) (34), bull frogs (*Rana catesbeiana*) (77), and rats (40). In the present study, treatment with GnIH resulted in inhibition of GnRH-induced LH secretion at late but not at mid gonadal recrudescence (summarized in **Tables S3**, **S4**). This is consistent with previous findings in goldfish both *in vivo* and *in vitro* (36). GnIH inhibition of gonadotropin gene expression has also been shown in cinnamon clownfish (*Amphiprion melanopus*) (33). In common carp (*Cyprinus carpio* L.), GnIH treatment reduced FSH and LH subunits expression, and minimum GnIH transcript levels were observed in the hypothalamus during spawning season (78). In the cichlid fish (*Cichlasoma dimerus*), treatment with cd-LPQRF-1 inhibited FSH and LH release, while cd-LPQRF-2 stimulated FSH release in cultured pituitary cells (34). In the flatfish, treatment with ssGnIH-2 did not affect LHβ expression but ssGnIH-3 lowered LHβ transcript levels (79). These studies provide evidence of species-specific changes in GnIH response depending on reproductive season. The general response elicited by GnIH in the female goldfish is largely inhibitory on gonadotropes and stimulatory on somatotropes in a seasonally related manner. Thus, both GnRH and GnIH are contributing factors in multifactorial regulation of seasonal growth and reproduction in female goldfish. Further studies investigating pituitary receptors of GnRH and GnIH could

explain the seasonal changes in GH and LH observed in this study. Basal GnIH receptor mRNA levels in goldfish pituitary did not show significant changes during any seasonal gonadal stage (35). However, treatments of exogenous GnIH and GnRH affected GnIH receptor transcripts in mid and late recrudescence goldfish (36). It is possible that fluctuations in GnIH and GnRH receptor ratio may have important influence on seasonal regulation of somatotropes and gonadotropes in goldfish.

Thyroid hormones are also important in the control of reproduction in fish and other vertebrates (52, 53, 55, 56, 80). In goldfish, serum concentrations of T3 are high during summer when fish have maximum growth rates; circulating T3 then follows a decreasing trend from fall through spring, reaching minimum levels in the spawning period (60). In the present study, treatment with T3 was inhibitory on basal GH levels during mid recrudescence as well as reducing both GnRH- and/or GnIH-elicited GH responses during sexually regressed and late recrudescence stages. At mid and late recrudescence, T3 injection reduced GnRH-induced serum LH increases. However, co-injection with GnIH reduced inhibitory T3 effects on GnRH-induced LH response in mid recrudescence (summarized in **Tables S2–S4**). Previous results have demonstrated that T3 treatment, *in vitro*, reduced LH subunit mRNA levels during early recrudescence in mixed sex fish without affecting GH or gonadotropin subunits transcript levels at other stages of gonadal recrudescence in goldfish (51). Furthermore, injections with T3 at mid recrudescence stage were shown to have no effects on gonadotropin mRNA levels in female goldfish, but reduced LH subunit mRNA levels in male goldfish after 36 h (54). In addition, injections with T3 were shown to reduce ERα and Cyp19a transcript levels in the ovary of mid recrudescent fish (54). Taken together, T3 exerts inhibitory effects on HPG axis, and the control of pituitary somatotrope activities with seasonal variations. Thus, these results support the hypothesis that T3 is an important contributor to seasonal control of GH and LH in female goldfish. Furthermore, in view of the previously described progressive decrease in circulating T3 levels as gonadal recrudescence advances, the reduction of the negative T3 influence on the HPG axis, as well as on pituitary GH release, is likely an important neuroendocrine event for the seasonal switch from somatic growth to gonadal maturation.

It is important to note that goldfish cannot be sexed accurately based on secondary sex characteristics, especially during the regressed gonadal stage. During the experiment, fish were housed in tanks that contained mature males and females that were randomly distributed. At the time of dissection, it was possible to accurately sex the fish. Due to this potential limitation, there were variations among the replicate numbers for different treatment groups. However, the majority of groups contained large replicate numbers as stated for each figure. Another possible limitation of this study was variation in size of fish in different seasons. All fish were post-pubertal and sexually mature and expected to have similar hormonal profiles and responsiveness to same treatments regardless of variations in size. However, we cannot ignore the influence that size may have and consider variations in size of the fish as a limitation of this study.

The present study investigated tissue- and season-specific transcript levels of several genes related to growth and reproduction in the liver and ovary. The results show similar seasonal pattern for ERα and ERβI mRNA levels in the female liver and ovaries with the highest level of these transcripts at mid recrudescence in both tissues. A similar seasonal pattern of ERα and ERβI transcript levels were also observed in male goldfish liver, although peak levels of ERα and ERβI mRNA in testes where different during late recrudescence (72).

Both ERα and ERβI are involved in stimulating vitellogenesis in female fish (80–82). Vtg production starts during early recrudescence and continues through mid-recrudescence until oocytes are fully mature in late recrudescence. This cycle is clearly seen in female liver ERα and ERβI transcript levels and correlates with higher Vtg mRNA levels in mid and late recrudescence. Interestingly, intermediate levels of ERα and ERβI transcript levels were seen in the regressed and late recrudescence states in the liver and ovary, respectively. The intermediate levels of ER expression in the liver during earlier stage of gonadal recrudescence could be the result of T3 priming effect for vitellogenesis (61). The Vtg gene is also found in males but is not usually expressed at significant levels due to lack of sufficient circulating levels of estrogens. Never the less, Vtg level was found to follow a similar seasonal pattern as female liver, although, the levels of ERα and Vtg mRNA in male liver are much lower compared females (72).

The present results also revealed higher levels of ERα and ERβI transcript levels in ovaries at the mid recrudescence stage which corresponds with higher expression in ovarian aromatase and FSHR transcript levels. In goldfish and other teleosts, FSH also has steroidogenic activity (83, 84) and thus the changes in FSHR expression at mid recrudescence is not solely related to gametogenesis, but is also relevant for the increase in total steroidogenic capacity at this reproductive stage. ERs and estrogen synthesis are crucial components of oocyte maturation and female reproduction (85, 86). FSH effects on increasing follicular cell activities and cell proliferation are largely mediated through estrogens and ERs, and E2 positive feedback on follicular cell proliferation further increases steroidogenic capacity of the maturing follicles (85). The increasing FSH and E2 levels during early recrudescence triggers liver vitellogenesis, and as the oocyte reaches final maturation, FSH and E2 levels decrease (85). E2 and GPR30 (a membrane estrogen receptor) has been demonstrated to inhibit spontaneous oocyte maturation in fish (86). In goldfish gonads, estradiol stimulates ERs in a time and dose dependant manner (81). Estrogens and ERs have been shown to be involved in testicular development (87, 88) and undergo seasonal variations together with ERβI, FSHR, and aromatase mRNA which are produced at higher levels during late recrudescence stage compared to other seasons (72). It should be noted that testicular aromatase transcript levels are generally lower than ovarian aromatase transcript levels (72). The observed higher mRNA levels of ERα, ERβI, and aromatase in the ovary compared to testis is likely the result of the roles these factors play in the regulation of ovarian function and development. Transcript level for GH, LH subunits, LHR and a number of other hormones and

receptors were not investigated in the present study. There is already information on GHR mRNA levels in goldfish at mid-late and regressed gonadal stages (73). Circulating levels of E2 have also been extensively measured throughout the seasonal cycles of goldfish (51, 60, 89, 90). We did not study these factors in the present study, but recognize that quantifying these receptors, and serum E2 levels in response to treatments would have helped to better understand and link the hormonal control of reproduction and growth between pituitary, ovary, and liver.

Results in the present study demonstrated seasonal fluctuations in TRαI, TRβ, and IGF-I transcript levels, which were similar to those found in male goldfish liver (72). This is consistent with previous observations in goldfish (91). TRαI transcript levels in female liver were found to be higher in mid recrudescence, which correlated with higher IGF-I transcript levels. TRβ transcript level was observed to be higher in regressed and mid recrudescence stages compared to late recrudescence. It was shown previously that thyroid hormones play a role in stimulating the vitellogenic capacity of liver by elevating ERα expression and through actions on both TRα and TRβ (61). Higher TRβ expression during earlier gonadal recrudescence stages possibly contributes to T3 priming effect in the liver of female goldfish discussed earlier (61).

We further examined transcript levels of the same genes in response to treatments with GnRH and GnIH in the liver and ovaries of goldfish. However, the majority of the results do not demonstrate clear correlation with changes in LH and GH serum levels. Notably, although changes in ovarian ERs, FSHR and aromatase transcript levels in GnRH- and/or GnIH-treated fish were correlated with one another at mid recrudescence and reflected the close relationships between these elements in the neuroendocrine regulation of ovarian steroidogenesis and development at this reproductive stage (84), these changes mirrored neither serum LH and GH concentrations in the treatment groups. In addition at mid recrudescence, a time when ovarian steroidogenesis and yolk incorporation should be increasing (89), GnRH paradoxically exerted inhibitory effects on the levels of these ovarian gene transcripts, and liver Vtg mRNA levels were reduced by single injection of GnRH both in the absence and in the presence of GnIH. It is possible that direct actions of GnRH and GnIH on peripheral tissues, including liver and gonads, as well as changes in pituitary LH and GH release may have contributed to these complex results. Teleost fish lack a hypothalamo-hypophyseal portal system, and GnIH and GnRH directly act on the pituitary cells by innervations (92). Circulating levels of GnRH or GnIH are undetectable or lower than 1 pg/ml and therefore unlikely to elicit peripheral effects via endocrine actions (93, 94). However, with injections, we introduced higher levels of GnIH and GnRH in the circulation and it would be possible for these peptides to exert direct actions on the liver and ovaries. In fish and other vertebrates, the expression of GnRH and GnIH and their receptors, as well as their direct action have been demonstrated in peripheral tissues including liver and gonads (95–104). For example, although *in vitro* GnRH treatments of goldfish ovarian follicles in culture stimulated germinal vesicle breakdown, GnRH also attenuated gonadotropin- and progesterone-induced oocyte meiosis and steroidogenesis (105–107). GnIH direct inhibition of steroidogenesis in follicles could also contribute to changes in transcript level of various genes (108, 109). On the other hand, partial correlations were observed between ovarian ERα, ERβI, and FSHR transcript levels and GnRH-stimulated LH responses during late recrudescence in the present study. Consistent with the known ability of GH to directly stimulate IGF-I production in hepatocytes (110), increased GH serum concentrations induced by GnRH in late recrudescence was correlated with increased IGF-I mRNA levels in the liver. Thus, the observed responses in the different transcripts in liver and ovary to hormone treatments could be the consequence of changes in circulating GH and LH levels in addition to direct actions on the liver and ovary.

Dopamine is another important factor that contributes to the seasonal shift between growth and reproduction because it is known to reciprocally stimulate somatotropes and inhibit gonadotropes in goldfish (66, 111–117). Maximum somatotrope responsiveness to dopamine was demonstrated during growth seasons and lowest response was seen in sexually mature fish (114). Dopamine is possibly a key factor in reciprocal regulation of basal and GnRH and GnIH regulation of gonadotropes and somatotropes. Whether and how dopamine and other neuroendocrine factors known to exert direct reciprocal influences on gonadotropes and somatotropes, such as norepinephrine (118–120) and serotonin (121–123), co-ordinate and interact with GnRH, GnIH, and thyroid hormones in the integrated neuroendocrine control of growth and reproduction would be important areas for future studies.

## CONCLUSION

In summary, the present study demonstrates that GnRH and GnIH are factors in the regulation of LH and GH levels. GnIH inhibits GnRH-induced LH and GH release in fish at late stage of recrudescence. T3 exerts mainly inhibitory action on basal and GnRH- and/or GnIH-induced GH release. T3 inhibitory action on GnRH-induced LH release is reduced in the presence of GnIH in a gonadal state-dependent manner. Overall regulation of growth and reproduction is multifactorial and involve GnRH, GnIH, and T3. These results help to better understand the reciprocal regulation of seasonal reproduction and growth in female goldfish and other seasonally reproducing animals.

## DATA AVAILABILITY STATEMENT

All datasets generated for this study are included in the article/**Supplementary Material**.

## ETHICS STATEMENT

The animal study was reviewed and approved by University of Calgary Ethics Committee for use and care of experimental animals.

## AUTHOR CONTRIBUTIONS

YM, CL, and HH designed research. Research performed by YM and CL. YM analyzed data. CL, JC, and HH provided intellectual input on the data analysis. YM, JC, and HH wrote the paper. JC provided funding and oversight on radioimmunoassays. HH provided funding, intellectual input, and oversight on experimental design and data analysis.

## FUNDING

This work was supported by the funding from Natural Sciences and Engineering Research Council of Canada to HH (NSERC Discovery Grant; project no. 1254045) and to JC (NSERC Discovery Grant; project no. 121399). YM and CL were supported by NSERC grants to HH. YM was also supported by Queen Elizabeth II scholarship.

## ACKNOWLEDGMENTS

Authors would like to thank Mr. Enezi Khalid (UofA) and Mr. George Kinley (UofA) for their technical assistance and guidance on radioimmunoassays.

## SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2020.00175/full#supplementary-material

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 10 September 2020
doi: 10.3389/fendo.2020.00656

# Molecular Mechanisms of Pituitary Cell Plasticity

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The mechanisms that mediate plasticity in pituitary function have long been a subject of vigorous investigation. Early studies overcame technical barriers and challenged conceptual barriers to identify multipotential and multihormonal cell populations that contribute to diverse pituitary stress responses. Decades of intensive study have challenged the standard model of dedicated, cell type-specific hormone production and have revealed the malleable cellular fates that mediate pituitary responses. Ongoing studies at all levels, from animal physiology to molecular analyses, are identifying the mechanisms underlying this cellular plasticity. This review describes the findings from these studies that utilized state-of-the-art tools and techniques to identify mechanisms of plasticity throughout the pituitary and focuses on the insights brought to our understanding of pituitary function.

**Keywords: pituitary, plasticity, multihormonal cells, multipotential, leptin, mRNA translation, Musashi, single cell**

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***Edited by:***
*Finn-Arne Weltzien, Norwegian University of Life Sciences, Norway*

***Reviewed by:***
*Giampaolo Trivellin, University of Milan, Italy*
*Anderson O. L. Wong, The University of Hong Kong, Hong Kong*

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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *04 June 2020*
***Accepted:*** *11 August 2020*
***Published:*** *10 September 2020*

***Citation:***
*Childs GV, MacNicol AM and MacNicol MC (2020) Molecular Mechanisms of Pituitary Cell Plasticity. Front. Endocrinol. 11:656. doi: 10.3389/fendo.2020.00656*

## INTRODUCTION

The pituitary system orchestrates appropriate behavioral responses to fluctuating physiological and/or pathological signals, through controlled production and secretion of diverse signaling peptide hormones. The levels of hormone that must be secreted to meet effective serum levels for the intended response, is vast relative to the small size of the pituitary itself, and this defined size limits the number of cells that can be utilized in effecting any one of the many responses. These opposing challenges of limited cell numbers and diverse, large required outputs are resolved through plasticity in allocation of cell resources to each particular function (see **Figure 1**). The mechanisms controlling pituitary cell plasticity are a source of tremendous interest since these mechanisms first began to be revealed and evidence of malfunction in pituitary plasticity under diverse genetic and disease states has further motivated study to understand these mechanisms [for reviews see (1, 2)]. Over the last several decades, an ongoing procession of cutting edge, novel techniques have been developed and embraced in the effort to understand pituitary function and plasticity. In this mini review, we identify the multiple fortuitous links that have occurred between the need for information about a particular aspect of pituitary function and the identification, discovery and/or development of a new technique or tool that provided that information. We follow the pathway of discovery made with early cell microscopy

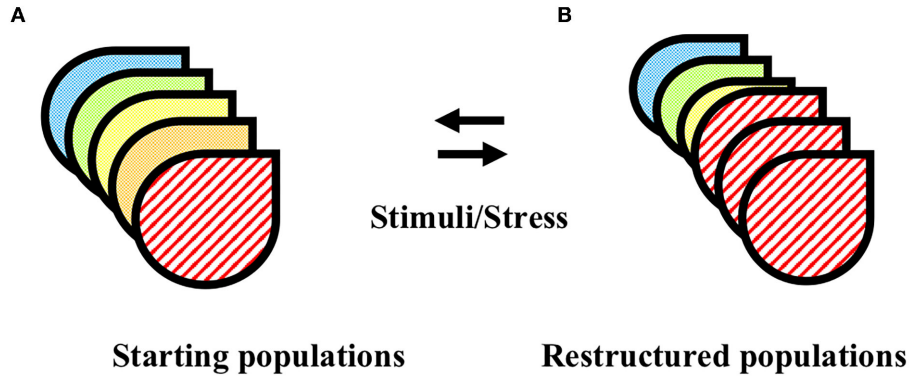


**FIGURE 1 |** Pituitary cell populations are restructured in response to physiological stimuli and pathological stressors. **(A)** The pituitary is comprised of multiple distinct functional cell types (designated by different image fill patterns). **(B)** In response to physiological stimuli or pathological stress, the pituitary cell populations undergo restructuring. The double arrows imply the potential reversibility of this process. See text for details and relevant references.

technologies through the ongoing development and utilization of complex and specific animal models to the emerging utilization of molecular and computational techniques that together identify cell-specific information. We will primarily focus on discoveries made through those tools and techniques that we have utilized ourselves, with the expectation that this overview will give a representative example of the pathways that have led to our current understanding of pituitary plasticity.

## BREAKTHROUGH DISCOVERIES; VISUALIZATION LEADING TO CELL IDENTIFICATION

The development of multiplexing technologies, simultaneously identifying multiple proteins and/or both proteins and mRNAs in the same cell population enabled a conceptual breakthrough in identification of the mechanisms of pituitary plasticity. The identification of more than one hormone in a single cell or the identification of cell surface activating receptors and distinct intracellular hormones in the same cell, suggested the presence of multipotential cells, a novel concept in the face of an existing paradigm of pituitary organization that indicated each hormone is produced by a dedicated cell population (3). The experimental proof of *bona fide* multipotential cells, as opposed to the uptake of proteins to one cell type from another cell type, required development and utilization of complex staining protocols along with novel microscopy technologies (4). These early studies, including those utilizing biotin-streptavidin directed staining, *in situ* hybridization, and immunogold electron microscopy, enabled identification of specific populations within the mature adult pituitary that express the mRNA, receptors and/or hormones indicative of multiple distinct cell types. Studies using calcium signaling identified populations of pituitary cells that appeared to be fully differentiated toward a specific cell type, yet retained the capacity to respond to multiple releasing hormones of other distinct cell types (5). These findings introduced the concept of multihormonal/multipotential cells within the adult pituitary, with the capacity to contribute to functional plasticity through generation of whichever cell type was needed to serve hormone demand. The origin of these multipotential cells continues to hold fascination in the study of pituitary plasticity. The mechanisms by which they arise, either from an existing subpopulation of immature progenitor cells or through a transdifferentiation of existing hormone-producing, mature cells remains an open question (6–8) (see **Figure 2**).

As the identification of multipotential/multihormonal cell types was hampered by technical barriers in distinguishing these cells from their monohormonal counterparts, so conceptual barriers further occluded their identification. Models of pituitary organization based upon embryological studies showed the ventral to dorsal gradients of tissue differentiation factors including Bmp2 and Gata2, that stimulate generation of gonadotropes in ventral regions and restrict expression of the opposing differentiation factor Pou1f1 (also named Pit-1), which is required for the generation of somatotropes, thyrotropes and lactotropes (9, 10). The decreased levels of Bmp2 in dorsal regions is thus required to allow the differentiation of somatotropes as Pou1f1 binds to Gata2 and prevents it from activating factors needed for gonadotrope development. These findings together support a model in which monohormonal somatotropes and gonadotropes develop in separate regions of the embryonic pituitary with the balance between expression of Pou1f1 and Gata2 restricting specification of each cell type (11–13). This developmental model appeared to preclude the presence of cells that produce multiple hormones, such as those that would require both Pou1f1 and Gata2, e.g., somatotogonadotropes (14).

Continued efforts in the field, however, revealed a model in which pituitary plasticity utilizes all members of a cell population, including multihormonal/multipotential subtypes, in response to challenges by stimuli that require multiple hormone responses. This coordinated multi cell-type response was demonstrated through affinity cytochemical studies showing the estradiol-mediated increase in gonadotropin releasing

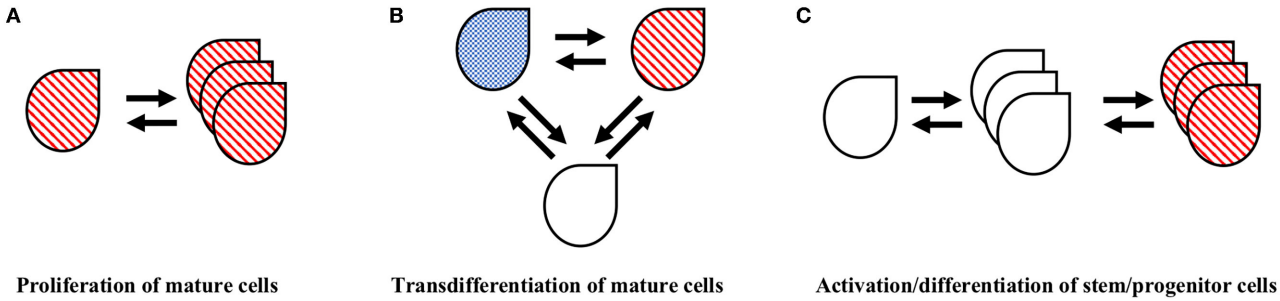


**FIGURE 2 |** Cellular mechanisms that may contribute to pituitary population restructuring. There is evidence that pituitary populations undergo restructuring through multiple cellular mechanisms including **(A)** mitotic proliferation of existing hormone cells, **(B)** transdifferentiation of mature hormone cells and **(C)** activation and differentiation of stem/progenitor cells. The double arrows imply a potential reversibility of these cellular processes. See text for details and relevant references.

hormone receptor (GnRHR) production by somatotropes, as well as by gonadotropes, with the somatotropes defined by their expression of growth hormone (GH) protein and mRNA (15). Gonadotropin releasing hormone (GnRH) was thus found to stimulate an increase in production of the "gonadotrope-specific" hormones, luteinizing hormone (LH) and follicle-stimulating hormone (FSH) by both gonadotropes and a subset of somatotropes/somatogonadotropes to support the high levels of LH and FSH that are needed to effect estrous cycle surges. A role for multihormonal cells in mediating pituitary plasticity was further proposed to facilitate the high levels of adrenocorticotropic hormone (ACTH), beta-endorphin, and thyroid stimulating hormone (TSH) that are required for the response to extreme or prolonged cold (16). The coordination of these multihormonal responses appear to require contributions from cells capable of producing multiple hormones in response to distinct neuropeptide secretagogues as has been demonstrated through cold stress-induced, arginine vasopressin (AVP) stimulation of "thyrocorticotropes" to produce both ACTH and TSH (17, 18). As these cells also respond to thyrotropin-releasing hormone (TRH) and corticotropin-releasing hormone (CRH), both of which enhance AVP receptor expression, these multipotential target cells would serve to amplify the pituitary response to stress.

The identification of mechanisms underlying pituitary functional plasticity has benefitted from the development and use of a number of techniques to isolate distinct pituitary cell subpopulations. These are reviewed in a recent publication (19). One early method developed in our laboratory included counterflow elutriation that took advantage of the different size and morphologies of distinct pituitary cell types to obtain subpopulations that were 90% pure (20). However, higher levels of purity are obtained with approaches that involve the expression of cell type-specific fluorescent proteins such as the enhanced green fluorescent protein (eGFP), linked to the gene promoter of a cell-type specific protein, such as somatotrope GH, enabled the use of fluorescent activated cell sorting technologies (FACS) that provide a pure population of live cells for downstream analyses (21). Development of pituitary cell-lineage tracing mouse models have identified a population of *Sox2*-expressing stem cells in the adult pituitary that can differentiate to produce all hormone-expressing cell types (22–26). Although activation of this cell population has been observed under conditions of acute experimental stress, e.g., hormone cell type-specific cell oblation and organ loss, the extent to which this mechanism contributes to pituitary homeostasis and functional plasticity is unclear (27). Adult stem cells have also been implicated in mediating pituitary neo-plastic growth, and this pathological aspect of pituitary plasticity has been extensively covered in recent excellent reviews (28, 29).

Continuing developments in microscopy, including the use of live cell imaging and electrophysiological tools, along with the development of analysis software has further enabled the identification of pituitary cell morphology plasticity and the observation of pituitary cell process motility and remodeling (30). Together, these cell properties have been shown to contribute to the formation of complex, three-dimensional heterotypic and homotypic pituitary cell networks that functionally contribute to plasticity of response (31, 32). Identification of the distinct subpopulations within a specific hormone cell type has been further facilitated through use of teleost transgenic models that demonstrate the role of heterotypic network communication in mediating gonadotrope function and plasticity (33). The ongoing development of pituitary tissue and cell culture techniques and the *in vitro* growth of pituitary stem/progenitor cells as "organoids" is directed toward development of an experimental model that is being utilized to address mechanisms of pituitary function that occur on an intermediate timescale, between the short term of cell culture and the lifetime of animal models (34–36). Organoid model developments include the use of human induced pluripotent stem cells (iPSCs) and the co-differentiation of hypothalamic and pituitary tissues in patient-specific organoids (37, 38). These human cell-based models complement the use of transgenic models in revealing the mechanisms underlying pituitary cell plasticity.

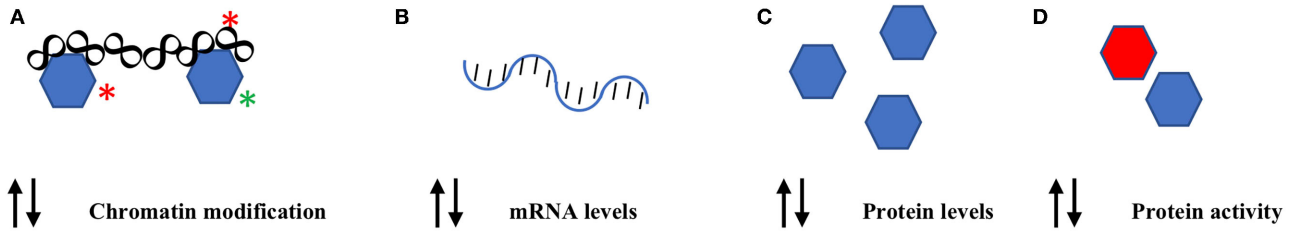


**FIGURE 3 |** Molecular mechanisms that may contribute to pituitary population restructuring. Pituitary cell populations may utilize multiple molecular mechanisms for restructuring including **(A)** chromatin remodeling **(B)** gene transcriptional control **(C)** control of mRNA translation to protein and **(D)** control of protein function. The double arrows indicate the potential for both activating (positive) and inhibitory (negative) effects upon these molecular mechanisms. See text for details and relevant references.

## CONTINUING DISCOVERIES; MECHANISMS MEDIATING CELL PLASTICITY

The utilization of transgenic animal models has greatly facilitated the study of pituitary function and plasticity. Many diverse, genomic models have been employed in identification of the roles of specific mediators of pituitary physiology and disease through the identification of effects upon whole animal physiology [for review see (2)]. Through the use of genetic models of pituitary cell type-specific leptin receptor knock-out, we have identified the mechanisms by which energy stores, as indicated through serum leptin signaling, influence pituitary function to optimize growth and reproduction. Findings from these studies have revealed the direct influence that leptin signaling has upon pituitary cell plasticity and the maturation required for hormone protein synthesis and secretion from pituitary somatotropes and pituitary gonadotropes (19, 39). Findings from these studies include the observation of decreased levels of growth hormone gene transcription (*Gh* mRNA) in pituitary somatotropes under conditions of loss of leptin signaling to the pituitary, thus indicating a link between leptin signaling to the pituitary and specific activator (s) of the *Gh* gene regulatory machinery (19).

Gene expression detection techniques have further resulted in the discovery of the absence of altered gene transcription under conditions in which it is expected, e.g., under conditions in which an increase in levels of GnRHR is observed in pituitary gonadotropes in response to leptin stimulation, that is not coincident with an increase in *Gnrhr* mRNA (39). This observed lack of concordance between changes in protein levels and changes in cognate mRNA levels suggests that a post-transcriptional mechanism mediates this regulatory process (40). We have recently found that the translation of the *Gnrhr* mRNA is repressed through the action of the RNA binding protein Musashi1 through direct association with the *Gnrhr* mRNA 3′untranslated region (41). Leptin stimulation is proposed to inhibit Musashi1 function, allowing de-repression and translation of the *Gnrhr* mRNA (39, 41). A recent study has demonstrated an opposing role for the RNA binding protein ELAVL1 through the post-transcriptional enhancement of *Gnrhr* mRNA stability (42). Since Musashi1 and ELAVL1 are found in common mRNA ribonucleoprotein complexes, Musashi1 and ELAVL1 may be coordinately regulated to differentially govern *Gnrhr* mRNA translation and thus gonadotrope remodeling throughout the estrus cycle (43). Several recent transcriptomics analyses of the pituitary at the cell type-specific and at the single cell level, have revealed an extraordinary level of variation in cell identity (44–47). The potential for plasticity at the cellular level, as defined by expression of genes associated with multiple hormone-producing cell types has been identified in a significant percentage of adult pituitary cells (47). These technologies greatly contribute to the comprehensive development of a model of pituitary cell functional plasticity. Findings from these analyses have definitively identified a multihormonal-expressing population within the adult pituitary that undergoes a high level of plasticity in hormone gene expression in response to the physiological stresses (47). The relevance of these data to mechanisms of pituitary plasticity will continue to be revealed as new 'omics data are obtained and bioanalysis tools are developed (48, 49) (see **Figure 3**).

## CONCLUSIONS AND FUTURE DIRECTIONS; BIG DATA TO MOLECULAR MANIPULATION

The understanding of pituitary function is relevant to diverse biomedical fields. Pituitary plasticity is fundamental to reproductive and endocrine function and impinges upon the control of metabolic disease, cancer and cell replacement therapies. From early imaging and cell morphology assays to current 'omics analyses, the array of methodologies directed toward an understanding of pituitary function stands as an exemplar of biomedical research capabilities (see **Figure 4**). The study of pituitary plasticity continues to utilize state of the art and emerging tools that provide data relevant to endocrine function and disorders over a wide biological scale from whole population patient data sets to molecular mechanisms revealed by novel animal models and single cell analyses. Ongoing and future studies are expected to combine targets revealed through these analyses with experimental interventions (35, 50). The recognition of the limitations of existing methods has driven

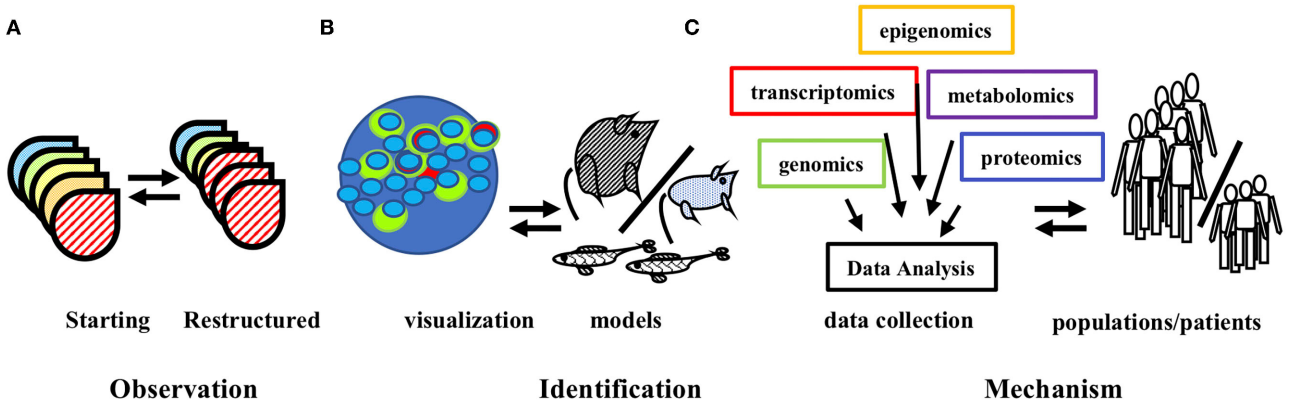


**FIGURE 4 |** Technological innovations contribute to understanding pituitary plasticity. **(A)** Early observations identified remodeling of pituitary hormone cell populations in response to stimuli and stress. **(B)** Microscopic observations and genetic models identified the cell populations that mediate restructuring. **(C)** The complimentary use of diverse data set analyses provides insight into the molecular mechanisms of pituitary population restructuring and suggest targets for therapeutic options for patients. The double arrows imply the information feedback of these complimentary technologies. See text for details and relevant references.

the development and use of novel technologies in pursuit of an understanding pituitary function. Similar to the creative courage that is required to propose new hypotheses, so, experimental courage is often required to test them; the employment of these together, will ensure that our ongoing investigations of pituitary function and plasticity will continue in new and exciting directions.

## AUTHOR CONTRIBUTIONS

GC and MM planned and wrote the paper. MM designed the figures. GC, AM, and MM edited the paper. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was funded by National Institutes of Health, 1R01DK113776-01 (GC, AM, and MM) and R01HD093461 (AM, GC and MM), and R01HD087057 (GC and AM).

## ACKNOWLEDGMENTS

The brief format of this review necessitated a limited representation of the field, and we apologize to the authors of the many studies that were not presented.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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ORIGINAL RESEARCH
published: 27 November 2020
doi: 10.3389/fendo.2020.603538

# Differential Regulation of the Expression of the Two Thyrotropin Beta Subunit Paralogs by Salmon Pituitary Cells *In Vitro*

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**Specialty section:**
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

**Received:** *07 September 2020*
**Accepted:** *15 October 2020*
**Published:** *27 November 2020*

**Citation:**
*Fleming MS, Maugars G, Martin P, Dufour S and Rousseau K (2020) Differential Regulation of the Expression of the Two Thyrotropin Beta Subunit Paralogs by Salmon Pituitary Cells In Vitro. Front. Endocrinol. 11:603538. doi: 10.3389/fendo.2020.603538*

We recently characterized two paralogs of the thyrotropin (TSH) beta subunit in Atlantic salmon, *tshβa* and *tshβb*, issued from teleost-specific whole genome duplication. The transcript expression of *tshβb*, but not of *tshβa*, peaks at the time of smoltification, which revealed a specific involvement of *tshβb* paralog in this metamorphic event. *Tshβa* and *tshβb* are expressed by distinct pituitary cells in salmon, likely related to TSH cells from the *pars distalis* and *pars tuberalis*, respectively, in mammals and birds. The present study aimed at investigating the neuroendocrine and endocrine factors potentially involved in the differential regulation of *tshβa* and *tshβb* paralogs, using primary cultures of Atlantic salmon pituitary cells. The effects of various neurohormones and endocrine factors potentially involved in the control of development, growth, and metabolism were tested. Transcript levels of *tshβa* and *tshβb* were measured by qPCR, as well as those of growth hormone (*gh*), for comparison and validation. Corticotropin-releasing hormone (CRH) stimulated *tshβa* transcript levels in agreement with its potential role in the thyrotropic axis in teleosts, but had no effect on *tshβb* paralog, while it also stimulated *gh* transcript levels. Thyrotropin-releasing hormone (TRH) had no effect on neither *tshβ* paralogs nor *gh*. Somatostatin (SRIH) had no effects on both *tshβ* paralogs, while it exerted a canonical inhibitory effect on *gh* transcript levels. Thyroid hormones [triiodothyronine (T3) and thyroxine (T4)] inhibited transcript levels of both *tshβ* paralogs, as well as *gh*, but with a much stronger effect on *tshβa* than on *tshβb* and *gh.* Conversely, cortisol had a stronger inhibitory effect on *tshβb* than *tshβa*, while no effect on *gh*. Remarkably, insulin-like growth factor 1 (IGF1) dose-dependently stimulated *tshβb* transcript levels, while it had no effect on *tshβa*, and a classical inhibitory effect on *gh*. This study provides the first data on the neuroendocrine factors involved in the differential regulation of the expression of the two *tshβ* paralogs. It suggests that IGF1 may be involved in triggering the expression peak of the *tshβb* paralog at smoltification, thus representing a potential internal signal in the link between body growth and smoltification metamorphosis.

**Keywords: *tshβ* paralogs, *gh*, thyroid hormones, cortisol, IGF1, CRH, *Salmo salar*, pituitary cells *in vitro***

## HIGHLIGHTS

- Atlantic salmon *tshβa* and *tshβb* paralog transcripts are differentially regulated by neuroendocrine factors *in vitro*
- Thyroid hormones have a stronger inhibitory effect on *tshβa* than on *tshβb* expression
- CRH specifically stimulates *tshβa* but not *tshβb* expression, while TRH and SRIH have no effect on both paralogs
- IGF1 specifically stimulates *tshβb* but not *tshβa* expression
- IGF1 may represent an internal cue linking growth and smoltification

## INTRODUCTION

The thyrotropic axis is the major neuroendocrine axis involved in the control of development and metabolism in all vertebrates. It also triggers larval metamorphosis, as investigated in depth in anuran amphibians [for review: (1)], and also shown in some teleosts such as flatfishes [for review: (2)]. Together with other neuroendocrine axes, it may be involved in the control of other life history transitions, such as smoltification (parr-smolt transformation) in salmonids. Smoltification is a crucial late developmental event, which triggers the downstream migration to the sea of the juvenile salmon and preadapts it to seawater conditions, where it will complete its oceanic growth phase before returning to its natal river to spawn [for reviews: (3–5)]. Smoltification is referred to as "secondary metamorphosis" by some authors, by comparison to the "primary" or "true" larval metamorphosis [for reviews: (6–8)].

Classically, in mammals, the thyrotropic axis comprises a cerebral neuropeptide named thyrotropin-releasing hormone (TRH), which acts on the pituitary to induce the synthesis and release of thyrotropin (thyroid-stimulating hormone; TSH). This pituitary hormone stimulates the production by the thyroid gland of thyroid hormones, thyroxine (T4) and triiodothyronine (T3), which act on a variety of peripheral target organs. The thyroid hormones also exert negative feedbacks on the brain and pituitary to regulate TSH production [for reviews: (9, 10)]. In non-mammalian vertebrates (amphibians and birds), some variations are observed in the thyrotropic axis, notably concerning the central control of TSH, which can also involve corticotropin-releasing hormone (CRH) [for review: (11)], a neuropeptide initially discovered in mammals for its role in the control of pituitary corticotropin and stress axis [for reviews: (12, 13)].

TSH, like gonadotropins, LH, and FSH, is a pituitary glycoprotein composed of two subunits, the alpha subunit (glycoprotein hormone alpha subunit, gp*a*), in common with gonadotropins, and a specific beta subunit (*tshβ*), which confers the hormone specificity [for review: (14)]. Due to teleost-specific whole genome duplication [TSWGD; also referred to as "3R," for "third round of whole genome duplication" (15)], teleosts possess two *tshβ* paralogs, *tshβa* and *tshβb* (16).

Our recent studies in Atlantic salmon, *Salmo salar*, revealed a remarkable functional divergence of the duplicated *tshβ* paralogs, with a striking peak of pituitary *tshβb* transcripts at the time of smoltification, in early April, concomitantly with the change in rheotaxism and initiation of downstream migration, whereas no change was observed for *tshβa*. While previous studies by many authors in salmonids had only concerned the "classical" *tshβa* paralog, the demonstration of another paralog and its expression peak at smoltification provided the first evidence for the involvement of TSH in smoltification metamorphosis (17).

Furthermore, as shown by *in situ* hybridization, the two Atlantic salmon *tshβ* paralogs are expressed by two different cell populations in the pituitary: *tshβa* cells are abundant and located in the anterior adenohypophysis, while *tshβb* cells are less numerous, well detected only at the time of smoltification, and located in the dorsal adenohypophysis near to the pituitary stalk (17). This differential localization and abundance of TSH cells in salmon could be compared to the situation in mammals and birds, which present a "classical" TSH cell population in the pituitary *pars distalis* (PD) and a less numerous TSH cell population in the *pars tuberalis* (PT) adjacent to the pituitary stalk [mammals (18–20); birds (21)]. In birds and mammals, PT-TSH acts retrogradely on hypothalamic area and is involved in the brain regulation of seasonal life-traits, such as reproduction, migration and hibernation [for reviews: (22–24)]. A similar potential role in the seasonal regulation of smoltification could be hypothesized for the *tshβb* paralog in salmon (17). While in birds and mammals, both pituitary TSH cell populations express the same single *tshβ* gene, the two TSH cell populations in Atlantic salmon express distinct *tshβa* and *tshβb* paralogs. This differential localization of the two paralogs, together with their sequence divergence [33% identity; 48% similarity; (17)] and their striking differential regulation at smoltification, illustrate a typical case of subfunctionalization (17).

The present study aims at investigating whether the expression of the two *tshβa* and *tshβb* paralogs are under differential central and peripheral controls. As for our previous investigations (17), this study was performed on Atlantic salmon from the Loire-Allier population, the last extant population from long-river in Western Europe, currently endangered, and under a conservation program at the Conservatoire National du Saumon Sauvage (CNSS). The regulation and timing of smoltification is an especially critical issue for this long-river population, as compared to short-river-ones, as smoltification occurs up to 900 km upstream, and smolts need to perform a long downstream migration and reach the estuary in a narrow window of favorable physiological and environmental conditions.

We used primary cultures of Atlantic salmon pituitary cells to study the direct pituitary control of the expression of both *tshβ* genes. While in birds and mammals, the same *tshβ* gene is expressed in both PD and PT cells, here we take advantage of the expression of distinct paralogs, *tshβa* and *tshβb*, by the two TSH cell populations in salmon, which enables us to follow their differential expression in whole mixed pituitary cell cultures. We tested the effects of central neurohormones, known to be

involved in TSH regulation in vertebrates (TRH, CRH, and somatostatin, SRIH) as well as peripheral hormones reported to increase before and/or during smoltification [thyroid hormones, insulin-like growth factor (IGF1), and cortisol] [for reviews: (6, 25)]. For comparison and validation, we also followed the regulation of the expression of growth hormone, *gh*, which may share some common regulatory factors with TSH in vertebrates, and which is involved in the regulation of osmoregulatory changes at smoltification in salmonids [for review: (26)].

# MATERIAL AND METHODS

## Animals

Atlantic salmon (*Salmo salar*) from the Loire-Allier population raised indoor under natural water, temperature, and photoperiod conditions, at the Conservatoire National du Saumon Sauvage (CNSS), Chanteuges, France (Agreement N° B43 056 005; according to the ARRETE N° DDCSPP/CS/2016/40), were used. They were anesthetized with an overdose of ms222 (0.4 ml/L; Sigma-Aldrich, St. Louis, MI, USA) and killed by decapitation in accordance with guidelines and regulations according to the protocol approved by Cuvier Ethic Committee, France. For each cell culture, pituitaries from 40 to 100 juvenile salmon were collected in cell culture medium at CNSS, and immediately transferred on ice to MNHN, Paris, where cell dispersion and cultures were performed. In order to be able to detect *tshβb* in pituitary cell cultures, salmon pituitaries were collected at different times in April (years 2015 and 2018) during the peak of its expression (17). For *tshβa* expression data, some additional experiments have been performed from January to April (years 2015, 2016, and 2018) with similar results as in April.

## Hormones

Thyroid hormones and cortisol were purchased from Sigma-Aldrich (Saint-Quentin Fallavier, France; triiodothyronine, T3, catalog number: T2752; thyroxine, T4, catalog number: T2501; cortisol, F, catalog number: H4001), and used at similar doses as in previous *in vitro* studies in the eel (27, 28), covering circulating ranges.

Bovine corticotropin-releasing hormone (CRH), which was shown to be effective on primary culture of pituitary cells from other teleost species [eel: (29); turbot: (30)], was purchased from Sigma-Aldrich (catalog number: C2671), and used at similar doses as in previous studies.

Thyrotropin-releasing hormone (TRH; catalog number: P1319) and somatostatin (SRIH; catalog number: S1763), fully conserved in vertebrates, were purchased from Sigma-Aldrich, and used at similar doses as in previous *in vitro* studies in other teleost species [eel: (29, 31); turbot: (30)].

Recombinant human insulin-like growth factor 1 (IGF1), which was shown to be effective on primary culture of pituitary cells from other teleost species [eel: (32); turbot: (33)], was purchased from R and D Systems (Lille, France; catalog number: 291-G1), and used at similar doses as in previous studies.

As in our previous studies, T3 and T4 were dissolved in NaOH 1N, cortisol in ethanol, CRH, TRH and SRIH in sterile water and IGF1 in sterile PBS to obtain stock solutions ($10^{-3}$ M), which were stored at −20°C.

## Primary Culture of Salmon Pituitary Cells

### Dispersion and Culture

Dispersion and primary culture of pituitary cells were performed using an enzymatic and mechanical method adapted from [eel (34); salmon (35)]. Briefly, 40 to 100 pituitaries were incubated at 25°C in a solution of 0.8 mg porcine type II trypsin (Sigma-Aldrich)/ml dispersion buffer (DB: Dulbecco's saline phosphate buffer without $Ca^{2+}$ and $Mg^{2+}$, with 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml fungizone; Gibco, Thermo Fisher Scientific, Villebon-sur-Yvette, France). After 30 min, the trypsin solution was replaced by a solution of 1 µg DNase (Sigma-Aldrich) and 1 mg soya bean trypsin inhibitor (Sigma-Aldrich)/ml DB for 10 min. Pituitary slices were then washed with DB (Gibco) and mechanically dispersed in DB by repeated passages through a plastic transfer pipette (Falcon, Thermo Fisher Scientific). Cell suspensions were filtered through nylon mesh (30 µm pore size), harvested by centrifugation at 200 g for 10 min, resuspended in DB, and counted with a Malassez cytometer. The number of viable cells was estimated by Trypan Blue coloration exclusion (Sigma-Aldrich) and was about 90%. Cells were plated on 96-well plates (~60,000 cells/well) pre-coated with a solution of 0.1 mg/ml poly-L-lysine (Sigma-Aldrich). Cultures were performed in serum-free culture medium (Medium 199 with Earle's salt and sodium bicarbonate buffer, 100 U/ml penicillin, 100 µg/ml streptomycin, and 250 ng/ml fungizone; Gibco) at 18°C under 3% $CO_2$ and saturated humidity in tissue culture incubator (Galaxy 170R, Eppendorf, France).

### In Vitro Treatments

Treatments were started 24 h after the beginning of culture to allow cell attachment (Day 0). Replicates of five wells for control and each treated group were used. Stock solutions ($10^{-3}$M) were diluted in culture medium just before addition to the culture wells. Culture medium was changed and treatment added to the cells on Day 0, Day 3, and Day 7. Cultures were stopped on Day 10, according to our previous protocol (36–38). The effects of treatments were tested in three to five independent experiments performed on different cell preparations from different batches of fish. Figures display the results of representative experiments.

### Cell RNA Extraction and cDNA Synthesis

Total RNA was directly extracted from cells in culture wells using the Cell-to-cDNA II Kit (Ambion Inc. Austin, TX, USA) according to the manufacturer's recommendations. Cells were washed with PBS (Gibco) and lysed with Cell Lysis II Buffer (80 µl/well). The lysates were digested with RNase-free DNase I (Roche Ltd., Basel, Switzerland). Four µl of RNA solution of each samples was then reverse transcribed with the SuperScript III First Strand cDNA Synthesis Kit (Invitrogen Cergy-Pontoise, France). The samples obtained were stored at −20°C until qPCR.

## Real-Time Quantitative PCR (qPCR)

Gene specific primers were previously designed based on the nucleotide sequences of the Atlantic salmon thyrotropin-*β* subunit paralog a and paralog b (*tshβa* and *tshβb*) and *β-actin* (17), the latter being used as reference gene (**Table 1**). For growth hormone, *gh*, as the two paralogs, *gh1* and *gh2*, issued from salmonid-specific WGD (40) have highly similar sequences and were regulated in the same manner in our pilot experiments (as tested on pooled samples, data not shown), common *gh* specific primers (**Table 1**) were designed for Atlantic salmon, using the Primer3 Software (Whitehead Institute/Massachusetts Institute of Technology, Boston, MA, USA). Forward and reverse primers were located in different exons to prevent amplification of genomic DNA. To optimize the assay, different annealing temperatures were tested according to the melting temperature (Tm) of primers. To check their specificity, amplification products were sequenced at GATC Biotech (Mulhouse, France).

Quantitative PCR assays were performed using the LightCycler® System (Roche) with SYBR Green I sequence-unspecific detection as previously described (17, 38). The qPCRs were prepared with 4 µl of diluted cDNA template, 2 µl of PCR grade water, 2 µl of SYBR Green master mix, and 1 µl of each forward and reverse primer (500 nM each at final concentration). The protocol was an initial step of polymerase activation for 10 min at 95°C; then 41 cycles (*β-actin*, *gh* and *tshβa*) of 10 s at 95°C for denaturing, 5 s at 60°C for annealing, 10 s at 72°C for primer extension, and a single final extension step of 5 min at 72°C. For *tshβb*, the protocol was an initial step of polymerase activation for 10 min at 95°C; 51 cycles of 10 s at 95°C, 5 s at 62°C, 10 s at 72°C, and a single final extension step of 5 min at 72°C. Each program ended with a melting curve analysis by slowly increasing the temperature (0.01°C/s) up to 95°C with a continuous registration of changes in fluorescent emission intensity. Serial dilutions of cDNA pools of pituitary cells were used as a standard curve. One chosen dilution was also included in each run as a calibrator. Each qPCR run contained a non-template control (cDNA was substituted by water) for each primer pairs to confirm that reagents were not contaminated. The efficiency of all primers was tested, and the specificity of each reaction was assessed by melting curve analysis to ensure the presence of only one product. Each sample was analyzed in duplicate by qPCR. Normalization of data was performed using *β-actin* mRNA level and results expressed as arbitrary units, relatively to mean value of control group, considered as 1.

**TABLE 1** | Primer sequences used in qPCR amplifications.

| Primers | 5'-3' sequence (bp) | |
|---|---|---|
| *actin*-F | CCAAAGCCAACAGGGAGAAG | Olsvik et al. (39) |
| *actin*-R | AGGGACAACACTGCCTGGAT | Fleming et al. (17) |
| *tshβa*-F | CTCCTTTGCCTGCTCTTCAG | Fleming et al. (17) |
| *tshβa*-R | GGCCAGCTCCTTCATGTTAC | Fleming et al. (17) |
| *tshβb*-F | TTGCCGTCAACACCACCAT | Fleming et al. (17) |
| *tshβb*-R | GGGATGATAGACCAGGGAGTG | Fleming et al. (17) |
| *gh*-F | AGAAGCTCAGCGACCTCAAA | This study |
| *gh*-R | TGTCATCCAGGCTCAGTACG | This study |

*The table provides the sequences of forward (F) and reverse (R) primers used for qPCR of Atlantic salmon β-actin, tshβa, tshβb, and gh.*

## Statistics

Results are given as mean ± SEM (n = 5 wells/treatment; 60,000 cells/well; cell culture from 40–100 pituitaries). Means were compared by one-way ANOVA Tukey's multiple comparison test using Instat (GraphPad Software Inc., San Diego, CA, USA). Differences are considered significant when $P < 0.05$.

# RESULTS

## Detection of *tshβa* and *tshβb* Transcripts by qPCR in Pituitary Cell Cultures

Transcript levels of *tshβa* paralog could be well detected by qPCR in primary cultures of pituitary cells (~60,000 cells/well) with similar expression levels in juvenile Atlantic parr or smolt, as tested in January (parr), April (smolt), and June (post-smolt) (data not shown). In contrast, transcript levels of *tshβb* paralog in cell cultures were under qPCR detection limit in January and June and could be measured in April. In April, basal expression levels of *tshβb* were still lower than that of *tshβa*, as suggested by the difference in the average quantification cycle values (Cq) (20 Cq for *tshβa* and 28 Cq for *tshβb*, which would correspond to about 500-fold difference in abundance).

## Effects of Thyroid Hormones (T3 and T4)

The effects of various concentrations of T3 and T4 (from $10^{-11}$ to $10^{-7}$ M) were tested (**Figure 1**). Both T4 and T3 dose-dependently downregulated *tshβa* mRNA levels (**Figure 1A**). Their effects were significant at $10^{-11}$ M (10% inhibition as compared to controls, $P < 0.05$ for T3 and 18% inhibition, $P < 0.001$ for T4) and reached for both hormones more than 85% inhibition at $10^{-9}$ M ($P < 0.001$) and more than 95% inhibition at $10^{-7}$ M ($P < 0.001$).

T3 and T4 also dose-dependently decreased *tshβb* mRNA levels, but with a lower inhibitory effect than on *tshβa* (**Figure 1B**). For T3, the effect was significant at $10^{-9}$ M (24% inhibition, $P < 0.01$) and reached 34% inhibition at $10^{-7}$ M, as compared to controls. For T4, the effect was significant at $10^{-7}$ M with 25% inhibition.

T3 and T4 dose-dependently inhibited *gh* expression (**Figure 1C**). For T3, the effect was significant at $10^{-9}$ M (25% inhibition, $P < 0.01$) and reached 42% inhibition at $10^{-7}$ M ($P < 0.001$) as compared to controls. For T4, the effect was significant at $10^{-7}$ M with 32% inhibition ($P < 0.001$).

## Effects of Cortisol (F)

The effects of various concentrations of F (from $10^{-11}$ to $10^{-7}$ M) were tested (**Figure 2**).

Low doses ($10^{-11}$ and $10^{-9}$ M) of F were ineffective in regulating *tshβa* mRNA levels, but $10^{-7}$ M had a significant inhibitory effect (44% inhibition; $P < 0.001$, as compared to controls) (**Figure 2A**).

Low doses ($10^{-11}$ and $10^{-9}$ M) of F had no effect on *tshβb* mRNA levels, while $10^{-7}$ M was able to strongly reduce them (72% inhibition; $P < 0.001$) (**Figure 2B**).

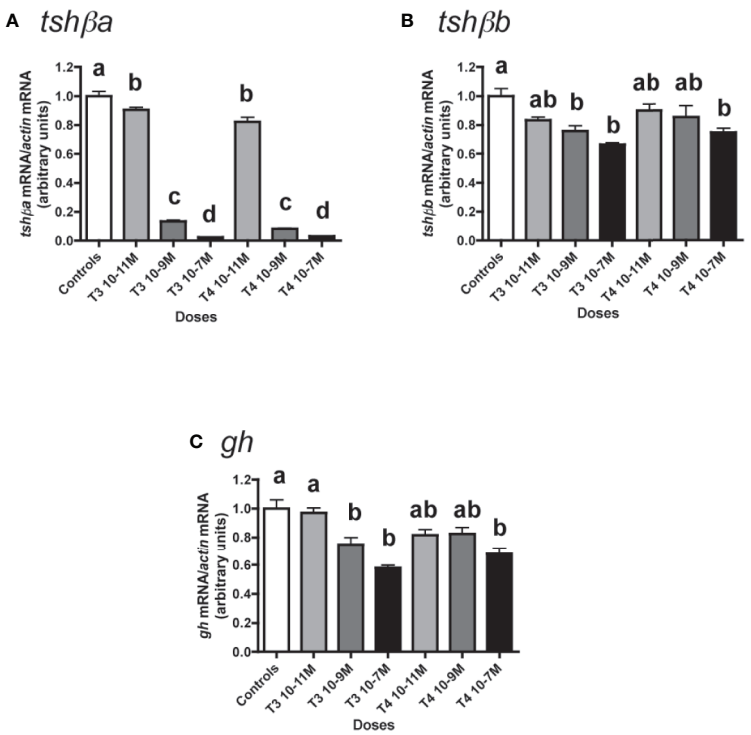


**FIGURE 1** | Effects of thyroid hormones (T3 and T4) on *tshβa*, *tshβb*, and *gh* transcript levels in primary cultures of Atlantic salmon pituitary cells. Pituitary cells from juvenile Atlantic salmons were treated with various concentrations of T3 or T4 for 10 days. The mRNA levels of *tshβa* **(A)**, *tshβb* **(B)**, and *gh* **(C)** were quantified by qPCR. Data were normalized against *β-actin*. The Figure displays the results from a representative experiment of three (*tshβb*) or five (*tshβa* and *gh*) independent cell culture experiments. Mean ± SEM; n = 5 well replicates. Different letters indicate significant differences, ANOVA.

In contrast, none of the doses of F tested had any effect on *gh* mRNA levels (**Figure 2C**).

## Effects of CRH and TRH Alone

The effects of CRH and TRH ($10^{-8}$ and $10^{-6}$ M) were tested (**Figure 3**). Both doses of CRH significantly increased the mRNA levels of *tshβa* (×1.6 for $10^{-8}$ M; ×1.7 for $10^{-6}$ M; $P < 0.001$ for both doses, as compared to controls) (**Figure 3A**). In contrast, TRH had no effect on *tshβa* mRNA levels at both doses (**Figure 3A**).

None of the two tested doses of CRH and TRH had any effect on *tshβb* mRNA levels (**Figure 3B**).

Both doses of CRH increased the mRNA levels of *gh* (×1.3; $P < 0.001$ for both doses) (**Figure 3C**). TRH had no effect on *gh* mRNA levels at both doses (**Figure 3C**).

## Effects of CRH and TRH in the Presence of T3

The potential stimulatory effects of CRH or TRH on *tshβ* paralogs were further investigated by testing the effects of CRH and TRH in combination with the inhibitory effect of T3 (**Figure 4**).

As observed above (**Figure 3A**), $10^{-6}$ M CRH alone stimulated *tshβa* mRNA levels (×1.3; $P < 0.001$), while $10^{-6}$ M TRH alone had no effect (**Figure 4A**). T3 at $10^{-8}$ M, as observed in **Figure 1C**, inhibited *tshβa* mRNA levels by more than 90% inhibition ($P < 0.001$) (**Figure 4A**). In the presence of $10^{-8}$ M T3, the stimulation of *tshβa* mRNA levels by $10^{-6}$ M CRH was greater (×8.8; $P < 0.001$) than when tested alone, while $10^{-6}$ M TRH had still no effect (**Figure 4A**).

Concerning the *tshβb* paralog, as observed above (**Figure 3B**), $10^{-6}$ M of CRH or TRH alone had no effect on *tshβb* mRNA levels (**Figure 4B**). $10^{-8}$ M T3, as observed in **Figure 1C**, inhibited *tshβb* mRNA levels (26% inhibition) (**Figure 4B**). In the presence of T3, $10^{-6}$ M of CRH and TRH had still no effect on *tshβb* mRNA levels (**Figure 4B**).

In order to further assess the stimulatory effect of CRH on *tshβa* mRNA levels, different doses of CRH ($10^{-10}$, $10^{-8}$, and $10^{-6}$ M) were tested in the presence of different doses of T3 ($10^{-10}$, $10^{-9}$, and $10^{-8}$ M) (**Figure 4C**). T3 ($10^{-10}$ M) inhibited *tshβa* mRNA levels by 27% compared to controls ($P < 0.001$). CRH dose-dependently stimulated *tshβa* mRNA levels in the presence of $10^{-10}$ M T3 (up to ×1.8 at $10^{-6}$ M; $P < 0.001$, as compared to $10^{-10}$ M T3 alone), which reached values higher than in non-treated controls (×1.3; $P < 0.001$; as compared to controls). T3 ($10^{-9}$ M) inhibited *tshβa* mRNA levels by 84% compared to controls ($P < 0.001$). CRH dose-dependently stimulated *tshβa* expression in the presence of $10^{-9}$ M T3 (up to ×3.2 at $10^{-6}$ M; $P < 0.001$, as compared to $10^{-9}$ M T3 alone). T3 at $10^{-8}$ M inhibited *tshβa* mRNA levels by more than 90% compared to controls ($P < 0.001$). CRH dose-dependently stimulated *tshβa* mRNA levels in the presence of $10^{-8}$ M T3 (up to ×17 at $10^{-6}$ M; $P < 0.001$, as compared to $10^{-8}$ M T3 alone), but which still remained lower than in non-treated controls. Thus, the

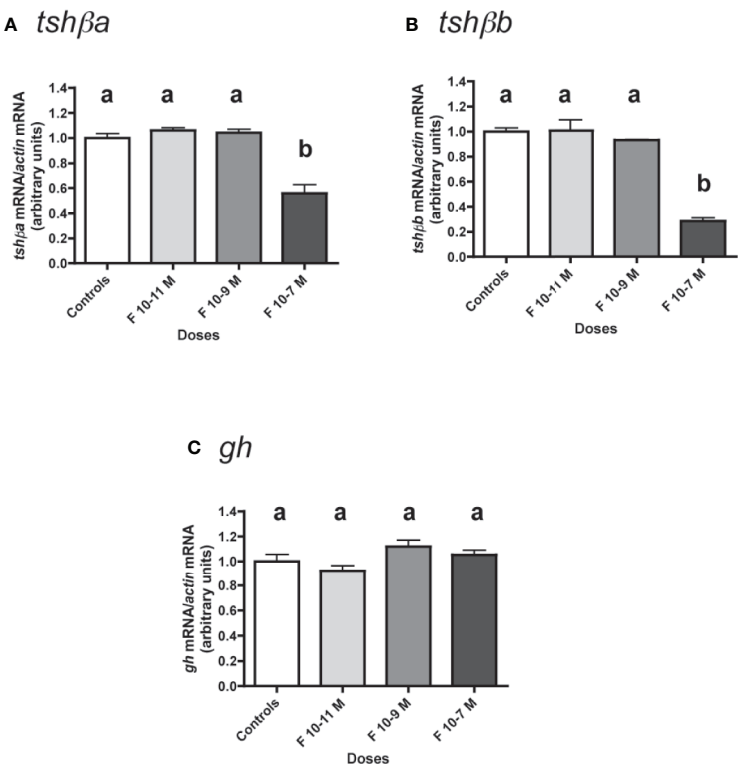


**FIGURE 2** | Effects of cortisol (F) on the expression of *tshβa*, *tshβb*, and *gh* transcript levels in primary cultures of Atlantic salmon pituitary cells. Pituitary cells from juvenile Atlantic salmons were treated with various concentrations of F for 10 days. The mRNA levels of *tshβa* **(A)**, *tshβb* **(B)**, and *gh* **(C)** were quantified by qPCR. Data were normalized against *β-actin*. The Figure displays the results from a representative experiment of three independent cell culture experiments. Mean ± SEM; n = 5 well replicates. Different letters indicate significant differences, ANOVA.

stimulatory effect of CRH on *tshβa* was largely enhanced in the presence of high inhibitory doses of T3.

## Effects of CRH and TRH in the Presence of F

Similarly, the potential stimulatory effects of CRH ($10^{-6}$ M) or TRH ($10^{-6}$ M) were tested in combination with the inhibitory effect of $10^{-7}$ M F (**Figure 5**).

As observed above (**Figures 3A** and **4A**), $10^{-6}$ M CRH alone stimulated *tshβa* mRNA levels (×1.6; $P < 0.001$), while $10^{-6}$ M TRH had no effect. F at $10^{-7}$ M, as observed in **Figure 2A**, moderately inhibited *tshβa* mRNA levels (25% inhibition). In the presence of F, the stimulation of *tshβa* mRNA levels by $10^{-6}$ M CRH was also found (×1.8; $P < 0.001$, as compared to $10^{-7}$ M F alone), while $10^{-6}$ M TRH had no effect (**Figure 5A**).

As observed above (**Figures 3B** and **4B**), $10^{-6}$ M of CRH or TRH alone had no effect on *tshβb* mRNA levels. F at $10^{-7}$ M, as shown in **Figure 2B**, strongly inhibited *tshβb* mRNA levels (76% inhibition). In the presence of F, $10^{-6}$ M of CRH and TRH had still no effect on *tshβb* mRNA levels (**Figure 5B**).

## Effects of SRIH

The effect of SRIH ($10^{-8}$ and $10^{-6}$ M) was tested (**Figure 6**). None of the two tested doses of SRIH had any effect on *tshβa* and *b* mRNA levels (**Figures 6A, B**). In contrast, *gh* mRNA levels were strongly downregulated by SRIH (72% inhibition at $10^{-8}$ M and 74% inhibition at $10^{-6}$ M; $P < 0.001$ for both doses, as compared to controls) (**Figure 6C**).

## Effects of IGF1

The effects of various concentrations of IGF1 (from $10^{-12}$ to $10^{-7}$ M) were tested (**Figure 7**). None of the six tested doses of IGF1 had any significant effect on *tshβa* mRNA levels (**Figure 7A**).

In contrast, IGF1 dose-dependently and strongly stimulated mRNA levels of the other paralog, *tshβb* (**Figure 7B**). The stimulatory effect of IGF1 was significant from $10^{-10}$ M (×1.6; $P < 0.05$, as compared to controls) and reached ×4.2 at $10^{-7}$ M ($P < 0.001$, as compared to controls).

On the opposite, *gh* mRNA levels were downregulated in a dose-dependent manner by IGF1 (**Figure 7C**). The inhibitory effect of IGF1 was significant at $10^{-10}$ M (47% inhibition) and reached a plateau with more than 85% inhibition at $10^{-9}$ M ($P < 0.001$ for all doses).

# DISCUSSION

In the present study, we investigated the differential regulation of the duplicated *tshβa* and *tshβb* paralogs in the juvenile Atlantic

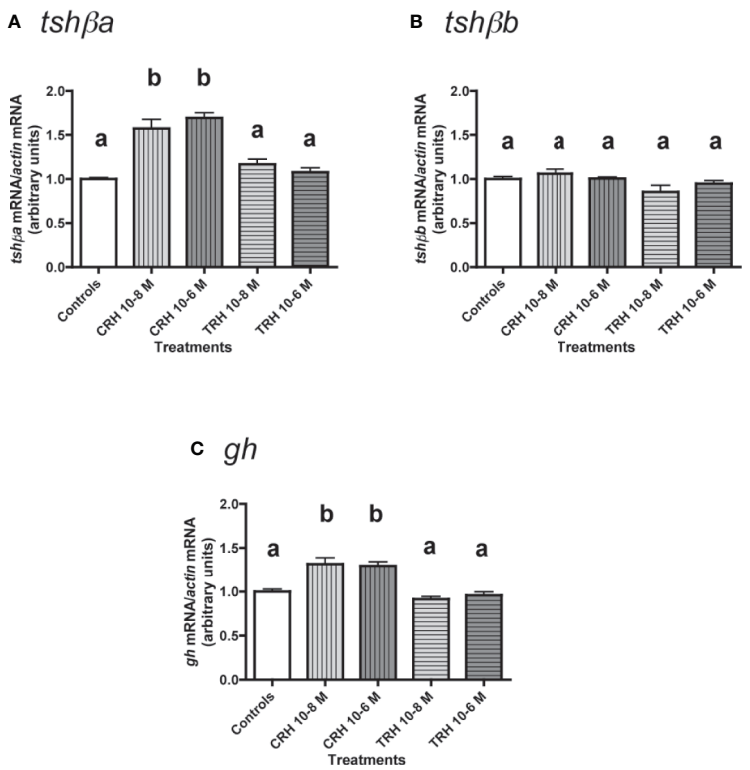


**FIGURE 3** | Effects of CRH and TRH on *tshβa*, *tshβb*, and *gh* transcript levels in primary cultures of Atlantic salmon pituitary cells. Pituitary cells from juvenile Atlantic salmons were treated with $10^{-8}$ and $10^{-6}$ M of CRH or TRH for 10 days. The mRNA levels of *tshβa* **(A)**, *tshβb* **(B)**, and *gh* **(C)** were quantified by qPCR. Data were normalized against *β-actin*. The Figure displays the results from a representative experiment of three independent cell culture experiments. Mean ± SEM; n = 5 well replicates. Different letters indicate significant differences, ANOVA.

salmon by various central and peripheral neuroendocrine factors. We were especially interested in deciphering whether one of these factors could be potentially involved in the regulation of the striking peak of *tshβb* transcript expression observed at the time of smoltification (17).

## Expression of *tshβa* and *tshβb* Paralogs in Primary Cultures of Atlantic Salmon Pituitary Cells

We investigated the regulatory effects of neurohormones and hormones, as exerted directly at the pituitary level, by using primary cultures of Atlantic salmon pituitary cells. In mammals and birds, the same single *tshβ* gene is expressed by the two distinct pituitary PD- and PT-TSH cell populations, that requires to micro-dissect the PT and PD pituitary regions in order to investigate potential differential regulations in cell cultures. A series of investigations have been performed on primary cultures of ovine PT cells (41–44), but to our knowledge, these studies did not address the regulation of *tshβ* expression. Here, we took advantage of the *tshβa* and *tshβb* paralogs issued from the teleost whole genome duplication, each one being expressed in distinct anterior and dorsal pituitary TSH cell populations in Atlantic salmon. This makes it possible to evaluate their differential transcriptional regulation in the same experiments and the same conditions, in primary cultures of mixed pituitary cells.

Our culture system is based on a low number of pituitary cells per well (~60,000 cells/well), as previously developed for the eel, *Anguilla anguilla* (31, 32), in order to limit the number of fish being used, in agreement with the international recommendations for animal experimentation (Reduce, Refine, Replace), and especially relevant for species concerned by biodiversity conservation. In these conditions, both *tshβa* and *tshβb* transcripts could be measured by qPCR in primary cultures of pituitary cells of Atlantic salmon smolts, as performed in April at the time of smoltification climax. In contrast, *tshβb* mRNA levels were under qPCR detection limit in pituitary cell cultures from parr (January) and post-smolts (June). This is in agreement with our previous studies on the annual expression profiles of *tshβa* and *tshβb* paralogs *in vivo*, as measured by qPCR on whole pituitaries. *In vivo*, *tshβa* pituitary transcript levels remained unchanged from December to June, including the period of smoltification. In contrast, *tshβb* transcript levels were very low in juvenile salmon parr, in December and January, started increasing in February–March to reach a peak in early April at the time of smoltification-related rheotaxism inversion, and dropped in May to reach back very low levels in June in post-smolts (17). Similarly, the lower

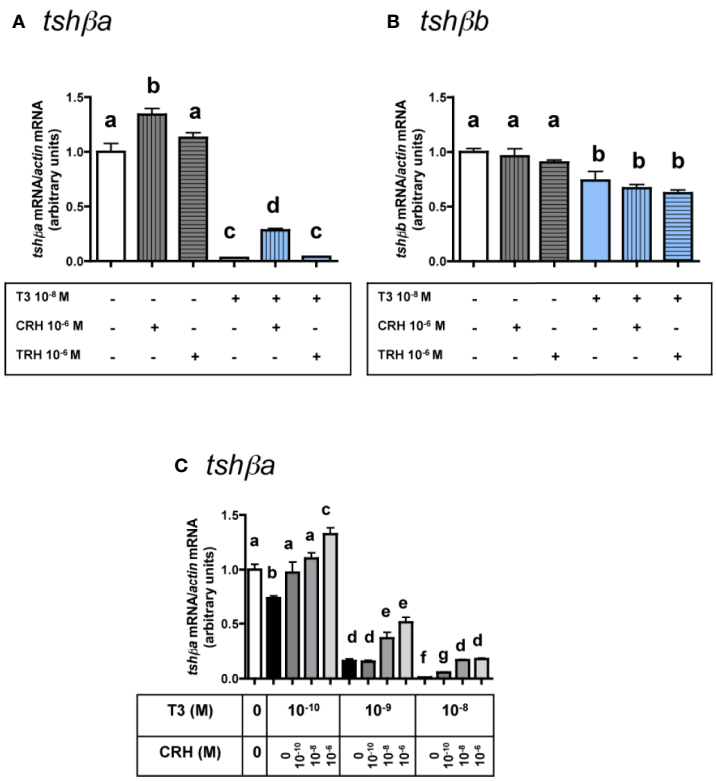


**FIGURE 4** | Effects of CRH and TRH on *tshβa* and *tshβb* transcript levels in the presence of T3 in primary cultures of Atlantic salmon pituitary cells. Pituitary cells from juvenile Atlantic salmons were treated for 10 days with $10^{-6}$ M of CRH or $10^{-6}$ M TRH in the presence of $10^{-8}$ M of T3, and mRNA levels of *tshβa* **(A)** and *tshβb* **(B)** were quantified by qPCR. In **(C)**, pituitary cells were treated for 10 days with various doses of CRH ($10^{-10}$, $10^{-8}$, and $10^{-6}$ M) in the presence of various doses of T3 ($10^{-10}$, $10^{-9}$, and $10^{-8}$ M) and mRNA levels of *tshβa* were quantified by qPCR. The Figure displays the results from a representative experiment of three independent cell culture experiments. Mean ± SEM; n = 5 well replicates. Different letters indicate significant differences, ANOVA.

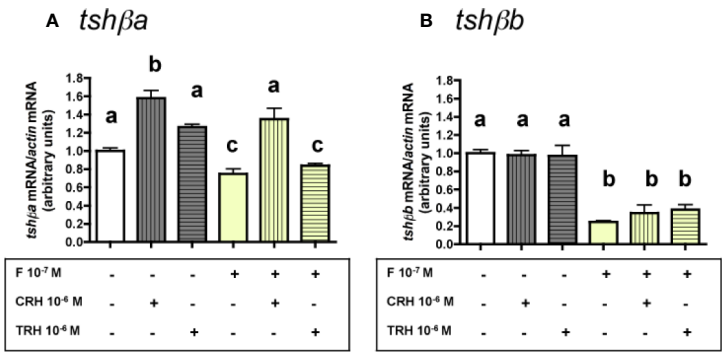


**FIGURE 5** | Effects of CRH and TRH on *tshβa* and *tshβb* transcript levels in the presence of cortisol in primary cultures of Atlantic salmon pituitary cells. Pituitary cells from juvenile Atlantic salmons were treated for 10 days with $10^{-6}$ M of CRH or $10^{-6}$ M TRH in the presence of $10^{-8}$ M of cortisol (F) and mRNA levels of *tshβa* **(A)** and *tshβb* **(B)** were quantified by qPCR. Data were normalized against *β-actin*. The Figure displays the results from a representative experiment of three independent cell culture experiments. Mean ± SEM; n = 5 well replicates. Different letters indicate significant differences, ANOVA.

abundance of *tshβb* than *tshβa* basal transcript levels in cell cultures even in April, as suggested by the comparison of their respective qPCR quantification cycle values (Cq), is in agreement with our previous results by qPCR on whole pituitaries and previous observation by *in situ* hybridization (17). This large difference in transcript abundance is in agreement with the hypothesis (17) of a local role and retrograde action toward the brain of the *tshβb* paralog, as for PT-TSH in amniotes, in contrast

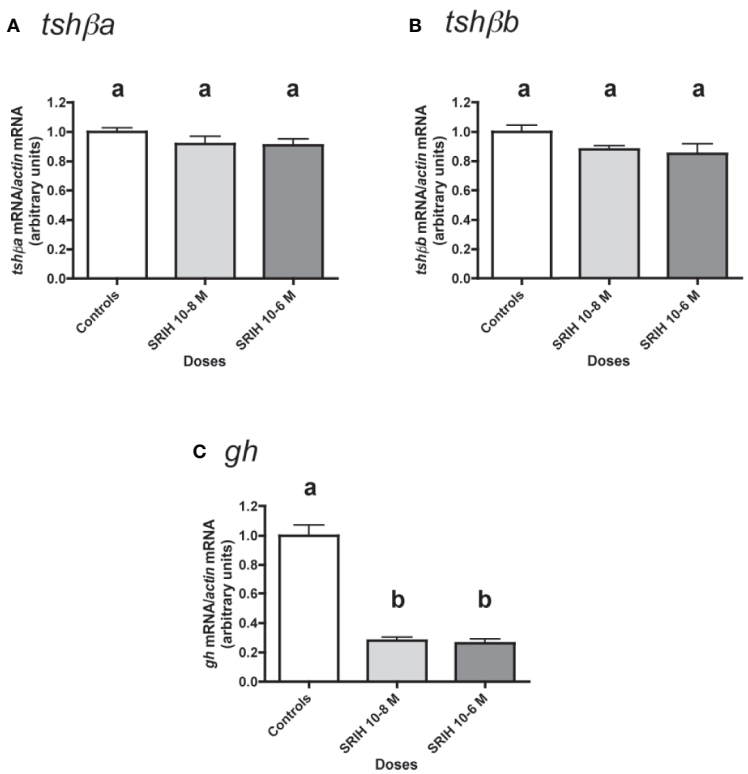


**FIGURE 6** | Effects of SRIH on *tshβa*, *tshβb*, and *gh* transcript levels in primary cultures of Atlantic salmon pituitary cells. Pituitary cells from juvenile Atlantic salmons were treated with $10^{-8}$ and $10^{-6}$ M of SRIH for 10 days. The mRNA levels of *tshβa* **(A)**, *tshβb* **(B)**, and *gh* **(C)** were quantified by qPCR. Data were normalized against *β-actin*. The Figure displays the results from a representative experiment of three independent cell culture experiments. Mean ± SEM; n = 5 well replicates. Different letters indicate significant differences, ANOVA.

to the endocrine role *via* the general blood circulation of the *tshβa* paralog, as for PD-TSH in amniotes.

## Stronger Inhibition by Thyroid Hormones of *tshβa* Than *tshβb* Expression in Atlantic Salmon

In salmonids, a rise of plasma T4 and/or T3 levels is observed at the time of smoltification [for reviews: (6, 45); Atlantic salmon, T4 and T3 (46); coho salmon *Oncorhynchus kisutch*, T4 (47, 48); coho, chinook salmon and steelhead trout, T4 and T3 (49); coho salmon, T4 and T3 (50, 51); masu salmon *Oncorhynchus masou*, T4 and T3 (52); masu salmon and amago salmon, *Oncorhynchus rhodurus*, T4 (53); masu salmon, T4 (54)]. As part of the thyrotropic axis, thyroid hormones (T3 and T4) are well-known to exert a negative feedback on the pituitary, downregulating TSH production in mammals and other vertebrates [for reviews: (9, 10)].

In line with this negative feedback, our study showed that thyroid hormones dose-dependently inhibited the expression of the transcripts of both salmon *tshβ* paralogs. Remarkably, the inhibitory effect was much stronger on *tshβa*, with up to more than 95% inhibition, than on *tshβb*, with a maximal inhibition of 25%.

For comparison, we measured the impact of thyroid hormones on *gh* transcript expression in Atlantic salmon pituitary cells. We also observed a moderate inhibitory effect of T4 and T3 on transcript expression, reaching a maximal inhibitory effect of 30%. Our previous studies in the eel also showed an inhibitory regulation of GH synthesis and release by thyroid hormones, exerted directly at the pituitary level (28), and which may represent an ancestral and largely conserved crosstalk between somatotropic and thyrotropic axes in vertebrates [for review: (55)].

In teleosts, before the availability of bioassays for directly quantifying TSH, one investigation mean was to look by histological methods at the activity of TSH cells. Baker thus observed that the addition of T4 to the culture medium of pituitary trout and eel culture prevents the hyperactivity of TSH cells *in vitro* [cytoplasmic degranulation (56); increase of uridine uptake (57)] suggesting that T4 has a direct negative effect on these cells. This cytological result was confirmed in guppy *Poecilia reticulata* (58, 59). *In vivo* experiments by Peter using implants of T4 in the pituitaries of goldfish and measuring radioactive iodine uptake by the thyroid gland also supported the existence of this negative feedback by T4 on the pituitary (60, 61). Later on, various *in vivo* studies in other teleosts confirmed these findings *via* direct measurements of *tshβ* mRNA levels using

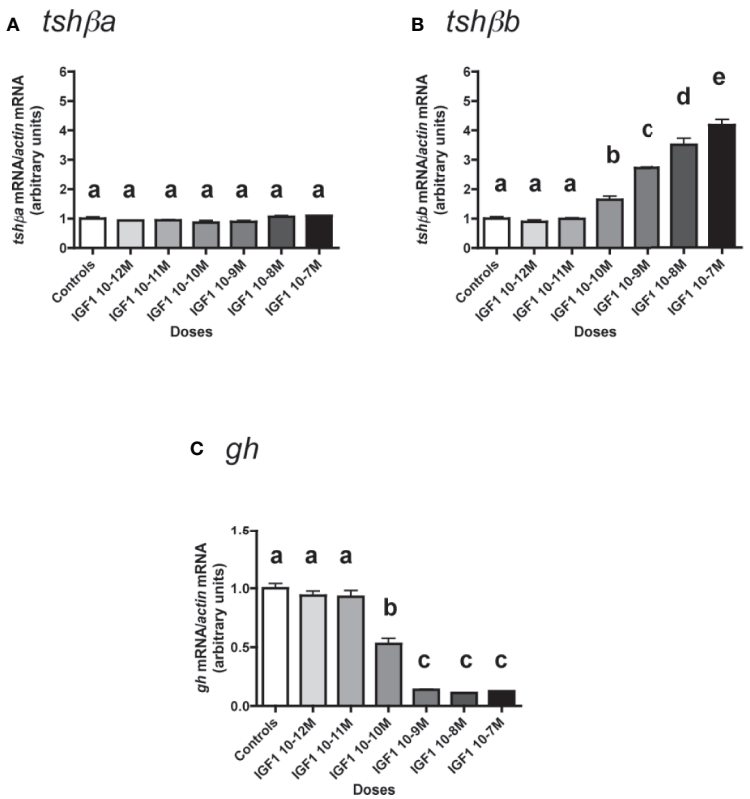


**FIGURE 7** | Effects of IGF1 on *tshβa*, *tshβb* and *gh* transcript levels in primary cultures of Atlantic salmon pituitary cells. Pituitary cells from juvenile Atlantic salmons were treated with various concentrations of IGF1 for 10 days. The mRNA levels of *tshβa* **(A)**, *tshβb* **(B)**, and *gh* **(C)** were quantified by qPCR. Data were normalized against *β-actin*. The Figure displays the results from a representative experiment of three independent cell culture experiments. Mean ± SEM; n = 5 well replicates. Different letters indicate significant differences, ANOVA.

RNase protection assay [coho salmon (62)], Northern-blot [European eel (63); goldfish (64), turbot, *Psetta maxima* (65); red drum *Sciaenops ocellatus* (66)], or RT-PCR and qPCR [fathead minnow *Pimephales promelas* (67); bighead carp *Aristichthys nobilis* (68, 69); Japanese eel *Anguilla japonica* (70); red drum (71)]. Using *in vitro* primary culture of eel pituitary cells, Pradet-Balade and collaborators provided direct evidence that the negative feedback by T3 and T4 on *tshβ* mRNA levels was situated at the pituitary level (63).

All these former studies in teleosts, including salmonids, only concerned the "classical" *tshβa* paralog. In contrast, in our study, we also investigated the second paralog, *tshβb*, and showed that both T3 and T4 inhibited the transcript expression of the second paralog, but to a much lesser extent than for the "classical" *tshβa* paralog. We may suggest that the lower inhibitory effect of T3 and T4 on *tshβb* thus might not prevent the peak of *tshβb* transcript levels to happen during smoltification (17), despite an increase in plasma thyroid hormone concentrations at that time [Atlantic salmon, T4 and T3 (46)].

In the rat, Bockman and collaborators also reported a differential effect of thyroid hormones *in vivo* on the regulation of *tshβ* transcript levels in the PT (likely equivalent to salmon *tshβb*) and in the PD (likely equivalent to salmon *tshβa*) (72). T4 treatment did not affect the expression of *tshβ* in the PT, while it downregulated the expression of *tshβ* in the PD (72). The lack of effect of T4 on PT *tshβ* transcription could be related by the authors to the lack of detection of thyroid hormone receptor TR*β* mRNA in PT, while the receptor is expressed in the PD (72). However, other *in vivo* studies indicated that PT-TSH cells may be more sensitive to thyroid hormones during fetal development than in adulthood in rats. In fetal rats indeed, TSH-positive PT-specific cells react to changes in thyroid function similarly to PD thyrotropes: PT-TSH cell nuclei were enlarged in fetus from females treated with an antithyroid drug (PTU) and smaller in fetus from T4-treated females (73). In contrast, in adult rats, no ultrastructural alterations are observed after thyroidectomy (74) or treatment with PTU (75). As compared to mammals, thyroid hormone receptors have been duplicated through teleost and salmon specific whole genome duplications. Future studies may aim at determining which thyroid hormone receptor(s) are expressed by the two TSH pituitary cell types in Atlantic salmon and the regulation of their expression at the time of smoltification. This would also assess whether the actions of thyroid hormones are exerted directly or indirectly on each type of TSH cells.

## Stronger Inhibition by Cortisol of *tshβb* Than *tshβa* Expression in Atlantic Salmon

Cortisol, in most mammals and ray finned fish, and corticosterone, in most birds, amphibians, and reptiles, are the major glucocorticoids in vertebrates [for review: (76)]. In addition to its role in the control of metabolism and stress response in all vertebrates, cortisol is involved in osmoregulation in teleosts, and in particular in seawater adaptation of smolts in salmonids [for reviews: (7, 77)].

In our study, cortisol at the highest dose tested ($10^{-7}$ M) was able to inhibit the mRNA levels of both *tshβ* paralogs, with a stronger effect on *tshβb* (about 70% inhibition) than *tshβa* (about 40% inhibition). To our knowledge, no study is available in teleosts concerning the effect of cortisol on TSH, even on the "classical" *tshβa* paralog. No effect was observed on *gh* transcript levels.

In human, it is known for a long time that glucocorticoid excess suppresses TSH secretion (78–80), while deficiency increases it (78, 81, 82). In rodents, early *in vivo* studies also reported a suppressive effect of glucocorticoids on basal TSH release (83–85). However, *in vitro* studies on rat pituitary cells in culture reported no changes in basal TSH levels after corticosterone treatment (86). In chicken, corticosterone inhibits *in vivo*, but not *in vitro*, sensitivity of thyrotropes to CRH at embryonic stage, suggesting an indirect mechanism of glucocorticoids on TSH in sauropsids (87).

In Atlantic salmon, plasma cortisol levels are low in winter and early spring (less than 10 ng/ml) and peak in May up to 40 to 100 ng/ml (88, 89), values largely compatible with the cortisol dose effective in our study. Same observation was made in coho salmon (90). The fact that cortisol strongly reduces *tshβb* mRNA levels in our study suggests that this hormone may potentially contribute to the termination of the *tshβb* peak observed at the end of April and May (17).

## Stimulation by CRH of *tshβa* But Not *tshβb* Expression, and No Effect of TRH on Both Paralogs in Atlantic Salmon

We compared the effects of CRH and TRH, both main central regulators of thyrotropic axis in vertebrates, on Atlantic salmon *tshβ* a and b paralog expression.

We showed a slight (about 1.5-fold increase), but significant, stimulatory effect of CRH on the transcript levels of *tshβa*, but not on those of *tshβb*. In the presence of T3 or cortisol, which downregulated the levels of both *tshβ* paralogs, CRH kept its stimulatory effect on *tshβa* mRNA levels, but still had no effect on *tshβb*. Furthermore, the effect of CRH on *tshβa* mRNA levels was largely enhanced in the presence of high inhibitory doses of T3, reaching up more than 15-fold increase (as compared to T3 alone), in the presence of $10^{-8}$ M T3. In contrast, TRH has no effect on either *tshβa* nor *tshβb* in the absence or presence of inhibitors. These results demonstrated a stimulatory effect of CRH specifically on *tshβa*, but not *tshβb*, paralog expression.

For comparison, we also analyzed the effects of CRH and TRH on *gh* transcript levels. CRH, but not TRH, had a slight but significant stimulatory effect on *gh* mRNA levels. Our previous studies reported a stimulatory effect of CRH on GH release in the European eel, which suggested a key role of CRH in the control of several neuroendocrine (corticotropic, somatotropic, and thyrotropic axes) (29).

In mammals, TRH was discovered for its stimulatory role on TSH secretion by the adenohypophysis (91). Concerning the PT, *in vivo* treatment in the rat showed a stimulatory effect of TRH on PD-*tshβ* transcript levels (likely equivalent to salmon *tshβa*), but no effect on PT-*tshβ* (likely equivalent to salmon *tshβb*) (72). The authors were able to relate this differential regulation to the expression of TRH receptor transcripts in the PD but not in the PT. Future studies may aim at investigating the expression of CRH receptor(s) in salmon pituitary *tshβa*- and *tshβb*-cells, taking into account that multiple CRH receptors are present in teleosts [for reviews: (92, 93)]. This would also allow to decipher the mechanisms of enhanced effect of CRH on *tshβa* expression in the presence of T3: whether basal *tshβa* expression in cell culture is almost maximal and needs to be inhibited for revealing potential stimulatory actions, as shown for instance for GH production by pituitary cell cultures in turbot (30); or whether T3 inhibits *tshβa* expression but also stimulates CRH receptor expression.

In teleosts, few studies have yet investigated the potential role of CRH on the thyrotropic axis. Two studies reported a stimulatory effect of CRH on thyrotropic axis, one *in vivo* on thyroid activity in goldfish (94) and one *in vitro* on TSH secretion by coho salmon pituitary cell culture (95). Another study reported no change of *tshβ* mRNA levels after CRH treatment in common carp (*Cyprinus carpio*) pituitary cell culture (96). Concerning TRH, it became apparent that in non-mammalian vertebrates, notably in teleosts, TRH not always possesses a TSH-releasing role [for reviews: (10, 11)]. The first studies reporting no effect of TRH on TSH release were performed *in vivo* and used indirect measurements. Wildmeister and Horster showed that administration of TRH did not induce exophthalmos in goldfish, while treatment with TSH did (97). In this species, treatment with TRH was indeed ineffective in affecting plasma T4 concentrations (98). In the African lungfish *Protopterus ethiopicus*, radioiodine uptake by the thyroid gland was not affected by TRH injection, but increased by TSH (99). However, other studies using the same kind of indirect measurements did show a stimulatory role of TRH [for review: (100)]. Bromage even described an inhibitory effect of TRH injection to guppies on thyroid and TSH cell activities (101). It is only later that *in vitro* experiments directly demonstrated species-specific differences in the role of TRH on TSH in teleosts. While TRH is able to increase *tshβ* mRNA levels in bighead carp (68, 69) and Japanese eel (70), it is not the case in common carp (96, 102). In coho salmon, CRH stimulated TSH secretion, while TRH had no effect (95).

CRH ability to stimulate TSH secretion in anuran amphibians was demonstrated by *in vitro* (103) and *in vivo* studies (104–106), and when comparison was made, a greater potency of CRH than TRH on TSH was observed [for reviews: (1, 11, 107)]. A stimulatory effect of CRH on TSH release was also found in chelonian sauropsids, as investigated in turtles (108, 109). In

birds also, *in vivo* and *in vitro* studies reported a stimulatory effect of CRH on TSH (87, 110, 111).

CRH neurogenesis has been shown to increase from parr to early-smolts in anadromous Atlantic salmon (112). In landlocked salmons receiving thyroxine to augment thyroid hormone plasma levels to those of anadromous fish, the rate of CRH neurogenesis is elevated to anadromous fish levels (112). CRH has been shown to stimulate locomotor activity in juvenile salmon (113). The present study shows that CRH exerts a specific stimulatory effect on *tshβa* expression, but has no effect on *tshβb*. It thus demonstrates that neither CRH (nor TRH which has no effect on both tsh*β* paralogs) would be involved in the triggering of the remarkable peak of pituitary *tshβb* paralog expression occurring at smoltification (17).

## No Effect of SRIH on *tshβa* and *tshβb* Expression in Atlantic Salmon

Somatostatin (or somatotropin-releasing inhibitory hormone, SRIH) was discovered for its inhibitory role on GH release in mammals (114–116), a role well conserved in vertebrates including teleosts [for review: (55)]. In addition to the inhibition of GH, SRIH may also exert an inhibitory effect on basal or TRH-induced TSH secretion in mammals [rat (115); human (117)].

In our study, using primary culture of salmon pituitary cells, SRIH showed its classical inhibitory action on GH, inducing more than 70% inhibition of *gh* mRNA levels. In contrast, SRIH had no effect on both *tshβa* and *tshβb* mRNA levels.

In teleosts, early pioneer studies in goldfish addressed the potential inhibitory control of the thyrotropic axis. Pituitary transplantation (61), pituitary stalk sectioning, or hypothalamic lesioning (118) stimulated thyroid activity (measurement of conversion ratio, cell height, and radioiodine uptake). These studies provided indirect evidence for the existence of a brain TSH-inhibitory factor (TIF). Peter and McKeown (100) then investigated the potential role of SRIH as TIF in goldfish. They showed that injection of SRIH inhibited thyroid radioiodine uptake and suggested that SRIH action was mediated by inhibitory effect on TSH release. To our knowledge, no other studies investigated the potential role of SRIH on TSH in teleosts.

In birds, *in vivo* and *in vitro* studies showed an inhibitory effect of SRIH on TRH- or CRH-stimulation of TSH secretion (111, 119). This inhibition was likely mediated by SRIH receptors sstr2 and sstr5, which are expressed by the thyrotropes (120, 121). In amphibians, SRIH does not affect basal TSH secretion by bullfrog pituitary cells in culture (122).

## Specific Stimulation by IGF1 of *tshβb* Expression in Atlantic Salmon

IGF1 is an endocrine growth factor mainly produced by the liver under the control of pituitary GH, and acting on many target tissues to promote growth in mammals as well as in the other vertebrates [for review: (123)]. As a component of the somatotropic axis, IGF1 exerts a negative feedback on pituitary GH. IGF1, as well as GH itself, is also involved in the regulation of other functions such as reproduction, and osmoregulation in teleosts [for reviews: (124, 125)]. IGF1 has also been proposed as one important internal signal in the cross-talks between growth, metabolism and triggering of puberty in mammals as well as in teleosts [eel (32); for reviews: (124, 126)].

In salmonids, IGF1 circulating levels have been shown to steadily increase from before to during smoltification [Atlantic salmon (127, 128); chinook salmon (129); masu salmon (130); for reviews: (6, 45)], and IGF1, together with GH and cortisol, has been proposed to regulate in smolt the adaptation of gills to osmoregulation in seawater [for reviews: (131, 132)].

In the present study, we found a dose-dependent inhibitory effect of IGF1 on pituitary *gh* transcripts (up to more than 85% inhibition), in agreement with the conservation of IGF1 negative feedback on the somatotropic axis throughout vertebrates.

Strikingly, our study revealed that IGF1 dose-dependently stimulates *tshβb* transcript expression (up to 5-fold increase, according to the experiments). In contrast, no effect was observed on *tshβa* mRNA levels, indicating that IGF1 stimulatory effect is specifically exerted on the *tshβb* paralog.

To our knowledge, our study is the first to report a direct pituitary regulatory role of IGF1 on *tshβ* mRNA levels in any vertebrate. IGF1 receptors are expressed in the ovine PT (133) and IGF1 has been shown to regulate mitogen-activated protein kinase in primary cultures of ovine PT cells (134). Three IGF1-receptors paralogs have been identified in salmon (135). Future studies may investigate which of these receptors is expressed in the pituitary, and more specifically by cells expressing the *tshβb* paralog, to decipher whether the IGF1 stimulatory effect is exerted directly on *tshβb* cells.

We recently demonstrated that *tshβb* transcript levels peak during smoltification in Atlantic salmon in April concomitantly with rheotaxism inversion initiating downstream migration (17). Considering our present data on the specific stimulatory effect of IGF1 on this *tshβb* paralog, we can hypothesize that the increase of plasma IGF1 levels, which may start as early as February and lasts until May (127, 128), may be involved in the induction of smoltification-related *tshβb* expression.

In juvenile salmon, smoltification is preceded by a marked increase in body growth rate (130). As for puberty, where IGF1 is one of the internal cues linking body growth to activation of the gonadotropic axis, IGF1 may represent a key internal signal linking body growth to triggering of smoltification-related neuroendocrine changes in salmon.

In teleosts including salmons, as in other vertebrates, IGF1 is not only produced and released in the blood by the liver, as an endocrine factor, but also expressed locally in various tissues, such as gonad, gill, muscle, fat, heart, kidney, spleen, brain, and pituitary itself [for review: (136)], where it exerts local paracrine/autocrine actions. In tilapia, *igf1* transcripts are expressed in hypothalamic neurons with IGF1-immunoreactive axons projecting to the pituitary; *igf1* transcripts are also expressed by some pituitary cells including some gonadotropes, and the number of *igf1*-expressing gonadotropes increases at puberty as well as during seasonal reproduction (137). This led the authors to propose that pituitary IGF1 may act as paracrine/autocrine stimulator of gonadotropic cells during puberty and reproductive season

[(137); for review: (124)]. We may suggest a similar role for IGF1 on *tshβb* thyrotropic expressing-cells during smoltification in salmon. Future studies will aim at investigating the localization and regulation of the *igf1* transcript in salmon brain and pituitary cells, during smoltification, to infer whether the stimulatory control on *tshβb* expression may be exerted by locally produced IGF1.

IGF1 has been proposed to play a key role in the mediation of vertebrate life-traits [for reviews: (138, 139)]. Our present findings suggest a role of IGF1 in the smoltification step during the life history of the long-river Loire-Allier salmon. This opens new research perspectives on the interactions between internal and environmental cues in the induction of smoltification in salmonids.

## CONCLUSION

In conclusion, this study provides the first data on the neuroendocrine factors involved in the differential regulation of the expression of the two *tshβ* paralogs in teleosts. Future studies, including localization of neurohormone and hormone receptors on different cell types of the salmon pituitary, should aim at investigating whether these regulations are exerted directly on *tshβa* or *tshβb*-cells, or indirectly *via* interactions with other pituitary cells. Thyroid hormones had a stronger inhibitory effect on *tshβa* than *tshβb* in salmon, similar to the differential regulation by thyroid hormones of *tshβ* from PD *versus* from PT in the rat. CRH stimulated the expression of *tshβa* with no effect on *tshβb*, which can also be compared to the stimulatory effect of TRH on *tshβ* from PD but not from PT, in the rat. This is the first report of IGF1 direct regulatory role on pituitary *tshβ* in any vertebrate species. Strikingly, IGF1 specifically stimulated the expression of *tshβb*, allowing us to infer a potential key role of IFG1 in the triggering of the *tshβb* peak at smoltification and crosstalk between body growth and smoltification metamorphosis.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Cuvier Ethic Commitee France.

## AUTHOR CONTRIBUTIONS

SD, PM, KR: design of experiments. PM: supervision of fish husbandry. MF, GM, KR: cell culture. MF, GM, KR: qPCR. SD, KR: writing the manuscript. All authors: discussion of the results and reviewing the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by grants from the French National Research Agency SALTEMP-BIOADAPT ANR-12-ADAP-0021-01 and from the European community, Innovative Training Network, ITN IMPRESS MSCA-ITN-2014-ETN N° 642893. MF was recipient of a PhD fellowship from ITN IMPRESS.

## ACKNOWLEDGMENTS

We are grateful to Chanteuges CNSS team for fish husbandry and to Dr. AG Lafont (MNHN, BOREA) for designing primers for *gh*.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer PGV declared a past co-authorship with one of the authors SD, to the handling editor.

frontiers in Endocrinology

REVIEW
published: 07 December 2020
doi: 10.3389/fendo.2020.605068

# Direct and Indirect Effects of Sex Steroids on Gonadotrope Cell Plasticity in the Teleost Fish Pituitary

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The pituitary gland controls many important physiological processes in vertebrates, including growth, homeostasis, and reproduction. As in mammals, the teleost pituitary exhibits a high degree of plasticity. This plasticity permits changes in hormone production and secretion necessary to meet the fluctuating demands over the life of an animal. Pituitary plasticity is achieved at both cellular and population levels. At the cellular level, hormone synthesis and release can be regulated *via* changes in cell composition to modulate both sensitivity and response to different signals. At the cell population level, the number of cells producing a given hormone can change due to proliferation, differentiation of progenitor cells, or transdifferentiation of specific cell types. Gonadotropes, which play an important role in the control of reproduction, have been intensively investigated during the last decades and found to display plasticity. To ensure appropriate endocrine function, gonadotropes rely on external and internal signals integrated at the brain level or by the gonadotropes themselves. One important group of internal signals is the sex steroids, produced mainly by the gonadal steroidogenic cells. Sex steroids have been shown to exert complex effects on the teleost pituitary, with differential effects depending on the species investigated, physiological status or sex of the animal, and dose or method of administration. This review summarizes current knowledge of the effects of sex steroids (androgens and estrogens) on gonadotrope cell plasticity in teleost anterior pituitary, discriminating direct from indirect effects.

**Keywords: estrogen, androgen, adenohypophysis, brain, gonads, plasticity, pituitary, steroids**

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***Edited by:***
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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *11 September 2020*
***Accepted:*** *12 October 2020*
***Published:*** *07 December 2020*

***Citation:***
*Fontaine R, Royan MR, von Krogh K, Weltzien F-A and Baker DM (2020) Direct and Indirect Effects of Sex Steroids on Gonadotrope Cell Plasticity in the Teleost Fish Pituitary. Front. Endocrinol. 11:605068. doi: 10.3389/fendo.2020.605068*

## INTRODUCTION

Teleost fish comprise the largest vertebrate group with close to 30,000 species (1), including well established model species such as zebrafish (*Danio rerio*) and Japanese medaka (*Oryzias latipes*), which provide valuable tools for basic research on vertebrate physiology (2, 3). In addition, numerous teleosts, such as salmonids, seabreams, basses, tilapia, and other species with regional importance, have commercial and ecological or societal value and are subjects of applied research related to aquaculture or conservation.

Compared to tetrapods, teleosts experienced an additional whole genome duplication, known as the 3R (4). Some teleosts, such as the salmonids, have also had a fourth duplication event (4R) (5). Therefore, teleost fish can potentially possess 2 to 4 times more genes than other vertebrates, and although many of the duplicated genes have been lost through teleost evolution, some have been conserved and developed new or

expanded functions. Fish reproductive physiology has been extensively investigated over the last decades, due to the high economic interest of controlling fish reproduction in aquaculture species and for evolutionary aspects as the main regulatory mechanisms are conserved among vertebrates.

In all vertebrates, the reproductive function is controlled through the physiological connections of the brain - pituitary - gonadal (BPG) axis, where the pituitary gonadotropes play a central role (6, 7). Located in the anterior pituitary (adenohypophysis), gonadotropes produce and release into the blood circulation the two gonadotropins (follicle-stimulating and luteinizing hormones, Fsh and Lh, respectively) which stimulate gonadal gametogenesis and steroidogenesis. Gonadotropins are heterodimeric proteins consisting of an α-subunit, common to both Lh and Fsh, and a unique β-subunit that confers the biological specificity (8). Interestingly, contrary to mammals and birds, in teleosts, the two gonadotropins are generally produced by discrete gonadotrope cell types; Lh cells and Fsh cells (9, 10).

Located below the hypothalamus, the pituitary is composed of two main parts with different developmental origins. The neurohypophysis (posterior pituitary) originates from a down-growth of the diencephalon and contains projections from neuroendocrine cells mainly located in the preoptic-hypothalamic region of the brain. The anterior pituitary originates from the placodal ectoderm at the anterior neural ridge which invaginates and subsequently separates from the stomodeum, a thickening of the ectoderm that forms the epithelium of the oral cavity (11). The anterior pituitary contains several hormone producing cells, including the gonadotropes which are localized to the *proximal pars distalis* (PPD).

Unlike in mammals where the different endocrine cell types are mosaically distributed in the adult anterior pituitary, in teleosts they are spatially discrete through the entire lifespan (6, 11). However, in both mammals and teleosts, the anterior pituitary shows high plasticity at both cellular and population levels, allowing the anterior pituitary to meet the demands for hormonal production as they change over the life cycle of an animal (12). At the cellular level, cellular activity (hormone production and release) can be modified by varying regulatory ligand sensitivity through the presence and number of receptors, or by altering rates of hormone synthesis and secretion, the latter corresponding to the hormone release as defined by Jena (13) (**Figure 1A**). At the population level, the number of cells of each endocrine cell type can change (**Figure 1B**). This can be due to proliferation of the endocrine cells (**Figure 2A**), differentiation of progenitor cells (**Figure 2B**), phenotypic conversion (transdifferentiation) of an endocrine cell into another cell type (**Figure 2C**), or cell death (apoptosis) (**Figure 2D**).

The pituitary endocrine cell population with the highest capacity for plasticity is likely the gonadotropes. Gonadotrope plasticity (cell activity and cell number) is regulated by a myriad of brain factors primarily released from the preoptic-hypothalamic region. The main brain factors in this regard are gonadotropin-releasing hormone (Gnrh), dopamine (DA), and Kiss in teleosts (**Figure 3**; for review see (7, 14, 15)). Gnrh serves as the main stimulator of gonadotropes (7, 16). The neuro-hypothalamic Kiss system also plays an important stimulatory role (15). In contrast, DA appears to be the main gonadotrope inhibitor (7, 17). Other neuroendocrine factors regulating gonadotropes include Gnih, neuropeptide Y, GABA (7), but these will not be discussed in this review.

In addition to signals from the brain, the plasticity of gonadotropes is also modulated by negative and positive feedback *via* endocrine signals from peripheral organs. Among these signals are the sex steroids. Sex steroids are synthesized from cholesterol, predominantly by the steroidogenic cells of the gonads, and circulate at different levels in males and females (18). Two major classes of sex steroids, androgens and estrogens, were classically delineated as male- and female-specific hormones as they were mainly synthesized by the testes and ovaries, respectively, and found to promote male and female secondary sex characteristics. We now know that both androgens and estrogens are essential regulators in both males and females.

Steroidogenesis primarily occurs in the gonads, in testicular Leydig cells in males, and ovarian granulosa and theca cells in females (19). However, granulosa cells are not strictly steroidogenic but process steroid precursors from theca cells. For instance, testosterone (T) from theca cells is aromatized in granulosa cells to 17β-estradiol (E2), the most prevalent and potent form of circulating estrogen in fish, a process regulated through maturation-dependent levels of aromatase. Aromatase is a member of the P450 cytochrome enzyme superfamily and encoded by the *cyp19a1* gene, which exists in two forms in teleosts: *cyp19a1a* and *cyp19a1b*, the former expressed in the ovary and the latter in the brain and pituitary of both sexes (20, 21). While T and the even more potent, non-aromatizable, hormone 5α-dihydrotestosterone (DHT) are the active androgens in mammals, the non-aromatizable 11-ketotestosterone (11-KT) is the main active androgen in most teleosts (22, 23). However, DHT is also found in the circulation in both male and female fathead minnow (*Pimephales promelas*) (24, 25), and is the predominant steroid produced by urohaze-goby (*Glossogobius olivaceus*) testis tissue *in vitro* (26). Moreover, activity of 5-α reductase, which converts T to DHT, has been detected in many tissues, including the brain and pituitary, in several teleost species (25, 27), indicating that there may be a still undescribed biological role of DHT in teleosts. Finally, there is a third class of vertebrate sex steroids, the progestogens, which can be converted through several steps to T, 11-KT and E2, cortisol, and other steroids (18), but will not be further covered in this review.

Interestingly, in vertebrates, sex steroids can also be produced in other tissues, including the central nervous system, either *via de novo* synthesis from cholesterol or from other steroid intermediates produced in the periphery, thus allowing the tissue to autonomously utilize and modulate local steroid signaling (28,

**Abbreviations:** 11- HA, 11β-hydroxyandrosterone; 11-KT, 11-ketotestosterone; Acth, Adrenocorticotropin; Ar, Androgen receptor; BPG, Brain-pituitary-gonad; D1, Type 1 dopamine receptors; D2, Type 2 dopamine receptors; DA, Dopamine; DHT, 5α-dihydrotestosterone; E2, 17β-estradiol; EE2, 17α-ethinylestradiol; Esr, Estrogen receptor; Fsh, Follicle-stimulating hormone; GDX, Gonadectomy; Gh, Growth hormone; Gnrh, Gonadotropin-releasing hormone; Lh, Luteinizing hormone; Msh, Melanocyte-stimulating hormone; MT, Methyltestosterone; OVX, Ovariectomy; PI, Pars intermedia; POA, Preoptic area; Pomc, Pro-opiomelanocortin; PPD, Proximal pars distalis; Prl, Prolactin; RPD, Rostral pars distalis; Sl, Somatolactin; T, Testosterone; Th, Tyrosine hydroxylase; Tsh, Thyrotropin.

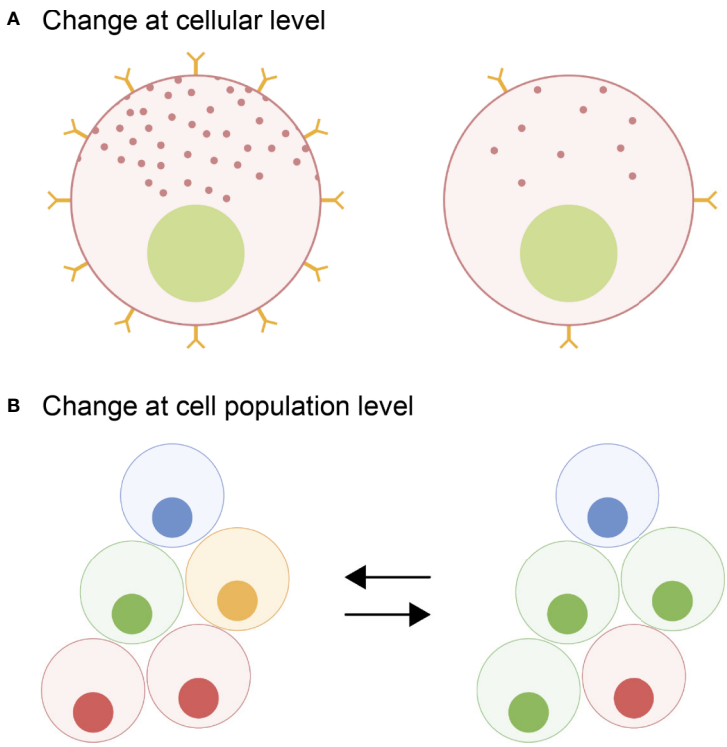


**FIGURE 1** | Schematic representation of the plasticity of the pituitary cells leading to a change in hormone production quantity. At the cellular level **(A)**, the activity of the endocrine cell (hormone synthesis and release) can be modulated through the regulation of the number of different receptors thus changing sensitivity of the pituitary cells to inputs and/or by changing the hormone production rates. At the population level **(B)**, the number of specific cell types can be modified changing the proportion of the different endocrine cell types in the pituitary.

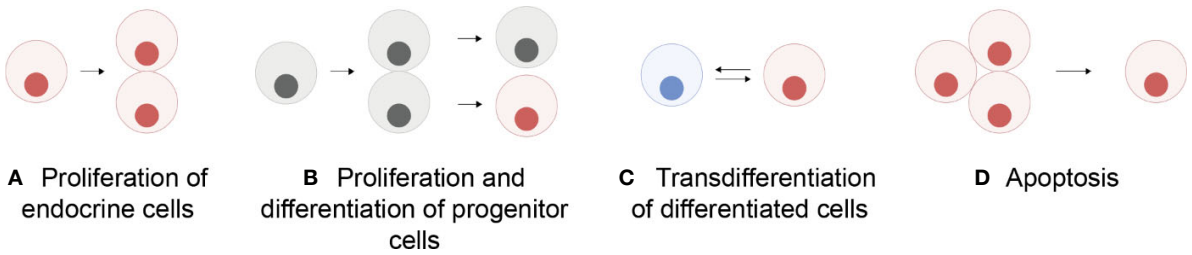


**FIGURE 2** | Schematic representation of the mechanisms allowing a change in the number of a specific endocrine cell type in the pituitary: proliferation (mitosis) of endocrine cells themselves **(A)**, proliferation of progenitor cells followed by their differentiation **(B)**, transdifferentiation (phenotypic conversion) of other differentiated cells **(C)**, and cell apoptosis **(D)**. Grey cells represent undifferentiated progenitor cells and colored cells represent differentiated cells.

29). In teleosts, expression of numerous enzymes, including aromatase, involved in sex steroid biosynthesis has been described in several brain areas (30, 31).

In target cells, sex steroids bind both nuclear and transmembrane receptors driving complex signaling responses (for reviews see (32–34)). In most vertebrates, genome duplications have led to two cytoplasmic estrogen receptors (ESR; ESR1 and ESR2), and one androgen receptor (AR). Teleost fish, however, often possess multiple paralogs of each receptor due to the additional whole genome duplications that occurred before and within the teleost group (3R/4R), adding complexity to sex steroid receptor signaling. Indeed, in most teleost species, while a single Esr1 has been maintained, two Esr2 (Esr2a and Esr2b), and two Ar (Arα and Arβ) have been conserved (35, 36).

Considering the progress in the field made in recent years, this review aims to summarize current knowledge regarding the effects of androgens and estrogens on gonadotrope activity and number in teleosts. Because these steroids can act on all levels of the BPG axis, we also aim to delineate their indirect effects *via* the brain from their direct effects on the gonadotropes.

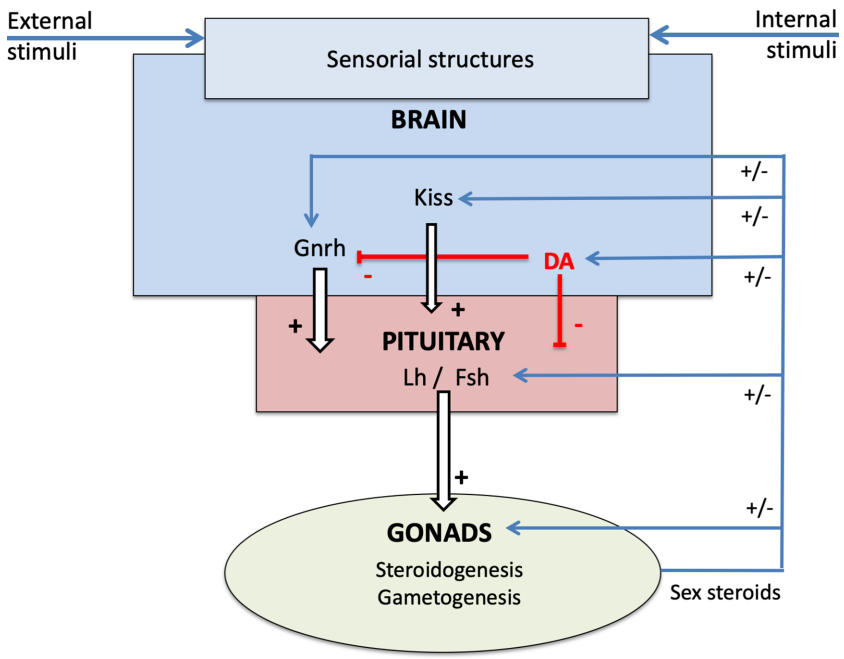


**FIGURE 3** | Schema of the neuroendocrine control of reproduction in teleosts, presenting the brain-pituitary-gonad (BPG) axis and the role of sex steroids in the retro-controls at the different levels.

# SEX STEROIDS ARE MEDIATORS OF GONADOTROPE PLASTICITY

## Effects on Gonadotrope Activity

### Effects on Sensitivity to Regulatory Signals

Sex steroids can alter gonadotrope activity by changing gonadotrope cell sensitivity to regulatory signals, through modulation of the levels of Gnrh or DA receptors. Although molecular mechanisms by which steroids regulate Gnrh and DA receptors are well documented in mammals, much less is known in fish.

In mammals, GnRH signaling occurs through two paralogous receptors (37), whereas additional paralogs have been identified in teleosts, with up to six receptors in Atlantic salmon (38). In spite of this genomic difference, effects of sex steroids on GnRH receptor expression has been found across taxa. For example, E2 up-regulated pituitary *GnRH-R* expression in several mammalian studies, including in cows (39, 40), rats (41, 42), and ewes (43, 44), while T also up-regulated pituitary *GnRH-R* in rats (45). In teleosts, the effects of sex steroids on *gnrhr* expression have been investigated in only a limited number of species which have yielded disparate results. In the European sea bass (*Dicentrarchus labrax*), Gnrh receptor transcript levels in gonadotropes strongly increased at the time of spawning, when E2 is elevated (46), suggesting a stimulatory effect of E2 as seen in mammals. Conversely, in African catfish (*Clarias gariepinus*), castration increased pituitary Gnrh receptor content (47). This effect was reversed by treatment with androstenedione (AS), but not with non-aromatizable 11β-hydroxyandrostenedione (11β-OHA4). Finally, in goldfish (*Carassius auratus*), T-enhanced Lh-responsiveness to Gnrh was shown to be independent of changes in pituitary Gnrh receptor affinity or number (48).

Interestingly, among teleost species studied, only one of the multiple paralogs appears to be directly up-regulated by sex steroids in each species. Furthermore, if we apply the nomenclature and molecular phylogeny from (38) for *gnrhr* genes to the published studies, we find that it is the same isoform across species. For example, E2 treatment in female Nile tilapia (*Oreochromis niloticus*), increased mRNA levels of both *gnrhr2ba1* (*gnrhr1*, according to the authors) and *gnrhr2ba2* (*gnrhr3) in vivo*, but only *gnrhr2ba1* (*gnrhr1) in vitro*, suggesting E2 directly regulates *gnrhr2ba1* but indirectly regulates *gnrhr2ba2* (49). Similarly, in the male black porgy (*Acanthopagrus schlegeli*), E2 and T increased the expression of *gnrhr2ba1* (*gnrhrI*) (50, 51), but not *gnrhr1cb* (*gnrhrII*) (50). Finally, in pituitary cultures from Atlantic cod (*Gadus morhua*), E2 and T stimulated the expression of *gnrhr2ba1 (gnrhr2a)* but not *gnrhr1cb* (*gnrh1b)* in mature and post-spawning fish, and DHT increased expression of only *gnrhr2ba1* in post-spawning fish (52). While three species cannot represent the entire diversity of teleosts, they suggest that this receptor acquired a sensitivity to sex steroids in a common ancestor and this has been conserved through evolution.

In mammals, it is well established that E2 modulates pituitary expression of type 2 dopamine receptors (D2), as demonstrated in rats (53–55). Regarding DA receptors, two DA receptor families have been described in vertebrates: type 1 dopamine receptors (D1) and D2. Similar to Gnrh-r, multiple paralogs within each family are found in teleosts (56). *In vitro* experiments have demonstrated that pituitary D2, but not D1, plays a role in the dopaminergic inhibition of gonadotropin synthesis and

secretion in several teleost species (17). D2 has been localized to the PPD in many teleosts, and to Lh cells in rainbow trout (*Oncorhynchus mykiss*) (57) and zebrafish (58). Sex steroid regulation of pituitary D2 has been studied in few teleost species, with divergent effects. In rainbow trout, D2 antagonist decreased the stimulatory effect of Gnrh3 on Lh cells (59), whereas in Nile tilapia, *d2* mRNA levels increased in females following E2 treatment, both *in vivo* and *in vitro* (49). In European eel, neither E2 nor T affected pituitary *d2* levels (60).

### Effects on Hormone Synthesis and Secretion

Gonadectomy (GDX) experiments and *in vivo* or *ex vivo* steroid treatments in a wide variety of species have demonstrated the significant role of the gonadal feedback loop in regulating gonadotropin synthesis and secretion in teleosts, with sex steroids exerting both negative and positive effects (**Table 1**).

Clearly, the effects of sex steroids on Lh and Fsh synthesis depend on developmental stage, reproductive status, sex, and even the duration and dose of the experimental treatment. For instance, in goldfish, *lhb* mRNA levels increased in juveniles, but not adults, following T and E2 treatments (79, 82). Sex-specific effects have been seen in European eel (*Anguilla anguilla*), where intraperitoneal E2 injections strongly increased pituitary Lh levels in immature eels of both sexes, while T strongly stimulated pituitary Lh level only in males (75). Similar sex-specific effects were observed for *fshb* transcripts in coho salmon (*Oncorhynchus kisutch*), where E2 was found to inhibit *fshb* in males but not in females (74). Dose-specific responses were reported in medaka, where high concentrations of E2 decreased *lhb* mRNA levels (90, 91) while more physiological levels stimulated *lhb* synthesis (9). Interestingly, in coho salmon, pituitary Fsh levels increased following administration of the non-aromatizable androgen 11-ketoandrostenedione (11-KA, a precursor of 11-KT), whereas T suppressed pituitary Fsh in summer but stimulated it in autumn (113), suggesting season-specific functions of T and an important role of aromatase in mediating negative feedback.

Secretion of Lh and Fsh are also affected by sex steroids, with differential effects depending on the species, sex, and/or maturational stage of the animal (**Table 1**). For instance, in goldfish, Lh release was stimulated by T in mature females and by E2 in early recrudescence in males (80). Studies in rainbow trout reported that T increased Fsh plasma levels in immature fish (103) but decreased in mature fish (104–106). Opposite effects were found in mature male Atlantic salmon (*Salmo salar*), in which T inhibited Fsh release in summer during gonadal maturation, but stimulated Fsh release during the autumn spawning period (68). Sex steroids can also influence gonadotrope cell activity by modulating gonadotrope response to Gnrh. For instance, in Atlantic croaker (*Micropogonias undulates*), a study found no effect of E2 on basal plasma Lh levels but an inhibitory effect on Gnrh-induced Lh release in mixed-sex adults (64). In goldfish, E2 and T potentiated the Gnrh agonist (Gnrh-a) effect on Lh secretion *in vivo* in a season-dependent manner (83)

Gonadotropin synthesis and release can be differentially affected by sex steroids within the same organism, as shown for example in rainbow trout where E2 stimulated Lh synthesis but did not affect Lh release (93, 99, 101–103). Finally, Lh and Fsh can be oppositely regulated within in the same species, both at the secretion and expression levels. For instance, in previtellogenic female rainbow trout, E2 implants increased plasma Lh level but decreased plasma Fsh (57), and in male coho salmon, E2 increased pituitary mRNA levels of *lhb* but decreased *fshb* (74).

## Effects on Gonadotrope Cell Populations

In addition to changes in gonadotrope cell activity, plasticity also results from changes in gonadotrope numbers and pituitary reorganization (**Figure 2**). For example, in European sea bass, while gonadotropes are only located in the PPD in immature fish, they tend to also colonize the periphery of the *pars intermedia* (PI) during maturation (46). Such population-level plasticity can make it difficult to discern whether steroid-induced changes in hormone mRNA or protein levels, detected by quantitative approaches such as ELISA or qPCR on the whole tissue, are due to changes in cell activity or cell number. Gonadotrope population changes in the pituitary can be due to proliferation of gonadotropes (**Figure 2A**), differentiation of progenitor cells, (**Figure 2B**) transdifferentiation (**Figure 2C**), and cell death (**Figure 2D**). These mechanisms underlie many changes in the rates of synthesis and release of Fsh and Lh by the pituitary, and there is increasing evidence that the sex steroids may play a role in these processes.

### Proliferation

In teleosts, the pituitary grows throughout the lifespan. Evidence that pituitary endocrine cells are mitotically active has been illustrated at the electron microscope level in the mosquito fish (*Gambusia affinis*) (114). More specifically, the number of gonadotropes can change according to life stage or other factors, such as social status. For example, Lh cell number increased in juvenile male African catfish as spermatogenesis progressed (61, 115), and in both male and female medaka, the numbers of both Lh and Fsh cells increased between juvenile and adult stages (91, 116). In Nile tilapia, Fsh cell numbers were higher in dominant than subordinate males (117).

Furthermore, some studies demonstrate that gonadotrope proliferation can be controlled by sex steroids. In medaka, for instance, gonadotrope proliferation following steroid treatment was documented using double staining with proliferation markers Pcna and BrdU (91, 116). Exposure to T or E2, but not 11-KT, stimulated Lh and Fsh cell proliferation in both males and females, suggesting a positive effect of E2 on gonadotrope cell proliferation. In contrast, in zebrafish larvae, the number of Fsh cells was significantly lower in E2-treated fish than in controls (118), suggesting that E2 inhibits Fsh cell proliferation during early development in zebrafish. An earlier study in juvenile male African catfish showed that the number of Lh gonadotropes with numerous Lh-containing granules increased following T treatment (61). However, the authors did not detect differences in pituitary cell proliferation between androgen-treated and control fish and therefore speculated that androgens might activate quiescent gonadotropes. Whether these divergent results are due to species or stage differences remains to be investigated.

### Differentiation of Progenitor Cells

Differentiation of progenitor cells may also increase gonadotrope cell numbers, but the evidence of a role of steroids in this process

**TABLE 1 |** Effects of gonadectomy or sex steroid treatments *in vivo* or *ex vivo* in different teleost species.

| Species | Stages/sex | Pituitary synthesis | | Secretion | | Reference |
|---|---|---|---|---|---|---|
| | | Lh | Fsh | Lh | Fsh | |
| African catfish (*Clarias gariepinus*) | immature M | GDX ↓ Lh T ↑ Lh (not 11-KT) | | | | (61) |
| | mature M | GDX ↓ Lh<br>E2 estrone T and AS, ↑ Lh (not DHT or 11β-OHA4) | | GDX ↑; T and AS ↓ (not E2, E1, DHT or 11β-OHA4) | | (62) |
| | | GDX ↓ *lhb* 11-KT ↑ *lhb* | | | | (63) |
| Atlantic croaker (*Micropogonias undulates*) | maturing mix M/F | E2 ↑ *lhb* | E2 ↓ *fshb* | | | (64) |
| | Late maturing/mature mix M/F | No effect of E2 or GDX on *lhb* | GDX ↑ *fshb*<br>*E2* ↓ *fshb* | No effect of GDX or E2 on basal Lh level<br>GDX ↑, and T and E2 ↓ Gnrh-induced stimulation | | |
| | M | | | E2 ↑ (not T, DHT) | | (65) |
| | immature M and F | T ↑ Lh | | | | (66) |
| Atlantic salmon (*Salmo salar*) | maturing M | T and 11-KA ↑ Lh | Summer: GDX ↑ Fsh T and 11-KA ↓ Fsh | | Summer: GDX ↑ T and 11-KA ↓ | (67) |
| | mature M | GDX ↓ Lh<br>T and 11-KA ↑ Lh | GDX ↓ Fsh 11-KA ↑ Fsh<br>Summer: T ↓ Fsh<br>Autumn: T ↑ Fsh | GDX ↓<br>T ↑ (not 11-KA) | GDX ↓ 11-KA ↑<br>Summer: T ↓<br>Autumn: T ↑ | (68) |
| Black porgy (*Acanthopagrus schlegelii*) | immature M | E2 ↑ *lhb* | E2 ↑ *fshb* | | | (51) |
| | 2 years old M | | | E2 ↑ (not T, 11-KT) | | (69) |
| | mature M | E2 ↑ *lhb* | | E2 ↑ (not T, 11-KT) | | (70) |
| | M (spawning season) | E2 ↑ Lh (not T) | | E2 ↑ (not T) | | (71) |
| | protandric transition | | | E2 ↑ | | (72) |
| | M and F | | | E2 ↑ (not T, 11-KT) | | (73) |
| Coho salmon (*Oncorhynchus kisutch*) | M and F | T and E2 ↑ *lhb* and Lh | E2 ↓ *fshb* in M (not in F)<br>No effect of E2 or T on Fsh | | T and E2 ↓ | (74) |
| European eel (*Anguilla anguilla*) | silver (immature) F | E2 ↑ Lh (not T) | | no effect of E2 or T | | (75) |
| | | T and E2 ↑ *lhb* | | | | (76) |
| | | E2 ↑ *lhb* (not T or DHT) | no effect of E2, T, or DHT on *fshb* | | | (77) |
| | silver M | T and E2 ↑ Lh | | | | (75) |
| Goldfish (*Carassius auratus*) | immature mixed sex | T and 11-HA for 12 or 24h ↓ while 48,72 and 96h ↑ *lhb* | | | | (78) |
| | | E2 and T ↑ *lhb* (not 11-KT) | *E2, T and 11-KT* ↓ *fshb* | | | (79) |
| | early recrudescent M and F | 11-KT ↓ *lhb*<br>E2, and T in F, ↑ *lhb* | E2,T and 11-KT ↓ *fshb* | E2 ↑ in M only (not T or 11-KT) | | (80) |

*(Continued)*

**TABLE 1** | Continued

| Species | Stages/sex | Pituitary synthesis | | Secretion | | Reference |
|---|---|---|---|---|---|---|
| | | Lh | Fsh | Lh | Fsh | |
| | mature M and F | E2 ↑ *lhb* in M only (not T or 11-KT) | No effects of T, E2, or 11-KT on *fshb* | *T* ↑ in F only (not E2 or 11-KT) | | (80) |
| | early recrudescent and mature F | no effect of GDX, T, E2, or 11-KT on lhb | *GDX* ↑ *fshb*<br>*E2, T, and 11-KT* ↓ *fshb* | | | (79) |
| | mature F | | | E2 ↓ | | (81) |
| | | no effect of GDX, T, or E2 on Lh | | GDX ↑ T and E2 ↓ | | (82) |
| | | | | no effect of E2 or T | | (83) |
| | mature mixed sex | T and 11-HA ↑ *lhb* | | | | (78) |
| | mature F + M (*in vivo* and *ex-vivo*) | E2 ↑ *lhb* and Lh | E2 ↑ *fshb* | | | (84) |
| Hybrid striped bass | female (mid-vitellogenesis) | no effect of GDX on Lh<br>GDX ↑ *lhb* E2 ↓ *lhb* and Lh | GDX ↑ *fshb*<br>E2 ↓ *fshb* | | | (85) |
| Indian catfish (*Heteropneustes fossilis*) | F (preparatory phase) | GDX ↓ lhb E2 ↑ lhb | GDX ↑ *fshb* E2 ↓ *fshb* | | | (86) |
| | F (resting phase) | no effect of GDX or E2 on *lhb* | GDX ↑ *fshb* E2 ↓ *fshb* | | | |
| | mature F | | | GDX ↑ | | (87) |
| Japanese eel (*Anguilla japonica*) | silver | E2 ↑ Lh | | E2 ↑ | | (88) |
| Masu salmon (*Oncorhynchus masou*) | yearling and 2 years old | no effect of GDX on Lh<br>MT ↑ Lh | | GDX ↑ | | (89) |
| Medaka (*Oryzias latipes*) | mature F | E2 and 11KT ↑ *lhb* | E2 and 11KT ↓ *fshb* | | | (9) |
| | mature F and M | E2 ↓ *lhb* in F<br>no effect of E2 on *lhb* in M | | | | (90) |
| | mature M and F | E2 ↓ *lhb* | | | | (91) |
| Nile tilapia (*Oreochromis niloticus*) | F (early vitellogenesis) | E2 ↓ *lhb* | E2 ↓ *fshb* | E2 ↓ | No effect of E2 | (49) |
| Orange-spotted grouper (*Epinephelus coioides*) | mature F | no effect of GDX or E2 on *lhb* | GDX ↑ *fshb* E2 ↓ *fshb* | | | (92) |
| Ricefield eel (*Monopterus albus*) | *in vitro* pituitary fragments from F | E2, T and 11-KT ↑ *lhb* | no effect of E2, T, or 11-KT on *fshb* | | | (21) |
| Rainbow trout (*Oncorhynchus mykiss*) | F (early vitellogenesis) | E2 ↑ Lh | | no effect of E2 | | (93) |
| | mature M (spermiation) | | | GDX ↑ E2, T, and 11-KT ↓ | | (94) |
| | mature M (early spermatogenesis) | | | GDX ↑ E2 and T ↓ | | |
| | mature M (resting phase) | | | GDX and T ↑ | | |

*(Continued)*

**TABLE 1** | Continued

| Species | Stages/sex | Pituitary synthesis | | Secretion | | Reference |
|---|---|---|---|---|---|---|
| | | Lh | Fsh | Lh | Fsh | |
| | mature M (late spermatogenesis) | | | No effect of GDX, T, or E2 | | (94) |
| | immature M and F | T ↑ *lhb* and Lh | | T ↑ Gnrh-induced stimulation | | (66, 95, 96) |
| | mature F (late vitellogenesis) | | | GDX ↑<br>no effect of E2 | | (97) |
| | mature F (post ovulatory) | | | no effect of GDX or E2 | | |
| | mature F (germinal vesicle migration) | | | GDX ↑ E2 ↓ | | |
| | Immature M and F | T only in F, and E1, E2, and others* ↑ Lh | | | | (98) |
| | organ culture from immature F | MT and E2 ↑ Lh | | no effect of MT or E2 | | (99) |
| | immature M | | | T ↑ | | (100) |
| | immature F | E2 ↑ Lh | | No effect of E2 | | (101) |
| | triploid (immature) F | T and E2 ↑ Lh | | T ↑ (not E2) | | (102) |
| | immature yearlings | T and E2 ↑ Lh | T ↑ Fsh E2 ↓ Fsh | T↑ (not E2) | T ↑ (but not E2) | (103) |
| | vitellogenic F | | | No effect of GDX or E2 | GDX ↑ E2 ↓ | (104) |
| | immature F | | | No effect of E2 | E2 ↓ | |
| | mature F | | | | T (and E2) ↓ | (105) |
| | previtellogenic F | | | E2 ↑ | E2 ↓ Fsh | (57) |
| | F | | | No effect of GDX or E2 | GDX ↑ E2 ↓ | (106) |
| Red sea bream (*Pagrus major)* | immature M | T ↓ *lhb* (not E2 or 11-KT) | 11-KT and T ↓ *fshb* (not E2) | T ↓ (not E2 or 11-KT) | | (107) |
| | mature M | 11-KT ↑ *lhb* (not E2 or T) | No effect of E2, T, or 11-KT on *fshb* | No effect of E2, T or 11-KT | | |
| Sablefish (*Anoplopoma fimbria*) | prepubertal F | E2 and T ↑ *lhb* | no effect of E2 or T on *fshb* | | | (108, 109) |
| Sea bass (*Dicentrarchus labrax*) | mature M and F | E2 T or DHT ↑ Lh;<br>T ↑ *lhb* (not DHT or E2) | E2, T or DHT ↓ *fshb* | no effect of E2, T or DHT | | (110) |
| | mature F and M | no effect of GDX or T on *lhb* | (GDX ↑ *fshb*) T ↓ *fshb* | | | (111) |
| Striped bass (*Morone saxatilis*) | immature F | T ↑ Lh | | no effect of T | | (112) |

*M, Males; F, Females; 11β-OHA4, 11β-Hydroxyandrostenedione; 11-HA, 11-hydroxyandrosterone; AS, androstenedione; E1, Estrone; 11-KA, 11-ketoandrostenedione; Others* include estriol, MT, testosterone propionate [11-oxygenated steroids, 11-KT, and 11-HA]; []: high concentrations are needed to provide significant differences.*

is not conclusive. In mammals, *Sox2*-expressing cells were shown to comprise a pool of pluripotent progenitor cells that proliferate and differentiate to either replenish pituitary cell populations or increase the absolute numbers of cells, including gonadotropes (119–122). These multipotent progenitor cells have been found in the pituitary cleft of mice (123–125) and rats (125, 126), lining the intraglandular structure bordering the adenohypophysis and neurohypophysis. While E2 treatment inhibited *SOX2* expression in human embryonic stem cells *in vitro* (127), other studies suggest that $SOX2^+$ cell proliferation is stimulated by E2 treatment (128). This indicates that progenitor stem cell proliferation, and perhaps differentiation, may be regulated by sex steroids in mammals. In teleosts, however, multipotent *sox2*-expressing cells have only been detected in the brain (129–132) and retina (133). Pituitary *sox2*-immunoreactive cells have, to our knowledge, only been identified in one teleost study, where they were localized at the junction of the adenohypophysis and neurohypophysis in medaka (91). However, evidence that these cells are pluripotent and contribute to endocrine tissue renewal in teleosts is lacking.

Another marker, S-100, has been widely used to identify follicular stellate (FS) cells, which are non-endocrine cells networked by gap junctions throughout the anterior pituitary in mammals [for review, see (134, 135)]. FS cells are thought to be progenitor cells and thus involved in pituitary cell renewal and plasticity (136, 137). Interestingly, mammalian FS cells have been found to be sex steroid sensitive. Indeed, in male rats, GDX decreased the number of gap junctions, but T replacement maintained their numbers (138, 139). Similar observations were made in females where OVX reduced the gap junction number while E2, and to a lesser extent T, partly restored it (140). However, the role of sex steroids in FS cell proliferation or differentiation remains unknown in mammals.

Pituitary non-secretory (agranular) cells have been described in several teleost species, including the southern mouth-brooder (*Pseudocrenilabrus philander*) (141), sailfin molly (*Poecilia latipinna*) (142), ironfish (hybrid between the Funa (*Carassius carassius*) and goldfish) (143), stickleback (*Pungitius pungitius L*) (144), European eel (145), Mediterranean yellowtail (*Seriola dumerilii*) (146), grey mullet (*Mugil cephalus*) (147), Arabian toothcarp (*Aphanius dispar*) (148), and white seabream (*Diplodus sargus*) (149). In Nile tilapia, FS cells were observed to network *via* gap junctions (150). In the Japanese eel (*Anguilla japonica*), it was demonstrated that aromatase-positive cells, most likely corresponding to FS cells, express the proliferation marker PCNA (151). Such cells are suspected to have the same origin as the aromatase positive radial glial cells in the brain that act as progenitors throughout life (152). These results suggest that the proliferation of such cells could be sex steroid dependent, but this remains to be systematically investigated.

### Transdifferentiation

The third mechanism that remodels gonadotrope populations is known as transdifferentiation, defined by (153–155) as the change from one hormone producing cell type into another. Transdifferentiation may allow the pituitary to appropriately respond to certain physiological and pathological conditions (156). While experiments have generated evidence of the phenomenon, the molecular mechanisms mediating such transformations are still enigmatic.

In teleosts, a recent study in medaka demonstrated that Fsh cells commenced *lhb* production *in vitro*, indicating the capability of a fully differentiated cell to transdifferentiate into another cell type (116). However, transdifferentiation between other pituitary cell types has not been reported in teleosts, and the role of sex steroids in transdifferentiation has not been investigated to date.

In contrast, several examples of pituitary endocrine cell transdifferentiation have been described in mammals. For instance, a study in adult mice found that stem-somatotropes can populate the pituitary with both somatotropes and lactotropes (157, 158). Similarly, studies in rats (159, 160) and humans (161) suggest that the proportions of somatotropes, lactotropes and mammosomatotropes ($GH^+/PRL^+$) in the adenohypophysis vary among non-pregnant, pregnant and lactating females due to cell transdifferentiation. More direct evidence of transdifferentiation of somatotropes into lactotropes is provided by *in vitro* studies (162, 163). In mammalian species, reversible interconversion has also been observed between somatotropes and lactotropes (164), somatotropes and thyrotropes (130, 153, 165–167), and somatotropes and gonadotropes (168).

While direct evidence for the role of sex steroids in cell phenotypic interconversion is also limited in mammals, there is some indirect evidence. In female dogs, estrogen deficiency due to ovarian dysfunction leads to an increase in gonadotropes, attributed in part to transdifferentiation (169). In rats, the production of *Fshb* and *Lhb* by somatotropes was coincident with a dramatic increase in *Esrβ* expression (168), suggesting that estrogen may regulate gonadotrope population remodeling *via* transdifferentiation.

### Cell Death

Pituitary cell apoptosis is considered necessary to ensure the balance between cell renewal and cell loss and permit optimal response to physiological demands (170). Although experimental evidence of sex steroids regulating endocrine pituitary cell apoptosis is lacking in teleosts, there is ample evidence in mammals of both androgens and estrogens modulating apoptosis in such cells, including gonadotropes (170, 171). For instance, in proestrus rats, E2 has been reported to increase anterior pituitary cell apoptosis, predominately in gonadotropes, both *in vitro* and *in vivo* (172, 173). Another study in female rats reported that gonadotrope proliferation is lowest during diestrus (when E2 is lowest) before rising gradually until the estrus phase (174), suggesting that E2 may exert either anti-proliferative or apoptotic action to maintain an appropriate gonadotrope population in mammals. Whether apoptosis regulates the number of endocrine cells in teleosts remains to be elucidated.

## Conclusion

As shown above, it is now well established that sex steroids participate in the regulation of gonadotrope plasticity. The effects mainly occur at the cellular level by modulating the sensitivity of the endocrine cells to ligands through the regulation of the number of receptors, or by regulating endocrine cell activity (hormone synthesis and release). It also appears that in at least some species, increased gonadotropin production can result from gonadotrope cell division or progenitor cell differentiation. However, these and other population-level processes

remain poorly characterized in teleosts. Therefore, there is a need for further studies to elucidate the underlying mechanisms regulating gonadotrope cell number and the potential role of sex steroids.

## SEX STEROIDS MEDIATE GONADOTROPE PLASTICITY DIRECTLY AND *VIA* THE BRAIN

Because of the strong regulation of gonadotropes by the brain, it can be difficult to distinguish whether signaling molecules act directly at the pituitary, or instead modulate the gonadotrope regulatory systems in the brain. For example, in *ex vivo* medaka brain and pituitary preparations, Fsh cells exhibited a calcium response to Gnrh stimulation (175), but in dissociated cell cultures Fsh cells did not (176), suggesting an indirect effect of Gnrh on Fsh cells. For sex steroids, it is particulary complicated as sex steroids can simultaneously act at all levels of the BPG axis, making it difficult to determine whether the observed effects are directly on gonadotropes, mediated through the brain or another pituitary cell type, or both. Therefore, a combination of *in vivo* or *ex vivo* and *in vitro* techniques can help discriminate direct from indirect effects. In addition, investigation of Esr and Ar expression in brain and pituitary cells *in vivo* have identified some direct targets of sex steroids and thus have helped to decipher the pathways used for the regulation of gonadotrope plasticity, although much work remains.

### Brain Mediated Effects

ESRs, and to a lesser extent ARs, have been found to be widely expressed in the brains of vertebrates (177, 178), including teleosts (**Table 2**). While *esr*s and *ar*s are expressed in many

**TABLE 2** | Literature on the expression of androgen and estrogen receptors in the brain and the pituitary of teleosts.

| Species | PITUITARY | | | | | BRAIN | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | ARα | ARβ | ESR1 | ESR2a | ESR2b | ARα | ARβ | ESR1 | ESR2a | ESR2b |
| African cichlid (*Astatotilapia burtoni*) | (179, 180) | | | (180) | | (179, 180) | | (180) | | |
| Atlantic croaker (*Micropogonias undulates*) | | | | | | | | (181, 182) | | |
| Eelpout (*Zoarces viviparus*) | | | | | | | | (183) | | |
| European eel (*Anguilla anguilla*) | (184) | | | | | | | | | |
| Fathead minnow (*Pimephales promelas*) | | | | (185) | | | | (185) | | |
| Goldfish (*Carassius auratus*) | *(186) (??)* | | (187–189) | | | *(186) (??)* | | *(190) (??)* (189, 191) | | |
| Medaka (*Oryzias latipes*) | (192) | | (91) | | | | | (193) | | |
| | | | | | | | | (194, 195) | | |
| Midshipman (*Porichthys notatus*) | | | (196) | | | GENE LOST | (197) | (196, 198) | | |
| Orange-spotted grouper (*Epinephelus coioides*) | | | | | | | | (92) | | |
| Oyster toadfish (*Opsanus tau*) | | | *(199) (??)* | | | | | *(199) (??)* | | |
| Paradise fish (*Macropodus opercularis*) | | | *(200) (??)* | | | | | *(200) (??)* | | |
| Pejerrey (*Odontesthes bonariensis*) | | | | | | | | (201) | | |
| Platyfish (*Xiphophorus maculatus*) | | | | | | | | *(202) (??)* | | |
| Rainbow trout (*Oncorhynchus mykiss*) | | | (203–205) | (205) | | | | (204, 206–210) | | |
| Ricefield eel (*Monopterus albus*) | | | (211) | (212) | | | | | (212) | |
| Sablefish (*Anoplopoma fimbria*) | (213) | | (213) | | | (213) | | (195, 214) | | |
| Sea bass (*Dicentrarchus labrax*) | | | (215) | (216) | | | | (215) | (216) | |
| | | | | | | | | (217) | | |
| Sea bream (*Sparus auratus*) | | | (218) | | | | | | | |
| Zebrafish (*Danio rerio*) | | | (219) | | | GENE LOST | (220) | (219) | | |

*Italic references followed by (??) show identification of receptor without subtype specificity.*

different brain areas, numerous teleost studies have demonstrated that both *esr*s and *ar*s are highly expressed in the classical neuroendocrine regions of the brain such as the POA and the mediobasal and caudal hypothalamus (221). The high expression of *esr*s and *ar*s in the brain provides histological support for the sex steroid feedback control on gonadotropin synthesis/secretion and gonadotrope proliferation which may be relayed through regulators from the brain, notably Gnrh, DA, and kisspeptins. Therefore, any change in neuronal activity (synthesis or secretion) or cell number (neurogenesis or neurodegeneration) in the populations producing such factors might affect gonadotrope activity.

## Gnrh System

The Gnrh system is the main stimulator of gonadotrope cell activity. Gnrh exerts its effects through three Gnrh paralogs, classified according to lineage: Gnrh1, Gnrh2, and Gnrh3 (222, 223). While both Gnrh receptors and Gnrh3 or Gnrh2 fibers have been observed in the retina and the pineal gland respectively, hypophysiotropic Gnrh1, or Gnrh3 in those teleost species lacking Gnrh1, serves as the main stimulator of gonadotropes by projecting in close proximity to gonadotrope cells in the pituitary [for review, see (15)].

Gnrh is regulated by sex steroids in both mammals [for review, see (224) and (225)] and fishes. In teleosts, sex steroids can stimulate or inhibit the activity of Gnrh neurons, thus indirectly regulate gonadotrope function, as shown in **Table 3** and previously discussed in (240). The effects of sex steroids seem to depend on the specific Gnrh cell population and the maturation stage of the fish. For instance, in yearling masu salmon (*Oncorhynchus masou*), castration in under-yearling precocious males increased *gnrh3* mRNA levels in the ventral telencephalon but not in the POA, suggesting that the Gnrh3 cell populations are differentially regulated by gonadal steroids (89). In medaka, E2 significantly suppressed *gnrh* expression in embryos (233) but not in adults (90), suggesting that the effect might be stage-specific in some species. Interestingly, in the spotted scat (*Scatophagus argus)*, E2 inhibited *gnrh1* expression in a dose-dependent manner and this effect was abolished by a broad spectrum Esr antagonist or an Esr1-specific antagonist, but not by an Esr2 antagonist (238), which suggests that Esr1 mediates the inhibitory effect of E2 in this species.

In mammals the limited expression of Esrs in Gnrh neurons indicates that steroids do not exert significant feedback directly to these neurons (for review, see 149 and 150). Similarly, *esr*s were not found in Gnrh neurons in the rainbow trout (241). However, a few studies have reported the presence of Esrs and Ars in teleost Gnrh neurons, suggesting the possibility of a direct sex steroid feedback. The *er*$\alpha$ paralog was found to be expressed in Gnrh3 neurons in medaka (195) and in Gnrh1, 2, and 3 neurons in Nile tilapia (242), while both *ar*$\alpha$ and *ar*$\beta$ were found in Gnrh1 neurons in the cichlid *Astatotilapia burtoni* (179). Thus, it is possible that sex steroids might regulate Gnrh activity and proliferation directly in teleosts, although as described below there is more evidence of indirect pathways *via* effects on Gnrh regulatory factors (e.g., kisspeptin and dopamine), as seen in mammals.

## Kiss System

Kisspeptin (Kiss), a member of the (RF)-amide peptide family, has been recognized as an important regulator of reproduction in vertebrates. In mammals, Kiss neurons in the POA and the mediobasal hypothalamus are believed to stimulate the synthesis and secretion of Gnrh and mediate feedback by sex steroids (243–245). However, in teleosts, a study in striped bass showed that Kiss regulates gonadotropes in a Gnrh-independent manner (246). Recently, a study in zebrafish reported that Kiss directly stimulates *lhb* and *fshb* expression in pituitary *in vitro* culture, therefore suggesting that Kiss more directly regulates gonadotropes in teleosts than in mammals [(247) and reviewed in (15)].

While *Kiss* expression is stimulated by E2 in rodents (248, 249), the existence of *kiss* paralogs (*kiss1* and *kiss2*) in teleosts (250, 251) substantially increases the complexity of E2 regulation of *kiss* genes in these species (**Table 3**). Furthermore, which of the paralogs plays a role in the regulation of the BPG axis and steroid feedback may vary by species. For instance, in female medaka, *kiss2* expression in POA does not vary with reproductive state or after OVX (193). However, *kiss1* cell number was higher in reproductive fish compared to that in non-reproductive fish, and decreased significantly after OVX, which suggests sex steroids may exert positive feedback on *kiss1* in this species. In contrast, in the orange-spotted grouper (*Epinephelus coioides*), the expression of *kiss2*, but not *kiss1*, significantly increased in OVX females, which was reversed with E2 treatment (92). However, *in situ* hybridization showed that both *kiss1* and *kiss2* neurons express *esr1*, *esr2a*, and *esr2b*, indicating that E2 may potentially regulate both *kiss1* and *kiss2* in this species. Esrs have also been described in kisspeptin neurons in other teleost species: *er*$\alpha$ in medaka (193) and European seabass (217), and *esr1*, *esr2a* and *esr2b* in goldfish (191, 252), suggesting that Kiss neurons may be directly regulated by estrogens in teleosts as in mammals.

Interestingly, androgens also modulate *kiss* expression with different effects depending on the species, sex, or reproductive stage (**Table 3**). For instance, a study in female European seabass reported that during mid-vitellogenesis, but not during early recrudescence, both GDX and T treatment after GDX significantly lowered *kiss1* expression, but that *kiss2* expression decreased only after T treatment in GDX animals (237). In the orange-spotted grouper, during MT-induced sex reversal from female to male, hypothalamic *kiss2* transcript levels were significantly lower 1 week after methyltestosterone (MT) implantation in females (234). Levels remained low in the 2$^{nd}$ and 3$^{rd}$ weeks, but increased significantly in the 4$^{th}$ week, compared to controls. Interestingly, a second MT implant at the 3$^{rd}$ week significantly enhanced *kiss2* expression. These results suggest that MT may stimulate *kiss2* in males but suppress it in females in this species.

## Dopaminergic System

Dopamine (DA), a catecholamine, is also known to modulate the levels of Gnrh through D2 type DA receptors (253), which subsequently regulate the activity and proliferation of gonadotropes as well as gonadotropin synthesis (17, 254). In teleosts, neurons that produce the tyrosine hydroxylase (Th) enzyme (the rate-limiting enzyme in catecholamine biosynthesis) and project to the pituitary have been localized in the POA, in close proximity of Gnrh neurons,

**TABLE 3 |** Effects of sex steroids on the brain and the main neuroendocrine factors involved the regulation of gonadotrope function: Gonadotropin-releasing hormone (Gnrh), Kiss and Dopamine (DA).

| Species | Stage | Gnrh | Kiss | DA |
|---|---|---|---|---|
| Black porgy (*Acanthopagrus schlegelii*) | immature M | E2, no effects on Gnrh1 (51) | | |
| Indian Catfish (*Heteropneustes fossilis*) | pre-spawning F but not in resting phase | | | E2 ↑ DA (226) |
| Asian catfish (*Clarias Batrachus*) | juvenile M and F | | | EE2 ↑ th and DA; MT ↓ th and DA (227) |
| European eel (*Anguilla anguilla*) | F silver eels | E2 ↑ IRGnrh (228)<br>E2 ↑ Gnrh1; T and Androstenedione ↓ Gnrh2 (229) | | |
| | prepubertal F | | | T and DHT (not E2) ↑ th (230) |
| Goldfish (*Carassius auratus*) | adult F | | E2 ↑ Kiss2 (191) | |
| | sexually regressed and recrudescent F | | | T and E2 ↑ pituitary DA turnover in sexually regressed fish but only T in recrudescent fish (231) |
| Masu salmon (*Oncorhynchus masou*) | yearling M | MT ↑ Gnrh3 (232) | | |
| | yearling F | MT, no effects on Gnrh3 (232) | | |
| | immature F | MT ↑ Gnrh3 (89) | | |
| Medaka (*Oryzias latipes*) | embryos | E2 ↓ Gnrh expression (233) | | |
| | adult M and F | E2, no effects (90) | | |
| | adult F | | E2 ↑ Kiss1 but not Kiss2 (193) | |
| Orange-spotted grouper (*Epinephelus coioides*) | adult F | | E2 ↓ kiss2 but not kiss1 (92) | |
| | MT-induced M | | MT ↑ *kiss2* (234) | |
| | immature F | E2 and T ↓ Gnrh (235) | MT ↓ *kiss2* (234) | |
| Rainbow trout (*Oncorhynchus mykiss*) | immature triploid F | E2 and T ↑ Gnrh3 (102) | | |
| | immature fish | E2, no effect on Gnrh1 or Gnrh2 (236) | | |
| | immature M | T ↑ Gnrh (100) | | |
| | vitellogenic F | | | E2 ↑ Th (106) |
| | recrudescent F | | | E2 ↑ DA and DA metabolites (93) |
| Sea bass (*Dicentrarchus labrax*) | vitellogenic F | E2 ↓ Gnrh1 (111) | E2, no effects on Kiss1 or Kiss2 (111) | |
| | mid-vitellogenic F but not in early recrudescence | | T, unclear effects on Kiss1 and Kiss2 (237) | |
| | recrudescent M | T, no effects on Gnrh1 (111) | T ↓ Kiss2 (111) | |
| Spotted scat (*Scatophagus argus*) | adult F | E2 ↓ Gnrh1 (238) | | |
| Zebrafish (*Danio rerio*) | immature fish | | E2 ↑ Kiss1 and Kiss2 (239) | |

*M, Males; F, Females.*

in several species including goldfish (255), rainbow trout (206), European eel (256), zebrafish (257), and the cichlid *A. burtoni* (253).

Several studies have demonstrated the effects of estrogens and androgens on *th* expression or DA levels in teleosts (**Table 3**). The effects of sex steroids on the dopaminergic system seem to also depend on the maturation state. For instance, in the Asian catfish (*Heteropneustes fossilis)*, OVX or E2 replacement in 4-week OVX fish did not significantly affect the DA system during the resting phase, but in the pre-spawning phase, OVX significantly decreased while E2 replacement increased DA levels (226). However, due to the limited number of studies and species investigated, it remains unknown whether the sex steroid regulation of DA is sex- or species-dependent.

Nevertheless, there is evidence that *esr*s are expressed in DA neurons in the POA of rainbow trout brain (206) which

suggests a direct role of estrogens on the DA system. However, it is difficult to know whether the observed effects of DA on gonadotropes result from a direct action of DA or if they are indirectly mediated through the Gnrh system, as Gnrh neurons are also controlled by DA.

### Apoptosis and Proliferation

Sex steroids have been shown to play important roles regulating certain neuronal cell populations in the brain through neurogenesis (29) and neurodegeneration (258) in vertebrates. Published research on the role of sex steroids on cell survival and apoptosis in the teleost brain is limited to a single study in adult male zebrafish, which found that cell survival was slightly reduced in several brain areas after E2 treatment (132).

There is more evidence of the effects of sex steroids on cell proliferation in the teleost brain. For instance, in adult male zebrafish, E2 treatment inhibited cell proliferation in several brain areas, whereas an aromatase inhibitor treatment tended to stimulate cell proliferation, although the effect was not significant in all regions studied (133). However, the same authors reported that fish treated with a high affinity Esr antagonist had higher numbers of proliferative cells in several brain regions, suggesting that E2 inhibits, rather than stimulates, neuronal cell proliferation in this species. An anti-proliferative effect of E2 was also seen in adult female zebrafish, where the number of proliferating cells labelled with BrdU decreased in several neurogenic brain regions, including the POA (214). However, opposite effects were observed in juvenile black porgy treated with E2, where levels of brain aromatase and numbers of proliferative cells increased (259). A significant reduction in brain cell proliferation was observed after treatment with an aromatase inhibitor, while castration did not affect the number of brain cells. This suggests that T itself may inhibit neurogenesis, but local E2 synthesis from aromatization of T may promote neurogenesis in this species.

Changes in specific neuroendocrine cell populations in teleosts due to sex steroids was first investigated in sex-reversing fishes. In bluehead wrasse (*Thalassoma bifasciatum*), the number of Gnrh neurons in the POA was higher in males at the terminal phase of sex transformation than females or initial-phase males (260, 261). Additionally, the number of Gnrh neurons was shown to increase in females and initial-phase males, but not in terminal phase males, following 11-KT treatment, demonstrating a role of sex steroids on Gnrh neuron number (260, 261). However, whether this increase was due to cell proliferation or recruitment was not investigated. In Mozambique tilapia, Gnrh3 neurons are more numerous in males, and treatment with 11-KT or methyltestosterone (MT, a potent synthetic androgen), but not E2, increased the number of Gnrh3 neurons in females to a level similar to that in males, and modified the fish behavior (262). Recently, the same group showed that this phenomenon was due to proliferation by identifying newly formed Gnrh3 neurons after androgen treatment (263). In larval zebrafish, treatment with the synthetic estrogen 17α-ethinylestradiol (EE2) increased the numbers of forebrain Gnrh3 cells (264). The authors suggest that EE2 accelerated Gnrh3 neuron development as 5 dpf larvae treated with EE2 had similar numbers of Gnrh3 neurons as 20 dpf control fish. Similar effects of sex steroids were seen in immature African catfish, where testosterone increased the number of Gnrh1 neurons (265).

There is also some evidence that sex steroids may also affect kiss neuron number in teleosts. In medaka, the number of *kiss1* neurons was observed to decrease after OVX in some brain regions, but was maintained with E2 treatment (193). However, it is not known whether this was due to a decrease of *kiss1* expression in some neurons after OVX or if E2 treatment had a positive effect on cell survival. Finally, information on the effects of sex steroids on DA cell number is to date still lacking.

## Direct Effects on Gonadotropes

Because no direct effects of sex steroids have been demonstrated on gonadotrope proliferation, transdifferentiation or cell death *in vitro*, this section will address the question of gonadotrope plasticity by considering changes in their activity only.

### The Pituitary: A Target of Sex Steroids

As in other vertebrates, Esrs have been identified in the pituitary of many teleost species while Ars have been described in only a few species (**Table 2**).

Interestingly, in male wild sablefish (*Anoplopoma fimbria*), pituitary *esr1* and *ara* mRNA levels were positively correlated with those of *lhb*, whereas *esr2a* and *esr2b* were correlated with *fshb* transcripts during gametogenesis (213). However, whether the alterations in *esr* or *ar* levels occurred in gonadotropes remains to be determined. Indeed, the specific cell types within the pituitary that express these receptors have only been investigated in a few teleost species. In European sea bass, high expression of *esr1*, *esr2a*, and *esr2b* mRNAs was localized to the PPD and PI, and double label *in situ* hybridization demonstrated that both Fsh and Lh cells express *esr1*, *esr2a*, and *esr2b* transcripts (215, 216). In ricefield eel (*Monopterus albus*), *esr1*, but not *esr2*, was expressed in Lh cells (211, 212). In medaka, Lh cells expressed all three isoforms, with *esr1* and *esr2b* most predominately expressed (91). In contrast, nothing is known regarding the presence of *ar*s in teleost gonadotropes.

In summary, the localization of both Esrs and Ars in the pituitary, and the presence of Esr in gonadotropes support a direct effect of sex steroids on gonadotropes. Yet, further studies are needed to identify which pituitary cell types express which receptors and thereby provide stronger evidence of direct signaling, and to determine whether expression patterns vary among species and with stage of sexual maturity.

### Aromatization of Androgens in the Pituitary: A Production Site of Estrogens

Using aromatase inhibitors (51, 211, 266) or a combination of aromatizable and non-aromatizable androgens (62, 79, 91, 116), several studies have clearly demonstrated that aromatase, by converting T into E2, plays a role in the cellular responses to T observed in teleosts.

Aromatase has been identified in the pituitary of all major vertebrate groups from fishes to mammals. However, experiments in goldfish, toadfish (*Opsanus tau*), and sculpin (*Myoxocephalus octadecimspinosus*) showed that teleost pituitaries have aromatase levels l00 –1000 times greater than those in mammals and other vertebrates (27, 267, 268). Since then, aromatase expression or activity has been demonstrated in the pituitary of many other teleost species such as African catfish (269), Atlantic salmon (270), Mozambique tilapia (*Oreochromis mossambicus)* (271), rainbow

trout (210), channel catfish (*Ictalurus punctatus*), and zebrafish (272), midshipman fish (*Porichthys notatus*) (196), Atlantic cod (273), killifish (*Fundulus heteroclitus*) (274), black sea bass (*Centropristis striata*) (275), a neotropical cichlid fish (*Cichlasoma dimerus*) (276), sablefish (213), brown ghost knifefish [*Apteronotus leptorhynchus*) (277)], and black porgy (51).

Interestingly, in pejerrey (*Odontesthes bonariensis*), aromatase (*cyp19a1b*) expressing cells labeled by immunohistochemistry or *in situ* hybridization were found close to blood vessels in the pituitary (278). Moreover, aromatase has been located in pituitary cells in the Japanese eel (152) and larval zebrafish (264), in Lh cells, but not Fsh cells, in ricefield eels (211), and in both Lh and Fsh cells in medaka (91, 116). While no sex differences were detected in pituitary aromatase mRNA levels or enzyme activity in the pejerrey (278) and the Japanese eel (279), respectively, aromatase activity in European seabass (280), and *cyp19a1b* expression in the yellow perch (281) and a South American catfish (282), were higher in male pituitaries. This might explain the differential sex responses of gonadotropes observed following steroid treatment in some species. In larval zebrafish, EE2 exposure did not affect *cyp19a1b* levels in the pituitary, despite increasing levels in the forebrain (264).

### Direct Effects on Hormone Synthesis and Release

In 1983, pituitary grafts (transplantation of pituitaries to another tissue) in rainbow trout revealed positive effects of T on pituitary and plasma Lh levels, indicating a stimulatory role of T on Lh synthesis and release (283). Stimulatory effects of androgens and estrogens have also been observed in studies using whole pituitary or fragments in cultures from rainbow trout (99) and ricefield eel (211). However, as paracrine signaling occurs between pituitary cells in vertebrates (284), including in teleosts as shown for instance in goldfish (285) and Grass carp (*Ctenopharyngodon idella*) (286), and pituitary endocrine cells can be stimulated by neuroendocrine factors despite the absence of cognate receptors, through cell-cell communication (176), using whole pituitary or fragments could mask potential direct effects from the steroids. Therefore, we argue that dissociated cell culture is the only suitable technique to investigate the direct effect of sex steroids (or other factors) on gonadotropin synthesis and secretion.

Studies using dissociated pituitary cell cultures from several teleost species have yielded convincing evidence for a direct role of sex steroids in the regulation of gonadotropes, mostly by stimulating *lhb* and *fshb* expression (**Table 4**). However, divergent effects of treatment duration have reported in juvenile eel pituitary cells for example, where no effects on *lhb* mRNA levels were observed after 24 h E2 treatment while a 72 h E2 treatment decreased it (288). In African catfish, both T and E2 inhibited *lhb* transcription after 24 h treatment but simulated *lhb* levels after 48 h treatment (287).

Interestingly, in the channel catfish, both E2 and T enhanced the expression of *lhb*, but the effect of T was abolished by an aromatase inhibitor (266), indicating an important role of aromatase and E2. This result is supported by other studies where T, but not non-aromatizable androgens, gave a similar effect on gonadotropin gene expression as did E2 treatment (77, 293). Several studies using dissociated pituitary cell cultures have also demonstrated a direct effect of sex steroids on Gnrh-induced Lh release (**Table 4**). For instance, E2 treatment increased Gnrh3-stimulated Lh release from female rainbow trout pituitary cells (57, 295).

Again, sex steroid effects on gonadotropes were found to vary with stage of sexual maturity and sex. For example, T stimulated *fshb* in cells from maturing Atlantic cod, had no effect in cells from mature fish and decreased *fshb* from post-spawning fish (52). In cells from masu salmon, the combination of Gnrh3 and E2 increased *lhb* mRNA levels and decreased those of *fshb* in males, but had no effect on *lhb* or *fshb* in females (297). These results indicate that E2 and Gnrh3 signaling differentially modulate gonadotropin synthesis and that effects might be sex-specific in this species.

Further evidence of the ability of sex steroids to directly modulate gonadotropin transcription is provided by the identification of steroid response elements (SRE) in gonadotropin promoters and by *in vitro* reporter assays. SRE are short, palindromic nucleotide sequences in target genes where steroid receptors bind to regulate transcription of those genes. Full-site and half-site estrogen response elements (ERE) have been identified upstream of the *lhb* gene in chinook salmon (298, 299), but only half-site EREs have been found in both *fshb* and *lhb* genes in Nile tilapia [*lhb* (300); *fshb* (301)] and goldfish [*lhb* (80); *fshb* (302)], and the *fshb* gene in chinook salmon (303) and sea bass (304). A half-site androgen response element (ARE) was also been identified upstream of *fhb* in sea bass (304). Using an *in vitro* reporter assay, the ricefield eel *lhb* promoter was activated highly by E2, and to a lesser extent by T and 11-KT, indicating the presence of functionally active ERE and ARE in the *lhb* promoter. Conversely, neither E2 or androgens activated the *fshb* promoter (211).

## Conclusion

As shown above, it is clear that sex steroids act at both the brain and gonadotrope levels. However, there is still a need to further decipher direct from indirect effects of sex steroids. So far, the use of dispersed cell cultures has been the only way to confirm a direct effect of sex steroids on gonadotropes. However, precaution should be taken as gonadotropes have been shown to change phenotype after dissociation and seeding. Indeed, in a medaka study, Fsh cells which were shown to not possess Gnrh receptors *in vivo* and to not respond to Gnrh treatment *in vitro* 24 h after dissociation, did respond to Gnrh after 3 days in culture (116), suggesting that they begin to express Gnrh receptors during incubation. Therefore, the direct effects of sex steroids in dissociated cell cultures should be confirmed by the localization of sex steroid receptors *in vivo* in the cells of interest.

A lot of work remains to identify the cell types that express *esr* and *ar*, in both the brain and the pituitary which will help to elucidate the regulatory pathway. While Gnrh, Kiss and DA are the primary gonadotrope regulators, they are complemented by, and themselves controlled by, other factors including sex steroids. New techniques such as multicolor *in situ* hybridization and single cell transcriptomics will accelerate such research.

Finally, most of the direct effects observed on gonadotrope plasticity are about the activity (cell sensitivity and hormone synthesis and release). No data exist on the direct effect of sex steroids on the regulation of cell proliferation or cell death, which are difficult to address in *in vitro* work. Therefore, new approaches are needed to address these questions in the future.

**TABLE 4 |** Direct effects of sex steroids demonstrated by *in vitro* studies using dissociated pituitary cells from teleosts.

| Species | Stages | Synthesis | | Secretion | References |
|---|---|---|---|---|---|
| | | Lh | Fsh | Lh | |
| African catfish (*Clarias gariepinus*) | mature M | 24 h treatment E2 and T ↓ *lhb*<br>48 h treatment: T and E2 ↑ *lhb* (not DHT) | | | (287) |
| Atlantic cod (*Gadus morhua*) | maturing mix F/M | No effect of T, DHT, or E2 on *lhb* | T ↑ *fshb* (not E2 or DHT) | | (52) |
| | mature mix F/M | DHT ↑ *lhb* (not T or E2) | E2 DHT ↑ *fshb* (not T) | | |
| | Post-spawning mix F/M | No effect of T, DHT, or E2 on *lhb* | T ↓ *fshb* (not E2 or DHT) | | |
| Black porgy (*Acanthopagrus schlegelii*) | mature M | | | no effects of E2, T, 11-KT on basal levels<br>11-KT and E2 ↑ Gnrh-induce stimulation | (69) |
| Channel catfish (*Ictalurus punctatus*) | mature F | E2 and T ↑ *lhb* | | | (266) |
| European eel (*Anguilla anguilla*) | silver F | T, DHT and 3α-diol ↑ *lhb* and Lh<br>24 h treatment: no effect of E2 on *lhb* or Lh<br>72 h treatment: E2 ↓ *lhb* | | T, DHT and 3α-diol ↑ (not E2) | (288) |
| | | T ↑ *lhb* and Lh (not E2) | | | (289) |
| | | T and DHT ↑ *lhb* (not E2) | E2 ↑ *fshb* (not T or DHT) | | (77) |
| Goldfish (*Carassius auratus*) | mature or sexually regressed | no effect of T on *lhb* | no effect of T on *fshb* | | (290) |
| | immature | *T* ↑ *lhb* | no effect of T on *fshb* | | |
| Hybrid tilapia (*Oreochromis niloticus* × *O. aureus*) | immature M | No effect of T on *lhb* | *T* ↑ *fshb* | | (291) |
| Marine medaka | mature mix M/F | E2 ↑ *lhb* | E2 ↑ *fshb* | | (292) |
| Masu salmon (*Oncorhynchus masou*) | F and M | E2 and T ↑ *lhb* (not 11-KT) | | E2 and T ↑ (not 11-KT) | (293) |
| | | E2 ↑ *lhb* and Lh | no effect of E2 on *fshb* or Fsh | | (294) |
| Nile tilapia (*Oreochromis niloticus*) | Maturing F | E2 ↑ *lhb* | no effect of E2 on *fshb* | | (49) |
| Rainbow trout (*Oncorhynchus mykiss*) | immature | T ↑ Lh | | T ↑ | (283) |
| | Adult F | | | E2 ↑ Gnrh-induce stimulation | (295) |
| | Immature F | | | E2 ↑ Gnrh-induce stimulation | (57) |
| Ricefield eel (*Monopterus albus*) | ?? | E2 ↑ *lhb* | no effect of E2 on *fshb* | | (212) |
| Zebrafish (*Danio rerio*) | mature mix F/M | T and E2 ↑ *lhb* | T and E2 ↑ *fshb* | | (296) |

## GENERAL CONCLUSION AND FUTURE PERSPECTIVES

To conclude, any attempt to draw generalizations in teleosts, or to propose hypotheses on evolutionarily conserved mechanisms or ecological, developmental or sex-specific adaptations, is difficult due to an insufficient body of evidence. Indeed, although many studies have been performed on several species, the information is often conflicting, with differential effects of sex steroids among species, sexes, developmental stages, and reproductive states. Because effects can vary between males and females, or over the reproductive cycle within one species, it is evident that the signaling pathways can change according to the unique molecular environment. It must be recognized that teleost fish diverged over their 300 MY evolutionary history, and there is enormous variability among its nearly 30,000 species, in terms of both the environmental parameters of their habitats (salinity, light, temperature, etc.) and their reproductive strategies (iteroparity vs. semelparity, daily spawning vs. seasonal spawning, etc.). Thus, it is not surprising to find diversity among teleosts regarding the roles of sex steroids in mediating gonadotrope plasticity and the pathways by which they exert these effects. This high complexity of sex steroid signaling necessitates further research addressing the molecular

mechanisms behind the observed responses. One mechanism underlying such variation is likely the differences in steroid levels across stages and between sexes, which has been extensively reviewed [e.g. (18, 305–307)]. A second mechanism is likely due to variation in the steroid modifying enzymes (e.g., aromatase) in the pituitary, leading to local changes in levels of specific steroids. A third mechanism is likely differences in the receptor subtypes and their numbers in target tissues, which might be differentially regulated by environmental and internal factors.

Fortunately, we have a wealth of powerful tools available to investigate the role of gonadal sex steroids. For instance, GDX, which is still used today in teleosts and adapted to new species of interest (308), is a powerful technique when combined with steroid replacement to investigate the role sex steroids play in tissue plasticity. However, GDX does not remove all sex steroids, as some can be produced by interrenal cells and adipose tissue (309), as well as in the brain and pituitary as described above. Therefore, future studies are needed to investigate the potential roles of the extra-gonadal sources of sex steroids. Administration with exogenous hormones is certainly an appropriate approach to evaluate physiological effects, but the method of administration, time and duration, and concentration can influence response. For example, recent studies in medaka showed that E2 may be best administered through feeding as it is convenient and effectively mimics the diurnal E2 changes in this species, whereas fish exposed to E2 in tank water exhibited blood E2 concentrations exceeding those of environmental water, suggesting that E2 bioconcentrates (310). In addition, as shown in the female ricefield eel for example (211), when testing for effects of steroids *in vivo*, the use of a non-aromatizable androgen such as 11-KT and an aromatase inhibitor in addition to androgen and estrogen treatments will help to clearly identify the roles of estrogens vs. androgens, which is still unclear.

Finally, because teleosts possess many paralogous sex steroid receptors, it is important that future studies investigate the role of each. Recently, transgenesis techniques such as TALEN and CRISPR/Cas9 have provided new approaches for such investigations. In medaka, for example, TALEN was used to develop an Esr1 knockout (KO) fish (311). Using these animals, the authors demonstrated the dispensable role of Esr1 for development and reproduction in medaka. Using the CRISPR/Cas9 technique, three mutant transgenic zebrafish lines have been created for each of the *esr* present in the zebrafish genome, as well as all possible double and triple knockouts of the three *esr*s (312). The authors did not observe any reproductive dysfunction for the three single *esr* mutant fish lines, which suggests functional redundancy among Esrs. However, double and triple knockouts showed that *esr2a* and *esr2b* were essential for reproduction in females and maintenance of the female sex phenotype as the double mutant sex-change from female to male. While these techniques show a great number of benefits, some limitations still exist. Knocking out gene expression from the one cell stage, as it is currently performed in most teleost experiments, may activate compensatory mechanisms (312). Also, as sex steroid receptors, aromatase, and other steroidogenic enzymes are widely expressed in the brain, pituitary and gonads, it is impossible to identify the precise origin of the effects observed after a KO is made. However, techniques allowing spatial and/or temporal control of the KO have recently been established in fish (313). Such techniques might be a promising tool for future investigations of the molecular, cellular and physiological roles of specific sex steroid receptors, and thus their roles in gonadotrope plasticity.

Thus, we are hopeful that more light will be shed on these topics as they will provide important information to better understand the role of sex steroids and the pathway they use to regulate gonadotrope plasticity.

## AUTHOR CONTRIBUTIONS

RF, DB, and F-AW planned the manuscript. RF and DB wrote the paper with the help of MR and KK. All authors contributed to the article and approved the submitted version.

## FUNDING

This study was funded by the Norwegian University of Life Sciences. DB was supported by the Waple Professorship at the University of Mary Washington.

## ACKNOWLEDGMENTS

We thank Dr. Olivier Kah for the interesting discussion and comments which have helped us to write this review, and Anthony Peltier for the illustrations used in the figures.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 11 January 2021
doi: 10.3389/fendo.2020.605111

# Effects of Melatonin on Anterior Pituitary Plasticity: A Comparison Between Mammals and Teleosts

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OPEN ACCESS

**Edited by:**
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**Specialty section:**
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

**Received:** 11 September 2020
**Accepted:** 12 November 2020
**Published:** 11 January 2021

**Citation:**
Ciani E, Haug TM, Maugars G, Weltzien F-A, Falcón J and Fontaine R (2021) Effects of Melatonin on Anterior Pituitary Plasticity: A Comparison Between Mammals and Teleosts. Front. Endocrinol. 11:605111. doi: 10.3389/fendo.2020.605111

Melatonin is a key hormone involved in the photoperiodic signaling pathway. In both teleosts and mammals, melatonin produced in the pineal gland at night is released into the blood and cerebrospinal fluid, providing rhythmic information to the whole organism. Melatonin acts *via* specific receptors, allowing the synchronization of daily and annual physiological rhythms to environmental conditions. The pituitary gland, which produces several hormones involved in a variety of physiological processes such as growth, metabolism, stress and reproduction, is an important target of melatonin. Melatonin modulates pituitary cellular activities, adjusting the synthesis and release of the different pituitary hormones to the functional demands, which changes during the day, seasons and life stages. It is, however, not always clear whether melatonin acts directly or indirectly on the pituitary. Indeed, melatonin also acts both upstream, on brain centers that control the pituitary hormone production and release, as well as downstream, on the tissues targeted by the pituitary hormones, which provide positive and negative feedback to the pituitary gland. In this review, we describe the known pathways through which melatonin modulates anterior pituitary hormonal production, distinguishing indirect effects mediated by brain centers from direct effects on the anterior pituitary. We also highlight similarities and differences between teleosts and mammals, drawing attention to knowledge gaps, and suggesting aims for future research.

**Keywords: melatonin, adenohypophysis, photoperiod, melatonin receptors, seasonal reproduction, plasticity, endocrinology, light**

## INTRODUCTION

Our environment is constantly changing. While some variations are fast and unpredictable (e.g. meteorological phenomena), others, such as solar cycles, moon phases, and seasons follow regular patterns. Photoperiod, the alternation of light and darkness, is the most reliable (noise-free, characterized by predictable rhythms over a long period of time) signal, allowing animals to synchronize their biological rhythms with both daily and seasonal changes. Photoperiod is conveyed by two types of signal: a neural message from photoreceptive structures to specific signaling centers in the brain, and a hormonal message (1, 2).

Melatonin is the key hormone that conveys rhythmic information from the environment, including photoperiod and temperature, to the organism. Circulating blood levels of melatonin exhibit a daily rhythm with higher levels during night than during day, and a seasonal rhythm with longer duration of the high level period during winter, as a consequence of the longer dark phase (**Figure 1**). Additionally, variations in temperature fine-tune those rhythms by modulating the amplitude of melatonin production. Duration and amplitude of melatonin release therefore provide clear information regarding time of the day and the year, and allow the synchronization of metabolic, physiological, and behavioral events, including growth, reproduction, and migration (3, 4).

Melatonin is synthesized from tryptophan in four enzymatic steps (4, 5). Tryptophan is first converted into 5-hydroxytryptophan by the tryptophan hydroxylase, then converted into serotonin by the 5-hydroxy-tryptophan decarboxylase. Afterwards, serotonin is acetylated by the arylalkylamine *N*-acetyltransferase (AANAT), producing *N*-acetylserotonin, which is finally converted into melatonin by the hydroxyindole-*O*-methyl transferase. AANAT has been reported to be the limiting enzyme driving the rhythm of melatonin production (6). It has been hypothesized that the functional shift of AANAT from amine detoxification to melatonin synthesis played a critical role in the evolution of melatonin as a night-time signal (7–9).

While the general mechanism of melatonin synthesis is conserved across vertebrates, the number of genes encoding the different enzymes differs between mammals and fish, as a consequence of whole genome duplications that occurred in the vertebrate lineage. Indeed, after the two successive whole genome duplications (referred to as 1R and 2R) which occurred at the base of the vertebrate lineage (10–12), a third one (3R) occurred at the base of the teleost fish lineage (13), and a fourth one (4R) occurred independently in both the cyprinid and salmonid lineages (14, 15). Following a genome duplication, one of the paralogous genes may be lost or duplicated paralogues may acquire differential specialized functions over time, and an increase in the number of paralogues, expands the hormone-receptor combinations (16). In contrast to mammals, all actinopterygians, including the teleosts, possess at least two *aanat* genes (*aanat1* and *aanat2*) (9, 17), resulting probably from the whole genomic duplications that occurred in the vertebrate lineage (18). Additionally, *aanat1* and *aanat2* have also been duplicated during the 3R (18). While one of the *aanat2* paralogues was lost early after the 3R, this was not the case for the *aanat1* paralogues and, to date, some fish possess two Aanat1 isoforms (*aanat1a* and *aanat1b*) or either one of them. While *aanat1* genes are mostly expressed in the retina, brain, and peripheral tissues, *aanat2* expression is specific to the pineal gland (19, 20), the site of production of circulating melatonin in both mammals (21) and teleosts (22, 23). Melatonin is then released from the pineal gland into the blood and cerebrospinal fluid to be transported to its target organs.

Melatonin acts through several different receptors (MTNR), belonging to the G-protein coupled receptor superfamily (24). Four sub-groups of Mtnr, arising from the 1R and 2R, have been characterized in vertebrates: MTNR1A (Mel1a or MT1), MTNR1B (Mel1b or MT2), MTNR1C (Mel1c or GPR50), and MTNR1D (Mtnr1A-like or Mel1d) (25–28). In mammals, melatonin action is mediated only through two MTNR paralogues, MTNR1A and MTNR1B, since the *Mtnr1d* gene was lost in the mammalian lineage and MTNR1C lost its ability to bind melatonin (28). Teleosts may possess up to 7 Mtnr paralogues (excluding the polyploid cyprinids), arising from the 3R and 4R (25, 28). MTNR affects different intracellular signaling pathways, including cAMP/PKA, *via* $G_i$ proteins (MTNR1A and MTNR1B) (29, 30), PLC/PKC *via* $G_q$-proteins (MTNR1A and MTNR1C) (31) and cGMP *via* $G_{i/o}$ proteins (MTNR1B) (32, 33). In medaka (*Oryzias latipes*), all four Mtnr subtypes are functional and decrease cAMP in response to melatonin exposure (27). Interestingly, melatonin receptors in Atlantic salmon (*Salmo salar*) increase cAMP when activated by melatonin (25). The broad distribution of MTNR expression in the central nervous system (including the pituitary) and peripheral tissues suggests melatonin can have widespread effects (28, 34).

The pituitary is a key endocrine gland in all vertebrates, involved in the regulation of many important physiological processes (35). These include growth, puberty, seasonal sexual maturation, metabolism, and homeostasis, which exhibit cycling components over the day, the year and the life cycle. Located below the hypothalamus, the pituitary is composed of two main parts with different developmental origins (36): the anterior pituitary (adenohypophysis) and the posterior pituitary (neurohypophysis) (**Figure 2**). The neurohypophysis originates from a down-growth of the diencephalon and is mainly composed of nerve terminals from

**Abbreviations:** 1R to 4R, 1st to 4th whole genome duplication; AANAT, Arylalkylamine N-acetyltransferase; AC, Adenylyl cyclase; ACTH, Adrenocorticotropic hormone; ARC, Arcuate nucleus; ATP, Adenosine 5'-triphosphate; cAMP, Cyclic adenosine 5'-monophosphate; cGMP, Cyclic guanosine monophosphate; CREB, Calcium/cAMP response element binding protein; Cry1, Cryptochrome1; DIO2/3, Deiodinase 2/3; ER, Endoplasmatic reticulum; EYA, Eyes absent homologue; FSH/FSHB, Follicle-stimulating hormone/FSH beta subunit; GH, Growth hormone; GnIH, Gonadotropin inhibiting hormone; GnRH, Gonadotropin releasing hormone; GnRHR, Gonadotropin releasing hormone receptor; LP/SP, Long photoperiod/Short photoperiod; LH/LHB, Luteinizing hormone/LH beta subunit; LL/DD, Constant light/Constant darkness; ME, Median eminence; MSH, Melanocyte-stimulating hormone; MTNR, Melatonin receptors; PD, Pars distalis; Per1, Period1; PI, Pars intermedia; PKA, Protein kinase A; PKC, Protein kinase C; PLC, Phospholipase C; POA, Preoptic area; POMC, Pro-opiomelanocortin; PRL, Prolactin; PT, Pars tuberalis; RFRP, RFamide related peptide; SCN, Suprachiasmatic nucleus; SL, Somatolactin; T3, Triiodothyroxine; T4, Thyroxine; TEF, Thyrotroph embryonic factor; TH, Thyroid hormone; TRH, Thyrotropin-releasing hormone; TSH/TSHB, Thyroid stimulating hormone/TSH beta subunit.

**Gene and protein nomenclature:** The present review follows the ZFIN nomenclature conventions for protein and gene names in mammals and fish (e.g. Mammalian protein: LHB; Mammalian gene: Lhb; fish protein: Lhb; fish gene: lhb).

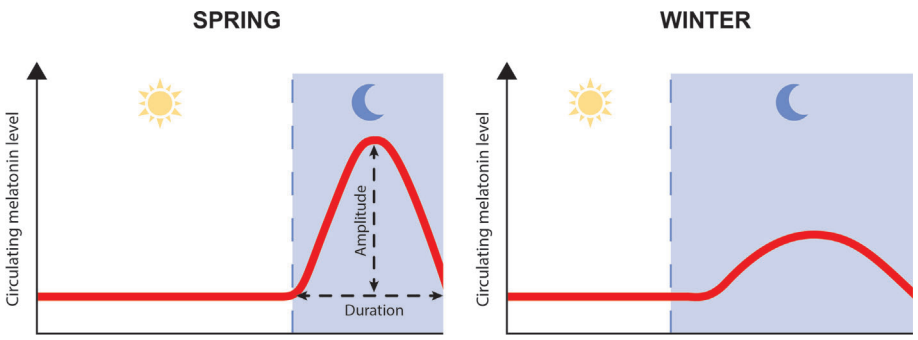


**FIGURE 1** | Schematic representation of daily and seasonal fluctuation in plasma melatonin levels.

neuroendocrine cells in the preoptic area (POA) and the hypothalamus of the brain, which are considered today as two distinct regions (37). The adenohypophysis originates from an upgrowth of the pharyngeal ectoderm and endoderm (38) and can be histologically divided in the *pars intermedia* (PI), the *pars distalis* (PD), and the *pars tuberalis* (PT), the latter present in mammals but not in teleosts. The adenohypophysis hosts several hormone-producing cell types: gonadotropes (producing the gonadotropins: follicle-stimulating and luteinizing hormones, FSH and LH), lactotropes (prolactin, PRL), somatotropes (growth hormone, GH), thyrotropes (thyrotropin, TSH), corticotropes (adrenocorticotropin, ACTH), and melanotropes (melanocyte-stimulating hormone, MSH) (39). Teleosts also possess one additional cell type, the somatolactotropes responsible for the production of somatolactin (Sl) (40).

The activity of pituitary endocrine cells is constantly changing over time, adjusting the hormonal production to changing physiological needs. It is controlled by factors produced from signaling centers in the brain, mainly the POA and hypothalamus, and from peripheral organs, which provide positive and negative feedback to these centers and to the pituitary (41, 42). In mammals, POA/hypothalamic neurons project to the median eminence (ME) of the hypothalamus, releasing their hormones into the hypophysial portal system where they are transported *via* the blood stream to the pituitary endocrine cells (42). Teleosts, on the other hand, lack the hypophysial portal system, and instead the POA/hypothalamic neurons innervate the pituitary, releasing their neurohormones directly at target cells or into pituitary blood vessels (41, 43). Pituitary hormonal production is regulated through both modulation of the activity of individual cells, and regional reorganizations of the anterior pituitary in terms of structure or cell composition, as discussed previously for gonadotropes (38, 44).

While pituitary plasticity is influenced by environmental factors, the role that melatonin plays in translating fluctuations of environmental conditions into pituitary hormonal production

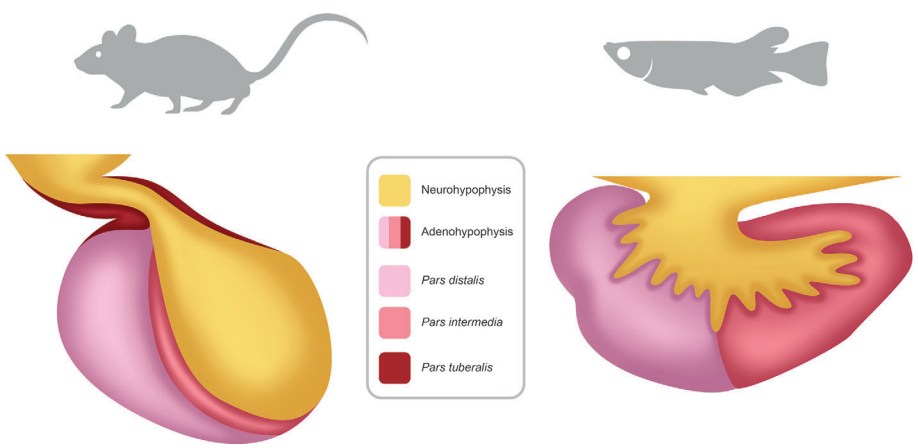


**FIGURE 2** | Schema of the pituitary in mammals and teleosts. The pituitary is composed of two main parts: the neurohypophysis (posterior pituitary) and the adenohypophysis (anterior pituitary). The neurohypophysis is mainly composed of neuron terminals from neuroendocrine cells with cell soma located in the preoptic-hypothalamic region of the brain. The adenohypophysis contains different hormones producing cell types and can be anatomically divided in *pars distalis*, *pars intermedia* and, in mammals but not in teleosts, *pars tuberalis*.

is not always clear. In addition, the mechanisms of melatonin action are complex, as both direct effects on pituitary endocrine cells and indirect effects through neuro/hormonal signaling centers combine to regulate pituitary activity. In this review, we describe the known pathways through which melatonin modulates anterior pituitary hormonal production, distinguishing between indirect effects mediated by brain centers and direct effects on the anterior pituitary. We also highlight similar and divergent features between teleosts and mammals, and emphasize important unsolved questions for future research.

# BRAIN-MEDIATED EFFECTS OF MELATONIN ON ANTERIOR PITUITARY ENDOCRINE CELLS

## Mammals

Endocrine pituitary cells are primarily controlled by brain signaling centers, mainly the preoptic and hypothalamic area (42) (**Figure 3**, **Table 1**), which integrate nervous and hormonal signals of different origins. These brain regions are characterized by the presence of numerous melatonin binding sites as shown in rodents and ruminants (60–66). Although the suprachiasmatic nucleus (SCN) of the hypothalamus drives the rhythmic production of melatonin in mammals (67), the present review will focus on known effects of melatonin on brain centers directly regulating pituitary endocrine production, as discussed below.

### Gonadotropes

Gonadotropes are the most investigated pituitary cell type in relation to melatonin, due to the high scientific and economic interest around the seasonal control of reproduction. Indeed, gonadotropes produce the two gonadotropins (FSH and LH), key hormones in the control of reproduction, which are heterodimeric glycoproteins composed of a common α-subunit (GPHα, also shared with TSH) and a hormone-specific β subunit (LHβ or FSHβ) conferring the specific biological activity (42).

#### *GnRH*

Mammalian gonadotropin-releasing hormone (GnRH1 or mGnRH-I), a 10-amino acid neuropeptide produced from POA/hypothalamic neurons, is the main regulator of gonadotropin synthesis and secretion (42). Most mammals also possess a second form (GnRH2 or cGnRH-II), expressed in the midbrain and other organs, which is primarily involved in other functions than regulating gonadotropin release. Vertebrates also possess two major types of GnRH receptors (type I with the GnRHR1a and II with the GnRHR2c) (68), however in many mammalian species, GnRHR2c receptor is not functional (69).

Melatonin influences GnRH production and thus the reproductive axis in seasonal breeders. Melatonin administration and short photoperiod (SP) cause testicular regression in the male summer breeder jerboa (*Jaculus orientalis*), a desert hibernating rodent, by inhibiting GnRH release (47). In contrast, melatonin administration in the ewe (*Ovis aries*, a winter breeder) increases the pulsatile GnRH secretion from hypothalamus, and pituitary LH

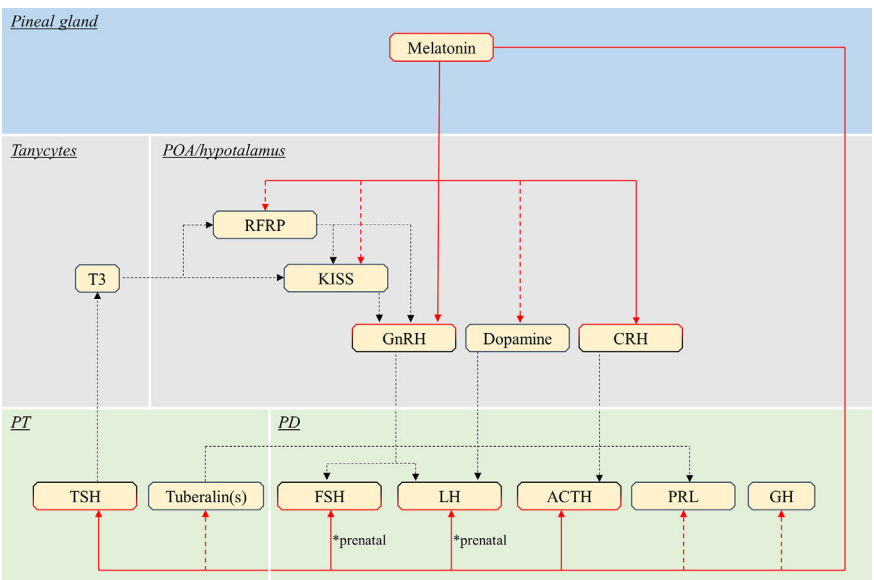


**FIGURE 3** | Schematic view of the putative pathways through which melatonin influence pituitary endocrine activity in mammals. Red continuous lines indicate cell types directly targeted from melatonin. Dashed red lines indicate cells influenced by melatonin *via* yet unidentified interneurons, paracrine signals or MTNR. Note that melatonin might act only on a few of the illustrated pathway, in different species (see text). Black dashed lines indicate all other interactions between brain and pituitary. POA, preoptic area; PT, *pars tuberalis*; PD, *pars distalis*; T3, triiodothyronine; RFRP, RFamide-related peptide; KISS, kisspeptin; GnRH, gonadotropin-releasing hormone; CRH, corticotropin-releasing hormone; TSH, thyroid-stimulating hormone; FSH, follicle-stimulating hormone; LH, luteinising hormone; ACTH, adrenocorticotropic hormone; PRL, prolactin; GH, growth hormone.

**TABLE 1** | Summary of the known effects of melatonin POA/hypothalamic neurons controlling pituitary hormonal production in mammals.

| Target | Effect of melatonin | Species | Breeding season/ Photoperiod | Description | Reference |
|---|---|---|---|---|---|
| **Mammals** | | | | | |
| GnRH | Stimulates | Sheep | Winter/SP | Melatonin administration increases GnRH secretion | Bittman et al. (45) Viguié et al. (46) |
| | Inhibits | Jerboa | Summer/LP | Short photoperiod and melatonin administration downregulate GnRH release | El Qandil et al. (47) |
| | Inhibits | GT1-7 mouse GnRH cell line | | Melatonin reduces GnRH mRNA and protein levels in GT1-7 cell line | Roy et al. (48) |
| | Modulates | Rat | non-seasonal breeder | Melatonin augments/reduces GABA-induced currents in GnRH neurons in a sex dependent way | Sato et al. (49) |
| KISS | Inhibits | Syrian hamster | Summer/LP | Melatonin reduces KISS1 mRNA | Revel et al. (50) Ansel et al. (51) |
| | | Turkish hamster | Summer/LP | Melatonin reduces KISS1 mRNA | Piekarski et al. (52) |
| | | Striped hamster | Summer/LP | Melatonin reduces KISS1 mRNA | Li et al. (53) |
| | | Rat | non-seasonal breeder | Melatonin reduces KISS1 mRNA | Oliveira et al. (54) |
| RFRP | Inhibits | Syrian hamster | Summer/LP | Melatonin (and SP) reduces RFRP-3 mRNA and protein | Mason et al. (55) Revel et al. (56) |
| | | Siberian hamster | Summer/LP | Melatonin (and SP) reduces RFRP-3 mRNA and protein | Ubuka et al. (57) Revel et al. (56) |
| Dopamine | Stimulates | Syrian hamster | Summer/LP | Melatonin administration stimulates tyrosine hydroxylase activity in males | Alexiuk et al. (58) |
| | Inhibits | Sheep | Winter/SP | Melatonin implants inhibit tyrosine hydroxylase activity | Viguié et al. (59) |

secretion (45, 46). While suggesting a connection between melatonin level and GnRH production, these *in vivo* experiments do not reveal whether melatonin acts directly on GnRH neurons, indirectly *via* interneurons or through a combination of both. However, *in vitro* experiments using the GT1-7 mouse hypothalamic GnRH cell line reveal that GnRH neurons express MTNR1A (MT1) and MTNR1B (MT2) (48) and demonstrate that melatonin inhibits both GnRH mRNA expression and protein secretion (48, 70).

Melatonin action might be modulated in a sexually dimorphic way in rodents as higher mRNA levels of *Mtnr1a* (MT1) are detected in male than female rat (*Rattus norvegicus*) GnRH neurons, while *Mtnr1b* (MT2) is not expressed in either sex (71). Sexual dimorphism of the melatonin response in GnRH neurons is supported by another *in vitro* study, where melatonin augmented the membrane current induced by gamma-aminobutyric acid ($GABA_A$) in 70% and attenuated it in 18% of neurons from adult males, while it augmented the current in only 25% and attenuated it in 61% of the neurons from adult females (49). Nevertheless, the physiological relevance of the direct actions of melatonin on GnRH neuron activity *in vivo* remains controversial, as melatonin might additionally act on upstream signals, such as KISS1, RFRP3 and T3, as discussed below.

### *RFRP3 (GnIH)*

RFamide related peptide3 (RFRP3) is the mammalian orthologue of avian GnIH, which was originally identified in birds as an inhibitory factor of gonadotropin synthesis and release, by acting on both GnRH neurons and gonadotropes. RFRP3 neurons are located in the paraventricular nucleus of the hypothalamus (42). Interestingly, the effects of RFRP3 on gonadotropin synthesis are deeply influenced by sex and timing of administration in mammals. For instance, in Syrian hamsters (*Mesocricetus auratus*), RFRP3 inhibits gonadotropin secretion in ovariectomized females (72) while it stimulates GnRH and gonadotropin secretion in males (73). In male Siberian hamster (*Phodopus sungorus*), RFRP3 directly injected into the third ventricle inhibits LH release when applied under LP, but has excitatory effects under SP (57), suggesting that melatonin might differentially influence the activity of RFRP3 neurons over the seasons.

In summer breeders, such as Siberian and Syrian hamster, both SP and melatonin injection reduces RFRP3 protein and mRNA levels, as well as decrease RFRP3 fibre density and number of projections to GnRH neurons (55–57). These studies also show that pinealectomy abolishes the effects of photoperiod manipulation, while subsequent melatonin exposure re-establishes them. While sex steroids are known to induce positive or negative feedback on hypothalamic signaling centers, the SP-induced reduction in RFRP3 protein and mRNA levels observed in male hamsters is not a consequence of reduced circulating steroid levels, since neither castration nor testosterone implants alter RFRP3 synthesis. These data therefore strongly suggest that melatonin is responsible for the inhibition of RFRP3.

In winter breeders, such as sheep, SP decreases both RFRP3 mRNA and protein levels, and RFRP3 neuron projections to GnRH neurons (74, 75). Similarly, in brushtail possum (*Trichosurus vulpecula*) females, the number of RFRP3 neurons decreases during winter (76). Interestingly, in the laboratory Wistar rat, a non-photoperiodic breeder, no effect of photoperiod manipulation was detected on RFRP3 neurons (56). These results suggest that the photoperiodic control of melatonin on RFRP3 is conserved among mammals, with inhibiting effects in both summer and winter breeders, while the downstream effects of the RFRP3 system on gonadotropin secretion might diverge to adapt to long-day or short-day breeding strategies.

Whether melatonin acts directly on RFRP cells in mammals requires further investigations as there is still a lack of evidence for

colocalization with MTNR, or studies clearly demonstrating a direct action of melatonin on RFRP3 neurons, as previously discussed by Kriegsfeld and collaborators (77).

### *Kisspeptin*

KISS neurons produce the neuropeptide kisspeptin (KISS) and stimulate GnRH synthesis and secretion, thereby regulating gonadotrope cell activity (78). Located in two discrete hypothalamic nuclei, the arcuate nucleus (ARC) in all mammals and the anteroventral periventricular area around the 3rd ventricle in rodents or the POA in non-rodent mammals, the activity and number of KISS neurons display a marked photoperiodic/seasonal pattern, as shown below.

In the winter breeding sheep, SP upregulates both ARC *KISS1* mRNA and protein, and increases the number of both ARC KISS neurons and synaptic connections from KISS to GnRH neurons (75). In contrast, melatonin inhibits the activity of KISS neurons in summer breeders. For instance, using a combination of photoperiod manipulation, pinealectomy and melatonin administration Revel and colleagues (50) and Ansel and colleagues (51) demonstrated that melatonin clearly reduces ARC *KISS1* mRNA in Syrian hamsters, an effect further modulated by the negative steroid feedback. Similar inhibitory effects of melatonin on *KISS1* mRNA were detected in Turkish (*Mesocricetus brandti*) (52) and striped (*Cricetulus barabensis*) (53) hamsters but also in the rat, a non-seasonal breeder (54). Interestingly, in the Siberian hamster, ARC *KISS1* mRNA levels are lower under LP due to a robust negative sex steroid feedback overriding the melatonin signal, since castration in LP animals restores high *KISS1* mRNA levels (79). Therefore, the role of melatonin among different species, or different reproductive stages, might be difficult to identify considering the impact of steroid feedbacks on ARC KISS neurons.

Although a direct effect of melatonin on *KISS1* mRNA levels was detected in a hypothalamic cell line from rat (80), *Mtnr* expression has not been found in sheep KISS neurons, neither during the breeding nor during the non-breeding season (81). These results suggest that the effects of melatonin on KISS neurons could be mediated upstream.

### *Dopamine*

The activity of the dopaminergic neurons located within the POA/hypothalamic area, which are involved in the inhibition of gonadotropin synthesis and release (82), also appears to be regulated by melatonin. For instance, in the ewe, a winter breeder, melatonin implants inhibit the activity of tyrosine hydroxylase (the rate-limiting enzyme in the dopamine synthesis pathway) in the median eminence, while stimulating LH release (59). In contrast, in male Syrian hamsters, in which SP elicits testicular regression, melatonin administration stimulates tyrosine hydroxylase activity in the median eminence, increasing dopamine synthesis and release (58).

## Other Endocrine Cells in the Pars Distalis

Melatonin plays a role in the regulation of other pituitary endocrine cell types in mammals by regulating their main hypophysiotropic factors.

Corticotrope cells produce ACTH, a hormone involved in various physiological processes including the stress response (promoting the release of cortisol from the adrenal gland) and the control of numerous daily and seasonal physiological rhythms (including sleep) (83). Corticotropes are mainly regulated by corticotropin releasing hormone (CRH) neurons located in the paraventricular nucleus. Melatonin exerts a stress-protective effect in mammals (84, 85). Daily melatonin administration reduces the ACTH secretory response to acute and chronic stress in rat (86, 87). In humans (*Homo sapiens*), oral melatonin administration in blind individuals normalizes the temporal pattern of ACTH and cortisol plasma concentrations during sleep, suppressing the pituitary-adrenal activity during early sleep and activating it during late sleep (88). Melatonin might modulate ACTH production by acting directly on hypothalamic CRH neurons, which express the MTNR1A in humans (89).

Lactotrope cells produce PRL, a peptide hormone involved in reproduction (lactation), moulting, metabolism, and immune responses. PRL secretion is stimulated by releasing factors from the PT and inhibited by dopamine secreted by tubero-infindibular dopaminergic neurons located in the dorsomedial arcuate nucleus (90–92). Exogenous melatonin administration and SP decrease the PRL secretion in ruminants. For instance, oral melatonin administration inhibits PRL secretion in lactating ewes (93). SP reduces PRL secretion in cows [*Bos taurus* (94)], while melatonin oral administration reduces PRL release in both prepubertal (95) and mature (96) cows. The pathway involved in the melatonin-mediated PRL inhibition seems to be mediated through a dopamine-independent mechanism, since melatonin administration inhibits PRL release even in rams where the hypothalamo-pituitary connection has been surgically disrupted (97, 98) and melatonin implants reduce PRL secretion without altering dopamine content in ewe (59).

Despite the involvement of both somatotropes and thyrotropes in seasonal physiological activity, there is no clearly established role for melatonin signaling to their POA/hypothalamic regulators. Somatotrope cells produce GH, a peptide hormone involved in numerous physiological processes including growth, metabolism and cellular proliferation. The main hypothalamic regulators of somatotropes are growth hormone releasing hormone (GHRH) and somatostatin, which stimulates and inhibits GH production, respectively (99). Thyrotrope cells produce TSH, a heterodimeric glycoprotein hormone, composed of an α- (GPHα) and a β- (TSHβ) subunit, involved in different seasonal physiological functions including reproduction and growth (100). Two distinct populations of thyrotropes, with distinct morphology and secretory activity are located in the PT and PD (101–103). Thyrotropin-releasing hormone (TRH) produced by hypothalamic neurons is the main regulator of PD TSH synthesis (104). By contrast, TRH has no effect on PT thyrotrope activity (105), which is controlled by other signals including melatonin, as discussed in section 3 below on direct effects of melatonin.

# Teleosts

In teleosts, endocrine pituitary cells are also mainly controlled by brain signaling centers in the preoptic and hypothalamic areas (41), which are characterized by the presence of numerous melatonin binding sites (106–108). The effects of melatonin on

these brain centers, and thus indirectly on pituitary activity, have been studied mainly in the context of reproduction in teleosts, such as in the salmon (109) and eel (110) where melatonin has been shown to play an important role in puberty. Therefore, the available knowledge and thus the discussion in the present review, only concern gonadotropes (**Figure 4**, **Table 2**).

## Gonadotropes

In contrast to mammals, the teleost gonadotropes mainly produce Lh and Fsh in distinct cells, with only a small portion of gonadotropes producing both hormones in some species (43). However, like in mammals, pituitary gonadotropin synthesis and release are regulated by POA/hypothalamic signaling centers, including Gnrh, Gnih, Kiss, and dopamine neurons (41, 123, 124).

### *Gnrh*

Fish possess up to three genes encoding Gnrh (*gnrh1*, *gnrh2*, *gnrh3*) (16, 41). In some teleost species, melatonin stimulates gonadotropin production by upregulating Gnrh expression. For instance, in adult female zebrafish (*Danio rerio*), melatonin exposure *via* immersion increases the mRNA levels of brain *gnrh3* and pituitary *lhb* (111). In a second study on adult zebrafish females, brain *gnrh3* mRNA levels were increased in both constant light (LL) and constant darkness (DD) as compared to normal light-dark cycles (117). The inconsistent responses of *gnrh* highlight the importance of the experimental conditions, and suggest the presence of different regulatory mechanisms activated by melatonin exposure and photoperiod manipulation, as mentioned in the introduction.

In other teleost species, melatonin inhibits gonadotropin production by downregulating Gnrh expression. In Nile tilapia (*Oreochromis niloticus*), whose development and reproduction are suppressed under SP (125, 126), intraperitoneal melatonin injections simultaneously reduce *gnrh1* mRNA in the brain and both *lhb* and *fshb* mRNA in the pituitary (112). Several studies were performed in male sea bass (*Dicentrarchus labrax*). Both intraperitoneal melatonin injections (114) and melatonin implants (115) downregulate brain mRNA levels of the two hypophysiotropic forms of Gnrh, *gnrh1* and *gnrh3*. Remarkably, these genes show natural daily variations in mRNA levels, with lower levels during the mid-dark phase, when plasma melatonin is highest (114). Melatonin implants also decrease pituitary mRNA levels of Gnrh receptors (named *gnrhr2ba1* and *gnrhr1cb*, according to recent phylogeny, (68) but named *gnrhr-II-1a* and *gnrhr-II-2b*, respectively, in the study), as well as *fshb* (115). Interestingly, pituitary Gnrh1 protein content shows daily variation with minimum levels during night time, under both natural photoperiod and LP (127). While downregulating the Gnrh system, melatonin implants also reduce plasma gonadotropins (Lh and Fsh) and androgens (testosterone, T and 11-keto-testosterone,

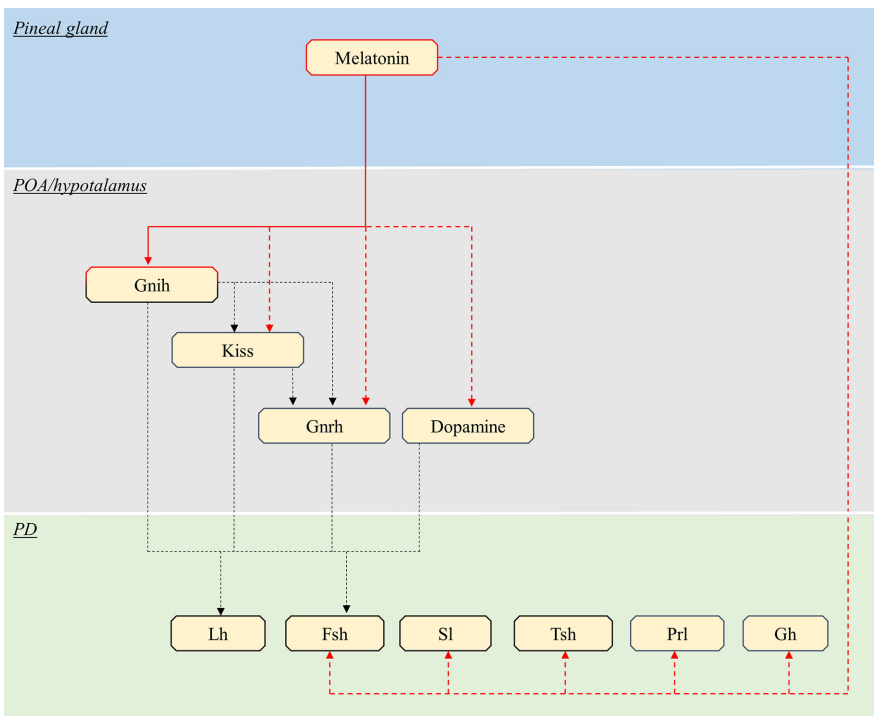


**FIGURE 4** | Schematic view of the putative pathways through which melatonin influence pituitary endocrine activity in teleosts. Red continuous lines indicate cell types directly targeted from melatonin. Dashed red lines indicate cells influenced by melatonin *via* yet unidentified interneurons, paracrine signals or Mtnr Note that melatonin might act only on a few of the illustrated pathway, in different species (see text). Black dashed lines indicate all other interactions between brain and pituitary. POA, *preoptic area*; PD, *pars distalis*; Kiss, kisspeptin; Gnrh, gonadotropin-releasing hormone; Lh, luteinising hormone; Fsh, follicle-stimulating hormone; Sl, somatolactin; Tsh, thyroid-stimulating hormone; PRL, prolactin; GH, growth hormone.

**TABLE 2 |** Summary of the known effects of melatonin POA/hypothalamic neurons controlling pituitary hormonal production in teleosts.

| Target | Effect of melatonin | Species | Spawning season/ Photoperiod | Description | Reference |
|---|---|---|---|---|---|
| **Teleosts** | | | | | |
| Gnrh | Stimulates | Zebrafish | Spring/LP (Daily in captivity) | Melatonin exposure *via* water upregulates brain *gnrh3* expression (adult females) | Carnevali et al. (111) |
| | Inhibits | Nile tilapia | Spring/LP | Melatonin injections downregulate brain *gnrh1* expression | Kim et al. (112) |
| | | Masu salmon | Autumn/SP | Oral melatonin administration (50 μg/g feed) decreases Gnrh release | Amano et al. (113) |
| | | Sea bass | Spring/LP | Melatonin injections downregulate brain *gnrh1* and *gnrh3* expression | Servili et al. (114) |
| | | | | Melatonin implants downregulate brain gnrh1 and gnrh3 and gnrhr-II-1a -2b | Alvarado et al. (115) |
| | None | European eel | Spring/LP | Melatonin implants have no effects on *gnrh* expression | Sébert et al. (116) |
| Gnih | Stimulates | Nile tilapia | Spring/LP | Melatonin injections upregulate brain *gnih* expression | Kim et al. (112) |
| | Inhibits | Zebrafish | Spring/LP (Daily in captivity) | Melatonin downregulates *gnih* expression in cultured hypothalamus | Yumnamcha et al. (117) |
| Kiss | Stimulates | Zebrafish | Spring/LP (Daily in captivity) | Melatonin exposure *via* water upregulates brain *kiss1* and *kiss2* expression (adult females) | Carnevali et al. (111) |
| | Inhibits | Sea bass | Spring/LP | Melatonin implants downregulate brain *kiss1* and *kiss2* expression | Alvarado et al. (115) |
| Dopamine | Inhibits | European eel | Spring/LP | Melatonin implants stimulate brain tyrosine hydroxylase activity | Sébert et al. (116) |
| | | Carp | Spring/LP | Melatonin inhibits dopamine release in cultured hypothalamus | Popek (118) |
| | | | | Melatonin injections inhibit brain dopamine release | Popek et al. (119) |
| | | Asian catfish | Spring/LP | Melatonin inhibits hypothalamic tyrosine hydroxylase activity during preovulatory phase in female | Chaube and Joy (120) |
| | | | | A higher dose of melatonin has no effect on tyrosine hydroxylase activity | Senthilkumaran and Joy (121) |
| | | Rainbow trout | Autumn/SP | Melatonin decreases hypothalamic-pituitary dopamine turnover | Hernández-Rauda et al. (122) |

11KT) levels, thus impairing sexual maturation (115). Servili and collaborators (128) show that in sea bass, the non-hypophysiotropic Gnrh2 neurons send their projections to the pineal gland, and directly stimulate melatonin secretion. Taken together, these results suggest that melatonin in male sea bass, downregulates the production of the hypophysiotropic Gnrh forms (Gnrh1 and Gnrh3) and their release in the pituitary. This, combined with the reduction of Gnrh receptors in the pituitary, result in gonadotropin downregulation. The use of different concentrations of exogenous melatonin can modulate its effects on Gnrh and gonadotropin content. For example, in underyearling masu salmon (*Oncorhynchus masou*), oral administration of melatonin (50 μg/g feed) under LP increases Fsh and T plasma content but has no effect on Lh (129), suggesting that mimicking SP by melatonin administration stimulates testicular development. However, a 10-fold higher dose (500 μg/g feed) decreases pituitary Gnrh and Lh content together with plasma T levels (113). In contrast, the Gnrh system does not respond to melatonin in some teleost species such as European eel (*Anguilla anguilla*) where melatonin implants had no effects on brain *gnrh1* and *gnrh2* mRNA levels (116).

The specific pathways through which melatonin affects Gnrh are largely unknown. In sea bass, the effects of melatonin on Gnrh neuron activity are most likely mediated *via* interneurons (114), since the distribution of melatonin receptors does not match the distribution of *gnrh1* and *gnrh3* cells (106). In masu salmon, melatonin binding sites were localized in the POA (113), however no colocalization study was performed to investigate their presence in Gnrh neurons.

### *Gnih*

Melatonin modulates the activity of Gnih neurons by stimulating or inhibiting Gnih expression in different species. In adult zebrafish females, exogenous melatonin treatment reduces *gnih* mRNA levels in cultured whole brain, while DD decreases *in vivo* brain *gnih* and increases both *lhb* and *fshb* mRNA in the pituitary (117). In contrast, in Nile tilapia (mixed sex), brain *gnih* mRNA levels increase during the night, in parallel with plasma melatonin levels in mature fish (130). Additionally, intraperitoneal melatonin injections increase *gnih* and *mtnr1c* mRNA in the brain and simultaneously decrease *lhb* and *fshb* mRNA in the pituitary.

Kim and colleagues (130) suggest that, like in birds, melatonin might act on Gnih neuron activity *via* Mtnr1c (131). Indeed, in the cinnamon clownfish (*Amphiprion melanopus*), Gnih neurons express Mtnr1c (named from the authors MT-R1) (132). However, whether this is a species-specific case, or a general condition for all teleosts, remains to be investigated.

### *Kiss*

In teleosts, contrary to in mammals, Kiss neurons directly regulate pituitary endocrine cells rather than acting through Gnrh neurons (133). Teleosts possess two genes encoding kisspeptins (*kiss1*, *kiss2*) (16, 134). Melatonin is also involved in the control of *kiss* expression in teleosts. In adult female zebrafish, melatonin exposure *via* immersion increases mRNA transcript levels of both *kiss1* and *kiss2* in the brain, and *lhb* in the pituitary (111). While *kiss1* does not respond to photoperiod manipulations, *kiss2* mRNA is induced under LL when melatonin plasma levels are at their minimum

(117). In contrast to in zebrafish, prolonged exposure to melatonin *via* implants decreases brain mRNA levels of *kiss1* and *kiss2* in male sea bass (115). The heterogeneity of *kiss* response, as seen for *gnrh*, highlight the influence of experimental conditions and suggest the possible involvement of different pathways influenced by the hormonal and nervous photoperiodic signal.

It is not known whether melatonin acts directly on kiss neurons or operates *via* interneurons in teleosts. In sea bass, Kiss1 and Kiss2 immunoreactive neurons were identified in the lateral tuberal nucleus and parvocellular nucleus, respectively (135), two locations that also express melatonin receptors (106). However, a clear colocalization of the melatonin receptors in Kiss neurons has not been demonstrated.

#### *Dopamine*

In several teleost species, dopaminergic neurons from the POA exert a strong negative control on gonadotropes, especially Lh-producing cells (82). Melatonin, in turn, influences the activity of hypophysiotropic dopaminergic neurons. In European eel melatonin implants stimulate the dopaminergic system in the POA, increasing tyrosine hydroxylase activity, the rate-limiting enzyme of dopamine synthesis (116). As a consequence, this treatment downregulates both *lhb* and *fshb* mRNA levels. In contrast to the eel, melatonin inhibits the dopaminergic system in other fish species. For instance, in mature female carp (*Cyprinus carpio*), melatonin decreases dopamine release, both in *in vitro* cultured hypothalamus (118) and *in vivo* by direct injection into the third cerebral ventricle (119). The *in vivo* inhibition of dopamine release was registered during the spawning period in summer, but not during sexual regression in winter, suggesting that the effect of melatonin on the dopaminergic system might depend of the maturation stage. Inhibiting effects of melatonin on the dopaminergic systems were also observed in other species, including the threespot wrasse (*Halichoeres trimaculatus*), where intraperitoneal melatonin injections downregulate brain dopamine content (136), and rainbow trout (*Oncorhynchus mykiss*), where melatonin decreases the hypothalamic-pituitary dopamine turnover (122). In preovulatory female catfish (*Heteropneustes fossilis*), melatonin injections for three days inhibit tyrosine hydroxylase enzymatic activity in the hypothalamus (120). However, in a previous study using the same species, reproductive phase and melatonin injection dose, but administrated over a longer period (20 days), failed to affect hypothalamic dopamine turnover (121). This indicates that the length of the treatment with melatonin might affect the response of the dopaminergic system.

Melatonin binding and *mtnr* mRNA distribution studies in rainbow trout (108) indicate that it is very unlikely that Mtnr is present on hypophysiotropic dopaminergic neurons. Therefore, the effects of melatonin might be mediated by interneurons in this species. Studies in Atlantic salmon (137) goldfish (*Carassius auratus*) (138) and Japanese catfish (*Silurus asotus*) (139), identify melatonin binding sites in the POA, where hypophysiotropic dopaminergic neurones are located (82); however, a clear colocalization has not been demonstrated.

## Summary

Melatonin affects pituitary hormonal production in both mammals and teleosts by regulating upstream brain factors. As gonadotropes play a crucial role in the control of reproduction, which often depends on environmental conditions, it is not surprising that especially their response to indirect melatonin signaling has been studied extensively.

In mammals, melatonin modulates gonadotropin expression by acting on POA/hypothalamic signaling centers. It downregulates KISS and GnRH production and stimulate dopaminergic activity in summer breeders, while it upregulates KISS and GnRH production and inhibits dopaminergic activity in winter breeders. Interestingly, melatonin reduces GnIH neuronal activity in both summer and winter breeders, indicating downstream differences in the signaling cascade.

In teleost fish, melatonin affects these brain signaling hubs in a more complex manner, both inhibiting and activating the gonadotrope axis, depending on the species. In some species, melatonin activates the gonadotrope axis by simultaneously stimulating the production of Gnrh and Kiss, while inhibiting Gnih; in others, melatonin exerts a negative action on reproduction. Melatonin may downregulate dopamine production resulting in increased gonadotropin synthesis, or it can stimulate the dopaminergic system and inhibit gonadotropin production. Nevertheless, in both teleosts and mammals, a clear picture of the cell types directly targeted by melatonin in the brain is still scarce. In light of such opposing forces, it is urgent to identify the specific cell types targeted by melatonin in both mammals and teleosts, by determining which ones express MTNR. This is a requirement before being able to fully elucidate the mechanisms involved in the integration of environmental signals in the brain neuroendocrine centers.

# DIRECT EFFECTS OF MELATONIN ON THE ANTERIOR PITUITARY

## Mammals

In addition to the effects mediated by the brain, melatonin can act directly on the pituitary gland in mammals (**Figure 3**, **Table 3**).

### Melatonin Receptors in Mammalian Pituitary

The main target for melatonin within the mammalian pituitary is the PT, as indicated by the important presence of melatonin binding sites in all mammalian species investigated so far. Those include, for instance, Siberian hamster (154, 155); Syrian hamster, (154–156); rat, (154, 155, 157, 158); red deer (*Cervus elaphus*) (159); ferret (*Mustela putorius furo*) (160), rhesus monkeys (*Macaca mulatta*) (161) and human (89). MTNR1A (MT1) is the main form of melatonin receptor present in the PT. *Mtnr1a* mRNA was detected by *in situ* hybridization in the PT of sheep, Siberian hamster and rat (154) and in primary PT cell cultures of sheep (162). MTNR1A has also been detected in human PT, *via* immunostaining (89). Identification of the specific cell types expressing melatonin receptors is a key requisite to discriminate the direct effects mediated by melatonin. Double labelling combining *in situ* hybridization and immunohistochemistry shows that Mtnr1a is expressed in most, but not all, thyrotrope cells within the PT, while it is absent from the other endocrine cells types in the anterior

**TABLE 3 |** Summary of the known effects of melatonin on pituitary *in vitro* and *ex vivo* cultures in mammals.

| Species | Type of preparation | Effects of melatonin | Reference |
|---|---|---|---|
| **Mammals** | | | |
| Sheep | PT cell culture | Acute: inhibits forskolin-induced secretion of tuberalin | Morgan et al. (140) |
| | PT cell culture | Acute: inhibits forskolin-induced cAMP | Hazlerigg et al. (141) |
| | PT cell culture | Prolonged: increase basal and forskolin-induced cAMP | Hazlerigg et al. (141) |
| | PT cell culture | Reduces *Mtnr1a* mRNA | Barret et al. (142) |
| | PT explants | Reduces *Mtnr1a* mRNA | Fustin et al. (143) |
| | PT explants and cell culture | Reduces *Egr1* expression | Fustin et al. (143) |
| Rat (neonatal) | PD organ cultures | Inhibits LH and FSH release | Martin and Klein (144)<br>Martin and Sattler (145) |
| | PD cell culture | Inhibits GnRH-induced Ca2+ signal and LH secretion | Vaněček and Klein (146) |
| | PD cell culture | Inhibits GnRH-induced cFOS | Sumova et al. (147) |
| | Organ cultures | No effect on TRH-induced TSH/PRL or SRIF-induced inhibition of GH | Martin and Klein (144)<br>Martin and Sattler (145) |
| Rat (maturing) | PD cell culture | No effect on GnRH-response | Rivest et al. (148) |
| Rat (Adult) | TP/ME explants | Inhibits Lh release | Nakazawa et al. (149) |
| | GH3/GH4 cell line | Inhibits secretion of PRL and GH (no effect on cAMP) | Griffiths et al. (150) |
| | GH3 cell line | Inhibits basal and forskolin-induced PRL secretion and expression | Ogura-Ochi et al. (151) |
| Baboon | PD cell culture | No effect on LH or FSH secretion | Ibáñez-Costa et al. (152) |
| Baboon | PD cell culture | Stimulates GH and PRL expression and release | Ibáñez-Costa et al. (152) |
| | PD cell culture | Increases expression of receptors for GhRH and ghrelin, decreases receptors for somatostatin | Ibáñez-Costa et al. (152) |
| | PD cell culture | No effect on ACTH or TSH | Ibáñez-Costa et al. (152) |
| Mouse | ATt20 cell line | Inhibits cAMP and ACTH release | Tsukamoto et al. (153) |

pituitary in European hamster (*Cricetus cricetus*) (163) and rat (157). This suggests that thyrotropes of the PT are the main pituitary target of melatonin action in mammalian species.

Interestingly, the expression of Mtnr within the PT varies across the year, in response to different neuroendocrine factors (34), modulating the endocrine response to melatonin between the different seasons. For instance, Mtnr expression increases during the reproductive season, peaking under LP in summer breeders or SP in winter breeders. Simulating winter season in the summer breeder Syrian hamster, using artificial SP (164) or melatonin injections (156, 165) induces a marked decrease in MTNR density in the PT of Syrian hamster. Melatonin injections decrease MTNR1A density in both Syrian and Siberian hamster PT (156). Similar effects of SP were shown in European hamster (163, 166) and hedgehog, *Erinaceus europaeus* (167). On the contrary, in winter breeders such as the mink (*Mustela vison*), the density of melatonin binding sites in the PT plummets in July and peaked in October, in concomitance with reactivation of sexual activity (168).

Melatonin and the pituitary also regulate daily physiological rhythms. As a consequence, variations in the presence of MTNR within the PT are not limited to annual cycles, but also occur within the span of a single day. Mtnr1a mRNA levels vary during the day in the PT of Siberian and Syrian hamster, peaking during daytime and decreasing at night (156).

Finally, in the PD of foetal rat, *Mtnr1a* mRNA was also identified by *in situ* hybridization before activation of the GnRH system, but not in postnatal stages (169). Bae and colleagues (170) demonstrate that *Mtnr1a* transcripts are present in the mice αT3-1 gonadotrope cell line, but their expression is downregulated after exposure to GnRH. The activation of the GnRH system in postnatal stages might therefore be responsible for the lack of MTNR in the adult PD reported in rat. Melatonin binding sites were also detected in the PD of adult sheep (171, 172), red deer (159) and ferret (160). A weak staining of *MTNR1A* was detected in human PD by *in situ* hybridization (89). To date, the identity of the cellular targets of melatonin within the PD and their contribution to the hormonal regulation of the gland remain largely unknown.

## Effects of Melatonin on the *Pars Tuberalis*

As mentioned above, melatonin pituitary binding primarily takes place in the PT. Through these cells, melatonin seems to regulate the PD activity *via* at least two different routes, a retrograde route from the PT to the brain (**Figure 5**) and an anterograde route from the PT to the PD, as discussed below.

### *Retrograde Route*

Studies in rodents demonstrate the inhibitory effects of melatonin on PT-TSH synthesis (173) *via* MTNR1A (174). During the long days in spring/summer, PT-TSH rises independently from TRH stimulation (175) due to reduced circulating melatonin levels. In the retrograde route (**Figure 5**), the expression of PT-TSH is rapidly induced, after LP exposure, by the transcription factor EYA3, which works with the circadian transcription factor thyrotroph embryonic factor (TEF) (176, 177). Melatonin acutely inhibits *Eya3* expression, but at the same time induces a peak of *Eya3* 12 h later. This leads to a strong morning peak of *Eya3* (and subsequently TSH) during long days. Although several other collaborating transcription factors are involved in the precise circannual regulation of TSH secretion from PT, EYA3 seems to be the one regulated by photoperiod *via* melatonin.

PT-TSH then reaches the brain where it binds to its receptors in tanycytes, specialized ependymal cells within the mediobasal hypothalamus, thus regulating the enzymatic activity of local deiodinases (Dio2-Dio3) (173, 178). This controls local thyroid hormone (TH) metabolism by converting thyroxine (T4) to the bioactive triiodothyroxine (T3), serving as key regulator of

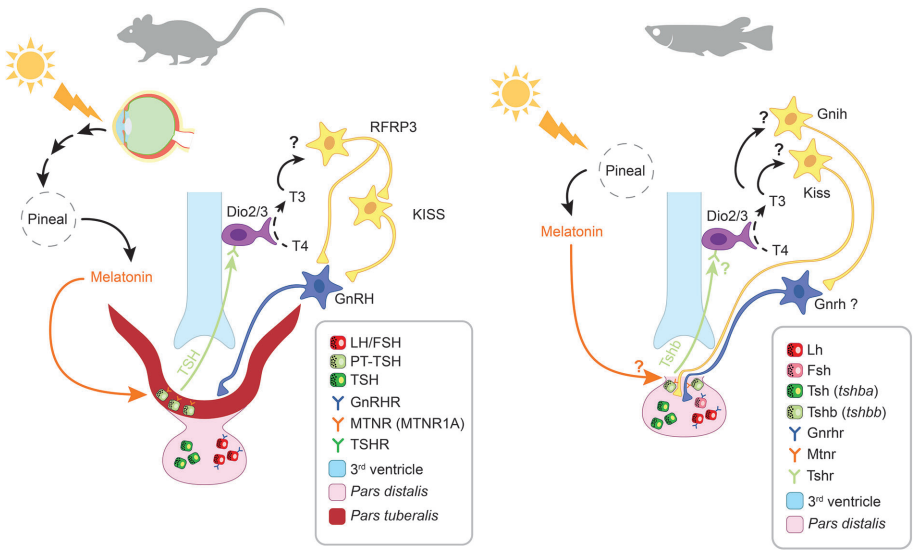


**FIGURE 5** | Melatonin-induced retrograde signaling in mammals and teleosts. Thyrotrope and gonadotrope cells are respectively represented as green and red squares (see legend). Question marks (?) indicate putative pathways not yet demonstrated. In mammals, the photic signal perceived from the retina reaches the pineal gland after being processed from different brain centers (including the suprachiasmatic nucleus, SCN) thus regulating the rhythmic release of melatonin at night. Circulating melatonin acts on *pars tuberalis* (PT) thyrotropes (PT-TSH) *via* MTRN1A, thus inhibiting PT-TSH release. In spring, when melatonin levels decrease, PT-TSH secretion is stimulated. PT-TSH, guided by tissue specific glycosylation, binds on its receptors on tanycytes located in the third ventricle of the hypothalamus. Here, PT-TSH regulates local deiodinases (Dio2/Dio3) influencing thyroid hormone metabolism by promoting the conversion of T4 into the bioactive T3. T3 in turn activates *arcuate nucleus* (ARC) kisspeptin (KISS) neurons *via* a still unknown mechanism. The following increase in gonadotropin releasing hormone (GnRH) release, stimulates gonadotropes activity in the *pars distalis* (PD). In teleosts the photic signal is directly perceived from photoreceptive structures within the pineal gland, thus regulating the rhythmic release of melatonin at night. Recent studies suggest that melatonin might regulate the release of a retrograde signal from the pituitary also in teleosts. A distinct population of thyrotrope cells (expressing a second Tsh paralogue, *tshbb)* located near the pituitary stalk, drastically increase *tshbb* expression under long photoperiod, a similar response to the one occurring in mammalian PT-TSH. Although melatonin receptors have been described in teleost pituitary and found to display daily and seasonal regulation, their presence in this thyrotrope population as well as the inhibition of Tsh synthesis and release in response to melatonin, remain to be demonstrated.

seasonality (173). Ikegami and collaborators (175) demonstrate that specific post-translational glycosylations allow PT-TSH to exclusively target the hypothalamus, and not the thyroid. While the cellular and molecular targets remain to be clearly identified, recent findings indicate that the increase of T3 in the mediobasal hypothalamus acts on KISS1 and RFRP3 neurons, which in turn modulate GnRH secretion (78). The molecular pathway from melatonin to T3 production appears to be conserved in mammalian species regardless of their reproductive strategy as summer or winter breeders (67, 179). Therefore, species-specific differences might occur downstream of this common pathway.

### *Anterograde Route*

In the anterograde route, melatonin regulates PRL synthesis and secretion in the PD by inhibiting the release from the PT of one or more PRL-releasing factors named "tuberalin" (67). To date, the PT-specific factor(s) are still undetermined, as more than 30 different factors are known to stimulate PRL secretion (92). Several candidates have been proposed including tachykinin-1 and neurokinin A in sheep (180) or endocannabinoids in hamster (179, 181). Notably, these factors might act through folliculo-stellate cells to regulate lactotropes (182).

Tuberalin secretion can indeed be stimulated in ovine PT cell cultures by forskolin (140), an activator of adenylyl cyclase (AC), the enzyme catalysing the conversion of ATP to cyclic AMP (cAMP). The forskolin-induced secretion of tuberalin from PT cells was assessed by adding medium from the PT culture to a PD culture and measuring the amount of PRL secreted in response. Melatonin acutely inhibited the forskolin-induced secretion of tuberalin but had no effect alone. In support, melatonin inhibited the forskolin-induced cAMP production in ovine PT cells (141). Furthermore, melatonin downregulated the expression of its own receptor in PT cells from rat (183) and sheep (142). In ovine PT cells, the downregulation of *Mtnr* expression involves the cAMP signaling pathway (143). Together, these results imply that melatonin works through the MTNR/Gi/cAMP pathway to inhibit tuberalin secretion and subsequently regulating PRL production in the PD. Interestingly, incubation with melatonin for 16 h sensitizes AC, increasing both basal and forskolin-induced cAMP production (141). After the prolonged melatonin exposure, acute application of melatonin still inhibits the forskolin-induced cAMP increase.

For melatonin to inhibit secretion of tuberalin, there must be a stimulating factor that melatonin can oppose. This "tuberalin releasing factor" has not been identified but was named StimX by Morgan and Williams (184). Dardente and colleagues (67) proposed dopamine as a promising candidate for StimX, arguing that it might act through the dopamine receptor D1 expressed in PT

cells, whose activation increases the intracellular cAMP level in neurons.

Downstream of cAMP, melatonin up-regulates or down-regulates the expression of a range of genes (67). Several of them are clock genes, including Period1 (*Per1*) and Cryptochrome-1 (*Cry1*) (185). This implies that PT cell activity might be regulated by an internal clock and that the clock itself may be modulated by melatonin. Interestingly, the same clock genes are not affected by melatonin in the suprachiasmatic nucleus (SCN) of the hypothalamus, indicating that the SCN clock is working more independently than peripheral clocks (186–188). In ovine PT cell cultures and explants, the expression of the immediate early gene *Egr1* is acutely suppressed by melatonin, which otherwise follows a daily rhythm (143). EGR1 in turn regulates several genes, some being upregulated, such as *Cry1*, others downregulated such as *Mrnt1a*. In contrast to *Mtnr1a*, the expression of *Cry1* was not affected by changes in cAMP levels.

Other PT endocrine cells beside thyrotropes might also be regulated by melatonin. Nakazawa and collaborators (149) found that melatonin inhibits LH release from male rat tissue explants (consisting of PT and median eminence) in a dose-dependent manner. This in turn increases the release of GnRH from the median eminence part of the explant.

### Effects of Melatonin on the *pars distalis*

#### *Gonadotropes*

In rodents, the effects on PD gonadotropes seem highly age-specific, with clear inhibitory effects in neonatal animals and no effects in adults. Melatonin (1–10 nM) reduces the GnRH-induced LH and FSH release in pituitary organ cultures from neonatal rats (144, 145). Furthermore, Vanecek and Klein (146) demonstrate that melatonin (10 nM) reduces the GnRH-induced $Ca^{2+}$ signal and subsequently LH secretion. Similarly, Pelisek and Vanecek (189) demonstrated that melatonin (2 nM) reduces GnRH-induced LH release, as well as the forskolin-induced cAMP production, in cell cultures from neonatal rats. Melatonin (1 nM) directly inhibits GnRH-induced $Ca^{2+}$ signaling in neonatal gonadotropes, both *via* plasma membrane $Ca^{2+}$ channels and endoplasmic reticulum $Ca^{2+}$ release channels (190). The inhibitory effect of melatonin on the GnRH-induced $Ca^{2+}$ oscillations might not be uniform over the gonadotrope cell population as the responses differ between cells, indicating a complex regulatory pathway (191, 192). The role of MTNR in neonatal PD may not be limited to gonadotrope regulation. In light of the previously described role of melatonin on the regulation of clock genes, Johnston and colleagues (169) suggest that *Mtnr1a* expression may reflect a developmental requirement for circadian synchronization between tissues before mature regulatory pathways become established. Additionally, the promoter region of rat *Mtnr1a* contains response elements for transcription factors involved in pituitary cell differentiation and regulation (169). Melatonin might therefore be involved in the correct development of the embryonic PD. Indeed, melatonin (100 nM) inhibits GnRH-induced increase of *cFos* (a proto-oncogene involved in cellular proliferation and differentiation) immunoreactivity in neonatal rat pituitary PD culture (147).

During development and maturation, melatonin binding is reduced, and in adults, melatonin does not have the same direct effect on the pituitary. Rivest and collaborators (148) found that melatonin incubation (5 nM) of pituitary cell cultures from sexually maturing rats does not modify the GnRH response. Likewise, Ibáñez-Costa and colleagues (152) found no effect of melatonin (pM to μM range) on the FSH and LH secretion in primary pituitary cultures from adult female baboons.

#### *Other Endocrine Cells in the pars distalis*

Regarding the effects of melatonin on other PD endocrine cells, the results are even scarcer. Melatonin ($10^{-8}$ to $10^{-6}$ M) reduces the production and secretion of both PRL and GH from the rat pituitary cell line GH4C1, but has no effect on basal or stimulated cAMP levels (150). Similarly, Ogura-Ochi and collaborators (151) show that melatonin suppresses both basal and forskolin-induced PRL secretion and mRNA abundance in the closely related GH3 cell line. In contrast, in primary pituitary cell cultures from adult female baboons, melatonin increases GH and PRL expression and release in a dose-dependent manner, an effect blocked by somatostatin (152). Both the common (AC/PKA/Ca-channels) and distinct (PLC/Ca-release) pathways seem to be involved. Melatonin (10 nM) also affects the expression of GHRH receptors, ghrelin and somatostatin, but not expression or release of ACTH or TSH. Also in pituitary organ cultures from neonatal rats, melatonin has no effect on TRH-induced TSH/PRL release or somatostatin-induced inhibition of GH release (144, 145). In the mice corticotrope cell line AtT20, melatonin reduces the levels of ACTH, alongside a reduction in cAMP (153).

## Teleosts

As in mammals, melatonin can also act directly on the pituitary gland in teleosts (**Figure 4**, **Table 4**).

### Melatonin Receptors in Teleosts Pituitary

Multiple Mtnr paralogues are expressed in the pituitary of teleosts (28). For instance, qPCR analysis detected the mRNA of four Mtnr paralogues in the pituitary of medaka (27). Three were described in Senegalese sole (*Solea senegalensis*) (200), goldfish (201), and Atlantic salmon (25). Two were detected in chum salmon (*Oncorhynchus keta*) (202) and pike (*Esox lucius*) (196). One was detected in European sea bass (203), suggesting possible multiple effects of melatonin which might also vary between species.

The exact location of melatonin receptor/binding sites in teleosts is not clear. Despite the aforementioned identification of *mtnr* mRNA in goldfish pituitary, Martinoli and colleagues (107) observe no specific binding of melatonin. On the other hand, rainbow trout (197) and pike (196) pituitaries have 2-[$^{125}$I]iodo-melatonin binding sites. However, the assay used was aimed at characterizing the binding capacity rather than their localization within the pituitary, although a regional distribution was reported, with binding sites clustering together in close proximity.

Like in mammals, the abundance of pituitary Mtnr in teleosts varies with the season and the day, suggesting a correlation with physiological state. The Senegalese sole (200) shows seasonal fluctuations with higher *mtnr* mRNA levels during the summer spawning period. While a first study in Atlantic salmon carried out in autumn indicated the absence of melatonin binding sites

**TABLE 4** | Summary of the known effects of melatonin on pituitary *in vitro* and *ex vivo* cultures in teleosts.

| Species | Type of preparation | Effects of melatonin | Reference |
|---|---|---|---|
| **Teleosts** | | | |
| Goldfish | Primary cell culture | No effect on AC activity | Deery et al. (193) |
| | Perfused fragments | No effect on secretion of Lh, Fsh or Gh. | Somoza and Peter (194) |
| European eel | Primary cell culture | No effect on *fshb* and *lhb* mRNA levels | Sébert et al. (116) |
| Atlantic croaker | Perfused fragments | Stimulates Lh (GthII) secretion | Khan and Thomas (195) |
| Pike | Whole pituitary | Inhibits forskolin-induced cAMP | Gaildrat and Falcón (196) |
| Rainbow trout | Primary cell culture | Low dose: inhibits forskolin-induced cAMP and Gh secretion | Falcón et al. (197) |
| | Primary cell culture | High dose: stimulates Gh secretion | Falcón et al. (197) |
| | Primary cell culture | High dose: inhibits Prl secretion | Falcón et al. (197) |
| European sea bass | Primary cell culture | Increases *Cry1* and *Cry2* mRNA levels | Herrero and Lepesant (198) |
| Medaka | Whole pituitary organ culture | Reduces *fshb*, *tshb* and *sl* mRNA levels (not *lhb*, *gh*, *prl* or *pomc)* | Kawabata-Sakata et al. (199) |

(137), a later study shows that Mtnr exhibits both seasonal and daily fluctuations in the male parr pituitary (25). In spring during sexual maturation, pituitary *mtnr* mRNA peaks during the day and drops at night, while in autumn minimal levels are constantly maintained throughout the day. In medaka, where SP inhibits reproduction, pituitary *mtnr1a, mtnr1c*, and *mtnr1d*, but not *mtnr1b* mRNA levels show daily fluctuations with higher levels at night under LP but not SP (27). The presence of multiple Mtnr paralogues whose concentrations vary across the season indicates a complex role of melatonin in teleost pituitary physiology. It is not yet clear precisely which cell types express *mtnr* in teleosts. Indeed, the seasonal and daily variations in *mtnr* mRNA levels observed in sole, sea bass, salmon, and medaka suggest a relation with the reproductive status of the fish. These fluctuations also highlight the importance of both the timing of sampling and the application of different techniques for the successful identification of the cells expressing Mtnr within the pituitary.

## Effects of Melatonin in Teleosts Pituitary

### *Putative Retrograde Signal*

Unlike mammals, teleosts do not possess an anatomically distinct PT. Nakane and Yoshimura (204) propose that, in salmonids, the translation of the photoperiodic signals into pituitary hormonal messages might instead take place in the *saccus vasculosus* (SV), a secretory organ located posterior to the pituitary and directly connected to the third ventricle of the hypothalamus. In masu salmon, specialized cells within the SV, the coronet cells, possess all the components involved in the regulation of seasonal reproduction *via* the Tsh pathway (Tshβ, Tsh receptors and Dio2). However, the exact photoperiodic role of the SV (and the involvement of melatonin in it), remains controversial. First, the SV signal pathway is presumably directly activated by photo-transduction through photoreceptive pigments without the requirement of melatonin (205). Additionally a recent study in juvenile Atlantic salmon reported no photoperiodic effects on SV *tshb* and *dio2b* mRNA levels (206). Finally, the SV is not present in all fish [e.g. it is absent in zebrafish (207, 208)]. Therefore, a different mechanism might be involved in the photoperiodic control of seasonality in fish.

Several teleosts possess two *tshb* paralogues resulting from the 3R, *tshba* and *tshbb* (209) (named by the authors *tshβ* and *tshβ3*). In Atlantic salmon, both are expressed in the pituitary, but only *tshbb* is expressed in the SV (210). Interestingly, while pituitary *tshba* mRNA levels are relatively constant, *tshbb* mRNA level vary profoundly, with a peak concomitant with the onset of downstream migration, in spring. Since the two paralogs also show expression at distinct locations in the pituitary, Fleming and colleagues (210) propose that *tshbb*-expressing cells, located near the pituitary stalk, are analogous to the mammalian PT-TSH cells and possibly regulate the retrograde diffusion of Tsh to the hypothalamus (**Figure 5**).

Irachi and collaborators (206) demonstrate a significant increase in pituitary *tshbb* mRNA levels followed by a rise in *dio2b* mRNA levels in the midbrain/optic tectum and hypothalamus in response to increased daylength, providing additional evidence that the pituitary Tshb (formed by the *tshbb* subunit) is a key contributor to photoperiodic signaling in fish, similar to the mammalian PT-TSH. However, whether like in mammals, melatonin signal is directly integrated by the pituitary Tshb (*tshbb*-expressing) cells in teleosts, is not known.

### *Other Endocrine Cells in the Pars Distalis*

The direct effects of melatonin on other endocrine cell types have not been extensively investigated in teleosts and the few studies available indicate different effects between species.

In goldfish, melatonin ($10^{-6}$–$10^{-3}$ M) has no effect on AC activity in homogenized pituitary samples (193). Although AC is part of the most common signaling pathway triggered by melatonin receptors, this result does not rule out the possibility that melatonin may trigger effects *via* other pathways. The conclusion that melatonin most probably had no direct effect on the pituitary was further supported by a study on perfused goldfish pituitary fragments, from which melatonin did not affect secretion of gonadotropins or Gh (194). Similarly, Sébert and colleagues (116) report from unpublished *in vitro* experiments that melatonin has no effect on *fshb* and *lhb* mRNA levels in primary pituitary cultures from European eel.

However, effects of melatonin have been observed in other species. Melatonin (0.2 ng/ml) stimulates Lh (GtH II) secretion from pituitary fragments from mature Atlantic croaker (195). In trout pituitary organ- and cell cultures, high concentration of melatonin [close to the night-time circulating levels as determined by Gern and colleagues (211)] induces a dose-dependent Gh secretion in the absence of forskolin, along with a decrease in the secretion of Prl (197). In contrast, low melatonin

concentration (close to daytime circulating levels) inhibits the forskolin-induced increase in cAMP levels and Gh secretion. Finally, melatonin exposure ($10^{-5}$ M) in *ex vivo* whole pituitary organ culture from sexually mature medaka, decreases *fshb*, *tshba* and *sl* mRNA levels but has no significant effects on *lhb*, *gh*, *prl*, and *pomc*, (199).

### Summary

The presence of melatonin receptors indicates that melatonin can directly regulate pituitary cells. According to the receptor localization, the PT thyrotropes are the main targets of melatonin in the mammalian pituitary. A few mammalian species also have melatonin binding sites in the PD, suggesting a possible direct control of melatonin on PD endocrine production. The teleost pituitary also expresses several Mtnr paralogues, however their localization is not clear.

In mammals, most of the direct effects of melatonin on the pituitary take place in the PT, in particular on thyrotropes (**Figure 5**). Here, melatonin acting *via* MTNR1A (MT1) affects the expression levels of a set of clock genes that in turn regulate synthesis and secretion of TSH and the hitherto unidentified tuberalin, for retrograde and anterograde seasonal regulation of gonadotropes and lactotropes in the PD, respectively. The signaling pathway from MTNR1A activation to transcriptional regulation seems to mainly be *via* inhibition of AC/cAMP and possibly CREB. Studies on the direct effects of melatonin in the PD are still scarce, but there is solid evidence that melatonin inhibits LH secretion from PD gonadotropes in neonatal stage, but not in adults.

Fewer studies on the direct effects of melatonin on the teleost pituitary have been performed. They report different effects depending on the species and the experimental conditions. Nevertheless, recent works described a new pituitary Tsh population responding to variation in photoperiod similarly to PT-TSH in mammals, suggesting that a Tsh retrograde signaling might also occur in teleosts. It is therefore crucial that future research investigate the presence of melatonin receptors and the responsiveness to melatonin in this cell type to verify whether, similar to in mammals, melatonin signal is directly integrated by Tshb (*tshbb*-expressing) cells in teleosts.

## DISCUSSION

As shown in the present review, the response of the pituitary to melatonin is highly plastic and differs between seasons, time of the day, physiological status, and reproductive strategies. The comparison between mammals and teleosts reveals a greater knowledge gap in the latter group, leaving many open questions on the role of melatonin in regulating the pituitary hormonal production. In both mammals and teleosts however, the exact pathways and cell types targeted by melatonin are largely unknown. It is therefore crucial to clearly describe the integration of the melatonin signal, and identify the cell types expressing melatonin receptors, in the pituitary as well as in the brain. Multi-color *in situ* hybridization or immuno-labelling and single cell transcriptomic approaches are relevant techniques that can be applied to identify the cell types directly targeted by melatonin. To validate the effects of melatonin on the different cell types a combination of *in vivo*, *ex vivo* and *in vitro* studies will be necessary, as no single approach can produce a complete and reliable picture by itself. For instance, caution should be taken when investigating dissociated endocrine pituitary cells in culture, as a recent study shows that dissociation leads to a quick cellular phenotypic change in the pituitary cells (212). Additionally, when deciding on animal model and experimental conditions, one should also consider the important biological differences that might influence, or be influenced by, a time-keeping hormone as melatonin, such as nocturnal versus diurnal habits, different reproductive seasons or hibernation periods. Indeed, the response in animals adapted during numerous generations to stable laboratory conditions, such as mice, rats, zebrafish and medaka, might diverge from the ones in the wild.

In addition, given the seasonality of the processes regulated by melatonin, the localization of MTNR appears to be heavily influenced by factors such as physiological status, season, but also time of the day. It is therefore reasonable to assume that the expression of MTNR in some key cell types might be characteristic of specific physiological conditions and might still remain undetected when analyzed outside a particular time window.

Finally, when investigating the effects of melatonin on neuroendocrine system activity, teleosts show a remarkable plasticity as their response is more sensitive to variation in environmental conditions as compared to mammals (1, 213). As a consequence, it is harder to draw a general picture of the role of melatonin on teleosts brain and pituitary, as the choice of the season or the time of the day to perform the experiment appear to influence the response of the pituitary gland in teleosts.

Because of this sheer number of physiological and environmental variables, as well as significant inter-specific variation, unravelling the full impact of melatonin on the pituitary gland remains a challenge.

## AUTHOR CONTRIBUTIONS

EC, TH, and RF contributed to the conception and design of the review. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by the Norwegian University of Life Sciences (NMBU), Ås, Norway, and the University of Oslo (UiO), Oslo, Norway, with the support of the Marie Curie Alumni Association.

## ACKNOWLEDGMENTS

The authors are grateful to Anthony Peltier for the illustrations (**Figures 1**, **2**, and **5**) and to Dr. Christiaan Henkel for having reviewed our manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 19 January 2021
doi: 10.3389/fendo.2020.614999

# From Pituitary Stem Cell Differentiation to Regenerative Medicine

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The anterior pituitary gland is comprised of specialized cell-types that produce and secrete polypeptide hormones in response to hypothalamic input and feedback from target organs. These specialized cells arise during embryonic development, from stem cells that express SOX2 and the pituitary transcription factor PROP1, which is necessary to establish the stem cell pool and promote an epithelial to mesenchymal-like transition, releasing progenitors from the niche. Human and mouse embryonic stem cells can differentiate into all major hormone-producing cell types of the anterior lobe in a highly plastic and dynamic manner. More recently human induced pluripotent stem cells (iPSCs) emerged as a viable alternative due to their plasticity and high proliferative capacity. This mini-review gives an overview of the major advances that have been achieved to develop protocols to generate pituitary hormone-producing cell types from stem cells and how these mechanisms are regulated. We also discuss their application in pituitary diseases, such as pituitary hormone deficiencies.

**Keywords: stem cells, pituitary, iPSCs, colonies, SOX2, differentiation**

OPEN ACCESS

***Edited by:***
*Karine Rizzoti,*
*Francis Crick Institute,*
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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *07 October 2020*
***Accepted:*** *01 December 2020*
***Published:*** *19 January 2021*

***Citation:***
*Camilletti MA, Martinez Mayer J, Vishnopolska SA and Perez-Millan MI (2021) From Pituitary Stem Cell Differentiation to Regenerative Medicine. Front. Endocrinol. 11:614999. doi: 10.3389/fendo.2020.614999*

## INTRODUCTION

Compelling evidence during the last decades has demonstrated the existence of stem cells (SCs) within the vertebrate's pituitary with capacity to divide and self-renew, and later to differentiate into specialized cell types. These stem cells express the transcription factor SOX2 and the pituitary transcription factor PROP1, which is necessary to establish the stem cell pool and promote an epithelial to mesenchymal-like transition, releasing progenitors from the niche (1). Pituitary SCs are essential during development and throughout the postnatal life, being able to differentiate according to physiological demand or in response to damage of the tissue in a highly plastic and dynamic manner (2, 3).

In this present review, a summary of the key findings amidst the localization of pituitary stem cells in different vertebrate model systems is presented featuring the major approaches employed for their discovery. Finally, we will discuss research advances to generate pituitary hormone-producing cell types from progenitor cells and their possible applications in regenerative therapies in hypopituitarism patients.

## CHARACTERIZATION OF PITUITARY STEM CELLS IN VERTEBRATES

One of the main anatomical differences between the mammalian and the teleost pituitary is the hypothalamic innervation. In mammals, a portal system carries the hypothalamic signals to the pituitary parenchyma, whereas in fish, the pituitary cells are directly innervated by the hypothalamus (4).

Studies in two different species of teleosts, the Japanese rice fish Medaka (*Oryzias latipes*) and the zebrafish (*Danio rerio*) have demonstrated the existence of potentially multipotent SC within the pituitary of these organisms. In Medaka, a significant number of cells expressing the marker Sox2 have been found localized in the dorsal region of the adenohypophysis in close proximity to the *pars nervosa*, with only a few cells dispersed throughout the parenchyma, consistent with its reported location in mammals (5) (**Figure 1**). Specifically, Medaka Sox2+ cells were found in regions of active proliferation evidenced by BrdU incorporation and Proliferating Cell Nuclear Antigen staining (PCNA+) (6). In zebrafish, *in situ* hybridization studies have shown the presence of Sox2+ cells in the anterior pituitary (7) only after 48–72 h postfertilization, suggesting a temporal pattern-expression during development. Although further work is necessary, it is likely that Sox2+ cells found in zebrafish pituitaries were pluripotent stem cells as shown in other tissues including the brain (12) and the retina (13–15).

Several genes found to be essential for pituitary development in mice including the Pou domain transcription factor *Pou1f1*, the protein phosphatase Eya1, and mediators of the Notch signaling were also found in zebrafish, making these models ideal for molecular studies (7, 16–19). However, other factors related to pituitary functions, like Dmrt5, appear to be exclusively found in zebrafish (20).

Compared to other vertebrate model systems, the chicken model (*gallus gallus domesticus*) displays several advantages to study prenatal developmental events, as the relatively large size of its eggs allows the possibility to observe the progress of embryo development in a time-controlled environment. The avian pituitary gland contains several hormone-producing cell types distributed along the caudal and cephalic lobes of the anterior pituitary (AP). These specialized cells arise gradually during development simultaneously with the inflow of hematopoietic cells (21).

Work by Fu et al. in the embryonic chick demonstrated that cells expressing the fibroblast growth factor 10 (Fgf10+) act as both hypothalamic and potential pituitary progenitors. Interestingly, while a group of Fgf10+ progenitors are required for the development of the anterior and mammillary domains, a population of Fgf10+ cells persists in a quiescent state in the tuberal infundibulum which gives rise to the posterior pituitary. In agreement with previous reports in mice (22), hypothalamic factors from the sonic hedgehog (shh) pathway were also found to regulate the differentiation of pituitary cells (8). In addition, transcriptome analysis of the chicken pituitary at 21, 22, and 45 days post-hatching showed a significant enrichment in genes coding for transcription factors known to be critical for pituitary cell development and differentiation such as Pou1f1 and LIM Homeobox proteins 2 (LHX2) and 3 (LHX3) (23).

Particularly in the specification of the Pou1f1 lineage (thyrotrophs, somatotrophs and lactotrophs), microarray and sequencing analysis suggested the involvement of *Ras-dva* in the chicken embryo (24), which is a target of Hesx1, a transcription factor required for proper pituitary development in mice (25). Consistently, further studies by Ellestad et al. showed that corticosterone and Pou1f1 were the main regulators of Ras-dva expression (26).

The identification of pituitary stem cells in non-mammalian animal models is a burgeoning field of research. These cells express pluripotency markers known to have a role in mammalian pituitary development and were found in similar locations as in mammals. However, whether they are multipotent progenitors with the ability to differentiate into hormone-producing cells remains to be defined. In this regard, most convincing evidence comes from studies on mice.

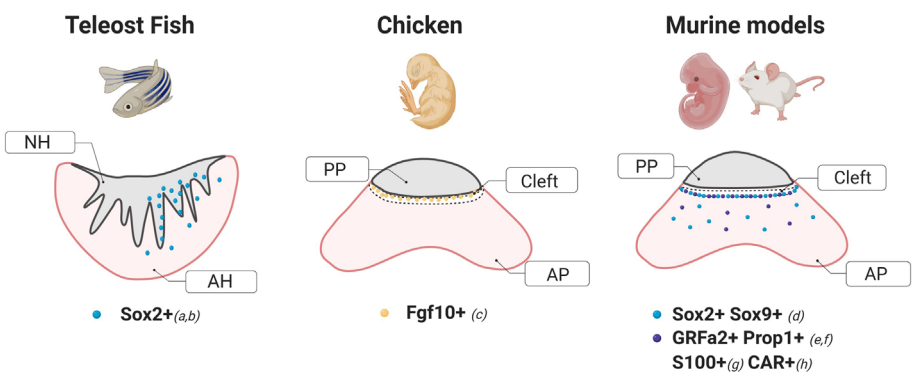


**FIGURE 1** | Schematic diagrams of stem cells markers and their localization within a coronal section of the pituitary of different animal models. These cartoons were done based on the following studies: a) Fontaine et al. (6), b) Nakahara et al. (7), c) Fu et al. (8), d) Fauquier et al. (5), e) Garcia-Lavandeira et al. (9), f) Pérez Millán et al. (1), g), Lepore et al. (10), H) Chen et al. (11). NH, Neurohypophysis; AH, Adenohypophysis; AP, Anterior Pituitary; PP, Posterior Pituitary.

## ADULT PITUITARY STEM CELLS MARKERS

Several studies have reported mouse pituitary stem cell populations with focus on different markers, mainly: S100, GFRalpha2, Prop1, Sox2, Sox9, Nestin, E-Cadherin and CXCL12/CXCR4. These proteins were proposed as pituitary stem cell markers, and their expression often overlaps within the same cell population.

One of the first approaches used to identify pituitary adult stem cells was based on cell cultures of dispersed mice intermediate and anterior pituitary lobes at low cell density. An experimental observation was that most cells failed to attach to the dish, but some formed small colonies (**Figure 2**). The cells within the colonies resembled folliculo-estellate cells (FS), which express S-100 (10). The presence of S-100+ or GFAP+ cells in the colonies was highly suggestive of FS-cells as a possible source for SC in the pituitary (31). Colony-forming ability of the cells also increased when sorting by intake of $\beta$-Ala-Lys AMCA, a fluorescent dipeptide derivative that FS-cells incorporate. AMCA+ cells were found scattered across the anterior pituitary, consistent with the reported location of FS-cells, but also importantly in the marginal epithelial cell layer, thus indicating that a different cell-type shared the ability to import AMCA with the FS-cells. Transplantation of these AMCA+ pituitary colony-forming cells into an *in-vivo* system demonstrated a capacity to differentiate into growth hormone (GH) producing cells (32).

Another set of colony-forming SC is the so-called GPS cells (GRFa2/Prop1/Stem) described by Garcia-Lavandeira et al. These cells exhibit a round morphology and form scattered cultures when grown directly on the plates but form more compact colonies when grown on a feeder layer (9). Continued amplification of undifferentiated GPS cells was achieved for several generations, demonstrating their robust self-renewal capacity, a necessary hallmark of stem cells. Interestingly, SOX2 and PROP1 are co-expressed in the pituitary of the mouse embryo and in adult pituitaries (33). PROP1 is a marker of precursors of all hormone-producing cells of the pituitary, and its role in epithelial-to-mesenchymal-like transition (EMT) has been demonstrated (1).

In this context, chemokines were recognized originally for their ability to dictate the migration and activation of bone marrow cells and many cell types, including T cells, dendritic cells, astrocytes, and endothelial cells (34). CXC chemokine ligand 12 (CXCL12, also known as stromal cell-derived factor-1) and its receptor CXCR4 are the first chemokine and receptor that have been shown to be critical for developmental processes, including the pituitary gland (35). Horiguchi et al. found that FS cells secreted CXCL12 and that the CXCL12/CXCR4 axis induces migration, due to chemoattraction, in the anterior pituitary in the formation of the FS cell network. CXCL12/CXCR4 interaction has also been described in human normal anterior pituitary, in both FS and hormone secreting cells (35, 36). They play an important role in GH production and secretion, and in the proliferation of somatotrophs.

Early in the search for pituitary stem cells Chen et al. showed that the primary culture of dispersed AP under neurosphere forming conditions gives rise to *"pituispheres"* (**Figure 2**). The mixing of dispersed APs from wild type and eGFP expressing

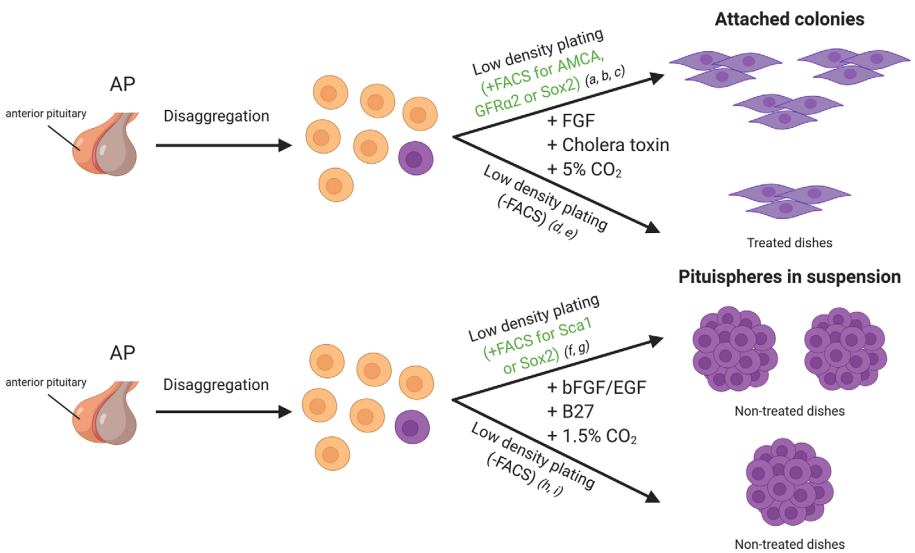


**FIGURE 2** | *In vitro* approaches for adult stem cell identification in the mice pituitary gland. Cells of the anterior lobe are dissociated into single cells, and they can be sorted by FACS based on specific characteristics or plated all together. Side population cells display verapamil-sensitive Hoechst dye efflux capacity and/or a stem cell marker such as SOX2, SCA1 and GFRa2. Cells can be seeded in dishes with medium enriched in growth factors (DMEM/Ham's F12 supplemented with growth factors). References listed correspond to the following articles: a) Lepore et al. (10), b) Andoniadou et al. (27), c) Garcia-Lavandeira et al. (9), d) Gleiberman et al. (28), e) Pérez Millán et al. (1), f) Chen et al. (29), g) Rizzoti et al. (3), h) Chen et al. (30), i) Fauquier et al. (5). FACS, Fluorescence-activated cell sorting.

mice resulted in either eGFP+ or non-fluorescent pituispheres. The absence of heterogeneous eGFP expression implied a clonal origin of the spheres (30). A high percentage of the side population described expressed SOX2 and SOX9, and this subset seemed to be responsible for the formation of the pituispheres described earlier. Strikingly, all pituitary hormones were detected after culturing them with differentiation media, while SOX2 expression faded (29). In some cases, multiple hormones were observed in the same sphere, further supporting the capacity of these cells to give rise to most of the hormonal lineages.

In mice and rats, SOX2+ cells were found distributed in two types of niches, the region lining the AP and the posterior pituitary, also known as the marginal cell layer, and in dense clusters in the parenchyma (5, 37, 38), able to self-renew and give rise to all hormone-producing cell types (3, 27, 39, 40). Further studies in the rat model identified an additional marker associated with the stem cell niche in this region, the Coxsackievirus and adenovirus receptor (CAR) (**Figure 1**). CAR-positive cells showed a similar histological distribution as that observed for SOX2 during development and postnatally, displayed an increased proliferation capacity evidenced by KI67 and expressed epithelial-to-mesenchymal markers like E-cadherin and Vimentin. CAR signals were not detected in the hormonal cell-lineage at any period (embryonic or postnatal), but co-localized with S100β-positive folliculo-estellate cells during adulthood (11).

A pool of SOX2+ cells was also shown to form pituispheres by Fauquier et al. The spheres initially displayed co-expression of SOX2 and E-cadherin, but not S100 or SOX9. However, after a week in culture, both later markers were detectable (5), supporting the hypothesis that FS-like cells observed in early colony formation assays were most likely progenitors and not "true" stem cells. Again, cells from these spheres could be differentiated into all hormone producing cells *in vitro*. Interestingly SOX9+ cells, as well as SOX2+ cells, can form pituispheres, since FACS sorting for these markers from either transgenic mice $Sox2^{EGFP/+}$ or $Sox9^{iresGFP/iresGFP}$ APs produced fluorescent pituispheres (3).

To this day there are multiple important unanswered questions about the pituitary gland development and maintenance during adult life, in particular regarding the participation of embryonic and adult stem cell population in this process. This is due to the limitations of the *in vitro* models. In 2019 Cox et al. established a protocol to generate organoids from pituitary tissue, a great contribution to the *in vitro* experimental models that allows to study the characteristics of pituitary stem cells and the foundations of pituitary organogenesis (41).

These experiments have therefore clearly demonstrated that adult SCs are multipotent, although the essential factors required for multipotency remain unclear due to the heterogeneity of cultured primary pituitary stem cells. Fortunately, the development of human induced pluripotent stem cell-derived pituitary organoids has allowed further dissection of key regulators of stemness, as discussed in the following section.

## PITUITARY STEM CELLS IN REGENERATIVE MEDICINE

The pluripotent state of the stem cells allows them to differentiate into several other cell types, both *in vivo* and *in vitro*. However, the origin of these stem cells is embryonic, which raises some ethical issues regarding their harvest and use. In this context, Takahashi et al. first developed in 2007 a protocol for reprogramming somatic human cells into an induced pluripotent status by retroviral transduction of Oct3/4, Sox2, c-Myc and Klf4. The iPSCs (induced Pluripotent Stem Cells), obtained from human dermal fibroblasts, resembled human embryonic stem cells in morphology and gene expression patterns, and were able to differentiate into other cell types (42). This first work opened the door for generating patient and disease specific stem cells for research in pathophysiology pathways and personalized treatments. In the following years, other methods using episomal vectors were also established for obtaining iPSC without relying on retroviral/lentiviral vectors (43, 44). This approach enabled the generation of patient specific iPSC without the integration of exogenous DNA, avoiding mutations by DNA integration, therefore bringing us a step closer to the creation of clinical grade iPSCs.

Suga et al. 2011 designed a protocol to differentiate ACTH producing cells from mice stem cells. Briefly, cell aggregations of stem cells were differentiated by incubations with BMP4 into a three-dimensional sphere of hypothalamic tissue cells surrounded by oral ectoderm cells (Pitx1/2+). Upon activation of the Shh pathway using *smoothened* agonist (SAG), some of these oral ectoderm cells began to express Lhx3 and invaginated into the cell aggregate, simulating Rathke's Pouch development. These Lhx3+ cells later differentiated into all hormone producing cell lineages, and upon addition of Notch inhibitor DAPT, a greater proportion of ACTH producing cells was obtained. Ectopically transplanted cells into the kidney subcapsule of hypophysectomized mice, produced higher ACTH blood levels, spontaneous locomotor activities and longer overall survival in comparison to non-transplanted mice. A similar protocol was then performed for differentiating pituitary cells from human embryonic stem cells (45).

A major caveat in regenerative medicine is that human endocrine cells derived from iPSCs often do not fully recapitulate the functioning levels of normal tissues (46). However, using the previous approach for obtaining ACTH producing cells, Kasai et al. 2020, demonstrated that these cells compared favorably with adult mice adenohypophysis cells, showing that the combined differentiation of pituitary and hypothalamic cells recapitulates the hormonal crosstalk between the two structures (47).

Attempts for obtaining pituitary cells in 2D cultures were also developed. A combination of BMP4, SHH, FGF8, and FGF10 stimuli was used to obtain hormone producing cells in monolayer stem cells in shorter times than 3D cultures; however, this approach relies on cell sorting based on GFP expression coupled to SIX1, challenging the process to adapt this protocol into obtaining pituitary cells from patients' iPSCs (48, 49).

The use of human cell lines, especially human SC, allows for an expansion in modeling human disease, particularly when there is a lack of an appropriate animal model or when the somatic cell types involved in the disease are difficult to isolate or grow in culture (50). Human-derived pituitary cells differentiated from iPSCs have been used for understanding mechanisms of human pathology such as autoimmune hypopituitarism by anti-Pit-1 antibodies (51).

Mutations in genes like *PROP1, LHX3, LHX4, HESX1, POU1F1, GLI2*, and *OTX2* are found in patients with combined pituitary hormone deficiency. Many genetic variants found in rare diseases are novel, and found in sporadic cases, where it is not possible to show variant segregation along the family, so most of these variants remain to be of uncertain significance in relation to the pathology. Functional testing of these variants is vital then to assess pathogenicity. Matsumoto et al. 2020, used iPSCs derived from patients' lymphocytes to functionally study a heterozygous variant in *OTX2*. They showed that the patient derived iPSCs were incapable of differentiating into hormone producing cells in contrast to control iPSCs. However, correcting this mutation by CRISPR/Cas-9 methodology modified the capacity of the iPSCs to fully differentiate into pituitary hormonal cells, validating that this variant was the one causing the hormone deficiency (52).

Finally, patient-specific iPSCs could be used for making therapeutic decisions in the context of personalized or precision medicine. Current treatments for hormone deficiencies or pituitary dysfunction mostly rely on recombinant hormone replacement. Though this has significantly reduced patient morbidity and mortality, it still presents some limitations, as drug administration cannot precisely recapitulate the hormonal changes by circadian and/or stress-induced requirements. This is mostly because the healthy pituitary responds to positive and negative regulatory feedback which regulates the amount of secreted hormones, which exogenous hormone replacement cannot address. Regenerative medicine, mostly involves the transplant of differentiated stem cells to correct the genetic or cell defects in patients, thus is a promising future treatment approach. On the other hand, patient derived iPSCs could be used to test pharmacological treatments for correcting the disease or phenotype observed (50).

However, these approaches mentioned before for obtaining pituitary cells in both 2D and 3D cultures still need to be perfected for use broadly in the clinical setting. The main setback with both techniques is the inability to generate the specific hormone producing cells required to combat an individual patient's pituitary hormone deficiency. Both approaches are fairly good in differentiating ACTH cells, with the other cell types varying in proportion. Gonadotropes are especially difficult to differentiate as they are often found in low frequency. In 2D culture, different proportions of BMP2 and FGF8 added to the medium can promote the change in proportion of pituitary cells. High BMP2 and low FGF8 promotes LH and FSH cells; equal concentration of both BMP2 and FGF8 has a greater proportion of GH and TSH producing cells, while high FGF8 and low BMP2 stimulate POMC+ cells (49). However, the exact *in vitro* conditions to produce a specific pituitary hormone expressing cell are still underdeveloped. We need a higher insight into the molecular mechanisms that regulate pituitary development. These development-in-a-dish models have already helped us understand some of the factors involved in the process of human pituitary development, so they are sure to help us move closer into that objective.

## CONCLUSION AND PERSPECTIVES

In summary, several studies support the existence of multipotent stem cells in the adult pituitary from fish to human. Even if not all these studies arrive to the same conclusions, several features, including Sox2 expression, characterization of the side population nature, and the marginal zone configuration, seem to characterize the phenotype of pituitary stem cells. We believe that further characterization of pituitary stem cells could allow a better understanding of the biological basis of some pituitary pathologies including hypopituitarism and adenoma development. While many obstacles remain to be overcome, we envision that the therapeutic potential of iPSCs will attract further research investments in this area to resolve the challenges and allow the identification of novel targets for disease treatments.

## AUTHOR CONTRIBUTIONS

All authors planned and wrote the paper. MC, JM, and SV designed the figures. All authors contributed to the article and approved the submitted version.

## FUNDING

PICT 2017-0002 to MP-M. PICT 2018-4239 to MIPM.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 27 January 2021
doi: 10.3389/fendo.2020.619352

# Functional Pituitary Networks in Vertebrates

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The pituitary is a master endocrine gland that developed early in vertebrate evolution and therefore exists in all modern vertebrate classes. The last decade has transformed our view of this key organ. Traditionally, the pituitary has been viewed as a randomly organized collection of cells that respond to hypothalamic stimuli by secreting their content. However, recent studies have established that pituitary cells are organized in tightly wired large-scale networks that communicate with each other in both homo and heterotypic manners, allowing the gland to quickly adapt to changing physiological demands. These networks functionally decode and integrate the hypothalamic and systemic stimuli and serve to optimize the pituitary output into the generation of physiologically meaningful hormone pulses. The development of 3D imaging methods and transgenic models have allowed us to expand the research of functional pituitary networks into several vertebrate classes. Here we review the establishment of pituitary cell networks throughout vertebrate evolution and highlight the main perspectives and future directions needed to decipher the way by which pituitary networks serve to generate hormone pulses in vertebrates.

**Keywords: pituitary, networks, plasticity, vertebrates, evolution**

OPEN ACCESS

***Edited by:***
*Romain Fontaine, Norwegian University of Life Sciences, Norway*

***Reviewed by:***
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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *20 October 2020*
***Accepted:*** *07 December 2020*
***Published:*** *27 January 2021*

***Citation:***
*Santiago-Andres Y, Golan M and Fiordelisio T (2021) Functional Pituitary Networks in Vertebrates. Front. Endocrinol. 11:619352. doi: 10.3389/fendo.2020.619352*

## INTRODUCTION

Biological variation is a defining characteristic in the process of species evolution and adaptation. Variation in morphology, physiology, or behavior within species is a prerequisite for evolutionary processes, like natural selection, to occur. In an ever-changing natural world, the organisms able to respond more efficiently to those changes are better fitted to their environment and could be selected (1). In this context, highly plastic systems are also adaptive since the information from both external and internal environments is better integrated. An example of such a highly plastic system is the pituitary gland, which secretes several key hormones that maintain homeostasis in all vertebrates (2).

The pituitary gland is embryonically derived from Rathke's pouch and is therefore considered to be of ectodermal origin (3, 4), although at least in fish, a small percentage of pituitary endocrine cells have endodermal origins (5). The pituitary of all vertebrates consists of three anatomically and developmentally distinct structures called the neurohypophysis or posterior pituitary, the adenohypophysis, or anterior lobe and the intermediate lobe, all of which function in close interdependence with the hypothalamus. The neurohypophysis comprises the neural lobe

(mainly oxytocin- and vasopressin-secreting neuroendocrine terminals in mammals and their homologous isotocin and vasotocin in non-mammalian vertebrates), the median eminence (the functional link between the hypothalamus and the anterior lobe), and the infundibulum. The intermediate lobe is mainly composed of melanotrophs that regulate skin pigmentation. The anterior lobe of the pituitary, which is the main focus of this review, is populated by five types of secretory cells: somatotrophs, gonadotrophs, lactotrophs, thyrotrophs, and corticotrophs, that may be identified by their hormone composition and their response to hypothalamic neurohormones. In mammals, these cell types follow a similar pattern of stimulus-secretion coupling that regulates the neuroendocrine axes and allows the preservation of homeostasis (6).

In all vertebrates, the main driver of pituitary secretion is the hypothalamic input. Factors released from hypothalamic neuron terminals into the pituitary portal system bind to their cognate receptors on target pituitary cells and elicit a signaling cascade that induces release (or inhibition) of hormone secretion from the pituitary (7) into the vascular system in a pulsatile manner, the pattern of which is critical in regulating the activity of the downstream target organs (2). Yet the individual cellular dynamics *in vitro* do not recapitulate the whole gland pulse generation needed for physiological actions. For example, *in vivo*, GH-secreting cells produce massive GH pulses (up to 1,000-fold rise) in response to physiological cues (8, 9), whereas these pulses are significantly weaker *in vitro* (10). The same dependence of hormone pulse generation on the tissue context has been observed in gonadotrophs (11–14), corticotrophs (15–17), and lactotrophs (18, 19). Thus, in order to generate this fine-tuned and coordinated secretion, a large-scale communication system must exist within the anterior pituitary that is critical for the ability to generate hormone surges.

In recent years, the use of high resolution imaging in combination with large-scale cell activity recordings has revealed the homotypic and heterotypic network arrangement of the mammalian pituitary cells (i.e., intercellular connectivity between the same secretory cell type or between different cell types, respectively). Research in this field has pointed out the relevance of the network organization in coordinating the synchronized and rhythmic secretion of hormones (16, 20). Intriguingly, the existence of cell networks has been identified in all major groups of vertebrates. As an example, in fish, which represent the most basal group of vertebrates, when the gonadotroph network connectivity is disrupted, the hormone secretion is compromised, as well as its biological significance (21, 22). It can be concluded from these facts that a) pulse generation is achieved thanks to cell networks, b) the importance of the pulse mode of secretion is revealed by studies in which its production is limited, and is followed by physiological perturbations in the target organs of regulation, and c) cell network formation, which is essential for the organization of these pulses, is conserved throughout vertebrate evolution (2).

Despite the clear importance of pituitary homotypic and heterotypic networks in the coordination of hormone pulsatility, research into this subject has been limited. Here, we review the current knowledge and methodological approaches to assess the function of pituitary networks throughout vertebrate evolution. The mechanisms involved in pulse generation, as an adaptive and plastic process that promotes the rhythmic secretion of hormones in vertebrates, will be discussed from a cell network perspective. We then explore the evolution of these networks in different groups of vertebrates, from fish to mammals and discuss the importance of the cellular networks in the phenotypic plasticity. We conclude that a comparative approach to the study of pituitary networks is fundamental to the comprehension of the system both under normal and pathological conditions. It is important to clarify that factors such as the well-established hypothalamic drive, the peripheral tissues and the environment are fundamental in the generation of hormone pulses and should be integrated in order to understand the system as a whole. However, the pituitary gland has its own local and plastic modes of autonomic regulation and its interaction with the hypothalamus is a complex two-ways regulation.

## HYPOTHALAMIC CONTROL OF THE PITUITARY GLAND IN VERTEBRATES

Over the course of vertebrate evolution, the general morphology of the pituitary gland has remained constant, with three main lobes whose function depends on the integration of signals originating from hypothalamic nuclei and from the systemic circulation (23). While a general anatomic plan is observed in the pituitary gland of all vertebrates (**Figure 1**), the specific arrangement of the endocrine cells (**Figure 2**), as well as the way by which their communication with the brain is executed, show great variation across taxa.

Teleost pituitaries in particular exhibit an important deviation from the masterplan of the tetrapod pituitary in respect to the anatomy of the hypothalamus-pituitary interface. Studies of fish pituitaries found that in ancient forms such as cartilaginous fish, sturgeons, and gars the hypothalamic fibers terminate and release their content onto the basal membrane separating the neurohypophysis from the adenohypophysis. The secreted hypothalamic factors then diffuse through the connective tissue and are uptaken by the pituitary vascular system that delivers them to their pituitary target cells (24). This neuro-vascular mode of delivery was perpetuated in the tetrapod evolution and is the dominant mode by which the hypothalamus exerts its effect on pituitary cells in all known tetrapods (24) (see **Figure 1**). However, studies of teleost pituitaries revealed a unique characteristic that was not found in any other vertebrate: hypothalamic fibers crossed the basement membrane that separates the neuronal tract from the adenohypophysis and formed terminals adjacent to endocrine cells (see teleost fish in **Figure 3**) (25–31). This unique characteristic drew so much attention, that a direct, neuroglandular mode of regulation was considered the main mode operating in teleosts.

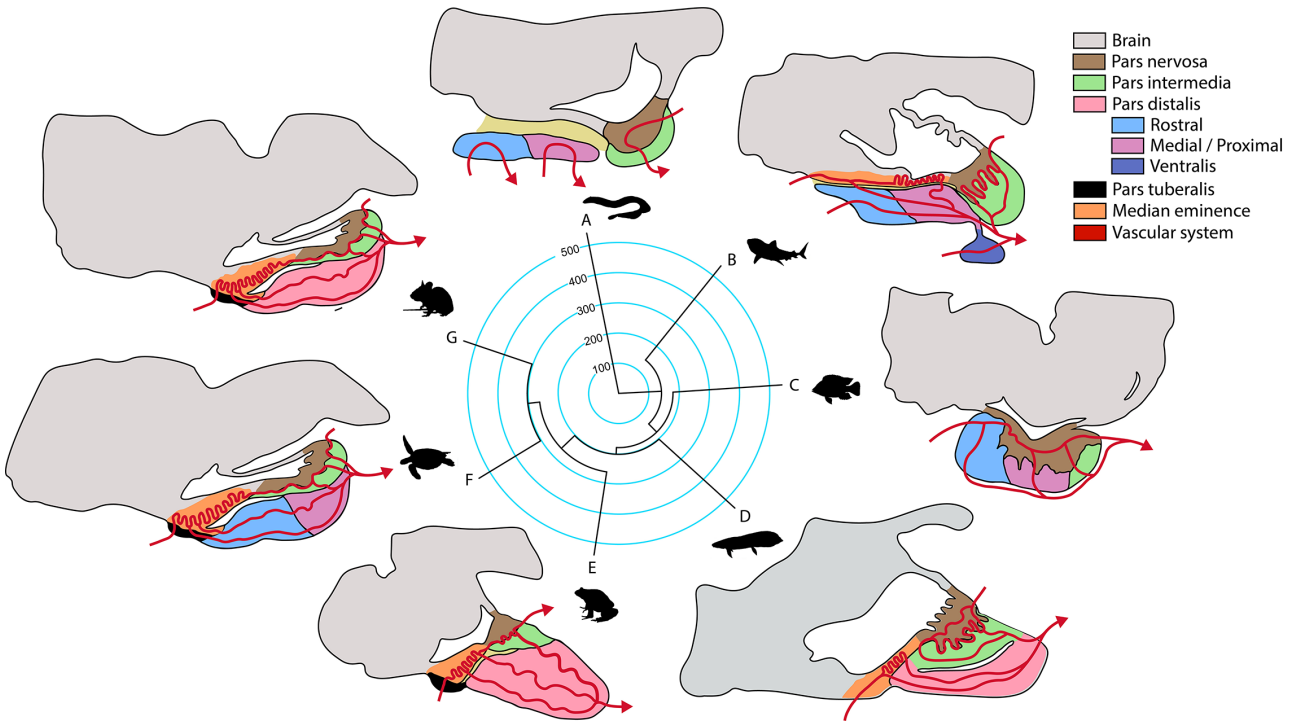


**FIGURE 1** | Phylogeny of the principal groups of vertebrates and anatomical pituitary organization (anterior-caudal-dorso-ventral orientation). Terminal branches of the phylogenetic tree show a representative organism of each clade (**A**, jawless fish; **B**, cartilaginous fish; **C**, ray-finned fish; **D**, lungfish; **E**, amphibian; **F**, reptile that includes birds; **G**, mammal) and their sagittal representation of the main structures in the pituitary gland according to (23). Median divergence times from the TimeTree database were used to construct the tree, numbers in the phylogeny are million years.

The development of transgenic fish with labeling of three components of the HP axis (GnRH3 cells, gonadotropes and blood vessels) enabled researchers to re-visit the existing dogma. Upon the examination of the GnRH tracts in pituitaries labeled for gonadotropes it became clear that the direct neuroglandular mode of regulation cannot be considered as the dominant route of gonadotrope regulation. This is because not all gonadotropes are contacted by GnRH terminals and because the vast majority of these terminals are not in the vicinity of the gonadotropes (32). By using transgenic fish with labeled blood vessels it became apparent that the GnRH terminals are in much closer association with the pituitary afferent vessels than with the gonadotropes (see the teleost fish in **Figures 3** and **5**). This organization strongly suggests that, at least in zebrafish, GnRH signals reach their pituitary targets through the blood or through diffusion rather than *via* direct neuro-glandular contacts. A dominant neuro-vascular interaction is also observed in other species and other hypothalamus-pituitary axes (31–36).

The distinction between a direct, neuroglandular mode of regulation *versus* an indirect, neuro-hemal mode of hypothalamic factor delivery has important consequences over the functional physiology of the hypothalamic pituitary axis. A direct, neuroglandular mode of communication would require considerably smaller concentrations of hypothalamic factors to be secreted since the secretagogues will be delivered directly to the target cell. Such a direct communication mode may also affect the timescale of the regulation as a very temporally precise signal could be delivered to the target cells resembling the timescales of communication between neurons. Moreover, if the anatomical interactions between terminals and cells are very precise, it would make the variety of hypophysiotropic signals largely redundant as the specificity of the regulation could be achieved by controlling the type of neuron contacting a specific pituitary cell type rather than by different secretagogues. To date, there is no evidence to suggest that teleost pituitaries differ in any of these aspects from the more widely studied mammalian pituitary.

While it is admittedly difficult to evaluate the functional contribution of the different delivery modes, at least from a quantitative point of view, the direct, synaptic-like contacts between terminals and their pituitary targets seem to play a less important role than previously thought in the regulation of pituitary secretion. Instead, diffusion from terminals through the pituitary tissue to the target cells (either passive, or, like in tetrapods, assisted by local blood flow), appears to be the dominant way by which pituitary endocrine cells receive their hypothalamic signals. Therefore, the anterior neurohypophysis of teleosts may act as a functional median eminence that is embedded into the pituitary in teleosts. The evolution of the hypothalamo-pituitary axis has recently been thoroughly reviewed by Trudeau and Somoza (37) and interested readers are encouraged to refer to this excellent review for more detail.

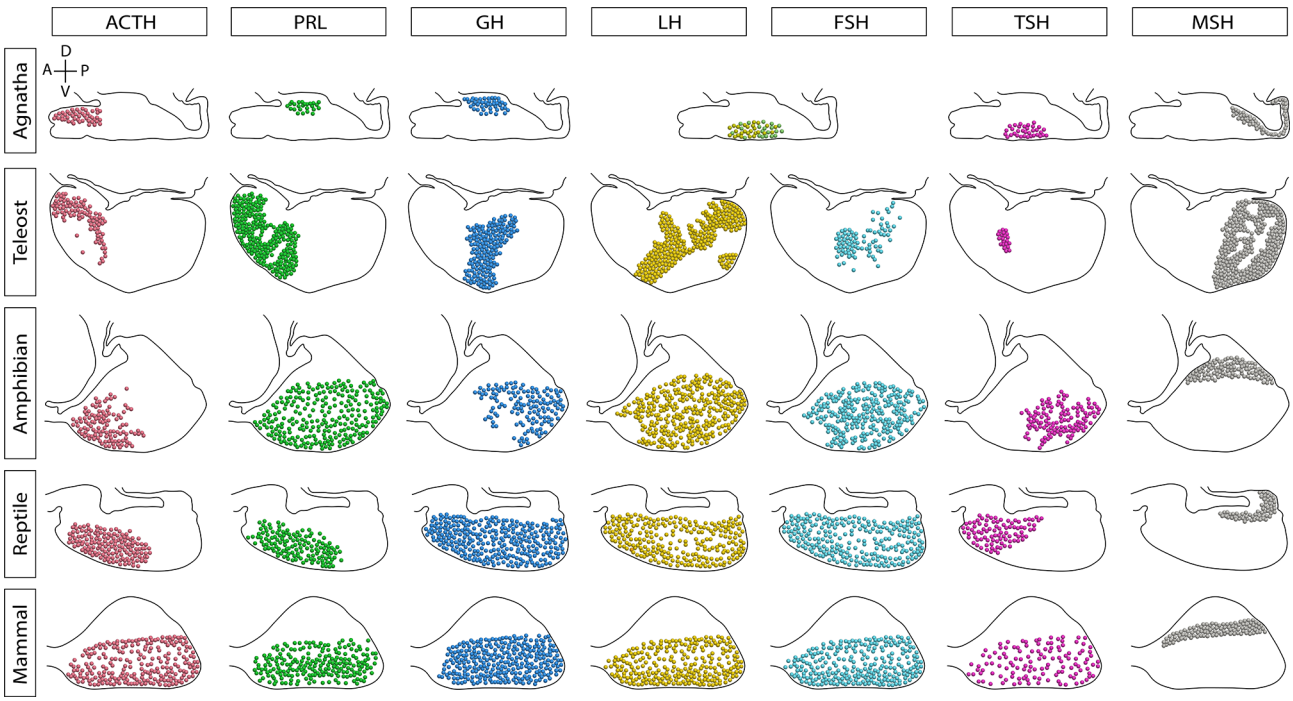


**FIGURE 2** | Sagittal representation of the endocrine cells distribution in the pituitary gland of vertebrates. The distribution of cell types has been modified over the evolution of vertebrates. In agnathans and teleosts, here represented by the sea lamprey and Nile tilapia, respectively, cells are markedly regionalized. Amphibians and reptiles present a regionalized distribution of some cell types, but others are scattered through the pars distalis. In contrast, the mammalian secreting cells are widely scattered throughout the lobe. In lampreys there is only one cell type producing glycoprotein hormone (GPH, a gonadotropin homologue) and thyrostimulin (TSN, a TSH homologue). Additionally, teleost fish possess a unique cell type that produces somatolactin, the somato-lactotroph, not shown. A, anterior or rostral; P, posterior or caudal; D, dorsal; V, ventral.

In the next section we briefly cover the principal mechanisms involved in the maintenance of homotypic and heterotypic cellular networks in the pituitary, by means of cell-cell contacts or paracrine communication. Those mechanisms are present in all vertebrates but their contribution may vary depending on the communication with the hypothalamus, vascular arrangement of the pars distalis, distribution of cell types in the gland (regionalized or scattered), and formation of heterotypic networks (see **Figures 1** and **2**).

## MODES OF COMMUNICATION

### Gap Junctions

Gap-junctions (GJ) are transmembrane channels that allow the free cell-to-cell exchange of cytoplasmic molecules of <1,000 daltons molecular weight. The intercellular communication through GJ is an important source in the transport of cytoplasmic constituents (including water and ions), metabolic substrates (such as sugars, amino acids, nucleotides), and second messengers (mainly calcium, $IP_3$, and cAMP) between cells. In the pituitary gland, gap junctions are an important mode of communications in all studied vertebrates. For example, gap junctions have been shown to couple fish pituitary cells (38, 39). In the rat, cells show a high level of intercellular coupling through gap junctions, where lucifer yellow diffusion was found up to 300-microns away from its site of injection (40). In addition, the transport between cells was primarily in lactotrophs, somatotrophs and folliculostellate cells (FS cells). Only a few LH, TSH, and ACTH cells were labeled (40). In all vertebrates gap junctions are considered a critical component in the coupling of pituitary cells and allow a coherent transmission of signals and the formation of cell networks (2, 19, 22, 41–48).

In terms of cell activity, Guérineau and collaborators have demonstrated the fundamental role of gap junctions in the synchronization of electrical activity (49). By multicellular measurement of spontaneous intracellular calcium mobilization in tissue preparations, the authors found the presence of clusters of pituitary cells that were in close proximity to each other and rhythmically coactive. As small molecules (Lucifer yellow, 457 Da), but not large molecules (Texas Red, 3 000 Da) spread between the co-active cells, in combination with the observation that a gap-junction blocker reduces the spread of synchronization, it was concluded that coordination between neighboring secretory cells is mediated by gap junctions. Of particular importance, the connected cells were principally of the same phenotype but not all the adjoining cells from the same phenotype were coupled. This fact indicated that the global response of secretory cells during hypothalamic stimulation involves, as we shall see, other mechanisms of cell communication that are not mutually exclusive.

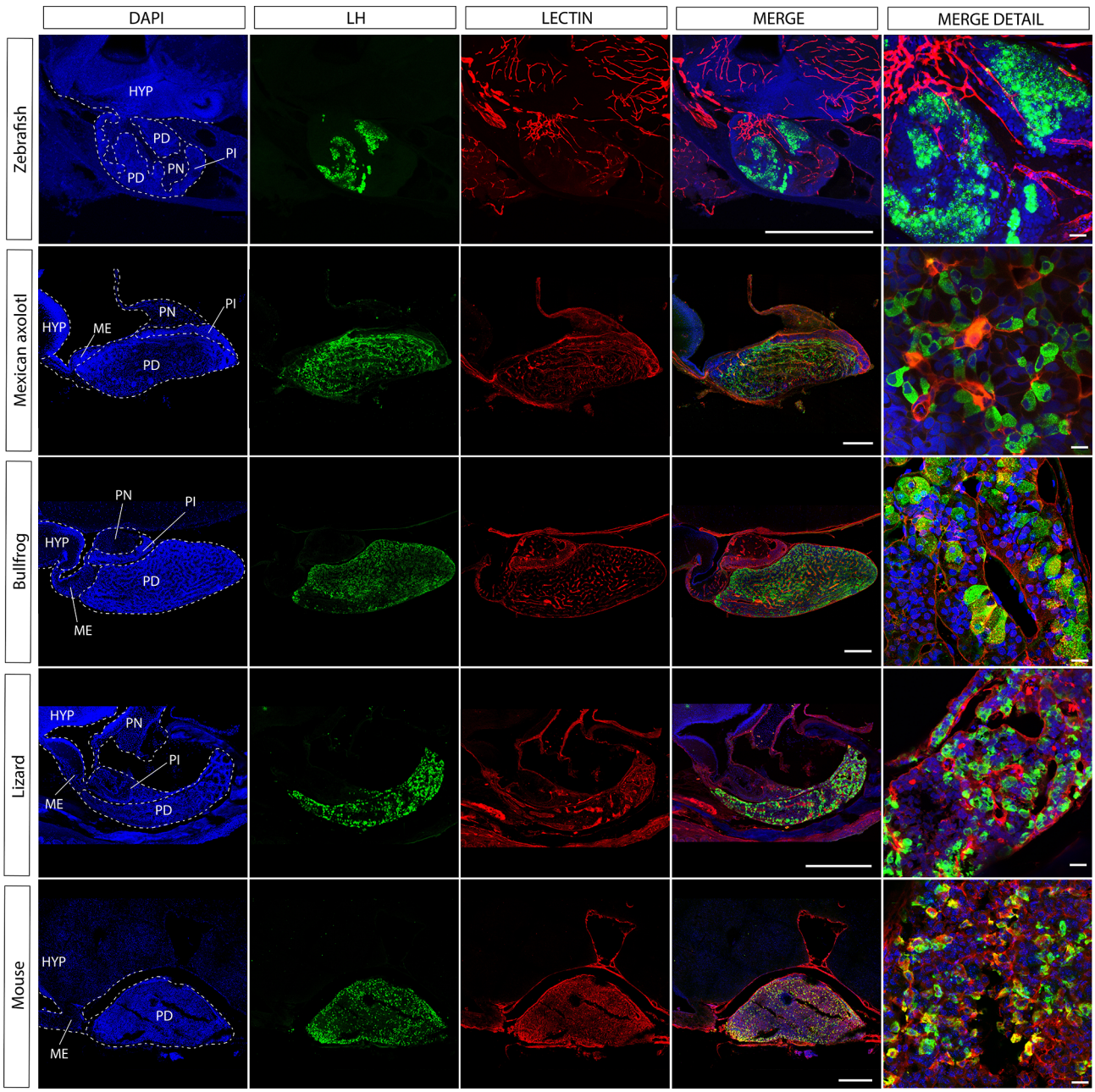


**FIGURE 3** | The gonadotroph network and the relation with the hypophyseal portal system in vertebrates. Midsagittal view of the pituitary gland in relation to the hypothalamus (anterior at the left, dorsal to the top) using confocal microscopy. Shown are immunostainings for LHβ (green) in five different species of vertebrates (zebra fish, *Danio rerio*; axolotl, *Ambystoma mexicanum*; bullfrog, *Lithobates catesbeianus*; lizard, *Sceloporus aeneus*; mouse, *Mus musculus*). In contrast to tetrapods, where LH-containing gonadotrophs are well distributed in the pars distalis, in teleost these cells are confined to the rostral pars distalis. The merge detail column is a power magnification of the precedent, showing that gonadotrophs establish cell-cell contact to form an interdigitated network and reveal that this pattern of cell organization is extended to all vertebrates. Note that the vascular network pervades the pars distalis and gonadotrophs form process to the capillaries (red, lectin-rhodamine), with the exception of zebrafish. DAPI counterstain is shown in blue. Dashed lines mark the three main regions of the pituitary. HYP, hypothalamus; PD, pars distalis; PI, pars intermedia; PN, pars nervosa; ME, median eminence. Scale bars represent 400 μm in whole-gland views and 20 μm for merge detail.

Changes in gap junction expression patterns have been linked to plasticity in the pituitary gland. Modifications in connexins 43 and 26 expression (subunits of gap junctions), have been reported in rodents, following physiological challenges and aging (50–54). For example, GJ expression is modified according to the stage of the estrous cycle, probably reflecting the plasticity of cell connectivity during the generation of hormone pulses. Indeed, other authors have reported that

estrogens play an important role in their regulation, since the absence of testosterone and estradiol downregulate the number of gap junctions by a quarter or more compared to intact rats (46, 55–57). Other experiments show that factors such as hydrocortisone, have a suppressive effect on the GJ formation between FS cells and between endocrine pituitary cells (58).

## Folliculostellate Cells

A population of glial-like cells, the FS cell network, is present in the pituitary gland of all major taxa of living vertebrates, and in mammals these non-endocrine cells comprise about 5–10% of the anterior pituitary cell mass (39, 59–62). These agranular cells have a characteristic stellate shape with long cytoplasmic processes, and arranged as a 3D anatomical network that extends over the whole gland in mammals (48, 53), aves (62), amphibians (63), and in fish (39). FS cells respond to a large variety of external and internal stimuli and produce growth factors and cytokines, such as fibroblast growth factor (FGF)-2, vascular endothelial growth factor (VEGF)-A, follistatin, and IL-6, all of which potentially have paracrine effects and regulate the activity of neighboring hormone-secreting pituitary cells (64).

The FS cell network has been suggested to wire the endocrine cells of the pituitary by relaying relevant signals throughout the gland. Secretory cells in the pituitary are intertwined through the vasculature and the FS network. The wave propagation of ions as $Ca^{2+}$, and small molecules as $IP_3$ or cAMP, through GJ within the FS cell network, allow synchronized excitability within the anterior pituitary gland (48, 65). Although, the distance and velocity in which the information can propagate to parenchymal cells has not been investigated yet, there is evidence supporting that the anterior pituitary possesses an intrinsic system of communication, that allows long-distance transmission of information within its parenchyma (66, 67). FS cells may have a central role in this process due to their gap-junction mediated network properties, and the expression of a large variety of signaling molecules. Hence, distant endocrine cells might receive coordinated information from both systemic molecules through the vasculature and paracrine signals primarily coming from the FS-cell network (48).

In mammals, it has been reported that FS cells can generate different responses in groups of endocrine cells surrounded by their cytoplasmic processes, thus forming functional clusters (66–68). Interestingly, it has been observed that GnRH-neuron projections converge not only at the blood capillaries, they are also adjacent to FS cells in the pars tuberalis (68, 69). Still, gap junctions between the GnRH-neuron terminals and FS cells, as well as GnRH-induced calcium response in FS cells have been recognized (69–71). In this sense, in addition to the vascular system, cell signaling in the FS network generated by hypothalamic stimuli, may be a route to propagate signals throughout the pituitary (66–69, 72, 73). In this respect, the unique anatomy of the teleost pituitary may provide interesting insights, since stellate cells have been reported to form dense networks around the neurohypophysis that penetrates deep into the parenchyma of the adenohypophysis (39). As a hypothesis to be proved, some endocrine cells may receive the signals only from the pituitary portal system and others could additionally integrate signals propagated by the FS-cell network, according to their distribution in the pituitary (70) (see **Figure 2**).

## Paracrine Communication

Paracrinicity is the process of short-distance communication between cells by substances released within the same tissue. The substance reaches its target by diffusion in the extracellular space or by direct contact formation (juxtacrine factors). More than 100 compounds in the pituitary have been identified as paracrine or autocrine factors (64). They include the neurotransmitters, growth factors, cytokines tissue factors such as annexin-1 and follistatin, hormones, ATP, NO, among others (64). The majority of paracrine or juxtacrine molecules, act through G-protein coupled receptors, with inhibitory (Gi) or excitatory (Gs) action, activating in most cases voltage gated channels activity and intracellular $[Ca^{2+}]$, as well as a whole host of other receptor types capable of affecting down-stream kinase and protein phosphorylation status (74). Paracrine signaling is a slow diffusion process, which occurs over seconds to minutes. This process of molecular diffusion is one of the main scenarios for possible organization and communication presumably sufficient to account for coordinated endocrine cell population activity and surge hormone output (74). However, this diffusion process seems to lack the directionality that would allow the formation of homo- or heterotypical networks, unless evidence is found to show that it could be a process directed through, for example, the interaction of molecules with the extracellular matrix. A more spatially precise intercellular signaling may be accomplished in the form of juxtacrine factors, as annexin 1 (formerly lipocortin-1), a phospholipid-binding protein which is predominantly expressed by the FS network (74).

In mammals, an extended review by Denef summarizes the paracrine interactions in the pituitary gland (64). The first evidence of the possible paracrine relationship between endocrine cells was established from gonadotrophs and lactotrophs, where the stimulation of gonadotrophs secretion with gonadotropin-releasing hormone (GnRH) triggers prolactin secretion (PRL) (75). More complex relationships were established between gonadotrophs and somatotrophs, in which it was observed that in aggregates of these two cell types, the GnRH stimulation produce an initial inhibition of GH release, whereas after GnRH stimulation, a rapid rebound secretion of GH was observed, suggesting that GnRH had both inhibitory and stimulatory actions on GH release (76). Gonadotrophs have a large number of secreted peptides who are potential candidates for a paracrine action. Some of these peptides modulate corticotroph activity when added exogenously to pituitary cell preparations (64). An inverse relation between lactotrophs and gonadotrophs was also established by the inhibition of LH secretion in hyperprolactinemia condition (77).

Other possible paracrine interactions imply coordination between different pituitary endocrine cells. For instance, the hypothalamic-pituitary-adrenal axis that is activated during stress. In this condition, there is a need to increase the metabolic rate in order to maintain body temperature, and this

process is regulated by the hypothalamic-pituitary-thyroid axis (78). Further example of crosstalk with the HPT axis is during pregnancy and lactation, in which energy consumption has to be adapted. The mechanisms involved in the regulation of these homeostatic changes are still largely unknown but paracrine interactions are likely to be involved in the heterotypic network communication (64).

In fish, paracrine interactions between pituitary cells have been shown to comprise an important regulatory layer of pituitary cell function and probably operate at a slower timescale than gap-junction mediated transmission. Perhaps one of the most interesting aspects of this communication mode in fish is the interplay between the growth and reproductive axis through the paracrine effects of LH on GH cells. Studies in grass carp have shown that both GH and LH have a stimulatory effect on GH expression and release whereas GH has an inhibitory effect on LH release (79). Cross communication between the growth and reproductive axes in fish is also achieved *via* the activin/inhibin/follistatin pathway as these genes are expressed in the pituitary and have known effects on gonadotropin expression (80–83) and the GH-LH interaction (84). The expression and secretion of somatolactin (SL), a member of the GH/prolactin family, has been shown to be autoregulated by the two SL isoforms (85, 86) as well as by kisspeptin (87), a hypothalamic hormone that in fish is also produced in the pituitary gland (88). Other proposed autocrine and paracrine effects between different axes in the fish pituitary have been proposed such as the effect of NKB produced in gonadotropes (89) on lactotrophs and somato-lactotrophs (90). Secretoneurin-a, a potent stimulator of gonadotropes in goldfish is produced in lactotrophs of the rostral pars distalis and may link the two axes *via* paracrine effects on the gonadotropes of the proximal pars distalis (91). Recent findings in long-term culture adult medaka female pituitary, showed that, Fsh cells which do not possess Gnrh receptors start to express them, allowing Fsh cells to produce Lh. These observations suggest that *in vivo*, a paracrine signal inhibits Gnrh receptor expression in Fsh cells and maintains their mono-hormonal identity (21, 35, 92).

It is also possible that paracrine factors not just mediate homotypic and heterotypic network formation, but also could have a priming effect on axis function because in PRL cells, the increased organization associated with lactation persists for months after weaning and leads to enhanced function (93). Despite a large body of evidence, we are still far from understanding the full effect of paracrinicity on network formation, because it is technically difficult to isolate this process from others such as communication between GJ or electrical conduction in FS cells.

## FUNCTIONAL PITUITARY NETWORKS IN VERTEBRATES

### Fish

One of the unique features of the fish pituitary is a high level of regionalization in the distribution of endocrine cells [(37, 94) and **Figures 2** and **3**]. This compartmentalization of the cells results in homotypic clusters of cells that favor direct cell-cell interactions and the formation of homotypic cell networks. Yet despite this unique topology, the study of cell networks in fish pituitaries has lagged behind that of mammals. The first evidence for direct cell-cell communication in fish pituitaries was the discovery of electrotonic cell coupling in the tilapia pituitary (38). Using double-patch electrophysiology on live pituitary slices, this groundbreaking work has shown for the first time the existence of gap-junction mediated communication between teleost pituitary cells. However, at the time, the type of coupled cells was unknown. Since then, many of the new insights regarding pituitary networks in fish were drawn from three model species: the zebrafish (*Danio rerio*), the Japanese medaka (*Oryzias latipes*), and Nile tilapia (*Oreochromis niloticus*). In these species, the generation of transgenic fish with labeled pituitary cells (35, 95–98) was an important turning point as it provided the opportunity to study topological cells networks in 3D and opened the way to functional studies of cell-cell interactions. Interestingly, in all three species, the main study of pituitary networks has concentrated on the gonadotrope cells of the anterior pituitary, leaving future studies to focus on other cell types. One of the reasons for this specific focus on gonadotropes stems from the fact that in fish, as opposed to tetrapods, LH and FSH are secreted from discreet cell populations rather than from a single cell type. This feature makes teleosts a particularly attractive model to study the differential regulation of LH and FSH secretion.

Studies in these three teleost species confirmed the existence of functional gonadotrope networks in fish. In tilapia and zebrafish, we have shown that LH cells are connected by gap junctions (22). In medaka, Karigo and co-authors used calcium imaging to show that in the intact gland LH cells exhibit a high level of synchronization in their activity (98) although the mode by which the cells communicate was not investigated. More recently, working with pituitary slices from medaka, Hodne *et al.* have used precise calcium uncaging in individual LH cells to show that the calcium signal can propagate to neighboring cells in a both homo-and heterotypic manner, leading to the conclusion that LH cells act as a relay for GnRH signals to FSH cells (35). The importance of the functional coupling has been revealed by its effect on hormone secretion from tilapia pituitaries. Application of gap junction blockers decreased GnRH-induced LH release from perfused pituitary fragments seven-fold (22), underscoring the role of the network in mounting an efficient response to hypothalamic stimuli. Interestingly, while tilapia LH cell output is highly attenuated by application of gap-junction blockers, FSH release remains largely unaffected. In medaka, calcium activity of FSH cells also seems to be less synched compared to their LH counterparts suggesting that the lower level of coupling between FSH cells may be a common feature of fish FSH gonadotropes. The notion that FSH cells act individually while LH cells mount coordinated responses is also evident in their secretion patterns since the induction of gonadotropin secretion *in vivo* causes a considerably stronger fold increase over basal levels in LH compared to FSH (89, 99–101).

Long extensions have been described in fish gonadotropes both *in situ* and in culture (32, 95, 102, 103). It is difficult to postulate as to the function of these processes but two plausible options are that these appendages are used for cell-cell communication or as a bridge between the cells and the vasculature. One line of evidence from cultured medaka cells (102) suggests that these processes do not relay signals between cells but are instead used for cell motility which serve to bring the cells together thus promoting cell-cell contacts and the formation of a network. It is hard to evaluate whether the processes serve the same role *in vivo* as in the adult medaka pituitary the cells are highly clustered yet the projections are still observed (102). Findings from the zebrafish model suggest that these processes are more likely to operate as extensions of the cells toward the vasculature than for intercellular communication. In the developing zebrafish, FSH cell extensions are clearly oriented in one direction: toward the primary plexus of vessels entering the pituitary (32). The aforementioned cytoplasmic processes are all directed toward the afferent vessels and terminate on their endothelium. This finding is even less surprising when considering the fact that not only metabolites are acquired from the blood, but also GnRH signals. At this early developmental stage no contact between gonadotrope and GnRH terminal exists. However GnRH fibers do reach the primary plexus, thus their output is carried through the blood to reach the developing gonadotropes (32). In addition to receiving blood-borne signals through the processes, these projections can also be used to deposit hormones to the circulation. In fact, medaka LH cell projections have been shown to develop large varicosities around the process-vessel junctions (102). It therefore seems more likely that the gonadotrope extensions are used to access the circulation than to mediate cell communication, though further investigations into this aspect are required.

While the study of pituitary cell networks in fish is still in its early stages, it is clear today that these networks developed early during vertebrate evolution and their function was conserved in higher vertebrates. In order to fully appreciate the role and function of pituitary networks in fish, future studies will have to extend the focus onto other pituitary cell types and probe the plasticity of these networks in response to physiological challenges that require the modulation of pituitary output.

## Amphibians

### Metamorphosis

The process of metamorphosis in vertebrates is well represented by the post-embryonic morphological remodeling in amphibians, where changes in physiology and behavior also occur. During metamorphosis, the neuroendocrine system plays a major role orchestrating the modifications at cellular and molecular levels in tissues by thyroid hormones (104–106). Particularly, a peak of thyroid hormone secretion marks the beginning of the process and the thyrotrophs, from the hypothalamus-pituitary-thyroid axis, exert a direct action over the production of this peak (107). As thyroid hormone continues to stimulate tissue remodeling, a continuous activity of thyrotrophs in the pituitary is also needed to maintain the secretion levels of the former (108, 109). Thus, thyrotrophs must shift plastically the secretion pattern by remodeling their structure and their relation with other cellular types within the pituitary and feedback loops in the axis (108, 110).

It is noteworthy that TSH secretion during amphibian metamorphosis is stimulated by CRF, the hypothalamic factor from the stress axis and not by TRH (111, 112). It is now understood that in amphibians, TRH activates TSH secretion in thyrotrophs of metamorphosed individuals but not in larval ones as reported in the bullfrog, reflecting that the thyrotroph plasticity is modified ontogenetically (113, 114). This phenomenon implies that thyrotrophs express receptors for TRH and/or that corticotrophs may regulate TSH secretion through paracrine communication (110, 115, 116). To date, the physiological and structural relationship between corticotrophs and thyrotrophs in the process of metamorphosis and whether a heterotypic network is formed remains to be elucidated, but it is possible to speculate about their intimate relationship, based on the shared main hypothalamic factor that regulates these cells types and also that the stress axis participates actively during the metamorphosis and its developmental plasticity (110, 117–120). Furthermore, cell plasticity in other pituitary cell types is also observed during metamorphosis. For example, when the salamander *Hynobius retardatus* was arrested prior to metamorphosis, an increase in the number of TSH-secreting cells was observed as well as an increase in bihormonal phenotypes such as TSH-PRL and TSH-GH, presumably involving transdifferentiation (121, 122).

The hypothalamus-pituitary-thyroid axis is also intertwined with the reproductive axis, where amphibian TSH in combination with gonadotropins has been demonstrated to trigger gonadal maturation (122, 123). Metamorphosis may modify gonadal maturation under environmental influence as demonstrated in urodels with neoteny, the heterochronic process where the larval phenotype is retained but organisms are sexually mature (124). Studies comparing the modification in the pituitary networks before and after metamorphosis in pedomorphic species (e.g., *Ambystoma mexicanum*; see **Figure 3**) with those with direct development (e.g., the bullfrog; see **Figure 3**) may reveal some of the molecular mechanisms involved in network plasticity, but also about its interaction with the hypothalamus (125–127).

### MSH Cells

Melanotrope cells are essential for amphibian camouflage *via* secretion of alpha-melanophore-stimulating hormones (α-MSH), a hormone derived from the POMC protein (128, 129). The melanotroph cells are restricted to the pars intermedia; in contrast to other secretory cells in anterior pituitary that are heterogeneously distributed and where heterotypic contacts are formed. The cluster organization of melanotrophs, must permit a fine coordination in response and α-MSH secretion, and probably involves gap junctions since expression of connexins (Cx43) has been reported (130, 131). The vascular system is a second candidate for melanotrope cells organization and

remodeling. In fact, in normal conditions, the pars intermedia is poorly vascularized compared to the adenohypophysis (see **Figure 3**), the capillary networks are present at the borders between the neural lobe and intermediate lobe (**Figure 1**). However, in transgenic frogs (*Xenopus laevis*) that overexpress VEGF-A in the pars intermedia, there is a complex formation of a vascular network (132). VEGF-A, the vascular endothelial growth factor, is involved in the formation of the vascular system during the development of the hypothalamus-pituitary system, and in the transgenic *Xenopus*, the increase of the vascular network has several consequences in the pars intermedia. First, these new capillaries are distributed around the lobules formed by melanotrophs. Second, the intermediate lobe is up to 3.5-fold greater than in control frogs as a consequence of melanotroph hypertrophy and hyperplasia, but the pars distalis is not reorganized. The implication of these results pointed to the active role of the vascular network in pituitary function and homeostasis. Further studies will be required to understand the effect of the vasculature in the pars distalis where cell networks are differentially disposed to the capillaries (see **Figure 3**). Specific modifications in the vascular system of animal models, as the example offered here, may also reveal how endocrine diseases emerge as vascular problems (2, 133).

## Reptiles and Birds

The establishment of cellular networks in the reptilian pituitary has not been studied directly, but the available literature indicates that homotypic and heterotypic networks may exist. For example, close contact between cells from the same lineage (including thyrotrophs), cluster formation, and cytoplasmic extensions have been reported by immunohistochemical studies (134–136). Due to their phylogenetic position between amphibians and mammals, reptiles gain relevance in the study of evolutionary transitions in the pituitary structure and function. Of particular importance may be the investigation of the chronological appearance of pituitary cell types, a process where ontogeny probably does not recapitulate phylogeny. Based on the work of Yamaki and collaborators, it has been demonstrated that the sequential development of endocrine cells in the mammalian pituitary varies largely in other vertebrates without a clear pattern, even close species show diversity in pituitary developmental programming (137). As an example, the establishment of the gonadotroph network upon the corticotroph scaffold, as described in mammals (138) may not be the case in turtles or fish, where corticotroph cell distribution is principally restricted to the rostral pars distalis, although gonadotrophs are widely distributed in the lobe (**Figures 2** and **3**). Comparative cell lineage in combination with other methods, such as lineage tracing, single RNA sequencing, and functional studies will reveal the mechanisms underlying the structure of pituitary networks, the heterochronic appearance in vertebrate development and their plasticity.

## Mammals

Of all vertebrates, pituitary networks in mammals, and particularly in mice, have been studied in the most comprehensive manner and their existence has been demonstrated in lactotrophs, gonadotrophs, somatotrophs, corticotrophs, and folliculostellate cells (2, 16, 19, 20, 48). The modes by which mammalian pituitary cells communicate have been described earlier. Unlike in other vertebrate classes, works in mammals have focused not only on the existence of pituitary cell networks, but also on the way by which these networks modulate their function to meet the changing demands of the animal.

### Lactotrophs

The process of lactation requires a big amount of prolactin production and secretion from pituitary by lactotrophs. Milk production requires a 10–50-fold increase in prolactin secretion and involves a marked decrease of dopamine production by neurons in the hypothalamus (18, 139). Dopamine normally inhibits prolactin secretion through dopamine receptors coupled to inhibitory G-proteins that block the voltage gated calcium L-channel and change the membrane potential, but also through a short-loop feedback mediated by prolactin itself. However, during lactation DA-producing neurons switch from inhibiting prolactin secretion to promoting its release by secreting enkephalin (139). Although necessary, these hypothalamic changes do not account for the major alterations that support the increase in prolactin secretion (2).

In 2012, Hodson and collaborators reported the existence of physiological and morphological plasticity in the lactotroph network that reinforce the secretion activity of these cells and were not directly dependent on hypothalamic activity (19). First, from experiments of intracellular calcium measurements in lactotrophs, it was shown that during lactation, the network functional connectivity is more robust as both calcium coactivity between pairs of cells and correlation in calcium profiles increase. Remarkably, in virgin mice the network connectivity of lactotrophs is low and only few cells harbor the majority of these connections. In contrast, in lactotrophs from lactating mice there is an increase in the proportion of significantly correlated cells and new nodes emerge. This last characteristic of the network persists after weaning, even months after the lactation event is over. Second, increase in connectivity is not a direct result of the decrease in dopamine production, since in virgin mice treated with a dopamine-receptor antagonist, an increase in functional connectivity is not observed. Furthermore, the interruption or decrease in suckling stimulus during lactation significantly reduces this functional network at values to those observed in virgin mice. Third, the coordinated functional activity in the network is directly dependent on structural connectivity due to lactotroph hypertrophy and gap junctions. When a gap junction blocker was applied to the lactotroph network from a lactating female, the connectivity resembled the network of virgin mice. Finally, the plastic transition of this network remains assembled and is crucial in subsequent lactation events that are characterized by a prolactin secretion improvement (19). The effects of the lactotroph network reconfiguration on other networks, for example gonadotrophs and reproductive axis, and the heterotypic interactions remain open questions, but their physiological relevance is clear.

## Gonadotrophs

Another process where the hypothalamic regulation of pituitary does not account for the shift in hormone release is offered by gonadotrophs and LH secretion throughout the estrous cycle. LH and FSH secretion are necessary for follicle maturation and ovulation in females but they are differentially secreted through the reproductive cycle (140). For example, in proestrus the pattern of LH pulse secretion turns into a surge mechanism that has been largely seen as a consequence of the GnRH surge, the main stimulating factor of gonadotrophs in the hypothalamus. However, GnRH stimulus *per se* is not the causal phenomena for the significant increase in responsiveness and secretion by gonadotrophs (140, 141).

*In vitro*, it has been reported that during diestrus, the gonadotroph response to GnRH is limited and just a small proportion of the population present intracellular calcium activity (142). High concentrations of GnRH (100 to 1,000 nM) are necessary in this state of the cycle to produce the whole-population response, but the amount of LH secretion does not increase significantly. On the other hand, in proestrus, low GnRH concentrations are sufficient to trigger a whole population response and, more importantly, the majority of gonadotrophs secrete a larger amount of LH. Despite the increase in gonadotroph responsiveness and LH secretion during proestrus that depend upon GnRH receptor expression, these processes also involve the gonadotroph network reconfiguration and its relation with vasculature (143).

From experiments *ex vivo*, where tissue interactions are preserved, the gonadotroph network reveals dynamic and plastic adaptation in proestrus through an increase in cell number (probably involving cell proliferation and transdifferentiation). Gonadotrophs also increase cell-cell contacts (involving cell motility), and the increase of protrusions toward other gonadotrophs and to the vascular systems (141, 143). Taken together, these cell modifications and changes in responsiveness to GnRH during the estrous cycle, account for the regulation in the rhythm and mode of LH secretion at different phases of the reproductive cycle. The plasticity in gonadotrope cells at the molecular, cellular and population levels and a comparison of shared mechanisms between fish and mammals has been recently reviewed and for a more in-depth description the reader can refer to (21, 144).

Plasticity in gonadotrophs is canalized in a context-dependent manner: inputs from the reproductive axis as well as from the environment are interpreted within the network to activate or attenuate the system output. For example, testosterone, estradiol, and GnRH have been reported to stimulate the LH secretion, cell proliferation, and GnRH receptor expression when present at certain concentrations and rhythmicity, but may desensitize the network and inhibit the LH production and secretion when present for prolonged periods of time or higher concentrations (21, 145).

Most factors involved in the network establishment are necessary but not sufficient for a complete explanation of the mechanisms involved in the gonadotroph function as a network. GnRH is an element that triggers the network response since the intrinsic activity of gonadotrophs is not particularly synchronized and is not associated with gonadotropin secretion (see **Figure 4**) (147). Furthermore, the amount of gonadotropin secretion is influenced by the balance of hormones in the reproductive axis and includes a reconfiguration in the network connectivity and this change is associated but is not a consequence of the GnRH stimulus (141, 143). Such connectivity is principally constructed through soma-soma contact and cytoplasmic extensions in which gap junction-mediated intercellular communication or cytosolic connection are involved. However, if the gonadotroph network is wired only by cell-cell contact, one could expect that cells in close proximity to each other, should present a similar pattern of response and form clusters where degrees of synchronization may be highest (19, 47). Interestingly, the analysis of clusters by correlation in the patterns of calcium mobilization shows that distant cells respond to GnRH similarly, but cells in contact or surrounded by capillaries may exhibit different responses, indicating that other mechanisms such as paracrine communication or connectivity with FS cells play an important role in the network function (64, 148, 149) (**Figure 4**). In addition, the functional and structural connection in the gonadotroph network is plastic and remodels throughout the estrous cycle, probably following the physiological demand during the proestrus of an LH surge (143). The variety of molecules and processes involved in the network formation, their relative contribution, as well as their degree of plasticity requires further investigation.

## Heterotypic Interactions

The homotypic network organization of gonadotrophs and lactotrophs discussed before provide evidence for its physiological relevance and plasticity. GH-secreting cells are the third population in pituitary that are organized in a homotypic network. This network is reconfigured during puberty with a sexually dimorphic pattern, probably as a result of different and dynamic feedback exerted by gonadal steroids (8, 150, 151). To date, the homotypic GH-cell network is the most extensively studied population in the anterior pituitary. There is evidence demonstrating the intimate interaction of the somatotroph network with other cellular types forming exquisitely arranged heterotypic networks, and when these interactions are disrupted, there are important repercussions at the gland-level hormone secretion (150, 152–155). For example, the ablation of somatotrophs at different degrees, compromises the pituitary production of TSH, PRL, LH, and ACTH, resulting in a modified ultrastructural composition of the cells and pituitary hypoplasia (152, 156, 157). Importantly, GH-cell ablation also impairs cell-cell communication between this homotypic network and other cell types, instead of causing GH reduction. The mechanisms of buffering the complete loss of somatotrophs in this model suggests an overproduction of the lineage from progenitor cells and a reduction in the generation of other lineages (152, 156, 157). Other possibilities could reflect transdifferentiation from different cell types since lactotrophs have been reported to decrease in number in these conditions, and it may also reflect the plastic function of multiresponsive

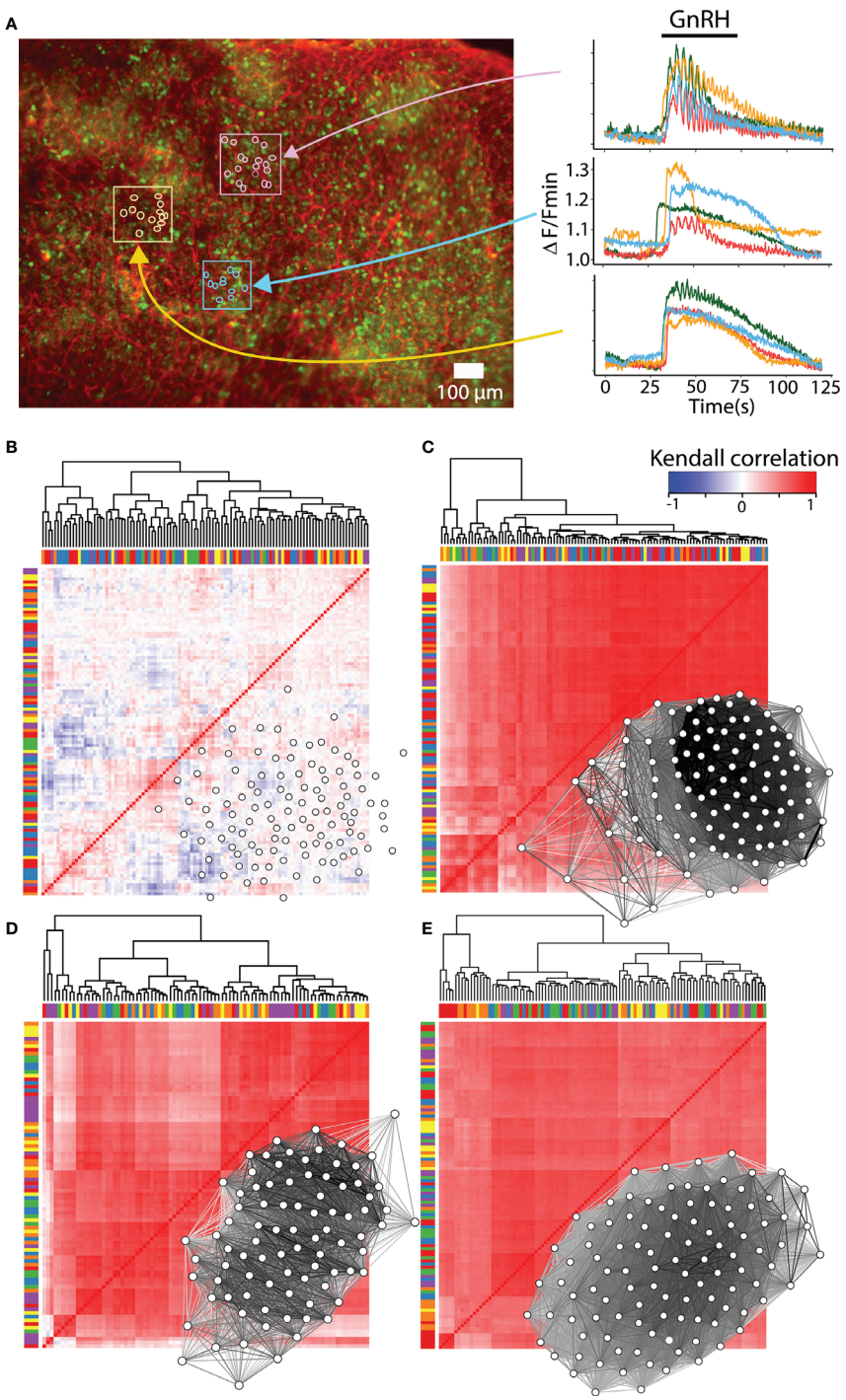


**FIGURE 4** | Reconfiguration of the gonadotroph network throughout the reproductive cycle. **(A)** Intracellular calcium recordings in *ex vivo* preparations of mouse pituitary loaded with the calcium sensor Fluo 4-AM (green) and rhodamine for vasculature (red). Monitoring of calcium activity was performed on the ventral side of the gland under an epifluorescence stereomicroscope and continuously perfused with Ringer's solution [methods as in (146)]. Color traces show the intracellular calcium activity of individual gonadotrophs in response to 10mM GnRH, indicating clusters of gonadotrophs surrounded by vasculature with a similar (pink and yellow boxes), or heterogenous (blue box) calcium response. **(B)** Heatmap and network organization of basal calcium activity of correlated pairs of gonadotropes from a male mouse. Gonadotrophs stimulated with GnRH in male mouse **(C)**, female mouse in diestrus **(D)**, and proestrus **(E)** showed an increase in synchronized calcium activity compared to basal activity. Notice that the overall correlation values are similar between diestrus and proestrus, as well as their connectivity. Bars in the heatmaps indicate sorting of gonadotropes by correlation but not by spatial proximity, while groups of gonadotrophs surrounded by vasculature are represented with the same color in bars. Network maps were plotted using qgraph and significant pairs (edges) of correlated cells (nodes) are shown ($P < 0.05$).

cells that have been observed to increase under several physiological challenges (158, 159). However, the mechanisms involved in this process remain to be explored.

Less understood is the ontogeny of the homotypic and heterotypic networks in the pituitary gland. The observation of the complex arrangement of networks leads to the question regarding the developmental origin of cellular interactions and two options are possible. First, it is possible that at the point of cell proliferation of one lineage, cells never lose the contacts (such as cytonemas) and form a continuum allowing high synchronicity of activity and identity prevalence of the homotypic networks, as observed in the first stages of spermatogenesis (160). Alternatively, the cells might appear isolated, establish a position in the gland, and after that, the lineages could acquire the connectivity observed in adulthood. The available literature revealed that the second option is observed during development in the corticotroph and gonadotroph networks (138). Corticotrophs and gonadotrophs first appear in the ventral side of the pituitary as small cells without cytoplasmic projections, and isolated from other cells of the same lineage. Then, corticotrophs reshape their morphology, penetrate into the adenohypophysis, and interconnect with other corticotrophs and with the vasculature through cytoplasmic projections (138). Gonadotrophs follow the same pattern and it is reasonable to speculate that the organization of other networks in the gland also appears just after terminal differentiation (16, 138).

The sequential differentiation of cell types in the pituitary also suggests that homotypic interactions establish first and the heterotypic networks appear later, although the lineages that are developmentally older have a repercussion over the newborn cells and lineages (156, 161). For example, LH-positive cells first appear in the ventral surface of the pituitary at embryonic day 17.5 when the homotypic network of corticotrophs is already established. LH cells then form a homotypic network using the corticotroph network as a scaffold. In fact, blocking cell differentiation of the POMC lineage leads to an increase in the number of LH-expressing gonadotrophs that are mainly located where corticotrophs should be distributed (138, 162).

One may ask whether the increase of the gonadotroph population after the interruption of corticotroph establishment during development is due to a physical or morphogenetic constraints exerced from corticotrophs to the other populations. The idea that corticotrophs and gonadotrophs probably arise from a common precursor, suggests that the extended gonadotrophs lineage in deficient POMC cell differentiation is more than expansion of the former in an "empty niche," reflecting a complex feedback between networks. For example, the transcription factor Tpit, expressed exclusively by POMC cells, exerts a negative effect over SF1, the transcription factor associated with the establishment of the gonadotroph phenotype (162, 163). This and other molecular interactions (e.g., pit-1 blocking the expression of the gonadotroph-like phenotype in thyrotrophs throughout GATA-2), suggests that a molecular interplay exists between cell lineages and results in an equilibrium of cell populations (16, 138, 164). The dynamic equilibrium achieved during ontogeny could persist to adulthood and shift to different points of equilibrium in a context-dependent manner, with the possibility of return to previous states, revealing the substrate of a highly plastic gland with cell populations that are able to navigate toward alternative phenotypes [see for example Sox2-expressing cells in adult organisms (156, 159, 165, 166)].

To date, other heterotypic interactions between the five mammalian adenohypophyseal populations have not been investigated but there are reasons to speculate about their relation and interdependence. First, the interlocked cell arrangement of gonadotrophs and corticotrophs was uncovered recently only by 3D image analysis that is hidden under classical 2D microscopy analysis (138). In this sense, thyrotrophs, the less abundant secretory population, may reveal a 3D continuous distribution in the pituitary [but see (130)]. Second, under diverse physiological challenges, transdifferentiation between cell types occurs independently of their ontogenetic proximity. For these reasons, the proportion of multi-responsive and multi-hormonal cells are constantly fluctuating, and different phenotypes such as lacto-somatotrophs, somato-gonadotrophs, gonado-lactotrophs have been observed (144). Finally, as we have mentioned before in this work, paracrine communication may play an important role in heterotypic communication, although its full implications are not yet clear (64).

## CONCLUSIONS

Despite variations in the hypothalamus-pituitary communication and the organization of the cell populations within the gland, pituitary cell networks have persisted throughout evolution, revealing their pivotal role in the ability of the pituitary to mount effective responses to hypothalamic and systemic stimuli. Two major elements allow the formation of networks in the pituitary gland: cell-cell contacts and diffusible factors, both present in vertebrate species, although with different preponderance. For example, in fish, where cell types are present in clusters and hypothalamic innervation invades the pituitary gland (see **Figure 5**), the secreted factors by these neurons near the vasculature reach their pituitary targets by diffusion. However, as not all cells in a cluster (see gonadotrophs from **Figure 5**) are in close proximity to neuron terminals and capillaries, a synchronized response is also achieved by gap junctions. Furthermore, the relevance of the vascular systems may reside in the delivery of nutrient and oxygen supply as well transport of pituitary outputs to the blood circulation, meanwhile paracrine and FS cells could produce a balance between distinct secretory cells and neuroendocrine axes. In contrast, in mammals, where the endocrine cells are distributed along the gland and the hypothalamic neurons secrete to the vasculature in the median eminence, a smaller proportion of the network could receive hypothalamic factors by diffusion (**Figure 5**). It seems that homo and heterotypic communication allow coordinated propagation of signals

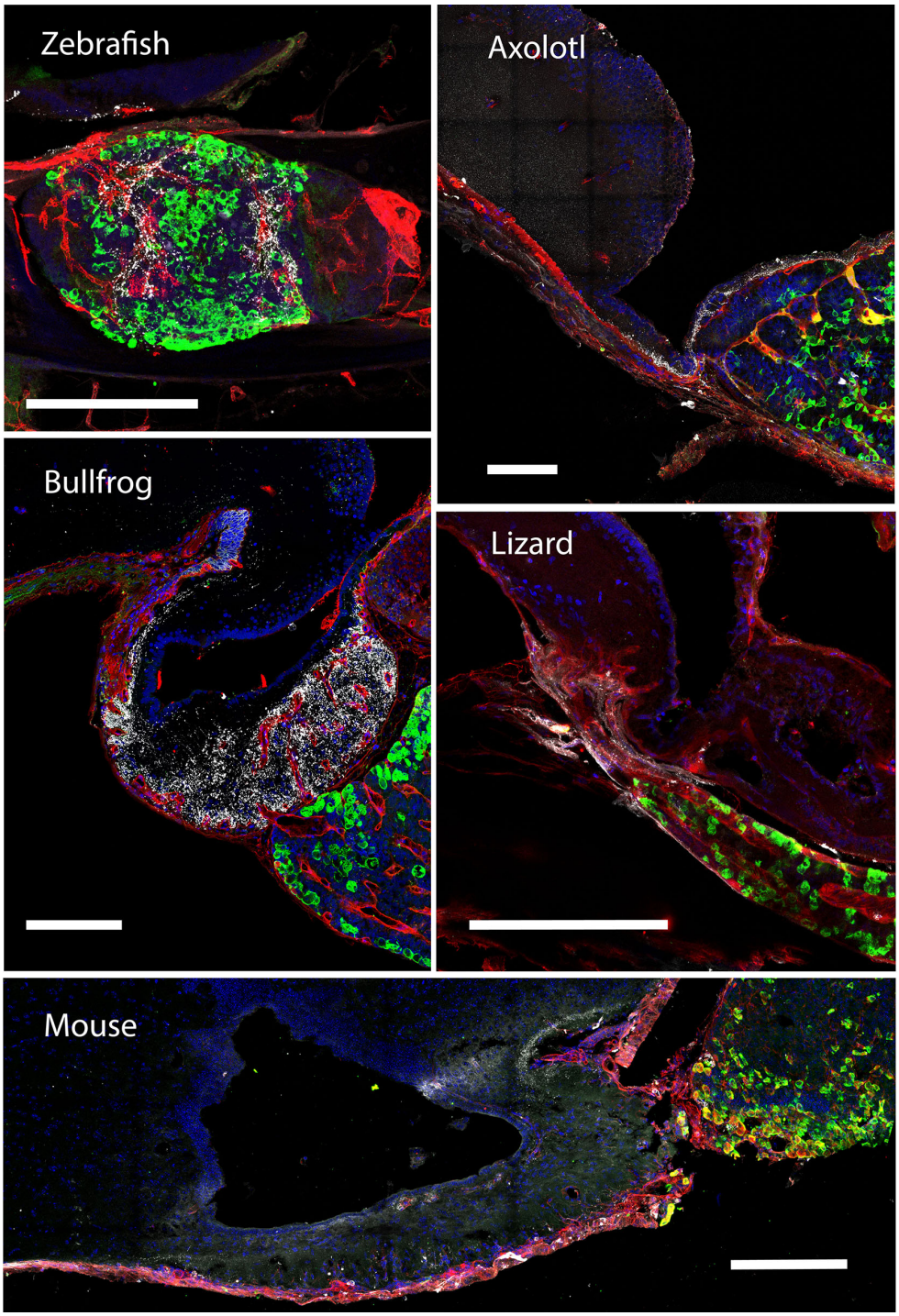


**FIGURE 5** | A multimodal communication between the hypothalamus and pituitary cell networks. Shown are sagittal sections (20 μm) of 4% paraformaldehyde fixed tissue, anterior at the left, dorsal to the top. Immunocytochemistry for gonadotrophs containing the LHβ subunit (green) and GnRH neurons (gray), species as in **Figure 3**. The distribution of gonadotrophs in the pars distalis, proximal to the hypothalamus, have remarkable cell-cell contacts in the tetrapod species, in teleost these cells are exclusively found in the rostral pars distalis forming a clear-cut cluster. The gonadotroph network arrangement is highly influenced by the vascular system (red; lectin-rhodamine), since these cells have cytoplasmic extensions to the capillaries. In tetrapods, the GnRH-producing neurons extend their projections to the median eminence where GnRH is secreted to the capillaries. In contrast, the GnRH neurons in teleost unfold throughout the pituitary and establish at the boundaries of the gonadotroph patch, following the vascular system. Note the close proximity between gonadotrophs and GnRH at the median eminence of tetrapods. DAPI is shown in blue for counterstain. Scale bars represent 200 μm.

between distant cell subpopulations, that may be enhanced through FS cells, paracrine factors and gap junction in small spaces (100 μm). Moreover, in other cell types that are less proximal to the median eminence (e.g., thyrotrophs), probably the hypothalamic signals are initially delivered through the vasculature and propagated by FS afterward. In summary, plasticity and evolution of the pituitary gland is the tinkering of existing parts combined in new ways rather than the creation of new parts.

In all vertebrate species, different physiological changes such as development, metamorphosis, reproductive cycles, stress, and metabolism require different pituitary outputs, not limited to large changes in the amount of secreted hormones, but also in timing and pulse frequency. Therefore, the gland and its interactions with the hypothalamus, organs, and tissues of the body are constantly being remodeled. The integration of all signals and the generation of hormone pulses, is the result of the plasticity of pituitary cell homotypic and heterotypic networks. Therefore, the interaction between these endocrine networks, are an important driver of the organism homeostasis.

The plasticity of hormone secretion in the pituitary gland has largely been seen as the result of changes in the hypothalamic input and pituitary-intrinsic modulations such as cell proliferation, cell size modifications, transdifferentiation, and multi-responsiveness. However, this review and other authors have contributed to the discussion of the elements that support the complexity of hypophysial plasticity. Several elements are involved to achieve this plasticity and have been discussed in detail (16, 20), these are: a) *gain of function*, by integration of stimuli from neuroendocrine axis and systemic information in the interaction of axes, as GH and PRL-networks, whose coordinated response to GHRH and TRH, respectively, is not seen when cells are dispersed; b) *plasticity for hormone secretion*, during changes in physiological demand, as seen in puberty when the GH axis is highly active and there is an increase in male somatotroph clustering associated with large amplitude of GH pulses; c) *experience-dependent response*, that is improved as a consequence of repeat demand, as mentioned in lactotrophs during the process of lactation; d) *network support of transcriptional synchronization*, when gene transcription follows a rhythmic pattern at the population level that is lost in dispersed cells, as it happens in lactotrophs; e) *redundancy through modularity*, although all the cells in a homotypic network are connected, there are subgroups of cells connected more strongly than other, this fact promotes certain independence of activity, and perturbations in a given region not necessarily affecting the whole network given the possibility of local homeostasis.

It is noteworthy that, despite their enormous functional importance, the study of pituitary networks is still in its infancy. In the last years there has been more literature focusing on the study of the pituitary as a system with functional networks. The term "network(s)" associated with the pituitary gland, as a main topic, first appeared in scientific literature in 1990, with the study of avian adenohypophysis and the effect of a serotonin precursor on thyrotrophs and the follicle-stellate network (167). In the next years, the concept of pituitary networks became popular and just in the last 10 years, 890 publications have been reported using the term network as a topic, according to The Web of Science Core Collection. Research of pituitary networks refers to different levels of interaction including protein-protein, protein-DNA, protein-metabolite, and cell homotypic and heterotypic networks (20). Accordingly, this young research field is highly productive and growing rapidly, showing the relevance of the cellular interactions when studying plasticity of the gland under diverse physiological demand.

So far research has focused mainly on the mammalian model of mice and, more recently, on fish. Research in both of these organisms has relied heavily on transgenic animal models that allow the identification, monitoring and manipulation of specific cell populations in live tissue as well as 3D imaging of the cell networks. Extending the study of functional pituitary networks to other vertebrate classes will be greatly assisted by the generation of transgenic animals in these taxa.

The development of genetically encoded calcium indicators (168) and *in vivo* imaging techniques (169) are expected to play an important role in advancing our understanding of the function and plasticity of pituitary cell networks in the coming years. These methods will allow researchers to extend the study of these networks from slices to freely moving and behaving animals and will therefore significantly increase the physiological relevance of our insights. Furthermore, single-cell transcriptomics (5, 170) and precise genome editing through viral delivery (171) will allow researchers to reveal the molecular machinery that drives the function and plasticity of pituitary cell networks, the relative role played by the elements that establish these networks and the relevance of the multimodal communication in the hypothalamus-pituitary system.

## AUTHOR CONTRIBUTIONS

TF and MG: Conception of the line of work, structure of ideas, and writing. YS-A: Writing, immunocytochemistry, calcium recordings, and figures. All authors contributed to the article and approved the submitted version.

## FUNDING

Authors were supported by grants from CONACyT and the Agence Nationale de la Recherche (273513, Repro-Net) and PAPIIT (IN222613 and IN227416). YS-A was supported by CONACyT master funding.

## ACKNOWLEDGMENTS

We would like to thank Edgar Jiménez-Díaz and the LANSBIODYT-UNICUA-UNAM (CONACYT) for equipment

and the technical support in the acquisition of confocal images. The amphibian and lizard for immunohistochemistry shown in this manuscript were kindly provided by Sebastian Zúñiga-Lagunes and Oscar Flores-Villela, the authors are deeply grateful. We would like to thank Berta-Levavi Sivan for her permission to use the zebrafish pituitary images. YS-A would like to thank the Posgrado en Ciencias Biológicas (UNAM) for the academic formation through its Master's degree program in Biological Sciences.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 29 January 2021
doi: 10.3389/fendo.2020.604519

# Pituitary Remodeling Throughout Life: Are Resident Stem Cells Involved?

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The pituitary gland has the primordial ability to dynamically adapt its cell composition to changing hormonal needs of the organism throughout life. During the first weeks after birth, an impressive growth and maturation phase is occurring in the gland during which the distinct hormonal cell populations expand. During pubertal growth and development, growth hormone (GH) levels need to peak which requires an adaptive enterprise in the GH-producing somatotrope population. At aging, pituitary function wanes which is associated with organismal decay including the somatopause in which GH levels drop. In addition to these key time points of life, the pituitary's endocrine cell landscape plastically adapts during specific (patho-)physiological conditions such as lactation (need for PRL) and stress (engagement of ACTH). Particular resilience is witnessed after physical injury in the (murine) gland, culminating in regeneration of destroyed cell populations. In many other tissues, adaptive and regenerative processes involve the local stem cells. Over the last 15 years, evidence has accumulated that the pituitary gland houses a resident stem cell compartment. Recent studies propose their involvement in at least some of the cell remodeling processes that occur in the postnatal pituitary but support is still fragmentary and not unequivocal. Many questions remain unsolved such as whether the stem cells are key players in the vivid neonatal growth phase and whether the decline in pituitary function at old age is associated with decreased stem cell fitness. Furthermore, the underlying molecular mechanisms of pituitary plasticity, in particular the stem cell-linked ones, are still largely unknown. Pituitary research heavily relies on transgenic *in vivo* mouse models. While having proven their value, answers to pituitary stem cell-focused questions may more diligently come from a novel powerful *in vitro* research model, termed organoids, which grow from pituitary stem cells and recapitulate stem cell phenotype and activation status. In this review, we describe pituitary plasticity conditions and summarize what is known on the involvement and phenotype of pituitary stem cells during these pituitary remodeling events.

**Keywords: pituitary, plasticity, stem cells, organoids, regeneration, maturation, aging**

OPEN ACCESS

***Edited by:***
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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *09 September 2020*
***Accepted:*** *25 November 2020*
***Published:*** *29 January 2021*

***Citation:***
*Laporte E, Vennekens A and Vankelecom H (2021) Pituitary Remodeling Throughout Life: Are Resident Stem Cells Involved? Front. Endocrinol. 11:604519. doi: 10.3389/fendo.2020.604519*

## INTRODUCTION

One of the defining characteristics of the pituitary gland is its ability to plastically adapt its cell composition to fulfill changing endocrine demands of the body throughout life. The gland occupies a central position in the endocrine system, upstream receiving regulatory signals from the hypothalamus and downstream sending hormonal messages to endocrine organs throughout the body (such as adrenal and thyroid glands, ovaries and testes), thereby regulating the production of specific hormones by these target glands. Tight control of pituitary hormonal release is maintained *via* a strict balance between cues from the hypothalamus and negative feedback loops from the peripheral target hormones. The major endocrine part of the gland (i.e., anterior pituitary, AP) contains five endocrine cell types, each dedicated to produce (a) specific hormone(s). Somatotropes synthesize and secrete growth hormone (GH), generally involved in bone and organ growth and regeneration; lactotropes produce prolactin (PRL), playing an essential role in pregnancy and lactation; gonadotropes generate follicle stimulating hormone (FSH) and luteinizing hormone (LH), controlling fertility and reproduction; adrenocorticotropic hormone (ACTH) is produced by corticotropes and necessary in stress and immune responses; and thyrotropes make thyroid-stimulating hormone (TSH) which is indispensable in metabolism control (1, 2). Apart from these endocrine cells, the AP also houses non-hormonal cell types encompassing endothelial, immune, and folliculostellate (FS) cells. Existence of stem cells in the pituitary gland was theorized for many decades until their convincing disclosure 15 years ago and thorough description since then, along with the identification of a number of stem cell markers, positioning SOX2 at the head of the list (3–7). From the multiple studies set out to unveil the biological significance of this stem cell population, it is at present perceived that these cells, at least in the basal adult gland, are highly quiescent. Thorough insight into their function(s) is still not firmly achieved (2, 8).

During postnatal life, several physiological processes ask for adaptations in hormone balances and thus pituitary output. The gland shows the essential flexibility to alter hormonal production by remodeling its function and cellular composition in these conditions. For onset and development of puberty, GH and gonadotropins (LH and FSH) are needed to drive and regulate pubertal growth spurt and gonad maturation (through the sex steroid hormones testosterone and estradiol), respectively (9). Increased levels of PRL are needed during pregnancy and lactation [to enlarge and prepare mammary glands for milk production (10–12)], and elevated ACTH concentrations are necessary to cope with stress (13–15). Pituitary cell remodeling is also seen in early-postnatal life, when the (rodent) pituitary gland undergoes prominent growth and maturation (2, 16, 17). In contrast, remodeling capacity may be compromised at aging concurrent with pituitary functional decline (18). Finally, injury in the gland during postnatal life triggers a local regenerative remodeling process culminating in regeneration of tissue cells and hormonal function (19–22). In general, it is only poorly understood how the specific cellular changes during these remodeling events are brought about, and whether and how pituitary stem cells are involved.

In this review, we summarize dynamic adaptations in the pituitary cell landscape at key time points of postnatal life and during specific (patho-)physiological processes, and discuss the current knowledge regarding involvement of the resident stem cells in these remodeling processes. To gain deeper insight into stem cell phenotype and role, appropriate, malleable and reliable research models are indispensable. Therefore, we also give an overview of *in vitro* pituitary (stem cell) study models and the important improvements that have recently been achieved in this field, in particular by establishing organoids.

## PITUITARY STEM CELLS DURING KEY PHYSIOLOGICAL EVENTS OF POSTNATAL LIFE

### Pituitary Stem Cells During Neonatal Maturation

When born, although all hormonal cell types are specified in the pituitary, the gland still needs to further expand and mature (23, 24). Before birth, during embryonic development [extensively reviewed elsewhere (25–27)], the pituitary initially appears as a thickening and subsequent invagination of the oral ectoderm called Rathke's pouch (RP), occurring in the mouse at embryonic day (E)8.5. From this first recognizable "pituitary" structure, the AP and intermediate lobe [containing the melanocyte stimulating hormone (MSH)-producing melanotropes] eventually develop. Around E12.5, the pouch disconnects from the oral roof and forms a closed entity around a central lumen (the future cleft). The marginal zone (MZ) around this lumen houses the progenitor cells of the embryonically developing pituitary which proliferate and then start to colonize the nascent AP where they generate three main lineages from which the different hormonal cell types develop, i.e., the PIT1 lineage giving rise to somatotropes, lactotropes and thyrotropes; the SF1/GATA2 lineage from which the gonadotropes develop; and the TBX19 lineage turning into corticotropes (25, 26, 28). This genesis of cell lineages and cell types is steered by a specific interplay between evolutionarily conserved signaling factors, such as bone morphogenetic proteins (BMP) and fibroblast growth factors (FGF), and sonic hedgehog (SHH), wingless-type MMTV integration site (WNT) and NOTCH family members, resulting in tightly regulated spatiotemporal expression of transcriptional regulators which govern the development of the distinct cell types (25, 26, 29, 30).

During the first postnatal weeks, the rodent pituitary almost doubles in size. This expansion and maturation process is driven by increased cell proliferation [including the re-entry of embryonically committed cells into the cell cycle (31)] and expansion of cell size because of endocrine differentiation with accumulation of hormone-containing secretory granules. In the rat, the proportion of proliferating cells in the postnatal pituitary is highest in the first week after birth to subsequently decline in the coming weeks toward very low levels at adulthood [i.e., from

400 dividing cells/mm$^2$ in the first week to 50 dividing cells/mm$^2$ at 8 weeks of age (17, 32)]. The basal adult pituitary indeed shows low turnover, with hormonal cells being replaced only every 60–70 days [as estimated in the young-adult rat (33, 34)]. Proliferation during neonatal maturation is observed not only in freshly differentiated, granular hormone-producing cells, representing 70–80% of the dividing population (32, 35–37), but also in non-hormonal agranular cells, at least partly identified as FS cells based on morphology and expression of the FS cell marker S100 (17, 38, 39). Nowadays, it is known that the heterogenous FS cell population encompasses pituitary stem cells (4, 5, 40).

Following the discovery of pituitary stem cells, several findings were reported that point to their potential involvement in neonatal pituitary growth and maturation. First, the proportion of stem cells is highest during the first postnatal week [i.e., postnatal day (P)1-7 *versus* P21, marking the end of the growth wave in mice, and *versus* adult age], as explored by both functional "side population" (SP) phenotype [a protective efflux capacity of stem cells (3, 4)] and molecular SOX2$^+$ nature (16). The topography of SOX2$^+$ cells at birth shows multicellular layers in the MZ, particularly prominent in the merging region of the anterior and intermediate lobe (referred to as the wedges) where "streams" of SOX2$^+$ stem cells appear to move into the developing AP (16). Moreover, the stem cell compartment shows a higher activation status involving a larger proportion of proliferating SOX2$^+$ cells. In accordance, neonatal pituitary stem cells show increased sphere formation capacity, further demonstrating their intensified functionality. Spheres (referred to as pituispheres) have been shown to develop from pituitary stem cells and sphere formation capacity is used as a readout of stem cell functionality and activation (3–5) (see further below). Additional support for the activated phenotype of neonatal pituitary stem cells includes their enhanced expression of stemness and embryogenesis-related genes (e.g., *Sox2, Sox9, Prop1* and components of NOTCH, WNT and SHH pathways), and swifter differentiation into hormonal cells in pituisphere culture as compared to the adult pituitary stem cells (16). PROP1 is a transcription factor formerly considered only essential for the development of the PIT1-dependent cell lineages during pituitary embryonic development but nowadays endowed with a more general function in embryonic stem/progenitor cells, governing their migration from the MZ to the nascent AP (41). It was shown *in vivo* (using mutant mice) and *in vitro* (using stem cell-derived colony culture; see below) that PROP1 drives the stem/progenitor cells into epithelial-mesenchymal transition (EMT) which is considered to underlie their migratory movement toward the developing AP (42). PROP1 disappears during this transition from stem/progenitor cell state towards committed endocrine cell (41). The factor remains expressed in SOX2$^+$ cells in the (rat) MZ acutely after birth, but the number of PROP1$^+$/SOX2$^+$ cells decreases during further postnatal maturation, with PROP1-expressing cells becoming rare in the adult pituitary (43), although this issue is still not fully settled since another study showed persistent expression (40). Also, NESTIN, a classic neural stem cell marker, has been identified in pituitary stem cells (3–5, 44). The proportion of NESTIN$^+$ cells rapidly decreases after birth [from 12% just before birth to 2% in the adult gland, as determined in rat (45)], whereas some of these cells remain proliferating which has led to the proposal that they represent the small proliferative fraction of the pituitary stem cells. The majority (70–80%) of the neonatal NESTIN$^+$ cells co-express PIT1 as well as dependent hormones (especially GH and PRL), suggesting that new hormonal cells might be differentiating from these NESTIN$^+$ progenitor cells (45). PROP1$^+$ and NESTIN$^+$ stem/progenitor cells thus also appear to be involved in the neonatal growth and maturation phase of the (rat) pituitary gland, both strongly fading in abundance at later age.

Together, higher proportion and activation status of the resident stem cells suggest a role in neonatal development of the pituitary. Possible mechanisms include direct generation of new endocrine cells and/or paracrine stimulation of committed progenitor cells to proliferate and differentiate or of differentiated hormonal cells to proliferate and expand in number (**Figure 1**). The first hypothesis is supported by the finding of swifter differentiation pace of neonatal stem cells in pituisphere culture (16) and of more significant contribution of stem cells (SOX2$^+$ or SOX9$^+$) to hormonal cells when transgenic lineage tracing is started in newborns (and analyzed 4 weeks later) *versus* in adulthood (6). Intriguingly, the largest contribution of traced stem cells was found in the gonadotrope population (~35%), which was proposed to be due to the use of tamoxifen to induce the lineage tracing, a selective estrogen receptor modulator which may affect gonadotrope development (6). Taken together, a clear view on degree and type of participation of stem cells in the neonatal phase of pituitary growth is not yet sketched. Further intriguingly, ablation of SOX2$^+$ stem cells in the neonatal pituitary does not affect postnatal hormonal cell population development toward adult age (46). Increased proliferation in the remaining SOX2$^+$ cells acutely upon the ablation process may account for this rescue of hormonal cell development in the maturing gland. Alternatively, the ablation grade may have been too low (30%) to provoke an effect in eventual hormonal cell population evolution (46). Fascinatingly, the stem cell population itself did also not restore to normal (as analyzed 4–6 months later) (46) which might indicate a redundancy of stem cells for normal postnatal pituitary development. Of note, double SOX2$^+$/hormone$^+$ cells are not detected during neonatal maturation (neither in the basal adult gland), which may indicate that stem cells first need to downregulate or shut off SOX2 expression before hormone expression can be initiated (16).

At present, it is not clear which molecular mechanisms or regulatory networks underlie the activation status of the neonatal pituitary stem cell compartment [in detail reviewed elsewhere (30)]. Deletion of the NOTCH downstream effector *RbpJ* in embryonic *Prop1*$^+$ progenitor cells leads to a significant decrease in proliferating SOX2$^+$ cells in neonatal pituitary (from 30 to 15%), and almost complete absence of early-postnatal SOX2$^+$ cells (31). Moreover, re-entry of embryonically committed PIT1$^+$ cells into the cell cycle, as normally occurring shortly after birth,

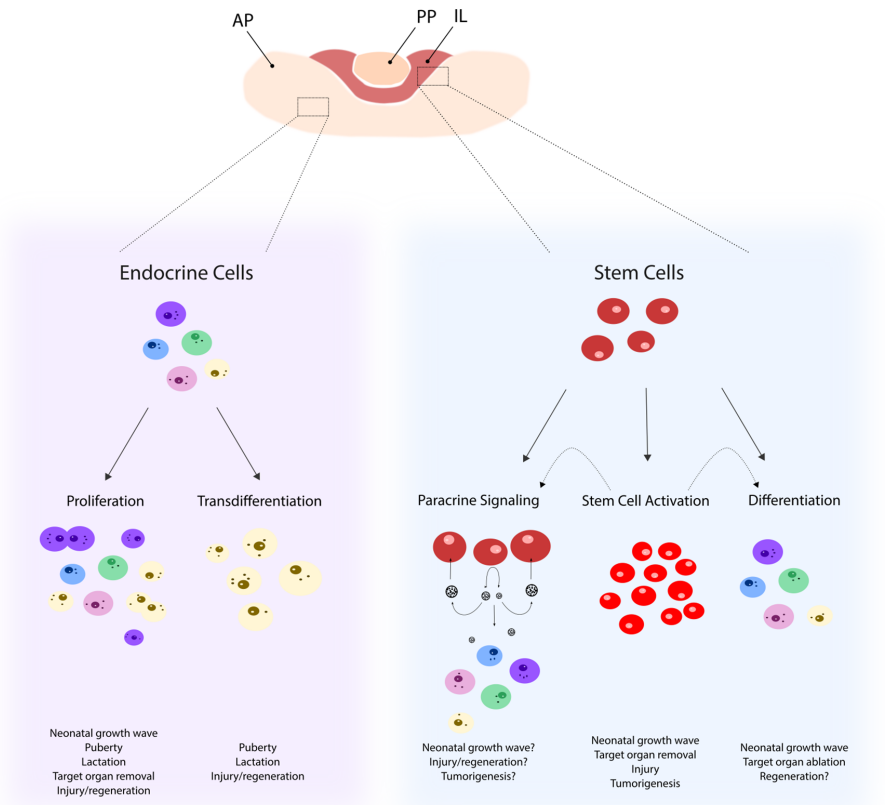


**FIGURE 1** | Possible mechanisms underlying remodeling of the pituitary cell landscape. Pituitary remodeling during postnatal (patho-)physiological processes may involve differentiated endocrine cells that either proliferate (as, for instance, observed in the neonatal growth wave) or transdifferentiate (as, for instance, seen during puberty). In addition, the stem cell compartment may become activated (involving increased proliferation and upregulated stemness factors and signaling pathways) as, for instance, observed following pituitary injury. Pituitary stem cells, activated or not, may contribute to new endocrine cell formation during the pituitary remodeling events through differentiation (as, for instance, after target organ removal such as adrenalectomy) and/or through sending paracrine signals to surrounding cells (as, for instance, likely to occur during tumorigenesis). AP, anterior pituitary; PP, posterior pituitary, IL, intermediate lobe.

is drastically reduced. At adult age, the pituitary of these mice is hypoplastic with a clear decrease in all endocrine cell types thereby supporting an important role for NOTCH signaling in early-postnatal growth and maturation of the pituitary toward its adult form through governing both the $SOX2^+$ cell self-renewal, maintenance and proliferation and the process and timing of $PIT1^+$ cell re-entry into the cell cycle (31). Genetic deletion of *Notch2*, one of the NOTCH receptors expressed in the pituitary stem/progenitor cells during development and later in life (47, 48), as well as postnatal inhibition of NOTCH activity using the γ-secretase inhibitor DAPT, resulted in a similar phenotype, with decreased proliferation of the stem/progenitor cells and declined expression of the stem cell markers *Sox2*, *Sox9* and Grainyhead-like 2 (*Grhl2*), a transcription factor found to be specifically expressed in the pituitary stem cells of the embryonic and early-postnatal gland (49, 50). In agreement, it has been shown that the NOTCH target gene *Hes1* is indispensable in the correct control of progenitor cell expansion, since pituitaries lacking *Hes1* exhibit decreased proliferation and increased cell cycle exit [with higher expression of cyclin-dependent kinase inhibitors such as p27 and p57 (51, 52)]. Furthermore, *Prop1* is a direct target of NOTCH signaling, thereby also assigning a role to PROP1 in the robust PIT1 lineage hormone cell expansion after birth (49).

Taken together, pituitary stem cells display an activated phenotype during the neonatal growth and maturation process of the gland. Despite emerging data and views, further research is needed to pinpoint their actual position in this active molding process, i.e., to decipher whether it involves direct contribution or stimulation of existing cells, or both, and to define the exact molecular regulatory mechanisms responsible for their activated status and function (**Figure 1**).

## Pituitary Stem Cells During Puberty

Puberty encompasses sexual maturation of body and gonads, and growth of skeleton, bones, and organs. This key developmental process is essentially steered by hormones, in particular the gonadotropins LH and FSH (further mediated by their downstream effectors estradiol and testosterone), and GH [further mediated by liver-derived insulin-like growth factor-1

(IGF-1)] which is responsible for the pubertal growth spurt (9, 53, 54). The hypothalamic-pituitary-gonadal axis is already created and active in pre- and postnatal development. Its activity transiently declines during childhood (so-called "juvenile pause") but is re-activated at the onset of puberty, signaling the start of pubertal development (55).

Surprisingly little is known about cell adaptations in the pituitary gland during this key phase of life. In mice, somatotropes and lactotropes reach their full cell size at the onset of puberty and ultrastructural features become similar to their counterparts in adult gland. Other (non-)endocrine cell types reach final cell size and morphology about one week after puberty (56). The increasing levels of GH coincide with a peak in somatotrope markers (e.g., *Pou1f1*, the gene encoding PIT1), although it is not clear yet whether there is higher expression of GH per somatotrope cell or whether somatotrope number expands (40). A study in children reaching puberty showed that rising GH levels are due to a rise in amplitude of the GH secretion peaks, not to an increase in pulse frequency (57). Whether pituitary stem cells are involved in remodeling of somatotropes during puberty, is not yet known.

Gonadotropes also change during puberty in number and activity. In medaka fish (*Oryzias latipes*), FSH- and LH-producing cells show hypertrophy and estradiol-driven proliferation during puberty (58). One study related to the involvement of stem/progenitor cells showed that genetically sustained *Prop1* overexpression in gonadotropes results in delayed puberty as marked by decreased gonadotrope differentiation (with nearly absent *Lhb* and *Fshb* expression), less developed seminal vesicles in male mice and delayed vaginal opening, smaller ovaries and thinner uteri in female mice, as well as belated growth (59, 60). On the other hand, also knock-out (KO) of the *Prop1* gene results in underdeveloped gonads (61). Human patients with *PROP1* mutations either do not enter or complete pubertal development, show growth failure and need sex hormone substitutions (62). Taken together, $PROP1^+$ pituitary stem/progenitor cells might play a role in normal pubertal development and the needed pituitary gonadotrope and somatotrope cell remodeling (**Figure 1**), but hard, convincing evidence is not available yet.

## Pituitary Stem Cells During Pregnancy and Lactation

Successful pregnancy and lactation largely depend on the action of, and regulation by PRL. Accordingly, PRL levels are substantially increased during these physiological states and processes (10–12). Whether this rise is substantially due to increasing numbers of lactotropes in the pituitary and if so, how new lactotropes emerge (i.e., proliferation of existing PRL-producing cells, transdifferentiation from somatotropes or differentiation from stem/progenitor cells), is still not unambiguously underpinned (**Figure 1**).

In rats, a peak in proliferating lactotropes is observed in late pregnancy (day 19–21 of gestation) resulting in an augmented proportion of $PRL^+$ cells during lactation when proliferative activity already returned to the (low) baseline level (63, 64). In humans, lactotrope hyperplasia occurs in various phases of pregnancy, assigned to both proliferation of pre-existing $PRL^+$ cells and transdifferentiation of somatotropes to lactosomatotropes [i.e., cells expressing both PRL and GH (65)]. In mice, no increase in lactotrope cell number was observed during lactation, but cell size of individual lactotropes was enlarged and cells formed long-lasting networks that enabled cell-cell communication and functional connectivity to coordinate elevated PRL secretion (10). Using $PRL^{eYFP/+}$ reporter mice, a 20% volume increase of the "enhanced yellow fluorescent protein"-positive ($eYFP^+$) cell population was found during lactation, but this study did also not observe a rise in the proportion of $PRL^+$ cells (66). Still, proliferation may have occurred in the lactotrope progenitor cell population, a possibility supported by another study that also did not perceive an increase in proliferating $PRL^+$ cells (neither in $SOX2^+$ stem cells) during lactation, but detected a large surge in proliferating $PIT1^+$ progenitor cells (31). Transdifferentiation between $PRL^{eYFP/+}$ and $GH^+$ cells was found to be low (less than 1%), excluding a major contribution of such conversion process during lactation in the mouse (66). Since a large volume increase of the pituitary (peaking at mid-lactation) was seen, which could not fully be explained by the increased volume of the $eYFP^+$ cell population, a non-cell specific, general increase in mitosis was suggested (66). In analogy, a general increase in proliferating AP cells toward the end of pregnancy was also observed by Zhu et al. (31), with $SOX2^+$ stem cells remaining mostly quiescent (31). On the other hand, estradiol, a regulator of PRL production during lactation, induces a small, 10% increase in dividing $SOX2^+$ cells (6).

A novel and powerful technology to unravel tissue cell landscape and dynamics is provided by single-cell RNA-sequencing (scRNA-seq). In a recent study interrogating the pituitary of 13-week old lactating mice and age-matched virgin control animals (67), expansion of the lactotrope cluster was observed in the lactating mice (36% as compared to 29% in control), also showing upregulated expression of the neuroendocrine vesicle secretory protein chromogranin B (*Chgb*). Upregulation of PRL levels during lactation thus may reflect the combined effect of an expanded lactotrope population and a more active PRL-secretory machinery. Authors further speculated that the newly formed lactotropes are derived from multi-hormonal cells, since the latter cluster is reduced in abundance during lactation (10% as compared to 13% in control), expression of *Prl* within this remaining multi-hormonal cluster significantly upregulated and expression of other hormone transcripts (e.g., *Lhb)* downregulated. In addition, transdifferentiation of somatotropes to lactotropes was also proposed to play a role since *Prl* expression was clearly higher in the somatotrope cluster of lactating animals. Importantly, whether stem cells are involved in the expansion of the lactotrope population was not looked at or speculated on. Interestingly, the stem cell cluster appeared to become smaller in the lactating animals [3.2% as compared to 4.5% in control (67)].

After weaning of the progeny, lactotrope cell number returns to normal steady-state levels, supposedly through a combination

of apoptosis and transdifferentiation away from the lactotrope phenotype. However, using the $PRL^{eYFP/+}$ reporter mouse line, transdifferentiation was only observed at very limited rate [i.e., $GH^+$ cells derived from lactotropes did not exceed 1% (66)].

Taken together, current data do not point to a significant role for stem cells in the generation of new lactotropes during pregnancy and lactation, but rather endow the committed progenitor cells with this function. Either, further and more detailed stem cell lineage tracing may indicate otherwise, or stem cells may rather play a nutritive, paracrine regulatory, or stimulatory role (**Figure 1**).

## Pituitary Stem Cells During Aging

Aging is associated with a deterioration in tissue homeostatic turnover and reparative capacity. In some tissues such as muscle, this waned behavior is causally associated with decreased stem cell number and functionality (68–71). Manifestations of aging are also observed in the endocrine system, including in pituitary output. Higher baseline TSH levels and altered ACTH dynamics have been observed in the aged population (18). In rat, thyrotrope cell number decreases with age, but $TSH^+$ cell area (volume density) and TSH serum levels increase (72), consistent with observations in humans (18). These findings suggest a desensitization of the pituitary-thyroid axis during aging. Gonadotropes also show aging-associated changes. In the male rat pituitary, gonadotrope cell number and size decrease with a phenotypic shift from large to small vesicles in the cells, the latter being the main type present in the female gland (73, 74). FSH and LH levels decrease with aging in male rats, whereas they remain constant or even rise in female rats. Signs of degeneration (such as pyknosis) are observed in the aging gland gonadotropes (73). One of the best known aging-associated endocrine events is the so-called somatopause, a progressive fall in basal GH levels and resultant circulating IGF1 (75). Especially the GH pulse amplitude, but not the frequency, is reduced in aged individuals (18). In rats, number of $GH^+$ cells was not found different between young (3-month old) and old (24-month old) animals (76). By contrast, in mice and humans $GH^+$ cell number decreases at aging (77, 78). In addition to these putative local pituitary causes of dropped GH levels, also regulators of GH production alter when getting older, *viz.* dropped levels of activators such as ghrelin and GHRH and rising concentrations of inhibitors such as somatostatin. Furthermore, changed sensitivity to these regulators may play a role including a gender-independent decline in GH cell responsiveness to GHRH and ghrelin (79, 80).

Although involvement of pituitary stem cells in the gland's aging phenotype has been regularly hypothesized (2, 21), this assumption is at present only scarcely supported. FS cells, known to encompass pituitary stem cells, are decreased in number in old rats [from 4.5 cells per reference area in young rats to 1.5 in old rats (81)]. However, in human pituitary, FS cell number was found to be expanded (82), an increase which might be grounded in the stem cell fraction of the heterogeneous FS cell population, but also in the stromal or immune subpopulations. In other tissues like muscle and heart, it has been observed that the stem cell population is negatively affected by age, presenting as a decline in number and in regenerative capacity (68–71). Interestingly, restorative capacity of the pituitary also fades at aging, in a quite speedily manner. Following infliction of injury in the gland of middle-aged (8- to 10-month old) mice using the GHCre/iDTR model (see below), regeneration—which plainly unfolds in young 8–12-week old animals—does not occur any longer. This regenerative failure in older mice coincides with a decline in pituitary stem cell number and fitness [i.e., decreased sphere formation capacity (21)].

Taken together, several changes occur in the pituitary's cell landscape and hormonal output during aging, but at present, barely anything is known about the impact of aging on the local stem cells and their participation in these changes. The least that is known is that their number, activity and fitness appear to decline, as occurring with stem cells in many other tissues (21, 68–71).

# PITUITARY STEM CELLS DURING PHYSIO-PATHOLOGICAL CONDITIONS

## Pituitary Stem Cells During Stress

Stress is a physiological coping mechanism marked by activation of the hypothalamic-pituitary-adrenal (HPA) axis and essentially resulting in increased levels of circulating ACTH and glucocorticoids (cortisol in humans and corticosterone in mice). Other pituitary hormones are also affected by stress with impact depending on severity and exposure time (13, 83).

In mice, acute (30 min) cold stress does not result in immediate changes of corticotrope cell number, although corticosterone levels rise (14). The relative abundance of the other AP cell types is increased at the expense of non-hormonal cells. Since these changes occur acutely after application of the stressor, it was proposed that the new endocrine cells develop through maturation of committed progenitor cells that already transcribe or translate minimal (undetectable) amounts of hormone and rapidly increase this expression upon stress. In contrast, a 30 min cold exposure in rats was found to result in an increase in $POMC^+$ and $ACTH^+$ cell number and size (15). Another study also showed an expansion of cells expressing *Pomc* mRNA (as analyzed by *in situ* hybridization) 2–3 h after applying a cold stressor in rats (84). Exposure to chemical stressors (such as formaldehyde) also results in a rise of $ACTH^+$ cell proportion as analyzed in mice (85). Following exposure of rats to increased ambient temperature, $ACTH^+$ cells show reduced volume density, likely due to elevated secretion of ACTH from the corticotrope cells, which is concordant with the higher levels of ACTH (and corticosterone) found in the circulation (86).

Apart from the corticotropes as logical stress target, also other pituitary hormonal cells are affected. Thyrotropes respond to acute cold in rats by expanding in number and size (15). Somatotrope dynamics change depending on the type of stressor. Pituitary GH protein levels in rats increase upon acute immobilization or restraint but not after repeated immobilization. However, size and density of $GH^+$ cells remain

largely comparable to control (87). Prenatal dexamethasone treatment in rats, resulting in intensified HPA activity in postnatal life—and thus a chronically elevated stress state—triggered long-term changes in FS cell morphology but not number, whereas not affecting the corticotrope population (88).

In the HPA axis, the adrenal gland negatively feeds back through corticoids to the hypothalamus and pituitary, thereby keeping ACTH production under control. Hence, adrenalectomy causes a rise in ACTH level and a transient increase in $ACTH^+$ cells in the pituitary (33). Mechanisms underlying this corticotrope remodeling include increased proliferation of existing $ACTH^+$ cells which thus re-enter the cell cycle [4.5% dividing $ACTH^+$ cells per unit area after adrenalectomy as compared to 0.5% in control (89)] and development of new corticotropes from stem cells, as demonstrated by SOX9 lineage tracing [i.e., 20% of the new $ACTH^+$ cells originate from the traced cells (6)]. Another study showed an increase in proliferating $TBX19^+$ corticotrope progenitor cells after adrenalectomy (90).

Overall, although pituitary cell remodeling during or following stress has been studied in different models, not much is known yet on whether and how pituitary stem cells play a role (**Figure 1**).

## Pituitary Stem Cells During Tumorigenesis in the Gland

Tumors in the pituitary gland, nowadays referred to as pituitary neuroendocrine tumors (PitNETs), represent 15% of all intracranial lesions and occur as symptom-causing tumors with a prevalence of 1 in 1,000 (1, 91). PitNETs can cause serious morbidity through hormone hypersecretion or, at the other side of the spectrum, reduced pituitary function (hypopituitarism) because of compressing healthy, neighboring pituitary tissue (1, 91). Not much is known on the underlying pathogenesis of PitNETs and their link with pituitary stem cells has so far only scarcely been studied. In search for so-called tumor stem cells (TSC), defined as cells driving tumor initiation, growth and heterogenic composition (thus displaying the orthodox stem cell properties of self-renewal and multipotency), and responsible for tumor re-growth after therapy because of their increased resistance, a SP was found in human and mouse PitNETs showing tumorsphere formation, expression of stemness markers and *in vivo* growth capacity, all pointing to a TSC character (92). Also other studies provided data proposing the existence of TSC in pituitary tumors [extensively reviewed in (93)]. Using a mouse model of lactotrope tumor (prolactinoma) formation in the pituitary (i.e., $Drd2^{-/-}$ mice), an increase in $SOX2^+$ cells was found in the tumorous gland, at least partly due to elevated proliferative activity (92). These findings suggest that the resident pituitary stem cells are activated in case of the "threatening" tumorigenic event in the gland. Whether this reaction is preventing fierce tumor growth progression, or is otherwise stimulating the process, is not known. Moreover, the $SOX2^+$ cells may not only represent activated tissue stem cells but also TSC developed from the(se) stem cells (or alternatively from other cell types by, for instance, dedifferentiation). However, direct descent of tumor cells from $SOX2^+$ cells was not supported in the $Drd2^{-/-}$ mouse using $SOX2^+$ lineage tracing (93), thus questioning their role as TSC. Rather, paracrine stimulation of tumor formation and growth may occur (**Figure 1**), as has also been reported in another pituitary-located tumor model (94). Transgenic expression of a genetically mutated, constitutively active form of the WNT signaling transducer β-catenin in embryonic pituitary progenitor cells (95) or in $SOX2^+$ cells (7) results in the development of lesions in the gland showing characteristics of adamantinomatous craniopharyngoma (ACP), a benign but burdening tumor mostly occurring in children (96). The tumors contained typical nucleocytoplasmic β-$catenin^+$ cell foci expressing the stem cell markers SOX2 and NESTIN (7, 95). However, $SOX2^+$ lineage tracing revealed that the tumor proper did not directly derive from the $SOX2^+$ stem cells, which, on the other hand, were characterized to produce several factors that could fuel tumor development and growth from the neighboring cells (7, 94–96). Intriguingly, tumors did not develop when β-catenin was transgenically expressed in committed ($PIT1^+$) or differentiated ($GH^+$, $PRL^+$) cells, indicating an essential (although indirect) role of stem cells in this tumorigenic process (95). In accordance with the aberrantly activated WNT pathway in the stem/progenitor cells of this ACP-resembling model, upregulated expression of WNT components has also been observed in the SP of human PitNETs (92). Other typical stem cell-regulating pathways that have been advanced to potentially play a role in pituitary tumorigenesis are the NOTCH pathway, which seems either activated or suppressed in the tumor (analyzed as a whole), depending on the pituitary tumor subtype (97), and the HIPPO pathway. Elevated expression of HIPPO pathway components (e.g., *YAP1*, *LATS2*) was detected in the SP of PitNETs (92). Moreover, genetically induced elevation of YAP/TAZ signaling in the $SOX2^+$ pituitary stem cells results in the development of non-secreting aggressive tumors (98). Intriguingly, in contrast to the $Drd2^{-/-}$ prolactinoma and ACP-resembling models described above, these tumors directly (and clonally) originated from deregulated $SOX2^+$ cells as investigated using $SOX2^+$ lineage tracing (98). However, these highly proliferative carcinoma-resembling tumors do not immediately draw a parallel with the typically benign tumors that occur in the pituitary. Finally, a recent study, clustering pituitary tumors by RNA-seq analysis in three groups coinciding with canonical lineage transcription factors [i.e., TBX19, NR5A1 (SF1) and POU1F1 (PIT1)], did not reveal a transcriptomic link with the (normal) stem cell population, but suggested that the "tumor progenitor cells" (TSC) derive from already (partially) committed cells expressing the respective transcription factor (99).

Taken together, although more and more studied, no clear view is shed yet on the connection between pituitary stem cells and tumorigenesis in the gland. It remains essential to decipher whether and how pituitary stem cells are implicated in order to advance our knowledge on pituitary tumorigenesis, at present only poorly understood. Of note, the local stem cells may become "activated" during tumorigenesis not only by genetic or molecular (signaling/growth factor) aberrations, but also by the accompanying physical tissue damage, a reaction indeed observed following inflicted injury in the gland.

## Pituitary Stem Cells Following Injury in the Gland

Hypopituitarism, involving deficiency in one or more pituitary hormones, results in serious morbidity given the gland's central position in the endocrine system (100). This hypofunction may be due to faults in embryonic development, or may be caused by damage occurring during postnatal life. Culprits include hemorrhagic necrosis in the gland (Sheegan's syndrome), physical damage by tumor growth as well as following its operational resection, and destructive impact on the gland through head trauma [traumatic brain injury (TBI)] as caused by traffic, sport or violence accidents. To define the behavior of the gland's stem cells following injury, a transgenic mouse model was created allowing the destruction of pituitary cells (19). Before, partial hypophysectomy had been applied which is technically challenging and difficult to standardize. An older study in rats reported an increase in "chromophobic" cells in the residual pituitary after partial tissue removal, which may represent the immature (stem) cells (22, 101). However, no cell regeneration or anatomical restoration was observed, even up to one year later (101). In the more recent transgenic pituitary injury model, specific cell populations are targeted for killing by diphtheria toxin (DT) treatment. In the GHCre/iDTR mouse model, expression of the DT receptor (DTR) is induced (hence, "inducible" or iDTR) through the activity of Cre recombinase, expressed under control of the GH promoter. DTR in the GH-expressing cells then makes these cells sensitive to DT-induced ablation. A 3-day DT injection results in major (80–90%) obliteration of the somatotrope cells, thereby realizing a controlled pituitary injury model (19). Interestingly, the resident stem cells promptly react to the cell-ablation damage in the gland. First, it was found that the stem cell population shows augmented proliferative activity and expands (about 2-fold) upon injury based on several read-outs including SP, FS cell and $SOX2^+$ phenotype and sphere-initiating capacity (19). Morphologically, especially the wedge regions show a prominent enlargement of the $SOX2^+$ stem cell zone (19). In addition, upregulated gene expression of factors typically associated with stem/progenitor cells and/or with pituitary embryogenesis (such as FGF, BMP, and components of the SHH, WNT, and NOTCH signaling pathways), further supported the activated status of the pituitary stem cell pool following injury. Similar facets of stem cell activation were observed in a complementary pituitary injury model killing the PRL-expressing cells (with 70% ablation grade) using the PRLCre/iDTR mouse model (20). Finally, a proliferative activation reaction was also detected in the remaining $SOX2^+$ cell compartment after DT-targeted $SOX2^+$ cell ablation (46). However, this response only unfolds in early-postnatal mice (1–4 weeks of age), not in adult animals (8–12 weeks), and does not result in restoration of $SOX2^+$ cell numbers as analyzed 4–6 months later (see above).

Interestingly, a significant restoration of the ablated cell population was noticed, thereby convincingly demonstrating for the first time that the adult pituitary gland possesses regenerative capacity. The number of somatotropes is restored to 50–60% after 4–5 months [GHCre/iDTR model (19)]. Also GH serum levels substantially re-lift [to 30% (21)]. However, regeneration does not reach higher levels, even following an extended follow-up period (19 months) after the somatotrope ablation insult, suggesting that full regeneration is not critical for survival and supportable life (21). Since the stem cell compartment is activated upon injury followed by tissue repair, the question presented whether stem cells are involved in the regenerative response. Co-expression of SOX2 and GH surfaces in several cells, not observed in the normal steady-state gland, thereby pointing to differentiation of (reacting) stem cells toward the somatotrope fate. No evidence was found for contribution of other mechanisms such as transdifferentiation from lactotropes (no increase in $PRL^+/GH^+$ cells) or proliferation of remaining somatotropes [dividing $GH^+$ cells are virtually non-existent (19)] (**Figure 1**). Nevertheless, direct demonstration of the descent of the newborn $GH^+$ cells from the $SOX2^+$ stem cells awaits lineage tracing, however at present technically difficult using a Cre-mediated approach. For instance, tamoxifen-induced SOX2CreERT-driven lineage tracing after GHCre-mediated somatotrope ablation cannot exclude simultaneous tracing of the remaining GH cells (also expressing Cre) and will thus not allow to discern between $reporter^+$ cells derived from $GH^+$ or $SOX2^+$ cells during the regenerative period. As an alternative mechanism underlying regeneration, the activated stem cells may act as restoration-stimulating signaling center, for instance driving the $PIT1^+$ progenitor cells into proliferation [as apparently also occurring in pregnancy/lactation and probably also during neonatal maturation, as discussed above (31)] (**Figure 1**). Involvement of stem cells in the regenerative reaction, whatever the mechanism, is further supported by upregulated gene expression in the stem cells of factors belonging to pathways typically involved in tissue regeneration such as the epidermal growth factor (EGF), FGF, EMT, and Hippo signaling systems (21), and of factors playing a role in pituitary embryogenesis (see above), suggesting recapitulation of the embryonic developmental program, as also reported in other regenerating tissues such as muscle (102). In the PRLCre/iDTR model, the lactotrope population shows a swifter restoration, reaching 60–70% after 6–8 weeks (20). The more efficient repair is likely due to the accumulated action of multiple regenerative processes in this cell lineage, including stem cell differentiation (increase in $SOX2^+/PRL^+$ co-expressing cells), amplification in lactotrope proliferation (as observed) and transdifferentiation of somatotropes to lactotropes [increase in cells co-expressing GH and PRL (20)] (**Figure 1**). Formerly, some signs of regeneration were also briefly reported following thymidine kinase-mediated obliteration of somatotropes and lactotropes (103). However, underlying mechanisms, and particularly the involvement of stem cells, were not investigated at that time. Moreover, this nucleotide (FIAU)-incorporating approach only kills cells that are actively dividing and therefore necessitated FIAU treatment from the embryonic till early-postnatal stage (103), thus excluding applicability of the technique for cell ablation and regeneration studies in the predominantly non-mitogenic adult pituitary.

TBI is increasingly recognized as an important cause of hypopituitarism with drop in hormone levels (especially GH and ACTH), possibly due to damage to the pituitary tissue, either directly or through hypothalamic or vascular impacts (104, 105). It has been found that hormone serum levels in TBI patients may restore after several (3 to 36) months (104, 106–108). Whether this recovery is due to compensatory behavior of the remaining pituitary cells or the hypothalamus-pituitary axis, to regained pituitary cell functionality, or to repair of the pituitary insult with new cell formation to replace damaged and destroyed cells, is not known (104, 105). Further in-depth investigation of the pituitary cell landscape upon TBI, for instance using scRNA-seq analysis, is expected to provide deeper insight into (stem) cell reaction and possible repair, and into TBI-induced hypopituitarism, which may eventually lead to new approaches to clinically deal with this prevalent endocrine deficiency condition.

Taken together, the resident stem cell compartment of the pituitary promptly reacts to injury in the gland, which may lead to ensuing regenerative processes. However, underlying mechanisms, whether it involves direct generation of new cells or indirect paracrine stimulation of cell neogenesis, still need to be clearly defined (**Figure 1**). In the longer run, this knowledge may pave the way to regenerative therapies in case of damage-induced hypopituitarism such as by tumor operation or head trauma. Pituitary stem cells could potentially be stimulated *in vivo* to drive new-formation of destroyed endocrine cell type(s), or may *ex vivo* be expanded, differentiated and transplanted, as proposed already a long time ago in a study in which chromophobes (freshly isolated from the pituitary) were shown to divide and differentiate when transplanted into the hypophysiotropic area of the rat (109). Alternatively, the secretome of activated pituitary stem cells, when functioning as signaling centers, may be defined and important factors applied as treatment *in vivo* (22) (**Figure 1**). As an important remark, it is still unclear whether also the human pituitary is capable of regenerating. Only very few studies addressed this issue. Following transsphenoidal electrocoagulation-mediated destruction of metastasized tumors in the gland (thus damaging the pituitary tissue), actively dividing chromophobic cells around the necrotic core and signs of glandular regeneration were observed in post-mortem pituitaries (110). As described above, restoration of pituitary function in terms of circulating hormone levels after TBI might also involve pituitary regeneration although at present not investigated. Clinical endurance of hypopituitarism, when brought about by local tumor growth or removal, does not immediately point to a restorative response. However, transient regenerative processes may occur which are stalled for one or the other reason, but which cannot be probed in human beings and will need innovative *in vitro* approaches.

# *IN VITRO* MODEL SYSTEMS TO STUDY PITUITARY STEM CELLS AND REMODELING

Although studies in experimental animals, in particular mice, have generated important information on pituitary development and functioning, there is a high need for appropriate *in vitro* research models to study pituitary stem cell biology and pituitary (cell) remodeling, and their cross-section. Over the years, several different pituitary *in vitro* models have been developed, ranging from 2D immortalized cell lines to 3D structures, each with own specific sets of advantages and shortcomings (**Table 1**). Here, we give a short overview of the different model systems and their potential as research tool to untangle pituitary remodeling and in particular the biology, role and regulation of the pituitary stem cells in these plastic adaptations.

## Pituitary Cell Lines

Cell lines have been developed that represent different pituitary cell types. The lines were derived by culturing pituitary tumors or by genetically transforming and immortalizing pituitary cells or specific cell types. Well-known examples are the mouse corticotrope AtT20 cell line and the rat lactosomatotrope GH3 cells. The cell lines proved valuable to study hormone regulation by hypothalamic and other factors (111, 112). FS cell lines have also been established [e.g., PDFS (113)] and have been instrumental to study cytokine regulation of AP cell functions (114, 115) and intrapituitary communication (111, 116).

Despite their value, cell lines have important shortcomings in mimicking pituitary (cell) physiology, in particular because of their transformed pheno-/genotype (usually aneuploid and neoplastic), their 2D culture format (contrasting with the 3D configuration and anchoring *in vivo*), and their poor or non-existing representation of the compound pituitary cell composition. On the other hand, the cell lines still appear to contain a (driving)? progenitor cell population. For instance, SP cells were identified in AtT20 and GH3 cells showing stemness functionality such as sphere formation and xenotransplant tumor growth (92).

## Model Systems Established From Primary Pituitary

### Models Starting From the Compound Pituitary

Primary cells from dissociated pituitary glands can be cultured as monolayers, either as original mixture or following enrichment of specific cell types using sedimentation gradients (117, 118) or counterflow centrifugation (119). Although easy to achieve, the resultant 2D cultured cells quickly (within one week) start to deteriorate, lose their normal physiological behavior and responsiveness (e.g., to hypothalamic hormones), and die.

Alternatively, primary pituitary tissue can be kept in culture as explants, frequently employed in the past (49, 120, 121). To do so, pituitary tissue fragments (e.g., halved glands) are cultured either submerged in optimized culture medium (composition differing between studies), or deposited at the air-liquid interface [e.g., RP explants (29, 122, 123)]. Although physiological responses are elicited in explant cultures, their "shelf life" is limited because of inadequate perfusion, lack of sufficient oxygenation and resulting necrosis in the core (after a few days in culture).

Above-mentioned shortcomings are overcome in an organotypic culture system of pituitary cell aggregates (44, 48,

**TABLE 1** | Pros and cons of *in vitro* pituitary study models.

| Model | Pros | Cons |
|---|---|---|
| ***Pituitary cell lines*** | - Readily available<br>- Easy to handle and manipulate | - Tumor-derived or genetically immortalized (not representative for normal tissue cells)<br>- Only represents a single cell type<br>- 2D format |
| **From primary compound pituitary** | | |
| ***Monolayer cell culture*** | - More pituitary cell types<br>- Easily established | - Limited expandability<br>- Quickly lose physiological behavior<br>- 2D format |
| ***Explant culture*** | - Representative (reflects tissue heterogeneity and physiological responses)<br>- Easily established<br>- 3D format | - Limited expandability – necrosis<br>- Limited experimental possibilities or manipulations |
| ***Cell aggregate culture*** | - Representative (reflects tissue heterogeneity and physiological responses)<br>- Easily established<br>- Long-term culture<br>- 3D format | - Limited expandability<br>- Difficult to enrich for FS/stem cells |
| **From primary pituitary stem cells** | | |
| ***Sphere culture*** | - Quite representative (differentiation into hormonal cells)<br>- Allows exploration of pituitary stem cell biology<br>- 3D format | - Limited expandability<br>- Limited application potential (e.g., to unravel differentiation processes) |
| ***Colony culture*** | - Quite representative (differentiation into hormonal cells)<br>- Allows exploration of pituitary stem cell biology | - Limited expandability<br>- Limited application potential (e.g., to unravel differentiation processes)<br>- 2D format |
| ***Organoid culture*** | - Extensive expandability of limited primary (stem) cells<br>- Quite representative (differentiation into specific hormonal cells)<br>- Allows exploration of pituitary stem cell biology<br>- High application potential (e.g., tumor/disease modeling, drug screening, …)<br>- Amenable to gene editing<br>- Cryopreservable<br>- 3D format | - Cost-intensive<br>- At present still limited differentiation<br>- Very hard to achieve from normal human pituitary |
| **From PSCs** | | |
| ***Organoid culture*** | - More representative (may reflect tissue heterogeneity)<br>- ESCs/iPSCs are readily available and expandable<br>- Model for human normal pituitary<br>- 3D format | - Labor intensive set-up<br>- Cost-intensive<br>- Limited expandability once differentiated into pituitary fate<br>- Human iPSCs known difficult to transfect |

124, 125). Dissociated primary pituitary cells (typically from rat or mouse) are cultured under constant gyratory movement in a pituitary-optimized, serum-free defined medium (SFDM), allowing the cells to re-aggregate and form histotypic cultures containing all main pituitary cell types [including FS and stem cells but lacking primary pituitary endothelial cells and housed lymphocytes and monocytes (48)], thereby reflecting the major pituitary cell composition as well as cell type-typical organization. Pituitary cell (re-)aggregates can be kept in culture for months without losing tissue and cell-type representation and functionality (i.e., hormone production capacity and regulation with responsiveness to hypothalamic hormones). As true in the *in situ* gland, not much cell proliferation is occurring in the aggregates, and cultures are not expanding but static in abundance. Although aggregates can be formed from enriched endocrine cell types, no aggregates have been established from isolated stem cells, and aggregates from enriched FS cells remain small and fragile (44, 48, 124, 125).

## Non-Organoid Models Derived From Pituitary Stem Cells

A common and essential shortcoming of the above-mentioned pituitary model systems is that at present none of them allows to specifically grow and study the stem cell compartment. *In vitro* study models for pituitary stem cells are essential to advance our knowledge on the behavior (self-renewal, multipotent

differentiation, activation), regulation and translational and clinical potential of this still under-defined pituitary cell population.

Clonal sphere formation is a general characteristic of stem cells, which was first defined for neural stem cells (126). Cultured in a specific growth medium including B27, EGF and/or basic FGF (bFGF/FGF2), stem cells clonally expand to form floating 3D spheres that can be serially passaged, based on the capacity of the sphere-initiating stem cell to self-renew and generate proliferative progenitor cells. Under specific culture conditions, differentiation to various cell types of the tissue of origin occurs in the spheres because of the (multipotent) differentiation capacity of the originating stem cell (126). Free-floating spheres can be derived from pituitary by culturing dissociated AP cells in SFDM (or other culture medium) supplemented with B27 and bFGF. These so-called pituispheres are composed of $SOX2^+$ stem cells [also expressing other pituitary stem cell markers such as NESTIN, E-cadherin, S100 and SOX9 (3–5)], originate from the SP [and not from the non-SP (3)] and from the $SOX2^+$ stem cells (6), and can be differentiated toward all pituitary hormonal cell types by culturing on an ECM (Matrigel)-coated surface in medium without stem cell growth factors. Pituispheres (when formed after 6 days of culture) can be dissociated and resultant cells re-seeded to grow new spheres, although the propagation efficiency gradually but quickly declines, being at present limited to 3–4 serial passages (3–5, 40). This stem cell model has proven its merits, mainly as read-out of stem cell functionality and activation (3–6, 16, 19, 31, 46, 48). For instance, more spheres are formed when starting from the damaged GHCre/iDTR pituitary, in line with the activated status of the stem cells upon injury [see above (19, 21)].

Another general test to probe stem cell phenotype and functionality is provided by the colony-forming assay, which, in contrast to the sphere assay, occurs in 2D format. Dissociated pituitary cells, cultured in medium containing serum, bFGF and cholera toxin, generate colonies after 6 days that express the stem cell markers NESTIN and SOX2, as well as limited levels of early pituitary development markers (LHX3), the latter increasing toward 14 days in culture (coinciding with the exit of the exponential growth phase in the colony). Eventually, the colonies contain cells of all five AP cell lineages, although at modest numbers (7, 96, 127).

Although valuable for specific readouts of stem cell functionality (probed by, for instance, colony-forming efficiency), the 2D format does not mimic the 3D anchoring and interaction of cells as present *in vivo*. Despite a 3D configuration, pituispheres remain limited in growth and application potential. Hence, a more versatile and efficient model system would definitely be instrumental to study pituitary and stem-cell biology *in vitro*. Over the last years, so-called organoid models have been developed, either derived from pluripotent stem cells (PSCs) or from pituitary stem cells.

## Organoid Models Established From Pluripotent and Pituitary Stem Cells

In their contemporary meaning, organoids represent composite cell configurations that *in vitro* grow from (single) stem cells that self-renew, proliferate and self-organize in 3D, eventually replicating key biological properties of the organ of origin (128, 129). Such present-day organoid modeling has been achieved starting from tissue stem cells and from PSCs.

Tissue stem cell-derived "organoid-ing" typically follows the principles that were laid down in the first successful development of such organoids (130). Tissue fragments or dissociated cells (encompassing the resident stem cells) are embedded in an ECM/basement membrane-mimicking 3D scaffold [such as Matrigel or basement membrane extract (BME)] and cultured in a cocktail of compounds encompassing generic stem cell regulators as well as factors operational in the stem cell niche of the specific tissue. The core of this blend is formed by EGF, Noggin and WNT activators. EGF is a potent mitogen that stimulates epithelial stem cell proliferation while Noggin acts as an inhibitor of BMP signaling which blocks stem cell differentiation. The WNT pathway is a well-known regulator of tissue stem cells, which in organoid culture is generally boosted by adding R-spondin 1 (RSPO1), a ligand of the leucine-rich repeat containing G protein-coupled receptor 5 (LGR5) which marks stem cells in multiple tissues, and which amplifies WNT signaling strength (128, 129, 131). Using this protocol, organoid models have meanwhile been developed from manifold tissues of both mouse and human origin such as stomach (132), liver (133), and endometrium (134, 135). A key asset of tissue stem cell-derived organoids is that they display strong, long-term expandability while robustly retaining their properties and remaining genomically stable (128, 129).

PSC-derived organoid models are achieved by mimicking the sequential steps that occur during embryogenesis of the specific tissue or organ. Embryonic stem cells (ESCs) and induced PSCs (iPSCs), either in 2D or 3D format, are treated with activators or inhibitors of specific embryogenic pathways in a consecutive manner. As an example, organoids mimicking the intestine were obtained by driving human PSC aggregates first toward definitive endoderm using activin A, and then toward hindgut endoderm using WNT3A and FGF4. The obtained floating spheroids were then embedded in Matrigel containing RSPO1, EGF, and Noggin to finally generate an intestinal organoid structure (136). Organoid modeling using PSCs is especially valuable for complex organs composed of several different structures or compartments and therefore difficult to sculpt using the tissue stem cells (which, moreover, are not always clearly identified for those organs), such as brain (137), liver (138), and kidney (139).

In general, organoids provide interesting and powerful application potential in both basic and translational research (**Table 1**). First, they represent a valuable tool to disentangle tissue development including stem cell biology. In addition, organoids can be used to model diseases (128, 129). For example, tumor tissue-derived organoids have been established from different sorts of cancer such as colorectal (140), breast (141), gastric (142), bladder (143), and endometrial cancers (135), resulting in large "living" (cryopreserved) organoid biobanks of patient-derived samples. This resource is highly instrumental for drug screening, which can give insight into inter-individual drug responses and may be translated into

personalized medicine. Furthermore, organoids can be applied for studying infectious diseases. Eye-catching examples are the application of brain organoids to decipher Zika virus infection (144) and the use of blood vessel, kidney and intestinal organoids to probe and unravel the currently raging SARS-CoV2 infestation (145, 146). In the field of regenerative medicine, organoids may also prove constructive to restore damaged or diseased tissue. For instance, it has been shown that human colon-derived organoids move to experimentally damaged areas in mouse colon where they implant and form new tissue with self-renewing crypts that are histologically and functionally normal, thereby displaying long-term engraftment (147). As another example, organoids from primary human hepatocytes engraft and proliferate extensively when transplanted in damaged mouse liver (148).

Both organoid model systems, whether tissue stem cell- or PCS-derived, have pros and cons (**Table 1**). Tissue stem cell-derived organoids are important models to study postnatal tissue stem cell biology (phenotype, regulation, function, activation) which is less straightforward in PSC-derived organoid models in which the adult stem cell phenotype is (as yet) not, or not fully, recapitulated, but instead embryonic progenitor cells are (and can be more effectively studied). Tissue stem cell-derived organoids are long-term and exponentially expandable, allowing to multiply minute tissue samples (e.g., human biopsies) for extensive downstream applications. In contrast, PSC-derived organoids represent an end-point situation, not being passageable, although the starting material (PSCs) can be infinitely expanded first, and large numbers of organoids can then be developed. As already mentioned, PSC-derived organoids are more apt to reproduce complex organs, and different tissue cell types are co-formed during the "organoiding", whereas adult stem cell-derived organoids solely reflect the tissue epithelial compartment. On the other hand, the latter reductionist model may be an asset in specific applications (such as simplified high-throughput screenings). Currently, strong efforts are being made to further advance the adult stem cell-derived organoid model by co-culturing epithelial (stem) cells with other cells present in the tissue microenvironment such as mesenchymal, endothelial and immune cells (149, 150). PSC-derived organoid models, by their very nature, more closely recapitulate the embryogenic path of the organ, thus providing simultaneous development of different intrinsic tissue-specific cell types, and in some cases (as described below for the pituitary) also extrinsic elements, i.e., neighboring structures that in real life co-develop with the tissue. Finally, both tissue stem cell- and PSC-derived organoids are amenable to gene editing (by, for instance, CRISPR/Cas9), although human iPSCs may show resistance to efficient transfection (151, 152).

## Pituitary Organoids Derived From Pluripotent Stem Cells

About 10 years ago, pituitary organoid development was achieved starting from PSCs (153). Based on knowledge of pituitary embryogenesis, mouse ESCs, brought in an aggregate formation, were first directed to oral and neural ectoderm in adjacent layers using a chemically defined medium lacking any growth factors. The aggregates started to express the hypothalamic markers *Rax* and *Sox1* in the inner layer. As occurring *in vivo* where an intimate, contact-dependent interplay is needed between oral and neural (prospective hypothalamus) ectoderm for pituitary embryogenesis, co-existence of both layers in the 3D ESC configuration was essential to eventually result in the formation of a RP-like structure. This process presented as a morphological invagination and expression of the early pituitary transcription factors PITX1 and LHX3 at the border of both layers upon SHH activation, thereby repeating what is occurring *in vivo*. Further development toward different pituitary cell lineages was then reached also based on knowledge of mouse pituitary embryogenesis. Corticotropes (visualized by *Tbx19* and ACTH expression) were obtained by NOTCH inhibition using DAPT. Driving cells into the somatotrope and lactotrope fate was achieved following WNT pathway stimulation and subsequent exposure to glucocorticoids (for somatotropes) or estradiol (for lactotropes). Functionality was shown for corticotropes which were most efficiently derived (approximately 35% of the non-neural cells within the ESC-derived structure, meaning ~3% of the total cell number). The differentiated ESC-derived aggregates responded *in vitro* to the hypothalamic releasing hormone CRH by increased ACTH release, and rescued *in vivo* ACTH levels and mouse lethality when subrenally transplanted into hypophysectomized mice (153). A few years later, this tour de force was repeated with *human* ESCs (154). Hormone-producing cells were generated with corticotropes developing spontaneously (approximately 12% of the $PITX1^+$ non-neural cells), somatotropes (and to a lesser extent lactotropes and thyrotropes) appearing after glucocorticoid exposure, and LH- and FSH-expressing gonadotropes arising following NOTCH inhibition. Thus, specific differentiation was found to be different in mouse and human cell cultures (see e.g., the effect of NOTCH inhibition), highlighting important caveats when translating mouse developmental principles to humans. Cells responded to hypothalamic releasing hormones and increased human ACTH expression was observed when aggregate structures were grafted subrenally in hypophysectomized mice (154).

A few years earlier, pituitary hormonal cells were also generated from human ESCs as well as their somatic cell-derived counterparts (iPSCs), although in a 2D format (155). Here as well, SHH signaling was crucial for pituitary specification. $ACTH^+$, $GH^+$ and $FSH^+$ hormonal cell types could be developed, with induction of corticotropes again found most efficient. NOTCH inhibition was necessary to increase the development of *PIT1*- and *GATA2*-expressing (gonadotrope) lineages. After subcutaneous grafting *in vivo* in immunodeficient mice, the differentiated cells showed long-term survival and production of human ACTH and GH. In a further refinement, dorsal-ventral patterning (as occurring in pituitary development *in vivo)* was imposed on the pituitary-directed human PSCs *in vitro* (156). Treatment with high BMP2 concentration resulted in upregulation of *FSHB* and *LHB*

expression and the detection of $FSH^+$ cells (being ventral cell types in embryonic development), whereas high FGF8 exposure yielded increased *POMC* expression and $POMC^+$ cells (dorsal cell type in development), both findings in accordance with knowledge on mouse pituitary embryogenesis in which an opposing BMP2-FGF8 signaling gradient determines dorsal-ventral patterning and regional cell type specification (157). Intermediate concentrations of both factors resulted in an increase in cells expressing *GH, PRL*, and *TSHB* as well as of $GH^+$ cells, which are *in vivo* also found in the transitional zone during development (158). Subcutaneous injection of the differentiated cells (embedded in Matrigel) in hypophysectomized mice showed functional somatotrope and corticotrope cells producing (human) GH and ACTH, respectively (156).

Some recent studies further built on the 3D pituitary-developing, human ESC-based organoid model of Ozone et al (154). After transposing the technique to human iPSCs, further endocrine maturation was achieved through creating "hypothalamic-pituitary units" by refining the culture method, but especially by prolonging the culture period (up to 500 days) allowing the hypothalamic neurons to further mature, which concurrently resulted in more progressed development of the pituitary corticotropes (159). Indeed, the resultant units were found to yield better differentiation of $ACTH^+$ cells with higher ACTH secretion ability (paralleling levels in adult mice), and were found functional, showing, for instance, physiological responses to hypoglycemic stress in releasing CRH and ACTH. One caveat, as reported, is that prominent results were only obtained with one iPSC line while three lines were tested showing wide variation in hypothalamus-pituitary induction efficiency (159). Interestingly, the pituitary-developing organoid model was also applied to study pathogenetic mechanisms underlying congenital pituitary hypoplasia (CPH), caused by mutations in orthodenticle homeobox 2 (*OTX2*), known to be important in the development of forebrain, eye and pituitary (160). *OTX2*-mutant CPH patient-derived iPSCs were subjected to the pituitary-developing organoid protocol which revealed weakened LHX3 expression, associated with increased apoptosis of the pituitary progenitor cells and impaired differentiation into AP. It was found that OTX2, expressed in the hypothalamic part of the 3D *in vitro* aggregate, regulates LHX3 expression in the oral ectoderm part *via* hypothalamic FGF10 expression, eventually essential for progenitor cell maintenance (160). The phenotype was rescued by correcting the mutation, whereas introducing the mutation in control iPSCs resulted in a similar apoptotic picture. Finally, Kanie et al (161). produced iPSCs from an anti-PIT1 syndrome (hypopituitarism) patient and control person which were subjected to the pituitary development protocol (161). Expression of LHX3 was observed after 40 days in culture and of GH, PIT1 and ACTH in some cells after 100 days. PIT1 was found to undergo antigenic processing and presentation; however, no difference was observed between patient and control model system. Together, these studies provide the first human pituitary organoid disease models, nicely illustrating the power and future direction in unraveling (genetic) human pituitary disease by using iPSC-derived organoid model systems.

Taken together, these novel PSC-derived research models provide important tools to study pituitary development which can especially advance our knowledge on *human* pituitary biology and embryogenesis (at present only concluded from snapshot or longitudinal imaging analyses of embryos or patients with aberrant pituitary development), and/or to generate pituitary endocrine cells for transplantation purposes, since PSCs are amenable to substantial expansion, and large numbers of pituitary endocrine cells can theoretically be generated (**Table 1**).

## Organoids Derived From Pituitary Stem Cells

Very recently, organoids were established from pituitary stem cells (162). Thorough evaluation of the typical organoid growth factors (see above) and of multiple pituitary embryogenesis-related signaling molecules such as FGF8, FGF10, and SHH led to the definition of an optimized medium in which organoids could be developed from (mouse) adult AP. The organoids originate from the $SOX2^+$ pituitary stem cells as shown by utilizing FACS-sorted $SOX2^{eGFP+}$ cells [from the SOX2 reporter mouse model $Sox2^{eGFP/+}$ (163)], whereas organoids do not form in cultures of the $SOX2^{eGFPneg}$ cell fraction. The obtained organoids represent the stem cell compartment, as they are completely composed of cells expressing pituitary stem cell markers (such as SOX2 and E-cadherin). After passaging (i.e., breaking the organoids up into fragments which are reseeded in culture, thereby expanding them), the organoids retain this stemness phenotype. Upon transplanting the organoids under the mouse kidney capsule, they engraft and differentiate toward endocrine lineages, albeit at a modest extent (162). To study pituitary stem cell biology and activation after injury, the designed organoid method was applied to the GHCre/iDTR damage-and-regeneration mouse model [as described above (19)]. The efficiency of pituitary organoid formation was found to be higher when starting from the damaged (GHCre/iDTR) than the control, undamaged (-/iDTR) pituitary, in agreement with the activated state of the pituitary stem cells after the inflicted injury (162). Increase in organoid formation efficiency was also observed when starting from the neonatal gland (162), again recapitulating the boosted activation status of the stem cell compartment (see above). RNA-seq analysis of the organoids revealed novel pituitary stem cell labels (such as *Krt8* and *Krt18*) and damage-upregulated stem cell markers [such as *Prrx1/2*; see also (164)] which were all confirmed *in vivo*, illustrating the high potential of this new organoid model as pituitary stem cell biology research tool with reliable *in vivo* correlate and translatability (162). The model system can be applied to unravel pituitary stem cell biology and activation, or inhibition, during the several conditions of pituitary remodeling as reported above. Moreover, this particular organoid technology can also be used to study pituitary diseases, in particular tumors from which organoids could be developed, and utilized to explore tumorigenic mechanisms and impact of existing medicines, or new pipeline drugs.

Taken together, pituitary organoid models have been developed from PSCs and pituitary stem cells. Both organoid

types represent interesting, complementary tools to study pituitary biology, each with own advantages and specific applications (**Table 1**). The PSC-derived model is most apt to study pituitary embryogenesis, moreover enabling the concomitant development of non-epithelial tissue cell types and of the neighoring hypothalamus. In addition, the model can be started from human PSCs, thereby providing a human *in vitro* pituitary model. The pituitary stem cell-derived organoid model is most suitable to study postnatal pituitary stem cell biology including in-depth characterization of phenotype and of regulatory and activating molecular networks. However, obtaining human normal pituitary tissue (e.g., from autopsy) is very hard to achieve. In contrast, human pituitary tumor samples can be readily obtained from surgery to be turned into tumor organoid models, while the PSC-derived technique is more apt to develop genetic disease models (as illustrated above). At present, the PSC-derived organoid model shows more efficient generation of hormone-producing cells, making it (currently) more apt for unraveling endocrine lineage development and maturation, and for potential regenerative purposes. Technically, setting up pituitary stem cell-derived organoids is less labor-intensive and achieved in a short period of time (within 10 days), whereas the PSC-derived model is more hands-on and takes a longer time to develop and mature (from 1 month to 500 days). Moreover, results should be confirmed in several different iPSC lines which are known to be considerably heterogenous in genomic make-up and biological behavior [see (159)].

## CONCLUSION

Plastic adaptation of its cell landscape to changing situations in life is an important property of the pituitary gland. Although these changes in cell constitution and hormonal output are known to happen, not much is understood yet on how they are brought about. Here, we looked at this question focusing on the stem cell position in this dynamic play. Although discovered more than a decade ago, many key questions regarding their regulation and biological function and significance remain today. Overall, their contribution to the dynamic cell adaptations as observed in the pituitary remains largely unresolved. With the emergence of new technologies including single-cell transcriptomics and pituitary organoid culturing, several of these questions can be tackled in the near future.

## AUTHOR CONTRIBUTIONS

EL collected all the information and wrote the manuscript. AV provided input on the aging pituitary. HV co-wrote, critically revised, and finalized the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by grants from the KU Leuven Research Fund and the Fund for Scientific Research (FWO) – Flanders (Belgium). EL and AV are supported by a PhD Fellowship from the FWO (11A3320N and 1141717N, respectivey).

## ACKNOWLEDGMENTS

The authors thank the funding agencies as mentioned above for making their research possible.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

MINI REVIEW
published: 04 February 2021
doi: 10.3389/fendo.2020.616053

# Plasticity of Anterior Pituitary Gonadotrope Cells Facilitates the Pre-Ovulatory LH Surge

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Gonadotropes cells located in the anterior pituitary gland are critical for reproductive fitness. A rapid surge in the serum concentration of luteinizing hormone (LH) secreted by anterior pituitary gonadotropes is essential for stimulating ovulation and is thus required for a successful pregnancy. To meet the requirements to mount the LH surge, gonadotrope cells display plasticity at the cellular, molecular and morphological level. First, gonadotrope cells heighten their sensitivity to an increasing frequency of hypothalamic GnRH pulses by dynamically elevating the expression of the GnRH receptor (GnRHR). Following ligand binding, GnRH initiates highly organized intracellular signaling cascades that ultimately promote the synthesis of LH and the trafficking of LH vesicles to the cell periphery. Lastly, gonadotrope cells display morphological plasticity, where there is directed mobilization of cytoskeletal processes towards vascular elements to facilitate rapid LH secretion into peripheral circulation. This mini review discusses the functional and organizational plasticity in gonadotrope cells including changes in sensitivity to GnRH, composition of the GnRHR signaling platform within the plasma membrane, and changes in cellular morphology. Ultimately, multimodal plasticity changes elicited by gonadotropes are critical for the generation of the LH surge, which is required for ovulation.

**Keywords: gonadotrope, gonadotropin releasing hormone receptor, plasticity, luteinizing hormone, luteinizing hormone surge, fertility**

OPEN ACCESS

***Edited by:***
*Finn-Arne Weltzien, Norwegian University of Life Sciences, Norway*

***Reviewed by:***
*Philippa Melamed, Technion Israel Institute of Technology, Israel*
*Trude Marie Haug, University of Oslo, Norway*

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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *10 October 2020*
***Accepted:*** *15 December 2020*
***Published:*** *04 February 2021*

***Citation:***
*Clay CM, Cherrington BD and Navratil AM (2021) Plasticity of Anterior Pituitary Gonadotrope Cells Facilitates the Pre-Ovulatory LH Surge. Front. Endocrinol. 11:616053. doi: 10.3389/fendo.2020.616053*

## INTRODUCTION

The anterior pituitary is the body's master endocrine gland and is, arguably, the most complex endocrine organ in the body. At maturity, this gland is responsible for the synthesis and secretion of hormones, which control homeostasis, growth, lactation, and reproduction in mammals (1). It is composed of five distinct endocrine cell types which secrete six different hormones: gonadotropes release luteinizing hormone (LH) and follicle-stimulating hormone (FSH); thyrotropes release

**Abbreviations:** GnRH, gonadotropin releasing hormone; GnRHR, gonadotropin releasing hormone receptor; LH, luteinizing hormone; FSH, follicle stimulating hormone; MAPK, mitogen activated protein kinase; ERK, extracellular signal regulated kinase.

thyroid-stimulating hormone (TSH); corticotropes release adrenocorticotropic hormone (ACTH); somatotropes release growth hormone (GH); and lactotropes release prolactin (Prl) (2). Collectively, anterior pituitary cells are under hypothalamic control and receive feedback from the periphery to adjust hormone secretion to keep the body in homeostatic balance (3).

In mammals, reproductive competence depends on the coordinated activation of gonadotrope cells in response to pulsatile hypothalamic gonadotropin releasing hormone (GnRH) (4, 5). Following release, GnRH is transported *via* the hypophysial portal vessels to the anterior pituitary where it binds to the GnRH receptor (GnRHR) on gonadotropes (6, 7). Stimulation of the GnRHR activates a complex array of intracellular signaling networks that culminates in the synthesis and secretion of the heterodimeric glycoproteins, LH and FSH, into systemic circulation where they regulate gonadal development and function (5, 8). The gonadal steroid hormones, estrogen, progesterone, and androgen, participate in a feedback loop with the hypothalamus and the anterior pituitary to regulate GnRH and gonadotropin secretion (9–11). Taken together, any substantive physiological response of gonadotrope cells represents a complex integration of multiple hormones and signaling pathways that culminate in the functional plasticity of distinct gene programs and protein expression over the course of the estrous cycle.

In females, a mid-cycle surge of LH is obligatory for ovulation. The "LH surge" is initiated by a rise in estradiol-17β (E2) production by the ovarian follicles, triggering a switch from negative feedback on the hypothalamus and pituitary gland to transient positive feedback (5, 11). E2 positive feedback is driven by activation of ERα in kisspeptin neurons in the hypothalamus, which stimulate GnRH neurons to increase GnRH pulse frequency (12–14). In addition, rising E2 levels prior to ovulation stimulate GnRHR expression in multiple model systems (15–17). The dynamic increase in GnRHR expression represents an important priming event, which increases the sensitivity of gonadotropes to GnRH in preparation for the LH surge (18–20). During this critical pre-ovulatory window, gonadotrope cells demonstrate both functional and organizational plasticity to induce the LH surge. At the simplest level, gonadotrope functional plasticity is illustrated by the changes in the intracellular stores of LH and FSH and the relative abundance of secretory granules following GnRH stimulation (21). Collectively then, changes in gonadotrope synthesis and secretion of LH are not only dependent on changes in GnRH pulse frequency, but also the number of GnRH receptors available for binding and, consequently, the responsiveness to a given dose of GnRH. Finally, GnRH and, perhaps, E2, initiate dynamic organizational plasticity within the gonadotrope population that results in the development of cellular processes or projections containing LH that extend toward capillary sinusoids (22, 23). In this mini review we will address each of these dynamic changes that underlie gonadotrope plasticity including altered sensitivity to hypothalamic GnRH, the GnRHR signaling platform, and morphological alterations in the gonadotrope network.

# GONADOTROPE FUNCTIONAL PLASTICITY

## The Gonadotrope Population

Gonadotropes are a minority population of cells representing between 5% and 15% of the anterior pituitary gland and are traditionally defined by a specific phenotype that includes expression of 4 unique genes, the common glycoprotein hormone α subunit, the unique LHβ and FSHβ subunits and the GnRHR (24). Gonadotropes are classically viewed as a single distinct cell lineage, however, as unbiased technological methods have advanced, it is increasingly clear that the gonadotrope population has greater complexity and plasticity with cell-type composition and hormonal expression than previously recognized (25). For example, gonadotropes can be classified as either mono or bihormonal based on their expression of LH and/or FSH (26, 27). Gonadotropes can further be classified by their size as "small", "medium", and "large" cells whose percentages display distinct changes during different stages of the reproductive cycle (28, 29).

Gonadotrope plasticity across the estrous cycle has been well documented (25, 30). For example, the percentage of bi-hormonal gonadotrope cells dynamically elevates during the pre-ovulatory period when large amounts of gonadotropin are released into systemic circulation (26, 31). Beyond changes in LH and FSH levels in gonadotropes, our current understanding of the pituitary expands beyond mutually exclusive cell lineages to include transdifferentiation. In support of this, somatotrope cells may transition into gonadotropes to support the LH surge by upregulating gonadotropin mRNA expression (32). Gonadotrope population plasticity is also supported by recent single cell RNA-seq analysis which described a unique cluster of cells containing Prl, Gh, Tshb, POMC, and Lhb mRNAs that represents approximately 5%–11% of the total anterior pituitary cell population in mice (33). This result is intriguing as traditionally gonadotropes and corticotropes derive as POU1F1 independent lineages during embryonic development (34). The developmental origins and transdifferentiation mechanisms of these multi-hormonal cell clusters is uncertain but clearly warrants further investigation especially considering mRNA levels do not necessarily correlate with protein expression.

Despite this unknown, it is interesting that the multi-hormonal pituitary cells represent a shift-able pool of cells that alter gene programs depending on the physiological state. For example, Prl expression in the population is significantly upregulated during pregnancy to initiate and sustain lactation postpartum. Concurrently, the multi-hormonal cells downregulate Lhb to impair ovulation and fertility during the lactation state (33). Thus, this multi-hormonal cell population is dynamic and functions to alter endocrine output to meet physiological demand. It is an intriguing possibility that these multi-hormonal cells may play a role in supporting the LH surge required for ovulation as estradiol has emerged as a stimulus for promoting transdifferentiation (35). Previous studies also found that multi-hormonal anterior pituitary cells are not only more abundant in females compared to males, but also display greater

plasticity (33, 36, 37). Thus, female specific plasticity may underlie a broader mechanism under which clusters of multi-hormonal cells adjust their output (LH) based on hypothalamic (GnRH) and sex steroid (E2) hormonal input prior to ovulation to collectively maximize LH production necessary for the surge. Taken together, it is clear that the gonadotrope population represents a complex heterogeneous cell network that integrates multiple physiological queues to modulate gonadotropin secretion.

## GnRH Receptor Expression

Ovulation is perhaps the most fundamental event in reproduction in virtually all female vertebrates. It requires a dramatic 20-30-fold surge of LH released by the anterior pituitary into the peripheral circulation to induce the release of the oocyte from the ovary (38). The dynamic change in GnRHR expression has received particular attention as an important mediator of the LH surge, since it functions to increase the sensitivity of gonadotropes to GnRH during the periovulatory period when serum concentrations of progesterone decline as a result of luteolysis and E2 levels rise with the development of the preovulatory follicle (39). The elevation of ovarian E2 leads to a 3–6 fold increase in GnRHR numbers in gonadotrope cells followed temporally by increased hypothalamic GnRHR secretion (20). It is also clear that the heightened expression of GnRHRs in response to E2 is a result of enhanced transcription of the *Gnrhr* gene (39). Although the mechanisms by which E2 stimulates *Gnrhr* gene expression are not clearly defined, it may be partially through a membrane-initiated signal proceeding through a cAMP-dependent mechanism (40).

Certainly, the loss or dysregulation of hypothalamic input to gonadotropes underlies a number of conditions of hypogonadotropic hypogonadism; however, it is becoming increasingly clear that diminished pituitary sensitivity to GnRH may also contribute to irregular ovulatory cycles. Of particular note is the inverse relationship between GnRHR levels and elevated BMI associated with obesity. Work in both humans and mice suggest that gonadotrope cells are regulated by metabolic signals, including the hormone leptin (41–43). More specifically, Gwen Childs research provides a direct link between leptin signaling and GnRHR expression in mice (41, 44). Leptin resistance, which is characteristic of an obese state, results in a loss of GnRHR expression on the plasma membrane that may reflect a post-transcriptional regulatory mechanism (41). Additional work by Nanette Santoro's group and others highlights that obesity reduces LH pulse amplitude in women without a corresponding change in LH pulse frequency (43, 45, 46). These women present with a relative hypogonadotropic hypogonadal condition that appears to reflect diminished pituitary sensitivity to GnRH. If correct, then changes in the metabolic state may alter the ability of E2 to either elicit enhanced GnRHR expression or to activate the key gene programs that effectively couple GnRHR activation and increased gonadotropin production. Unfortunately, as gonadotropes only reflect approximately 5%–15% of the total population of endocrine cells in the pituitary, it is difficult to probe the unique transcriptional events evoked by E2 in gonadotropes (24). Recently, however, targeted expression of EGFP followed by Fluorescence Activated Cell Sorting (FACS) and RNA-seq allowed for the first characterization of global changes in gene expression in response to E2. This opens new avenues to explore the impact of pathological conditions (e.g. metabolic state) on E2 regulation of gonadotropes in a non-rodent species (sheep) that displays an estrous cycle that closely mimics the menstrual cycle of women (47).

## GnRH Receptor Organization

Following plasma membrane expression, GnRH-occupied GnRHR couples to Gαq/11 leading to stimulation of phospholipase C, elevation of intracellular free calcium and activation of kinase C (PKC) isoforms (48, 49). These early events underlie GnRH activation of multiple mitogen activated protein kinase (MAPK) signaling cascades including extracellular signal regulated kinase (ERK) (50–52). The ERK signaling module has been studied extensively in gonadotropes and is linked to the transcriptional regulation of the *lhb* gene through induction of the immediate early gene Egr-1 (53–55). It is well documented that faster GnRH pulse frequencies generated during the pre-ovulatory period initiate distinct ERK activation patterns that are responsible for dynamic Egr-1 upregulation necessary for LHβ synthesis required for the LH surge (56, 57). *In vivo* work highlights that the lack of ERK signaling in gonadotrope cells results in an acyclic, anovulatory phenotype in female mice due to the loss of Egr-1 and LHβ subunit expression (55). Thus, GnRH induced activation of ERK is absolutely required for expression of LHβ and ovulation underscoring the importance of this signaling pathway within the female reproductive axis.

It is also clear that proper second messenger signaling in gonadotropes is dependent on GnRHR localization to highly compartmentalized microdomains in the plasma membrane (58). Membrane microdomains efficiently and spatially organize the initiation of intracellular signaling by co-localizing membrane receptors and their cognate downstream signaling components in complexes that, upon ligand activation, co-segregate to form transient signaling platforms (59, 60). Within gonadotropes, the GnRHR and downstream signaling intermediates including c-raf kinase, ERK, calmodulin and Gαq are detectable in microdomains in primary cultures of mouse pituitary cells (58). Additionally, the GnRHR co-immunoprecipitated with ERK from microdomain fractions prepared from mouse pituitary cells suggesting that GnRHR and ERK reside in the same microdomain (61). Functionally this is important because following disruption of gonadotrope microdomain organization, GnRHR is no longer capable of initiating ERK activation (58). Taken together, microdomains serve as a spatial platform that allows for precise organization of a multi-protein scaffold that underlies GnRH initiation of ERK activation. What awaits elucidation is the plasticity of the GnRHR signaling proteome. More specifically, how does composition of the GnRHR signaling platform dynamically change over the course of the estrous cycle?

Fluorophore-tagged GnRH receptors have allowed direct observations of the in-membrane behavior of unoccupied,

agonist occupied, and antagonist-occupied receptors (62, 63). Fluorescence resonance energy transfer methods demonstrated that agonist but not antagonist leads to self-association of GnRH receptors in the plasma membrane (62). Thus, GnRH (agonist) induced receptor self-association in gonadotropes likely facilitates assembling multiple discrete GnRHR containing membrane microdomains to transduce a GnRH signal that leads to functional ERK activation and subsequent LH synthesis in gonadotropes.

Dynamic assembly of microdomains within the plasma membrane following GnRH activation suggests a role for the cytoskeleton in mediating their aggregation. Consistent with this, many of the molecular components regulating the actin cytoskeleton have been shown to associate with membrane microdomains (64, 65). An emerging concept is microdomains serve as centers for organizing ligand mediated communication between the plasma membrane and the actin cytoskeleton to control nucleation (66). The cytoskeleton has critical importance in gonadotropes as inhibition of actin remodeling effectively blocks GnRH signaling to ERK but also more acute signaling events such as the opening of L-type calcium channels in the plasma membrane (61, 67). Additionally, the disruption of GnRH-induced actin remodeling events blocks LH secretion from primary murine gonadotrope cells (68). Taken together, there appears to be interdependence between actin reorganization, compartmentalized GnRHR signaling to ERK and GnRH-mediated secretory events. Thus, understanding GnRH regulation of actin dynamics has clear implications for both LH biosynthesis and GnRH mediated release of LH into the bloodstream. Both of these pituitary events are critical for generating the large increase in LH secretion necessary for ovulation.

## GONADOTROPE ORGANIZATIONAL PLASTICITY

### The *In Vivo* Gonadotrope Network

In gonadotropes, the cytoskeleton plays a critical role in the regulated release of LH following GnRH stimulation (68, 69). Following ligand binding, the actin network reorganizes to allow vesicles to fuse with the plasma membrane to secrete hormone into the extracellular space. Reserve vesicles then move along microtubules and actin to replenish those vesicles that successfully docked with the plasma membrane (70, 71). *In vivo* and *ex vivo* evidence suggests that GnRH-stimulated gonadotropes are also capable of developing processes that extend directionally toward blood vessels during LH secretory episodes (22, 72, 73). Additionally, our group has demonstrated that primary pituitary cells concentrate LHβ into areas of dynamic membrane reorganization after GnRH treatment and, as previously discussed, disruption of the actin cytoskeleton inhibits LH release (68). These observations suggest that GnRHR engagement of the cytoskeleton not only facilitates the exocytosis of LH granules but also organizes these cells into a favorable spatial orientation to achieve a rapid and pronounced increase in circulating LH *in vivo*.

Towards this end, gonadotrope cells are organized in homotypic and heterotypic cellular networks that are often embedded in connective tissue surrounded by rich vascular networks (74–76). Capillary endothelia are fenestrated facilitating transfer of materials between the interstitium and the blood. Depending on the physiological state of the animal, such as estrous cycle stage, gonadotrope cells display considerable surface area apposed to capillary endothelium (73, 77). At the population level, gonadotropes can undergo plasticity changes that allow for coordination with one another for efficient and robust delivery of gonadotropin into the capillary (22, 74, 78). Previous work suggests that steroid hormones can regulate structural plasticity and cell-to cell connectivity of pituitary cells. For example, following castration, somatotropes within a living pituitary slice were found more clustered and responsive to growth hormone within hours (79). In gonadotropes, pretreatment of *ex vivo* murine pituitary slices with 10 nm E2 was capable of significantly enhancing gonadotrope responsiveness to GnRH with respect to cellular process generation and extension (22). The stimulatory E2 effect on gonadotropes was evident in experiments with long-term (14 h) but not short-term (1.5 h) exposure (22). The time dependence is consistent with upregulation of E2 mediated increases in GnRHR synthesis and increased responsiveness to GnRH necessary to generate the LH surge (80). The timing is also consistent with E2 upregulation of a number of genes involved in cell movement and the cytoskeleton (47).

Lastly, if gonadotropes position themselves to maximize their secretory surface to capillary sinusoids then LH secretory granules would need to be polarized to the side of the gonadotrope nearest to a vascular sinusoid during the preovulatory period. Importantly, this appears to be the case. In ovine pituitary cells, LH granules were polarized in about 20-30% of gonadotropes with this number increasing to 80% during the preovulatory LH surge (77, 81). Additionally, positive feedback from E2 can directly affect the mobilization of LH secretory granules (82). The mechanisms by which GnRH and E2 contribute to LH polarization are unclear but gonadotropes appear "primed" organizationally to support the LH surge. Collectively gonadotrope priming reflects multiple events that include enhanced GnRH responsiveness, transport and polarization of LH granules and increased apposition to the vascular system.

## CONCLUSIONS

The dynamic changes exhibited by gonadotropes in preparation for the LH surge reflects a culmination of events following GnRH binding. These events include enhanced GnRHR responsiveness (driven by elevated E2), GnRHR aggregation, activation of ERK and upregulation of the immediate early gene Egr-1, synthesis and polarization of LH granules, engagement of the actin cytoskeleton, and increased apposition to pituitary vasculature. GnRH, in combination with E2, facilitates plasticity changes that not only underlie LH synthesis, but also to place the gonadotrope population containing LH secretory granules in close proximity to vasculature to maximize circulating LH concentrations. Thus,

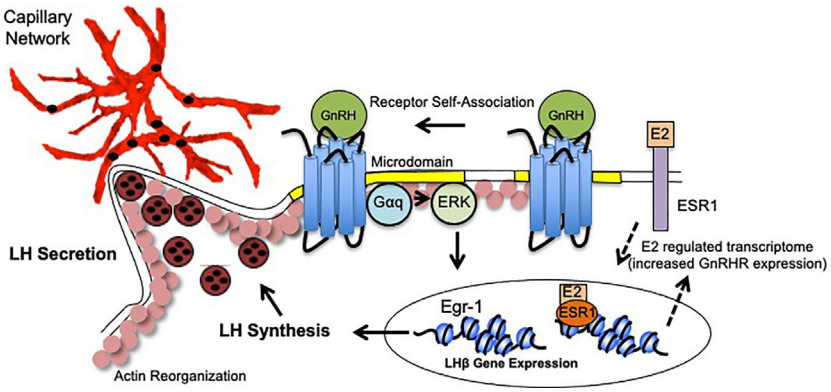


**FIGURE 1** | Model of Gonadotrope Dynamics Underlying the LH Surge. During the periovulatory period, ovarian estradiol-17(E2) levels rise. E2 activation of both plasma membrane associated and nuclear ESRI elicits expression of a unique gene program that includes enhanced expression of the GnRH Receptor (GnRHR), and therefore heightened sensitivity to hypothalamic GnRH (dashed arrows). Once expressed, the GnRHR is constitutively localized to microdomains in the plasma membrane (yellow). Following GnRH binding, the GnRHR self-associates to form dimers/oligomers. Second messengers including Gaq/1l and ERK are activated within the microdomains. Increased ERK activity leads to the activation of the immediate early gene Egr-1 which upregulates *lhb* gene expression. Functional LH is synthesized and packaged into secretory vesicles that embed in the cortical actin cytoskeleton (pink circles). GnRH activation also causes reorganization of the actin cytoskeleton which allows for the gonadotropes to appose the capillary endothelium (red) to release LH into circulation (black circles).

it is the collective functional and organizational plasticity induced by gonadotropes that mediate the preovulatory LH surge (**Figure 1**).

The mechanisms that underlie the surge in LH release from anterior pituitary gonadotropes have fascinated endocrinologists for decades. It is clear that GnRH and E2 are critical regulators of the LH surge, but mechanistic questions remain regarding how these and other hormones induce plasticity at the cellular, molecular, and gonadotrope network level. For example, how the gonadotrope network communicates *in vivo* to initiate directed movement towards vasculature is still unknown as is the potential role of pituitary cell transdifferentiation during the LH surge. Questions also remain about the role of other steroid regulators, such as progesterone (P4), in inducing the LH surge. Progesterone has historically been thought to suppress gonadotrope function and LH release, although some studies suggest that low P4 levels late in the follicular phase signal the hypothalamus to increase GnRH secretion (83). How this potential mechanism relates directly to gonadotropes requires further studies. Understanding the mechanism that control gonadotrope plasticity is not only of interest at a basic scientific level but also has profound clinical implications from the development of novel birth control and fertility treatments. Future studies will be critical to elucidate the combinatorial effects of endocrine, paracrine, autocrine, and metabolic stimuli on gonadotrope plasticity.

## AUTHOR CONTRIBUTIONS

All authors contributed to the article and approved the submitted version.

## FUNDING

This publication was funded by National Institutes of Health, P20GM103432 and R21HD090541 (BC, AN) and R01HD065943 (CC).

## ACKNOWLEDGMENTS

Our apologies to the many laboratories and investigators who have contributed to the field whose research we did not cite due to space limitations.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

ORIGINAL RESEARCH
published: 16 February 2021
doi: 10.3389/fendo.2020.615777



OPEN ACCESS

**Edited by:**
*Finn-Arne Weltzien, Norwegian University of Life Sciences, Norway*

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**Specialty section:**
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

**Received:** *09 October 2020*
**Accepted:** *15 December 2020*
**Published:** *16 February 2021*

**Citation:**
*Rojo-Ruiz J, Navas-Navarro P, Nuñez L, García-Sancho J and Alonso MT (2021) Imaging of Endoplasmic Reticulum $Ca^{2+}$ in the Intact Pituitary Gland of Transgenic Mice Expressing a Low Affinity $Ca^{2+}$ Indicator. Front. Endocrinol. 11:615777. doi: 10.3389/fendo.2020.615777*

# Imaging of Endoplasmic Reticulum $Ca^{2+}$ in the Intact Pituitary Gland of Transgenic Mice Expressing a Low Affinity $Ca^{2+}$ Indicator

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The adenohypophysis contains five secretory cell types (somatotrophs, lactotrophs, thyrotrophs, corticotrophs, and gonadotrophs), each secreting a different hormone, and controlled by different hypothalamic releasing hormones (HRHs). Exocytic secretion is regulated by cytosolic $Ca^{2+}$ signals ($[Ca^{2+}]_C$), which can be generated either by $Ca^{2+}$ entry through the plasma membrane and/or by $Ca^{2+}$ release from the endoplasmic reticulum (ER). In addition, $Ca^{2+}$ entry signals can eventually be amplified by ER release *via* calcium-induced calcium release (CICR). We have investigated the contribution of ER $Ca^{2+}$ release to the action of physiological agonists in pituitary gland. Changes of $[Ca^{2+}]$ in the ER ($[Ca^{2+}]_{ER}$) were measured with the genetically encoded low-affinity $Ca^{2+}$ sensor GAP3 targeted to the ER. We used a transgenic mouse strain that expressed erGAP3 driven by a ubiquitous promoter. Virtually all the pituitary cells were positive for the sensor. In order to mimick the physiological environment, intact pituitary glands or acute slices from the transgenic mouse were used to image $[Ca^{2+}]_{ER}$. $[Ca^{2+}]_C$ was measured simultaneously with Rhod-2. Luteinizing hormone-releasing hormone (LHRH) or thyrotropin releasing hormone (TRH), two agonists known to elicit intracellular $Ca^{2+}$ mobilization, provoked robust decreases of $[Ca^{2+}]_{ER}$ and concomitant rises of $[Ca^{2+}]_C$. A smaller fraction of cells responded to thyrotropin releasing hormone (TRH). By contrast, depolarization with high $K^+$ triggered a rise of $[Ca^{2+}]_C$ without a decrease of $[Ca^{2+}]_{ER}$, indicating that the calcium-induced calcium-release (CICR) *via* ryanodine receptor amplification mechanism is not present in these cells. Our results show the potential of transgenic ER $Ca^{2+}$ indicators as novel tools to explore intraorganellar $Ca^{2+}$ dynamics in pituitary gland *in situ*.

**Keywords: genetically encoded $Ca^{2+}$ indicator, transgenics, ER, calcium, organelle, aequorin, pituitary**

## INTRODUCTION

The anterior pituitary (AP) is a complex organ that controls a broad array of physiological functions such as growth, lactation, metabolism, or stress response (1). This functional heterogeneity is conferred by the heterogeneity in cell populations, both anatomically and functionally, that includes the core of the different axes of the endocrine system. The AP contains five endocrine cell types which control the secretion of different hormones. These include: growth hormone (GH, from somatotrophs), prolactin (PRL, from lactotrophs), follicle-stimulating hormone and luteinizing hormone (FSH and LH, from gonadotrophs), thyroid stimulating hormone (TSH, from thyrotrophs), and adrenocorticotropic hormone (ACTH, from corticotrophs).

According to the classical view, each AP cell type stores one single hormone, (or two, in the case of gonadotrophs), whose secretion is specifically regulated by a particular hypothalamic releasing hormone (HRH) (2, 3). However, distinct cell subpopulations expressing more than one single hormone have been reported (4–8). These multifunctional cells can be characterized by combining calcium imaging with labeling for multiple hormones by immunofluorescence (9). In addition to the polyhormonal cells, multi-responsive cells able to display $Ca^{2+}$ and secretory responses to more than one HRH have also been identified by some authors (4, 10, 11). The subpopulations of multifunctional cells exhibit a striking sexual dimorphism (9), with changes during sexual cycle (12), cold stress, and along lifespan (13). Multifunctional cells are also frecuently observed in pituitary human adenomas (14, 15) and its existence may provide the basis for the paradoxical secretion and transdifferentiation (4, 16–18). However, all the above studies have only been carried out in primary cultures of rat and mouse AP cells where the interactions among different cell types and with the extracellular matrix are lost.

Recent studies using single cell transcriptomics have expanded our current knowledge on the gene expression profile associated with specific cell subtypes or AP functions in mice, rats or humans (8, 19–25).

AP provides an excellent model for endocrine excitation-secretion coupling. In the last decades AP studies have provided seminal insights into the mechanisms involved in endocrine stimulus-secretion coupling and regulation by ion channels activity. Exocytic secretion is regulated by cytosolic $Ca^{2+}$ signals ($[Ca^{2+}]_C$), which can be generated either by $Ca^{2+}$ entry from the extracellular medium through the plasma membrane and/or by $Ca^{2+}$ release from the endoplasmic reticulum (ER) (26). $Ca^{2+}$ influx can occur as a consequence of the transient depolarization of the plasma membrane, which opens the voltage-activated $Ca^{2+}$ channels present in AP cells. The ER is the main $Ca^{2+}$ store in most cells, including AP cells. The resting ER $Ca^{2+}$ concentration ($[Ca^{2+}]_{ER}$) approaches $10^{-3}$ M, in contrast to the resting $[Ca^{2+}]_C$, which is ~$10^{-7}$ M (27). This high $[Ca^{2+}]_{ER}$ is maintained by the equilibrium between SERCA, pumping inside the ER, and passive $Ca^{2+}$ efflux from the ER to the cytosol through non-specific leak channels and/or through specific channels, such as the inositol trisphosphate receptor channels (IP3Rs) and/or ryanodine receptors (RyRs) (28). Binding of HRH to a G-protein coupled receptor (GPCR) leads to the activation of phospholipase Cβ (PLCβ) which hydrolyses phosphatidylinositol-4, 5- bisphosphate ($PIP_2$) to inositol-1, 4, 5-trisphosphate (IP3). Due to the large $Ca^{2+}$ gradient between the ER and the cytosol, IP3 releases $Ca^{2+}$ from the intracellular stores, and this elicits an increase in the $[Ca^{2+}]_C$ and secretion of the corresponding hormone.

At least 15 subtypes of Gq/11-coupled GPCRs have been described in AP cells, as well as several receptor tyrosine kinases, whose activation leads to the mobilization of intracellular $Ca^{2+}$ in an IP3-dependent manner (29). For example, lactotrophs and thyrotrophs are primarily activated by thyrotropin-releasing hormone (TRH) and gonadotrophs by luteinizing hormone releasing hormone (LHRH, also named GnRH). Other ligands that bind Gq/11-coupled receptors include ATP, acetylcholine, angiotensin, endothelin, serotonin, substance P, or vasoactive intestinal peptide/pituitary adenylate cyclase-activating peptide (26, 29, 30). Emptying of the ER $Ca^{2+}$ stores can trigger the subsequent opening of the store-operated $Ca^{2+}$ entry pathway in the plasma membrane (31, 32). This is supported by the findings that ER $Ca^{2+}$ emptying by inhibitors of the SERCA pump (33) or blockage of the store-operated $Ca^{2+}$ channel (32), both antagonized the secretion of adrenocorticotropin. In addition, after the initial $Ca^{2+}$ peak, some AP cell populations displayed an oscillatory $Ca^{2+}$ pattern. For example, in mammalian gonadotrophs, the initial $Ca^{2+}$ pulse triggered by LHRH is typically followed by a large baseline of $[Ca^{2+}]_C$ oscillations, which are dependent on IP3 (34, 35). In GH3 pituitary cells, emptying of the ER $Ca^{2+}$ stores with thapsigargin produced a sustained increase of $[Ca^{2+}]_C$ attributable to $Ca^{2+}$ release and activation of store-operated calcium entry. Besides, superimposed dihydropyridine-sensitive $[Ca^{2+}]_C$ oscillations attributable to L-channel activity are observed (31).

In addition to activation of GPCRs, ER $Ca^{2+}$ release can be generated by amplification of a small primary $Ca^{2+}$ influx through calcium-induced calcium release (CICR). In frog melanotrophs, it appears that spontaneous voltage-activated $Ca^{2+}$ influx is coupled to CICR, presumably through IP3Rs (36). Blocking $Ca^{2+}$ entry, by removing external $Ca^{2+}$ or adding a $Ca^{2+}$ channel blocker, for example, will also inhibit $Ca^{2+}$ release due to passive ER $Ca^{2+}$ depletion. Hence, monitoring exclusively cytosolic $Ca^{2+}$ does not unequivocally allow to discriminate between the two sources of $Ca^{2+}$. It is,

**Abbreviations:** ACTH, adrenocorticotrophic hormone; AP, anterior pituitary; CICR, $Ca^{2+}$-induced $Ca^{2+}$-release; CRH, corticotropin releasing hormone; ER, endoplasmic reticulum; FSH, follicle-stimulating hormone; GAP, GFP-Aequorin Protein; GECI, genetically encoded calcium indicator; GFP, green fluorescent protein; GH, growth hormone; GnRH, gonadotropin releasing hormone; HRH, hypothalamic releasing hormone; LH, luteinizing hormone; LHRH, luteinizing hormone releasing hormone; PRL, prolactin; SERCA, sarco-endoplasmic reticulum $Ca^{2+}$ ATPase; SNR, signal to noise ratio; TBH, 2,5-ditert-butylbenzohydroquinone; TRH, thyrotropin-releasing hormone; TSH, thyroid stimulating hormone; $[Ca^{2+}]_C$, $[Ca^{2+}]_{ER}$, $Ca^{2+}$ concentrations in cytosol or ER, respectively.

therefore, necessary to make use of specific $Ca^{2+}$ tools that unambiguously allow identification of the origin of $Ca^{2+}$ in response to a secretagogue. This need is even more evident in the case of endocrine AP cells, where the unique combination of ion channels, excitability mechanisms and signaling pathways determines hormone secretion in a cell specific manner. It is, therefore, crucial to monitor directly ER $Ca^{2+}$ dynamics to unveil the unique and diverse $Ca^{2+}$ signaling mechanisms underlying anterior pituitary cell-specific regulation.

We have recently described a new generation of ratiometric $Ca^{2+}$ indicators (GAP, for GFP-Aequorin-Protein) that can be targeted to various organelles (37). GAP3 is optimized for measuring intraluminal $Ca^{2+}$ in the ER matrix (38). Here we exploited the fact that erGAP3 transgenic mice express the ER-$Ca^{2+}$ indicator GAP3 in pituitary gland, to study the contribution of the ER $Ca^{2+}$ stores to the $Ca^{2+}$ signals elicited by a distinct hypothalamic secretagogue. By simultaneously imaging cytosolic- and ER $Ca^{2+}$ signals at the single cell level in an intact gland preparation, we compared the ER $Ca^{2+}$ responses to different releasing hormones in a variety of AP preparations. Our study demonstrates the potential of a genetically encoded $Ca^{2+}$ sensor expressed in transgenic mice for recording intraorganellar $Ca^{2+}$ responses in intact AP.

# METHODS

## Transgenic Mice

All the procedures concerning mice were approved by the animal care committee of the University of Valladolid. The generation of erGAP3 mice was described elsewhere (38). To target GAP3 to the ER (erGAP), the calreticulin signal peptide and the KDEL ER-retention peptide, were fused in frame to the 5'- and the 3'-end of the GAP gene, respectively. erGAP3 was controlled by the CAG-GS promoter (39). Mice were housed under specific pathogen-free (SPF) conditions. Tail DNA was routinely screened by PCR using two oligonucleotides, forward and reverse primers for GAP3, 5'-GATGGCAACATCCT CGGACA-3' and 5'-GTCCTTGCTCAGGGCTGATT-3' (234 bp product), respectively. Lines 1 and 10 of erGAP3 mice were used. Mice were maintained in heterozygosity.

## Immunofluorescence

Male and female mice were anesthetized with ketamine (80 mg/Kg) and xylazine (10 mg/Kg) and transcardially perfused with 0.9% physiological saline followed by a solution of 4% paraformaldehyde (~20 ml) and then overnight post-fixed in the same solution at 4°C. The tissue was cryoprotected in 30% sucrose and then processed for immunohistochemistry. Glands were cryosectioned (10–15 μm thick) and stored at −80 °C until use. Slides were permeabilized in PBS containing 0.5% Triton X-100 for 1 h and blocked in PBS with 10% goat serum. Slides were incubated overnight with specific antibodies against each hormone (ACTH, FSH, TSH, PRL, LH and GH) diluted (1:1,000; 1:100; 1:2,000 1:1,000; 1:1,000; 1:1,000; respectively) in fresh blocking buffer. Antisera was a generous gift from Dr. Parlow (National Hormone & Peptide Program Harbor-UCLA Medical Center, Torrance, CA). As a secondary antibody, an anti-rabbit antibody coupled to Alexa Fluor 568 (Roche) diluted 1:500 in fresh blocking buffer was used, which was incubated for 30 min. Staining controls with secondary antibody alone elicited no fluorescent signal. Nuclei were stained with Hoechst 33342. Fluorescence images were collected on a Zeiss upright Axioplan 2 microscope, using a 63X W "C-Apochromat" objective (N.A. 1.2). The fluorescence filters used were: red fluorescence: Ex 546/12, Em LP590; green fluorescence: Ex 470DF35, Em 535DF35; blue fluorescence: Ex 390/22, Em 460/50 nm. For cell quantification, positive cells randomly chosen within various fields per section corresponding to various sections per gland were analyzed using the ImageJ software. For each hormone, the percentage of labeled cells was calculated by dividing by the total number of cells, evaluated from the labeled nuclei.

## Calcium Imaging in Dissociated AP Cells

The basic protocol was previously described elsewhere (9). Briefly, mice were euthanized by cervical dislocation and the AP glands were quickly removed and digested with trypsin (1 mg/ml, Sigma) in Minimum Essential Medium (S-MEM; Gibco) for 30 min at 37°C. Dispersed cells were plated onto coverslips previously coated with poly-L-lysine-coated (0.01 mg/ml) and cultured in Dulbecco's modified Eagle's medium (DMEM; Gibco) supplemented with 10% fetal bovine serum and antibiotics. Experiments were performed after 2–6 h of culture. Imaging was performed as described below for slices.

## Calcium Imaging in Pituitary Slices or Entire Gland

Mice (P7-5 months) from transgenic line erGAP3 (L1 or L10) were sacrificed by cervical dislocation. AP gland was dissected out and sliced into 350–400 μm thick sections with a Mcllwain Tissue Chopper and quickly transferred to a fine-meshed membrane filter and maintained in artificial cerebrospinal fluid (ACSF) containing: 125 mM NaCl, 2.5 mM KCl, 1 mM $MgCl_2$, 26 mM $NaHCO_3$, 1 mM $CaCl_2$, 10 mM glucose, 1.25 mM $NaH_2PO_4$, pH 7.4, continuously bubbled with a 95% $O_2$/5% $CO_2$ gas mixture at 25 °C. Slices were mounted onto the stage of a Zeiss Axioplan upright microscope equipped with a 20X objective (W-achroplan, Zeiss; NA= 0.5) and a Zeiss AxioCam camera MRm (12 bit) connected through a software interface (Axiovision, Zeiss) to a Xenon fluorescent excitation source and a filter wheel. GAPs were sequentially excited at 405 and 470 nm and acquired at 518–553 nm. For simultaneous measuring of $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_C$, slices were incubated for 1 h at room temperature with 8–12 μM Rhod-2 AM in bubbled ACSF medium. Rhod-2 was excited at 545 nm (546/12) using a dichroic mirror FT580 and light emitted was recorded above 590 nm (LP590). Pituitary slices were sequentially excited at 405, 470 (GAP) and 540 nm (Rhod). All the experiments were performed at 22–25 °C in a custom-made chamber of 42 μl volume under constant perfusion at 3 ml/min with an 'extracellular-like solution' containing 145 mM NaCl, 5 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 10 mM glucose, and 10 mM Na-HEPES (pH 7.4). All stimuli were diluted in this extracellular-like

medium and perfused for 30 s or the time indicated. Imaging of erGAP3 in the whole pituitary gland were performed similarly. Output images were captured with the AxioVision Rel 4.6.3 (Zeiss) software and pixel-to-pixel ratio analysed with ImageJ (https://imagej.nih.gov/ij/). The erGAP3 ratio R (F470/F405) was used as an index of $[Ca^{2+}]_{ER}$, and was expressed as R/R0. F540 was an index of $[Ca^{2+}]_{C}$, expressed as F/F0. R0 (or F0) was computed as the mean of the ratios (or F540) obtained during the first five to 10 frames of each experiment.

## Statistical Analysis

The data were analysed using Origin 7 (OriginLab™) and excel. Results are expressed as mean ± SEM, as indicated

# RESULTS

## Transgenic Mice Express erGAP3 in AP Cells

In order to monitor $[Ca^{2+}]_{ER}$ in intact AP glands we used erGAP3 expressing transgenic mice in which the biosensor was controlled by the ubiquitous promoter CAG-GS (38). Using fluorescence stereomicroscopy, we easily detected the endogenous green fluorescence of erGAP3 in the pituitary gland of erGAP3 transgenic mice (**Figure 1**). The pars anterior (PA) displayed a strong green fluorescence, in contrast to the neurohypophysis (N), which was negative for erGAP. The transgene was expressed in two independent transgenic mouse lines (lines 1 and 10). Virtually all the cells were positive (mean ± SEM: 97% ± 0.2; 1,312 cells, line 10; and 788 cells, line 1). The erGAP3 fluorescence was visible both in newborn (9 days; **Figures 1A, B**) and in adult mice (3 months; **Figures 1C, D**), indicating that the transgene expression is stable along the lifespan of the mouse line.

The low $Ca^{2+}$ affinity GAP3 (Kd~ 489 μM) was specifically targeted to the ER using the well-established strategy based on adding the signal peptide of calreticulin and the KDEL retention motif to the N-terminal and the C-terminal of the GAP gene, respectively (37, 40). The GFP positive cells showed a reticular pattern that extended throughout the entire cell and was excluded from the nucleus, as expected for localization to the endoplasmic reticulum (**Figures 1B, D**). Importantly, the GFP fluorescence was homogeneously distributed throughout the ER and no precipitates or punctate fluorescence were visible.

The cells in the AP can be classified on the basis of the stored hormone into somatotrophs (50%), lactotrophs (20%–25%), corticotrophs (10%–20%), gonadotrophs (10%), and thyrotrophs (5%) (41, 42). No apparent structural alterations were found in the pituitary gland of the erGAP3 transgenic mouse. When we

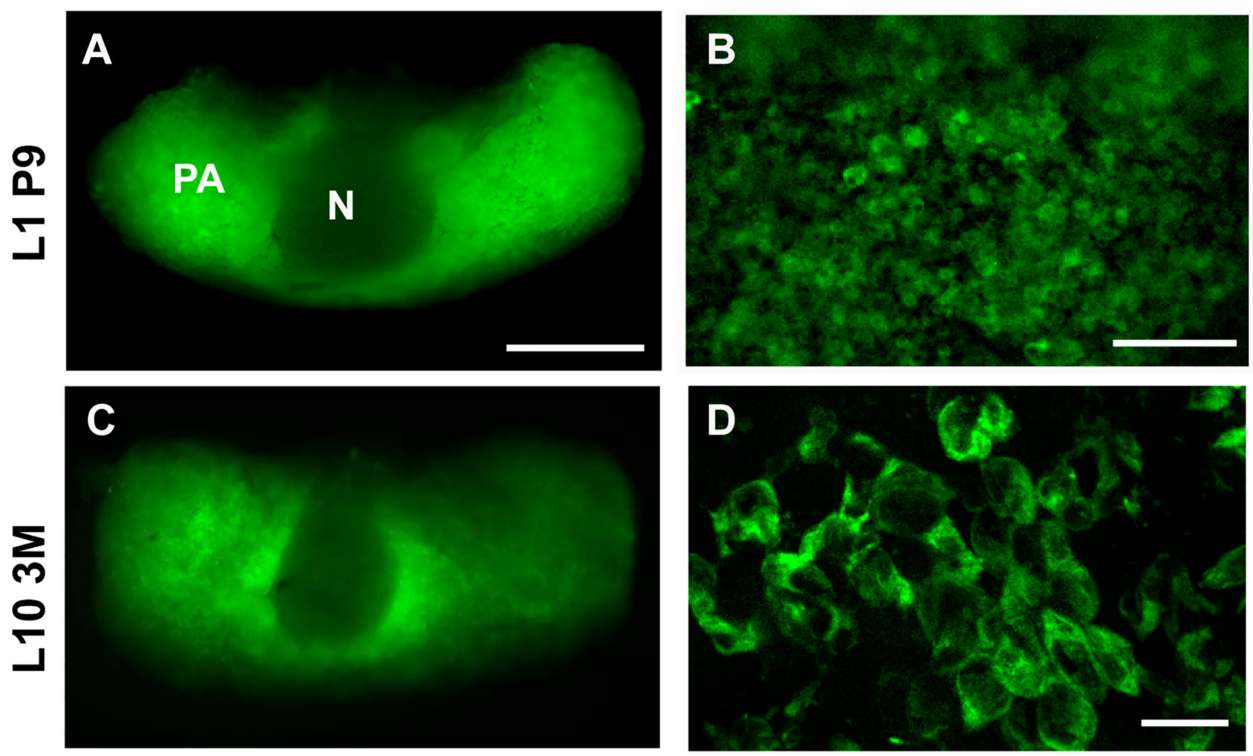


**FIGURE 1** | Expression of erGAP3 in the anterior pituitary (AP) of transgenic mice. **(A)** Dorsal view of an intact live pituitary isolated from a 9-day-old mouse of transgenic line L1. Image was taken at 470 nm excitation. N, neurohypophysis; PA, pars anterior. **(B)** Higher magnification view of **(A)**. **(C)** Dorsal view of intact live pituitary of a 3-months-old mouse of transgenic line L10 pituitary. **(D)** Confocal image of a cryosection (10 μm) of a fixed AP corresponding to **(C)**. Scale bar indicates 500 μm in **(A, C)**; 100 μm in **(B)**; and 20 μm in **(D)**.

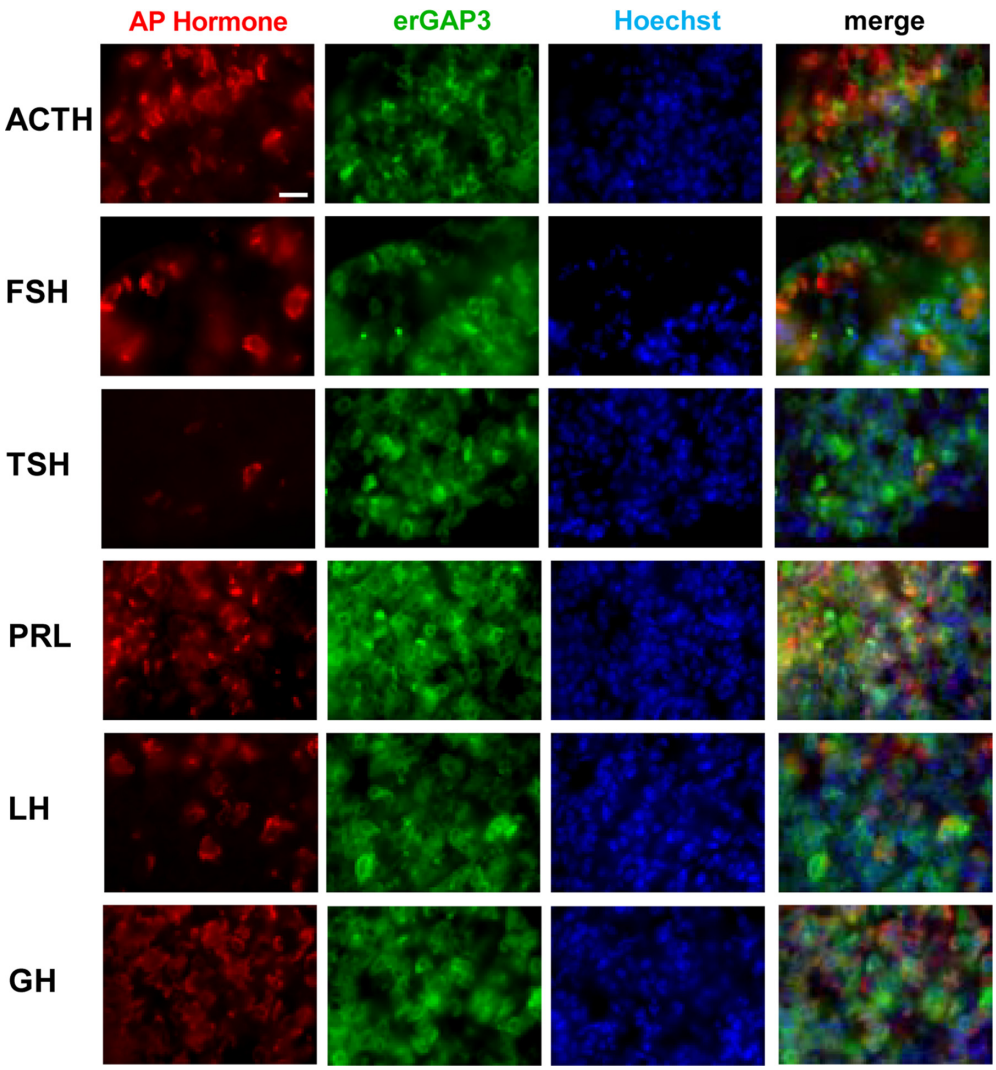


**FIGURE 2** | Immunohistochemical characterization of the pituitary in erGAP3 transgenic mice. An anterior pituitary (AP) cryosection was fixed and immunostained with specific antibodies against the AP hormones (ACTH, FSH, TSH, PRL, LH and GH). Nuclei were stained with Hoechst. Scale bar is 20 µm.

examined each cell type by immunofluorescence for the presence of the stored hormones, we found that the proportions were within the expected range (**Figure 2**). Importantly, the erGAP3 indicator was expressed in all five AP cell types (between 87 and 97% for each cell type).

## erGAP3 Is Functional in Dissociated AP Cells

The functionality of erGAP3 in pituitary cells was first examined in cultured cells dissociated from the pituitary gland of transgenic mice, since single cell imaging allows to record fluorescence changes with better optical conditions. Cultured cells displayed a robust erGAP3 expression in the ER. In the pituitary gonadotrophs, binding of LHRH to its receptor activates the $Ca^{2+}$/inositol phosphate signaling cascade (43, 44). Cell stimulation with LHRH (100 nM) provoked the expected reciprocal fluorescent signals of the two individual GAP excitation wavelengths, with an increase of the light emission when excited at 405-nm and a decrease when excited at 470-nm that reflects the decrease of $[Ca^{2+}]_{ER}$ (**Figures 3A, B**). Calculating the ratio between the two fluorescence emission values (F470/F405) yielded a net ER $Ca^{2+}$ decrease, a consequence of the release of $Ca^{2+}$ from the ER into the cytosol (**Figure 3C**). This response is expected for a factor coupled to the $Ca^{2+}$/inositol phosphate cascade. We observed a fractional decrease in the GAP3 ratio value of around 40%. The transient decrease returned to baseline levels upon agonist removal, demonstrating the reversibility of the response. In some cells, however, the ER $Ca^{2+}$ dropped but it did not recover the initial ER $Ca^{2+}$ level after washing. In other few cells, LHRH provoked a refilling of the ER $Ca^{2+}$ store (not shown). Taken together, these results show that erGAP3

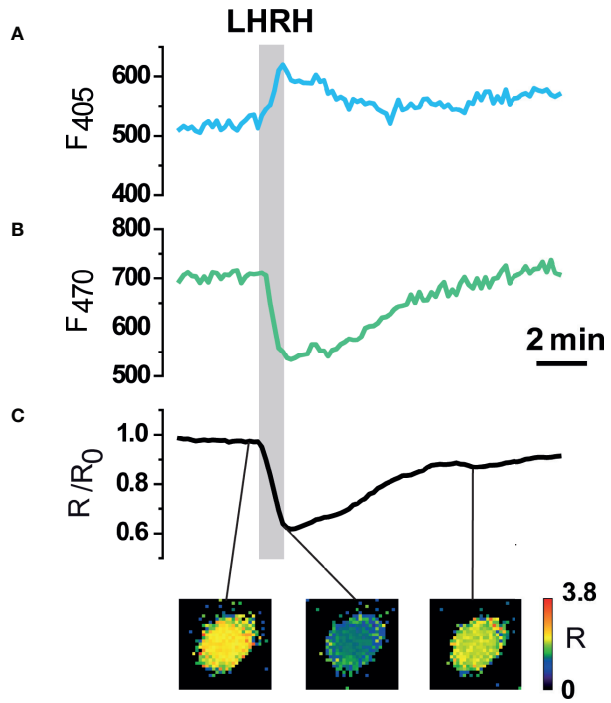


**FIGURE 3** | Functional expression of erGAP3 in dissociated anterior pituitary (AP) cells. **(A, B)** Individual excitation fluorescence channels (405 and 470 nm). **(C)** Ratiometric fluorescence response of erGAP3 (R) in dissociated pituitary cells isolated from transgenic line L10 (P6 mouse). Decrease of $[Ca^{2+}]_{ER}$ evoked by LHRH (100 nM). Note that the fluorescence signals at each wavelength change in the opposite direction. The decrease in the Ratio (R), expressed as R/Ro, indicates a decrease in the $[Ca^{2+}]_{ER}$. Representative trace of responses in a single cell (out of 266 erGAP3 expressing cells from 13 independent experiments from two mice) are shown. The pseudo-color images correspond to the R/R0 values at the time points in the graph (before stimuli; peak; after washing out).

expression in pituitary gland displays a performance of the $Ca^{2+}$ indicator comparable to that previously obtained in other cells types such as HeLa cells, HEK293 cells, astrocytes, or hippocampal neurones (38, 45–47).

## Simultaneous Imaging of $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_C$ in Acute Pituitary Slices

We next explored the ER $Ca^{2+}$ signals in acute AP slices obtained from erGAP3 transgenic mice, where tissue structure is better preserved than in dispersed single cell cultures. Also, we recorded $Ca^{2+}$ dynamics simultaneously in the ER and the cytosol by using erGAP3 in combination with Rhod-2, a high affinity cytosolic $Ca^{2+}$ indicator, whose red fluorescence is spectrally compatible with that of GAP3 (**Figure 4**). The results show that most of the cells analysed (97%; 69 of 71 cells; five slices; three mice) exhibited responses to LHRH (100 nM) with a strong and rapid decrease in $[Ca^{2+}]_{ER}$ (**Figure 4A**, gray trace) and a coordinated transient in the cytosolic $Ca^{2+}$ (**Figure 4A**, pale red trace). The addition of LHRH triggered an ER $Ca^{2+}$ release of ~30% (the R/R0 mean ± SEM decreased from 1 down to 0.77 ± 0.01; n=69 cells) and reached a lower steady-state level. Washout of LHRH during 5 min generally failed to refill the ER, which remained half-filled after the washout. This occurred even after the stimulation of $Ca^{2+}$ entry with a depolarizing pulse of high $K^+$ (80 mM), that provoked a large cytosolic transient with no ER $Ca^{2+}$ changes. The typical protocol finished with the perfusion of an ER depletion cocktail, used to determine the Rmin of erGAP3, that was composed of the sarco-endoplasmic reticulum $Ca^{2+}$-ATPase (SERCA) inhibitor 2,5-ditert-butyl-benzo-hydro-quinone (TBH; 10 μM) in $Ca^{2+}$-free medium. Interestingly, a quarter of the LHRH-responsive cells (25%; 17 of 71; five slices; three mice) also responded to thyrotropin-releasing hormone (TRH, 100 nM) with a rapid lumenal $Ca^{2+}$ release (R/R0 (mean ± SEM) decreased from 1 down to 0.88 ± 0.05; n=18 cells; **Figure 4A**, black trace) and a coordinated small cytosolic $Ca^{2+}$ increase (**Figure 4A**, red trace). In general, the ER $Ca^{2+}$ drop elicited by TRH was smaller than that provoked by LHRH and, after washing out, it recovered the basal $[Ca^{2+}]_{ER}$ value observed prior to the stimulus. Moreover, the amplitude of the LHRH-induced drop was smaller in these multireceptorial cells than that observed in the LHRH-responsive monoreceptorial cells (**Figure 4A**, black and gray drops). Furthermore, most LHRH positive cells did not exhibit any response to GHRH or to CRH, although a few cells showed a minute drop in the ER $Ca^{2+}$ not correlated with any cytosolic $Ca^{2+}$ changes (**Figure 4B**). We also found some exceptional cells that showed a response exclusively to TRH but not to LHRH (**Figure 4C**). Addition of acetylcholine (100 μM) triggered moderate opposing $Ca^{2+}$ transients in all cells, temporally coincident in the ER and the cytosol. It is noticeable that the depolarizing high $K^+$ medium induced an increase in the cytosolic $Ca^{2+}$ without exhibiting any significant change in the ER $Ca^{2+}$ store. We did not find any ER $Ca^{2+}$ release parallel to the increase in the cytosol, indicating that CICR mechanism acting through ryanodine receptors is not active in these cells.

Although no significant ER $Ca^{2+}$ changes were found upon addition of the corticotropin-releasing hormone (CRH), this factor provoked a small and sustained increment in the cytosolic $Ca^{2+}$ in a fraction of cells (F/F0 mean ± SEM; 1.03 ± 0.02; 24 of 76 cells; five slices; three mice; **Figure 5A**). This result is in agreement with the primary signaling cascade triggered by CRH, which is coupled to cAMP/PKA. Interestingly, the cells that showed a response to CRH also responded to TRH with a small but visible cytosolic $Ca^{2+}$ transient and to LHRH with a clear spike. This result indicates the presence of multireceptorial cells. These CRH-positive cells also displayed a large $Ca^{2+}$ spike in response to depolarization with high $K^+$ and to stimulation with acetylcholine. Finally, no responses were observed to growth-hormone releasing hormone (GHRH), neither in the cytosol nor in the ER. Spontaneous or HRH-induced cytosolic $Ca^{2+}$ oscillations, either with TRH or LHRH, were observed in some cells but no ER $Ca^{2+}$ changes were associated with them (**Figure 5B**).

## Imaging of $[Ca^{2+}]_{ER}$ in Intact Pituitary Gland

A higher level of tissue preservation was achieved by imaging ER $Ca^{2+}$ signals in the whole intact pituitary gland, where erGAP3 reported changes in 80% of the cells analysed (42 of 52 cells)

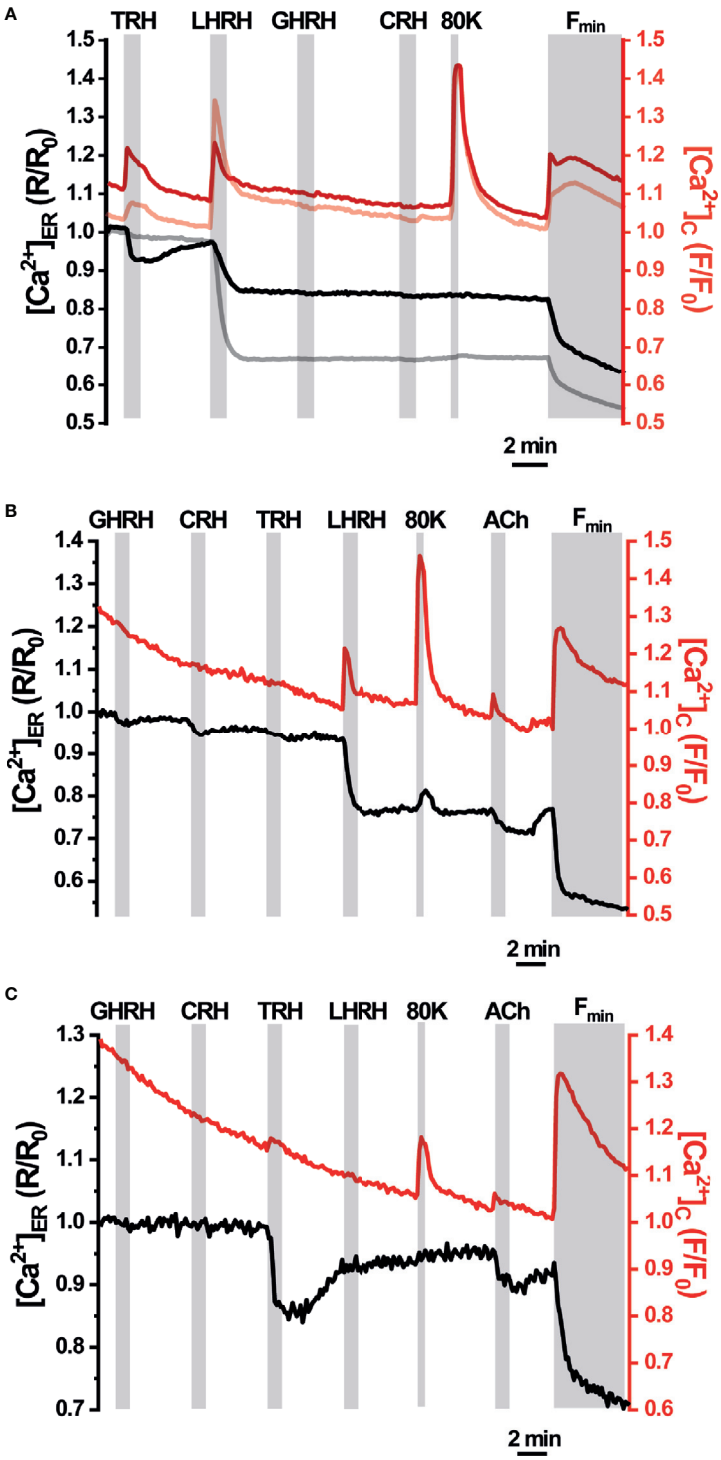


**FIGURE 4** | Simultaneous responses of $[Ca^{2+}]_{ER}$ and $[Ca^{2+}]_C$ in pituitary slices of the erGAP3 transgenic mice. Acute pituitary slices isolated from a 5-months-old erGAP3 transgenic mouse (L10) were loaded with Rhod-2. $[Ca^{2+}]_{ER}$ (black and gray traces) is represented as F470/F405 ratio (R) normalized to $R_0$ (erGAP3; $R/R_0$) and $[Ca^{2+}]_C$ represented as $F/F_0$ (Rhod-2; red traces). Single cell traces are representative for at least three similar experiments. GHRH, growth hormone releasing hormone; CRH, corticotropin-releasing hormone; TRH, thyrotropin-releasing hormone; LHRH, LH releasing hormone (each hormone, 100 nM); 80K, KCl (80 mM); ACh, acetylcholine (100 μM); Fmin, depletion cocktail composed of TBH (10 μM) in $Ca^{2+}$-free medium (EGTA 0.5 mM). **(A)** Mean traces (12 cells in the same field) that only exhibited ER $Ca^{2+}$ changes to LHRH (grey and pale red traces). Mean traces (five cells in the same field) that exhibited ER $Ca^{2+}$ changes to TRH and LHRH (black and intense red). **(B)** Representative traces of a LHRH-responsive cell exhibiting a small response to GHRH and to CRH. **(C)** Representative traces of a cell exclusively responsive to TRH in the same microscopic field as in B.

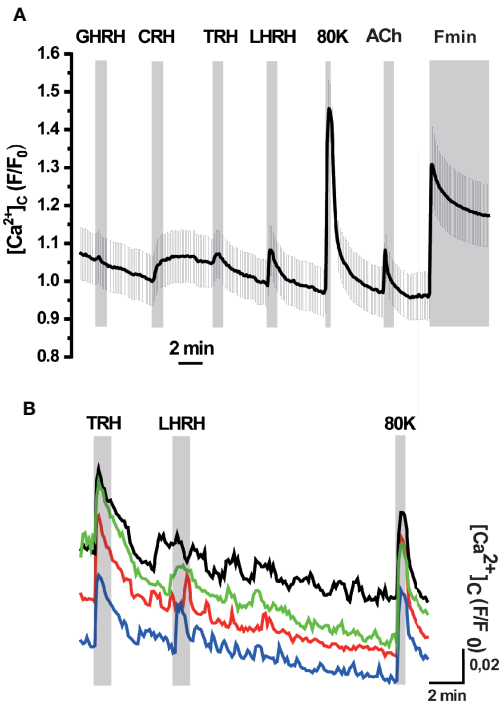


**FIGURE 5** | $[Ca^{2+}]_C$ responses in pituitary slices of erGAP3 transgenic mice. **(A)** Representative trace of $[Ca^{2+}]_C$ mean of the cells responsive to corticotropin-releasing hormone (CRH). Shading corresponds to SEM, n= 14 cells (out of 22 of the same experiment). Other experimental details as in **Figure 4**. **(B)** Example trace of cytosolic $Ca^{2+}$ oscillations displayed in some cells. Individual traces have been displaced vertically for a better visibility.

(**Figure 6**). Three distinct patterns of ER $Ca^{2+}$ release were observed. First, some cells (23%; 10 out of 42) responded exclusively to TRH with a rapid and large ER $Ca^{2+}$ drop and this decrease was reversible by washing out the stimulus, allowing the refilling of the ER. A second group of cells (57%; 24 out of 42) only responded to LHRH, but not to TRH. The amplitude of the ER $Ca^{2+}$ release triggered by LHRH is comparable to that of TRH (~30% R/R0 change) and the ER also recovered the basal $[Ca^{2+}]_{ER}$ (average ± SEM; t= 6.7 ± 0.8 min for LHRH and t =12.3 ± 5.8 min for TRH). A third population (12%; five cells out of 42) responded to both factors, TRH and LHRH, indicating the presence of multiresponsive cells bearing several types of HRH receptors. Addition of TRH as the first stimulus released a fraction of the stored ER $Ca^{2+}$, and the second stimulus, LHRH, released an additional fraction. Interestingly, the three types of cells responded to a high $K^+$ (80 mM) depolarization stimulus with a transient increase of 0.1 ± 0.01 (R/R0 mean ± SEM; n=42 cells), as a consequence of a transient $Ca^{2+}$ uptake into the ER, and the signal quickly returned to the resting ER levels. This last result indicates that the erGAP3 sensor was not saturated at resting ER level and no sign of CICR were observed, in agreement with the results shown in slices (**Figure 4A**).

## DISCUSSION

Dissecting $Ca^{2+}$ dynamics and elucidating the $Ca^{2+}$ signals interacting between organella require specific localization of the $Ca^{2+}$ sensors, especially in complex tissues and organs. Genetically encoded $Ca^{2+}$ indicators (GECIs) provide a powerful tool that

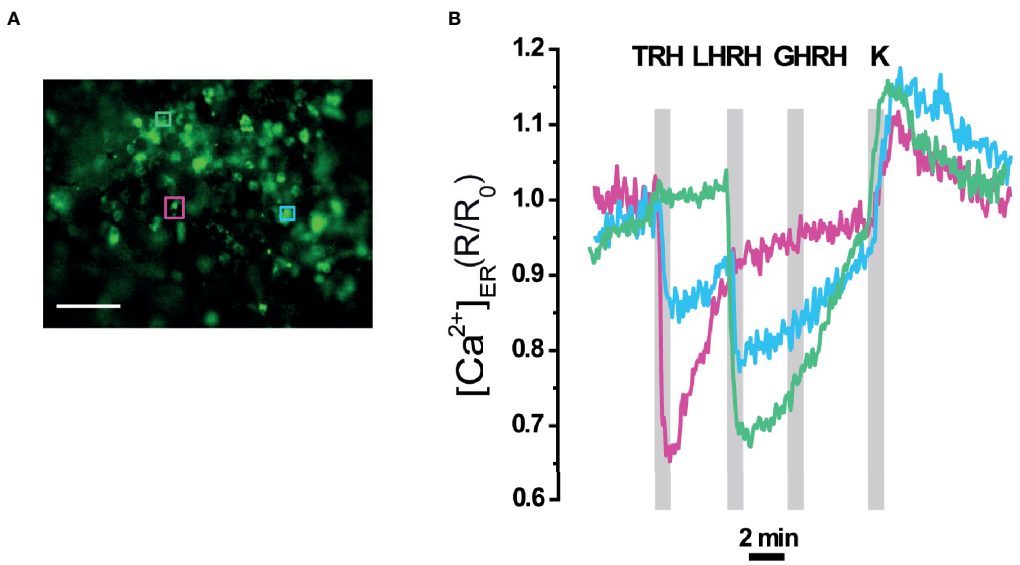


**FIGURE 6** | $[Ca^{2+}]_{ER}$ responses in intact pituitary gland of erGAP3 transgenic mice. **(A)** Fluorescence image (470 nm excitation) of the intact pituitary gland isolated of a P17 erGAP3 mouse. **(B)** Three different representative responses of ER $Ca^{2+}$ corresponding to the three cells are indicated in **(A)**. Note the presence of two cells (pink and green) that each responded to only one HRH and a third cell (blue) to two hypothalamic releasing hormones (HRHs). Other experimental details as in **Figure 4**.

overcomes some of the disadvantages of the synthetic probes, such as their lack of subcellular specificity or the difficulty of loading thick tissue preparations. Transgenic mice expressing GECIs have proven to be a particularly useful technology for being minimally invasive, its ease of use and its stability and width of expression. Although the number of GECIs have dramatically increased in recent years, those optimized for high $Ca^{2+}$ compartments such as the ER are more limited. Even less frequent is the application of low $Ca^{2+}$ affinity indicators to transgenics, which is mostly restricted to non-mammalian organisms (48). The generation of transgenic lines expressing functional $Ca^{2+}$ indicators can be problematic, especially in mammals. One of the drawbacks frequently encountered is the reduced sensitivity of the sensor in GECI transgenic lines in comparison with that obtained *in vitro*. Some indicator proteins displayed a punctate fluorescence, often visible as nuclear precipitates, a sign of immobile, sequestered, and non-functional indicators. One possible explanation is that many of the existing GECIs use calmodulin as $Ca^{2+}$-sensitive motifs. Calmodulin is a highly expressed protein with a wide array of effectors, and its overexpression can be problematic and can result in embryonic lethality or in insufficient signal-to-noise ratio (SNR) (49, 50). We used here a low-$Ca^{2+}$ affinity variant of the GAP indicators, based on the jellyfish aequorin instead of the mammalian calmodulin as the moiety providing the $Ca^{2+}$ binding sites. This property makes the binding or the sequestration of the indicator to endogenous proteins less likely, thus avoiding possible perturbations of the signal. In the two transgenic lines generated for erGAP3, expression of the indicator in pituitary gland was robust and SNR allowed readily imaging of $Ca^{2+}$ signals in a HRH–specific manner. The changes observed in the Rmin were close to those previously reported *in vitro* (**Figure 3**) (46). We did not observe abnormalities in the pituitary gland morphology and, importantly, no nuclear fluorescent precipitates were visible (**Figure 1**).

We show here that the erGAP3 transgenic mouse lines provide a useful and novel tool in the study of pituitary $Ca^{2+}$ dynamics. The advantages of imaging $[Ca^{2+}]_{ER}$ using these transgenic mice are several: simple tissue preparation and imaging procedures; preservation of pituitary gland organization; and sensitive, highly efficient and simultaneous ER $Ca^{2+}$ imaging of multiple pituitary cells. It is well known that cell to cell contact in the intact tissue is crucial to retain many of the $Ca^{2+}$ signaling patterns (51, 52). Most of previous work on pituitary excitability was undertaken on dissociated cells in short term primary cultures or in a variety of immortalized clonal cell lines, e.g., GH3 or AtT-20 cells (53). Given the heterogeneity of the pituitary gland and the cell to cell interactions, it is advantageous using an intact preparation that preserves the spatial architecture of the original gland. A few reports have been performed in acute slices (41, 54–56). In the present study, $Ca^{2+}$ imaging was performed both in gland slices and in whole intact gland. In both preparations we detected robust and reproducible ER $Ca^{2+}$ signals in a HRH specific manner (**Figures 4**–**6**). The combination of cytosolic and ER $Ca^{2+}$ measurements in pituitary slices demonstrated that the ER $Ca^{2+}$ is the main source of the cytosolic $Ca^{2+}$ response to TRH and LHRH, in accordance with the signaling cascade triggered by these factors (44, 57) (**Figures 4** and **5**). By contrast, ER $Ca^{2+}$ would contribute minimally to the signals elicited by GHRH or CRH. Finally, we identified some cells that released ER $Ca^{2+}$ in response to two secretagogues. This result was observed in the three preparations studied and confirmed the presence of multi-responsive cells bearing multiple types of receptors cells, as previously described by our and others groups (4, 10, 11). Interestingly, in a recent study using sc-transcriptome, the authors found a cell population with a unique multi-hormone gene expression profile that would reveal an unanticipated cellular complexity and plasticity in adult pituitary (8). Finally, our data indicate that ryanodine receptors are not operative in AP cells, a finding consistent with recent scRNAseq analyses of AP cells (58).

In this study, we did not focus on the exact quantification of each specific subpopulation within the gland. Instead other methods like immunohistochemistry and immunocytochemistry have been used to assess the proportions of each cell population. Probably due to limitations in the spatio-temporal resolution of our imaging equipment, our study might favor recording the LHRH-responsive cells. These cells are larger than other AP cells and displayed stronger fluorescence changes. These two factors probably led to a higher signal-to-noise ratio (SNR). In this context, the numbers of cells reported to respond to each HRH give an indication on the approximate fractions of each population but a more detailed study with a confocal or two-photon microscope would add spatial resolution required for an exact quantification. More recently, transcriptomics studies and single cell RNA-sequencing (sc-RNA) analysis of AP have proven to be excellent tools to gain insights into the expression profiles specific for each AP population (8, 19–25, 59). The combination of this powerful methodology with organellar $Ca^{2+}$ imaging will help to correlate specific expression patterns with $Ca^{2+}$ signaling pathways and will expand our present knowledge on the identities of AP cell types and their functions.

The identification of each of the five AP cell types during calcium imaging studies has proven to be a challenging task. In some protocols, cells are fixed and stained at the end of the calcium imaging experiment (9, 60). This method, although it has provided relevant insights into pituitary physiology, is technically challenging and it can affect the native features of the cell. More recently, an increasing number of studies have begun to exploit mouse models in which a specific cell type is genetically labeled with a fluorescent protein under the control of specific promoters. This allows the visualization of a specific cell type and its recording in real time, and does not require its posterior manipulation. One of the promoters often used is the proopiomelanocortin (POMC) promoter, that can label an ACTH population (14, 59, 60). However, some endogenous promoters might be too weak, limiting their usage, e.g., the gonadotropin-releasing hormone promoter, that formerly failed to generate a transgenic mouse for the $Ca^{2+}$ indicator inverse pericam due to a poor SNR (61). Given the good performance reflected here by the expression of transgenic erGAP3 controlled by a ubiquitous promoter, it seems worth generating transgenic lines of erGAP3 for specific pituitary subpopulations in future studies.

## DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

## ETHICS STATEMENT

The animal study was reviewed and approved by Comité de Ética en Experimentación y Bienestar animal de la Universidad de Valladolid (CEEBA).

## AUTHOR CONTRIBUTIONS

JR-R and PN-N performed the experiments and analyses and participated in preparing the manuscript figures. LN participated in the initial dispersed cultures. All the authors participated in the conception and design of the study. MA and JG-S wrote the manuscript. All authors contributed to the article and approved the submitted version.

## FUNDING

This work was supported by grants from the Spanish Ministerio de Economia y Competitividad (BFU2017-83066-P) and the Consejería de Educación, Junta de Castilla y León (GR175), and the Instituto de Salud Carlos III (TerCel; RD16/0011/0003).

## ACKNOWLEDGMENTS

We thank C. Rodriguez and J. Fernández for technical assistance; and I. López for technical assistance with cryosections and immunofluorescence experiments, as well as for assistance with mice. We also thank Dr. L. Senovilla (INSERM, Paris), Dr. C. Villalobos (IBGM, Valladolid) and Dr. T. Schimmang (IBGM, Valladolid) for their comments and suggestions on the manuscript.

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**Conflict of Interest:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

frontiers in Endocrinology

REVIEW
published: 23 February 2021
doi: 10.3389/fendo.2020.614981

# A Comparative Update on the Neuroendocrine Regulation of Growth Hormone in Vertebrates

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Growth hormone (GH), mainly produced from the pituitary somatotrophs is a key endocrine regulator of somatic growth. GH, a pleiotropic hormone, is also involved in regulating vital processes, including nutrition, reproduction, physical activity, neuroprotection, immunity, and osmotic pressure in vertebrates. The dysregulation of the pituitary GH and hepatic insulin-like growth factors (IGFs) affects many cellular processes associated with growth promotion, including protein synthesis, cell proliferation and metabolism, leading to growth disorders. The metabolic and growth effects of GH have interesting applications in different fields, including the livestock industry and aquaculture. The latest discoveries on new regulators of pituitary GH synthesis and secretion deserve our attention. These novel regulators include the stimulators adropin, klotho, and the fibroblast growth factors, as well as the inhibitors, nucleobindin-encoded peptides (nesfatin-1 and nesfatin-1–like peptide) and irisin. This review aims for a comparative analysis of our current understanding of the endocrine regulation of GH from the pituitary of vertebrates. In addition, we will consider useful pharmacological molecules (i.e. stimulators and inhibitors of the GH signaling pathways) that are important in studying GH and somatotroph biology. The main goal of this review is to provide an overview and update on GH regulators in 2020. While an extensive review of each of the GH regulators and an in-depth analysis of specifics are beyond its scope, we have compiled information on the main endogenous and pharmacological regulators to facilitate an easy access. Overall, this review aims to serve as a resource on GH endocrinology for a beginner to intermediate level knowledge seeker on this topic.

**Keywords: growth hormone, hormones, pituitary, somatotrophs cells, neuropeptides, vertebrates, cell signaling**

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***Edited by:***
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***Specialty section:***
*This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology*

***Received:*** *07 October 2020*
***Accepted:*** *31 December 2020*
***Published:*** *23 February 2021*

***Citation:***
*Vélez EJ and Unniappan S (2021) A Comparative Update on the Neuroendocrine Regulation of Growth Hormone in Vertebrates. Front. Endocrinol. 11:614981. doi: 10.3389/fendo.2020.614981*

## INTRODUCTION

Growth hormone (GH), originally isolated from bovine pituitaries in 1944 (1), is a key endocrine regulator of somatic growth. The main action of pituitary-derived GH is the stimulation of hepatic insulin-like growth factors (IGFs). The GH/IGF axis acts on different target tissues (**Figure 1**) including the muscle and adipose tissue, to regulate different physiological processes associated with growth promotion, protein synthesis, cell proliferation and metabolism. Therefore, dysregulation of the GH/IGF axis leads to growth disorders. In this regard, alterations in hypothalamic growth

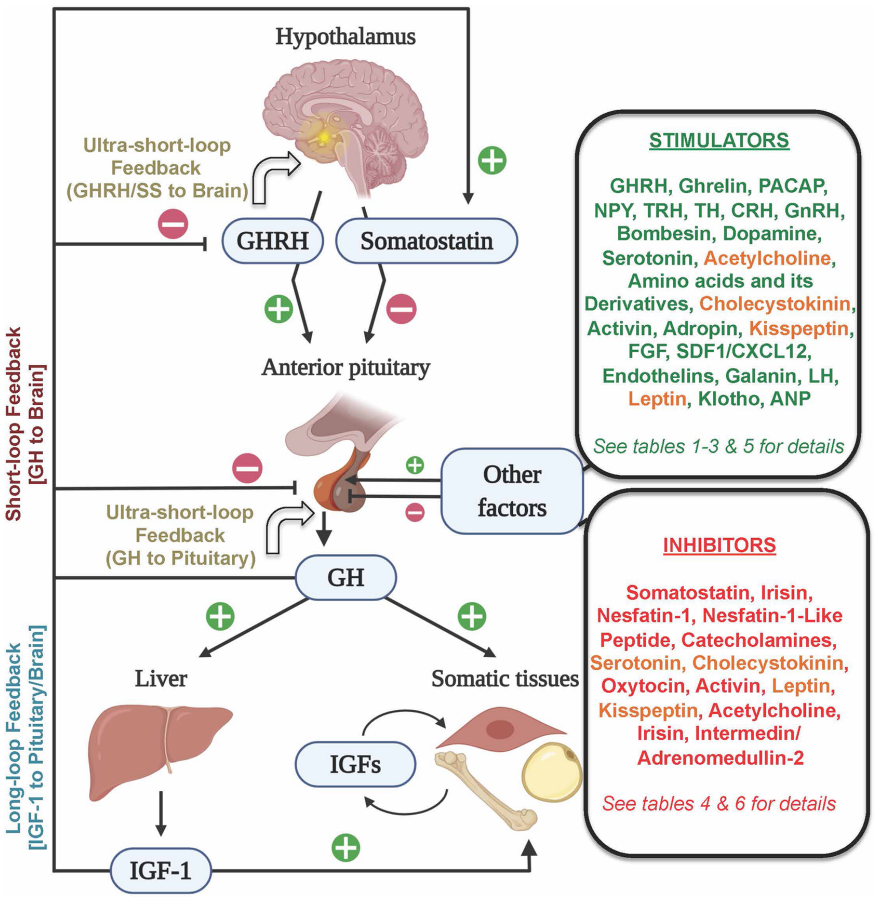


**FIGURE 1** | Schematic representation of the neuroendocrine (GHRH/Somatostatin–GH–IGF) axis and its main hormonal regulators. The hypothalamic stimulator GHRH and the inhibitor somatostatin mainly control GH synthesis and secretion by the pituitary somatotrophs. GH stimulates, mostly in the liver, the secretion of IGF–1, which acts in autocrine, paracrine, and endocrine manners in different somatic tissues to control diverse physiological processes, including protein synthesis, cell proliferation and metabolism. Both GH and IGF–1 could regulate its levels through the long–loop and short–loop feedback mechanisms, while GH and GHRH and somatostatin could regulate their levels via an ultra–short–loop feedback mechanism. Endogenous factors arising outside (shown in two boxes on the right side) of the hypothalamo–pituitary–liver axis could elicit stimulatory (green font), inhibitory (red font) or dual roles (orange font) to regulate pituitary GH. Figure created with BioRender.com tools.

hormone-releasing hormone (GHRH), one of the main stimulators of GH (reviewed in detail below), could affect the pituitary GH and consequently the GH/IGF axis. Moreover, the disorders of the pituitary transcription factors and other components of the GH/IGF axis [GH secretagogues; GHSs, GH- and IGF-receptors, and their signal transducers] also can alter GH production, secretion and responsiveness (2).

The hypersecretion of GH, which is mostly associated with benign pituitary adenomas, causes gigantism or acromegaly (3, 4). Besides, GH excess can increase the risk of developing cancer, cardiovascular diseases, diabetes and osteopathy, and is associated with a reduction in lifespan (5, 6). The leading medical therapies for excessive GH consist of the use of somatostatin receptor ligands (SRL), as somatostatin is the main GH inhibitor (reviewed in detail below), and in the limitation of GH actions using antagonists of the GH-receptors (5, 7). However, it has been reported that some acromegaly patients become "partially resistant" to SRL treatment (8). In GH deficiency, recombinant and long-acting GH formulations are commonly used as replacement therapies for growth disorders (6, 9). Moreover, recombinant human IGF-1 replacement has been useful in reversing the adverse conditions associated with GH deficiency or GH insensitivity in children (10, 11). GH treatment was also found useful in treating some catabolic conditions such as AIDS wasting and cystic fibrosis (2, 9, 12, 13). In addition to its clinical relevance as a key molecule of the GH/IGF axis (**Figure 1**), GH is a pleiotropic hormone involved in several vital processes in vertebrates. These processes include nutrition, metabolism, reproduction, physical activity, neuroprotection, immunity, osmoregulation and even social behavior (14–21). The biological actions of GH as a major growth and metabolic modulator has been utilized in different fields including the livestock industry (22) and aquaculture (23–25). These reinforce the multidisciplinary interest on GH and the need for progress in GH knowledge across vertebrates. The identification of additional novel regulators of somatotrophs,

GH synthesis and secretion has many beneficial outcomes. Some of the relatively recently identified stimulators of pituitary GH secretion or production include adropin, klotho and the fibroblast growth factors, and the inhibitors include irisin and the nucleobindin-encoded peptides nesfatin-1 and nesfatin-1–like peptide. The recent advancements in GH biology, including the regulation of GH receptors (GHR) and its signal transduction, as well as GH secretion, have been extensively reviewed in vertebrates including fish (5, 7, 14, 15, 18, 21, 26–29). The goal of this review is to serve as a one-stop resource for readers who seek beginner to intermediate level knowledge on the comparative aspects of GH endocrinology in vertebrates.

## GH SYNTHESIS

GH is mainly produced and secreted by the somatotrophs of the adenohypophysis (anterior pituitary). Generally, the modulation of these processes begins with the activation of G-protein coupled receptors (GPCRs) in the somatotrophs (**Figure 2**). The extracellular binding of GHRH to a transmembrane GPCR induces the intracellular linking of a heterotrimeric G protein (composed by α, β and γ subunits) to the GPCR (5, 30, 31). The binding of guanosine triphosphate (GTP) to the G protein induces the dissociation of the G protein and GPCR. That results also in the decoupling of Gα and Gβγ-subunits (32). In the case of a GH stimulator, the activated Gα-subunit (Gα), in turn, stimulates the adenylyl cyclase (AC) activity (33). Accordingly, the subunit involved is recognized as a stimulatory Gα (Gαs). Conversely, the binding of a GH suppressor (somatostatin) activates an inhibitory Gα-subunit (Gαi), which reduces the activity of AC (31, 33, 34). This enzyme catalyzes the conversion of adenosine triphosphate (ATP) into cyclic adenosine monophosphate (cAMP) (35). Once AC is activated, the rise of cAMP levels enables the binding of cAMP to the two regulatory subunits present in the tetrameric protein kinase A (PKA), allowing both the dissociation and activation of the two PKA-catabolic subunits (30). At this point, these activated catabolic subunits can act as serine-threonine kinases to phosphorylate a wide range of substrates, including membrane, cytosolic and nuclear proteins (30, 35, 36). Within these target substrates, the cAMP-responsive element-binding protein CREB stands out as a critical modulator of the cAMP-PKA-dependent transcriptional regulation in the somatotrophs (1, 31, 36). The phosphorylation of CREB at Ser-133 by PKA allows its binding with the cAMP response element (CRE) (30). CRE acts as a transcription factor of different cAMP-regulated genes, including the pituitary-specific positive transcription factor 1 (*pit-1*), which in turn stimulates the expression of GH gene (**Figure 2**) (15, 31, 37–39).

On the other hand, it has been reported that activated PKA can limit the levels of cAMP by either the stimulation of phosphodiesterases (PDE) (**Figure 2**), or through the desensitization of some GPCRs (36, 40–42). Besides, some GH regulators inhibit mRNA encoding their own receptors indicating that hormone desensitization also happens at the transcriptional level in somatotrophs, as observed in rats (43) and in a non–human primate (44). However, no such

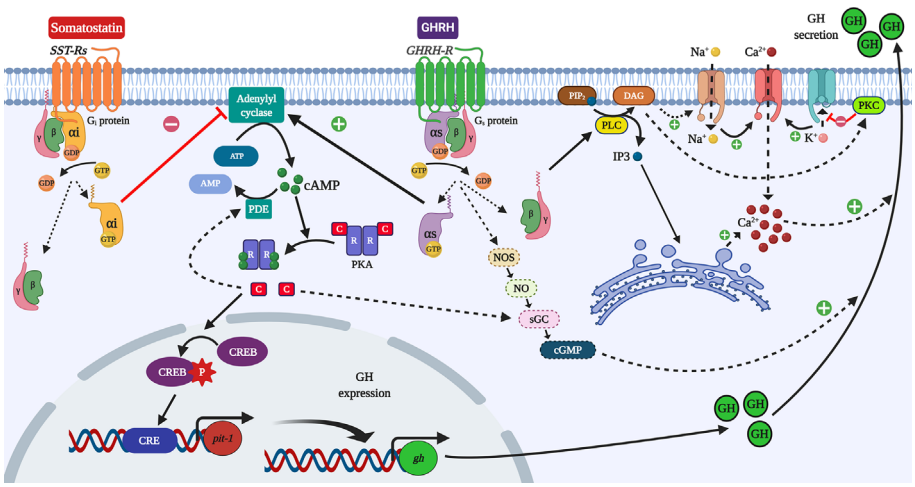


**FIGURE 2** | Simplified overview of the cAMP/PKA/CREB pathway in the control of GH synthesis and secretion in somatotrophs. The modulation of GH synthesis starts with the activation of GPCRs and the control of the activity of the adenylyl cyclase (AC) enzyme by the action of either stimulatory Gα (Gαs) or inhibitory (Gαi) subunits. The activation of AC increases cAMP levels, which activates the protein kinase A (PKA). CREB is one of its targets, and phosphorylated CREB can stimulate the expression of the transcription factor *pit–1*, which upregulates GH mRNAs. The stimulation of phosphodiesterases (PDE) by PKA could elicit a negative feedback, limiting cAMP levels. The activation of adenylyl cyclase and the protein lipase C (PLC) induced by GHRH causes the rise in either cAMP or IP3, respectively, stimulating the calcium ($Ca^{2+}$) influx, which in turn potentiates the exocytosis and release of GH. This mechanism involves the activation of $Na^{+}$ channels to depolarize the plasma membrane to regulate $Ca^{2+}$ influx by $Ca^{2+}$–channels, and the mobilization of $Ca^{2+}$ from the endoplasmic reticulum. It was reported that cGMP could stimulate GH release independently of cAMP. Otherwise, the limitation of cAMP levels and the activation of $K^{+}$ channels reduce the secretion of GH. Figure created with BioRender.com tools.

desensitization of a GH–inhibitory GPCR was observed in European eel (45), suggesting species–specific differences in how hypothalamic factors affect somatotrophs in mammals and teleost fish. Sex steroids regulate the fish responsiveness to both GH stimulators and inhibitors (14, 46, 47), and it is expected that the responsiveness of fish somatotrophs to hypothalamic factors follows a seasonal pattern associated with the sexual and gonadal maturation typical of each species. The distribution of pituitary endocrine cells in fish and the anatomical configuration of this gland allow the direct innervation from the hypothalamus (48, 49). This anatomical feature allows a large number of different neuroendocrine factors to be able to modulate the function of fish somatotrophs (14, 38, 50). For example, the innervation of the fish pituitary by adrenergic nerve fibers leads to the direct inhibition of GH secretion by norepinephrine in goldfish pituitary cells (51, 52). Meanwhile, in birds and mammals, norepinephrine only causes an indirect, minor effect on GH secretion that is likely dopamine–dependent (53). Thus, the regulation of somatotrophs evolved to be less complex during vertebrate evolution [reviewed by Gahete et al. (38)].

## GH SECRETION

The rise in cAMP induced by the activation of AC modulates the $Ca^{2+}$ channels to increase calcium influx, thus facilitating the exocytosis and release of GH during its stimulation (14, 35, 42, 50). During GH suppression, the negative regulation of AC blocks the $Ca^{2+}$–channels, reducing the release of GH (31, 33). The mechanism behind these is regulated by $Ca^{2+}$–channels. It lies in the activation of $Na^{+}$ channels to depolarize the plasma membrane, or in the activation of $K^{+}$ channels to hyperpolarize it, which is further regulated by the increase and decrease of cAMP, respectively (54, 55). In addition, decoupled Gβγ–subunits can modulate the protein lipase C (PLC). The activation of PLC leads to an increase in inositol triphosphate (IP3), stimulating the mobilization of $Ca^{2+}$ from the endoplasmic reticulum (56) and enhances GH release. Furthermore, PLC in turn activates protein kinase C (PKC) (35, 44), which will also contribute to increased calcium influx by the depolarization of the membrane (**Figure 2**) (57, 58). The PLC/PKC pathway seems to be the primary intracellular modulator of some of the stimulatory actions of GHSs in mammalian and fish somatotrophs (35, 44, 59–62). During GH inhibition, in addition to the negative regulation of AC already discussed, the PLC/PKC pathway is also used to block the $Ca^{2+}$ influx (8).

The stimulation of GH release is also mediated by cyclic GMP (cGMP) in a cAMP–independent mechanism, probably associated with nitric oxide (NO) levels (14, 50, 63). The NO/cGMP cascade could be linked to the AC/cAMP/PKA pathway as PKA can phosphorylate the soluble guanylyl cyclase (sGC) (64). Due to this, the NO/cGMP pathway also appears to be involved in the actions of GH inhibitors on somatotrophs (65). Other signaling pathways are involved in mediating the inhibition of GH secretion. Some examples include the phosphatidylinositol 3–kinase/protein kinase B (PI3K/AKT) and the mitogen–activated protein kinase (MAPK) pathways (26, 55). The different mechanisms involving cAMP, PLC/PKC, NO/cGMP, and PKA, contribute to modulate $Ca^{2+}$ influx and the secretion of GH in vertebrates including fish. The secretion of GH in both fish and mammals follows a pulsatile, circadian pattern, with relatively higher release during the dark phase (7, 13, 14, 28, 38). Besides, sexual dimorphism in GH secretion was observed in rats (66) and humans (67, 68), with large nocturnal GH pulses and low inter–peak levels in males, and a higher interpeak and more sustained secretion in females (38). This differential GH secretion is a key determinant of the gender–specific patterns of growth and metabolism in rodents (69). GH sexual dimorphism was also reported in fish (14). This could be explained, in part, by the sex differences in hormonal regulators of somatotrophs in various species.

## MAIN HORMONAL REGULATORS OF SOMATOTROPHS—GHRH AND SOMATOSTATIN

Two brain (hypothalamus)–derived modulating factors, the stimulator GHRH and the inhibitor somatostatin (70), act as the primary central regulators of both synthesis and secretion of pituitary GH (**Figure 1**) (1, 7, 14, 15, 20, 21, 26, 34, 38). The hypothalamic GHRH was initially discovered from a human pancreatic tumor associated with acromegaly (71, 72). Later, GHRH was identified in non–mammals, and it was shown that fish GHRH is homologous to mammalian GHRH (73). GHRH is mainly expressed in the brain and testes in numerous vertebrates, including reptiles, birds and mammals (74), as well as in fish (73). Its main receptor is the GHRH–R (42, 75). Although it is detected in different tissues in mammals, GHRH–R is mainly expressed in pituitary cells (42). In goldfish, GHRH–R is expressed in the brain and pituitary (73). It was initially thought that GHRH–like peptides do not affect GH secretion in fish (50). Later, it was discovered that those GHRH–like peptides are indeed homologs of the mammalian PACAP (73). In the same article, the real fish homologous to mammalian GHRH was reported, and it was observed that GHRH increased both cAMP and GH secretion in goldfish pituitary cells (73). Similar GH–stimulatory effects of GHRH was identified in amphibians (76), reptiles (77, 78), birds and mammals (42, 78, 79).

Like GHRH, somatostatin is also a hypothalamic peptide, but inhibits GH secretion *in vivo* and *in vitro* in rats (80). Different forms of somatostatin, including the mammalian homolog, cortistatin, have been identified in vertebrates (38, 81). As reviewed by Sheridan and Hagemeister (26), it is now recognized that various somatostatin forms are expressed in different tissues and it is not restricted to the hypothalamus. Somatostatin exerts its effects in somatotrophs through up to five subtypes of GPCRs, the SST–Rs (26, 34, 55). The inhibitory actions of somatostatin on GH release have been widely observed in different fish (14, 26). Somatostatin blocks the transcription and translation of GH in cultured somatotrophs from rainbow trout (82), as well as blunts the stimulatory effects of other factors such as GnRH, dopamine and PACAP (14). GH in fish is under a

dominant inhibitory control compared to mammals. That means, in the absence of somatostatin, the basal GH secretion reaches the maximum, and the stimulatory factors are ineffective, as observed in the turbot (83). In frogs and turtles, it was thought that somatostatin has no direct effects on somatotroph regulation, but it can block the *in vitro* stimulatory effects of TRH in amphibians and reptiles (38). Somatostatin strongly inhibits both GH mRNA and secretion from iguana pituitary *in vitro*, while the same dose and duration failed to modulate GH in chicken and rat pituitary cultures (78). In mammals, the role of somatostatin is a more complex topic. In male rats, somatostatin appears essential in generating GH secretion rhythmicity, as reviewed by Tannenbaum (84). Considering that somatostatin neurons can directly or indirectly inhibit the activity of GHRH neurons, MacGregor and Leng proposed a mathematical model to explain the hypothalamic control of GH secretion (85). However, results from other mammals, including female rats [Reviewed by Gahete et al. (38)], challenge the role of somatostatin in regulating GH rhythmicity. More recently, it has been demonstrated that somatostatin irregularly inhibits GHRH neurons in male and female mice, inducing sex–specific oscillatory patterns in the GHRH neural electrical activity (86). The sexual dimorphism in the GHRH oscillatory patterns induced by somatostatin seems dependent on the different actions of both glutamate and GABA neurons, and these differences could explain the distinctive GH secretion pattern between male and female mice (86). This topic certainly deserves further investigation in the future. In humans, it has been proposed that somatostatin regulates the magnitude of GH release but is not involved in controlling the rhythmicity of GH secretion (38, 87). In addition to their inhibitory effects, at both low and high doses, somatostatin stimulates the secretion of GH in primary porcine somatotrophs (38, 81, 88). On the other hand, SST–Rs can dimerize with other GPCRs such as ghrelin or dopamine receptors, altering the signaling of different factors and consequently, the regulation of GH (61, 89–91). Furthermore, it has been observed that somatostatin can modulate the secretion of GHRH (92), which contributes to this complex regulation. Overall, by eliciting multiple effects detailed above, somatostatin is recognized as the primary inhibitor of GH in vertebrates (7, 14, 15, 26, 38).

In addition to GHRH and somatostatin, there are several additional regulators of GH. For example, gonadal steroids can regulate GHRH effects in mice (69), contributing to the sexually dimorphic secretion of pituitary GH. Indeed, the gonadal steroids secreted during both sexual and gonadal maturation induce a clear seasonal pattern in the GH plasma levels in aquatic species (14, 93–95). Other factors including IGF–1, GH itself, ghrelin and synthetic GH secretagogs (GHSs) can modulate the synthesis and/or release of GH by somatotrophs in vertebrates (**Figure 1**) (5, 7, 15, 25, 59). The levels of IGF–1 in normal situations act as a sensor and feedback regulator of the GH/IGF system. IGF–1, which is mainly expressed in the hepatic tissue, can directly inhibit GH secretion in the somatotrophs of fish (18, 50, 96, 97), birds (98, 99) and mammals (28, 87, 92, 100), through a long–loop negative feedback (by acting on the pituitary and/or on GHRH in the brain, **Figure 1**), but also indirectly by enhancing the hypothalamic release of somatostatin (5). In addition, IGF–1 is involved in a wide range of physiological processes including protein synthesis, cell proliferation and differentiation (17, 101), and is considered the other major endocrine and local effector of the GH/IGF axis. Besides, GH itself can send feedback signals to the brain (short–loop feedback), or could act in an autocrine or paracrine manner within the pituitary (ultra–short–loop), to limit its synthesis and release by somatotrophs (**Figure 1**). Both GHRH and somatostatin are also capable of eliciting ultra–short–loop feedbacks within the brain. The exact identification of whether IGF–1 or GH induces the negative feedback in an *in vivo* model is a complex issue (38), but the inhibitory actions of GH in mammalian somatotrophs has been demonstrated both *in vitro* and *in vivo* (28). Although GH treatment increased GH in grass carp pituitary cells (50), other studies have demonstrated that GH inhibits GH release in rainbow trout pituitary *in vitro* (102). A recent *in vivo* study in gilthead sea bream showed that the administration of a sustained–release formulation of recombinant bovine GH significantly reduced pituitary GH mRNA (25). In that research, at 6 weeks post–injection, the reduction of GH mRNA was independent of circulating IGF–1 levels, supporting the negative feedback of GH in this species. To our knowledge, the ultra–short–loop has not been well characterized in the other groups of vertebrates, and future research will undoubtedly help to understand the auto–regulation of GH.

As mentioned earlier, the gradual decrease in complexity in the regulation of somatotrophs during vertebrate evolution (38) has led to a large number of factors regulating somatotrophs in fish, while a relatively shorter list of neuroregulators exists in mammals (14, 38). However, it is important to note that complexity exists in mammals. Somatostatin neurons can inhibit, directly or indirectly, the activity of GHRH neurons (103). It has been reported that GHS–receptors (GHS–Rs) can dimerize with other GPCRs, including the SST–Rs (61). The heterodimer formation (i.e. GHS–Rs:SST–Rs) could alter the signaling of the GPCRs, and thus its effects, as reported on the regulation of insulin release in rodent pancreatic cells (89). In this sense, chimeric molecules with the ability to bind with both SST–Rs and dopamine receptors induced more potent inhibition of GH release in human pituitary somatotroph adenoma cells (90, 91). Whether this enhanced potency is due to the heterodimerization of the receptors is still unknown. Additional research is needed to fully understand the implication of this mechanism in the regulation of GH secretion in somatotrophs cells along vertebrates. In addition to the dual regulation of mammalian somatotrophs (i.e. GHRH *vs*. somatostatin), a wide variety of other factors also modulate the synthesis and secretion of GH in vertebrates. The classical regulators of GH secretion in amphibians, reptiles, birds, humans and fish were reviewed by different authors in the past (14, 29, 38, 50), and more recently the knowledge on the effects of nutritional status, diet composition and environmental factors on the GH system in fish has been updated (21, 97, 104). In the present review, while revisiting the classic and main regulators of

somatotrophs function in vertebrates, our focus is also on new and emerging bioactive molecules and hormones that regulate GH synthesis and/or secretion. We considered the role of these secondary GH regulators and clustered them as groups of GH–stimulatory neurotransmitters (**Table 1**), neuropeptides (**Table 2**) and peripheral factors (**Table 3**), as well as the inhibitory molecules (**Table 4**). The "up" arrows in these tables indicate a stimulatory effect, while the "down" arrows point to an inhibition. We expand on some of the major regulators below. A very detailed discussion of specifics of each of these factors is beyond the scope of this review. The readers are encouraged to consult several recent reviews of specific topics, and some are cited in this article.

## OTHER STIMULATORS OF GH

### Pituitary Adenylate Cyclase–Activating Polypeptide (PACAP)

PACAP was originally isolated from the ovine hypothalamus due to its AC–stimulatory effects in rat pituitary cells (167). PACAP presents two molecular forms (PACAP27 and PACAP38) (167, 168) that are expressed in the brain and other peripheral tissues (169). Initially, it was thought that in fishes and other non–mammals, both GHRH and PACAP were encoded in the same gene, whereas in mammals, they originated from different precursors (14, 170). However, later it was reported that in both non–mammals and mammals, PACAP and GHRH are encoded in different genes (169). Regarding receptors, three different subtypes (i.e. PAC1–R, VPAC1–R, and VPAC2–R) have been identified, and they can activate diverse pathways, including the signaling through AC (169). PACAP is a key GH–release stimulator, which acts through the increase of $Ca^{2+}$ influx in fish and amphibians (15, 38, 96, 170–172). In this sense, PACAP has been postulated as the GHRH ancestor in less evolved vertebrates (50, 170, 173). Contrarily, GHRH exerts a stronger stimulation of GH release than PACAP in chicken somatotrophs (174), and the same occurs in mammals including humans (170). The role of PACAP in the secretion of GH in mammals is controversial (38, 173). It has been observed that while PACAP stimulates GH gene expression in birds, it has no such effects in rodents (78). Therefore, as previously reviewed by Gahete et al. (38), the role of GHRH and PACAP could have evolved during the evolution of

**TABLE 1** | Summary of positive regulators of pituitary GH: Neurotransmitters.

| Neurotransmitters | Groups | Roles | References |
|---|---|---|---|
| **Dopamine** | Fish | ↑ GH mRNA (PKA–dependent) in tilapia | (105) |
| | | ↑ GH secretion both directly and somatostatin–dependent in goldfish | (106, 107) |
| | | ↑ GH secretion in common carp | (108) |
| | Amphibian, reptiles and birds | Little ↑ or no effect on GH | (109, 110) |
| | Mammals | ↑ GH secretion in an isolated case of human acromegaly, but generally ↓ GH | (111), **Table 4** |
| **Serotonin** | Mammals | ↑ GH secretion (somatostatin/GHRH–dependent) | (28, 38) |
| | Fish and birds | Opposite results also observed | **Table 4** |
| **Acetylcholine** | Mammals | ↑ GH secretion | (112, 113–116) |
| | Birds | Opposite results observed in chicken | **Table 4** |
| **Amino acids and derivatives** | Fish | Glutamate ↑ GH secretion in rainbow trout | (117) |
| | | Cysteamine ↑ GH secretion through somatostatin–depletion in grass carp | (118, 119) |
| | Mammals | Argninine ↑ GH mRNA and secretion in rat | (120, 121) |
| | | Cysteamine ↑ GH secretion through somatostatin–depletion in sheep | (122) |

**TABLE 2** | Summary of positive regulators of pituitary GH: Neuropeptides.

| Neuropeptides | Groups | Roles | References |
|---|---|---|---|
| **Cholecystokinin (CCK)** | Fish | ↑ GH secretion directly and indirectly (by reducing somatostatin) in goldfish | (123–125) |
| | Mammals | ↑ *in vitro* GH secretion in rat | (126) |
| | | ↓ GH *in vivo* in sheep | **Table 4** |
| **Activin** | Fish | ↑ GH release in perifused pituitary fragments of goldfish | (127) |
| | | ↓ GH mRNA in zebrafish | **Table 4** |
| | Mammals | ↑ GH mRNA and secretion in rat, with exceptions | (128), **Table 4** |
| **Adropin** | Fish | ↑ GH mRNA in pituitary cells of tilapia | (129) |
| **Kisspeptin** | Fish | ↑ GH secretion in pituitary cells from goldfish | (130, 131) |
| | Mammals | ↑ GH secretion in peripubertal rats | (132) |
| | | ↑ GH secretion in fasted sheep (ghrelin–NPY dependent), but could also ↓ it | (133), **Table 4** |
| **Fibroblast Growth Factor (FGFs)** | Mammals | ↑ GH secretion in rat pituitaries and human adenoma cultures | (134) |
| **Chemokine derived factor 1 (SDF1, *aka* CXCL12)** | Mammals | ↑ GH mRNA and secretion in rat | (135, 136) |
| **Endothelins** | Mammals | ↑ GH secretion – ghrelin dependent– in bovine | (137, 138) |
| **Galanin** | Fish | ↑ GH release *in vivo* or *in vitro* in coho salmon and goldfish | (139) |
| | Birds | ↑ GH secretion acting directly on the pituitary | (140) |
| | Mammals | ↑ GH release directly and indirectly | (141–145) |
| **LH** | Fish | Essential for GH synthesis and release in grass carp | (50, 146) |

**TABLE 3 |** Summary of positive regulators of pituitary GH: Peripheral factors/other factors.

| Neuropeptides | Groups | Roles | References |
|---|---|---|---|
| **Leptin** | Fish | ↓ GH mRNA | **Table 4** |
| | Mammals | ↑ GH secretion directly and indirectly (i.e. somatostatin) in pig perifused pituitaries | (147, 148) |
| | | ↑ GH secretion in sheep | (149) |
| | | ↑ GH secretion in anterior pituitary explants of fasted bovids | (150) |
| | | The lack of leptin receptor ↓ both GH mRNA and protein in mice | (151) |
| | | Administration ↑ increases pituitary GH content in leptin–deficient obese mice model | (152) |
| **Klotho** | Mammals | ↑ GH secretion in *vitro* and *in vitro* in rodents, and in human GH–secreting adenomas | (134) |
| **Atrial and ventricular natriuretic peptides** | Fish | ↑ GH release in tilapia cultured pituitaries | (153) |

**TABLE 4 |** Summary of negative regulators of pituitary GH.

| Molecules | Groups | Roles | References |
|---|---|---|---|
| **Catecholamines** [norepinephrine (NE), epinephrine and dopamine] | Fish | NE and epinephrine ↓ basal GH release from pituitary cells of goldfish | (106) |
| | Birds | NE ↓ GHRH–effects in chicken pituitary | (110) |
| | Mammals | NE ↓ basal and GHRH–stimulated GH release in cultured ovine pituitary cells | (154) |
| | | Dopamine ↓ GH mRNA and secretion in sheep, cattle and human neonates, but opposite role also observed | (143, 155, 156), **Table 1** |
| **Serotonin** | Fish | ↓ GH *in vitro* secretion in goldfish | (157) |
| | Birds | ↓ GH secretion –hypothalamus–dependent– in chicken | (158) |
| | Mammals | Opposite role observed | **Table 1** |
| **CCK** | Mammals | ↓ GH in sheep, but opposite role observed in rat | (159), **Table 2** |
| | Fish | Opposite role observed in goldfish | **Table 2** |
| **Oxytocin** | Mammals | ↓ GH secretion in rat | (160) |
| **Activin** | Fish | ↓ GH expression in cultured pituitaries of zebrafish | (96) |
| | | Opposite role observed in goldfish | **Table 2** |
| | Mammals | ↓ GH mRNA in rat, but opposite role also observed | (161), **Table 2** |
| **Leptin** | Fish | ↓ GH mRNA in tilapia | (162) |
| | Mammals | Opposite role observed | **Table 3** |
| **Kisspeptin** | Mammals | Endogenous kisspeptin can ↓ GH secretion through GPR54 in sheep, but opposite role also observed | (163) |
| | Fish | Opposite role observed in goldfish | **Table 2** |
| **Irisin** | Fish | ↓ GH mRNA and secretion in tilapia *in vitro* | (164) |
| **Intermedin/Adrenomedullin–2** | Mammals | ↓ GHRH–stimulated GH release in rat dispersed pituitary cells | (165) |
| **Acetylcholine** | Birds | ↓ GH secretion –hypothalamus–dependent– in chicken | (168) |
| | Mammals | Opposite effect observed | **Table 1** |
| **Nesfatin–1 and NLP** | Mammals | ↓ GH mRNA and protein in rat pituitary cells | (166) |

vertebrates. While both GHRH and PACAP exert equipotent action regulating GH in amphibians and reptiles, in birds and mammals, PACAP only plays a secondary role, with GHRH being the main GH stimulator in those groups (170).

## Neuropeptide Y (NPY)

NPY was first isolated from the pig brain (175) and is a member of a family of peptides that includes three (i.e. peptide Y, peptide YY, and the pancreatic polypeptide) additional GPCR agonists (176–178). NPY was identified later in different fish species (179, 180). In mammals NPY has been located in the brain (176), although NPY immunoreactivity was detected in several tissues in vertebrates, including the fish pituitary (179, 180). NPY is recognized as one of the most important regulators of energy homeostasis and food intake in both fish and mammals (178, 180). Besides, NPY acts on fish somatotrophs to increase GH secretion both *in vitro* and *in vivo* (181–183). To our knowledge, there are no published reports on the involvement of NPY on GH regulation in birds, reptiles and amphibians. The role of NPY in mammals is controversial, and appears species–specific: stimulates GH secretion in swine (184) and cows (185), in rodents NPY reduced GH (186), increased it (187), or had no effects on the secretion of GH (188). A more recent work reported that NPY stimulates the secretion of GH through its action at the hypothalamic level by the control of GHRH and somatostatin in sheep (133). Additional work is necessary to clarify whether NPY exerts a direct action on mammalian somatotrophs.

## Thyrotropin–Releasing Hormone (TRH)

TRH, the first hypothalamic hypophysiotropic factor characterized, was initially isolated from the porcine and ovine hypothalamus in 1969 (189, 190). TRH is mainly expressed in the hypothalamus of fish, amphibians, birds and mammals, and has been detected in a number of peripheral tissues in reptiles (74). TRH binding was initially observed in the plasma membrane of the anterior pituitary extracted from cattle (191), and the receptor was later identified as a GPCR in mice (192). Although the main role of TRH is the

stimulation of the synthesis and release of the thyroid stimulating hormone (TSH) to control the thyroid gland (192), TRH influences the secretion of other pituitary hormones (38). In fact, TRH increases GH expression or secretion in some fish species (14, 50), amphibians (193), reptiles (78), birds (78, 194) and mammals (78, 195). In chickens, TRH stimulates GH with a potency similar to that of GHRH (99). On the other hand, TRH could also indirectly stimulate GH through thyroid hormones (see below).

### Thyroid Hormones (THs)

THs exist in two forms, the predominant circulating T4, and the biologically active T3 (196), which are essential components of the pituitary–thyroid axis (192). In mammals, it is well known that TRH induces the synthesis of TSH by the pituitary, which in turn induces the synthesis and release of T4 by the thyroid gland. Then, T4 can be enzymatically converted by deiodinases to T3 in different tissues, including the brain and liver (192, 196). THs can regulate the transcription of different target genes, mainly through their interaction with nuclear receptors (TRs) (196, 197). THs can also modulate gene expression through non–genomic actions involving the activation of different signaling pathways (196, 198). In fish, TRs have been found in the pituitary (50), and little evidence exists on the non–genomic actions of THs in these species [recently reviewed by Deal and Volkoff (196)]. The transcriptional regulation induced by the THs contributes to the modulation of various physiological processes, including development, growth and metabolism. In fact, THs stimulate the synthesis and release of pituitary GH in some fish (199, 200) and rats (201–204), though contrary or no effects have been observed in other fish species, reptiles or birds (15, 196). Not much is known about the TH regulation of GH in reptiles and amphibians (205), and the discrepancies in TH effects on GH regulation among fish have been recently summarized by Deal and Volkoff (196).

To our knowledge, the reasons behind the inconsistencies in the regulation of GH by THs are not fully understood. A combination of two different factors could have contributed to these contradictory results. As previously discussed by Giustina and Wehrenberg (206), the maintenance of basal GH secretion, to some extent, depends on the stimulation of somatotrophs by THs. Otherwise, when the concentration of THs exceeds the physiological level, it can increase the secretion of somatostatin and decrease GHRH, eventually causing a downregulation of pituitary GH (206). On the other hand, it has been reported that THs can stimulate the synthesis and release of hepatic IGF–1 in both fish and mammals (207–209), which through the long–loop negative feedback (**Figure 1**) could elicit a suppression of pituitary GH secretion. Based on these, the different effects of TH on GH observed in various species are likely caused by the high doses of THs used in those studies (i.e. excess vs. physiological levels), or by its effects on hepatic IGF–1. This aspect requires future confirmation through additional research.

### Corticotropin–Releasing Hormone (CRH)

CRH, also known as corticotropin releasing factor (CRF), was first identified in ovine hypothalamus (210) and later in other vertebrates, including fish and amphibians (211). Although recognized as a hypothalamic hormone, CRH was located in other human tissues (212, 213). CRH exerts its actions through GPCRs (214). In the pituitary, CRH stimulates the secretion of adrenocorticotropic hormone (ACTH) (180, 213) and can induce the release of other pituitary hormones such as α–melanocyte–stimulating hormone (α–MSH) and β–endorphin (215). Similarly, CRH is recognized as a GH–release stimulator in non–mammals (38). While CRH stimulates GH in reptiles (77) and in European eel, but had no effects in turbot (83). CRH is a potent stimulator of TSH release from the pituitary of amphibians, fish and birds [reviewed by De Groef (216)]. Some of the CRH effects could be attributed to its indirect action via thyroid hormone stimulation. Although CRH was not considered a stimulator of GH in mammals (217), a paradoxical increase of GH in response to CRH was observed in patients with pituitary adenomas (38) and this is a topic under consideration in current research (218, 219).

### Gonadotropin–Releasing Hormone (GnRH)

GnRH was first isolated in the early 1970s from the porcine hypothalamus (220). As recently reviewed by Duan and Allard (221), GnRH has been identified in a wide range of vertebrates, including fish, amphibians, reptiles, birds and mammals. This hypothalamic factor exerts its action in the pituitary cells through the activation of GPCRs and the signaling by PLC and cAMP pathways (222, 223). As a result, GnRH regulates the secretion of FSH and LH and is recognized as a critical modulator of the reproductive axis (224). Goldfish somatotrophs express GnRH receptors (225, 226). GnRH stimulates the secretion of GH in goldfish (227–229), tilapia (105) and Ricefield eel (230), but not in some others as found in African catfish or rainbow trout (231, 232). However, it was observed in pituitary cell culture of rainbow trout, GnRH stimulates GH secretion only in the presence of IGF–1 (233). GnRH could indirectly stimulate somatotroph function through the paracrine action of LH, which can also act as a stimulator of GH as reported in grass carp (50, 146) (**Table 2**). The regulation of GH by GnRH is a species–specific response depending on the presence of IGF–1 or other factors such as LH, or the type of receptor involved. Although it was postulated that the GH secretagogue actions of GnRH could be restricted to fish (38), recently it was found that GnRH stimulates GH secretion in the iguana, and both GH mRNA and GH secretion in chickens (78). Besides, GnRH combined with enkephalin increased GH secretion in rat pituitary cells (234), and long–term treatment with GnRH in humans caused an increase in height in precocious puberty (235).

### Bombesin (BB)

Bombesin was first isolated from the skin of frogs in 1970 (236). BB immunoreactivity was found in reptiles (237), and two homologs, GRP and neuromedin B, were found in birds and mammals (238, 239). BB expression was also found in different fish species (240, 241), as is the case of the forebrain and pituitary of goldfish (241). Among other functions, BB stimulates the secretion of gastric acid and pancreatic enzymes (238) and is involved in the modulation of the stress response (239). As an

activator of GPCRs (242), it has been reported that BB stimulates GH secretion in rat both *in vivo* (243) and *in vitro* (244). Contrarily, other authors found that BB reduced GH secretion in rats by the stimulation of somatostatin release (245). This controversial response could be associated with the presence of estrogens, as it was reported that bombesin inhibits GH secretion in normal rats but exerts stimulatory role in estrogenized rats (246). In contrast, BB stimulated GH secretion in cultured bovine pituitary cells (112). In goldfish, the perifusion of pituitary with BB significantly increased GH secretion (241). In the same species, BB increased GH release and inhibits the expression of somatostatin (123, 124), and it has been postulated that the actions of BB in the regulation of GH in these species could be mediated by somatostatin (14). However, whether BB can regulate GH synthesis and secretion in other fish, amphibians, reptiles and birds is unknown.

### Ghrelin

Ghrelin was originally reported in 1999 (247), as the first and only known endogenous ligand of the growth hormone secretagogue receptor 1a (GHS–R1a) (248). It was purified from the stomach extracts of rats, and was later identified in a number of species from humans to invertebrates. The N–terminal region of ghrelin is very highly conserved across species, and in most species, the third serine has an octanoyl group (249). This highly conserved region with the acyl group is considered to be the bioactive core of ghrelin, and it is critical for ghrelin binding to its receptor (249). GHS–R1a, currently known as the ghrelin receptor, is expressed in the pituitary somatotrophs and allows the direct action of ghrelin on these cells to induce GH synthesis and secretion. Ghrelin is known to stimulate GH secretion in many species including rats (250, 251), humans (252, 253), birds (103, 254), and fish (255–260). These effects are either *in vitro*, supporting the ability of ghrelin to act directly on somatotropes, or *in vivo*, by acting directly or through influencing the multitude of other GH regulators (14, 50). The binding of acylated ghrelin to its receptor triggers a cascade of intracellular events, including the stimulation of phospholipase C, inositol triphosphate and calcium pathways (261, 262). Overall, almost two decades since its discovery, ghrelin is now considered as one of the most important hormonal regulators of GH in vertebrates.

### Other Stimulators

In this review, we have tabulated the neurotransmitters (**Table 1**), neuropeptides (**Table 2**) and the peripheral factors (**Table 3**). Their specific effects in different species or groups are also furnished in these tables. In addition to the factors already discussed, a wide variety of other minor factors have been shown to exert a GH stimulatory role. For example, it has been recently reported that the peptide hormone adropin, which participates in the regulation of vascular function and energy homeostasis in mammals, stimulates GH gene expression in the pituitary of tilapia (129). To our knowledge, it is unknown whether adropin participates in the regulation of GH secretion in other groups of vertebrates. On the other hand, it has been observed that the transmembranal protein klotho, originally recognized as an ageing–suppressor in mice, increases GH secretion both *in vitro* and *in vitro* in rodents, as well as in human GH–secreting adenomas (134). Moreover, klotho is a modulator of the IGF–1 signaling pathway. It can inhibit the peripheral actions of IGF–1, and block the negative feedback of IGF–1 on pituitary GH secretion (92). Consequently, klotho has been postulated as a new player in the regulation of GH/IGF axis in mammals (92). However, the potential role of klotho in the regulation of GH secretion in other groups of vertebrates, including fish, is unknown. Besides, klotho can also regulate the signaling pathway of the fibroblast growth factor (FGF) (134). The same authors have also observed that FGF increased GH secretion in both rat pituitaries and human adenoma cultures (134). While it is unknown whether FGF exerts a direct action on GH regulation in other vertebrates, it has been recently observed that FGF increases the secretion of ghrelin in zebrafish (263). Thus, it is expected that FGF could also influence (at least indirectly) GH levels in fish. Certain amino acids, including aspartic acid, glutamic acid and arginine, although recognized as classical regulators of GH, was thought not to act directly on somatotrophs (264–266). However, new *in vitro* studies have shown that some amino acids exert their effects directly at the pituitary level (**Table 1**). However, it is important to note that some molecules may have species–specific roles and exert inhibitory actions, as detailed in **Table 4**. In addition to the endocrine regulators of GH discussed here, a large number of pharmacological compounds were employed to study the regulation of GH in somatotrophs. We have summarized the main pharmacological stimulators (**Table 5**) of the major signaling pathways involved in the regulation of both the synthesis and secretion (**Figure 2**) of GH. For further details on the use of these molecules, and the most effective doses or concentration ranges reported, please refer to the literature cited in the table.

## OTHER GH INHIBITORS

Other factors with an inhibitory role on GH are summarized in **Table 4**. Note that some molecules may have species–specific roles and exert the opposing actions, as detailed in **Tables 1–3**. For example, it has been reported that irisin, which is recognized as a metabolic peptide in mammals, inhibits both GH mRNA and secretion in cultured pituitary cells of tilapia (164). It is unknown whether irisin has a direct modulatory role on GH synthesis and secretion in other vertebrates. An inverse association between GH and irisin levels has been observed in humans, as the administration of recombinant human GH in young patients with Turner syndrome increased the circulating levels of irisin (281). We have reported that two novel metabolic peptides, nesfatin–1 and nesfatin–1–like peptide, are negative modulators of the synthesis of pituitary GH in mammals (166). Although their receptors are still unknown, it is expected that these peptides act through GPCR (282). It has been shown that both nesfatin–1 and nesfatin–1–like peptide regulate GH in the

**TABLE 5 |** Selection of GH signaling pathway stimulators.

| Target/category | | Molecules | Doses | References |
|---|---|---|---|---|
| **G protein** | Activator of stimulatory Gα subunits (Gαs) | Cholera toxin | 0.025–25 ng/mL, 3 nM | (267, 268) |
| | Blocker of inhibitory Gα subunits (Gαi) | Pertussis toxin | 10–300 ng/mL | (135, 165) |
| **Adenylyl cyclase** | | Forskolin | 0.01–10 μM | (43, 105, 146, 173, 267, 269, 270) |
| **PKA** | Cell permeable cAMP analogs | 8–bromo–cAMP | 0.3–5 mM | (172, 268, 271) |
| | | 8–pCPT–cAMP | 40–500 μM | (166, 272) |
| | Inhibitors of phosphodiesterases | IBMX | 0.001 mM–10 μM | (267, 269, 271) |
| | | Rolipram | 10 μM | (269) |
| **CREB** | | TUDCA | 200 μM | (273) |
| **Calcium levels** | Ionophores | A23187 | 3–30 μM | (172, 268) |
| | | Ionomycine | 10 μM | (171, 274) |
| | Voltage–sensitive calcium channels (VSCC) | Bay K8644 | 10 nM–10 μM | (172, 274) |
| | Inhibitors of $Ca^{2+}$–ATPase (SERCA) | Cyclopiazonic acid and BHQ | 10 μM | (274) |
| | | Thapsigargin | 100 nM | (88, 262) |
| | Activators of $Ca^{2+}$ release channels | Caffeine | 10 mM | (274) |
| | | Ryanodine | 0.01–100 nM | (275) |
| **Nitric oxide route** | | SNAP | 0.01–1000 nM | (148) |
| | | L–AME | 1 mM | (65) |
| **PLC** | | m–3M3FBS | 10 μM | (276) |
| **PKC** | | PMA | 0.1–1 μM | (59, 83, 277) |
| | | DiC8 | 10 μM | (278) |
| **PI3K** | | sc3036 | 10 μM | (279) |
| **JAK2** | | Coumermycin A1 | 1 μM | (280) |

rat somatotrophs through the AC/PKA/CREB signaling pathway (166), suggesting that the mechanism of action of nesfatin–1 and nesfatin–1–like peptide involves a GPCR associated with an inhibitory Gα–subunit (Gαi). As discussed in the previous section, numerous pharmacological inhibitors were also used for the study of GH and somatotrophs (**Figure 2**). These are listed in **Table 6**.

## PERSPECTIVES

While GH is a key endocrine regulator of somatic growth, it is also involved in the regulation of other vital processes in vertebrates. Thus, GH has implications in health, disease and even in animal production, and the fine–tuned control of GH synthesis and secretion is still a hot research topic more than 75 years after its discovery (1). Numerous GH

**TABLE 6 |** Selection of GH signaling pathway inhibitors.

| Target/category | | Molecules | Doses | References |
|---|---|---|---|---|
| **G protein** | Blocker of stimulatory Gα subunits (Gαs) | Suramin, and its analogs | 10 μM | (283) |
| | Activator of inhibitory Gα subunits (Gαi) | *Pasteurella multocida* toxin | 1 nM | (284) |
| **Adenylyl cyclase** | | MDL–12330A | 0.03–30 μM | (88, 172, 262) |
| **PKA** | Blockers | H89 | 100 nM–30 μM | (105, 172, 262, 268) |
| | | Rp–cAMP and DPT–PKI | 50 μM–1 mM | (230, 268) |
| | Phosphodiesterase activator | MR–L2 | 1–10 μM | (285) |
| **CREB** | | 2–naphthol–AS–E–phosphate | 25 μM | (286) |
| **Calcium levels** | Cell permeable $Ca^{2+}$–chelator | BAPTA–AM | 10–50 μM | (135, 171, 274) |
| | Voltage–sensitive calcium channels (VSCC) | Nifedipine and Verapamil | 1–100 μM | (130, 230, 262, 271) |
| | | $Ca^{2+}$ antagonists $CoCl_2$ and $CdCl_2$ | 0.1–2 mM | (88, 271) |
| | Activator of $Ca^{2+}$–ATPase (SERCA) | CDN1163 | 10 μM | (287) |
| | Inhibitors of $Ca^{2+}$ release channels | TMB–8 | 100 μM | (274) |
| | | Xestopongin C | 1 μM | (275) |
| **Nitric oxide route** | | NMMA | 0.3–1 mM | (148, 268) |
| | | NAME | 10 μM | (65) |
| **PLC** | | U–73122 | 5–50 μM | (88, 230, 262) |
| **PKC** | | GF109203X | 20 μM | (230) |
| | | Phloretin | 25 μM | (262) |
| | | BIM | 2 μM | (268) |
| **PI3K** | | Wortmannin | 10–100 nM | (146, 164) |
| | | LY294002 | 10 μM | (164) |
| **JAK2** | | AG490 | 100 μM | (146) |
| **MEK1/2** | | PD98059, U0126 | 10 μM | (62, 134, 164) |
| **p38 MAPK** | | SB202190 | 20 μM | (146) |
| | | SB203580, PD169816 | 10 μM | (164) |
| **Transcription** | | Actinomycin D | 8 μM | (129) |

regulators have been discovered and more progress in our knowledge on GH and somatotroph biology is expected in the future. Definitively, the progress in our knowledge of GH and its transfer and application will benefit the society in many ways. The same reasons support the need for more basic, clinical and comparative endocrinology research on GH biology in vertebrates.

## AUTHOR CONTRIBUTIONS

EV and SU prepared the manuscript, and EV created the tables and figures. All authors contributed to the article and approved the submitted version.

## FUNDING

EV is supported by postdoctoral fellowship (#4530) and a Top–Up Incentive Award (#5362) from the Saskatchewan Health Research Foundation (SHRF), and a postdoctoral fellowship (#2019MFE–429976–71377) from the Canadian Institutes of Health Research (CIHR). SU is a University of Saskatchewan Centennial Enhancement Chair in Comparative Endocrinology, and the research on hormones in his laboratory has been funded by grants from the Natural Sciences and Engineering Research Council (NSERC), CIHR, Canada Foundation for Innovation, SHRF, and Ferring Pharmaceuticals.

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