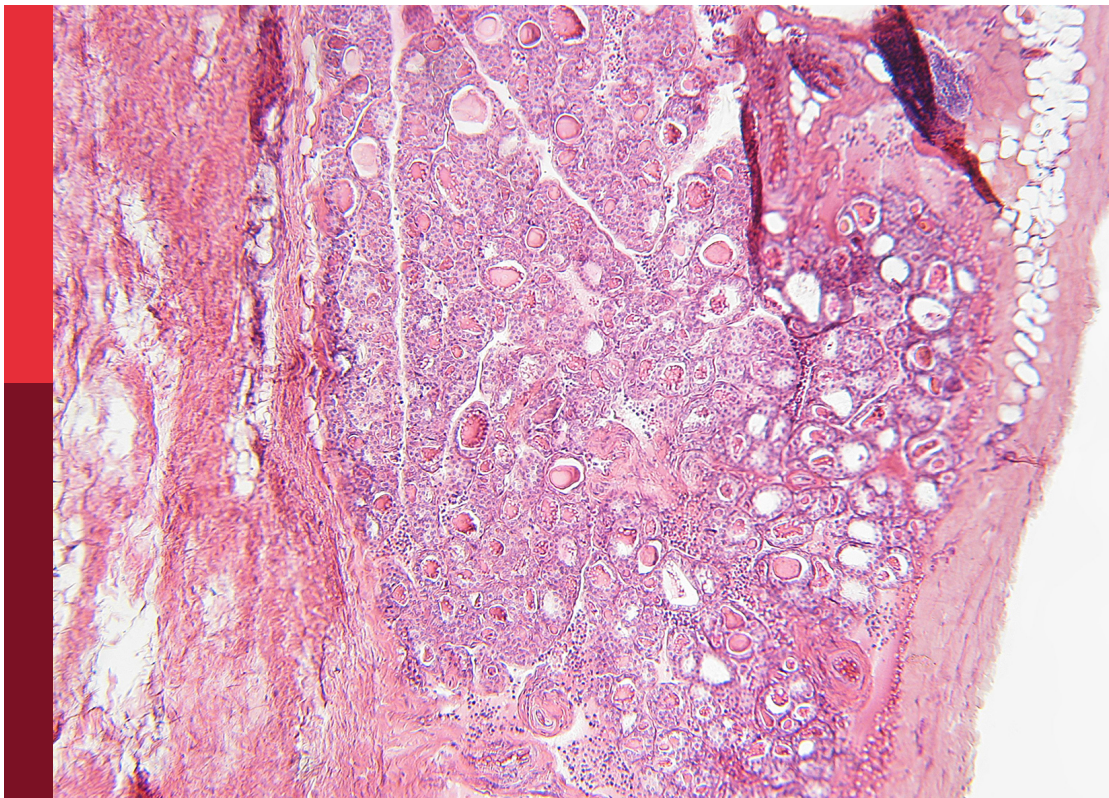
frontiers | Research Topics

Neuroendocrine regulation of feeding and reproduction in fish

Edited by
Shan He, José A. Muñoz-Cueto and Bin Wang

Published in
Frontiers in Endocrinology



FRONTIERS EBOOK COPYRIGHT STATEMENT

The copyright in the text of individual articles in this ebook is the property of their respective authors or their respective institutions or funders. The copyright in graphics and images within each article may be subject to copyright of other parties. In both cases this is subject to a license granted to Frontiers.

The compilation of articles constituting this ebook is the property of Frontiers.

Each article within this ebook, and the ebook itself, are published under the most recent version of the Creative Commons CC-BY licence. The version current at the date of publication of this ebook is CC-BY 4.0. If the CC-BY licence is updated, the licence granted by Frontiers is automatically updated to the new version.

When exercising any right under the CC-BY licence, Frontiers must be attributed as the original publisher of the article or ebook, as applicable.

Authors have the responsibility of ensuring that any graphics or other materials which are the property of others may be included in the CC-BY licence, but this should be checked before relying on the CC-BY licence to reproduce those materials. Any copyright notices relating to those materials must be complied with.

Copyright and source acknowledgement notices may not be removed and must be displayed in any copy, derivative work or partial copy which includes the elements in question.

All copyright, and all rights therein, are protected by national and international copyright laws. The above represents a summary only. For further information please read Frontiers' Conditions for Website Use and Copyright Statement, and the applicable CC-BY licence.

ISSN 1664-8714
ISBN 978-2-83251-815-1
DOI 10.3389/978-2-83251-815-1

About Frontiers

Frontiers is more than just an open access publisher of scholarly articles: it is a pioneering approach to the world of academia, radically improving the way scholarly research is managed. The grand vision of Frontiers is a world where all people have an equal opportunity to seek, share and generate knowledge. Frontiers provides immediate and permanent online open access to all its publications, but this alone is not enough to realize our grand goals.

Frontiers journal series

The Frontiers journal series is a multi-tier and interdisciplinary set of open-access, online journals, promising a paradigm shift from the current review, selection and dissemination processes in academic publishing. All Frontiers journals are driven by researchers for researchers; therefore, they constitute a service to the scholarly community. At the same time, the *Frontiers journal series* operates on a revolutionary invention, the tiered publishing system, initially addressing specific communities of scholars, and gradually climbing up to broader public understanding, thus serving the interests of the lay society, too.

Dedication to quality

Each Frontiers article is a landmark of the highest quality, thanks to genuinely collaborative interactions between authors and review editors, who include some of the world's best academicians. Research must be certified by peers before entering a stream of knowledge that may eventually reach the public - and shape society; therefore, Frontiers only applies the most rigorous and unbiased reviews. Frontiers revolutionizes research publishing by freely delivering the most outstanding research, evaluated with no bias from both the academic and social point of view. By applying the most advanced information technologies, Frontiers is catapulting scholarly publishing into a new generation.

What are Frontiers Research Topics?

Frontiers Research Topics are very popular trademarks of the *Frontiers journals series*: they are collections of at least ten articles, all centered on a particular subject. With their unique mix of varied contributions from Original Research to Review Articles, Frontiers Research Topics unify the most influential researchers, the latest key findings and historical advances in a hot research area.

Find out more on how to host your own Frontiers Research Topic or contribute to one as an author by contacting the Frontiers editorial office: frontiersin.org/about/contact

Neuroendocrine regulation of feeding and reproduction in fish

Topic editors

Shan He — Huazhong Agricultural University, China

José A. Muñoz-Cueto — University of Cádiz, Spain

Bin Wang — Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (CAFS), China

Citation

He, S., Muñoz-Cueto, J. A., Wang, B., eds. (2023). *Neuroendocrine regulation of feeding and reproduction in fish*. Lausanne: Frontiers Media SA.

doi: 10.3389/978-2-83251-815-1

Table of contents

frontiers | Frontiers in Endocrinology

TYPE Editorial
PUBLISHED 17 February 2023
DOI 10.3389/fendo.2023.1160378

Check for updates

OPEN ACCESS

EDITED AND REVIEWED BY
Cunming Duan,
University of Michigan, United States

*CORRESPONDENCE
José A. Muñoz-Cueto
munoz.cueto@uca.es

SPECIALTY SECTION
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 07 February 2023
ACCEPTED 08 February 2023
PUBLISHED 17 February 2023

CITATION
Wang B, He S and Muñoz-Cueto JA (2023) Editorial: Neuroendocrine regulation of feeding and reproduction in fish. *Front. Endocrinol.* 14:1160378. doi: 10.3389/fendo.2023.1160378

COPYRIGHT
© 2023 Wang, He and Muñoz-Cueto. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Editorial: Neuroendocrine regulation of feeding and reproduction in fish

Bin Wang[1,2], Shan He[3,4] and José A. Muñoz-Cueto[5,6,7]*

[1]Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture and Rural Affairs, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China, [2]Laboratory for Marine Fisheries and Food Production Processes, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao, China, [3]College of Fisheries, Huazhong Agricultural University, Wuhan, China, [4]Engineering Research Center of Green Development for Conventional Aquatic Biological Industry in the Yangtze River Economic Belt, Ministry of Education, Wuhan, China, [5]Department of Biology, Faculty of Marine and Environmental Sciences, University of Cádiz, Cádiz, Spain, [6]Marine Research Institute (INMAR), Marine Campus of International Excellence (CEIMAR) and Agrifood Campus of International Excellence (ceiA3), Cádiz, Spain, [7]The European University of the Seas (SEA-EU), Cádiz, Spain

KEYWORDS

feeding, reproduction, neuroendocrine factors, teleosts, aquaculture

Editorial on the Research Topic
Neuroendocrine regulation of feeding and reproduction in fish

The appropriate choice and intake of food and nutrients have a great beneficial impact on health, growth, reproduction and lifespan of fish. Nevertheless, nutritional requirements vary according to the life cycle, physiological and reproductive status of animals. Feeding is a complex behavior regulated by central and peripheral neuroendocrine/endocrine signals, in which hypothalamic neuroendocrine factors such as orexin, galanin, neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), cocaine- and amphetamine-regulated transcript (CART), among others, could play an important role (1–3). In addition to the "classical" neuroendocrine factors, e.g., gonadotropin-releasing hormone (GnRH), dopamine, γ-aminobutyric acid (GABA), serotonin and NPY, new actors as gonadotropin-inhibitory hormone (GnIH), kisspeptin (Kiss), spexin (SPX), neurokinin B/tachykinin (NKB/TAC) and secretoneurin (SN) have gained increasing importance in the regulation of fish reproduction over the last decade (4–13).

It is interesting to note that some of these neuroendocrine factors could modulate food intake as well as gonadal development/maturation and reproductive performance through complex interactions along the hypothalamic-pituitary-gonadal (HPG) axis (5, 14–16). The main objective of the present Research Topic is to provide a comprehensive and updated vision on the neuroendocrine control of feeding and reproduction in fish, focusing especially on the factors that could modulate both physiological processes. This Research Topic contains 13 contributions, 9 original research articles and 4 review papers, which reported recent advances in physiological actions, signaling pathways, evolution and transcriptional regulation of neuroendocrine systems modulating feeding and reproduction in fish.

Four manuscripts in the present Research Topic focused on the role of kisspeptins in fish reproduction and the interaction of stress and reproductive axes. The first review paper by Wang et al. summarized the research progresses of kisspeptin and its receptors (KissR) in teleost fish, with particular emphasis on molecular diversity, phylogenetic evolution, tissue distribution, physiological actions on reproduction, intracellular signaling pathways and regulatory mechanisms. They also highlighted some relevant aspects of the kisspeptinergic system in flatfish species. Predicted *kiss1* and *kissr3* gene sequences are found in the genomes of Senegalese sole, half-smooth tongue sole and turbot, which were previously thought to be lost during evolution in Pleuronectiformes (17, 18). Zhao et al. reported their investigation on the identification and physiological roles of kisspeptins (Kiss1, Kiss2) and their receptors (KissR2 and KissR3) in the control of reproduction in turbot. They confirmed the existence of *kiss1*/*kissr3* genes in this flatfish species by molecular cloning, and showed that both Kiss1 and Kiss2 stimulated pituitary gonadotropin gene expression. Zahangir et al. found in their original research article that Kiss2 significantly stimulated the expression of *kissr2*, *gnrh1* and gonadotropin subunits in a gonadal stage-dependent manner in male grass puffer, which has only a single pair of *kiss2* and *kissr2* (19). In addition, Bu et al. described the molecular mechanisms of glucocorticoid regulation of *kiss1* and *kiss2* genes in yellowtail clownfish. Cortisol stimulated mRNA levels of *kiss1*, *kiss2*, glucocorticoid receptor 1 (*gr1*) and 2 (*gr2*). Particularly, cortisol enhanced *kiss2* promoter activities *via* GRs, with GR1 being more effective than GR2. These data provide additional evidence for the involvement of kisspeptin in the regulation of stress-induced reproductive disorders.

The tachykinin/neurokinin B family of neuropeptides was addressed in a review manuscript and an original paper. The second review paper of this Research Topic is authored by Campo et al., and compared the similarities and differences of tachykinin systems and biological roles in the control of reproduction and food intake between mammals and teleosts. In humans, the tachykinin system comprises three *tac* genes (*tac1*, *tac3*, and *tac4*) which encode 10 different mature peptides and three TAC receptors (TACR1, TACR2, and TACR3). In fish, however, duplicates for *tac1* (*tac1a* and *tac1b*), *tac3* (*tac3a* and *tac3b*), and *tac4* (*tac4a* and *tac4b*) exist and 12 different mature peptides have been identified so far. In turn, up to six TACR types have been characterized in fish: two TACR1 (TACR1a and TACR1b), one TACR2, and three TACR3 (TACR3a1, TACR3a2, and TACR3b). In contrast to mammals, TAC3 peptides (NKB and NKBRP) have various effects on reproduction in teleosts, mainly depending on the species, the maturity stage, and the peptide tested. Further studies are urgently needed to clarify the direct actions of TAC peptides on the expression of central actors involved in the control of food intake in both mammals and teleosts. Zuo et al. investigated the reproductive function of the TAC3/TACR3 system in a catadromous teleost, the Japanese eel. They found that two *tac3* (*tac3a* and *tac3b*) and one *tacr3* (*tacr3a*) genes exist in this species, and confirmed that a mutation caused early termination of TACR3 protein, resulting in the loss of 35 amino acids at the C-terminal of the receptor. Thus, neither NKB nor NKBRP peptides encoded by *tac3* genes could increase CRE-luc and SRE-luc activities *via* their cognate receptor. However, NKB significantly stimulated *gnrh1*, *fshb* or *lhb* mRNA levels, perhaps *via* other receptors.

Two additional manuscripts of this Research Topic focused on hypothalamo-neurohypophyseal neuropeptides. The third review paper by Mennigen et al. discussed the reproductive physiology of the arginine vasopressin (AVP)/oxytocin (OXT) neuropeptide family in teleost fishes. They reviewed the current state of knowledge regarding this teleost nonapeptide system, such as structure, evolution, anatomy, receptor repertoire, regulation, among others. This article mainly focused on the reproductive function, with emphasis on reproductive behavior, reproductive cues, and actions on the HPG axis. Of note, nonapeptide homologues of AVP and OXT in bony fish are designated as vasotocin (VT) and isotocin (IT), respectively. Zhang et al. reported in pregnant lined seahorse that injection of VT intraperitoneally induced premature parturition, and up-regulated serum estrogen concentration and transcript levels of pituitary *fshb*/*lhb* and brood pouch G protein-coupled estrogen receptor (*gper*), however, down-regulated pituitary prolactin (*prl*) mRNA levels. These results suggest that VT could promote premature parturition of seahorse by regulating estrogen synthesis through the HPG axis.

The fourth review paper by Assan et al. is centered on advancements in food intake and feeding behavior regulation in fish, dietary selection and preference and the influence of some extrinsic factors, such as stress, temperature, hypoxia, photoperiod/light regime, circannual and circadian rhythms. They highlighted the physiological role of apelin, a novel appetite-regulating peptide (3), in the modulation of feeding, along with response to different nutritional status in various fish species. It has been well documented that GnRH is a highly conserved decapeptide that is essential for reproduction in vertebrates, but recent *gnrh* knockout studies in zebrafish and medaka suggest that the GnRH system may not be the sole "master" of reproduction in these two species, with its role apparently being less central than originally thought (11, 20). In their original research article, Li et al. proposed GnRH as a coupling factor to integrate the feeding metabolism and reproduction in teleosts. Both GnRH2 and GnRH3 significantly stimulated mRNA levels of pituitary reproduction-related genes (*ghta*, *lhb*, *fshb*, *inhba*, and *sg2*) through the AC/PKA, PLC/IP3/PKC, and Ca^{2+}/CaM/CaMK-II pathways, but reduced dopamine receptor 2 (*drd2*) gene expression *via* the Ca^{2+}/CaM/CaMK-II pathway. In addition, these two neuropeptides also enhanced transcript abundance of pituitary anorexigenic peptides (*pomcb*, *cart2*, *uts1*, *nmba*, and *nmbb*) through the AC/PKA, PLC/IP3/PKC, and Ca^{2+}/CaM/CaMK-II pathways. In turn, Ren et al. provided evidence in pompano for the participation of GnRH1 in the immune regulation of liver disease and the regulation of digestive and metabolic enzyme activities, suggesting that GnRH1 has also non reproductive related functions. Taken together, GnRH seems to be a candidate for the integration of reproduction and metabolism in fish.

Since the first discovery of gonadotropin-inhibitory hormone in the quail, the presence of GnIH orthologs has been reported in a variety of vertebrate species, including fish (12). Despite its functional significance and diversity, little information is available regarding the mode of GnIH action on target cells and the potential interaction with other neuroendocrine factors (21, 22). Wang et al. elucidated the intracellular signaling pathways mediating in sea bass GnIH actions and the interactions with sea bass kisspeptin

signaling. They found that sea bass GnIHR signals can be transduced through the PKA and PKC pathways, and GnIH can interfere with kisspeptin actions by reducing its signaling. On the other hand, spexin 1 (SPX1) has recently emerged as a neuropeptide with pleotropic functions in vertebrates, including the regulation of feeding and reproduction, but knowledge about the physiological role or biological action of SPX2 is still very limited in fish (6, 7). In this Research Topic, Wang et al. evaluated the effects of intraperitoneal injection of endogenous SPX2 on the expression levels of reproductive genes of the brain-pituitary axis in half-smooth tongue sole, providing pioneer evidence for the involvement of SPX2 in the regulation of reproduction in any vertebrate. The functional significance of SPX2, which only exists in non-mammalian species, warrant more investigation in depth.

Finally, we would like to express our most sincere thanks to the authors, the reviewers, and the journal staff for their efforts to make this Research Topic so fruitful.

Author contributions

BW and SH wrote the manuscript. JAM-C edited the manuscript. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by grants from National Natural Science Foundation of China 32072949 to BW, 32172951 to SH, PAIDI2020 (Consejería de Economía, Conocimiento, Empresas y Universidad. Junta de Andalucía. Grant no P18-RT-5152) to JAM-C.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Volkoff H. Fish as models for understanding the vertebrate endocrine regulation of feeding and weight. *Mol Cell Endocrinol* (2019) 497:110437. doi: 10.1016/j.mce.2019.04.017

2. Ronnestad I, Gomes AS, Murashita K, Angotzi R, Jonsson E, Volkoff H. Appetitecontrolling endocrine systems in teleosts. *Front Endocrinol (Lausanne)* (2017) 8:73. doi: 10.3389/fendo.2017.00073

3. Volkoff H. The neuroendocrine regulation of food intake in fish: A review of current knowledge. *Front Neurosci* (2016) 10:540. doi: 10.3389/fnins.2016.00540

4. Tsutsui K, Ubuka T, Ukena K. Advancing reproductive neuroendocrinology through research on the regulation of GnIH and on its diverse actions on reproductive physiology and behavior. *Front Neuroendocrinol* (2022) 64:100955. doi: 10.1016/j.yfrne.2021.100955

5. Shahjahan M, Kitahashi T, Parhar IS. Central pathways integrating metabolism and reproduction in teleosts. *Front Endocrinol (Lausanne)* (2014) 5:36. doi: 10.3389/fendo.2014.00036

6. Lim CH, Lee MYM, Soga T, Parhar I. Evolution of structural and functional diversity of spexin in mammalian and non-mammalian vertebrate species. *Front Endocrinol (Lausanne)* (2019) 10:379. doi: 10.3389/fendo.2019.00379

7. Ma A, Bai J, He M, Wong AOL. Spexin as a neuroendocrine signal with emerging functions. *Gen Comp Endocrinol* (2018) 265:90–6. doi: 10.1016/j.ygcen.2018.01.015

8. Blanco AM. Hypothalamic- and pituitary-derived growth and reproductive hormones and the control of energy balance in fish. *Gen Comp Endocrinol* (2020) 287:113322. doi: 10.1016/j.ygcen.2019.113322

9. Hu G, Lin C, He M, Wong AO. Neurokinin b and reproductive functions: "KNDy neuron" model in mammals and the emerging story in fish. *Gen Comp Endocrinol* (2014) 208:94–108. doi: 10.1016/j.ygcen.2014.08.009

10. Trudeau VL. Neuroendocrine control of reproduction in teleost fish: Concepts and controversies. *Annu Rev Anim Biosci* (2022) 10:107–30. doi: 10.1146/annurevanimal-020420-042015

11. Muñoz-Cueto JA, Zmora N, Paullada-Salmeron JA, Marvel M, Mañanos E, Zohar Y. The gonadotropin-releasing hormones: Lessons from fish. *Gen Comp Endocrinol* (2020) 291:113422. doi: 10.1016/j.ygcen.2020.113422

12. Muñoz-Cueto JA, Paullada-Salmeron JA, Aliaga-Guerrero M, Cowan ME, Parhar IS, Ubuka T. A journey through the gonadotropin-inhibitory hormone system of fish. *Front Endocrinol (Lausanne)* (2017) 8:285. doi: 10.3389/fendo.2017.00285

13. Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O. Neuroendocrinology of reproduction in teleost fish. *Gen Comp Endocrinol* (2010) 165(3):438–55. doi: 10.1016/j.ygcen.2009.04.017

14. Bedecarrats GY, Hanlon C, Tsutsui K. Gonadotropin inhibitory hormone and its receptor: Potential key to the integration and coordination of metabolic status and reproduction. *Front Endocrinol (Lausanne)* (2022) 12:781543. doi: 10.3389/fendo.2021.781543

15. Sivalingam M, Ogawa S, Trudeau VL, Parhar IS. Conserved functions of hypothalamic kisspeptin in vertebrates. *Gen Comp Endocrinol* (2022) 317:113973. doi: 10.1016/j.ygcen.2021.113973

16. Son YL, Ubuka T, Tsutsui K. Regulation of stress response on the hypothalamicpituitary- gonadal axis *via* gonadotropin-inhibitory hormone. *Front Neuroendocrinol* (2022) 64:100953. doi: 10.1016/j.yfrne.2021.100953

17. Mechaly AS, Viñas J, Piferrer F. The kisspeptin system genes in teleost fish, their structure and regulation, with particular attention to the situation in pleuronectiformes. *Gen Comp Endocrinol* (2013) 188:258–68. doi: 10.1016/j.ygcen.2013.04.010

18. Pasquier J, Kamech N, Lafont AG, Vaudry H, Rousseau K, Dufour S. Molecular evolution of GPCRs: Kisspeptin/kisspeptin receptors. *J Mol Endocrinol* (2014) 52(3):T101–117. doi: 10.1530/JME-13-0224

19. Shahjahan M, Motohashi E, Doi H, Ando H. Elevation of Kiss2 and its receptor gene expression in the brain and pituitary of grass puffer during the spawning season. *Gen Comp Endocrinol* (2010) 169(1):48–57. doi: 10.1016/j.ygcen.2010.07.008

20. Zohar Y, Zmora N, Trudeau VL, Muñoz-Cueto JA, Golan M. A half century of fish gonadotropin-releasing hormones: Breaking paradigms. *J Neuroendocrinol.* (2022) 34(5):e13069. doi: 10.1111/jne.13069

21. Wang B, Yang G, Xu Y, Li W, Liu X. Recent studies of LPXRFa receptor signaling in fish and other vertebrates. *Gen Comp Endocrinol* (2019) 277:3–8. doi: 10.1016/j.ygcen.2018.11.011

22. Tsutsui K, Ubuka T. Gonadotropin-inhibitory hormone (GnIH): A new key neurohormone controlling reproductive physiology and behavior. *Front Neuroendocrinol* (2021) 61:100900. doi: 10.1016/j.yfrne.2021.100900

frontiers in Endocrinology

REVIEW
published: 15 December 2021
doi: 10.3389/fendo.2021.798903

Fish Feed Intake, Feeding Behavior, and the Physiological Response of Apelin to Fasting and Refeeding

Daniel Assan[1,2,3,4,5], *Yanlin Huang*[1,2,3,4], *Umar Farouk Mustapha*[1,2,3,4], *Mercy Nabila Addah*[6], *Guangli Li*[1,2,3,4] *and Huapu Chen*[1,2,3,4,5]*

[1] Fisheries College, Guangdong Ocean University, Zhanjiang, China, [2] Guangdong Research Center on Reproductive Control and Breeding Technology of Indigenous Valuable Fish Species, Guangdong Ocean University, Zhanjiang, China, [3] Guangdong Provincial Engineering Laboratory for Mariculture Organism Breeding, Zhanjiang, China, [4] Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Zhanjiang, China, [5] Southern Marine Science and Engineering Guangdong Laboratory, Zhanjiang, China, [6] Department of Fisheries and Aquatic Resources Management, Faculty of Bioscience, University for Development Studies, Tamale, Ghana

Feed is one of the most important external signals in fish that stimulates its feeding behavior and growth. The intake of feed is the main factor determining efficiency and cost, maximizing production efficiency in a fish farming firm. The physiological mechanism regulating food intake lies between an intricate connection linking central and peripheral signals that are unified in the hypothalamus consequently responding to the release of appetite-regulating genes that eventually induce or hinder appetite, such as apelin; a recently discovered peptide produced by several tissues with diverse physiological actions mediated by its receptor, such as feed regulation. Extrinsic factors have a great influence on food intake and feeding behavior in fish. Under these factors, feeding in fish is decontrolled and the appetite indicators in the brain do not function appropriately thus, in controlling conditions which result in the fluctuations in the expression of these appetite-relating genes, which in turn decrease food consumption. Here, we examine the research advancements in fish feeding behavior regarding dietary selection and preference and identify some key external influences on feed intake and feeding behavior. Also, we present summaries of the results of research findings on apelin as an appetite-regulating hormone in fish. We also identified gaps in knowledge and directions for future research to fully ascertain the functional importance of apelin in fish.

Keywords: apelin, feeding behavior, feed intake, fish, orexigenic

OPEN ACCESS

Edited by:
Bin Wang,
Chinese Academy of Fishery Sciences (CAFS), China

Reviewed by:
Xu-Fang Liang,
Huazhong Agricultural University, China
Qiong Zhi Li,
Sichuan Agricultural University, China

***Correspondence:**
Huapu Chen
chpsysu@hotmail.com

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

Received: *20 October 2021*
Accepted: *18 November 2021*
Published: *15 December 2021*

Citation:
Assan D, Huang Y, Mustapha UF, Addah MN, Li G and Chen H (2021) Fish Feed Intake, Feeding Behavior, and the Physiological Response of Apelin to Fasting and Refeeding. Front. Endocrinol. 12:798903. doi: 10.3389/fendo.2021.798903

1 INTRODUCTION

Food is one of the foremost expenses of intensive fish farming, which fish farmers need to pay much attention to. Its availability in quantity and quality is significant for the appropriate growth and reproduction of fish (1). Feeding as determined by Metcalfe and colleagues (2) plays a vital role in animal life-sustaining activities. Evidence has it that, the regulation of feed intake, as in mammals, is well conserved in vertebrates, including some fish species (3, 4). The optimization of food intake can

lead to enhanced growth and body composition, with increased food conversion efficiency and reduced nutrient losses, which are major objectives in intensive fish farming (1).

The intake of feed is known to be regulated by complex interactions between the brain and peripheral appetite-regulating hormonal factors, including apelin (5). As indicated by Volkoff and colleagues (1), when the endocrine mechanisms controlling food intake in fish is understood, it will not only lead to the explicit modifications in fish-holding situations and feeding approaches such as temperature and time of feeding respectively but rather, it will also help to develop new procedures to improve food conversion efficiency as well as aquaculture growth.

In the past years, less attention has been given to apelin in the regulation of feed intake in vertebrates. With its uncertain role in mammals as a feed intake regulator, apelin has been identified to play an orexigenic role in vertebrates such as fish. It aids in several regulation of biological activities in fish, which most importantly includes the regulation of food consumption. The goal of this review is twofold; firstly, to examine the recent advances in our understanding of the feeding behavior, focusing more on dietary selection and preference of fish as well as analyzing the influence that some external factors have on feed intake and behavior. Secondly, we gather information from previous research studies on apelin, categorizing its specific role in fish as an appetite-regulating hormone and identifying gaps in knowledge and directions for future research regarding this important topic.

2 FEED INTAKE AND FEEDING BEHAVIOR IN FISH

The result of food intake is the alteration that lies between starvation, craving, and satiation. Starvation is the physiological necessity for food, including a strong incitement to feeding behavior; looking for food and consuming it. Satiation is the physiological and rational sense of "fullness" that happens after food intake whiles appetite or craving, on the other hand, is the desire to eat, which is commonly related to the material (find, fragrance, taste) perceptiveness of the food to be consumed (6).

Feed is among the most authoritative signals outside the fish's body that can arouse feeding behavior and growth (7–9). Its readiness and composition exert a key control of these processes, by acting principally on the hormones responsible for their endocrine control (9). Some central and peripheral appetite regulators in fishes are affected by a single meal, showing per-prandial fluctuations in their expression and/or secretion levels. Such changes in fishes have been identified in the brain hormone (10–13). The search for food and its intake in fish is girded by a series of behavioral acts matched through a supportive work between the nervous and endocrine systems (14). The control of feed ingestion behavior is a remarkable multifaceted development that comprises particularized interactions between the brain and marginal indications (15). The metabolic sensors located in the central nervous system of fishes provide room for the hypothalamic systems to receive nutritional information, allowing a qualitative control of food ingestion (16). The neural effectors of the hypothalamic origin facilitate the control of food consumed by the fish, thus, by integrating between hunger and satiety signals (17) which include apelin and neuropeptide Y for hunger hints (18, 19), and amylin and cocaine-and amphetamine-regulated transcript for satiety hints (20, 21). As important as it is, it interests more fisheries and aquaculture firms in curbing fish growth and reproduction by changing food and/or endocrine settings.

Fish feeding behavior is miscellaneous and has been broadly examined in both wild and farmed fish from their ecological perspectives (22, 23) whiles behavioral responses of fish to feeding have been associated with feeding approaches, feeding habits, feeding regularity, feed detection mechanisms and feed preferences (24). Feeding behavior and its regulation in fish comprises of external and internal environment information being analyzed by signaling molecules and receptors in the fish. Thus, the hypothalamus, assisted by other brain sections in the fish, integrates inbound indications (3, 14). As ascertained by Volkoff and colleagues, changes in dietary behavior and cravings are frequently related to changes in gene expression and/or protein content of the appetite regulators or their receptors. That is to say, changes in the mRNA/protein levels of a given hormone due to starvation or feeding have the probability of reflecting its physiological role in regulating feed ingestion. Nevertheless, it should be noted that, in the view of the multifactorial character of food regulation, there is a probability for compensative mechanisms in the manner or conduct of feeding to take place whiles fluctuations in available hormones might not essentially suggest variations in feed intake (1).

2.1 Dietary Selection and Preference in Fish

Fish do not consume all the food items they come across. Dietary selection has been broadly explored in mammals, which lessen their consumption of an imbalanced diet to avoid negative dietary impacts (25). Nourishment choice is based on the preface that animals, such as fish, have "quality dietary insight" and hence, select a diet that optimally restores a metabolic imbalance as a result of a nutritional challenge (26). Fishes are selective in the choice of food that contains the necessary nutrients for their survival, growth, and reproduction. This insinuates that fishes as in other animals have evolved from extraordinary diversity of means and challenges, being able to sense nutrients and the existence of precise hungriness to regulate the intake of specific nutrients (27). The source of nutrients can be recognized by gastrointestinal receptors during food digestion, as they are released interiorly in the stomach and pass into the digestive tract. Those receptors would trigger neural action and hormonal signals that would direct brain centers about the dietary properties of food and subsequently alter feeding behavior (28, 29).

Nutrition is a very significant inward factor in fish, as the foremost determination of fish feeding is to satisfy the protein and energy requirements of the fish, relating to feeding behavior

and feed consumption, and have the probability to unswervingly influence or impact the appetite of the fish, reflecting in its growth (30). Nutrients are biological composites involved in biochemical reactions that produce energy and are ingredients of cellular biomass (31). These nutrients have been categorized into two groups; macronutrients and micronutrients. Macronutrients are those that are required in comparatively large quantities since they are the key source to generate the energy required by organisms to live, grow, and reproduce (32). Micronutrients on the other hand are those needed in smaller quantities, even though they have several significant roles in cellular processes (33, 34). The figure below gives examples of key macronutrients and micronutrients and their importance in feed (**Figure 1**). Detailed information on feed and nutrient requirements for fish can be found in a book from the National Research Council (NRC) (34).

The quality of feed suggests the nutritional efficacy and the objective components of a feed making it pertinent to eat and digestible for the fish (42). Fish growth, good health, and maintenance are achieved only when the precise quantity of energy and vital nutrients are available in their adequate proportions in the feed, aiding in proficient feed intake (43, 44). Response to feed intake, its tastiness, and digestibility differ as a result of the difference in fish feed components (41). Signifying that, fish farmers should access the quality of feed they provide for their fish since it plays a key role in it being accepted by the fish, how appetizing it is, and its digestibility. Similarly, the composition of a diet is an additional nutritional aspect of a feed that needs quality attention since it influences appetite-regulating hormones. While the literature available on this is insufficient as stated by Bertucci and colleagues, several research studies have it that, when macronutrients are changed in the diet composition of the fish, it has a significant impact on either the secretion and/or the expression of appetite-regulating hormones. Thus, it's of great importance in fisheries and aquaculture since fluctuating diet and/or hormone milieu influence fish growth and reproduction (9).

Aside from the use of the 'feed intake' method which has extensively been used in animal nutrition, the use of 'self-feeders' in diet selection could be used as a great means to improve the understanding of the physiological approaches towards feeding behavior in fish. For example, studies on rainbow trout (*Oncorhynchus mykiss*) revealed that it could differentiate between diets varying in the vital amino acid content. It also showed that the fish had a precise preference for the whole diet over the balanced amino acids (45). Also, research conducted on self-selection of diets in sharp snout seabream (*Diplodus puntazzo*) and the Senegalese sole (*Solea senegalensis*) revealed that these species select macronutrients according to their dietary needs (29, 46).

According to da Silva and colleagues (27), there are numerous benefits to offering animals a free choice of nourishment, which is considered the most biological and moderate way of providing feed for fish. With time, fish can learn to select specific feeds following their nourishing requirements, as well as self-feed (46, 47). Equally, when the fish meets its goal of each specific nutrient consumption, it will provide its body with the optimal concentrations of nutrients required for proper growth and reproduction (26, 48).

2.2 Extrinsic Factors Influencing Feeding and Feeding Behavior in Fish

Generally, hunger stimulates the behavioral response of feeding fish. When feed is available, fish may initially feed at a faster rate and slowly decrease or stop with a gradual decline of appetite. Feeding behavior despite being influenced by intrinsic factors is extremely influenced by ecological or extrinsic factors (1, 49, 50). Below, we highlight some of these environmental factors that influence food ingestion and feeding behaviors in fish.

2.2.1 Stress

Stress has been defined as the disturbance of physiological or biological mechanisms due to internal and external factors, which are generally designated as stressors (51). These provoke a cataract of consistent behavioral and biological rejoinders in which a living organism makes efforts to reestablish homeostasis, consequently incapacitating the threat. In an aquaculture firm, cultured fish are restricted, captured, crowded, sedated, held, and transported during repetitive husbandry (51). In consequence, all these taken into consideration are ordinary events in fish farming and they are possible stressors that interrupt the behavioral and biological mechanisms of the organism. Thus, causing a

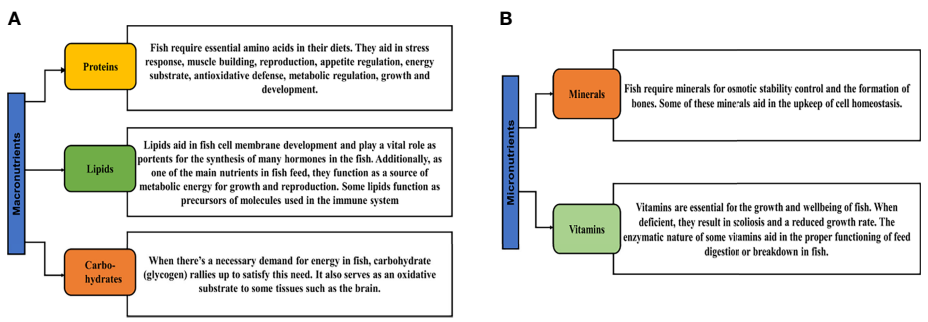


FIGURE 1 | The role and importance of key macronutrients **(A)** and micronutrients **(B)** in fish food. Proteins; (9, 32, 35), lipids; (9, 36–38), carbohydrates; (8, 39, 40), minerals; (9, 41) and vitamins; (41).

functional response crucial to recover the dynamic consistency (52). Reduction in feed ingestion has been described to be distinctive behavioral feedback to stress in fish (53, 54). Undeniably, stress can also disrupt several feeding conducts in fish, including the food search, finding, or capturing prey (16, 55, 56), leading to a decline in growth in several fish species (54). Fish under stressful conditions as compared to unstressed fish eat less and have slow growth. Even when food intake levels are maintained in fish, these conditions are known to persuade a decline in the conversion efficiency of feed consumed, leading to the decreased growth rate (57, 58). For example; a research by Lee and colleagues revealed that acute physical stress caused by cleaning once or thrice a week reduced the daily and cumulative feeding levels and feed conversion efficiency significantly in the sea bass (*Dicentrarchus labrax*) (59). Furthermore, these stressors have been known to adjust the control of endocrinal growth alliance in fish such as the secretion of pituitary growth hormone, among others (60–62). In the two subsections below, we discussed two key stressors that influence the well-being of fish, which needs keen attention.

2.2.1.1 Temperature

There have been several demonstrations of the relationship that exists between temperature and feeding in several fish species. Temperature is one of the most dominant factors influencing some key biological functions in fish, including feed ingestion and feeding behavior (63, 64). Relatively, despite the complex and species-specific effects of temperature in fish, the relation between feeding/feeding behavior and temperature is like a bell-shaped structure (65); at normal temperature conditions, the voluntariness of food intake also increases (65) and/or is maintained during the acclimatization period of temperature which is specific to a particular species. On the other hand, when there's a slight decrease in temperature, the fish adapts to the temperature and maintains its feeding rate for a short period. It has also been ascertained that before the ultimate maximal/minimal critical temperature for a species reaches, it will lose appetite, cease, and lastly stop feeding (66); see **Figure 2**. Examples given here revealed that, a research conducted on Atlantic cod (*Gadus morhua*) revealed that, when kept in a water temperature of 2°C for four weeks, there was a decrease in feed consumption compared to those kept in 11°C and 15°C water temperature (67). Also, research conducted on the red-spotted grouper (*Epinephelus akaara*) revealed that when the water temperature is around 25°C, there's an increase in its feeding and digestion level (68). However, it should be taken into consideration that when the optimal temperature of a particular fish species reaches and/or exceeds, it results in a gradual decline in feeding behavior (69, 70).

2.2.1.2 Hypoxia

Dissolved oxygen (DO) is among the most significant extrinsic factors in fish farming (71). It is known to be a key restrictive factor in aquaculture with the particular reason for the circumstance being that, fish have aerophilic absorption which requires DO at efficient levels (72). The depletion of DO concentration (hypoxia) in water bodies has been identified to be a stern extrinsic stress, which commonly occurs in high-density aquaculture (73). Reports have indicated that growth, survival, behavior as well as other physiological activities of some fish species are highly influenced by different degrees of hypoxia (72, 74) and is also known to be an endocrine disruptor (75).

Fish under severe hypoxia conditions experience reduced movement or feed intake to conserve energy (72). In research conducted on the Atlantic salmon (*Salmo salar*) with regards to the hypoxic period and its physiological activities, results revealed that there were behavioral changes associated with oxygen shortage and physiological stress in some groups. Also, the severity of hypoxia reduced the intake of feed in the fish (76). In a research study on tilapia (*Oreochromis niloticus*) it was discovered that fish kept in hypoxic conditions had significantly reduced feed intake, survival rate, and weight gain (71).

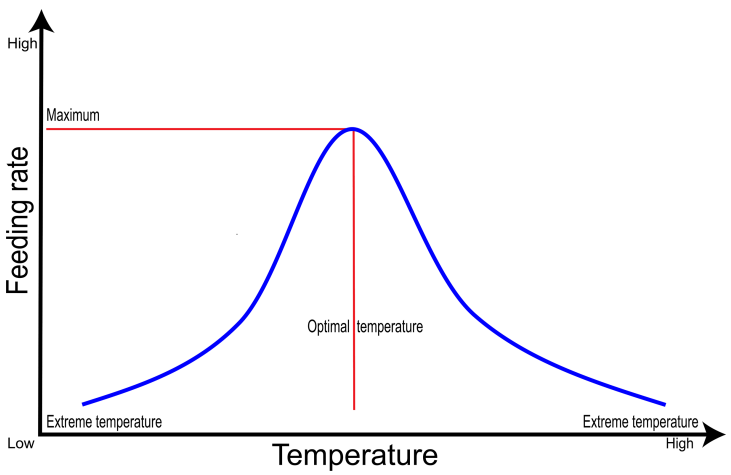


FIGURE 2 | A general relative relation between feeding rate and temperature of fish species. The feeding rate decrease and subsequently stops at higher or lower temperatures (extreme temperatures).

Additionally, a research study on rainbow trout (*Oncorhynchus mykiss*) demonstrated that hypoxia reduces feed consumption, growth rate, oxygen consumption, energy, and lipid contents (77). On the other hand, research conducted on tilapia (*Oreochromis niloticus*) comparing three DO (normal, low and medium) levels showed that, the final fish weight of those in the normal DO levels group were significantly higher as compared to those in the low and medium DO levels groups. Additionally, fish under the low DO level group demonstrated a lower feed intake rate. The low DO level group also revealed that fish in this group had a lower growth and feed utilization rate (78). This significantly demonstrates that when fed under enough DO levels, fish show good efficiency of their feed intake (79), which will most importanlt aid in good feed conversion ratio, fish growth and reproduction in the absence of any other stress. Hypoxia has been discovered to persuade primary, secondary, and tertiary stress responses in fish (80, 81). However, most fishes can adapt to the variations in DO levels but if severe hypoxia remains, fish will sooner or later die (82).

Cultured fish always face repetitive and chronic hypoxia stress especially from overcrowding which they can barely escape due to their confined environment. Therefore, it is suggested that DO levels should be checked and highly maintained near the saturation level. In doing so, it enhances feed intake, feeding behavior, fish growth as well as improves the overall wellbeing and performance of the fish, as the result of hypoxia on the biological or metabolic actions of farmed fish would be negatively affected. A deep look into an article by Abdel-Tawwab and colleagues (72) gives more insight into the effects of hypoxia on fish growth and physiological activities.

2.2.2 Photoperiod and Light Regime

Photoperiod has been known to influence and manipulate some biological functioning in fish (83). Research conducted on several fish species have revealed that photoperiod and the light regime influence their feeding activities. Photoperiod plays a significant part in the growth and survival of fish, thus influencing its feed intake and feeding behavior (84). It is known to have the ability to affect the general wellbeing and routine of fish (85).

The requirements of photoperiod and light concentration in fish are species-specific and differ for the several developing phases (84). Consequently, whiles this could be related to fish species specificity, when photoperiod is appropriately applied, it may aid in an advanced performance of the fish, thereby improving the productivity and sustainability of aquacultural practices. For example, a research study conducted on catfish (*Clarias gariepinus*) fingerlings cultured under three different photoperiod conditions; 24 hours (hrs) darkness, 24hrs light and 12/12hrs darkness and light revealed that those cultured under 24 hrs of darkness had significantly highest feed intake, best feed conversion ratio and lowest quantity of uneaten feed as compared to those cultured under 24hrs light and 12/12 hrs of darkness and light (85). Also, in a research study on the pacamã catfish (*Lophiosilurus alexandri*), it was revealed that 24hrs continuous light led to the highest feed intake (86). Going more further, in research conducted on the sharp-snout seabream (*Diplodus puntazzo*) it was concluded that although feeding behavior was strictly diurnal, 97% of feed demands were made during the light periods (87). A detailed look into how photoperiod affects fish species feed intake and feeding behavior will be of much importance.

2.2.3 Circannual and Circadian Rhythms

All these external factors that impact the feeding behavior in fish have periodic or recurring styles. Thus, they affect food intake unswervingly *via* cyclical and or 24-hourly rhythms (88) or ramblingly through rhythms in endocrine systems (89, 90). All animals, even fish, showcase natural behavioral rhythms, including the two principal feed intake rhythms in fish; the daily (circadian) and seasonal (circannual) rhythms (23, 91, 92).

Several organisms including fish, exhibit annual rhythms in physiological and behavioral factors, such as feeding, reproduction, body weight, hibernation, and movement. These factors are controlled by oscillations in the secretion of hormones. The timing of these annual rhythms is delimited by changes in day length, photoperiod, or temperature, which makes available a reliable and predictive indicator of seasonal changes in environmental conditions (93). The circannual (seasonal) rhythms in vertebrates (fish) associate meticulously with ambient environmental factors, thus environmental (water) temperature and the length of the day. During the spring and summer seasons, when the days are longer and the temperature of water bodies is higher, several fish species increase their feed intake as well as their feeding behavior (23). There is limited information on how these seasonal or circannual rhythms influence feed ingestion and feeding behavior in fish, making it complex to give a straightforward conclusion about feeding activities and associated seasonal changes. As it stands now, we recommend that more research be conducted on fish feeding and feeding behavior regarding the impact of the circannual rhythms.

The circadian rhythm is a natural rhythm that is regulated by a biological daily clock that proceeds in a steady setting. This biological clock is a 24-hour cycle in the biochemical, physiological, or behavioral processes of a live organism geared for maximizing cellular activities and recognizing solar day-related environmental obstacles (94). The 24-hourly rotations of behavior and physiology (example; feeding activity) have been established in all classes of craniates, including some fish species (95, 96). Several inward or endogenic clocks prompt these circadian cycles. They consist of an independent transcriptional-translational response grummet that encompasses the recurring circadian-regulative genes expression (97) and perseveres under continuous extrinsic circumstances, such as photoperiod (98).

As in many animals, fish species consume meals at specific times during the day or night. That is to say that in fish circadian rhythms, the natural daily food ingestion times differ among species (90). Some classify specifically as daytime feeders such as Atlantic salmon, *Salmo salar* (99), redbelly tilapia (*Tilapia zillii*) (100) rohu (*Labeo rohita*), and common carp (*Cyprinus carpio*) (101) whiles others are described as night time feeders, example; European catfish (*Silurus glanis*) (102) and Zebrafish (*Danio rerio*) (103). Additionally, several fish species have showcased ideal times of eating daily (day or night). For example, research

studies conducted on goldfish (*Carassius auratus*) and rainbow trout (*Oncorhynchus mykiss*) respectively revealed that the intake of food and the composition of the body is influenced by the time a single daily meal is delivered (104) whiles rainbow trout (*Oncorhynchus mykiss*) fed during their habitual or natural eating times have higher feed efficiency (105).

There are approximately a handful of known genes or hormones which regulate feed intake in fish species, including neuropeptide Y, peptide YY, ghrelin, galanin, apelin, among others. These appetite-regulating genes influence the intake of feed in two ways; feed intake inducer or inhibitor. The appetite-inducing hormones persuade or signal hunger in fish, thus causing them to search for food to eat (orexigenic factor). On the other hand, appetite-inhibiting hormones are the hormones in fish that signal their satisfaction (anorexigenic factor). Several external and internal factors affect the display of this physiological role in feed intake regulation in fish with regards to their specificity (106). As such, these factors regulate the roles of the gene either by playing opposite roles or not affect the fish at appropriate times (50). Below, we elaborate more and present summaries of the results of research findings on apelin as an appetite-regulating hormone in fish.

3 APELIN AND ITS PHYSIOLOGICAL ROLE IN REGULATING FEED INTAKE IN FISH

3.1 Isolation and Characteristics of Apelin

Apelin is a 36-amino acid (AA) peptide that was initially isolated from bovine stomach extracts (107). It is a recently discovered peptide known as a ligand for the APJ receptor, a putative receptor protein related to the type-1 angiotensin receptor, and a member of the family of seven transmembrane domains G-protein-coupled receptors (GPCRs) (108, 109). From the findings of researches conducted by the research teams of Langelaan and Malyszko, it revealed in mammals that, a 77 AA precursor, prepro-apelin, gives rise to numerous forms of apelin, which can be composed of 13–36 AA residues (110, 111), thus 36, apelin-17, and apelin-13 (112).

The apelin receptor, also called APJ or angiotensin receptor-like-1 which is currently known as the common receptor for apelin was primarily cloned in 1993 due to its robust sequence homology with the angiotensin II receptor (AT1) (54% in transmembrane spheres and 31% for the complete sequence) but APJ does not bind angiotensin II (113). It is known to be an orphan G-protein-coupled receptor that was originally secluded from a human genomic collection using the polymerase chain reaction (PCR) (113). Apelin which was originally described as an endogenous ligand for APJ as stated by (107) secreted as a 77 amino acid forerunner, prepro-apelin, which is differentially processed producing numerous smaller peptide fragments, which comprises apelin-12, apelin-13, apelin-17, and apelin-36 (107, 114, 115).

3.2 mRNA Expression of Apelin in Fish Tissues

Several research studies have revealed the presence of the apelin gene (*apelin*) in several tissues of some fish species such as the goldfish (*Carassius auratus*) red-bellied piranha (*Pygocentrus nattereri*) and cunner (*Tautogolabrus adspersus*) these include different brain regions such as the ladder, optic tectum/thalamus, olfactory bulbs, and the hypothalamus. It can also be found in the pituitary, as well as the peripheral tissues in the fish; spleen, kidney, liver; muscle, brain, gut, gonad, gill, and heart, with seemingly higher expression levels in spleen, kidney, brain, gonad, gill, and heart (19, 116, 117). Although weakly expressed, *apelin* was also identified in the hepatopancreas, eye, intestine, and skin of the Ya-fish (*Schizothorax prenanti*) (118). Also, in *Schizothorax davidi*, apelin mRNA was expressed in the spleen and heart, considerable levels in the brain (myelencephalon and telencephalon), liver, and trunk kidney (119), and pirapitinga, *Piaractus brachypomus*, *apelin* mRNA expression was revealed in the liver, stomach, pyloric caeca, foregut, hindgut, kidney, gill, skin, and muscle as well as in the brain and pituitary (117).

3.3 Non-Appetite Regulatory Role of Apelin

Apelin is known to control cardiovascular functions in mammals, including blood pressure and blood flow (109). The apelin/apj system plays important and several roles in the physiology and pathophysiology of many organs, including the regulation of blood pressure (120), cardiac contractility (121, 122), among others. It is known to be one of the most effective stimulators of cardiac contractility yet discovered and plays a role in cardiac tissue renovation in vertebrates (123–125).

3.4 Apelin as an Appetite-Regulating Hormone in Fish

Apelin, which has an uncertain role in the regulation of feeding in mammals is known to act as an orexigenic factor and might have several biological regulating roles in fish (19, 119, 126). Either its peripheral and/or central injections increased food intake in fish species that have been studied. For example; research conducted on the blind cavefish, *Astyanax fasciatus mexicanus* revealed that peripheral injection of *apelin* significantly increased food intake of the fish as compared to saline injections (127). In goldfish (*Carassius auratus*) both intraperitoneal (i.p.) and intracerebroventricular (i.c.v.) injection of *apelin-13* revealed an augmentation of its food intake (19). Also, in the Siberian sturgeon (*Acipenser baerii*) continuous i.p. injection of *apelin* demonstrated an increase in feed ingestion (126). Additionally, *apelin* i.p. injection in Ya-fish (*Schizothorax prenanti*) also stimulated the intake of feed (118).

3.5 The Response of Apelin to Fasting and Refeeding in Fish

Several hormones in fish as in other vertebrates control the intake of food. These hormones, known as appetite-regulating hormones are produced from the brain and or other marginal tissues in the body of the fish (50). These appetite-regulating hormones play roles either as a food inducer or inhibitor. Apelin's role on food intake in vertebrates like teleost is poorly understood (118). Nonetheless, researches have been conducted

to find out the role that apelin plays in some fish species' food intake, either as an orexigenic or an anorexigenic indicator. Here, we elaborate more on its response to feeding and fasting, indicating which role it fits well into.

Research conducted on common carp (*Cyprinus carpio*) discovered that starvation resulted in a significant upsurge in hypothalamus *apelin* and expression of *APJa* mRNA. It then returned to normal levels after the fish were refed. Also, the expression of *APJb* mRNA augmented after temporary starvation, thus within 2 and 4 days; nevertheless, there was no significant difference between fed fish and refed fish even after the starvation was prolonged. From this same research, there was a significant increase in *apelin*, *APJa*, and *APJb* mRNA expression levels in the foregut of the common carp, which then returned to normal levels after refeeding, either after a short-term or long-term fast (128). It was discovered from a research study that the abundance levels of *apelin* mRNA were greater in starved goldfish than in fed goldfish (*Carassius auratus*) in both hypothalamus and telencephalon (19). Another research study also revealed that *apelin* mRNA levels in the whole brain were higher at 1 hr after feeding than that of unfed Siberian sturgeon (*Acipenser baerii*). However, its expression returned to normal levels at 3 hrs after feeding (126). In this same research study, it was revealed that *apelin* has bidirectional effects on feeding regulation in the Siberian sturgeon (*Acipenser baerii*) thus, *apelin* acts as a satiety factor in the short-term feeding regulation and a hunger factor in long-term feeding regulation (126). Moreover, in research conducted on *Schizothorax davidi*, it was concluded that *apelin* expression of fed fish at + 1 hr and + 3 hrs after feeding was lower than that of unfed fish, and *apelin* expression in the hypothalamus of unfed fish augmented on the 5th and 7th days and when fasting fish were re-fed, *apelin* mRNA expressions disclosed a notable decrease from the 9th to the 14th day concerning the fed group (119). Furthermore, in the red-bellied piranha (*Pygocentrus nattereri*) fasting induced a significant increase in the mRNA expression of *apelin* in the brain (129).

Research conducted on Ya-fish (*Schizothorax prenanti*) revealed that there was about 2.5 and a 2-fold decrease in *apelin* mRNA expression in the hypothalamus of fed fish at 1 hr and 3 hrs post-feeding compared to unfed controls, respectively (118). Both the levels of *apelin* and *APJ* mRNA expressions had a decreasing trend hours before feeding. In this same research, concerning food deprivation, food-destitute Ya-fish (*Schizothorax prenanti*) had a noteworthy change of about 1.8-fold higher mRNA expression levels of *apelin* than 3, 5 and 7 days habitually fed controls. The mRNA expression of *apelin* was significantly decreased when 7-day fasted Ya-fish (*Schizothorax prenanti*) were re-fed, and the levels of the 7-day fed control group and fasted group of Ya-fish (*Schizothorax prenanti*) had an about 1.5- and 2.5-fold higher than the 7-day refed fish, respectively.

To sum up, the role of apelin is highly attributable to appetite regulation in fish. That is to say, the apelin hormone in starved or unfed fish induces hunger (up-regulated) and thus, persuades the fish to go after or search for its meal and there's a gradual or complete decrease in the expression of *apelin* as the fish eats or post-eating. A referral to **Figure 1** in an article publish by Assan and colleagues (50) gives a clear clue on the existing relationship between appetite-inducing genes and appetite-inhibiting genes.

Table 1 summarizes fish that have been used as models for *apelin* research studies as appetite-regulating factors.

3.6 The Interactions of Apelin With Other Appetite-Inducing Hormones in Fish

Recent researches have demonstrated that individual orexigenic molecules or hormones interact with each other. For example, in cavefish, *Astyanax fasciatus mexicanus*, *apelin* i.p. injections increased *orexin* brain expression but did not affect either *cholecystokinin* or *cocaine- and amphetamine-regulated transcript* expression, suggesting that *apelin* might increase food intake through the stimulation of the orexin system in cavefish (127). Additionally, it was demonstrated by *in vitro* and *in vivo* experiments that *apelin* could persuade important mRNA expression levels of appetite-related and growth-related genes, including *neuropeptide Y*, *agouti-related peptide*, and *orexin*. This suggests that *apelin* has the potential to control the food intake and development of common carp by regulating the expression of these vital genes (130).

3.7 Characteristic Similarities Within Appetite-Regulating Hormones

Besides the fish-species-specificity and other intrinsic and extrinsic factors antagonizing appetite-regulating hormones from exerting their full function on regulating feeding in

TABLE 1 | Summary of fish used as models to identify apelin as an appetite-regulating hormone.

Fish models	Treatment	Duration of treatment	Gene regulation	Tissues with the highest mRNA expression	Reference
Ya-fish (*Schizothorax prenanti*)	Starvation	7 days	Up-regulation	Heart, spleen, hypothalamus and kidney	(118)
Common carp (*Cyprinus carpio*)		8 days		Brain, pituitary gland, spleen, and kidney	(128)
Goldfish (*Carassius auratus*)		7 days		Spleen, kidney, brain, gonad, gill, and heart	(19)
Siberian sturgeon (*Acipenser baerii*)		15 days		Brain, spleen, stomach, and kidney	(126)
Red-bellied piranha (*Pygocentrus nattereri*)		7 days		Spleen, kidney, heart, and brain	(129)
Schizothorax davidi		14 days		Brain, heart, spleen, liver, and trunk kidney	(119)

TABLE 2 | List of other known appetite-inducing hormones in fish.

Other appetite-inducing hormones	Key expressed tissue	Reference
Ghrelin	Gastrointestinal tract	(131, 132)
Neuropeptide Y	Brain/Gastrointestinal tract	(12, 18)
Agouti Related Protein	Brain	(12, 133)
Orexins	Brain	(12, 134)
Galanin	Brain	(135, 136)
Growth hormone	Pituitary	(137)

teleost and other fish species, other genes play the same specific role in feed regulation in fish as apelin does. See **Table 2** for a list of other appetite-inducing hormones in fish.

Generally, appetite-inducing hormones (hunger or orexigenic hormones) serve as hunger signals, causing an increase in feed ingestion. That is to say, fasting or starvation causes an up-regulation of these appetite-inducing hormones in fish, for example; *ghrelin* (131, 132), *neuropeptide Y* and *orexin* (12, 18). On the other hand, appetite-inhibiting hormones (satiety or anorexigenic hormones) cause a reduction in food intake, thus fasting or starvation does not affect their expression but rather feed intake causes an up-regulation of these appetite-inducing hormones. Thus, appetite-inhibiting hormones in fish demonstrate pre-prandial decreases and postprandial increases in their concentrations. Example; *peptide YY* (50), *cholecystokinin* (138), and *cocaine- and amphetamine-regulated transcript* (20).

It has been demonstrated that the peripheral or central orexigenic hormone injections in fish persuade a significantly increase in food consumption rate as indicated in some research studies (15, 118, 126, 127, 139). Also, experiments demonstrating the acute and or chronic effect of anorexigenic hormone injections on either peripheral tissues or the brain of some fish species revealed that there were significant reductions in food ingestion for a short period in the acute and a long period all through the whole experiment for the chronic injection. Examples of such experiments include those conducted by (127, 140).

4 CONCLUSION

Data available on fish feeding regulations indicate that the fundamental mechanisms in regulating feeding behavior are conserved. Our knowledge about how extrinsic factors influence feed ingestion and feeding behavior has been simplified. However, it appears that the general scheme of feeding regulation in fishes is similar to that of other vertebrates in the sense that hunger and feeding are controlled by central feeding centers that are influenced by endocrine factors rising from both the brain or from marginal tissues. As a whole, we believe there is still limited information available in fish compared with other organisms regarding how these extrinsic factors influence fish feeding and feeding response.

The role of *apelin* is highly attributable to appetite regulation in fish species that have been studied. To date, most researches conducted on appetite-regulating hormones in fish species have been relatively short-term studies, thus, making it difficult to establish a relation between short-term and long-term appetite-related factors. Fish have been known to exhibit a wider range of feeding behaviors, feeding habits, and feeding adaptations, including fasting or starvation periods. Here we suggest research to be advanced on the mechanisms regulating feeding and appetite-regulating hormones and genes in fish. Also, research on the response of *apelin* to feeding, fasting, and re-feeding should be conducted based on the influence of these extrinsic factors, adding up to the existing studies.

AUTHOR CONTRIBUTIONS

DA designed the content and structure of the whole paper. UFM designed the figures. DA, YH, UFM, and MA wrote the review. GL and HC proofread and revised the paper. All authors contributed to the article and approved the submitted version.

FUNDING

This article was supported by grants from the Key Research and Development Program of Guangdong (2021B202020002), the Guangdong Basic and Applied Basic Research Foundation (2019A1515010958 and 2019A1515012042), the Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang) (ZJW-2019-06), The Open Project of the Key Laboratory of Utilization and Protection of Tropical Marine Biological Resources (Hainan Tropical Ocean University), the Ministry of Education (grant number UCTMB20201), and Guangdong South China Sea Key Laboratory of Aquaculture for Aquatic Economic Animals, Guangdong Ocean University (No. KFKT2019ZD07).

ACKNOWLEDGMENTS

We are thankful to the researchers whose contributions have been cited in this review paper, which have helped us to prepare a constructive review. Moreover, we apologize to those authors whose admirable work could not be cited due to space limitations.

REFERENCES

1. Volkoff H, Hoskins LJ, Tuziak SM. Influence of Intrinsic Signals and Environmental Cues on the Endocrine Control of Feeding in Fish: Potential Application in Aquaculture. *Gen Comp Endocrinol* (2010) 167:352–9. doi: 10.1016/j.ygcen.2009.09.001
2. Metcalfe NB, Huntingford FA, Thorpe JE. Seasonal Changes in Feeding Motivation of Juvenile Atlantic Salmon (Salmo Salar). *Can J Zool* (1986) 64:2439–46. doi: 10.1139/z86-364
3. Volkoff H. The Neuroendocrine Regulation of Food Intake in Fish: A Review of Current Knowledge. *Front Neurosci* (2016) 10:540. doi: 10.3389/fnins.2016.00540

4. Delgado MJ, Cerdá-Reverter JM, Soengas JL. Hypothalamic Integration of Metabolic, Endocrine, and Circadian Signals in Fish: Involvement in the Control of Food Intake. *Front Neurosci* (2017) 11:354. doi: 10.3389/fnins.2017.00354
5. Sunter D, Hewson AK, Dickson SL. Intracerebroventricular Injection of Apelin-13 Reduces Food Intake in the Rat. *Neurosci Lett* (2003) 353:1–4. doi: 10.1016/S0304-3940(03)00351-3
6. Hoskins LJ, Volkoff H. The Comparative Endocrinology of Feeding in Fish: Insights and Challenges. *Gen Comp Endocrinol* (2012) 176:327–35. doi: 10.1016/j.ygcen.2011.12.025
7. Sobrino Crespo C, Perianes Cachero A, Puebla Jiménez L, Barrios V, Arilla Ferreiro E. Peptides and Food Intake. *Front Endocrinol* (2014) 5:58. doi: 10.3389/fendo.2014.00058
8. Conde-Sieira M, Soengas JL. Nutrient Sensing Systems in Fish: Impact on Food Intake Regulation and Energy Homeostasis. *Front Neurosci* (2017) 10:603. doi: 10.3389/fnins.2016.00603
9. Bertucci JI, Blanco AM, Sundarrajan L, Rajeswari JJ, Velasco C, Unniappan S. Nutrient Regulation of Endocrine Factors Influencing Feeding and Growth in Fish. *Front Endocrinol (Lausanne)* (2019) 10:83. doi: 10.3389/fendo.2019.00083
10. Campos VF, Robaldo RB, Deschamps JC, Seixas FK, McBride AJA, Marins LF, et al. Neuropeptide Y Gene Expression Around Meal Time in the Brazilian Flounder Paralichthys Orbignyanus. *J Biosci* (2012) 37:227–32. doi: 10.1007/s12038-012-9205-7
11. Wall A, Volkoff H. Effects of Fasting and Feeding on the Brain mRNA Expressions of Orexin, Tyrosine Hydroxylase (TH), PYY and CCK in the Mexican Blind Cavefish (Astyanax Fasciatus Mexicanus). *Gen Comp Endocrinol* (2013) 183:44–52. doi: 10.1016/j.ygcen.2012.12.011
12. Volkoff H, Sabioni RE, Cyrino JEP. Appetite Regulating Factors in Dourado, Salminus Brasiliensis: cDNA Cloning and Effects of Fasting and Feeding on Gene Expression. *Gen Comp Endocrinol* (2016) 237:34–42. doi: 10.1016/j.ygcen.2016.07.022
13. Volkoff H, Sabioni RE, Coutinho LL, Cyrino JEP. Appetite Regulating Factors in Pacu (Piaractus Mesopotamicus): Tissue Distribution and Effects of Food Quantity and Quality on Gene Expression. *Comp Biochem Physiol Part A Mol Integr Physiol* (2017) 203:241–54. doi: 10.1016/j.cbpa.2016.09.022
14. Kuz'mina VV. The Regulatory Mechanisms of Feeding Behavior in Fish. *J Evol Biochem Physiol* (2019) 55:1–13. doi: 10.1134/S0022093019010010
15. Volkoff H, Unniappan S, Kelly SP. The Endocrine Regulation of Food Intake. *Fish Physiol* (2009) 28:421–65. doi: 10.1016/S1546-5098(09)28009-5
16. Conde-Sieira M, Aguilar AJ, López-Patiño MA, Míguez JM, Soengas JL. Stress Alters Food Intake and Glucosensing Response in Hypothalamus, Hindbrain, Liver, and Brockmann Bodies of Rainbow Trout. *Physiol Behav* (2010) 101:483–93. doi: 10.1016/j.physbeh.2010.07.016
17. Lin X, Volkoff H, Narnaware Y, Bernier NJ, Peyon P, Peter RE. Brain Regulation of Feeding Behavior and Food Intake in Fish. *Comp Biochem Physiol Part A Mol Integr Physiol* (2000) 126:415–34. doi: 10.1016/S1095-6433(00)00230-0
18. Aldegunde M, Mancebo M. Effects of Neuropeptide Y on Food Intake and Brain Biogenic Amines in the Rainbow Trout (Oncorhynchus Mykiss). *Peptides* (2006) 27:719–27. doi: 10.1016/j.peptides.2005.09.014
19. Volkoff H, Wyatt JL. Apelin in Goldfish (Carassius Auratus): Cloning, Distribution and Role in Appetite Regulation. *Peptides* (2009) 30:1434–40. doi: 10.1016/j.peptides.2009.04.020
20. Volkoff H, Peter RE. Characterization of Two Forms of Cocaine-and Amphetamine-Regulated Transcript (CART) Peptide Precursors in Goldfish: Molecular Cloning and Distribution, Modulation of Expression by Nutritional Status, and Interactions With Leptin. *Endocrinology* (2001) 142:5076–88. doi: 10.1210/endo.142.12.8519
21. Thavanathan R, Volkoff H. Effects of Amylin on Feeding of Goldfish: Interactions With CCK. *Regul Pept* (2006) 133:90–6. doi: 10.1016/j.regpep.2005.09.025
22. Houlihan D, Boujard T, Jobling M. *Food Intake in Fish*. Oxford: Blackwell Science (2001).
23. Gerking SD. *Feeding Ecology of Fish*. Elsevier (2014).
24. Lall SP, Tibbetts SM. Nutrition, Feeding, and Behavior of Fish. *Vet Clin North Am Exot Anim Pract* (2009) 12:361–72. doi: 10.1016/j.cvex.2009.01.005
25. Badman MK, Flier JS. The Gut and Energy Balance: Visceral Allies in the Obesity Wars. *Science (80-)* (2005) 307:1909–14. doi: 10.1126/science.1109951
26. Simpson SJ, Raubenheimer D. A Framework for the Study of Macronutrient Intake in Fish. *Aquac Res* (2001) 32:421–32. doi: 10.1046/j.1365-2109.2001.00593.x
27. da Silva RF, Kitagawa A, Sánchez Vázquez FJ. Dietary Self-Selection in Fish: A New Approach to Studying Fish Nutrition and Feeding Behavior. *Rev Fish Biol Fish* (2016) 26:39–51. doi: 10.1007/s11160-015-9410-1
28. Almaida-Pagán PF, Rubio VC, Mendiola P, De Costa J, Madrid JA. Macronutrient Selection Through Post-Ingestive Signals in Sharpsnout Seabream Fed Gelatine Capsules and Challenged With Protein Dilution. *Physiol Behav* (2006) 88:550–8. doi: 10.1016/j.physbeh.2006.05.027
29. Rubio VC, Boluda Navarro D, Madrid JA, Sánchez-Vázquez FJ. Macronutrient Self-Selection in Solea Senegalensis Fed Macronutrient Diets and Challenged With Dietary Protein Dilutions. *Aquaculture* (2009) 291:95–100. doi: 10.1016/j.aquaculture.2009.02.040
30. Zhou C, Xu D, Lin K, Sun C, Yang X. Intelligent Feeding Control Methods in Aquaculture With an Emphasis on Fish: A Review. *Rev Aquac* (2018) 10:975–93. doi: 10.1111/raq.12218
31. Efeyan A, Comb WC, Sabatini DM. Nutrient-Sensing Mechanisms and Pathways. *Nature* (2015) 517:302–10. doi: 10.1038/nature14190
32. Kestemont P, Baras E. Environmental Factors and Feed Intake: Mechanisms and Interactions. *Food Intake Fish* (2001) 131–56. doi: 10.1002/9780470999516.ch6
33. Freeman DW. Using the NRC to Manage Horse Nutrition (An Overview of Management Issues in the Nutrient Requirements of Horses. In: *Conference Sponsors (Citeseer)*, vol. 171. Washington DC: The National Academic Press. (2007).
34. NRC, N. R. C. *Nutrient Requirements of Fish and Shrimp*. Washington, DC: National academies press (2011).
35. Li P, Mai K, Trushenski J, Wu G. New Developments in Fish Amino Acid Nutrition: Towards Functional and Environmentally Oriented Aquafeeds. *Amino Acids* (2009) 37:43–53. doi: 10.1007/s00726-008-0171-1
36. Sheridan MA. Regulation of Lipid Metabolism in Poikilothermic Vertebrates. *Comp Biochem Physiol Part B Comp Biochem* (1994) 107:495–508. doi: 10.1016/0305-0491(94)90176-7
37. Tocher DR. Metabolism and Functions of Lipids and Fatty Acids in Teleost Fish. *Rev Fish Sci* (2003) 11:107–84. doi: 10.1080/713610925
38. Borges P, Oliveira B, Casal S, Dias J, Conceiçao L, Valente LMP. Dietary Lipid Level Affects Growth Performance and Nutrient Utilisation of Senegalese Sole (Solea Senegalensis) Juveniles. *Br J Nutr* (2009) 102:1007–14. doi: 10.1017/S0007114509345262
39. Soengas JL, Aldegunde M. Energy Metabolism of Fish Brain. *Comp Biochem Physiol Part B Biochem Mol Biol* (2002) 131:271–96. doi: 10.1016/S1096-4959(02)00022-2
40. Soengas JL. Contribution of Glucose-and Fatty Acid Sensing Systems to the Regulation of Food Intake in Fish. A Review. *Gen Comp Endocrinol* (2014) 205:36–48. doi: 10.1016/j.ygcen.2014.01.015
41. Craig S, Helfrich LA. Understanding Fish Nutrition, Feeds and Feeding, Cooperative Extension Service, Publication 420–256. Virginia State Univ USA (2002). 26–7.
42. Eriegha OJ, Ekokotu PA. Factors Affecting Feed Intake in Cultured Fish Species: A Review. *Anim Res Int* (2017) 14:2697–709.
43. Hepher B. *Nutrition of Pond Fishes*. Cambridge university press (1988).
44. Omitoyin BO. *Introduction to Fish Farming in Nigeria*. Ibadan, Nigeria: Ibadan University Press (2007).
45. Yamamoto T, Shima T, Furuita H, Suzuki N, Sánchez-Vázquez FJ, Tabata M. Self-Selection and Feed Consumption of Diets With a Complete Amino Acid Composition and a Composition Deficient in Either Methionine or Lysine by Rainbow Trout, Oncorhynchus Mykiss (Walbaum). *Aquac Res* (2001) 32:83–91. doi: 10.1046/j.1355-557x.2001.00007.x
46. Vivas M, Rubio VC, Sánchez-Vázquez FJ, Mena C, García García B, Madrid JA. Dietary Self-Selection in Sharpsnout Seabream (Diplodus Puntazzo) Fed Paired Macronutrient Feeds and Challenged With Protein Dilution. *Aquaculture* (2006) 251:430–7. doi: 10.1016/j.aquaculture.2005.06.013
47. Aranda A, Sánchez-Vázquez FJ, Zamora S, Madrid JA. Self-Design of Fish Diets by Means of Self-Feeders: Validation of Procedures. *J Physiol Biochem* (2000) 56:155–66. doi: 10.1007/BF03179782

48. Fortes-Silva R, Rosa PV, Zamora S, Sánchez-Vázquez FJ. Dietary Self-Selection of Protein-Unbalanced Diets Supplemented With Three Essential Amino Acids in Nile Tilapia. *Physiol Behav* (2012) 105:639–44. doi: 10.1016/j.physbeh.2011.09.023
49. van de Pol I, Flik G, Gorissen M. Comparative Physiology of Energy Metabolism: Fishing for Endocrine Signals in the Early Vertebrate Pool. *Front Endocrinol* (2017) 8:36. doi: 10.3389/fendo.2017.00036
50. Assan D, Mustapha UF, Chen H, Li Z, Peng Y, Li G. The Roles of Neuropeptide Y (Npy) and Peptide YY (Pyy) in Teleost Food Intake: A Mini Review. *Life* (2021) 11(6):547. doi: 10.3390/life11060547
51. Ramsay JM, Feist GW, Varga ZM, Westerfield M, Kent ML, Schreck CB. Whole-Body Cortisol Response of Zebrafish to Acute Net Handling Stress. *Aquaculture* (2009) 297:157–62. doi: 10.1016/j.aquaculture.2009.08.035
52. Aluru N, Vijayan MM. Stress Transcriptomics in Fish: A Role for Genomic Cortisol Signaling. *Gen Comp Endocrinol* (2009) 164:142–50. doi: 10.1016/j.ygcen.2009.03.020
53. Wendelaar Bonga SE. The Stress Response in Fish. *Physiol Rev* (1997) 77:591–625. doi: 10.1152/physrev.1997.77.3.591
54. Bernier NJ. The Corticotropin-Releasing Factor System as a Mediator of the Appetite-Suppressing Effects of Stress in Fish. *Gen Comp Endocrinol* (2006) 146:45–55. doi: 10.1016/j.ygcen.2005.11.016
55. Beitinger TL. Behavioral Reactions for the Assessment of Stress in Fishes. *J Great Lakes Res* (1990) 16:495–528. doi: 10.1016/S0380-1330(90)71443-8
56. Conde-Sieira M, Chivite M, Míguez JM, Soengas JL. Stress Effects on the Mechanisms Regulating Appetite in Teleost Fish. *Front Endocrinol* (2018) 9:631. doi: 10.3389/fendo.2018.00631
57. Paspatis M, Boujard T, Maragoudaki D, Blanchard G, Kentouri M. Do Stocking Density and Feed Reward Level Affect Growth and Feeding of Self-Fed Juvenile European Sea Bass? *Aquaculture* (2003) 216:103–13. doi: 10.1016/S0044-8486(02)00417-9
58. d'Orbcastel ER, Lemarié G, Breuil G, Petochi T, Marino G, Triplet S, et al. Effects of Rearing Density on Sea Bass (Dicentrarchus Labrax) Biological Performance, Blood Parameters and Disease Resistance in a Flow Through System. *Aquat Living Resour* (2010) 23:109–17. doi: 10.1051/alr/2009056
59. Leal E, Fernández-Durán B, Guillot R, Ríos D, Cerdá-Reverter JM. Stress-Induced Effects on Feeding Behavior and Growth Performance of the Sea Bass (Dicentrarchus Labrax): A Self-Feeding Approach. *J Comp Physiol B Biochem Syst Environ Physiol* (2011) 181:1035–44. doi: 10.1007/s00360-011-0585-z
60. Rotllant J, Balm PHM, Pérez-Sánchez J, Wendelaar-Bonga SE, Tort L. Pituitary and Interrenal Function in Gilthead Sea Bream (Sparus Aurata L., Teleostei) After Handling and Confinement Stress. *Gen Comp Endocrinol* (2001) 121:333–42. doi: 10.1006/gcen.2001.7604
61. Deane EE, Woo NYS. Modulation of Fish Growth Hormone Levels by Salinity, Temperature, Pollutants and Aquaculture Related Stress: A Review. *Rev Fish Biol Fish* (2009) 19:97–120. doi: 10.1007/s11160-008-9091-0
62. Saera-Vila A, Calduch-Giner JA, Prunet P, Pérez-Sánchez J. Dynamics of Liver GH/IGF Axis and Selected Stress Markers in Juvenile Gilthead Sea Bream (Sparus Aurata) Exposed to Acute Confinement: Differential Stress Response of Growth Hormone Receptors. *Comp Biochem Physiol Part A Mol Integr Physiol* (2009) 154:197–203. doi: 10.1016/j.cbpa.2009.06.004
63. Kasumyan AO, DÖving KB. Taste Preferences in Fishes. *Fish Fish* (2003) 4:289–347. doi: 10.1046/j.1467-2979.2003.00121.x
64. Handeland SO, Imsland AK, Stefansson SO. The Effect of Temperature and Fish Size on Growth, Feed Intake, Food Conversion Efficiency and Stomach Evacuation Rate of Atlantic Salmon Post-Smolts. *Aquaculture* (2008) 283:36–42. doi: 10.1016/j.aquaculture.2008.06.042
65. Volkoff H, Rønnestad I. Effects of Temperature on Feeding and Digestive Processes in Fish. *Temperature* (2020) 7:307–20. doi: 10.1080/23328940.2020.1765950
66. Shafland PL, Pestrak JM. Lower Lethal Temperatures for Fourteen Non-Native Fishes in Florida. *Environ Biol Fishes* (1982) 7:149–56. doi: 10.1007/BF00001785
67. Kehoe AS, Volkoff H. The Effects of Temperature on Feeding and Expression of Two Appetite-Related Factors, Neuropeptide Y and Cocaine-and Amphetamine-Regulated Transcript, in Atlantic Cod, Gadus Morhua. *J World Aquac Soc* (2008) 39:790–6. doi: 10.1111/j.1749-7345.2008.00215.x
68. Jeon E-J, Kim B-H, Lee C-H, Lee Y-D. Response of Appetite-Related Genes in Relation to the Rearing Water Temperature in Red Spotted Grouper (Epinephelus Akaara). *Fish Aquat Sci* (2020) 23:1–9. doi: 10.1186/s41240-020-00150-1
69. Walberg E. Effect of Increased Water Temperature on Warm Water Fish Feeding Behavior and Habitat Use. *J Undergrad Res Minnesota State Univ Mankato* (2011) 11:13.
70. Mizanur RM, Yun H, Moniruzzaman M, Ferreira F, Kim K, Bai SC. Effects of Feeding Rate and Water Temperature on Growth and Body Composition of Juvenile Korean Rockfish, Sebastes Schlegeli (Hilgendorf 1880). *Asian Australasian J Anim Sci* (2014) 27:690. doi: 10.5713/ajas.2013.13508
71. Lv H-B, Ma Y, Hu C-T, Lin Q-Y, Yue J, Chen L-Q, et al. The Individual and Combined Effects of Hypoxia and High-Fat Diet Feeding on Nutrient Composition and Flesh Quality in Nile Tilapia (Oreochromis Niloticus). *Food Chem* (2021) 343:128479. doi: 10.1016/j.foodchem.2020.128479
72. Abdel-Tawwab M, Monier MN, Hoseinifar SH, Faggio C. Fish Response to Hypoxia Stress: Growth, Physiological, and Immunological Biomarkers. *Fish Physiol Biochem* (2019) 45:997–1013. doi: 10.1007/s10695-019-00614-9
73. Refaey MM, Li D, Tian X, Zhang Z, Zhang X, Li L, et al. High Stocking Density Alters Growth Performance, Blood Biochemistry, Intestinal Histology, and Muscle Quality of Channel Catfish Ictalurus Punctatus. *Aquaculture* (2018) 492:73–81. doi: 10.1016/j.aquaculture.2018.04.003
74. Roman MR, Brandt SB, Houde ED, Pierson JJ. Interactive Effects of Hypoxia and Temperature on Coastal Pelagic Zooplankton and Fish. *Front Mar Sci* (2019) 6:139. doi: 10.3389/fmars.2019.00139
75. Wu RSS, Zhou BS, Randall DJ, Woo NYS, Lam PKS. Aquatic Hypoxia Is an Endocrine Disruptor and Impairs Fish Reproduction. *Environ Sci Technol* (2003) 37:1137–41. doi: 10.1021/es0258327
76. Remen M, Oppedal F, Torgersen T, Imsland AK, Olsen RE. Effects of Cyclic Environmental Hypoxia on Physiology and Feed Intake of Post-Smolt Atlantic Salmon: Initial Responses and Acclimation. *Aquaculture* (2012) 326–329:148–55. doi: 10.1016/j.aquaculture.2011.11.036
77. Magnoni LJ, Eding E, Leguen I, Prunet P, Geurden I, Ozório ROA, et al. Hypoxia, But Not an Electrolyte-Imbalanced Diet, Reduces Feed Intake, Growth and Oxygen Consumption in Rainbow Trout (Oncorhynchus Mykiss). *Sci Rep* (2018) 8:4965. doi: 10.1038/s41598-018-23352-z
78. Abdel-Tawwab M, Hagras AE, Elbaghdady HAM, Monier MN. Effects of Dissolved Oxygen and Fish Size on Nile Tilapia, Oreochromis Niloticus (L.): Growth Performance, Whole-Body Composition, and Innate Immunity. *Aquac Int* (2015) 23:1261–74. doi: 10.1007/s10499-015-9882-y
79. Abdel-Tawwab M, Hagras AE, Elbaghdady HAM, Monier MN. Dissolved Oxygen Level and Stocking Density Effects on Growth, Feed Utilization, Physiology, and Innate Immunity of Nile Tilapia, Oreochromis Niloticus. *J Appl Aquac* (2014) 26:340–55. doi: 10.1080/10454438.2014.959830
80. Bernier NJ, Gorissen M, Flik G. Differential Effects of Chronic Hypoxia and Feed Restriction on the Expression of Leptin and Its Receptor, Food Intake Regulation and the Endocrine Stress Response in Common Carp. *J Exp Biol* (2012) 215:2273–82. doi: 10.1242/jeb.066183
81. Segner H, Sundh H, Buchmann K, Douxfils J, Sundell KS, Mathieu C, et al. Health of Farmed Fish: Its Relation to Fish Welfare and Its Utility as Welfare Indicator. *Fish Physiol Biochem* (2012) 38:85–105. doi: 10.1007/s10695-011-9517-9
82. Cook DG, Herbert NA. The Physiological and Behavioural Response of Juvenile Kingfish (Seriola Lalandi) Differs Between Escapable and Inescapable Progressive Hypoxia. *J Exp Mar Bio Ecol* (2012) 413:138–44. doi: 10.1016/j.jembe.2011.12.006
83. Biswas AK, Takeuchi T. Effects of Photoperiod and Feeding Interval on Food Intake and Growth Rate of Nile Tilapia Oreochromis Niloticus L. *Fish Sci* (2003) 69:1010–6. doi: 10.1046/j.1444-2906.2003.00720.x
84. Nwosu BFM, Holzlöhner S. Effect of Light Periodicity and Intensity on the Growth and Survival of Heterobranchus Longifilis Val. 1840 (Teleostei: Clariidae) Larvae After 14 Days of Rearing. *J Appl Ichthyol* (2000) 16:24–6. doi: 10.1046/j.1439-0426.2000.00145.x
85. Adewolu MA, Adeniji CA, Adejobi AB. Feed Utilization, Growth and Survival of Clarias Gariepinus (Burchell 1822) Fingerlings Cultured Under Different Photoperiods. *Aquaculture* (2008) 283:64–7. doi: 10.1016/j.aquaculture.2008.07.020
86. Kitagawa AT, Costa LS, Paulino RR, Luz RK, Rosa PV, Guerra-Santos B, et al. Feeding Behavior and the Effect of Photoperiod on the Performance and Hematological Parameters of the Pacamã Catfish (Lophiosilurus

Alexandri). *Appl Anim Behav Sci* (2015) 171:211–8. doi: 10.1016/j.applanim.2015.08.025
87. Vera LM, Madrid JA, Sanchez-Vazquez FJ. Locomotor, Feeding and Melatonin Daily Rhythms in Sharpsnout Seabream (Diplodus Puntazzo). *Physiol Behav* (2006) 88:167–72. doi: 10.1016/j.physbeh.2006.03.031
88. Kulczykowska E, Sánchez Vázquez FJ. Neurohormonal Regulation of Feed Intake and Response to Nutrients in Fish: Aspects of Feeding Rhythm and Stress. *Aquac Res* (2010) 41:654–67. doi: 10.1111/j.1365-2109.2009.02350.x
89. Cowan M, Azpeleta C, López-Olmeda JF. Rhythms in the Endocrine System of Fish: A Review. *J Comp Physiol B* (2017) 187:1057–89. doi: 10.1007/s00360-017-1094-5
90. Isorna E, de Pedro N, Valenciano AI, Alonso-Gómez ÁL, Delgado MJ. Interplay Between the Endocrine and Circadian Systems in Fishes. *J Endocrinol* (2017) 232:R141–59. doi: 10.1530/JOE-16-0330
91. Boujard T, Leatherland JF. Circadian Rhythms and Feeding Time in Fishes. *Environ Biol Fishes* (1992) 35:109–31. doi: 10.1007/BF00002186
92. Bolliet V, Azzaydi M, Boujard T. Effects of Feeding Time on Feed Intake and Growth. *Food Intake Fish* (2001) 233–49. doi: 10.1002/9780470999516.ch10
93. Goldman BD. Mammalian Photoperiodic System: Formal Properties and Neuroendocrine Mechanisms of Photoperiodic Time Measurement. *J Biol Rhythms* (2001) 16:283–301. doi: 10.1177/074873001129001980
94. Al-Mousawi ZAH, Alallawee MHA, Ali SA. Circadian Rhythms and Hormonal Homeostasis in Animals : A Review. *Basrah J Vet Res* (2021) 20:128–37.
95. López-Olmeda JF, Sánchez-Vázquez FJ. Feeding Rhythms in Fish: From Behavioral to Molecular Approach. *Biol Clock Fish* (2010), 155–83. doi: 10.1201/b10170
96. Jobling M, Kadri S, Huntingford F, Alanärä A. Feeding Biology and Foraging. *Aquac Behav* (2012) 121–49. doi: 10.1002/9781444354614.ch5
97. Ishikawa T, Hirayama J, Kobayashi Y, Todo T. Zebrafish CRY Represses Transcription Mediated by CLOCK-BMAL Heterodimer Without Inhibiting Its Binding to DNA. *Genes to Cells* (2002) 7:1073–86. doi: 10.1046/j.1365-2443.2002.00579.x
98. Kojima S, Shingle DL, Green CB. Post-Transcriptional Control of Circadian Rhythms. *J Cell Sci* (2011) 124:311–20. doi: 10.1242/jcs.065771
99. Paspatis M, Boujard T. A Comparative Study of Automatic Feeding and Self-Feeding in Juvenile Atlantic Salmon (Salmo Salar) Fed Diets of Different Energy Levels. *Aquaculture* (1996) 145:245–57. doi: 10.1016/S0044-8486(96)01336-1
100. Negassa A, Getahun A. Food Habits and Diel Feeding Rhythm of Introduced Fish, Tilapia Zillii Gervais 1948 (Pisces: Cichlidae) in Lake Zwai, Ethiopia. *SINET Ethiop J Sci* (2004) 27:9–16. doi: 10.4314/sinet.v27i1.18216
101. Rahman MM, Verdegem M, Wahab MA, Hossain MY, Jo Q. Effects of Day and Night on Swimming, Grazing and Social Behaviours of Rohu Labeo Rohita (Hamilton) and Common Carp Cyprinus Carpio (L.) in Simulated Ponds. *Aquac Res* (2008) 39:1383–92. doi: 10.1111/j.1365-2109.2008.02007.x
102. Boujard T. Diel Rhythms of Feeding Activity in the European Catfish, Silurus Glanis. *Physiol Behav* (1995) 58:641–5. doi: 10.1016/0031-9384(95)00109-V
103. del Pozo A, Sánchez-Férez JA, Sánchez-Vázquez FJ. Circadian Rhythms of Self-Feeding and Locomotor Activity in Zebrafish (Danio Rerio). *Chronobiol Int* (2011) 28:39–47. doi: 10.3109/07420528.2010.530728
104. Noeske TA, Spieler RE. Circadian Feeding Time Affects Growth of Fish. *Trans Am Fish Soc* (1984) 113:540–4. doi: 10.1577/1548-8659(1984)113<540:CFTAGO>2.0.CO;2
105. Gelineu A, Médale F, Boujard T. Effect of Feeding Time on Postprandial Nitrogen Excretion and Energy Expenditure in Rainbow Trout. *J Fish Biol* (1998) 52:655–64. doi: 10.1111/j.1095-8649.1998.tb00810.x
106. Rønnestad I, Gomes AS, Murashita K, Angotzi R, Jönsson E, Volkoff H. Appetite-Controlling Endocrine Systems in Teleosts. *Front Endocrinol* (2017) 8:73. doi: 10.3389/fendo.2017.00073
107. Tatemoto K, Hosoya M, Habata Y, Fujii R, Kakegawa T, Zou M-X, et al. Isolation and Characterization of a Novel Endogenous Peptide Ligand for the Human APJ Receptor. *Biochem Biophys Res Commun* (1998) 251:471–6. doi: 10.1006/bbrc.1998.9489
108. Lee DK, Cheng R, Nguyen T, Fan T, Kariyawasam AP, Liu Y, et al. Characterization of Apelin, the Ligand for the APJ Receptor. *J Neurochem* (2000) 74:34–41. doi: 10.1046/j.1471-4159.2000.0740034.x
109. Llorens-Cortes C, Moos F. Opposite Potentiality of Hypothalamic Coexpressed Neuropeptides, Apelin and Vasopressin in Maintaining Body-Fluid Homeostasis. *Prog Brain Res* (2008) 170:559–70. doi: 10.1016/S0079-6123(08)00443-3
110. Malyszko J, Malyszko JS, Pawlak K, Mysliwiec M. Visfatin and Apelin, New Adipocytokines, and Their Relation to Endothelial Function in Patients With Chronic Renal Failure. *Adv Med Sci (De Gruyter Open)* (2008) 53:399–412. doi: 10.2478/v10039-008-0024-x
111. Langelaan DN, Bebbington EM, Reddy T, Rainey JK. Structural Insight Into G-Protein Coupled Receptor Binding by Apelin. *Biochemistry* (2009) 48:537–48. doi: 10.1021/bi801864b
112. Masri B, Knibiehler B, Audigier Y. Apelin Signalling: A Promising Pathway From Cloning to Pharmacology. *Cell Signal* (2005) 17:415–26. doi: 10.1016/j.cellsig.2004.09.018
113. O'Dowd BF, Heiber M, Chan A, Heng HHQ, Tsui L-C, Kennedy JL, et al. A Human Gene That Shows Identity With the Gene Encoding the Angiotensin Receptor Is Located on Chromosome 11. *Gene* (1993) 136:355–60. doi: 10.1016/0378-1119(93)90495-O
114. Hosoya M, Kawamata Y, Fukusumi S, Fujii R, Habata Y, Hinuma S, et al. Molecular and Functional Characteristics of APJ Tissue Distribution of mRNA and Interaction With the Endogenous Ligand Apelin. *J Biol Chem* (2000) 275:21061–7. doi: 10.1074/jbc.M908417199
115. Kawamata Y, Habata Y, Fukusumi S, Hosoya M, Fujii R, Hinuma S, et al. Molecular Properties of Apelin: Tissue Distribution and Receptor Binding. *Biochim Biophys Acta (BBA) Molecular Cell Res* (2001) 1538:162–71. doi: 10.1016/S0167-4889(00)00143-9
116. Hayes J, Volkoff H. Characterization of the Endocrine, Digestive and Morphological Adjustments of the Intestine in Response to Food Deprivation and Torpor in Cunner, Tautogolabrus Adspersus. *Comp Biochem Physiol Part A Mol Integr Physiol* (2014) 170:46–59. doi: 10.1016/j.cbpa.2014.01.014
117. Volkoff H. Cloning and Tissue Distribution of Appetite-Regulating Peptides in Pirapitinga (Piaractus Brachypomus). *J Anim Physiol Anim Nutr (Berl)* (2015) 99:987–1001. doi: 10.1111/jpn.12318
118. Lin F, Wu H, Chen H, Xin Z, Yuan D, Wang T, et al. Molecular and Physiological Evidences for the Role in Appetite Regulation of Apelin and Its Receptor APJ in Ya-Fish (Schizothorax Prenanti). *Mol Cell Endocrinol* (2014) 396:46–57. doi: 10.1016/j.mce.2014.08.009
119. Yuan D, Wang B, Tang T, Lei L, Zhou C, Li Z, et al. Characterization and Evaluation of the Tissue Distribution of CRH, Apelin, and GnRH2 Reveal Responses to Feeding States in Schizothorax Davidi. *Fish Physiol Biochem* (2021) 47:421–38. doi: 10.1007/s10695-020-00922-5
120. Chen MM, Ashley EA, Deng DXF, Tsalenko A, Deng A, Tabibiazar R, et al. Novel Role for the Potent Endogenous Inotrope Apelin in Human Cardiac Dysfunction. *Circulation* (2003) 108:1432–9. doi: 10.1161/01.CIR.0000091235.94914.75
121. Gerbier R, Alvear-Perez R, Margathe J, Flahault A, Couvineau P, Gao J, et al. Development of Original Metabolically Stable Apelin-17 Analogs With Diuretic and Cardiovascular Effects. *FASEB J* (2017) 31:687–700. doi: 10.1096/fj.201600784R
122. Gunter S, Solomon A, Tsang L, Woodiwiss AJ, Robinson C, Millen AME, et al. Apelin Concentrations Are Associated With Altered Atherosclerotic Plaque Stability Mediator Levels and Atherosclerosis in Rheumatoid Arthritis. *Atherosclerosis* (2017) 256:75–81. doi: 10.1016/j.atherosclerosis.2016.11.024
123. Chandrasekaran B, Dar O, McDonagh T. The Role of Apelin in Cardiovascular Function and Heart Failure. *Eur J Heart Fail* (2008) 10:725–32. doi: 10.1016/j.ejheart.2008.06.002
124. Japp AG, Newby DE. The Apelin–APJ System in Heart Failure: Pathophysiologic Relevance and Therapeutic Potential. *Biochem Pharmacol* (2008) 75:1882–92. doi: 10.1016/j.bcp.2007.12.015
125. Ladeiras-Lopes R, Ferreira-Martins J, Leite-Moreira AF. The Apelinergic System: The Role Played in Human Physiology and Pathology and Potential Therapeutic Applications. *Arq Bras Cardiol* (2008) 90(5):343–9. doi: 10.1590/s0066-782x2008000500012
126. Hao J, Liu Q, Zhang X, Wu Y, Zhu J, Qi J, et al. The Evidence of Apelin has the Bidirectional Effects on Feeding Regulation in Siberian Sturgeon (Acipenser Baerii). *Peptides* (2017) 94:78–85. doi: 10.1016/j.peptides.2017.05.007
127. Penney CC, Volkoff H. Peripheral Injections of Cholecystokinin, Apelin, Ghrelin and Orexin in Cavefish (Astyanax Fasciatus Mexicanus): Effects on Feeding and on the Brain Expression Levels of Tyrosine Hydroxylase,

Mechanistic Target of Rapamycin and Appetite-Related Hormones. *Gen Comp Endocrinol* (2014) 196:34–40. doi: 10.1016/j.ygcen.2013.11.015
128. Yan X, Qin C, Deng D, Yang G, Feng J, Lu R, et al. Apelin and Apj in Common Carp (Cyprinus Carpio L.): Molecular Characterization, Tissue Expression and Responses to Periprandial Changes and Fasting–Refeeding. *Aquac Res* (2020) 51:1012–25. doi: 10.1111/are.14447
129. Volkoff H. Appetite Regulating Peptides in Red-Bellied Piranha, Pygocentrus Nattereri: Cloning, Tissue Distribution and Effect of Fasting on mRNA Expression Levels. *Peptides* (2014) 56:116–24. doi: 10.1016/j.peptides.2014.03.022
130. Yan X, Qin C, Yang G, Deng D, Yang L, Feng J, et al. The Regulatory Role of Apelin on the Appetite and Growth of Common Carp (Cyprinus Carpio L.). *Animals* (2020) 10:2163. doi: 10.3390/ani10112163
131. Unniappan S, Canosa LF, Peter RE. Orexigenic Actions of Ghrelin in Goldfish: Feeding-Induced Changes in Brain and Gut mRNA Expression and Serum Levels, and Responses to Central and Peripheral Injections. *Neuroendocrinology* (2004) 79:100–8. doi: 10.1159/000076634
132. Matsuda K, Miura T, Kaiya H, Maruyama K, Shimakura S-I, Uchiyama M, et al. Regulation of Food Intake by Acyl and Des-Acyl Ghrelins in the Goldfish. *Peptides* (2006) 27:2321–5. doi: 10.1016/j.peptides.2006.03.028
133. Cerdá-Reverter JM, Peter RE. Endogenous Melanocortin Antagonist in Fish: Structure, Brain Mapping, and Regulation by Fasting of the Goldfish Agouti-Related Protein Gene. *Endocrinology* (2003) 144:4552–61. doi: 10.1210/en.2003-0453
134. Hagan JJ, Leslie RA, Patel S, Evans ML, Wattam TA, Holmes S, et al. Orexin A Activates Locus Coeruleus Cell Firing and Increases Arousal in the Rat. *Proc Natl Acad Sci* (1999) 96:10911–6. doi: 10.1073/pnas.96.19.10911
135. de Pedro N, Céspedes MV, Delgado MJ, Alonso-Bedate M. The Galanin-Induced Feeding Stimulation Is Mediated via α2-Adrenergic Receptors in Goldfish. *Regul Pept* (1995) 57:77–84. doi: 10.1016/0167-0115(95)91255-4
136. Volkoff H, Peter RE. Interactions Between Orexin A, NPY and Galanin in the Control of Food Intake of the Goldfish, Carassius Auratus. *Regul Pept* (2001) 101:59–72. doi: 10.1016/S0167-0115(01)00261-0
137. Johansson V, Winberg S, Björnsson BT. Growth Hormone-Induced Stimulation of Swimming and Feeding Behaviour of Rainbow Trout Is Abolished by the D1 Dopamine Antagonist SCH23390. *Gen Comp Endocrinol* (2005) 141:58–65. doi: 10.1016/j.ygcen.2004.11.014
138. Kurokawa T, Suzuki T, Hashimoto H. Identification of Gastrin and Multiple Cholecystokinin Genes in Teleost. *Peptides* (2003) 24:227–35. doi: 10.1016/S0196-9781(03)00034-2
139. Yokobori E, Azuma M, Nishiguchi R, Kang KS, Kamijo M, Uchiyama M, et al. Neuropeptide Y Stimulates Food Intake in the Zebrafish, Danio Rerio. *J Neuroendocrinol* (2012) 24:766–73. doi: 10.1111/j.1365-2826.2012.02281.x
140. Zhang X, Tang N, Qi J, Wang S, Hao J, Wu Y, et al. CCK Reduces the Food Intake Mainly Through CCK1R in Siberian Sturgeon (Acipenser Baerii Brandt). *Sci Rep* (2017) 7:1–13. doi: 10.1038/s41598-017-12646-3

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2021 Assan, Huang, Mustapha, Addah, Li and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers in Endocrinology

REVIEW
published: 22 March 2022
doi: 10.3389/fendo.2022.862614

Overview and New Insights Into the Diversity, Evolution, Role, and Regulation of Kisspeptins and Their Receptors in Teleost Fish

Bin Wang[1,2*†], *Alejandro S. Mechaly*[3,4*†] *and Gustavo M. Somoza*[5*†]

[1] Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture and Rural Affairs, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China, [2] Laboratory for Marine Fisheries and Food Production Processes, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao, China, [3] Instituto de Investigaciones en Biodiversidad y Biotecnología (INBIOTEC-CONICET), Mar del Plata, Argentina, [4] Fundación para Investigaciones Biológicas Aplicadas (FIBA), Mar del Plata, Argentina, [5] Instituto Tecnológico de Chascomús (CONICET-UNSAM), Chascomús, Argentina

OPEN ACCESS

Edited by:
Chad D. Foradori,
Auburn University, United States

Reviewed by:
Hélène Volkoff,
Memorial University of Newfoundland, Canada
Kataaki Okubo,
The University of Tokyo, Japan
Ya-Xiong Tao,
Auburn University, United States

***Correspondence:**
Bin Wang
wangbin@ysfri.ac.cn
Alejandro S. Mechaly
amechaly@inbiotec-conicet.gob.ar
Gustavo M. Somoza
somoza@intech.gov.ar

[†]These authors have contributed equally to this work

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

Received: 26 January 2022
Accepted: 21 February 2022
Published: 22 March 2022

Citation:
Wang B, Mechaly AS and Somoza GM (2022) Overview and New Insights Into the Diversity, Evolution, Role, and Regulation of Kisspeptins and Their Receptors in Teleost Fish. Front. Endocrinol. 13:862614. doi: 10.3389/fendo.2022.862614

In the last two decades, kisspeptin (Kiss) has been identified as an important player in the regulation of reproduction and other physiological functions in vertebrates, including several fish species. To date, two ligands (Kiss1, Kiss2) and three kisspeptin receptors (Kissr1, Kissr2, Kissr3) have been identified in teleosts, likely due to whole-genome duplication and loss of genes that occurred early in teleost evolution. Recent results in zebrafish and medaka mutants have challenged the notion that the kisspeptin system is essential for reproduction in fish, in marked contrast to the situation in mammals. In this context, this review focuses on the role of kisspeptins at three levels of the reproductive, brain-pituitary-gonadal (BPG) axis in fish. In addition, this review compiled information on factors controlling the Kiss/Kissr system, such as photoperiod, temperature, nutritional status, sex steroids, neuropeptides, and others. In this article, we summarize the available information on the molecular diversity and evolution, tissue expression and neuroanatomical distribution, functional significance, signaling pathways, and gene regulation of Kiss and Kissr in teleost fishes. Of particular note are recent advances in understanding flatfish kisspeptin systems, which require further study to reveal their structural and functional diversity.

Keywords: fish, kisspeptin, kisspeptin receptor, reproduction, signaling pathway, gene regulation

INTRODUCTION

Eighteen years have passed since the first paper on kisspeptin in a teleost species was published. In that study, the complementary DNA (cDNA) of a kisspeptin receptor (referred to then as *GPR54* and now as *kissr2*) was isolated in the Nile tilapia, *Oreochromis niloticus* (1). The interest in studying the kisspeptin system in fish came from its key role in mammalian reproduction (2–4). A clear example of this is that more than 250 papers have been published to date on kisspeptin and kisspeptin receptors in teleosts, as shown in the Scopus database.

It is now generally accepted that the kisspeptin system in most teleost species consists of two ligands, known as Kiss1 and Kiss2, and two receptors, Kissr2 and Kissr3. However, only one element of this system (either the ligand and/or the receptor) has been detected in Pleuronectiformes, such as *kissr2* in Atlantic halibut, *Hippoglossus hippoglossus* (5); both *kiss2* and *kissr2* in the Senegalese sole, *Solea senegalensis* (6, 7), half-smooth tongue sole, *Cynoglossus semilaevis* (8), and Japanese flounder, *Paralichthys olivaceus* (9). Of note, the European eel, *Anguilla anguilla*, is the only teleost species having two *kiss* genes (*kiss1* and *kiss2*) and three *kissr* types (*kissr1*, *kissr2*, and *kissr3*) reported to date (10).

In recent years, a considerable number of studies have suggested that the kisspeptin system is the "master system" controlling the BPG axis in mammals by exerting its action on gonadotropin-releasing hormone (Gnrh) neurons (3, 11, 12). However, in fish, several studies have considered the Gnrh system as the main system and, the kisspeptin system as a complementary system in controlling fish reproduction (13–15). First, the anatomical association of kisspeptin and Gnrh neurons is not obvious, or almost absent in many teleost species (16–21). Similarly, in zebrafish (*Danio rerio*) and medaka (*Oryzias latipes*) *kiss* and/or *kissr* knockouts display normal reproduction (20, 22, 23). However, it must be considered that, surprisingly, similar results have been obtained with *gnrh* knockouts because, at least in zebrafish, single *gnrh3* mutants and *gnrh3* plus 2 *kiss* gene triple mutants can normally reproduce (23–26). However, in the same species, laser ablation of Gnrh cells at the larval stage resulted in the loss of reproduction in adult fish (27), suggesting that the cellular integrity of Gnrh cells is essential and the Gnrh system is a key and essential player for normal reproduction. Then, it was suggested that the unaltered normal reproductive capacity of mutant fish is compensated by the action of other neuropeptides known to affect gonadotropin secretion (15, 24, 25). In this context, it would be interesting to investigate whether similar mechanisms occur in other teleost species and to clearly determine which peptide(s) are involved in these compensatory mechanisms.

The aim of this review is to examine the entire literature on the kisspeptin system in teleost fishes, with particular emphasis on diversity and evolution, central and peripheral distribution, physiological effects on reproduction, intracellular signaling pathways and regulatory mechanisms.

KISSPEPTIN GENES AND PEPTIDES

Kisspeptins were initially considered to be members of the RFamide peptide family (10). However, other studies demonstrated that kisspeptins are far from the RF-amide family and were proposed to be members of the Kisspeptin/Galanin/Spexin family (28). Given the low conservation of kisspeptin ligands among fish species, their characterization took longer compared to kisspeptin receptors (7). The first Kiss1 orthologs in fish to be characterized were those of zebrafish, spotted pufferfish (*Tetraodon nigroviridis*), Japanese pufferfish (*Fugu rubripes*) and medaka (29, 30). Shortly after, Kiss2 was characterized in zebrafish and medaka (31).

In humans, Kiss1 prepropeptide consists of 145 amino acids (aa) in length, with a major cleavage product of 54 aa (originally named as metastin) and three shorter peptides of 14, 13 and 10 amino acids in length. All these peptides bind to their cognate G protein-coupled receptor today known as kisspeptin receptor (32). It was then demonstrated that the 10 aa peptide was conserved across vertebrates (33), suggesting that it plays an important role in different taxa (34). However, the situation is not as conserved in teleosts. For example, the Kiss1 precursor contains a conserved putative cleavage site six amino acids upstream of the core sequence, suggesting that the mature form of Kiss1 is a pentadecapeptide (17, 35, 36). In addition, the *kiss2* gene produces a mature dodecapeptide in several species (17, 34–36). Moreover, several studies have shown that Kiss1-15 and Kiss2-12 peptides are more effective than Kiss1/2-10 for receptor activation in teleosts (34).

KISSPEPTIN RECEPTOR GENES AND PROTEINS

Kisspeptin receptors are membrane receptors that belong to the superfamily of G protein-coupled receptors (GPCRs) (37). These receptors have a highly conserved structure of seven transmembrane domains (TMDs) that has facilitated their cloning and characterization in vertebrates, including teleosts. As mentioned earlier, the first *kissr* to be characterized in fish was found in the Nile tilapia (1). Soon after, several *kissr2* were characterized in other fish species, such as cobia (*Rachycentron canadum*) (38), grey mullet (*Mugil cephalus*) (39), fathead minnow (*Pimephales promelas*) (40) and two flatfish species, Senegalese Sole (6) and Atlantic halibut (5). The zebrafish genome then helped Biran and coworkers (41) to identify for the first time two kisspeptin receptors in a fish species, then named *kiss1ra* and *kiss1rb* and now known as *kissr2* and *kissr3*, according to the nomenclature introduced by Pasquier et al. (10). Since then, two kisspeptin receptors have been discovered in most teleost fish species studied (15, 42, 43). However, not all teleost species have two kisspeptin receptors. For example, only *kissr2* has been found in the three-spined stickleback (*Gasterosteus aculeatus*), fugu (*Takifugu niphobles*), and spotted pufferfish (44). Mechaly and coworkers (5, 6) also failed to detect *kissr3* by PCR in Pleuronectiformes and suggested that *kissr3* may have been lost during evolution of this order (45).

EVOLUTION OF THE KISSPEPTINERGIC SYSTEMS IN FISH

The first phylogenetic studies on the kisspeptin system genes were essentially obtained from cloned and characterized sequences (1, 5–7, 31, 33, 46). However, advances in next-generation sequencing (NGS) technologies and genomics have

made available dozens of transcriptomes and genomes from a wide range of teleosts and allowed to re-examine the diversity, origin, and evolution of kisspeptins and their receptors not only in fish but also in other vertebrates (10, 44, 47). For example, sequences related to kisspeptin have been identified from genome databases in the European eel (44) and pejerrey fish, *Odontesthes bonariensis* (36), among others.

In teleosts, several studies have performed phylogenetic studies on the nucleotide and/or amino acid sequences of kisspeptins and kisspeptin receptors (10, 44). Since the appearance of this large dataset, more complete phylogenetic studies on the evolution of the kisspeptin systems have been conducted. The situation is similar for flatfishes, and the currently available genomes and/or transcriptomes from half-smooth tongue sole (48), turbot, *Scophthalmus maximus* (49), Senegalese sole (50, 51), common sole, *Solea solea* (50, 51), Japanese flounder (52), black flounder, *Paralichthys orbignyanus* (53), and more recently the available genomes of 11 flatfish species, representing 9 pleuronectiform families (54) have added data to understand kisspeptin phylogeny in teleosts. A summary of all the available information is shown in **Figure 1A**.

Within this framework, another aim of the review is to highlight some relevant aspects of the kisspeptinergic system in flatfishes, as conflicting information on the presence of kisspeptin genes has been reported in this group. One example is the absence of *kiss1* and *kissr3*, as suggested by Mechaly et al. (45) for the Senegalese sole and Atlantic halibut. However, using

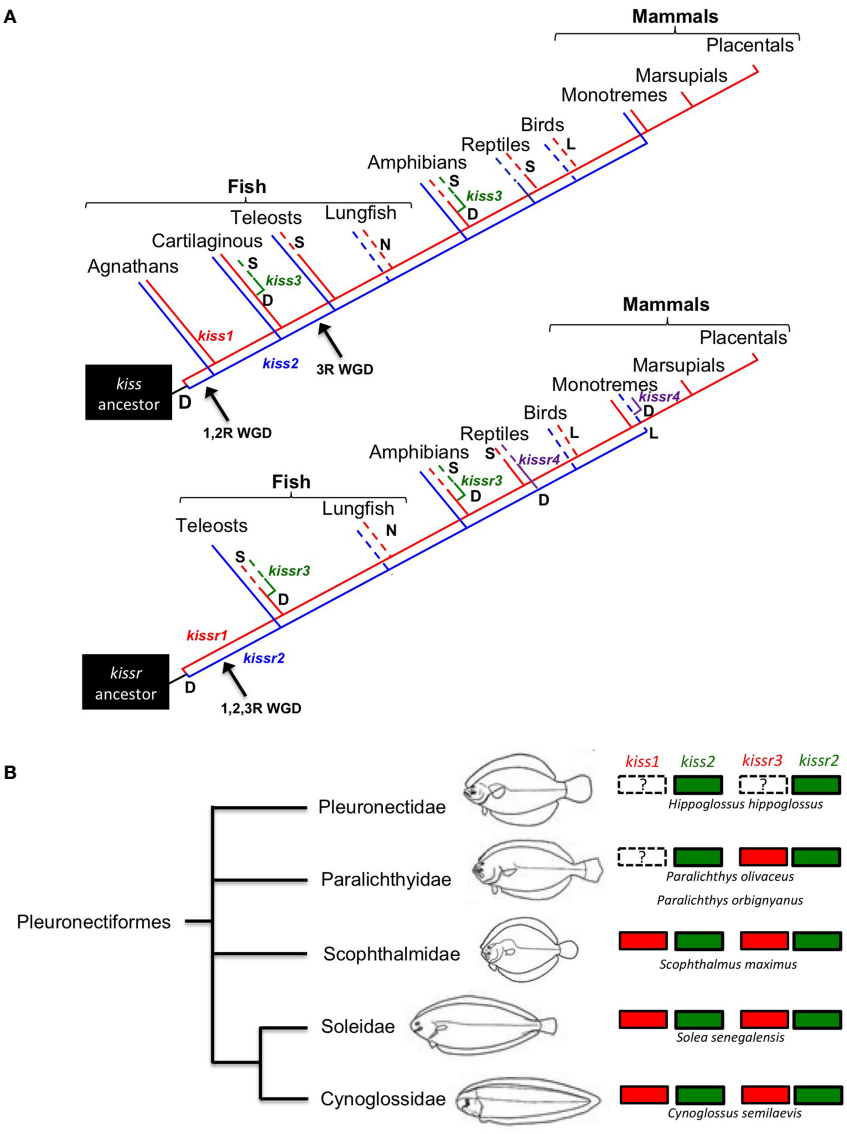


FIGURE 1 | **(A)** *kiss* and *kissr* gene evolution in vertebrates derived from available information. D = gene duplication, L = gene lost, S = gene lost in some species, N = gene not searched. (Modified from 7). **(B)** Consensus tree of flatfish relationships proposed by Chapleau (55), figure modified from Chanet et al. (56).

the current genomic information of both species, we found either complete or partial *kissr3* sequences in Senegalese sole (**Figure 1B**). However, we could not find *kiss1* sequences in the black and Japanese flounders. Both genomes have high sequencing coverage and identified 25,231 protein-coding genes in black flounder (53) and 21,787 protein-coding genes in the case of Japanese flounder (52). However, the absence of *kiss1* annotation in flounder genomes does not necessarily mean that this gene is missing in these species, as "missing" genes can often occur in unassembled reads or contigs (57).

To make a definitive conclusion about the Kiss1 situation, PCR analysis must be performed in both cases. However, it must be kept in mind that this does not guarantee the detection of the gene, as has already been shown in Senegalese sole and Atlantic halibut (5–7). To test whether *kiss1* and/or *kissr3* have been lost in some pleuronectiform species, a more comprehensive comparative sequence analysis needs to be performed. With this in mind, a syntenic analysis of *kiss* and *kissr* neighboring genes in Pleuronectiformes was performed (**Figure 2**). It is always possible that mutations and/or translocations may have occurred to explain the absence of a particular gene in the genome. For example, a *kiss1*-like transcript was already found in the red seabream, *Pagrus major* (58). A similar situation has also been observed in primates, where a *Kiss2*-like gene was in human, chimpanzee, and gorilla genome databases (59).

TISSUE EXPRESSION AND NEUROANATOMICAL DISTRIBUTION OF THE KISS/KISSR SYSTEMS IN FISH

While studies in mammals have shown that kisspeptin has pleiotropic effects (60, 61), the situation in teleosts has not been studied in detail, as most of the studies have focused on the central regulation of reproduction (15, 42, 45). However, kisspeptin transcripts and proteins are widely distributed in various brain areas and tissues of fish, but no clear roles have been associated with these extra brain kisspeptins. These tissues/organs, include the pituitary gland, the spinal cord, the intestine, the gonads, and the liver (62–65). The presence of kisspeptin in blood has been detected in mammals (66). However, to the best of our knowledge there are no available data on kisspeptins levels in fish plasma. This is also the case in flatfishes, where kisspeptin

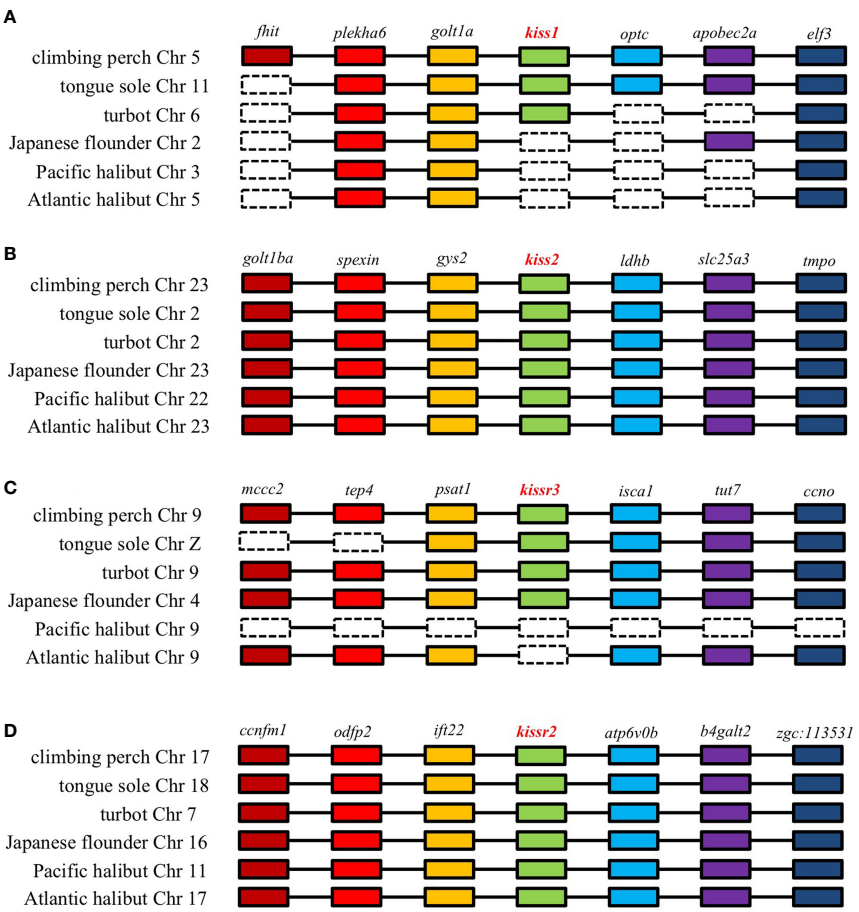


FIGURE 2 | Genomic synteny analysis of kisspeptin **(A, B)** and its receptor **(C, D)** genes in different teleost species.

elements have been detected in various tissues, organs and brain areas. As mentioned earlier, only *kiss2* and *kissr2* have been described in all flatfishes studied to date (45). However, to the best of our knowledge, no functions have been assigned to Kiss2 in these tissues/organs. With the genomes available today this situation has changed, as *kiss1* and *kissr3* sequences have been found in several pleuronectiform species, but no functional studies of *kiss1-kissr3* tissue expression have been performed to date. In this context, the advent of RNA sequencing technology (RNA-seq) will allow us to clarify this situation. However, future studies are needed to clarify the pleiotropic role of kisspeptins not only in Pleuronectiforms but also in other teleost species.

It is important to mention that alternative splice variants are frequently observed in mammals when analyzing GPCR tissue distribution (67), and spliced variants of kisspeptin have also been detected in several teleosts. For example, the presence of alternative splice variants for *kissr2* and/or *kissr3* was observed in Senegalese sole (6, 45), southern bluefin tuna, *Thunnus maccoyii* (68), yellowtail kingfish, *Seriola lalandi* (68), zebrafish (69), European eel (44) and pejerrey fish (65) through intron retention. Mechaly et al. (6, 45, 65) indicate that truncated and likely non-functional proteins are produced in this manner. In two species, yellowtail kingfish (68) and zebrafish (69) alternative spliced variants were also generated by deletion of exons. For a detailed description of the splicing events detected in kisspeptin receptors in fish see Mechaly et al. (45, 65). In this regards, future studies are needed to evaluate whether truncated proteins are generated and whether this represents regulation by unproductive splicing, as has been described for arginine-serine-rich (SR) splicing factors in several organisms (70). To the best of our knowledge, alternative spliced variants have been detected in a kisspeptin ligand in the specific case of Pleuronectiformes and identified only in the *kiss2* gene of Senegalese sole (7).

FUNCTIONAL SIGNIFICANCE OF THE KISS/KISSR SYSTEM IN FISH

In fishes, the functional roles of kisspeptinergic systems in regulating reproduction is not always clear and is sometimes contradictory. For example, Kiss1 has been shown to significantly increase the levels of messengers of luteinizing hormone *b* subunit *(lhb)*, growth hormone (*gh)* and prolactin *(prl)* in goldfish (*Carassius auratus*) pituitary cells (71). However, in European eel, four different kisspeptin analogues (Kiss1-10, Kiss1-15, Kiss2-10 and Kiss2-12) are able to specifically inhibit *lhb* expression in a dose-dependent manner without affecting follicle-stimulating hormone *b* (*fshb)* mRNA levels when acting on pituitary cells (72). But, as mentioned earlier, *kiss*/*kissr* mutated fish showed almost normal fertility and gonadal maturation, suggesting that kisspeptin systems are not strictly required for reproduction, at least in some teleosts (20, 22) or compensatory mechanisms may take over the role of kisspeptins in reproduction (20, 22, 43, 73, 74).

Moreover, connection of kisspeptin nerve terminals and Gnrh cells is not really clear in all teleost species. For example, in the Nile tilapia, a kisspeptin receptor has been shown to be expressed in Ghrh cells (1), and a small number of Gnrh neurons receive kisspeptin innervation in zebrafish (16, 21), striped bass (*Morone saxatilis*) (17) and the cichlid *Astatotilapia burtoni* (75). Conversely, in medaka (19) and European sea bass (*Dicentrarchus labrax*) (18), the presence of kisspeptin receptors on Gnrh neurons could not be detected. However, in zebrafish, Kiss2 nerve terminals reach the pituitary gland (76) and Kiss2 cell bodies and fiber-like projections are found in the *proximal pars distalis* (PPD) with a distribution like Gnrh3 nerve terminals (77), supporting the possibility of an intrapituitary kisspeptinergic regulation of pituitary function. Thus, the physiological significance and functions of kisspeptin in fish reproduction remain controversial. **Table 1** summarizes the physiological effects of kisspeptins in teleosts fish.

KISSPEPTINS' ACTIONS AT BRAIN LEVEL

The biological effects of kisspeptin on Gnrh neurons have been demonstrated in several teleost species at different levels. Kiss1 stimulates the electrical activity of terminal nerve-Gnrh3 neurons in adult medaka (97). Kiss1 also stimulates the electrical activity of the preoptic area (POA) and hypothalamic Gnrh3 neurons in adult zebrafish, while Kiss2 inhibits their neuronal activity (98). In the orange-spotted grouper, *Epinephelus coioides*, intraperitoneal (ip) injection of Kiss2 leads to upregulation of hypothalamic expression of *gnrh1* (91). Similarly, Kiss2, but not Kiss1, significantly stimulates *gnrh1* expression in striped bass brain slices (82). Stimulatory effects of Kiss2 on *gnrh1* expression in the brain and hypothalamus are also observed in the black porgy, *Acanthopagrus schlegelii* (99), Nile tilapia (92), and Japanese flounder (100). In hybrid bass, a differential and gonadal stage-dependent role of kisspeptins on *gnrh1* expression in the brain was observed; both Kiss1 and Kiss2 increase *gnrh1* expression in pre-pubertal fish, while Kiss2 reduces *gnrh1* expression in gonadal recrudescencing fish (17).

Chronic administration of Kiss1 and Kiss2 leads to a decrease in *gnrh1*, *gnrh2*, and/or *gnrh3* transcript levels in the brain of female striped bass (81). An inhibitory effect of Kiss1 and Kiss2 on *gnrh1* and *gnrh2* expression in the forebrain and midbrain is also found in male European sea bass (83). On the other hand, Kiss2 does not alter *gnrh2* and *gnrh3* mRNA expression in the hypothalamus of the half-smooth tongue sole *in vitro* (89). Likewise, injection with Kiss1 and/or Kiss2 peptides induce no significant differences in *gnrh* mRNA levels in other teleosts, such as zebrafish brain *gnrh2* and *gnrh3* (31), hybrid bass brain *gnrh2* and *gnrh3* (17), orange-spotted grouper hypothalamic *gnrh3* (91), yellowtail kingfish brain and hypothalamic *gnrh1* (88), lined seahorse hypothalamic *gnrh3* (93), European sea bass hypothalamic *gnrh1* and forebrain-midbrain *gnrh3* (83). However, Kiss2 has both stimulatory and inhibitory effects on *gnrh1* mRNA levels in the brain of female chub mackerel (*Scomber japonicus*), depending on the mode of

TABLE 1 | Summary of physiological effects of kisspeptins in fish.

Species (Common names)	Kisspeptin types	Peptide sequences	Physiological actions	References
Carassius auratus (Goldfish)	Kiss1-10	YNLNSFGLRY-NH2	Stimulation of pituitary LH, GH and PRL release and synthesis *in vitro*	Yang et al. (71)
			Stimulation of pituitary SLa release *in vitro*	Jiang et al. (78)
			Increase of plasma LH levels *in vivo*	Li et al. (33)
			Stimulation of brain and ovary *kiss1* synthesis *in vivo*	Valipour et al. (79)
	Kiss2-10	FNYNPFGLRF-NH2	No effect on LH release both *in vivo* and *in vitro*	Li et al. (33)
			Stimulation of pituitary *fshb* and *lhb* synthesis *in vivo*	Valipour et al. (80)
			Increase of plasma 17*b*-estradiol levels *in vivo*	Valipour et al. (80)
Danio rerio (Zebrafish)	Kiss1-10	YNLNSFGLRY-NH2	No effect on brain *gnrh2* and *gnrh3* synthesis as well as pituitary *fshb*, *lhb*, *gh1* and *prl* synthesis *in vivo*	Kitahashi et al. (31)
	Kiss2-10	FNYNPFGLRF-NH2	Stimulation of pituitary *fshb* and *lhb* synthesis *in vivo*	Kitahashi et al. (31)
			Stimulation of pituitary *fshb, lhb* and *prl1* synthesis in females *in vitro*	Song et al. (77)
Morone saxatilis × *Morone chrysopshy* (Hybrid bass)	Kiss1-15	QDVSSYNLNSFGLRY-NH2	Increase of plasma LH levels at gonadal recrudescence *in vivo*	Zmora et al. (17)
			Stimulation of brain *gnrh1* synthesis at prepuberty *in vivo*	Zmora et al. (17)
			Inhibition of brain *kissr2* synthesis at recrudescence *in vivo*	Zmora et al. (17)
	Kiss2-12	SKFNFNPFGLRF-NH2	Increase of plasma LH levels at prepuberty and gonadal recrudescence *in vivo*	Zmora et al. (17)
			Stimulation of brain *kissr2* and *gnrh1* synthesis at prepuberty *in vivo*	Zmora et al. (17)
			Inhibition of brain *kissr2* and *gnrh1* synthesis at gonadal recrudescence *in vivo*	Zmora et al. (17)
Morone saxatilis (Striped bass)	Kiss1-15	QDVSSYNLNSFGLRY-NH2	Inhibition of brain *gnrh1* and *gnrh2* synthesis *in vivo*	Zmora et al. (81)
			Inhibition of pituitary *gnrh1r* synthesis *in vivo*	Zmora et al. (81)
			Stimulation of pituitary *fshb* synthesis *in vivo*	Zmora et al. (81)
			Increase of oocyte diameter *in vivo*	Zmora et al. (81)
			Stimulation of pituitary *fshb* synthesis *in vitro*	Zmora et al. (82)
			Inhibition of pituitary *lhb* synthesis *in vitro*	Zmora et al. (82)
			Increase of FSH levels *in vitro*	Zmora et al. (82)
	Kiss2-12	SKFNFNPFGLRF-NH2	Inhibition of brain *gnrh1*, *gnrh2* and *gnrh3* synthesis *in vivo*	Zmora et al. (81)
			Inhibition of pituitary *gnrh1r* synthesis *in vivo*	Zmora et al. (81)
			Decrease of plasma LH and FSH levels *in vivo*	Zmora et al. (81)
			Stimulation of brain *gnrh1* synthesis *in vitro*	Zmora et al. (82)
			Stimulation of pituitary *fshb* synthesis *in vitro*	Zmora et al. (82)
			Increase of FSH and LH levels *in vitro*	Zmora et al. (82)
Dicentrarchus labrax (European sea bass)	Kiss1-10	YNLNSFGLRY- NH2	Increase of plasma LH levels *in vivo*	Felip et al. (46)
	Kiss1-15	QDVSSYNLNSFGLRY-NH2	Stimulation of *kissr2* synthesis in forebrain-midbrain *in vivo*	Espigares et al. (83)
			Inhibition of *gnrh1* and *gnrh2* synthesis in forebrain-midbrain *in vivo*	Espigares et al. (83)
			Increase of hypothalamic and pituitary GnRH1 content *in vivo*	Espigares et al. (83)
			Increase of plasma LH levels *in vivo*	Espigares et al. (83)
	Kiss2-10	FNFNPFGLRF-NH2	Increase of plasma LH and FSH levels *in vivo*	Felip et al. (46)
	Kiss2-12	SKFNFNPFGLRF-NH2	Stimulation of *kissr2* synthesis in forebrain-midbrain *in vivo*	Espigares et al. (83)
			Inhibition of *gnrh1* and *gnrh2* synthesis in forebrain-midbrain *in vivo*	Espigares et al. (83)
			Increase of hypothalamic GnRH1 content *in vivo*	Espigares et al. (83)
			Inhibition of pituitary *gnrhr-II-1a* synthesis *in vivo*	Espigares et al. (83)
			Increase of plasma LH, T and 11-KT levels *in vivo*	Espigares et al. (83)
			Increase of sperm motility parameters *in vivo*	Espigares et al. (83)
			Stimulation of pituitary LH and FSH release *in vitro*	Espigares et al. (84)
Scomber japonicus (Chub mackerel)	Kiss1-10	QDMSSYNFNSFGLRY-NH2	Inhibition of pituitary *lhb* synthesis in sexually immature adult females *in vivo*	Selvaraj et al. (85)
			Increase of plasma 11-KT levels in sexually immature adult males and E2 levels in females *in vivo*	Selvaraj et al. (85)
			Induction of spermiation and vitellogenic onset *in vivo*	Selvaraj et al. (85)
			Increase of plasma 11-KT and E2 levels in pre-pubertal males *in vivo*	Selvaraj et al. (86)
			Acceleration of spermatogenesis in pre-pubertal males *in vivo*	Selvaraj et al. (86)
	Kiss2-12	SNFNFNPFGLRF-NH2	Inhibition of brain *gnrh1* synthesis in sexually immature adult females *in vivo*	Ohga et al. (87)
			Stimulation of pituitary *fshb* and *lhb* synthesis in both sexes *in vivo*	Ohga et al. (87)
			Stimulation of brain *gnrh1* synthesis in sexually immature adult females *in vivo*	Selvaraj et al. (85)
			Increase of spermatocytes numbers in pre-pubertal males *in vivo*	Selvaraj et al. (86)

(Continued)

TABLE 1 | Continued

Species (Common names)	Kisspeptin types	Peptide sequences	Physiological actions	References
Seriola lalandi (Yellowtail kingfish)	Kiss1-10	YNLNSFGLRY-NH2	Stimulation of pituitary *kissr2* synthesis during the non-breeding season *in vivo*	Nocillado et al. (88)
			Stimulation of pituitary *fshb* synthesis during the breeding season *in vivo*	Nocillado et al. (88)
			Stimulation of pituitary *fshb* and *lhb* synthesis during the non-breeding season *in vivo*	Nocillado et al. (88)
			Stimulation of gonadal development regardless of the season *in vivo*	Nocillado et al. (88)
	Kiss2-10	FNFNPFGLRF-NH2	Stimulation of gonadal development during the non-breeding season *in vivo*	Nocillado et al. (88)
			Inhibition of brain *kissr2_v1* and *kissr2_v5* synthesis in pre-pubertal males *in vivo*	Nocillado et al. (68)
			Stimulation of *kissr2_v4* synthesis in pre-pubertal males *in vivo*	Nocillado et al. (68)
			Increase of plasma E2 levels in pre-pubertal females *in vivo*	Nocillado et al. (68)
Anguilla anguilla (European eel)	Kiss1-10	YNWNSFGLRY-NH2	Inhibition of pituitary *lhb* and *gnrhr-2* synthesis *in vitro*	Pasquier et al. (72)
	Kiss1-15	ENFSSYNWNSFGLRY-NH2		
	Kiss2-10	FNRNPFGLRF-NH2	Inhibition of pituitary *lhb* and *gnrhr-2* synthesis *in vitro*	Pasquier et al. (72)
	Kiss2-12	SKFNRNPFGLRF-NH2		
Cynoglossus semilaevis (Half-smooth tongue sole)	Kiss2-10	FNFNPFGLRF-NH2	Stimulation of hypothalamic *kiss2* and *lpxrfa* synthesis *in vitro*	Wang et al. (89)
			Inhibition of hypothalamic *kissr2* and *lpxrfa-r* synthesis *in vitro*	Wang et al. (89)
			Stimulation of pituitary *fshb* and *gtha* synthesis *in vitro*	Wang et al. (90)
Epinephelus coioides (Orange-spotted grouper)	Kiss2-10	FNFNPFGLRF-NH2	Stimulation of hypothalamic *gnrh1* synthesis *in vivo*	Shi et al. (91)
			Stimulation of pituitary *fshb* synthesis *in vivo*	Shi et al. (91)
Oreochromis niloticus (Nile tilapia)	Kiss2-10	FNYNPLSLRF-NH2	Stimulation of brain *gnrh1*, *fshb* and *lhb* synthesis *in vivo*	Park et al. (92)
			Increase of plasma 11-KT levels in males and E2 levels in females *in vivo*	Park et al. (92)
			Acceleration of spermatogenesis *in vivo*	Park et al. (92)
Hippocampus erectus (Lined seahorse)	Kiss2-10	FNVNPFGLRF-NH2	Stimulation of pituitary *fshb* and *lhb* synthesis *in vivo*	Zhang et al. (93)
			Increase of plasma testosterone levels *in vivo*	Zhang et al. (93)
Solea senegalensis (Senegalese sole)	Kiss2-10	FNFNPFGLRF-NH2	Increase of plasma FSH and LH levels *in vivo*	Oliveira et al. (94)
			Increase of plasma testosterone levels *in vivo*	Oliveira et al. (94)
Heteropneustes fossilis (Tinging catfish)	Kiss1-10	YNWNSFGLRY-NH2	Stimulation of hypothalamic, pituitary and ovarian *gnrh1* and *gnrh2* *in vivo*	Chaube et al. (95)
			Stimulation of pituitary *fshb* and *lhb* synthesis *in vivo*	Chaube et al. (95)
			Increase of plasma and ovarian E2, progesterone and 17,20b-dihydoxy-4-pregnen-3-one levels	Chaube et al. (95)
	Kiss2-10	FNFNPFGLRF-NH2	Stimulation of hypothalamic, pituitary and ovarian *gnrh1* and *gnrh2* *in vivo*	Chaube et al. (95)
			Stimulation of pituitary *fshb* and *lhb* synthesis *in vivo*	Chaube et al. (95)
			Increase of plasma and ovarian E2, progesterone and 17,20b-dihydoxy-4-pregnen-3-one levels	Chaube et al. (95)
Micropterus salmoides (Largemouth bass)	Kiss2-10	FNFNPFGLRF-NH2	Stimulation of brain *gnrh3* and *kissr2* synthesis *in vivo*	Li et al. (96)
			Stimulation of pituitary *fshb* and *lhb* synthesis *in vivo*	Li et al. (96)
			Stimulation of ovarian *er2* and testicular *ar* synthesis *in vivo*	Li et al. (96)
			Increase of plasma 17*b*-estradiol and testosterone levels *in vivo*	Li et al. (96)
			Acceleration of vitellogenesis and spermatogenesis *in vivo*	Li et al. (96)

administration. Subcutaneous and slow release of Kiss2 increases *gnrh1* expression (86), whereas intracerebroventricular (icv) administration of Kiss2 suppresses *gnrh1* expression (87). Taken together, these data suggest that the mode of actions of Kiss1 and Kiss2 on Gnrh neurons are different among fish species and depend not only on gonadal status but also on the way of administration.

On the other hand, the LPXRFa system (the piscine ortholog of gonadotropin-inhibitory hormone, Gnih) is also a target for the central effects of kisspeptin in fish. In hypothalamic explants of half-smooth tongue sole, Kiss2 exerts a stimulatory effect on *lpxra* transcript levels, while apparently reducing *lpxrfa-r* mRNA levels (89). To our knowledge, this is the first evidence for the involvement of kisspeptin in the LPXRFa system in any fish species investigated so far. In addition, autoregulation of the kisspeptin system has been observed in several teleosts. For details, see the section on neuropeptides (see below).

KISSPEPTINS´ ACTIONS ON THE PITUITARY

The physiological roles of both Kiss1 and Kiss2 do not appear to follow a common pattern in teleosts. Previous *in vitro* studies

indicate a direct stimulatory effect of kisspeptin on gonadotropins in different species. For example, Kiss1 significantly triggers Lh release from primary pituitary cell cultures of goldfish (71, 101), and Kiss2 has a stimulatory effect on both Lh and Fsh release in pituitary cells of European sea bass (84). In striped bass, both Kiss1 and Kiss2 stimulate Fsh release *in vitro*, whereas only Kiss2 is able to exert a stimulatory effect on Lh release (82). Moreover, ip injection of Kiss1, but not Kiss2, significantly increases serum Lh levels in goldfish (33). An increase in plasma Lh and Fsh levels is observed in European sea bass after injection of both Kiss1 and Kiss2 (46, 83). However, Kiss2 is more effective than Kiss1 in triggering gonadotropin secretion in this species (46). Similarly, intramuscular injection of Kiss2 stimulates secretion of Fsh and Lh in Senegalese sole of both sexes (94). Moreover, a differential and gonadal stage-dependent roles of kisspeptin on Lh release was observed in hybrid bass: Kiss1 increases plasma LH levels during gonadal recrudescence *in vivo*, whereas Kiss2 stimulates the release of LH during at pre-puberty and gonadal recrudescence (17).

In addition, injection of Kiss2 triggers an increase in pituitary *fshb* and *lhb* mRNA expression in zebrafish (31) and chub mackerel (87). Treatment of orange-spotted grouper with Kiss2 results in an increase in *fshb* mRNA abundance *in vivo* (91). Moreover, half-smooth tongue sole Kiss2 apparently induces an increase in *gtha* and *fshb* mRNA levels, without affecting *lhb* mRNA transcripts *in vitro* (90). In zebrafish, Kiss2 significantly stimulates *fshb* and *lhb* expression in the female pituitary gland *in vitro* (77).

However, other teleost studies have reported some inhibitory effects of kisspeptins on gonadotropins. For example, chronic treatment with Kiss2 results in a decrease in plasma Lh and Fsh levels *in vivo* in striped bass (81). Both heterologous and homologous kisspeptin peptides inhibit *lhb* mRNA levels *in vitro*, without affecting *fshb* expression in European eel (72, 102). An inhibitory effect of Kiss1 on *lhb* expression is also observed in striped bass (82) and female chub mackerel (85). However, no effect of Kiss1 treatment on the relative abundances of *lhb* and *fshb* is observed in zebrafish (31) and chub mackerel (86, 87). Kiss2 also dose not alter *lhb* and *fshb* mRNA levels in yellowtail kingfish (88), chub mackerel (85), striped bass (81) and European sea bass (84).

In addition to the effects on gonadotropins, kisspeptins have also been shown to be involved in regulating the synthesis and/or release of other pituitary hormones in fish. Kiss1 in goldfish directly stimulates the secretion of Prl and Gh as well as gene expression *in vitro* (71). Similarly, Kiss1 enhances the release of somatolactin-a (Sla) in goldfish pituitary cells (78). Kiss2 in zebrafish significantly stimulates the expression of *prl1* in the female pituitary *in vitro* without affecting the mRNA levels of *prl2*, pro-opiomelanocortin-a (*pomca*) and *pomcb* (77). At the pituitary level, injection of Kiss1, but not Kiss2, significantly increases pituitary levels of Gnrh1 in European sea bass (83). In addition, an inhibitory effect of kisspeptin on *gnrhr* expression is observed in European eel (72), European sea bass (83) and striped bass (81). As mentioned earlier, it is also important to emphasize that unidentified Kiss2 cells and projections are found in the PPD, as well as the distribution of Gnrh3 fibers (77), suggesting the possibility of a paracrine/autocrine intrapituitary kisspeptinergic system.

KISSPEPTINS´ ACTIONS ON THE GONADS

To date, there are a few reports on the effects or functions of kisspeptins at the gonadal level in teleosts. An initial study in yellowtail kingfish showed that chronic treatment with Kiss1 and Kiss2 could stimulate gonadal development in prepubertal males (103). Further studies in the same species showed that Kiss1 is more effective in stimulating gonadal development during the breeding season, while the effects of Kiss2 is more pronounced during the nonbreeding season (88). Kiss1 is also able to accelerate puberty onset in juvenile male white bass (*Morone chrysops*) (104). Plasma levels of 11-ketotestosterone (11-KT) and 17b-estradiol are increased, and spermatogenesis and the onset of vitellogenesis are observed in sexually immature adult chub mackerel over 6–7 weeks following subcutaneous implantation of Kiss1, but not Kiss2 (85). Furthermore, subcutaneous injection of Kiss1 also accelerates spermatogenesis in prepubertal male chub mackerel (86).

On the other hand, only Kiss2 stimulates plasma levels of testosterone (T) and 11-KT in male European sea bass, causing an increase in cumulative milt, sperm density and sperm motility parameters (83). Similarly, plasma levels of 11-KT in males and E_2 in females are significantly increased in immature Nile tilapia treated with Kiss2, and Kiss2 apparently accelerates the process of spermatogenesis (92). Recently, Kiss2 was shown to stimulate T secretion in both sexes of Senegalese sole (94). All these data suggest an effect on the gonads, probably mediated by gonadotropins, but a direct effect of kisspeptins on the gonads have been less considered.

It is now known that there is intra-gonadal expression of kisspeptins and kisspeptin receptors in fish gonads, suggesting a local action on fish gonads (36, 42, 62, 63, 105–110). In this context, the intra-gonadal roles of kisspeptin in fish are poorly understood. For example, Kiss1 was recently detected in the gonads of Asian catfish (*Clarias batrachus*) and it was suggested that it could locally regulate gonadal steroidogenesis (111, 112). In addition, Chaube et al. (95) found that kisspeptins in female stinging catfish, *Heteropneustes fossilis*, act not only at the brain or pituitary level but also on the ovary to stimulate ovarian maturation and ovulation, demonstrating the potential of these peptides for aquaculture.

Taken together, these results suggest that kisspeptins may regulate the reproductive axis by acting not only at the brain and pituitary level but also at the gonadal level in teleost species.

OTHER PHYSIOLOGICAL ROLES

Less explored and beyond the control of reproduction, kisspeptins are involved in other physiological processes in fish. For example, mammalian kisspeptin increases the

expression of pituitary *gh*, *sl*, melatonin receptor (*mt*), and hepatic insulin growth factor-1 (*igf-1)*, along with higher levels of plasma Gh, Igf-1, and melatonin in the cinnamon clownfish (*Amphiprion melanopus*), suggesting a role in controlling growth in this species (113). On the other hand, intracranial administration of Kiss1 suppresses the fear response elicited by an alarm substance (AS) in zebrafish, representing a unique role for the Kiss1 system in the brain of teleosts (114). Further studies in the same species showed that Kiss1 reduces the AS-triggered fear response *via* serotonin receptors (115).

However, whether and how kisspeptins are involved in the control of food intake and energy balance in fish remains unknown and represents a promising area for future research, as nutritional status has a profound effect on *kiss*/*kissr* gene expression in some teleosts (7, 65, 116).

SIGNALING PATHWAYS ACTIVATED BY THE KISS/KISSR SYSTEMS IN FISH

Despite the importance of studying the involvement of kisspeptins in the regulation of reproduction in fish, the detailed intracellular signaling pathways mediating the effects of the Kiss/Kissr systems have not been fully elucidated (2, 10, 117). In these studies, mainly heterologous mammalian cell lines transfected with fish cognate receptors were used together with cAMP-responsive element-dependent luciferase (CRE-luc) or serum responsive element-dependent luciferase (SRE-luc) reporter assays to investigate the possible involvement of the protein kinase A (PKA) or protein kinase C (PKC) pathways, respectively (118, 119).

Analysis of zebrafish Kissr3 signal transduction in COS-7 cells reveals a clear stimulation of CRE-luc activity and SRE-luc activity by Kiss1, suggesting that zebrafish Kissr3 signal can be transduced *via* both PKA and PKC pathways, whereas Kissr2 transduces its activity through the PKC pathway (41). Similarly, in zebrafish, both Kiss1 and Kiss2 induce a concentration-dependent increase in SRE-luc activity in CV-1 cells, CHO-K1 cells, and HEK293 cells expressing their cognate receptors (35, 69). In chub mackerel and medaka, however, Kissr3 activity is transduced *via* the PKC pathway, whereas Kissr2 signaling is transduced *via* both the PKA and PKC pathways (19, 120). Similar results are also observed for Kissr3 signaling in Pacific bluefin tuna, *Thunnus orientalis* and Japanese Spanish mackerel, *Scomberomorus niphonius* (121).

On the other hand, in striped bass, both Kissr2 and Kissr3 are signaling through the PKC pathway rather than the PKA pathway (82). Interestingly, in goldfish and European sea bass Kissr2 and Kissr3 signals can be transduced *via* both the PKA and PKC pathways (33, 122). To date, only the Kissr2 type has been identified in orange-spotted grouper, half-smooth tongue sole, yellowtail kingfish and Southern bluefin tuna, and differential activation of the signal transduction pathways has been demonstrated. In the case of orange-spotted grouper, Kiss2 activates the PKC pathway, but not the PKA pathway (91). However, in the other three species, Kissr2 signaling is shown to be transduced *via* both the PKC and PKA pathways (68, 123). In addition, blockade of the PKC and PKA pathways by specific inhibitors significantly reduces the stimulatory effects induced by half-smooth tongue sole Kiss2, further confirming the participation of these two signaling pathways in the action of Kissr2 (123).

It is worth noting that the coexistence of two Kiss/Kissr systems in a single fish species indicates differential ligand selectivity for the two cognate receptors. In general, Kissr2 and Kissr3 exhibit higher affinity for Kiss2 and Kiss1, respectively, as observed in zebrafish (69), chub mackerel (120), medaka (19), and European sea bass (122). However, in goldfish, Kissr3 is more efficiently activated by Kiss2, whereas Kissr2 is preferentially activated by Kiss1 (33). In striped bass, Kissr3 is activated almost equally by Kiss1 and Kiss2, and Kissr2 is activated more efficiently by Kiss2 than by Kiss1 (82). It is noteworthy that the longer ligand forms show a stronger efficacy in activating the receptors than the core decapeptide (35, 82, 120–122).

In addition, the possible involvement of intracellular Ca^{2+} was also evaluated among post-receptor signaling events evoked by kisspeptin, showing that all European eel kisspeptin forms are able to increase intracellular Ca^{2+} in CHO-K1 cells stably transfected with the rat Kissr1 (72). It should be noted that the European eel is the only teleost species that possesses three different kisspeptin receptors (Kissr1, Kissr2, and Kissr3) that have been studied to date. However, there is no information on the signaling pathways triggered by homologous Kiss peptides across each Kissr type of eel (10, 72). On the other hand, other studies were performed using primary cultured pituitary cells to investigate the molecular mechanisms of the effects of the Kiss/Kissr system on target cells. Consistent with the results obtained with the heterologous systems mentioned above, goldfish Kiss1 may act directly at the pituitary level to increase SLa release *via* the PKA and PKC pathways and subsequent activation of Ca^{2+}-dependent cascades (78). Goldfish Kiss1 also directly stimulates the secretion of Lh and Gh from primary cultures of pituitary cells in a Ca^{2+}-dependent manner (101). Moreover, Kiss2 is shown to increase phosphorylation levels of ERK and Akt in female pituitary explants in zebrafish (77).

Currently, there is limited information on the interaction between kisspeptins and other neuroendocrine factors in cell signaling (124). In zebrafish, none of the three LPXRFa peptides (LPXRFa-1, LPXRFa-2, and LPXRFa-3) alters SRE-luc activity in COS-7 cells transfected with any of the three cognate LPXRFa receptors (LPXRFa-R1, LPXRFa-R2, and LPXRFa-R3), however, both LPXRFa-2 and LPXRFa-3 exert an inhibitory effect on Kiss2 activation of Kissr2, which involves the PKC pathway (125). Moreover, LPXRFa-2, but not LPXRFa-3, also inhibits Kiss1 activation of Kissr3, which involves the PKC pathway (125). Similarly, half-smooth tongue sole LPXRFa-1 and LPXRFa-2 can also antagonize the action of Kissr2 by inhibiting the PKC pathway (90). Because half-smooth tongue sole LPXRFa-R is coupled to Gαi protein (126), whereas its Kissr2 is coupled to Gαs protein (123), thus LPXRFa-2 also exerts an inhibitory effect on Kissr2 signaling involving the PKA pathway (123). Of note, Kissr3, LPXRFa-R2, and LPXRFa-R3 all transduce their activity

through the PKA pathway in zebrafish (41, 125), but no comparative studies have been conducted. Given that activation of Kissr1 in mammals is coupled to multiple signals (10, 12, 127), further studies are needed to investigate previously unknown intracellular mechanisms by which kisspeptin exerts its physiological functions in teleosts, as well as possible interactions of kisspeptins with other factors (**Figure 3**).

REGULATION OF THE KISS/KISSR SYSTEMS IN FISH

Photoperiod (Melatonin)

In mammals, kisspeptin is recognized as a mediator of photoperiodic control of reproduction, and the effects of photoperiod are mainly by melatonin produced in the pineal gland during the night (128, 129). Nevertheless, studies on the effects of photoperiod on the kisspeptin system are still scarce and in some way contradictories in teleosts. For example, an initial study in Nile tilapia showed that continuous illumination reduces brain *kissr2* expression levels, suggesting a possible link between environmental stimuli and the kisspeptin system (130). In contrast, continuous light increases hypothalamic *kissr2* expression levels in Atlantic salmon (64). On the other hand, there is no clear relationship between *kiss2*/*kissr2* expression and photoperiod in Atlantic cod (131).

In medaka, a long-day (LD) breeder, the number of *kiss1* neurons located in the *Nucleus ventral tuberis* (NVT) in the LD condition is larger than that in the short-day (SD) condition, whereas the *kiss2* neurons located in the *Nucleus recessus lateralis* (NRL) are not altered (29, 132). On the contrary, *kiss2*, but not *kiss1*, transcript levels in the brain of striped bass/white bass hybrid, a SD spawner, increase in the SD regime compared to the LD regime (133). In zebrafish, a LD breeder, constant darkness increases brain melatonin concentrations, and melatonin stimulates *kiss1* and *kiss2* gene expression in the brain (134, 135). Similarly, melatonin elicits a significant increase in *kiss1*, *kiss2* and *kissr2* mRNA abundance in the hypothalamus of male European sea bass (136), while an inhibitory effect of melatonin on *kiss1* and *kiss2* mRNA levels is observed in the dorsal brain of male European sea bass (136) and in the whole brain of female sapphire devil, *Chrysiptera cyanea* (137). Furthermore, continuous light results in the loss of forebrain-midbrain *kiss1*/*kissr3* seasonal rhythms in male European sea bass, which apparently prevents further normal testicular development (138). Taken together, these results indicate that the effects of photoperiod mediated by melatonin can regulate the *kiss*/*kissr* systems. This appeared to be species- and tissue-specific, and the mechanisms of action remain to be studied in detail in fish.

Temperature

Temperature, especially in ectothermic vertebrates is one of the most important environmental factors regulating reproduction.

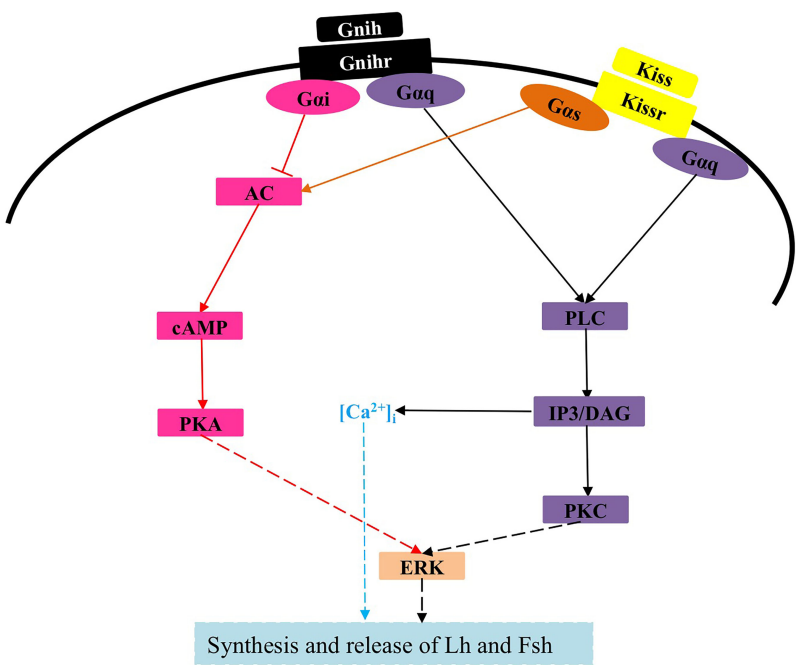


FIGURE 3 | Signaling pathway of Kissr and possible interaction with Gnih in teleosts. The solid lines represent confirmed effects, whereas the dashed lines indicate very limited evidence or possible pathways and interactions that merit further investigation. Kiss, kisspeptin; Kissr, Kiss receptor; Gnih, gonadotropin-inhibitory hormone; Gnihr, Gnih receptor; Gαq, Gαs and Gαi, heterotrimeric G proteins; PLC, phospholipase C; IP3, inositol 1,4,5-trisphosphate; DAG, diacylglycerol; PKC, protein kinase C; ERK, extracellular signal-regulated kinase; AC, adenylyl cyclase; PKA, protein kinase A; Lh, luteinizing hormone; Fsh, follicle-stimulating hormone.

However, the mechanism by which temperature affects reproduction remains unclear in teleosts. Preliminary evidence has indicated that kisspeptin systems may be involved in mediating the effects of temperature on reproduction. For example, in sexually mature male zebrafish temperature differentially modulated gene expression of the two kisspeptin systems (139). A low temperature of 15°C, but not a high temperature of 35°C, significantly increases *kiss1* mRNA levels in the whole brain, as well as *kissr3* mRNA levels in the habenula and the brain region containing nucleus of the medial longitudinal fascicle, the oculomotor nucleus, and the interpeduncular nucleus. However, *kiss2* mRNA levels in the whole brain and *kissr2* mRNA levels in the caudal zone of the periventricular hypothalamus and the *posterior tuberal nucleus* is significantly decreased when exposed to both low and high temperatures. Interestingly, *kissr2* mRNA levels in the nucleus of the medial longitudinal fascicle, the oculomotor nucleus, and interpeduncular nucleus show an increase when animals were exposed to low temperatures compared with the normal rearing temperature for this species, 27°C. These results suggest that the *kiss1*/*kissr3* system is activated by low temperatures, whereas the *kiss2*/*kissr2* system is inhibited by both low and high temperatures, suggesting that these two kisspeptin systems may be involved in different aspects of zebrafish physiology (139).

Similarly, an inhibitory effect on the expression of *kiss2* and *kissr2* genes is also observed in the diencephalon/midbrain of mature male grass puffer, that spawns on the beach in semilunar cycles during spring tide in early summer, when exposed to both low and high temperatures (140). Notably, although brain melatonin concentrations are significantly increased at high temperatures, high temperatures do not affect *kiss2* mRNA levels in the hypothalamus of adult male zebrafish (135). On the other hand, high temperature results in an increase in *kiss2* transcripts in the head of pejerrey larvae at week 4 after hatching. It is important to note that pejerrey is a fish with strong temperature-dependent sex determination, and high temperatures can result in 100% male offspring. These data suggest that *kiss2* may play an important role in the process of sex differentiation in this species (36).

Nutritional Status

In mammals, the reproductive axis is known that be regulated by energy balance, and the kisspeptin system appears to play a key role in linking energy balance and reproduction (141). Fasting has been shown to decrease hypothalamic *kiss1* and *kissr1* mRNA levels in mouse and rhesus monkey, *Macaca mulatta* (142, 143). Moreover, fasting in rat results in a concomitant decrease in hypothalamic *kiss1* and an increase in *kissr1* mRNA levels (144).

In teleosts, kisspeptin systems also appear to be associated with nutritional status. For example, in Senegalese sole, 15 days of starvation results in a significant increase in *kiss2* and *kissr2* mRNA levels in the hypothalamus, but no changes are observed for these two genes in the stomach (7). Similarly, two alternative variants for *kissr3* (*kissr3_v1* and *kissr3_v2*) and *kissr2* (*kissr2_v1* and *kissr2_v2*) are identified in pejerrey, and fasting also increases hypothalamic *kiss2* and *kissr2_v1* mRNA levels, whereas *kissr2_v2* shows no expression in the hypothalamus (65). However, food deprivation has no significant effect on the expression levels of *kissr2_v1* and *kissr2_v2* in the testis and habenula of pejerrey compared to the control group (65).

Also, a longer period of food restriction (14 months) results in an increase in mRNA levels of *kiss1*, *kiss2*, *kissr2* and *kissr3* in the hypothalamus of European sea bass (116). Overall, it appears that the neuroendocrine mechanisms mediating the effect of negative energy balance on reproduction may differ between mammals and teleosts. It is noteworthy that kisspeptin reduces appetite in several mammalian species (145–148). However, whether and how kisspeptins are involved in the regulation of food intake and energy balance in teleosts requires further investigation.

Sex Steroids

Sex steroids, estrogens and androgens, are important for the differential expression of the elements of the kisspeptin systems. For example, in female medaka, the number of *kiss1* neurons in the NVT, but not in the *nucleus preopticus periventricularis* (NPPv), is significantly reduced after ovariectomy (OVX) compared with the sham-operated group, and basal levels are restored after E_2 treatment (29). In addition, double-labeling *in situ* hybridization showed that estrogen receptor alfa (Erα) is expressed together with *kiss1* in NVT neurons, suggesting that these neurons are involved in the positive feedback regulation of the BPG axis in this species (132). However, the number of NRL *kiss2* neurons is not altered after OVX, and no ERα transcripts are detected in or in close association to the NRL *kiss2* neurons (132). In contrast, in goldfish, the number of *kiss2* neurons in the POA is downregulated after OVX and is restored by E_2 administration, and *kiss2* neurons in the POA express all three ER types (149).

In OVX orange-spotted grouper, the expression of *kiss2* but not *kiss1* is significantly increased in the brain, and E_2 substitution could reverse this effect (150). Bioinformatics analysis of the promoter of kisspeptins and kisspeptin receptors in yellowtail kingfish and zebrafish reveals high abundance of several regulatory elements such as AP-1, Sp1, ER, AR and PR (151), suggesting possible regulation of Kiss genes and their receptors by steroids, especially E_2. It was also demonstrated that E_2 is able to positively feedback on the expression of *kiss1* and *kiss2* in goldfish through different ERα pathways (152), and similar results are observed in orange-spotted grouper (150). On the other hand, E_2 treatment causes a significant increase in mRNA expression of *kiss1*, *kiss2*, and *kissr2* in zebrafish brain, but *kissr3* transcript levels are not altered (16). In addition, a positive effect of E_2 on the expression of *kiss2* but not *kiss1* is observed in the brain of the sapphire devil, *Chrysiptera cyanea* (153) and in the hypothalamus of the Dabry's sturgeon, *Acipenser dabryanus* (154).

Kisspeptin receptors are also regulated by gonadal steroids in fish. E_2 also increases expression of the *kissr2* and *kissr3* genes in the sapphire devil brain (155) and European sea bass pituitary cells (84). Hypothalamic *kissr3* but not *kissr2* transcripts are upregulated in Dabry's sturgeon after E_2 injection (154), whereas neither *kiss2* nor *kissr2* mRNA levels are altered by E_2 in the hypothalamus of half-smooth tongue sole (156). Interestingly, no significant changes in hypothalamic *kiss1*, *kiss2* and their receptors mRNA levels are observed in European sea bass by

E_2 treatment after OVX, as determined by qRT-PCR. However, the number of *kiss1* and *kiss2* expressing cells is reduced in some brain regions, and E_2 replacement prevents this effect, as revealed by *in situ* hybridization (157).

Androgens have also been shown to mediate feedback on the regulation of kisspeptin neurons. Transcript levels of *kiss1*, *kiss2*, and *kissr2* in the brain are reduced by T treatment of OVX female striped bass during mid-vitellogenesis (158). Similarly, T administration reduces mRNA levels of *kiss1*, *kiss2*, and *kissr2* in the brain of gonadectomized (GDX) at mid-gonadal development of male striped bass. In contrast, pubertal males responds to T replacement by up-regulation of *kiss1* and *kiss2*, whereas no changes are observed in juvenile and recrudescent males, suggesting a differential and gonadal stage-dependent role of T in regulating mRNA levels of *kiss1* and *kiss2* (133). On the other hand, a negative feedback effects of T on hypothalamic *kiss2* expression is observed in GDX European sea bass males, without affecting *kiss1*, *kissr2* and *kissr3* mRNA levels (157). However, T has no effect on the expression of the elements of kisspeptin system in the hypothalamus of half-smooth tongue sole (156) and midbrain of goldfish (159). A stimulatory effect of T on mRNA levels of *kissr2* and *kissr3* is detected in primary cultured pituitary cells of European sea bass (84). Taken together, these results suggest that the regulation of genes encoding kisspeptins and their receptor by gonadal steroids in teleosts depends on the species, tissue, gene, reproductive stage, and route of administration and that needs to be investigated in each individual species.

Neuropeptides Related to Reproduction

In teleosts, negative and positive feedbacks were described for kisspeptins on their own expression. For example, Kiss1 administration decreases the amount of *kiss1* mRNA in the habenula of zebrafish (160) and induces a higher expression of *kissr2* in the brain of fathead minnow (40). Similarly, Kiss2 stimulates *kissr2* mRNA levels in primary cultured brain cells of Japanese flounder (100). Both *kiss2* and *kissr2* transcript levels are significantly increased in the hypothalamus of black porgy, *Acanthopagrus schlegelii*, after injection with Kiss2 (99). In addition, exogenous administration of Kiss2 increases gene expression of reproduction-related genes (*gnrh3*, *kissr2*, *fshb*, *lhb*, *ar*, and *er2*), sex hormone levels (E_2 and T), and accelerates the onset of puberty in largemouth bass, *Micropterus salmoides* (96). On the other hand, Kiss2 increases hypothalamic *kiss2* expression in half-smooth tongue sole, and decreases *kissr2* mRNA levels (89). In addition, a negative effect of Kiss2 is found on the mRNA abundance of *kissr2_v1* and *kissr2_v5* in the brain of male yellowtail kingfish, while the mRNA levels of *kissr2_v4* are significantly increased (68).

Injection of Kiss2 does not alter *kissr2* mRNA levels in the hypothalamus of lined seahorse, *Hippocampus erectus* (93). Neither Kiss1 nor Kiss2 alters the transcript levels of *kissr2* and *kissr3* mRNAs in the hypothalamus of European sea bass, and *kissr3* mRNA levels in the forebrain-midbrain are not altered by these two peptides (83). However, *kissr2* gene expression is increased in the forebrain-midbrain after exposure to Kiss1 and Kiss2 (83). Interestingly, a differential and gonadal stage-dependent roles of Kiss1 and Kiss2 in regulating *kissr2* expression in hybrid bass brain is observed (17). Transcript levels of *kissr2* are increased only by Kiss2 in prepuberty, whereas a significant decrease in mRNA levels is observed after treatment with Kiss1 and Kiss2 in recrudescence (17).

LPXRFa may also induce differential effects on the expression of kisspeptins and their receptor in teleosts. Intracerebroventricular (icv) injection of LPXRFa-2 suppresses *kiss1*, *kiss2*, and *kissr3* transcripts in the brain of male European sea bass, without affecting *kissr2* expression (161). However, intramuscular (im) injection of LPXRFa-2 significantly increases *kissr3* expression and has no effect on the expression of the other genes (162). On the other hand, no changes in the expression of these four kisspeptin genes (*kiss1*, *kiss2*, *kissr2*, and *kissr3*) are observed after administration of LPXRFa-1 in the same studies (161, 162). In half-smooth tongue sole, neither LPXRFa-1 nor LPXRFa-2 alters hypothalamic *kiss2* mRNA levels *in vitro* (163). Similarly, im injection of LPXRFa-2 and LPXRFa-3 does not alter *kiss2* gene expression in the brain of Senegalese sole (164), and none of the three LPXRFa peptides alters hypothalamic *kiss1* and *kiss2* mRNA levels in orange-spotted grouper (165).

In mammals, kisspeptin is considered an upstream regulator of Gnrh secretion, and although the situation is clearly different in teleosts, Gnrh may exert feedback on gene expression of kisspeptin systems. A mammalian GnRH analog, [D-Ala^6, Pro^{9}Net]-mGnRHa, has a stimulatory effect on the expression of *kiss2* in European sea bass pituitary cell cultures but has no effect on the mRNA levels of *kissr2* and *kissr3* (84). Furthermore, no significant differences in hypothalamic abundance of *kiss2* and *kissr2* mRNAs are observed after exposure to the aforementioned GnRHa in half-smooth tongue sole (89). Similarly, treatment with GnRHa has no effects on the expression levels of *kissr2* in the brain, pituitary gland, and gonads in male yellowtail kingfish (88). Overall, these results suggest a complex control of the kisspeptin system, and each neuropeptide exerts a differential effect on kisspeptin gene regulation, which could depend on the species, sex, tissues, reproductive stages of the animals, peptides used, dose, route of administration, and elapsed time after treatment.

Other Factors

Thyroid hormones (T_3 and T_4) play an important role in the control of growth, morphogenesis, metabolism, and reproduction in several species, including fish (166, 167). Moreover, T_3 ip administration significantly increases hypothalamic *kiss2* gene in sexually mature male Nile tilapia, whereas this gene is suppressed under a hypothyroid condition induced by methimazole treatment (168).

Endocrine disrupting chemicals (EDCs) can also affect reproductive regulation, in part by affecting kisspeptins system, which is a clear example of neuroendocrine disruption (169). For example, bisphenol-A shows a greatly increased expression of *kiss1*, *kiss2*, and *kissr2* in the brains of pubertal Catla (*Catla catla)* without affecting mRNA levels of *kissr3* (170). In addition, bisphenol-F leads to an increase in the expression of *kiss1* and *kissr3* in the brain of zebrafish but has no effect on the mRNA levels of *kiss2* and *kissr2* (171). In adult male goldfish exposed to vinclozolin, a pesticide that acts as an antiandrogen and impairs

reproduction in mammals, *kiss1* but not *kiss2* mRNA levels are increased in the midbrain (159). Similarly, the antiandrogen flutamide also induces *kiss1* and *kiss2* gene expression in the midbrain of goldfish (159). All these data suggest that these EDCs act on steroid receptors and/or steroid balance.

On the other hand, semicarbazide (SMC), an industrially produced synthetic hydrazine compound, significantly downregulates mRNA expression of *kiss2* and *kissr2* in the brain of female Japanese flounder (172). An inhibitory effect of SMC on *kissr2* expression in the brain is also observed in male Japanese flounder (173). Moreover, mRNA levels of *kissr2* and *kissr3* are significantly reduced in the brain of adult female Japanese medaka after chronic exposure to Roundup, a glyphosate-based herbicide. However, neither *kiss1* nor *kiss2* transcripts are altered (174). Moreover, these EDCs may act on the kisspeptins system by mimicking the effects of gonadal steroids, as plasma E_2 and T levels can be altered by EDCs (93, 173).

Interestingly, other less studied factors, such as social status may also regulate mRNA levels of *kissr* in the entire brain of mouthbrooding cichlids, with higher mRNA levels of *kissr2* observed in high-status territorial males compared to non-territorial males (75).

CONCLUSIONS AND FUTURE DIRECTIONS

In fish, kisspeptins may exert their functions by acting at multiple levels of the brain-pituitary-gonadal axis. Two recent reviews focusing on fish and vertebrates highlighted the different pathways by which kisspeptins may be involved in reproduction, discussed the levels and nature of action, and interaction with Gnrh and other neuropeptides (15, 43). In this review, attention was focused on the whole reproductive brain-pituitary-gonadal axis. Unlike mammals, *kiss/kissr* null zebrafish and medaka can reproduce normally, suggesting that kisspeptin is either not essential for reproduction or that there are compensation mechanisms exerted by other neuropeptides. Teleost are known for their neuroplasticity and multifactorial control of reproduction, with new reproductive neuropeptides emerging (175–177).

With respect to Kiss/Kissr diversity and evolution, we focused particularly on Pleuronectiformes because this order is a good model from an evolutionary perspective and multiple genomes are currently available. Moreover, in Pleuronectiformes, previous studies have mentioned that the kisspeptin-1 system seems to have been lost during evolution (8, 15, 45), but recent synteny and phylogenetic analysis has shown that this is not so clear for all species in this group. In addition, four rounds of genome duplication are known to have occurred in salmonids (178), but no additional *kiss or kissr* have been found to date. Therefore, it will be interesting to search for orthologous pseudogenes in salmonid genomes.

Most studies on kisspeptin in fish have focused on reproduction, while the role of the kisspeptin system in peripheral tissues is still unclear and there are important questions to be addressed. For example, kisspeptin suppresses food intake in some mammalian species, such as mice, rats, and desert jerboas (145–148). Whether and how kisspeptin regulates appetite and energy balance in teleosts is not yet clear and requires further investigation. Further studies are also needed to elucidate the roles of the kisspeptin systems in development, metabolism, and behavior, as well as to explore the intracellular signaling pathways involved in kisspeptin actions and possible interactions with other neuroendocrinological factors in teleosts.

AUTHOR CONTRIBUTIONS

BW, ASM, and GMS contributed equally to the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This study was carried out with financial support from a project funded by the Agencia Nacional de Promoción Científica y Tecnológica (ANPCYT, Argentina) PICT-2015-2783 to GMS. and PICT-2017- 2839 to ASM, the National Natural Science Foundation of China 32072949 and the Laboratory for Marine Fisheries and Food Production Processes, Pilot National Laboratory for Marine Science and Technology (Qingdao) ZZ-B06 to BW, who also received a scholarship supported by the China Scholarship Council (CSC, File No. 201903260004).

REFERENCES

1. Parhar IS, Ogawa S, Sakuma Y. Laser-Captured Single Digoxigenin-Labeled Neurons of Gonadotropin-Releasing Hormone Types Reveal a Novel G Protein-Coupled Receptor (Gpr54) During Maturation in Cichlid Fish. *Endocrinology* (2004) 145:3613–8. doi: 10.1210/en.2004-0395
2. Tena-Sempere M, Felip A, Gomez A, Zanuy S, Carrillo M. Comparative Insights of the Kisspeptin/Kisspeptin Receptor System: Lessons From Non-Mammalian Vertebrates. *Gen Comp Endocrinol* (2012) 175:234–43. doi: 10.1016/j.ygcen.2011.11.015
3. Kirilov M, Clarkson J, Liu X, Roa J, Campos P, Porteous R, et al. Dependence of Fertility on Kisspeptin–Gpr54 Signaling at the GnRH Neuron. *Nat Commun* (2013) 4:2492. doi: 10.1038/ncomms3492
4. Ruohonen ST, Poutanen M, Tena-Sempere M. Role of Kisspeptins in the Control of the Hypothalamic-Pituitary-Ovarian Axis: Old Dogmas and New Challenges. *Fertil Steril* (2020) 114:465–74. doi: 10.1016/j.fertnstert.2020.06.038
5. Mechaly AS, Viñas J, Murphy C, Reith M, Piferrer F. Gene Structure of the Kiss1 Receptor-2 (Kiss1r-2) in the Atlantic Halibut: Insights Into the Evolution and Regulation of Kiss1r Genes. *Mol Cell Endocrinol* (2010) 317:78–89. doi: 10.1016/j.mce.2009.11.005
6. Mechaly AS, Viñas J, Piferrer F. Identification of Two Isoforms of the Kisspeptin-1 Receptor (*Kiss1r*) Generated by Alternative Splicing in a Modern Teleost, the Senegalese Sole (*Solea Senegalensis*). *Biol Reprod* (2009) 80:60–9. doi: 10.1095/biolreprod.108.072173
7. Mechaly AS, Viñas J, Piferrer F. Gene Structure Analysis of Kisspeptin-2 (Kiss2) in the Senegalese Sole (*Solea Senegalensis*): Characterization of Two

Splice Variants of Kiss2, and Novel Evidence for Metabolic Regulation of Kisspeptin Signaling in non-Mammalian Species. *Mol Cell Endocrinol* (2011) 339:14–24. doi: 10.1016/j.mce.2011.03.004
8. Song H, Wang M, Wang Z, Liu J, Qi J, Zhang Q. Characterization of Kiss2 and Kissr2 Genes and the Regulation of Kisspeptin on the HPG Axis in Cynoglossus Semilaevis. *Fish Physiol Biochem* (2017) 43:731–53. doi: 10.1007/s10695-016-0328-x
9. Song H, Wang M, Zhongkai W, Yu H, Wang Z, Zhang Q. Identification and Characterization of Kiss2 and Kissr2 Homologs in Paralichthys Olivaceus. *Fish Physiol Biochem* (2016) 42:1073–92. doi: 10.1007/s10695-016-0199-1
10. Pasquier J, Kamech N, Lafont AG, Vaudry H, Rousseau K, Dufour S. Molecular Evolution of GPCRs: Kisspeptin/kisspeptin Receptors. *J Mol Endocrinol* (2014) 52:T101–17. doi: 10.1530/JME-13-0224
11. Roa J, Aguilar E, Dieguez C, Pinilla L, Tena-Sempere M. New Frontiers in Kisspeptin/GPR54 Physiology as Fundamental Gatekeepers of Reproductive Function. *Front Neuroendocrinol* (2008) 29:48–69. doi: 10.1016/j.yfrne.2007.07.002
12. Pinilla L, Aguilar E, Dieguez C, Millar RP, Tena-Sempere M. Kisspeptins and Reproduction: Physiological Roles and Regulatory Mechanisms. *Physiol Rev* (2012) 92:1235–316. doi: 10.1152/physrev.00037.2010
13. Kah O. A 45-Years Journey Within the Reproductive Brain of Fish. *Gen Comp Endocrinol* (2020) 288:113370. doi: 10.1016/j.ygcen.2019.113370
14. Muñoz-Cueto JA, Zmora N, Paullada-Salmerón JA, Marvel M, Mañanos E, Zohar Y. The Gonadotropin-Releasing Hormones: Lessons From Fish. *Gen Comp Endocrinol* (2020) 291:113422. doi: 10.1016/j.ygcen.2020.113422
15. Somoza GM, Mechaly AS, Trudeau VL. Kisspeptin and GnRH Interactions in the Reproductive Brain of Teleosts. *Gen Comp Endocrinol* (2020) 298:113568. doi: 10.1016/j.ygcen.2020.113568
16. Servili A, Le Page Y, Leprince J, Caraty A, Escobar S, Parhar IS, et al. Organization of Two Independent Kisspeptin Systems Derived From Evolutionary-Ancient Kiss Genes in the Brain of Zebrafish. *Endocrinology* (2011) 152:1527–40. doi: 10.1210/en.2010-0948
17. Zmora N, Stubblefield J, Zulperi Z, Biran J, Levavi-Sivan B, Munoz-Cueto JA, et al. Differential and Gonad Stage-Dependent Roles of Kisspeptin1 and Kisspeptin2 in Reproduction in the Modern Teleosts, Morone Species. *Biol Reprod* (2012) 86:177. doi: 10.1095/biolreprod.111.097667
18. Escobar S, Servili A, Espigares F, Gueguen MM, Brocal I, Felip A, et al. Expression of Kisspeptins and Kiss Receptors Suggests a Large Range of Functions for Kisspeptin Systems in the Brain of the European Sea Bass. *PloS One* (2013) 8:e70177. doi: 10.1371/journal.pone.0070177
19. Kanda S, Akazome Y, Mitani Y, Okubo K, Oka Y. Neuroanatomical Evidence That Kisspeptin Directly Regulates Isotocin and Vasotocin Neurons. *PloS One* (2013) 8:e62776. doi: 10.1371/journal.pone.0062776
20. Nakajo M, Kanda S, Karigo T, Takahashi A, Akazome Y, Uenoyama Y, et al. Evolutionarily Conserved Function of Kisspeptin Neuronal System is Nonreproductive Regulation as Revealed by Nonmammalian Study. *Endocrinology* (2018) 159:163–83. doi: 10.1210/en.2017-00808
21. Ogawa S, Sivalingam M, Anthonysamy R, Parhar IS. Distribution of Kiss2 Receptor in the Brain and its Localization in Neuroendocrine Cells in the Zebrafish. *Cell Tiss Res* (2020) 379:349–72. doi: 10.1007/s00441-019-03089-5
22. Tang H, Liu Y, Luo D, Ogawa S, Yin Y, Li S, et al. The Kiss/Kissr Systems are Dispensable for Zebrafish Reproduction: Evidence From Gene Knockout Studies. *Endocrinology* (2015) 156:589–99. doi: 10.1210/en.2014-1204
23. Liu Y, Tang H, Xie R, Li S, Liu X, Lin H, et al. Genetic Evidence for Multifactorial Control of the Reproductive Axis in Zebrafish. *Endocrinology* (2017) 158:604–11. doi: 10.1210/en.2016-1540
24. Spicer OS, Wong TT, Zmora N, Zohar Y. Targeted Mutagenesis of the Hypophysiotropic Gnrh3 in Zebrafish (*Danio Rerio*) Reveals No Effects on Reproductive Performance. *PloS One* (2016) 11:e0158141. doi: 10.1371/journal.pone.0158141
25. Marvel M, Spicer OS, Wong TT, Zmora N, Zohar Y. Knockout of the Gnrh Genes in Zebrafish: Effects on Reproduction and Potential Compensation by Reproductive and Feeding-Related Neuropeptides. *Biol Reprod* (2018) 99:565–77. doi: 10.1093/biolre/ioy078
26. Whitlock KE, Postlethwait J, Ewer J. Neuroendocrinology of Reproduction: Is Gonadotropin-Releasing Hormone (GnRH) Dispensable? *Front Neuroendocrinol* (2019) 53:100738. doi: 10.1016/j.yfrne.2019.02.002
27. Abraham E, Palevitch O, Gothilf Y, Zohar Y. Targeted Gonadotropin-Releasing Hormone-3 Neuron Ablation in Zebrafish: Effects on Neurogenesis, Neuronal Migration, and Reproduction. *Endocrinology* (2010) 151:332–240. doi: 10.1210/en.2009-0548
28. Yun S, Kim DK, Furlong M, Hwang JI, Vaudry H, Seong JY. Does Kisspeptin Belong to the Proposed RF-Amide Peptide Family? *Front Endocrinol (Lausanne)* (2014) 5:134. doi: 10.3389/fendo.2014.00134
29. Kanda S, Akazome Y, Matsunaga T, Yamamoto N, Yamada S, Tsukamura H, et al. Identification of KiSS-1 Product Kisspeptin and Steroid-Sensitive Sexually Dimorphic Kisspeptin Neurons in Medaka (*Oryzias Latipes*). *Endocrinology* (2008) 149:2467–76. doi: 10.1210/en.2007-1503
30. van Aerle R, Kille P, Lange A, Tyler CR. Evidence for the Existence of a Functional Kiss1/Kiss1 Receptor Pathway in Fish. *Peptides* (2008) 29:57–64. doi: 10.1016/j.peptides.2007.10.018
31. Kitahashi T, Ogawa S, Parhar IS. Cloning and Expression of Kiss2 in the Zebrafish and Medaka. *Endocrinology* (2009) 150:821–31. doi: 10.1210/en.2008-0940
32. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The Metastasis Suppressor Gene KiSS-1 Encodes Kisspeptins, the Natural Ligands of the Orphan G Protein-Coupled Receptor GPR54. *J Biol Chem* (2001) 276:34631–6. doi: 10.1074/jbc.M104847200
33. Li S, Zhang Y, Liu Y, Huang X, Huang W, Lu D, et al. Structural and Functional Multiplicity of the Kisspeptin/GPR54 System in Goldfish (*Carassius Auratus*). *J Endocrinol* (2009) 201:407–18. doi: 10.1677/joe-09-0016
34. Ohga H, Adachi H, Kitano H, Yamaguchi A, Matsuyama M. Kiss1 Hexadecapeptide Directly Regulates Gonadotropin-Releasing Hormone 1 in the Scombroid Fish, Chub Mackerel. *Biol Reprod* (2017) 96:376–88. doi: 10.1095/biolreprod.116.142083
35. Lee YR, Tsunekawa K, Moon MJ, Um HN, Hwang JI, Osugi T, et al. Molecular Evolution of Multiple Forms of Kisspeptins and GPR54 Receptors in Vertebrates. *Endocrinology* (2009) 150:2837–46. doi: 10.1210/en.2008-1679
36. Tovar-Bohórquez MO, Mechaly AS, Hughes LC, Campanella D, Orti G, Canosa LF, et al. Kisspeptin System in Pejerrey Fish (*Odontesthes Bonariensis*). Characterization and Gene Expression Pattern During Early Developmental Stages. *Comp Bioch Physiol* (2017) 204A:146–56. doi: 10.1016/j.cbpa.2016.11.014
37. Clements MK, McDonald TP, Wang RP, Xie GC, O'Dowd BF, George SR, et al. FMRFamide-Related Neuropeptides are Agonists of the Orphan G-Protein-Coupled Receptor GPR54. *Biochem Biophys Res Commun* (2001) 284:1189–93. doi: 10.1006/bbrc.2001.5098
38. Mohamed JS, Benninghoff AD, Holt GJ, Khan IA. Developmental Expression of the G Protein-Coupled Receptor 54 and Three GnRH mRNAs in the Teleost Fish Cobia. *J Mol Endocrinol* (2007) 38:235–44. doi: 10.1677/jme.1.02182
39. Nocillado JN, Levavi-Sivan B, Carrick F, Elizur A. Temporal Expression of G-Protein-Coupled Receptor 54 (GPR54), Gonadotropin-Releasing Hormones (GnRH), and Dopamine Receptor D2 (Drd2) in Pubertal Female Grey Mullet, Mugil Cephalus. *Gen Comp Endocrinol* (2007) 150:278–87. doi: 10.1016/j.ygcen.2006.09.008
40. Filby AL, van Aerle R, Duitman J, Tyler CR. The Kisspeptin/Gonadotropin-Releasing Hormone Pathway and Molecular Signaling of Puberty in Fish. *Biol Reprod* (2008) 78:278–89. doi: 10.1095/biolreprod.107.063420
41. Biran J, Ben-Dor S, Levavi-Sivan B. Molecular Identification and Functional Characterization of the Kisspeptin/Kisspeptin Receptor System in Lower Vertebrates. *Biol Reprod* (2008) 79:776–86. doi: 10.1095/biolreprod.107.066266
42. Ohga H, Selvaraj S, Matsuyama M. The Roles of Kisspeptin System in the Reproductive Physiology of Fish With Special Reference to Chub Mackerel Studies as Main Axis. *Front Endocrinol (Lausanne)* (2018) 9:147. doi: 10.3389/fendo.2018.00147
43. Sivalingam OS, Trudeau VL, Parhar IS. Conserved Functions of Hypothalamic Kisspeptin in Vertebrates. *Gen Comp Endocrinol* (2022) 317:113973. doi: 10.1016/j.ygcen.2021.113973
44. Pasquier J, Lafont AG, Jeng SR, Morini M, Dirks R, van den Thillart G, et al. Multiple Kisspeptin Receptors in Early Osteichthyans Provide New Insights

Into the Evolution of This Receptor Family. *PloS One* (2012) 7:e48931. doi: 10.1371/journal.pone.0048931
45. Mechaly AS, Viñas J, Piferrer F. The Kisspeptin System Genes in Teleost Fish, Their Structure and Regulation, With Particular Attention to the Situation in Pleuronectiformes. *Gen Comp Endocrinol* (2013) 188:258–68. doi: 10.1016/j.ygcen.2013.04.010
46. Felip A, Zanuy S, Pineda R, Pinilla L, Carrillo M, Tena-Sempere M, et al. Evidence for Two Distinct KiSS Genes in non-Placental Vertebrates That Encode Kisspeptins With Different Gonadotropin-Releasing Activities in Fish and Mammals. *Mol Cell Endocrinol* (2009) 312:61–71. doi: 10.1016/j.mce.2008.11.017
47. Dufour S, Quérat B, Tostivint H, Pasqualini C, Vaudry H, Rousseau K. Origin and Evolution of the Neuroendocrine Control of Reproduction in Vertebrates, With Special Focus on Genome and Gene Duplications. *Physiol Rev* (2020) 100(2):869–943. doi: 10.1152/physrev.00009.2019
48. Chen S, Zhang G, Shao C, Huang Q, Liu G, Zhang P, et al. Whole-Genome Sequence of a Flatfish Provides Insights Into ZW Sex Chromosome Evolution and Adaptation to a Benthic Lifestyle. *Nat Genet* (2014) 46:253–60. doi: 10.1038/ng.2890
49. Figueras A, Robledo D, Corvelo A, Hermida M, Pereiro P, Rubiolo JA, et al. Whole Genome Sequencing of Turbot (*Scophthalmus Maximus*; Pleuronectiformes): A Fish Adapted to Demersal Life. *DNA Res* (2016) 23:181–92. doi: 10.1093/dnares/dsw007
50. Benzekri H, Armesto P, Cousin X, Rovira M, Crespo D, Merlo MA, et al. *De Novo* Assembly, Characterization and Functional Annotation of Senegalese Sole (*Solea Senegalensis*) and Common Sole (*Solea Solea*) Transcriptomes: Integration in a Database and Design of a Microarray. *BMC Genom* (2014) 15:952. doi: 10.1186/1471-2164-15-952
51. Manchado M, Planas JV, Cousin X, Rebordinos L, Claros MG. Current Status in Other Finfish Species: Description of Current Genomic Resources for the Gilthead Seabream (Sparus Aurata) and Soles (Solea Senegalensis and Solea Solea). In: S Mackenzie and S Jentoft, editors. *Genomics in Aquaculture*. Amsterdam: Elsevier (2016). p. 195–221. doi: 10.1016/B978-0-12-801418-9.00008-1
52. Shao C, Bao B, Xie Z, Chen X, Li B, Jia X, et al. The Genome and Transcriptome of Japanese Flounder Provide Insights Into Flatfish Asymmetry. *Nat Genet* (2017) 49:119–24. doi: 10.1038/ng.3732
53. Burguener G, Viñas J, Martinez P, Boccanfuso JJ, Somoza GM, Turjanski A, et al. Sequencing and First Draft Assembly of the Genome of the Flounder Paralichthys Orbignyanus. In: *IX Argentinian Congress of Bioinformatics and Computational Biology*. Mar del Plata, Argentina (2018).
54. Lü Z, Gong L, Ren Y, Chen Y, Wang Z, Liu L, et al. Large-Scale Sequencing of Flatfish Genomes Provides Insights Into the Polyphyletic Origin of Their Specialized Body Plan. *Nat Genet* (2021) 53(5):742–51. doi: 10.1038/s41588-021-00836-9
55. Chapleau F. Pleuronectiform Relationships: A Cladistic Reassessment. *Bull Mar Sci* (1993) 52(1):516–40.
56. Chanet B, Mondéjar Fernández J, Lecointre G. Flatfishes interrelationships revisited based on anatomical characters^{2+})/calmodulin-Dependent Cascades. *Int J Ichthyol* (2020) 44:009–18. doi: 10.26028/cybium/2020-441-002
57. Baptista RP, Kissinger JC. Is Reliance on an Inaccurate Genome Sequence Sabotaging Your Experiments? *PloS Pathog* (2019) 15:e1007901. doi: 10.1371/journal.ppat.1007901
58. Shimizu Y, Tomikawa J, Hirano K, Nanikawa Y, Akazome Y, Kanda S, et al. Central Distribution of Kiss2 Neurons and Peri-Pubertal Changes in Their Expression in the Brain of Male and Female Red Seabream Pagrus Major. *Gen Comp Endocrinol* (2012) 175:432–42. doi: 10.1016/j.ygcen.2011.11.038
59. Osugi T, Ohtaki N, Sunakawa Y, Son YL, Ohkubo M, Iigo M, et al. Molecular Evolution of Kiss2 Genes and Peptides in Vertebrates. *Endocrinology* (2013) 154:4270–80. doi: 10.1210/en.2012-2267
60. Uenoyama Y, Pheng V, Tsukamura H, Maeda KI. The Roles of Kisspeptin Revisited: Inside and Outside the Hypothalamus. *J Reprod Dev* (2016) 62:537–45. doi: 10.1262/jrd.2016-083
61. Wolfe A, Hussain MA. The Emerging Role(s) for Kisspeptin in Metabolism in Mammals. *Front Endocrinol (Lausanne)* (2018) 9:184. doi: 10.3389/fendo.2018.00184
62. Fairgrieve MR, Shibata Y, Smith EK, Hayman ES, Luckenbach JA. Molecular Characterization of the Gonadal Kisspeptin System: Cloning, Tissue Distribution, Gene Expression Analysis and Localization in Sablefish (*Anoplopoma Fimbria*). *Gen Comp Endocrinol* (2016) 225:212–23. doi: 10.1016/j.ygcen.2015.07.015
63. Saha A, Pradhan A, Sengupta S, Nayak M, Samanta M, Sahoo L, et al. Molecular Characterization of Two Kiss Genes and Their Expression in Rohu (*Labeo Rohita*) During Annual Reproductive Cycle. *Comp Biochem Physiol* (2016) 191B:135–45. doi: 10.1016/j.cbpb.2015.10.008
64. Chi L, Li X, Liu Q, Liu Y. Photoperiod Regulate Gonad Development via Kisspeptin/Kissr in Hypothalamus and Saccus Vasculosus of Atlantic Salmon (*Salmo Salar*). *PloS One* (2017) 12:e0169569. doi: 10.1371/journal.pone.0169569
65. Mechaly AS, Tovar-Bohórquez MO, Mechaly AE, Suku E, Perez MR, Giorgetti A, et al. Evidence of Alternative Splicing as a Regulatory Mechanism for Kissr2 in Pejerrey Fish. *Front Endocrinol (Lausanne)* (2018) 9:604. doi: 10.3389/fendo.2018.00604
66. Horikoshi Y, Matsumoto H, Takatsu Y, Ohtaki T, Kitada C, Usuki S. Dramatic Elevation of Plasma Metastin Concentrations in Human Pregnancy: Metastin as a Novel Placenta-Derived Hormone in Humans. *J Clin Endocrinol Metab* (2003) 88:914–9. doi: 10.1210/jc.2002-021235
67. Oladosu FA, Maixner W, Nackley AG. Alternative Splicing of G Protein–Coupled Receptors: Relevance to Pain Management. *Mayo Clin Proc* (2015) 90:1135–51. doi: 10.1016/j.mayocp.2015.06.010
68. Nocillado JN, Biran J, Lee YY, Levavi-Sivan B, Mechaly AS, Zohar Y, et al. The Kiss2 Receptor (Kiss2r) Gene in Southern Bluefin Tuna, Thunnus Maccoyii and in Yellowtail Kingfish, *Seriola Lalandi* - Functional Analysis and Isolation of Transcript Variants. *Mol Cell Endocrinol* (2012) 362:211–20. doi: 10.1016/j.mce.2012.06.024
69. Onuma TA, Duan C. Duplicated Kiss1 Receptor Genes in Zebrafish: Distinct Gene Expression Patterns, Different Ligand Selectivity, and a Novel Nuclear Isoform With Transactivating Activity. *FASEB J* (2012) 26:2941–50. doi: 10.1096/fj.11-201095
70. Lareau LF, Brenner SE. Regulation of Splicing Factors by Alternative Splicing and NMD is Conserved Between Kingdoms Yet Evolutionarily Flexible. *Mol Biol Evol* (2015) 32(4):1072–9. doi: 10.1093/molbev/msv002
71. Yang B, Jiang Q, Chan T, Ko WK, Wong AO. Goldfish Kisspeptin: Molecular Cloning, Tissue Distribution of Transcript Expression, and Stimulatory Effects on Prolactin, Growth Hormone and Luteinizing Hormone Secretion and Gene Expression via Direct Actions at the Pituitary Level. *Gen Comp Endocrinol* (2010) 165:60–71. doi: 10.1016/j.ygcen.2009.06.001
72. Pasquier J, Lafont AG, Denis F, Lefranc B, Dubessy C, Moreno-Herrera A, et al. Eel Kisspeptins: Identification, Functional Activity, and Inhibition on Both Pituitary LH and GnRH Receptor Expression. *Front Endocrinol (Lausanne)* (2018) 8:353. doi: 10.3389/fendo.2017.00353
73. Trudeau VL. Kiss and Tell: Deletion of Kisspeptins and Receptors Reveal Surprising Results. *Endocrinology* (2015) 156:769–71. doi: 10.1210/en.2015-1019
74. Trudeau VL. Facing the Challenges of Neuropeptide Gene Knockouts: Why do They Not Inhibit Reproduction in Adult Teleost Fish? *Front Neurosci* (2018) 12:302. doi: 10.3389/fnins.2018.00302
75. Grone BP, Maruska KP, Korzan WJ, Fernald RD. Social Status Regulates Kisspeptin Receptor mRNA in the Brain of Astatotilapia Burtoni. *Gen Comp Endocrinol* (2010) 169:98–107. doi: 10.1016/j.ygcen.2010.07.018
76. Song Y, Duan X, Chen J, Huang W, Zhu Z, Hu W. The Distribution of Kisspeptin (Kiss)1- and Kiss2-Positive Neurons and Their Connections With Gonadotrophin-Releasing Hormone-3 Neurons in the Zebrafish Brain. *J Neuroendocrinol* (2015) 27:198–211. doi: 10.1111/jne.12251
77. Song Y, Chen J, Tao B, Luo D, Zhu Z, Hu W. Kisspeptin2 Regulates Hormone Expression in Female Zebrafish (*Danio Rerio*) Pituitary. *Mol Cell Endocrinol* (2020) 513:110858. doi: 10.1016/j.mce.2020.110858
78. Jiang Q, He M, Ko WK, Wong AO. Kisspeptin Induction of Somatolactin-Alpha Release in Goldfish Pituitary Cells: Functional Role of cAMP/PKA-, PLC/PKC-, and Ca^{2+})/calmodulin-Dependent Cascades. *Am J Physiol Endocrinol Metab* (2014) 307:E872–884. doi: 10.1152/ajpendo.00321.2014

79. Valipour A, Heidari B, Asghari SM, Balalaie S, Rabouti H, Omidian N. The Effect of Different Exogenous Kisspeptins on Sex Hormones and Reproductive Indices of the Goldfish (*Carassius Auratus*) Broodstock. *J Fish Biol* (2021) 98(4):1137–43. doi: 10.1111/jfb.14645
80. Valipour A, Heidari B, Vaziri H, Asghari SM. Expression of Reproductive-Related Genes and Changes in Oocyte Maturation of Goldfish Broodstock (*Carassius Auratus*) Following Injection of Different Exogenous Kisspeptins. *Reprod Domest Anim* (2021) 56(10):1349–57. doi: 10.1111/rda.13998
81. Zmora N, Stubblefield J, Golan M, Servili A, Levavi-Sivan B, Zohar Y. The Medio-Basal Hypothalamus as a Dynamic and Plastic Reproduction-Related Kisspeptin-Gnrh-Pituitary Center in Fish. *Endocrinology* (2014) 155:1874–86. doi: 10.1210/en.2013-1894
82. Zmora N, Stubblefield JD, Wong TT, Levavi-Sivan B, Millar RP, Zohar Y. Kisspeptin Antagonists Reveal Kisspeptin 1 and Kisspeptin 2 Differential Regulation of Reproduction in the Teleost, Morone Saxatilis. *Biol Reprod* (2015) 93:76. doi: 10.1095/biolreprod.115.131870
83. Espigares F, Carrillo M, Gomez A, Zanuy S. The Forebrain-Midbrain Acts as Functional Endocrine Signaling Pathway of Kiss2/Gnrh1 System Controlling the Gonadotroph Activity in the Teleost Fish European Sea Bass (*Dicentrarchus Labrax*). *Biol Reprod* (2015) 92:70. doi: 10.1095/biolreprod.114.125138
84. Espigares F, Zanuy S, Gomez A. Kiss2 as a Regulator of Lh and Fsh Secretion via Paracrine/Autocrine Signaling in the Teleost Fish European Sea Bass (*Dicentrarchus Labrax*). *Biol Reprod* (2015) 93:114. doi: 10.1095/biolreprod.115.131029
85. Selvaraj S, Ohga H, Kitano H, Nyuji M, Yamaguchi A, Matsuyama M. Peripheral Administration of Kiss1 Pentadecapeptide Induces Gonadal Development in Sexually Immature Adult Scombroid Fish. *Zool Sci* (2013) 30:446–54. doi: 10.2108/zsj.30.446
86. Selvaraj S, Ohga H, Nyuji M, Kitano H, Nagano N, Yamaguchi A, et al. Subcutaneous Administration of Kiss1 Pentadecapeptide Accelerates Spermatogenesis in Prepubertal Male Chub Mackerel (*Scomber Japonicus*). *Comp Biochem Physiol A* (2013) 166:228–36. doi: 10.1016/j.cbpa.2013.06.007
87. Ohga H, Selvaraj S, Adachi H, Imanaga Y, Nyuji M, Yamaguchi A, et al. Functional Analysis of Kisspeptin Peptides in Adult Immature Chub Mackerel (*Scomber Japonicus*) Using an Intracerebroventricular Administration Method. *Neurosci Lett* (2014) 561:203–7. doi: 10.1016/j.neulet.2013.12.072
88. Nocillado JN, Zohar Y, Biran J, Levavi-Sivan B, Elizur A. Chronic Kisspeptin Administration Stimulated Gonadal Development in Pre-Pubertal Male Yellowtail Kingfish (*Seriola Lalandi*; Perciformes) During the Breeding and non-Breeding Season. *Gen Comp Endocrinol* (2013) 191:168–76. doi: 10.1016/j.ygcen.2013.06.005
89. Wang B, Liu Q, Liu X, Xu Y, Shi B. Molecular Characterization of Kiss2 Receptor and In Vitro Effects of Kiss2 on Reproduction-Related Gene Expression in the Hypothalamus of Half-Smooth Tongue Sole (*Cynoglossus Semilaevis*). *Gen Comp Endocrinol* (2017) 249:55–63. doi: 10.1016/j.ygcen.2017.04.006
90. Wang B, Yang G, Xu Y, Zhang Y, Liu X. *In Vitro* Effects of Tongue Sole LPXRFa and Kisspeptin on Relative Abundance of Pituitary Hormone mRNA and Inhibitory Action of LPXRFa on Kisspeptin Activation in the PKC Pathway. *Anim Reprod Sci* (2019) 203:1–9. doi: 10.1016/j.anireprosci.2019.01.009
91. Shi Y, Zhang Y, Li S, Liu Q, Lu D, Liu M, et al. Molecular Identification of the Kiss2/Kiss1ra System and its Potential Function During 17alpha-Methyltestosterone-Induced Sex Reversal in the Orange-Spotted Grouper, Epinephelus Coioides. *Biol Reprod* (2010) 83:63–74. doi: 10.1095/biolreprod.109.080044
92. Park JW, Jin YH, Oh SY, Kwon JY. Kisspeptin2 Stimulates the HPG Axis in Immature Nile Tilapia (*Oreochromis Niloticus*). *Comp Biochem Physiol B* (2016) 202:31–8. doi: 10.1016/j.cbpb.2016.07.009
93. Zhang H, Zhang B, Qin G, Li S, Lin Q. The Roles of the Kisspeptin System in the Reproductive Physiology of the Lined Seahorse (*Hippocampus Erectus*), an Ovoviviparous Fish With Male Pregnancy. *Front Neurosci* (2018) 12:940. doi: 10.3389/fnins.2018.00940
94. Oliveira CCV, Fatsini E, Fernández I, Anjos C, Chauvigné F, Cerdà J, et al. Kisspeptin Influences the Reproductive Axis and Circulating Levels of microRNAs in Senegalese Sole. *Int J Mol Sci* (2020) 21:9051. doi: 10.3390/ijms21239051
95. Chaube R, Sharma S, Senthilkumaran B, Bhat SG, Joy KP. Kisspeptins Stimulate the Hypothalamus - Pituitary - Ovarian Axis and Induce Final Oocyte Maturation and Ovulation in Female Stinging Catfish (*Heteropneustes Fossilis*): Evidence From *In Vivo* and *In Vitro* Studies. *Aquaculture* (2022) 548:737734. doi: 10.1016/j.aquaculture.2021.737734
96. Li W, Hu J, Sun C, Dong J, Liu Z, Yuan J, et al. Characterization of Kiss2/Kissr2 System in Largemouth Bass (Micropterus Salmoides) and Kiss2–10 Peptide Regulation of the Hypothalamic–Pituitary–Gonadal Axis. *Comp Biochem Physiol B* (2022) 257:110671. doi: 10.1016/j.cbpb.2021.110671
97. Zhao Y, Wayne NL. Effects of Kisspeptin1 on Electrical Activity of an Extrahypothalamic Population of Gonadotropin-Releasing Hormone Neurons in Medaka (*Oryzias Latipes*). *PloS One* (2012) 7:e37909. doi: 10.1371/journal.pone.0037909
98. Zhao Y, Lin MC, Mock A, Yang M, Wayne NL. Kisspeptins Modulate the Biology of Multiple Populations of Gonadotropin-Releasing Hormone Neurons During Embryogenesis and Adulthood in Zebrafish (*Danio Rerio*). *PloS One* (2014) 9:e104330. doi: 10.1371/journal.pone.0104330
99. Ma XL, Yuan BL, Zhou LB. The Kiss2/GPR54 System Stimulates the Reproductive Axis in Male Black Porgy, Acanthopagrus Schlegelii. *Gen Comp Endocrinol* (2019) 280:158–67. doi: 10.1016/j.ygcen.2019.04.024
100. Song H, Wang M, Qi J, Wang Z, Zhang Q. Native Recombinant Kisspeptin can Induce Gnrh1 and Kissr2 Expression in Paralichthys Olivaceus In Vitro. *Comp Biochem Physiol B* (2016) 200:36–43. doi: 10.1016/j.cbpb.2016.05.002
101. Chang JP, Mar A, Wlasichuk M, Wong AO. Kisspeptin-1 Directly Stimulates LH and GH Secretion From Goldfish Pituitary Cells in a Ca(2+)-Dependent Manner. *Gen Comp Endocrinol* (2012) 179:38–46. doi: 10.1016/j.ygcen.2012.07.028
102. Pasquier J, Lafont AG, Leprince J, Vaudry H, Rousseau K, Dufour S. First Evidence for a Direct Inhibitory Effect of Kisspeptins on LH Expression in the Eel, Anguilla Anguilla. *Gen Comp Endocrinol* (2011) 173:216–25. doi: 10.1016/j.ygcen.2011.05.019
103. Elizur A, Nocillado J, Biran J, Sivan B, Zohar Y. Advancement of the Onset of Puberty in Seriola Lalandi by Chronic Treatment With Kiss Peptides. *Indian J Sci Technol* (2011) 4:274–5. doi: 10.17485/ijst/2011/v4is.167
104. Beck BH, Fuller SA, Peatman E, McEntire ME, Darwish A, Freeman DW. Chronic Exogenous Kisspeptin Administration Accelerates Gonadal Development in Basses of the Genus Morone. *Comp Biochem Physiol A* (2012) 162:265–73. doi: 10.1016/j.cbpa.2012.03.019
105. Chaube R, Sharma S, Senthilkumaran B, Bhat SG, Joy KP. Identification of Kisspeptin2 cDNA in the Catfish Heteropneustes Fossilis: Expression Profile, *in Situ* Localization and Steroid Modulation. *Gen Comp Endocrinol* (2020) 294:113472. doi: 10.1016/j.ygcen.2020.113472
106. Marín-Juez R, Viñas J, Mechaly AS, Planas JV, Piferrer F. Stage-Specific Gene Expression During Spermatogenesis in the Senegalese Sole (*Solea Senegalensis*), a Fish With Semi-Cystic Type of Spermatogenesis, as Assessed by Laser Capture Microdissection and Absolute Quantitative PCR. *Gen Comp Endocrinol* (2013) 188:242–50. doi: 10.1016/j.ygcen.2013.04.015
107. Mechaly AS, Viñas J, Piferrer F. Sex-Specific Changes in the Expression of Kisspeptin, Kisspeptin Receptor, Gonadotropins and Gonadotropins Receptors in the Senegalese Sole (*Solea Senegalensis*) During a Full Reproductive Cycle. *Comp Biochem Physiol A* (2012) 162:364–71. doi: 10.1016/j.cbpa.2012.04.003
108. Selvaraj S, Kitano H, Fujinaga Y, Ohga H, Yoneda M, Yamaguchi A, et al. Molecular Characterization, Tissue Distribution, and mRNA Expression Profiles of Two Kiss Genes in the Adult Male and Female Chub Mackerel (*Scomber Japonicus*) During Different Gonadal Stages. *Gen Comp Endocrinol* (2010) 169:28–38. doi: 10.1016/j.ygcen.2010.07.011
109. Shahi N, Singh AK, Sahoo M, Mallik SK, Thakuria D. Molecular Cloning, Characterization and Expression Profile of Kisspeptin1 and Kisspeptin1 Receptor at Brain-Pituitary-Gonad (BPG) Axis of Golden Mahseer, Tor Putitora (Hamilto) During Gonadal Development. *Comp Biochem Physiol B* (2017) 205:13–29. doi: 10.1016/j.cbpb.2016.11.010
110. Tovar-Bohórquez MO, Mechaly AS, Elisio M, Chalde T, Canosa LF, Miranda LA, et al. Kisspeptins and Their Receptors in the Brain-Pituitary-Gonadal Axis of Odonthestes Bonariensis. Their Relation With Gametogenesis Along the Reproductive Cycle. *Gen Comp Endocrinol* (2017) 252:209–18. doi: 10.1016/j.ygcen.2017.06.028

111. Singh A, Lal B, Parhar IS, Millar RP. Seasonal Expression and Distribution of Kisspeptin1 (Kiss1) in the Ovary and Testis of Freshwater Catfish, Clarias Batrachus: A Putative Role in Steroidogenesis. *Acta Histochem* (2021) 123:151766. doi: 10.1016/j.acthis.2021.151766
112. Singh A, Lal B, Parkash J, Millar RP. Gametogenic and Steroidogenic Action of Kisspeptin-10 in the Asian Catfish, Clarias Batrachus: Putative Underlying Mechanistic Cascade. *Comp Biochem Physiol B* (2021) 256:110642. doi: 10.1016/j.cbpb.2021.110642
113. Kim NN, Choi YU, Park HS, Choi CY. Kisspeptin Regulates the Somatic Growth-Related Factors of the Cinnamon Clownfish Amphiprion Melanopus. *Comp Biochem Physiol A* (2015) 179:17–24. doi: 10.1016/j.cbpa.2014.08.020
114. Ogawa S, Nathan FM, Parhar IS. Habenular Kisspeptin Modulates Fear in the Zebrafish. *Proc Natl Acad Sci USA* (2014) 111:3841–6. doi: 10.1073/pnas.1314184111
115. Nathan FM, Ogawa S, Parhar IS. Kisspeptin1 Modulates Odorant-Evoked Fear Response via Two Serotonin Receptor Subtypes (5-HT1A and 5-HT2) in Zebrafish. *J Neurochem* (2015) 133:870–8. doi: 10.1111/jnc.13105
116. Escobar S, Felip A, Zanuy S, Carrillo M. Is the Kisspeptin System Involved in Responses to Food Restriction in Order to Preserve Reproduction in Pubertal Male Sea Bass (*Dicentrarchus Labrax*)? *Comp Biochem Physiol A* (2016) 199:38–46. doi: 10.1016/j.cbpa.2016.05.005
117. Gopurappilly R, Ogawa S, Parhar IS. Functional Significance of GnRH and Kisspeptin, and Their Cognate Receptors in Teleost Reproduction. *Front Endocrinol (Lausanne)* (2013) 4:24:24. doi: 10.3389/fendo.2013.00024
118. Cheng Z, Garvin D, Paguio A, Stecha P, Wood K, Fan F. Luciferase Reporter Assay System for Deciphering GPCR Pathways. *Curr Chem Genomics* (2010) 4:84–91. doi: 10.2174/1875397301004010084
119. Son YL, Ubuka T, Soga T, Yamamoto K, Bentley GE, Tsutsui K. Inhibitory Action of Gonadotropin-Inhibitory Hormone on the Signaling Pathways Induced by Kisspeptin and Vasoactive Intestinal Polypeptide in GnRH Neuronal Cell Line, GT1-7. *FASEB J* (2016) 30:2198–210. doi: 10.1096/fj.201500055
120. Ohga H, Fujinaga Y, Selvaraj S, Kitano H, Nyuji M, Yamaguchi A, et al. Identification, Characterization, and Expression Profiles of Two Subtypes of Kisspeptin Receptors in a Scombroid Fish (Chub Mackerel). *Gen Comp Endocrinol* (2013) 193:130–40. doi: 10.1016/j.ygcen.2013.07.016
121. Ohga H, Akase F, Sakanoue R, Matsushima A, Ohta K, Matsuyama M. Alanine Scanning and Characterization of Core Peptides in Scombridae Fish Family for Construction of Kiss1 Super Analog. *Gen Comp Endocrinol* (2020) 288:113356. doi: 10.1016/j.ygcen.2019.113356
122. Felip A, Espigares F, Zanuy S, Gomez A. Differential Activation of Kiss Receptors by Kiss1 and Kiss2 Peptides in the Sea Bass. *Reproduction* (2015) 150:227–43. doi: 10.1530/REP-15-0204
123. Wang B, Yang G, Liu Q, Qin J, Xu Y, Li W, et al. Inhibitory Action of Tongue Sole LPXRFa, the Piscine Ortholog of Gonadotropin-Inhibitory Hormone, on the Signaling Pathway Induced by Tongue Sole Kisspeptin in COS-7 Cells Transfected With Their Cognate Receptors. *Peptides* (2017) 95:62–7. doi: 10.1016/j.peptides.2017.07.014
124. Wang B, Yang G, Xu Y, Li W, Liu X. Recent Studies of LPXRFa Receptor Signaling in Fish and Other Vertebrates. *Gen Comp Endocrinol* (2019) 277:3–8. doi: 10.1016/j.ygcen.2018.11.011
125. Spicer OS, Zmora N, Wong TT, Golan M, Levavi-Sivan B, Gothilf Y, et al. The Gonadotropin-Inhibitory Hormone (Lpxrfa) System's Regulation of Reproduction in the Brain-Pituitary Axis of the Zebrafish (*Danio Rerio*). *Biol Reprod* (2017) 96:1031–42. doi: 10.1093/biolre/iox032
126. Wang B, Yang G, Liu Q, Qin J, Xu Y, Li W, et al. Characterization of LPXRFa Receptor in the Half-Smooth Tongue Sole (*Cynoglossus Semilaevis*): Molecular Cloning, Expression Profiles, and Differential Activation of Signaling Pathways by LPXRFa Peptides. *Comp Biochem Physiol A* (2018) 223:23–32. doi: 10.1016/j.cbpa.2018.05.008
127. Castaño JP, Martinez-Fuentes AJ, Gutierrez-Pascual E, Vaudry H, Tena-Sempere M, Malagon MM. Intracellular Signaling Pathways Activated by Kisspeptins Through GPR54: Do Multiple Signals Underlie Function Diversity? *Peptides* (2009) 30:10–5. doi: 10.1016/j.peptides.2008.07.025
128. Ciani E, Haug TM, Maugars G, Weltzien FA, Falcón J, Fontaine R. Effects of Melatonin on Anterior Pituitary Plasticity: A Comparison Between Mammals and Teleosts. *Front Endocrinol (Lausanne)* (2021) 11:605111. doi: 10.3389/fendo.2020.605111
129. Kitahashi T, Parhar IS. Comparative Aspects of Kisspeptin Gene Regulation. *Gen Comp Endocrinol* (2013) 181:197–202. doi: 10.1016/j.ygcen.2012.10.003
130. Martinez-Chavez CC, Minghetti M, Migaud H. GPR54 and Rgnrh I Gene Expression During the Onset of Puberty in Nile Tilapia. *Gen Comp Endocrinol* (2008) 156:224–33. doi: 10.1016/j.ygcen.2008.01.019
131. Cowan M, Davie A, Migaud H. Photoperiod Effects on the Expression of Kisspeptin and Gonadotropin Genes in Atlantic Cod, Gadus Morhua, During First Maturation. *Comp Biochem Physiol A* (2012) 163:82–94. doi: 10.1016/j.cbpa.2012.05.191
132. Mitani Y, Kanda S, Akazome Y, Zempo B, Oka Y. Hypothalamic Kiss1 But Not Kiss2 Neurons are Involved in Estrogen Feedback in Medaka (*Oryzias Latipes*). *Endocrinology* (2010) 151:1751–9. doi: 10.1210/en.2009-1174
133. Zmora N, Stubblefield J, Zulperi Z, Klenke U, Zohar Y. Kisspeptin-Photoperiod/Gonadal Steroid Relationships in the Brain of Two Perciforms, the Striped Bass and Hybrid Bass. *Indian J Sci Technol* (2011) 4:10–1. doi: 10.17485/ijst%2F2011%2Fv4iS8%2F30854
134. Carnevali O, Gioacchini F, Maradonna F, Olivotto I, Migliarini B. Melatonin Induces Follicle Maturation in Danio Rerio. *PloS One* (2011) 6:e19978. doi: 10.1371/journal.pone.0019978
135. Loganathan K, Moriya S, Parhar IS. High Melatonin Conditions by Constant Darkness and High Temperature Differently Affect Melatonin Receptor Mt1 and TREK Channel *Trek2a* in the Brain of Zebrafish. *Zebrafish* (2018) 15:473–83. doi: 10.1089/zeb.2018.1594
136. Alvarado MV, Carrillo M, Felip A. Melatonin-Induced Changes in Kiss/Gnrh Gene Expression Patterns in the Brain of Male Sea Bass During Spermatogenesis. *Comp Biochem Physiol A* (2015) 185:69–79. doi: 10.1016/j.cbpa.2015.03.010
137. Imamura S, Hur SP, Takeuchi Y, Badruzzaman M, Mahardini A, Rizky D, et al. Effect of Short- and Long-Term Melatonin Treatments on the Reproductive Activity of the Tropical Damselfish Chrysiptera Cyanea. *Fish Physiol Biochem* (2022) 48:253–62. doi: 10.1007/s10695-022-01051-x
138. Espigares F, Rocha A, Gomez A, Carrillo M, Zanuy S. Photoperiod Modulates the Reproductive Axis of European Sea Bass Through Regulation of Kiss1 and Gnrh2 Neuronal Expression. *Gen Comp Endocrinol* (2017) 240:35–45. doi: 10.1016/j.ygcen.2016.09.007
139. Shahjahan M, Kitahashi T, Ogawa S, Parhar IS. Temperature Differentially Regulates the Two Kisspeptin Systems in the Brain of Zebrafish. *Gen Comp Endocrinol* (2013) 193:79–85. doi: 10.1016/j.ygcen.2013.07.015
140. Shahjahan M, Kitahashi T, Ando H. Temperature Affects Sexual Maturation Through the Control of Kisspeptin, Kisspeptin Receptor, GnRH and GTH Subunit Gene Expression in the Grass Puffer During the Spawning Season. *Gen Comp Endocrinol* (2017) 243:138–45. doi: 10.1016/j.ygcen.2016.11.012
141. Navarro VM. Metabolic Regulation of Kisspeptin - the Link Between Energy Balance and Reproduction. *Nat Rev Endocrinol* (2020) 16(8):407–20. doi: 10.1038/s41574-020-0363-7
142. Luque RM, Kineman RD, Tena-Sempere M. Regulation of Hypothalamic Expression of KiSS-1 and GPR54 Genes by Metabolic Factors: Analyses Using Mouse Models and a Cell Line. *Endocrinology* (2007) 148:4601–11. doi: 10.1210/en.2007-0500
143. Wahab F, Aziz F, Irfan S, Zaman WU, Shahab M. Short-Term Fasting Attenuates the Response of the HPG Axis to Kisspeptin Challenge in the Adult Male Rhesus Monkey (*Macaca Mulatta*). *Life Sci* (2008) 83:633–7. doi: 10.1016/j.lfs.2008.09.001
144. Castellano JM, Navarro VM, Fernández-Fernández R, Nogueiras R, Tovar S, Roa J, et al. Changes in Hypothalamic KiSS-1 System and Restoration of Pubertal Activation of the Reproductive Axis by Kisspeptin in Undernutrition. *Endocrinology* (2005) 146:3917–25. doi: 10.1210/en.2005-0337
145. Dong TS, Vu JP, Oh S, Sanford D, Pisegna JR, Germano P. Intraperitoneal Treatment of Kisspeptin Suppresses Appetite and Energy Expenditure and Alters Gastrointestinal Hormones in Mice. *Dig Dis Sci* (2019) 65:2254–63. doi: 10.1007/s10620-019-05950-7
146. Saito R, Tanaka K, Nishimura H, Nishimura K, Sonoda S, Ueno H. Centrally Administered Kisspeptin Suppresses Feeding via Nesfatin-1 and Oxytocin in Male Rats. *Peptides* (2019) 112:114–24. doi: 10.1016/j.peptides.2018.12.003
147. Stengel A, Wang L, Goebel-Stengel M, Tache Y. Centrally Injected Kisspeptin Reduces Food Intake by Increasing Meal Intervals in Mice. *Neuroreport* (2011) 22:253–7. doi: 10.1097/WNR.0b013e32834558df

148. Talbi R, Laran-Chich MP, Magoul R, El Ouezzani S, Simonneaux V. Kisspeptin and RFRP-3 Differentially Regulate Food Intake and Metabolic Neuropeptides in the Female Desert Jerboa. *Sci Rep* (2016) 6:36057. doi: 10.1038/srep36057
149. Kanda S, Karigo T, Oka Y. Steroid Sensitive Kiss2 Neurones in the Goldfish: Evolutionary Insights Into the Duplicate Kisspeptin Gene-Expressing Neurones. *J Neuroendocrinol* (2012) 24:897–906. doi: 10.1111/j.1365-2826.2012.02296.x
150. Guo Y, Wang Q, Li G, He M, Tang H, Zhang H. Molecular Mechanism of Feedback Regulation of 17beta-Estradiol on Two Kiss Genes in the Protogynous Orange-Spotted Grouper (*Epinephelus Coioides*). *Mol Reprod Dev* (2017) 84:495–507. doi: 10.1002/mrd.22800
151. Nocillado JN, Mechaly AS, Elizur A. *In Silico* Analysis of the Regulatory Region of the Yellowtail Kingfish and Zebrafish Kiss and Kiss Receptor Genes. *Fish Physiol Biochem* (2013) 39:59–63. doi: 10.1007/s10695-012-9642-0
152. Wang Q, Sham KW, Ogawa S, Li S, Parhar IS, Cheng CH. Regulation of the Two Kiss Promoters in Goldfish (*Carassius Auratus*) by Estrogen via Different ERalpha Pathways. *Mol Cell Endocrinol* (2013) 375:130–9. doi: 10.1016/j.mce.2013.04.023
153. Imamura S, Hur SP, Takeuchi Y, Badruzzaman M, Mahardini A, Rizky D, et al. The mRNA Expression Patterns of Kisspeptins, GnRHs, and Gonadotropins in the Brain and Pituitary Gland of a Tropical Damselfish, Chrysiptera Cyanea, During the Reproductive Cycle. *Fish Physiol Biochem* (2020) 46:277–91. doi: 10.1007/s10695-019-00715-5
154. Yue HM, Ye H, Ruan R, Du H, Li CJ, Wei Q. Feedback Regulation of 17beta-Estradiol on Two Kisspeptin Genes in the Dabry's Sturgeon (*Acipenser Dabryanus*). *Comp Biochem Physiol B* (2019) 230:1–9. doi: 10.1016/j.cbpb.2019.01.006
155. Imamura S, Hur SP, Takeuchi Y, Bouchekioua S, Takemura A. Molecular Cloning of Kisspeptin Receptor Genes (Gpr54-1 and Gpr54-2) and Their Expression Profiles in the Brain of a Tropical Damselfish During Different Gonadal Stages. *Comp Biochem Physiol A* (2017) 203:9–16. doi: 10.1016/j.cbpa.2016.07.015
156. Wang B, Liu Q, Liu X, Xu Y, Song X, Shi B. Molecular Characterization of Kiss2 and Differential Regulation of Reproduction-Related Genes by Sex Steroids in the Hypothalamus of Half-Smooth Tongue Sole (*Cynoglossus Semilaevis*). *Comp Biochem Physiol A* (2017) 213:46–55. doi: 10.1016/j.cbpa.2017.08.003
157. Alvarado MV, Servili A, Moles G, Gueguen MM, Carrillo M, Kah O, et al. Actions of Sex Steroids on Kisspeptin Expression and Other Reproduction-Related Genes in the Brain of the Teleost Fish European Sea Bass. *J Exp Biol* (2016) 219:3353–65. doi: 10.1242/jeb.137364
158. Klenke U, Zmora N, Stubblefield J, Zohar Y. Expression Patterns of the Kisspeptin System and GnRH1 Correlate in Their Response to Gonadal Feedback in Female Striped Bass. *Indian J Sci Technol* (2011) 4:33–4. doi: 10.17485/ijst/2011/v4is.29
159. Golshan M, Habibi HR, Alavi SM. Transcripts of Genes Encoding Reproductive Neuroendocrine Hormones and Androgen Receptor in the Brain and Testis of Goldfish Exposed to Vinclozolin, Flutamide, Testosterone, and Their Combinations. *Fish Physiol Biochem* (2016) 42:1157–65. doi: 10.1007/s10695-016-0205-7
160. Ogawa S, Ng KW, Ramadasan PN, Nathan FM, Parhar IS. Habenular Kiss1 Neurons Modulate the Serotonergic System in the Brain of Zebrafish. *Endocrinology* (2012) 153:2398–407. doi: 10.1210/en.2012-1062
161. Paullada-Salmeron JA, Cowan M, Aliaga-Guerrero M, Morano F, Zanuy S, Munoz-Cueto JA. Gonadotropin Inhibitory Hormone Down-Regulates the Brain-Pituitary Reproductive Axis of Male European Sea Bass (*Dicentrarchus Labrax*). *Biol Reprod* (2016) 94:121. doi: 10.1095/biolreprod.116.139022
162. Paullada-Salmeron JA, Cowan M, Aliaga-Guerrero M, Lopez-Olmeda JF, Mananos EL, Zanuy S, et al. Testicular Steroidogenesis and Locomotor Activity are Regulated by Gonadotropin-Inhibitory Hormone in Male European Sea Bass. *PloS One* (2016) 11:e0165494. doi: 10.1371/journal.pone.0165494
163. Liu Q, Wang B, Liu X, Xu Y, Shi B, Liu Z. Effects of Gonadotropin-Inhibitory Hormone Peptides on the Reproduction-Related Gene Expression in the Hypothalamus of Half-Smooth Tongue Sole (*Cynoglossus Semilaevis*). *Prog Fish Sci* (2017) 38:56–62. doi: 10.11758/yykxjz.20160805001
164. Aliaga-Guerrero M, Paullada-Salmeron JA, Piquer V, Mananos EL, Munoz-Cueto JA. Gonadotropin-Inhibitory Hormone in the Flatfish, Solea Senegalensis: Molecular Cloning, Brain Localization and Physiological Effects. *J Comp Neurol* (2018) 526:349–70. doi: 10.1002/cne.24339
165. Wang Q, Qi X, Guo Y, Li S, Zhang Y, Liu X, et al. Molecular Identification of GnIH/GnIHR Signal and its Reproductive Function in Protogynous Hermaphroditic Orange-Spotted Grouper (*Epinephelus Coioides*). *Gen Comp Endocrinol* (2015) 216:9–23. doi: 10.1016/j.ygcen.2015.04.016
166. Habibi HR, Nelson ER, Allan ER. New Insights Into Thyroid Hormone Function and Modulation of Reproduction in Goldfish. *Gen Comp Endocrinol* (2012) 175:19–26. doi: 10.1016/j.ygcen.2011.11.003
167. Tovo-Neto A, da Silva Rodrigues M, Habibi HR, Nobrega RH. Thyroid Hormone Actions on Male Reproductive System of Teleost Fish. *Gen Comp Endocrinol* (2018) 265:230–6. doi: 10.1016/j.ygcen.2018.04.023
168. Ogawa S, Ng KW, Xue X, Ramadasan PN, Sivalingam M, Li S, et al. Thyroid Hormone Upregulates Hypothalamic Kiss2 Gene in the Male Nile Tilapia, Oreochromis Niloticus. *Front Endocrinol (Lausanne)* (2013) 4:184. doi: 10.3389/fendo.2013.00184
169. Waye A, Trudeau VL. Neuroendocrine Disruption: More Than Hormones are Upset. *J Toxicol Environ Health B* (2011) 14:270–91. doi: 10.1080/10937404.2011.578273
170. Faheem M, Jahan N, Khaliq S, Lone KP. Modulation of Brain Kisspeptin Expression After Bisphenol-A Exposure in a Teleost Fish, K. *Fish Physiol Biochem* (2019) 45:33–42. doi: 10.1007/s10695-018-0532-y
171. Qiu W, Fang M, Liu J, Fu C, Zheng C, Chen B, et al. *In Vivo* Actions of Bisphenol F on the Reproductive Neuroendocrine System After Long-Term Exposure in Zebrafish. *Sci Total Environ* (2019) 665:995–1002. doi: 10.1016/j.scitotenv.2019.02.154
172. Yue Z, Yu M, Zhao H, Wang J, Zhang X, Tian H, et al. The Anti-Estrogenicity of Chronic Exposure to Semicarbazide in Female Japanese Flounders (*Paralichthys Olivaceus*), and its Potential Mechanisms. *Mar Pollut Bull* (2018) 129:806–12. doi: 10.1016/j.marpolbul.2017.10.081
173. Yue Z, Yu M, Zhang X, Wang J, Ru S. The Anti-Androgenic Effect of Chronic Exposure to Semicarbazide on Male Japanese Flounder (*Paralichthys Olivaceus*) and its Potential Mechanisms. *Comp Biochem Physiol C* (2018) 210:30–4. doi: 10.1016/j.cbpc.2018.04.005
174. Smith CM, Vera MKM, Bhandari RK. Developmental and Epigenetic Effects of Roundup and Glyphosate Exposure on Japanese Medaka (*Oryzias Latipes*). *Aquat Toxicol* (2019) 210:215–26. doi: 10.1016/j.aquatox.2019.03.005
175. Mitchell K, Zhang WS, Lu C, Tao B, Chen L, Hu W, et al. Targeted Mutation of Secretogranin-2 Disrupts Sexual Behavior and Reproduction in Zebrafish. *Proc Natl Acad Sci USA* (2020) 117:12772–83. doi: 10.1073/pnas.2002004117
176. Tanaka S, Zmora N, Levavi-Sivan B, Zohar Y. Vasoactive Intestinal Peptide Indirectly Elicits Pituitary Lh Secretion Independent of GnRH in Female Zebrafish. *Endocrinology* (2022) 163:113. doi: 10.1210/endocr/bqab264
177. Trudeau VL. Neuroendocrine Control of Reproduction in Teleost Fish: Concepts and Controversies. *Annu Rev Anim Biosci* (2022) 10:18.11–24. doi: 10.1146/annurev-animal-020420-042015
178. Lien S, Koop BF, Sandve SR, Miller JR, Kent MP, Nome T, et al. The Atlantic Salmon Genome Provides Insights Into Rediploidization. *Nature* (2016) 533:200–5. doi: 10.1038/nature17164

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Wang, Mechaly and Somoza. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 02 May 2022
doi: 10.3389/fendo.2022.869021

Physiological Effects and Transcriptomic Analysis of sbGnRH on the Liver in Pompano (*Trachinotus ovatus*)

Xilin Ren[1,2], *Jinlei Liu*[3], *Charles Brighton Ndandala*[1], *Xiaomeng Li*[1], *Yuwen Guo*[1], *Guangli Li*[1] *and Huapu Chen*[1,2*]

[1] Guangdong Research Center on Reproductive Control and Breeding Technology of Indigenous Valuable Fish Species, Guangdong Provincial Engineering Laboratory for Mariculture Organism Breeding. Guangdong Provincial Key Laboratory of Pathogenic Biology and Epidemiology for Aquatic Economic Animals, Fisheries College, Guangdong Ocean University, Zhanjiang, China, [2] Southern Marine Science and Engineering Guangdong Laboratory, Zhanjiang, China, [3] Guangdong Havwii Agricultural Group Co., Ltd, Zhanjiang, China

OPEN ACCESS

Edited by:
Bin Wang,
Chinese Academy of Fishery Sciences (CAFS), China

Reviewed by:
Alejandro S. Mechaly,
Instituto de Investigaciones en Biodiversidad y Biotecnología (INBIOTEC) (CONICET), Argentina
Dizhi Xie,
South China Agricultural University, China

***Correspondence:**
Huapu Chen
chpsysu@hotmail.com

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

Received: 03 February 2022
Accepted: 15 March 2022
Published: 02 May 2022

Citation:
Ren X, Liu J, Ndandala CB, Li X, Guo Y, Li G and Chen H (2022) Physiological Effects and Transcriptomic Analysis of sbGnRH on the Liver in Pompano (Trachinotus ovatus). Front. Endocrinol. 13:869021. doi: 10.3389/fendo.2022.869021

Pompano (*Trachinotus ovatus*) is one of the important economic marine fishes in the south coast of China. At present, the research on the basic biology of pompano is relatively weak, which has seriously affected the development of this economic important fish. The liver is an important digestive and metabolic organ of fish which plays an important regulatory role in its growth and development. It is necessary to clarify the effects of sea bream gonadotropin releasing hormone (sbGnRH) on liver physiology and metabolic enzyme activity. The effects of sbGnRH peptides (10 ng/gbw) on the physiological and biochemical indices and metabolic enzyme activities of pompano liver were studied. It was found that after injection of 10 ng/gbw sbGnRH peptides, the contents of albumin, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, glucose, creatine kinase, iron, magnesium, aspartate aminotransferase, alanine aminotransferase and creatinine increased, while of cholesterol and calcium contents decreased. The activities of amylase, lipase, pyruvate kinase, acyl CoA oxidase, superoxide dismutase, phospholipid hydroperoxide glutathione peroxidase, catalase, glucose-6-phosphate dehydrogenase, fatty acid synthase and lipoprotein lipase increased, while the activities of malic enzyme, carnitine acyl, carnitine translocation, acetyl CoA carboxylase and malondialdehyde decreased. Three hours after the injection of different concentrations of sbGnRH peptides (0 and 10 ng/gbw), the transcriptome sequences of the two groups of livers were sequenced. After quality control and removal of some low-quality data, clean reads of 21,283,647、19,427,359、21,873,990、21,732,174、23,660,062 and 21,592,338 were obtained respectively. In this study, 99 genes were screened and identified as differentially expressed genes, including 77 up-regulated genes and 22 down-regulated genes. According to the Kyoto Encyclopedia of Genes and Genomes (KEGG) and Gene Ontology (GO) pathway analyses, these pathways and the typical genes involved can be divided into cellular processes, environmental information

processing, genetic information processing, diseases, metabolism and organismal systems. The results from this study provide a the oretical basis for studying the effects of sbGnRH on the physiology, biochemistry and metabolic enzyme activities of liver in pompano.

Keywords: *Trachinotus ovatus*, sbGnRH, transcriptome, liver, physiology

1 INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a key hormone produced in hypothalamus and plays an important role in reproductive regulation, regulating the development of vertebrates. In teleosts, mature GnRH is synthesized in hypothalamic neuronal cells, temporarily stored in secretory cells at its nerve endings, and transferred to the pituitary after release. It binds with its receptor in the pituitary and acts on gonadotropin cells to trigger the synthesis and release of gonadotropins (GTHs). Synthetic GTHs reaches the gonad through blood, which can promote steroid hormone production and gametogenesis (1). GnRH produced in the hypothalamus can affect vertebrate reproduction at many levels, such as pituitary and gonad. The most classic pathway is through pituitary mediated signal pathway. GnRH stimulates pituitary synthesis and release of GTHs, and then stimulates a series of downstream reproductive related activities (2–4). It can also adjust the content of local GTHs and the level of sex steroid hormones in the blood by paracrine or autocrine, so as to change the sexual behavior of animals (5–7). GnRH acts through the Gonadotropin releasing hormone receptor (GnRHR) of the 7-transmembrane G protein-coupled receptor (GPCR) superfamily. It indicates that GnRH signal transduction can induce the expression of lh by protein kinase C (PKC) cascade mediated immune regulation mechanism, the expression of fsh by cAMP/protein kinase A (PKA) cascade mediated. Regulation of gonadotropin expression is a key factor in inducing and completing gonadal maturation. Kisspeptin-Kissr can also control hypothalamic GnRH release by activating PLC pathway and intracellular Ca^{2+} mobilization. Kisspeptin also stimulates GnRH neurons through mitogen-activated protein kinase (MAPK), especially ERK1/2, p38 and phosphatidylinositol 3-kinase (PI3K)/Akt activation. The activation of these pathways is also considered necessary for progesterone synthesis and preovulation follicles. This suggests that kisspeptin-Kissr signaling may not be necessary in teleost species, but may be a compensation for the successful development and maturation of normal reproductive axis.

In addition, many studies have also found GnRH or similar peptides in central nervous system, peripheral nerve and sympathetic ganglia by using immunoenzymatic localization, immunohistochemistry, *in situ* hybridization and radioimmunoassay, and GnRH receptor (GnRHR) is also found in these tissues. (8). GnRH is widely distributed in the reproductive system, neural network, endocrine system, immune system and digestive system. By integrating and transmitting the information of each system, they can coordinate and cooperate to achieve the best physiological state (9). In different tissues, GnRH may play different biological functions and maintain the stability of the internal environment. In addition, GnRH can also participate in the regulation of the immune system through paracrine and autocrine. In the digestive system, GnRH participates in metabolic activities as a paracrine regulator; and in cancer cells as autocrine regulators. Among them, sbGnRH mRNA in many fish, such as pompano (*Trachinotus ovatus*) (10), turbot (*Scophthalmus maximus*) (11) and rainbow trout (*Oncorhynchus mykiss*) (12), shows a common tissue expression pattern, and its expression can be detected in different tissues, indicating that sbGnRH has a variety of physiological functions. It can regulate a variety of physiological activities, including reproduction.

The liver is an important digestive and metabolic organ in the growth and development of teleost fish. Studies have shown that the expression of sbGnRH in the fish liver is high, and the exertion of various physiological pathways may be related to the function of sbGnRH, which regulates the metabolism of substances and energy (13, 14). It plays a key role in nutrient utilization, lipid, carbohydrate and protein metabolism, endocrine and immune homeostasis regulation (15). However, there are few studies on the comprehensive evaluation of the effects of sbGnRH on the physiological and metabolic patterns of oval nematode liver, which is of great significance to explore the interaction mechanism between liver and sbGnRH. In this study, the differences of liver physiological indexes of pompano after injection of different concentrations of sbGnRH peptides were detected. Further comparative transcriptome analysis was carried out in order to provide a global view of the role of sbGnRH in liver biology and explore the potential role of sbGnRH in non-reproductive systems. These data are valuable for further elucidating the various physiological functions of sbGnRH in this species.

2 MATERIALS AND METHODS

2.1 Fish and Sample Preparation

Adult pompano (*T. ovatus*) (body weight, 300 ± 20 g, body length: 22 ± 3 cm) were purchased from the Dongfeng Market (Zhanjiang, Guangdong, China), female fish were selected according to the observed gonads. The sbGnRH peptide used for this study was purchased from GL Biochem (Shanghai, China). sbGnRH peptides were dissolved in physiological saline. All the fish were anesthetized with 100 mg/L tricaine methane sulfonate (MS-222, Sigma,St. Louis, MO, USA) and dissected. Animal experiments were conducted in accordance

with the guidelines approved by the Animal Research and Ethics Committees of Fisheries College of Guangdong Ocean University, Zhanjiang, China.

The dose and sampling time of sbGnRH peptides injection were selected according to our previous research results (10). Female adult fish were intraperitoneally injected with 10 ng/g of sbGnRH peptides according to the average body weight, while the control group was intraperitoneally injected with physiological saline. Each group contained three fishes. Three hours after injection, the selected fish were anesthetized and the liver was collected, frozen in liquid nitrogen and later stored at -80°C.

2.2 Measurement of Physiological and Biochemical Indexes and Enzyme Activities of Liver

Liver samples were accurately weighed and homogenized (diluted 1:10) in Phosphate buffered saline (PBS) (Solarbio, Beijing, China) with a tissue crusher. The homogenization was 900 × g centrifuge for 10 min at 4°C and retained the supernatant. The protein content of the homogenate was measured using Folin-phenol reagent (16, 17). The contents of albumin (ALB), cholesterol (CHO), high-density lipoprotein cholesterol (HDLC), low-density lipoprotein cholesterol (LDLC), glucose (GLUC), creatine kinase (CK), iron (IRON), magnesium (MG), calcium (CA), phosphate (PHOS), alkaline phosphatase (ALP), aspartate aminotransferase (AST), alanine aminotransferase (ALT) and creatinine (CREA) were detected by Roche Cobas C311 automatic biochemical analyzer (Shanghai, China). The kits used were Cobas C311 products.

According to the product instructions, amylase (AMS), glucose-6-phosphate dehydrogenase (G-6-PD), malic enzyme (ME), pyruvate kinase (PK), acyl CoA oxidase (ACO), lipase (LPS), fatty acid synthase (FAS), carnitine acyl carnitine translocation (CACT), acetyl CoA carboxylase (ACC), lipoprotein lipase (LPL), superoxide dismutase (SOD), Phospholipid hydroperoxide glutathione peroxidase (GSH-PX), catalase (CAT) and malondialdehyde (MDA) were detected. See **Table S1** for product numbers.

2.3 RNA-Seq and Transcriptomic Analysis

Six liver samples (3 replicates per group) were used to prepare a transcriptome (RNA-seq) sequencing libraries by the Biomarker Technologies. The RNA-seq process was performed as follows: 1 μg RNA per sample was used as input material for the RNA sample preparations. Sequencing libraries were generated using NEBNext®Ultra™ RNA Library Prep Kit for Illumina® (NEB, USA) following the manufacturer's recommendations and index codes were added to attribute sequences to each sample. Briefly, mRNA was purified from total RNA using poly-T oligo-attached magnetic beads. Fragmentation was carried out using divalent cations under elevated temperature in NEBNext First Strand Synthesis Reaction Buffer (5X). First strand cDNA was synthesized using random hexamer primer and M-MuLV Reverse Transcriptase. Second strand cDNA synthesis was subsequently performed using DNA Polymerase I and RNase H. Remaining overhangs were converted into blunt ends *via* exonuclease/polymerase activities. After adenylation of 3' ends of DNA fragments, NEBNext Adaptor with hairpin loop structure was ligated to prepare for hybridization. The library fragments were purified with AMPure XP system (Beckman Coulter, Beverly, USA). Then 3 μl USER Enzyme (NEB, USA) was used with size-selected, adaptor-ligated cDNA at 37°C for 15 min followed by 5 min at 95°C before PCR. PCR was performed with Phusion High-Fidelity DNA polymerase, Universal PCR primers and Index (X) Primer. After which, PCR products were purified (AMPure XP system) and library quality was assessed on the Agilent Bio analyzer 2100 system. After cluster generation, the library preparations were sequenced on an Illumina Hiseq 2000 platform and paired-end reads were generated. Transcriptome assembly was accomplished based on the left.fq and right.fq using Trinity (18) with the min_kmer_cov set to 2 by default and all other parameters set to default.

UniGene sequence was compared with Non-Redundant protein (NR) (19), Swiss prot (20), Clusters of Orthologous Group (COG) (21), Eukaryotic Orthologous Groups (KOG) (22) and evolutionary genealogy of genes: Non-supervised Orthologous Groups (eggNOG 4.5) (23) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (24) database alignment by using diamond (25) software. Kobas (26) was used to obtain the KEGG ontology results of UniGene in KEGG. Interproscan (27) used the database integrated by interpro to analyze the Gene Ontology (GO) (28) ontology results of new genes. After predicting the amino acid sequence of UniGene, HMMER (29) software was used to compare results obtained with Protein Families (Pfam) (30) database to obtain the annotation information of UniGene. Bowtie (31) was used to compare the sequenced reads with the UniGene library. According to the comparison results, the expression level was estimated in combination with RNA-Seq by Expectation-Maximization (RSEM) (32). The expression abundance of the corresponding UniGene was expressed by fragments per kilobase of exon model per million reads mapped (FPKM) value. In the process of differential expression analysis, the recognized and effective Benjamin Hochberg method is used to correct the significance p-value obtained from the original hypothesis test. The corrected p-value, FDR (false discovery rate), is then used as the key index of differential expression gene screening to reduce the false positive caused by independent statistical hypothesis test on the expression value of a large number of genes (33). In the screening process, p-value < 0.01 and the difference multiple FC (fold change) ≥ 1.5 are used as the screening criteria.

2.4 Real-Time Quantitative PCR (RT-qPCR) Validation

The expression pattern of differentially expressed genes (DEGs) in RNA-seq analysis was verified by RT-qPCR (34). Total RNA was extracted from liver tissues of the control group and sbGnRH peptides treated group using Trizol according to manufacturer's instructions (Invitrogen, CA, USA). Each group had 3 repetitions. According to the PrimeScript RT Master Mix Perfect Real -Time Kit (Takara, China) manufacturer's

instructions, each sample has the same amount of RNA for reverse transcription (35, 36). RT-qPCR was performed with the LightCycle 480 system (Roche, Basel, Switzerland) using SYBR Premix Ex Taq II (TaKaRa Bio Inc., Shiga, Japan). Primer pairs used in this study were shown in **Table 1**. The reference gene *β-actin* was used as an internal reference to normalize the mRNA level. The relative gene expression was calculated by $2^{-\Delta\Delta Ct}$ method.

2.5 Statistical Analysis

Data were expressed as the mean ± standard error (SE). All statistical tests were performed using Statistical Package for the Social Sciences (SPSS) 19.0 (SPSS, Chicago, IL, USA). Significant differences in the data among groups were tested by one-way analysis of variance (ANOVA), followed by Duncan's *post-hoc* test. The probability level lower than 0.05 ($P < 0.05$) indicated significance difference and lower than 0.01 ($P < 0.01$) is extremely significance.

3 RESULTS

3.1 Effects of sbGnRH Peptides on Liver Physiological and Biochemical Indexes *In Vivo*

The indexes of protein metabolism showed that there was no significant difference in the content of liver ALB in pompano injected with different concentrations of sbGnRH peptides ($P > 0.05$). Compared with the control group, the content of ALB in the treatment group increased (**Figure 1A**). In lipid metabolism, the content of CHO in the treatment group decreased significantly ($P < 0.05$), the content of HDLC increased significantly ($P < 0.05$), and the content of LDLC increased, but there was no significant difference between the treatment group and the control group ($P > 0.05$) (**Figure 1B**). In terms of energy metabolism, the contents of GLUC and CK in the treatment group increased significantly ($P < 0.05$) (**Figure 1C**). There was no significant difference in the contents of IRON, MG, CA and PHOS in the inorganic components ($P > 0.05$), but the contents of IRON, MG and PHOS increased and CA decreased in the treatment group (**Figure 1D**). The content of ALP in the liver function index increased, which had no significant difference with the control group ($P > 0.05$); The contents of AST and ALT were significantly higher than those in the control group ($P < 0.05$) (**Figure 1E**). The content of CREA in the renal function index increased significantly compared with the control group ($P < 0.05$) (**Figure 1F**).

TABLE 1 | Primer sequences used in RT-real time quantitative PCR (RT-qPCR).

Gene	Primer name	Primer sequence (5′ -3′)	Purpose
β-actin	*β-actin*-F	GAGAGGTTCCGTTGCCCAGAG	Reference gene
	β-actin-R	CAGACAGCACAGTGTTGGCGT	Reference gene
vtg	*vtg*-F	CTGTGCTGATGGTGCTCTGTTGA	qPCR
	vtg-R	CAACAGAGCACCATCAGCACAGA	qPCR
gpr1	*gpr1*-F	GTGGTTGCTCAATCTTGCGATGG	qPCR
	gpr1-R	ATAATGTGTATCATGGCTGCTGTATGC	qPCR
egr1	*egr1*-F	AGAAGCCAGTGGTGGAGCAGAC	qPCR
	egr1-R	TGAGGAAGAGGTAGAAGAGGAAGAAGTG	qPCR
rbm34	*rbm34*-F	GAAGAAGAGGAAGGCGTCAGAGTTG	qPCR
	rbm34-R	GTCACTCGGTCCACTCGGATGT	qPCR
tep1	*tep1*-F	GGAATGTGAGAAGGAGGAGGAGAAGA	qPCR
	tep1-R	GGCTGAGATGACGGTGCTGTTG	qPCR
bcl9l	*bcl9l*-F	TTCTCTGGAGGACAGGTGGAAGG	qPCR
	bcl9l-R	GATGAGGAGGAGGCACTGAAGGA	qPCR

3.2 Effects of sbGnRH Peptides on Liver Digestive and Metabolic Enzyme Activities *In Vivo*

After injection of sbGnRH peptides at the concentration of 10 ng/gbw for 3 h, the comparison results of digestive enzyme activities showed that the activities of AMS and LPS in the liver decreased, and there was no significant difference with the control group ($P > 0.05$) (**Figure 2A**). In terms of enzymes related to carbohydrate metabolism, the activities of PK and ACO decreased, but there was a significant difference in ACO compared with the control group ($P < 0.05$) (**Figure 2B**). In terms of antioxidant defense, GSH-PX activity decreased, and there was no significant difference with the control group ($P > 0.05$); SOD ($P < 0.05$) and CAT ($P < 0.01$) activities decreased significantly (**Figure 2C**). In terms of lipid metabolism, LPL activity decreased ($P > 0.05$), G-6-PD and FAS activities decreased significantly compared with the control group ($P < 0.05$), while HL activity increased ($P > 0.05$), CACT ($P < 0.05$), ME and ACC ($P < 0.01$) activities increased significantly compared with the control group (**Figure 2D**). In addition, a significant increase in MDA content reflecting oil rancidity was observed ($P < 0.05$) (**Figure 2E**).

3.3 Sequencing and Assembly Results of the Liver Transcriptome of Pompano

Based on Sequencing by Synthesis (SBS) technology, the liver tissue of pompano was sequenced 3 hours after *in vivo* injection of 10 ng/gbw (treatment group) sbGnRH peptides using Illumina hiseq high-throughput sequencing platform. Raw reads were obtained from C-1, C-2, C-3, T-1, T-2, and T-3. After quality control and removal of some low-quality data, clean reads of 21,283,647、19,427,359、21,873,990、21,732,174、23,660,062 and 21,592,338 were obtained respectively. The sequencing quality results show that the Q20 value (percentage of sequences with a sequencing error rate less than 1%) of each group of samples exceeds 97%, the Q30 value (percentage of sequences with a sequencing error rate less than 0.1%) exceeds 92%, and the GC content was about 46.92% - 47.80% (**Table 2**).

3.4 Gene Function Annotation

UniGene sequences were compared with COG, GO, KEGG, KOG, Pfam, Swiss-Prot, eggNOG and NR databases to obtain the annotation information of UniGene (**Table 3**). 25,715 Unigene with annotation information were obtained. Among the results of all database alignment genes, 4527 (17.60%) were

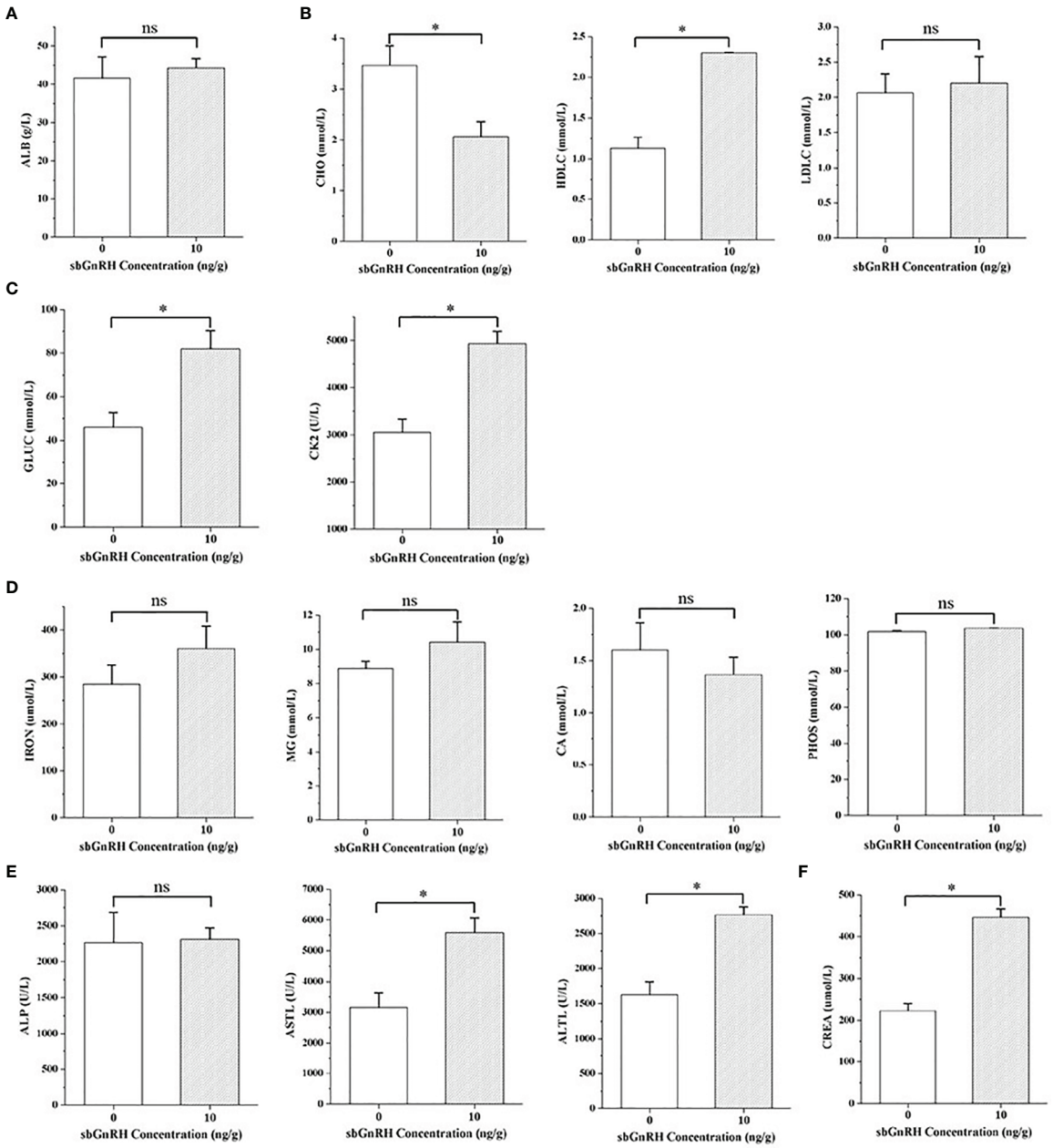


FIGURE 1 | Comparative evaluation of physiological and biochemical indexes in the liver of pompano. **(A)** protein metabolism; **(B)** lipid metabolism; **(C)** energy metabolism; **(D)** inorganic component; **(E)** liver function; **(F)** renal function. Data are expressed as mean ± standard error (SE) (n = 3), and the statistical significance (compared with the control group) was calculated using one-way analysis of variance (ANOVA), followed by Duncan's *post hoc* test. * indicate statistical differences at $P < 0.05$, respectively. ns, not significant ($P > 0.05$).

annotated in the COG database, 22280 (86.64%) were annotated in the GO database, 20570 (79.99%) were annotated in the KEGG database, 15706 (61.08%) in KOG database, 17050 (66.30%) in Pfam database, 11992 (46.63%) in Swiss-Prot database, 21702 (84.39%) in eggNOG database and 24797 (96.43%) in NR database.

UniGene sequences were aligned into the NR database. The species with the largest number were *Seriola dumerili* (7274, 29.33%), followed by *Seriola lalandi* (4534, 18.28%), *Lates calcifer* (2550, 10.28%), *Danio rerio* (1401, 5.65%), *Echeneis naurates* (812, 3.27%), *Scophthalmus maximus* (473, 1.91%), *Larimichthys crocea* (419, 1.69%), *Morone saxatilis* (300, 1.21%), *Epilephelus lanceolatus* (257, 1.04%), *Stegastes partitus* (231, 0.93%). This indicates that the species with the closest genetic relationship compared with the liver transcriptome sequencing of pompano is *Seriola dumerili* (**Figure 3**).

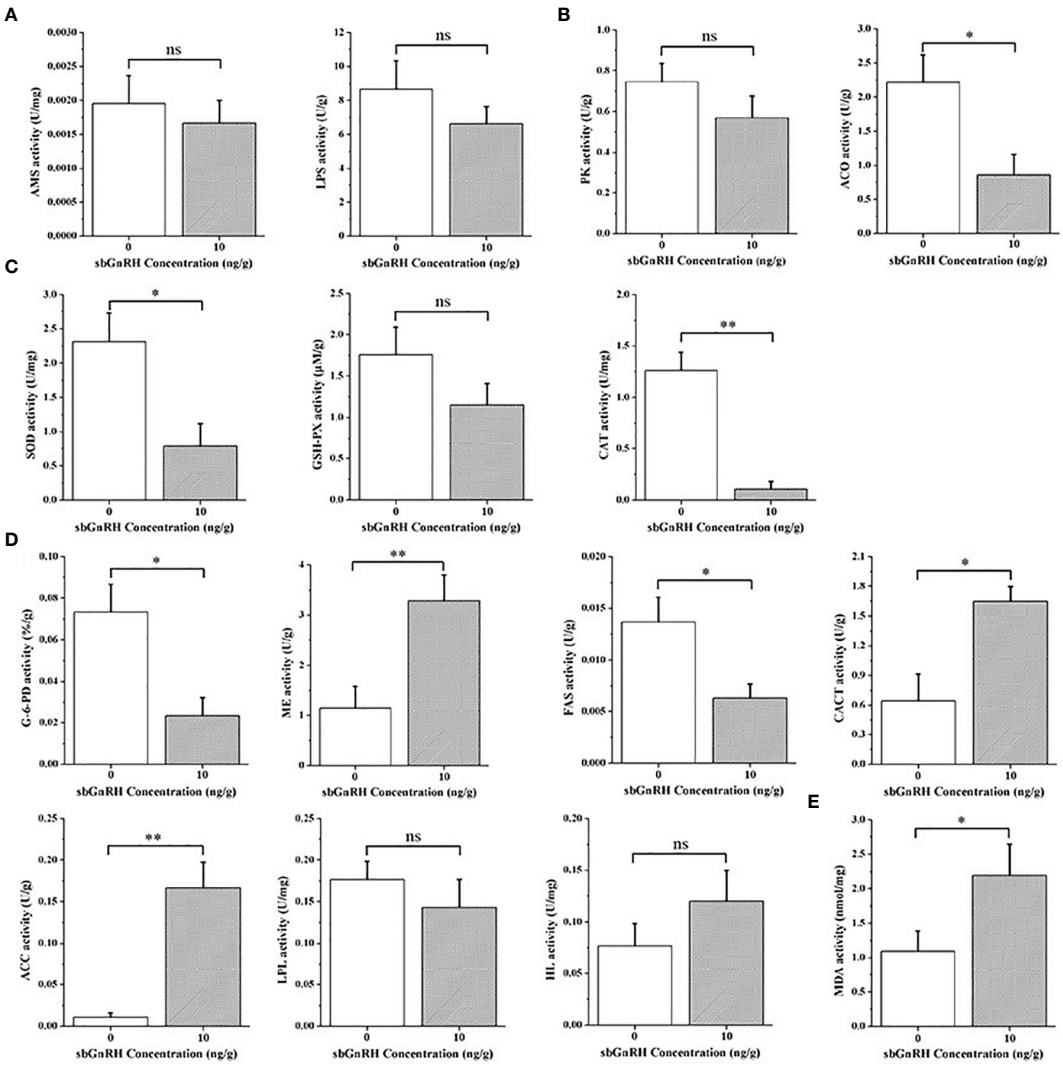


FIGURE 2 | Comparative evaluation of related liver enzyme activities between control group and treatment group. **(A)** digestion; **(B)** carbohydrate metabolism-related enzymes; **(C)** antioxidant defense; **(D)** lipid metabolism; **(E)** Membrane lipid peroxidation. Data are expressed as mean ± standard error (SEM) (n = 3), and the statistical significance (compared with the control group) was calculated using one-way analysis of variance (ANOVA), followed by Duncan's *post hoc* test. * and ** indicate statistical differences at $P < 0.05$ and $P < 0.01$, respectively. ns, not significant ($P > 0.05$).

TABLE 2 | Summary of transcriptome sequencing data of liver in pompano.

Sample	Clean Reads	Clean ReadsQ20 (%)	Clean ReadsQ30 (%)	GC Content (%)
C-1	21,283,647	97.88	94.02	46.92
C-2	19,427,359	98.00	94.29	47.60
C-3	21,873,990	97.66	93.50	47.70
T-1	21,732,174	97.33	92.95	47.36
T-2	23,660,062	98.22	94.78	47.80
T-3	21,592,338	98.05	94.40	47.39

TABLE 3 | Statistics of database annotation information.

Database	COG	GO	KEGG	KOG	Pfam	Swiss-Prot	eggNOG	NR
Numbers	4527	22280	20570	15706	17050	11992	21702	24797
Ratio	17.60%	86.64%	79.99%	61.08%	66.30%	46.63%	84.39%	96.43%

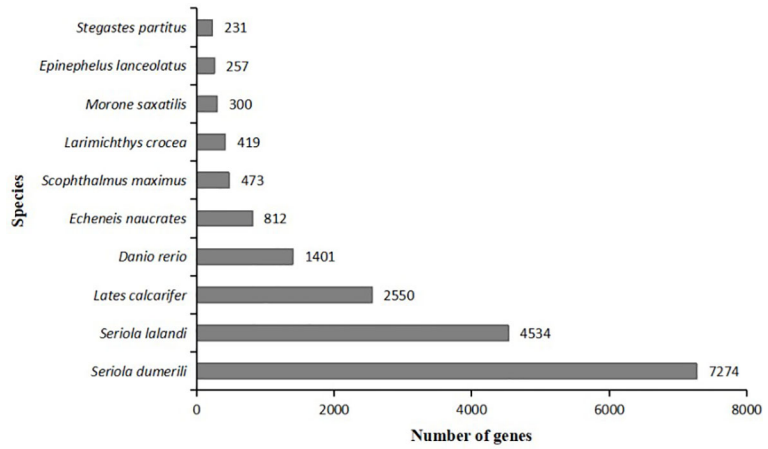


FIGURE 3 | Comparative distribution of NR homologous species. The Y-coordinate represents species; the X-coordinate represents the number of genes.

3.5 Analysis of Differentially Expressed Genes

In this study, 99 genes were screened and identified as differentially expressed genes, including 77 up-regulated genes and 22 down-regulated genes (**Figure 4A**). Hierarchical cluster analysis was performed on the screened differentially expressed genes. The results showed that the biological repeat individuals of liver tissue after treatment with different concentrations of sbGnRH peptides could gather respectively, indicating that the repeatability of the sample was more reliable (**Figure 4B**). Based on the expression amount of genes in different samples, the identified differentially expressed genes were annotated. The number of differentially expressed genes annotated was 88, including 21 in COG database, 74 in GO database, 74 in KEGG database, 50 in KOG database, 72 in Pfam database and 56 in Swiss-Prot database, eggNOG database annotation to 76, NR database annotation to 86 (**Table 4**).

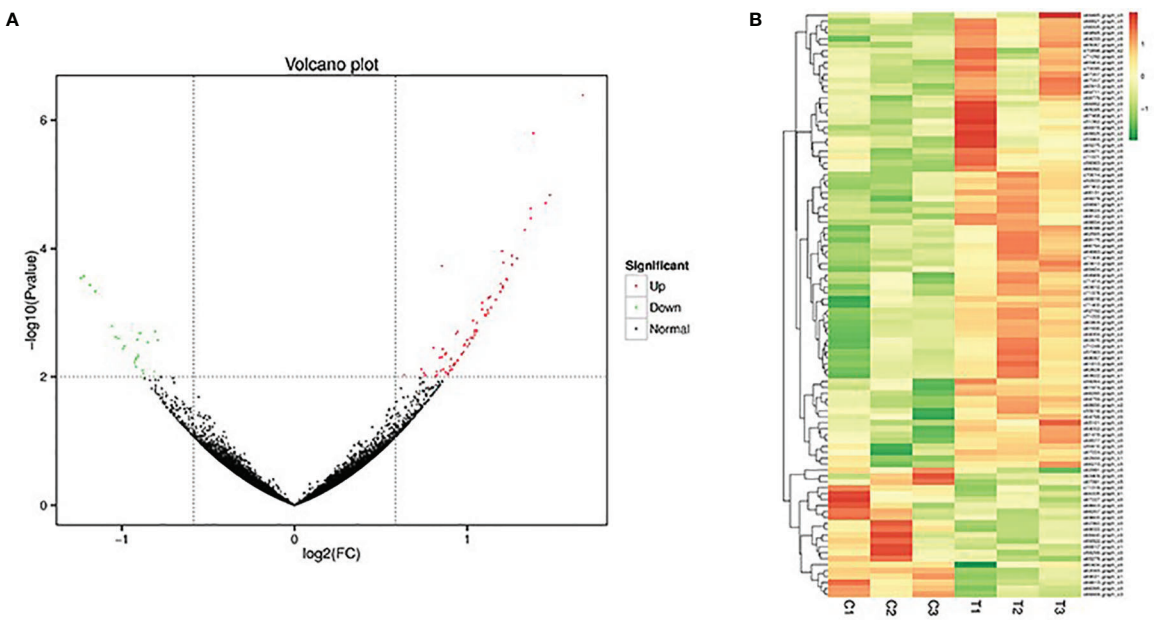


FIGURE 4 | Differential gene expression in the liver of pompano between control group and treatment group. **(A)** Volcano plot of the differences in gene expression. Each dot represents a gene. The green and red dots in the figure represent genes with significant expression differences, green represents down-regulation of gene expression, red represents up-regulation of gene expression, and black dots represent genes with no significant expression differences. **(B)** Heatmap of the hierarchical cluster of DEGs for illustrating the overall pattern of gene expression among different liver samples.

TABLE 4 | Number statistics of differentially expressed genes annotated.

DEG Set	Annotated	COG	GO	KEGG	KOG	Pfam	Swiss-Prot	eggNOG	NR
Numbers	88	21	74	74	50	72	56	76	86

3.6 The Enriched GO Terms and KEGG Pathways

According to GO enrichment analysis, DEGs are divided into three main functional categories: cellular component (CC), molecular function (MF) and biological process (BP), including 15, 12 and 18 subcategories respectively (**Figure 5**). Among them, the DEGs in the CC category were significantly enriched in cell (GO: 0005623), cell part (GO: 0044464) and organelle (GO: 0043226) GO terms. In the MF category, the DEGs were significantly enriched in catalytic activity (GO: 0003824) and binding (GO: 0005488) GO terms. The majority of DEGs in the BP category were associated with cellular processes (GO: 0009987), single-organism process (GO: 0044699) and biological regulation (GO: 0065007) GO terms.

The KEGG pathway enrichment analysis results of liver DEGs show that 41 pathways are enriched, of which the first 20 pathways with the most reliable enrichment significance (the smallest q-value) are shown in **Figure 6A**. These pathways and the typical genes involved can be divided into cellular processes, environmental information processing, genetic information processing, diseases, metabolism and organismal systems (**Figure 6B**). Among them, the following KEGG pathways related to disease immunity were significantly enriched: Dilated cardiomyopathy, Renal cell carcinoma, Salmonella infection and Pathways in cancer; the metabolism related KEGG pathway is significantly enriched: Fatty acid metabolism, Pyruvate metabolism, Glycosphingolipid biosynthesis - lacto and neolacto series, Fatty acid elongation, Carbon metabolism, Glyoxylate and dicarboxylate metabolism, Glycerophospholipid metabolism, Inositol phosphate metabolism, Ether lipid metabolism, Folate biosynthesis, Amino sugar and nucleotide sugar metabolism, Glycosaminoglycan biosynthesis - keratan sulfate, Oxidative phosphorylation and Retinol metabolism (**Figure 6B**). These functional classifications provide a theoretical basis for revealing the effects of sbGnRH on liver diseases, immune and metabolic functions of pompano.

3.7 Validation of RNA-Seq Data With qRT-PCR

In this experiment, six genes (*vtg*, *gpr1*, *egr1*, *tep1*, *bcl9l* and *rbm34*) were randomly screened for qPCR verification, in order to verify the accuracy of transcriptome sequencing results. The results showed that the expression of *vtg*, *gpr1* and *egr1* genes was

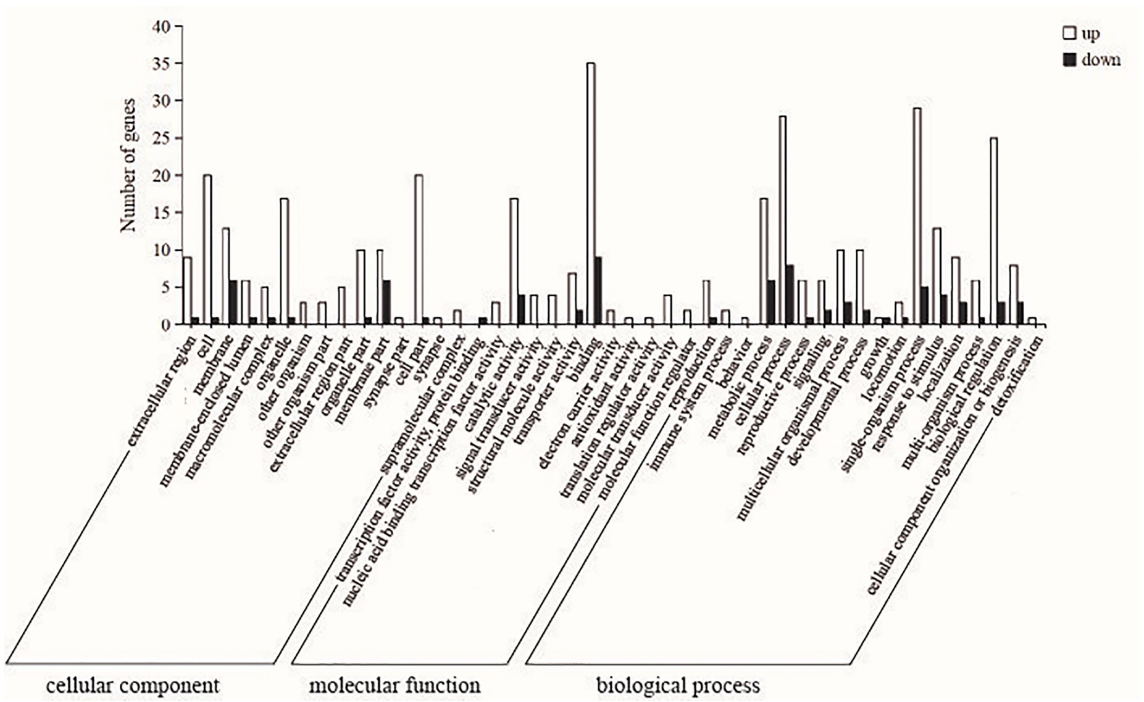


FIGURE 5 | Enrichment analysis of liver differentially expressed genes (DEGs) gene ontology (GO) between control and sbGnRH injection groups. White bars indicate up-regulated genes; Black bars indicate down-regulated genes; the Y-coordinate represents the number of genes; the X-coordinate represents the name of the pathway.

FIGURE 6 | The top 20 significantly enriched KEGG pathways of differentially expressed genes (DEGs). **(A)** The pathways and rich factor are shown in the vertical and the horizontal axis, respectively. The dot size indicates the number of genes and the color indicates the q-value. **(B)** Classification of differentially expressed genes KEGG. The vertical axis is the name of KEGG metabolic pathway, the left part is the specific pathway name, and the right part is the classification category corresponding to each pathway. And the horizontal axis is the Annotated Genes. The number on the column is the number of differentially expressed genes related to this pathway. The same column color represents the same category.

up-regulated and the expression of *tep1*, *bcl9l* and *rbm34* genes was down-regulated after sbGnRH peptides treatment. The qPCR results were consistent with the sequencing results, indicating the accuracy and specificity of transcriptome analysis (**Figure 7**).

4 DISCUSSION

Fish biochemical indexes can judge adaptation and nutritional information (37). The protein content in fish is related to metabolism, energy consumption and immune strength. When

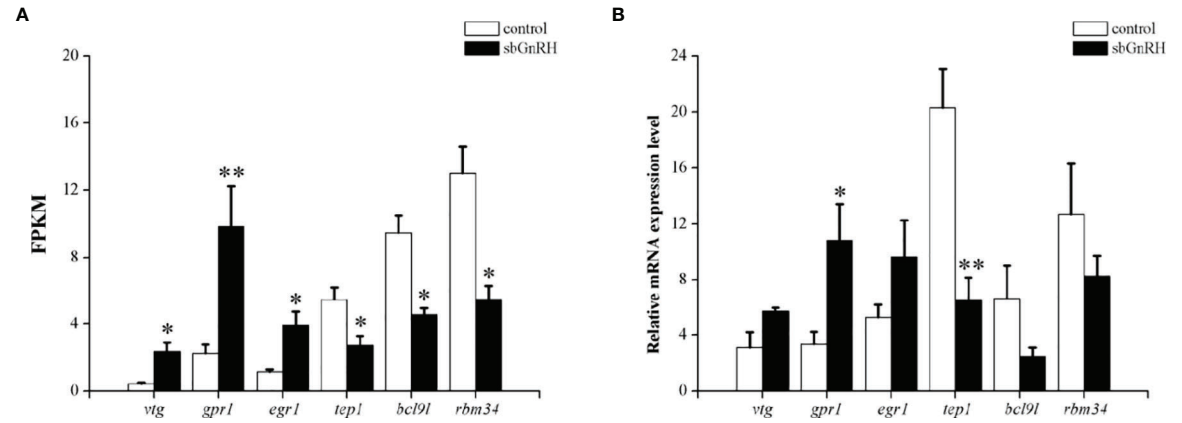


FIGURE 7 | Expression of the gene in the liver of pompano in control and sbGnRH injection groups. **(A)** Transcriptome data (n=3) and **(B)** quantitative polymerase chain reaction (qPCR) results (n=3). Data are presented as mean ± standard error (SEM). * and ** indicate statistical differences at $P < 0.05$ and $P < 0.01$, respectively. The statistical significance (compared with the control group) was calculated using one-way analysis of variance (ANOVA), followed by Duncan's *post hoc* test.

subjected to external stress, metabolism is strengthened and energy consumption is accelerated, resulting in the decrease of protein content and immune ability (38). After the appropriate concentration of sbGnRH peptides was injected *in vivo*, the ALB content in the liver of pompano increased, and there was no significant difference between the groups. The increase of ALB content may affect the enhancement of the immune level. The relative stability of total protein content is the performance of self-regulation of fish body, which is also related to the experiment, This is similar to the results of *Epinephelus coioides*, *Ctenopharyngodon idella*, *Cyprinus carpio* and *Mylopharyngodon piceus* (39, 40). Triglyceride, cholesterol, low-density lipoprotein and high-density lipoprotein are the four commonly used blood lipid indexes. Any of the first three exceeding the standard belongs to hyperlipidemia, but the low content indicates malnutrition and slow growth, the results of these indices of pompano are consistent with those of *Acipenser schrencki* and *Epinephelus coioides* (41). Combined with transcriptome analysis, the significant increase of HDLC may be related to fatty acid metabolism and glycerol phospholipid metabolism. The expression of ZGC: 55413 protein gene and uncharacterized protein loc791752 isoform X1 gene involved in these metabolisms are up-regulated. Constant glucose concentration plays an important role in maintaining the normal life activities of fish. Usually, glycogen is the core energy storage, and the mode of glycogen utilization of fish carbohydrates is located in the liver and muscle, (42). Among the indicators of energy metabolism, the contents of GLUC and CK increased significantly, which may be related to the beta-1,4-galactosyltransferase 4 gene in glycosaminoglycan biosynthesis and glycosphingolipid biosynthesis and the malate synthase gene in carbon metabolism. After injection of sbGnRH peptides, its expression was up-regulated. AST and ALT are important aminotransferases, which are widely distributed in the cell membrane, cytoplasm and mitochondria. They are often used as factors to evaluate hepatopancreatic function (43). Under normal conditions, the activities of both enzymes are not high, but when tissue cells, especially liver and heart cells, are damaged, the activities of these enzymes increase significantly. Therefore, AST and ALT activities can reflect the damage of fish physiological function (44). The results showed that the contents of AST and ALT increased after injection of 10 ng/gbw sbGnRH peptides, suggesting that sbGnRH may be involved in the immune regulation of liver disease. Creatinine is mainly the final product of creatine and creatine phosphate metabolism and an indicator of gill and renal function. Therefore, creatinine content is closely related to muscle activity (45, 46). Creatinine excretion can reflect the function of the kidney. When kidney disease occurs, creatinine excretion is blocked and the content of creatinine in the blood increases significantly (47).

sbGnRH is widely distributed in the nervous, endocrine, reproductive, digestive and immune systems. By transmitting information, all systems are coordinated and unified, and sbGnRH in different tissues has different biological functions (9). Aquatic animals obtain nutrition and energy almost entirely through feeding, digestion and absorption. The absorption and utilization of nutrients strongly depend on the activity of digestive enzymes and make a positive contribution to the growth ability of fish (48). In this study, ME activity and ACC activity in lipid metabolizing enzymes were enhanced, further indicating that sbGnRH may participate in pyruvate metabolism, glyoxylate and dicarboxylate metabolism, resulting in the up regulation of malate synthase gene expression. It has been shown that the higher growth performance of male tilapia (*Oreochromis niloticus*) may be due to its strong ability to digest and metabolize nutrients (49). It has been well confirmed that exogenous steroid treatment has different effects on energy distribution patterns. For example, the change of androgen level in the reproductive season is related to the change of energy distribution (50); Elevated plasma testosterone levels cause changes in liver

metabolism, which may be related to the process of energy redistribution (51). Combined with the metabolic enzyme activity index and liver transcriptome analysis of pompano, sbGnRH may be involved in non-reproductive processes such asdigestion, lipid metabolism and antioxidant defense of pompano. However, the mechanism of GnRH in the digestive system and immune system is still a relatively unexplored research field. Our preliminary findings will pave the way for a more comprehensive understanding of this complex action system.

RNA-seq is an important method for quantitative transcriptional expression to clarify the response of environmental factors such as salinity, temperature, pH, dissolved oxygen and sex hormones in species or organisms (52–56). In this study, the mRNA function of liver response to sbGnRH injection was studied by detecting the liver RNA-seq of pompano injected with 10 ng/gbw concentrations of sbGnRH peptides. The changes of DEGs in the liver of sbGnRH injection group and control group were analyzed by transcriptome and qPCR. Three hours after injection of 10 ng/gbw sbGnRH peptides, we identified DEGs in pompano and detected its expression pattern. 99 DEGs were identified in the control group and sbGnRH treatment group. In this study, GO enrichment analysis showed that after injection of sbGnRH peptides into pompano, DEGs were significantly enriched in cells, cell parts, binding, metabolic processes, cellular processes and biological regulation. In teleosts, it has been identified to be related to the regulation of GnRH (57–59). The results of this study show that KEGG pathway related to disease immunity and metabolism is significantly enriched, suggesting that sbGnRH plays a non-reproductive related function in different tissues (60–62). In this study, sbGnRH injection significantly up-regulated genes related to reproductive regulation,early growth response protein 1, cytoplasmic polyadenylation element-binding protein 1 and ribosomal protein S6 kinase alpha-3 isoform X2. sbGnRH is involved in the regulation of oocyte meiosis, progesterone mediated oocyte maturation and GnRH signaling pathway. Therefore, it is necessary to further study the reproductive and non-reproductive functions of sbGnRH in pompano.

5 CONCLUSIONS

In this study, transcriptome sequencing technology was used as a tool to explore the function of sbGnRH in regulating physiological and biochemical indexes and metabolic enzyme activities in the liver of pompano. The results show that sbGnRH is involved in the immune regulation of liver disease and the regulation of digestive and metabolic enzyme activities, suggesting that sbGnRH has non reproductive related functions. sbGnRH may play different biological functions in the immune system and digestive system, to maintain the homeostasis of the body. Therefore, we know that GnRH has many functions and can regulate many physiological processes. It not only plays an important role in the reproductive system, but also regulates the non-reproductive system. The data of this study are of great significance to further clarify the non-reproductive related function of sbGnRH.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. This data can be found here: NCBI: SRX14408583, SRX14408582, SRX14408581, SRX14408580, SRX14408579, SRX14408578.

ETHICS STATEMENT

The animal study was reviewed and approved by the Animal Research and Ethics Committees of Fisheries College of Guangdong Ocean University. Written informed consent was obtained from the owners for the participation of their animals in this study.

AUTHOR CONTRIBUTIONS

XR: Investigation, Data curation, Formal analysis, Writing–original draft. JL: Data curation, Formal analysis, Resources. XL: Data curation. CN: Investigation. YG: Investigation, Methodology. GL: Investigation. HC: Data curation, Funding acquisition, Methodology, Resources, Supervision, Writing–original draft, Writing - re-view and editing. All authors contributed to the article and approved the submitted version.

FUNDING

This article was supported by grants from the Key Research and Development Program of Guangdong (2021B202020002), National Natural Science Foundation of China (41706174), The talent team tender grant of Zhanjiang marine equipment and biology (2021E05035), the Guangdong Basic and Applied Basic Research Foundation (2019A1515010958 and 2019A1515012042), the Southern Marine Science and Engineering Guangdong Laboratory (Zhanjiang) (ZJW-2019-06).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.869021/full#supplementary-material

REFERENCES

1. Gorbman A, Sower SA. Evolution of the Role of GnRH in Animal (Metazoan) Biology. *Gen Comp Endocrinol* (2003) 134(3):207–13. doi: 10.1016/j.ygcen.2003.09.018
2. Andreuvieyra CV, Habibi HR. Factors Controlling Ovarian Apoptosis. *Can J Physiol Pharmacol* (2000) 78(12):1003–12. doi: 10.1139/cjpp-78-12-1003
3. Andreuvieyra CV, Buret AG, Habibi HR. Gonadotropin-Releasing Hormone Induction of Apoptosis in the Testes of Goldfish (*Carassius Auratus*). *Endocrinology* (2005) 146(3):1588–96. doi: 10.1210/en.2004-0818
4. Soverchia L, Carotti M, Andreu-Vieyra C, Mosconi G, Cannella N, Habibi H, et al. Role of Gonadotropin-Releasing Hormone (GnRH) in the Regulation of Gonadal Differentiation in the Gilthead Seabream (*Sparus Aurata*). *Mol Reprod Dev* (2007) 74(1):57–67. doi: 10.1002/mrd.20484
5. Leung PC, Steele GL. Intracellular Signaling in the Gonads. *Endocr Rev* (1992) 13(3):476–98. doi: 10.1210/edrv-13-3-476
6. Nabissi M, Soverchia L, Polzonettimagni AM, Habibi HR. Differential Splicing of Three Gonadotropin-Releasing Hormone Transcripts in the Ovary of Seabream (*Sparus Aurata*). *Biol Reprod* (2000) 62(5):1329–34. doi: 10.1095/biolreprod62.5.1329
7. Asa SL, Ezzat S. The Pathogenesis of Pituitary Tumours. *Nat Rev Cancer* (2002) 2(11):836–49. doi: 10.1038/nrc926
8. Fan WJ, Li CJ, Chen L, Zhou X. Advances on Research and Application of Gonadotropin-Releasing Hormone (GnRH). *Heilongjiang J Anim Reprod* (2020) 28(4):1005–2739. doi: 10.19848/j.cnki
9. Ye D, Pan JW, Liao MJ, Zhang ZH, Zhu MY. The Structure and Biological Functions of Gonadotropin-Releasing Hormone (GnRH). *Prog Biochem Biophys* (2003) 30(1):49–53. doi: 10.3321/j.issn:1000-3282.2003.01.010
10. Ren XL, Huang YL, Li XM, Li ZY, Yang H, He RQ, et al. Identification and Functional Characterization of Gonadotropin -Releasing Hormone in Pompano (*Trachinotus Ovatus*). *Gen Comp Endocrinol* (2022) 316:113958. doi: 10.1016/j.ygcen.2021.113958
11. Anderson E, Fjelldal PG, Klenke U, Vikingstad E, Taranger GL, Zohar Y, et al. Three Forms of GnRH in the Brain and Pituitary of the Turbot, *Scophthalmus Maximus*: Immunological Characterization and Seasonal Variation. *Comp Biochem Physiol Part B* (2001) 129:551–8. doi: 10.1016/S1096-4959(01)00363-3
12. Uzbekova S, Lareyre JJ, Madigou T, Davail B, Jalabert B, Breton B. Expression of Prepro-GnRH and GnRH Receptor Messengers in Rainbow Trout Ovary Depends on the Stage of Ovarian Follicular Development. *Mol Reprod Dev* (2002) 62:47–56. doi: 10.1002/mrd.10065
13. Eisthen HL, Delay RJ, Wirsig-Wiechmann CR, Dionne VE. Neuromodulatory Effects of Gonadotropin Releasing Hormone on Olfactory Receptor Neurons. *J Neurosci* (2000) 20(11):3947–55. doi: 10.1523/JNEUROSCI.20-11-03947.2000
14. Kawai T, Abe H, Akazome Y, Oka Y. Neuromodulatory Effect of GnRH on the Synaptic Transmission of the Olfactory Bulbar Neural Circuit in Goldfish, Carassius Auratus. *J Neurophysiol* (2010) 104(6):3540–50. doi: 10.1152/jn.00639.2010
15. Fu BD, Wang XH, Feng X, Yu XM, Tong JG. Comparative Transcriptomic Analyses of Two Bighead Carp (*Hypophthalmichthys Nobilis*) Groups With Different Growth Rates. *Comp Biochem Physiol Part D Genomics Proteomics* (2016) 20:111–7. doi: 10.1016/j.cbd.2016.08.006
16. Lowry OH, Rosebrough NJ, Farr AL, Randall RJ. Protein Measurement With the Folin Phenol Reagent. *J Biol Chem* (1951) 193(1):265–75. doi: 10.1016/S0021-9258(19)52451-6
17. Chen HP, Jiang DN, Li ZY, Wang YR, Yang XW, Li SF, et al. Comparative Physiological and Transcriptomic Profiling Offers Insight Into the Sexual Dimorphism of Hepatic Metabolism in Size-Dimorphic Spotted Scat (*Scatophagus Argus*). *Life* (2021) 11(6):589. doi: 10.3390/life11060589
18. Grabherr MG, Haas BJ, Yassour M, Levin JZ, Thompson DA, Amit I, et al. Full Length Transcriptome Assembly From RNA-Seq Data Without a Reference Genome. *Nat Biotechnol* (2011) 29(7):644–52. doi: 10.1038/nbt.1883
19. Deng YY, Li JQ, Wu SF, Zhu YP, Chen YW, He FC. Integrated Nr Database in Protein Annotation System and its Localization. *Comput Eng* (2006) 32(5):71–4. doi: 10.1109/INFOCOM.2006.241
20. Apweiler R, Bairoch A, Wu CH, Barker WC, Boeckmann B, Ferro S, et al. UniProt: The Universal Protein Knowledgebase. *Nucleic Acids Res* (2004) 32 (Database issue):D115 –9. doi: 10.1093/nar/gkh131
21. Tatusov RL, Galperin MY, Natale DA, Koonin EV. The COG Database: A Tool for Genome Scale Analysis of Protein Functions and Evolution. *Nucleic Acids Res* (2000) 28(1):33–6. doi: 10.1093/nar/28.1.33
22. Koonin EV, Fedorova ND, Jackson JD, Jacobs AR, Krylov DM, Makarova KS, et al. A Comprehensive Evolutionary Classification of Proteins Encoded in Complete Eukaryotic Genomes. *Genome Biol* (2004) 5(2):R7. doi: 10.1186/gb-2004-5-2-r7
23. Huerta-Cepas J, Szklarczyk D, Forslund K, Cook H, Heller D, Walter MC, et al. eggNOG 4.5: A Hierarchical Orthology Framework With Improved Functional Annotations for Eukaryotic, Prokaryotic and Viral Sequences. *Nucleic Acids Res* (2016) 44(1):286–93. doi: 10.1093/nar/gkv1248
24. Kanehisa M, Goto S, Kawashima S, Okuno Y, Hattori M. The KEGG Resource for Deciphering the Genome. *Nucleic Acids Res* (2004) 32(Database issue):277–80. doi: 10.1093/nar/gkh063
25. Buchfink B, Xie C, Huson DH. Fast and Sensitive Protein Alignment Using DIAMOND. *Nat Methods* (2015) 12(1):59–60. doi: 10.1038/nmeth.3176
26. Xie C, Mao XZ, Huang JJ, Ding Y, Wu JM, Dong S, et al. KOBAS 2.0: A Web Server for Annotation and Identification of Enriched Pathways and Diseases. *Nucleic Acids Res* (2011) 39:316–22. doi: 10.1093/nar/gkr483
27. Jones P, Binns D, Chang HY, Fraser M, Li WZ, McAnulla C, et al. InterProScan 5: Genome-Scale Protein Function Classification. *Bioinformatics* (2014) 30(9):1236–40. doi: 10.1093/bioinformatics/btu031
28. Ashburner M, Ball CA, Blake JA, Botstein D, Butler H, Cherry JM, et al. Gene Ontology: Tool for the Unification of Biology. *Nat Genet* (2000) 25(1):25–9. doi: 10.1038/75556
29. Eddy SR. Profile Hidden Markov Models. *Bioinformatics* (1998) 14(9):755–63. doi: 10.1093/bioinformatics/14.9.755
30. Finn RD, Bateman A, Clements J, Coggill P, Eberhardt RY, Eddy SR, et al. Pfam: The Protein Families Database. *Nucleic Acids Res* (2014) 42(1):222–30. doi: 10.1093/nar/gkt1223
31. Langmead B, Trapnell C, Pop M, Salzberg SL. Ultrafast and Memory-Efficient Alignment of Short DNA Sequences to the Human Genome. *Genome Biol* (2009) 10(3):R25. doi: 10.1186/gb-2009-10-3-r25
32. Li B, Colin ND. RSEM: Accurate Transcript Quantification From RNA-Seq Data With or Without a Reference Genome. *BMC Bioinform* (2011) 12:323. doi: 10.1186/1471-2105-12-323
33. Love MI, Huber W, Anders S. Moderated Estimation of Fold Change and Dispersion for RNA-Seq Data With Deseq2. *Genome Biol* (2014) 15(12):550. doi: 10.1186/s13059-014-0550-8
34. Ru XY, Shi HJ, Wang T, Liu QQ, Jiang DN, Peng YH, et al. Effects of 17beta-Estradiol on Growth-Related Genes Expression in Female and Male Spotted Scat (*Scatophagus Argus*). *Comp Biochem Physiol Part B Biochem Mol Biol* (2020) 250:110492. doi: 10.1016/j.cbpb.2020.110492
35. Jiang DN, Mustapha UF, Shi HJ, Huang YQ, Si-Tu JX, Wang M, et al. Expression and Transcriptional Regulation of *Gsdf* in Spotted Scat (*Scatophagus Argus*). *Comp Biochem Physiol Part B Biochem Mol Biol* (2019) 233:35–45. doi: 10.1016/j.cbpb.2019.04.002
36. Chen HP, Cui XF, Wang YR, Li ZY, Tian CX, Jiang DN, et al. Identification, Functional Characterization, and Estrogen Regulation on Gonadotropin-Releasing Hormone in the Spotted Scat, Scatophagus Argus. *Fish Physiol Biochem* (2020) 46(5):1–15. doi: 10.1007/s10695-020-00825-5
37. Chen JJ, Cao JL, Luo YJ. Effects of Starvation on Blood Physiological and Biochemical Indices in Clarias Fuscus. *J Anhui Agric Sci* (2009) 37(5):2014–5. doi: 10.13989/j.cnki.0517-6611.2009.05.131
38. Feng GN, Yang WP, Wang AM, Li J, Xu HF, Han GM, et al. Effects of Starvation Stress on Body Shape, Chemical Composition and Blood Physiological of Cyprinus Carpio. *J Shanghai Ocean Univ* (2011) 20(6):814–9. doi: 10.1016/S1671-2927(11)60313-1
39. Gui YM, Wu Y, Zhu GQ, Shen CG. Changes on Physiological and Biochemical Index for ChineseCarPsin Winter I - Blood Index and Energy Metabolism. *J Dalian Fish Coll* (1994) 9(3):15–27. doi: 10.16535/j.cnki.dlhyxb.1994.03.003
40. Chen J, Xiong BX, Gu QH, Huang J. The Effect of Endogenous and Environment Factors on Hematology Physiological and Biochemical Indices of Fish. *Hubei Agric Sci* (2011) 50(9):1861–5. doi: 10.14088/j.cnki.issn0439-8114.2011.09.062
41. Chen WQ, Liu ZM, Wu L, Wang YH, Ma JZ, Wu HX. The Influences of Light Color on Growth, Haematological and Biochemical Indices of Juvenile

Plectropomus Leopardus. *Chin J Ecol* (2016) 35(7):1889–95. doi: 10.13292/j.1000-4890.201607.002
42. Du ZY, Liu YJ, Tian LX, Cao JM, Liang GY, He JG. Effects of Starvation on Visceral Weight and Main Biochemical Composition of the Muscle, Liver and Serum in the Japanese Sea Bass (*Lateolabrax Japonicus*). *Acta Zool Sin* (2003) 49(4):458–65. doi: 10.369/j.iss.1674-5507.2003.01.006
43. Lin L, Zeng XL, Zhang J. Effect of Profenofos Poisoning on Liver Lipid Peroxidation and Liver Function in Rabbits. *Chin J Tissue Eng Res* (2004) 8 (21):4380–1. doi: 10.3321/j.issn:1673-8225.2004.21.058
44. Du YS, Yi MM, Xiao P, Meng LJ, Li X, Sun GX, et al. The Impact of *Aeromonas Salmonicida*, Infection on Innate Immune Parameters of Atlantic Salmon (*Salmo Salar*, L). *Fish Shellfish Immunol* (2015) 44(1):307–15. doi: 10.1016/j.fsi.2015.02.029
45. Al-Salahy MB. Some Physiological Studies on the Effect of Onion and Garlic Juices on the Fish, Clarias Lazera. *Fish Physiol Biochem* (2002) 27(2):129–42. doi: 10.1023/B:FISH.0000021913.60189.76
46. Han NN, Shi CY. The Application of Blood Indexes in Ichthyological Research. *J Anhui Agric Sci* (2010) 38(33):18877–8. doi: 10.13989/j.cnki.0517-6611.2010.33.158
47. Patriche T, Patriche N, Bocioc E, Coada MT. Serum Biochemical Parameters of Farmed Carp (*Cyprinus Carpio*). *Aquaculture* (2011) 4(2):137–40.
48. Thongprajukaew K, Kovitvadhi U. Effects of Sex on Characteristics and Expression Levels of Digestive Enzymes in the Adult Guppy Poecilia Reticulata. *Zool Stud* (2013) 52(3):25–32. doi: 10.1186/1810-522X-52-3
49. Toguyeni A, Fauconneau B, Boujard T, Fostier A, Kuhn ER, A Mol K, et al. Feeding Behaviour and Food Utilisation in Tilapia, *Oreochromis Niloticus*: Effect of Sex Ratio and Relationship With the Endocrine Status. *Physiol Behav* (1997) 62(2):273–9. doi: 10.1016/S0031-9384(97)00114-5
50. Leonard JBK, Iwata M, Ueda H. Seasonal Changes of Hormones and Muscle Enzymes in Adult Lacustrine Masu (*Oncorhynchus Masou*) and Sockeye Salmon (O. Nerka). *Fish Physiol Biochem* (2001) 25(2):153–63. doi: 10.1023/A:1020512105096
51. Sangiao-Alvarellos S, Polakof S, Arjona FJ, García-López A, del Río MPM, Martínez-Rodríguez G, et al. Inflfluence of Testosterone Administration on Osmoregulation and Energy Metabolism of Gilthead Sea Bream *Sparus Auratus*. *Gen Comp Endocrinol* (2006) 149(1):30–41. doi: 10.1016/j.ygcen.2006.05.003
52. Liu SK, Wang XL, Sun FY, Zhang JR, Feng JB, Liu H, et al. RNA-Seq Reveals Expression Signatures of Genes Involved in Oxygen Transport, Protein Synthesis, Folding, and Degradation in Response to Heat Stress in Catfish. *Physiol Genomics* (2013) 45(12):462–76. doi: 10.1152/physiolgenomics.00026.2013
53. Cao DY, Li JF, Huang BS, Zhang JD, Pan CH, Huang JS, et al. RNA-Seq Analysis Reveals Divergent Adaptive Response to Hyper- and Hypo-Salinity in Cobia, Rachycentron Canadum. *Fish Physiol Biochem* (2020) 46(5):1–15. doi: 10.1007/s10695-020-00823-7
54. Saetan W, Tian CX, Yu JW, Lin XH, He FX, Huang Y, et al. Comparative Transcriptome Analysis of Gill Tissue in Response to Hypoxia in Silver Sillago (*Sillago Sihama*). *Animals* (2020) 10(4):628. doi: 10.3390/ani10040628
55. Tian CX, Lin XH, Saetan W, Huang Y, Shi HJ, Jiang DN, et al. Transcriptome Analysis of Liver Provides Insight Into Metabolic and Translation Changes Under Hypoxia and Reoxygenation Stress in Silver Sillago (*Sillago Sihama*). *Comp Biochem Physiol Part D Genomics Proteomics* (2020) 36:100715. doi: 10.1016/j.cbd.2020.100715
56. Shi HJ, Ru XY, Pan SH, Jiang DN, Huang Y, Zhu CH, et al. Transcriptomic Analysis of Pituitary in Female and Male Spotted Scat (*Scatophagus Argus*) After 17β-Estradiol Injection. *Comp Biochem Physiol Part D Genomics Proteomics* (2021) 41:100949. doi: 10.1016/j.cbd.2021.100949
57. Kirk CJ, Bottomley L, Minican N, Carpenter H, Shaw S, Kohli N, et al. Environmental Endocrine Disrupters Dysregulate Estrogen Metabolism and Ca^{2+} Homeostasis in Fish and Mammals *via* Receptor-Independent Mechanisms. *Comp Biochem Physiol Part A Mol Integr Physiol* (2003) 135 (1):1–8. doi: 10.1016/S1095-6433(02)00366-5
58. Zhang DP, Popesku JT, Martyniuk CJ, Xiong HL, Duarte-Guterman P, Yao LH, et al. Profiling Neuroendocrine Gene Expression Changes Following Fadrozole-Induced Estrogen Decline in the Female Goldfish. *Physiol Genomics* (2009) 38(3):351–61. doi: 10.1152/physiolgenomics.00051.2009
59. Martyniuk CJ, Kroll KJ, Doperalski NJ, Barber DS, Denslow ND. Environmentally Relevant Exposure to 17α-Ethinylestradiol Affects the Telencephalic Proteome of Male Fathead Minnows. *Aquat Toxicol* (2010) 98(4):344–53. doi: 10.1016/j.aquatox.2010.03.007
60. Hong Y, Kwai WC, Hsiao-Lin H, Chun P, Nelly A, Peter CK. Leung, Expression of the Messenger RNA for Gonadotropin-Releasing Hormone and its Receptor. *Life Sci* (1998) 62(22):2015–23. doi: 10.1016/S0024-3205(98)00173-8
61. Huang WQ, Yao B, Sun L, Pu RL, Wang L, Zhang RQ. Immunohistochemical and *in Situ* Hybridization Studies of Gonadotropin Releasing Hormone (GnRH) and its Receptor in Rat Digestive Tract. *Life Sci* (2001) 68 (15):1727–34. doi: 10.1016/S0024-3205(01)00968-7
62. Siler-Khodr T, Marcia Grayson MA. Salmon GnRH and its Analogues Bind the Human Placental Receptor. *J Soc Gynecol Invest* (2001) 8(4):233–8. doi: 10.1177/107155760100800408

Conflict of Interest: Author JL is employed by Guangdong Havwii agricultural Group Co., LTD.

The remaining 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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Ren, Liu, Ndandala, Li, Guo, Li and Chen. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 15 June 2022
doi: 10.3389/fendo.2022.883608

New Evidence for the Existence of Two Kiss/Kissr Systems in a Flatfish Species, the Turbot (*Scophthalmus maximus*), and Stimulatory Effects on Gonadotropin Gene Expression

Chunyan Zhao[1,2,3], *Bin Wang*[4], *Yifan Liu*[2,3,5], *Chengcheng Feng*[2,3], *Shihong Xu*[2,3], *Wenqi Wang*[1], *Qinghua Liu*[2,3] *and Jun Li*[2,3,6]*

[1] School of Marine Science and Engineering, Qingdao Agricultural University, Qingdao, China, [2] The Key Laboratory of Experimental Marine Biology, Centre for Ocean Mega-Science, Institute of Oceanology, Chinese Academy of Sciences, Qingdao, China, [3] Laboratory for Marine Biology and Biotechnology, Pilot National Laboratory for Marine Science and Technology, Qingdao, China, [4] Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture and Rural Affairs, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China, [5] National Engineering Research Center for Marine Aquaculture, Zhejiang Ocean University, Zhoushan, China, [6] Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou, China

OPEN ACCESS

Edited by:
Yong Zhu,
East Carolina University, United States

Reviewed by:
Agata Żmijewska,
University of Warmia and Mazury in Olsztyn, Poland
Hironori Ando,
Niigata University, Japan
Satoshi Ogawa,
Monash University Malaysia, Malaysia

***Correspondence:**
Jun Li
junli@qdio.ac.cn

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

Received: *25 February 2022*
Accepted: *10 May 2022*
Published: *15 June 2022*

Citation:
Zhao C, Wang B, Liu Y, Feng C, Xu S, Wang W, Liu Q and Li J (2022) New Evidence for the Existence of Two Kiss/Kissr Systems in a Flatfish Species, the Turbot (Scophthalmus maximus), and Stimulatory Effects on Gonadotropin Gene Expression. Front. Endocrinol. 13:883608. doi: 10.3389/fendo.2022.883608

Seasonal reproduction is generally controlled by the hypothalamus-pituitary-gonadal (HPG) axis in fish. Previous studies have demonstrated that the kisspeptin (Kiss)/kisspeptin receptor (Kissr) system, a positive regulator of the HPG axis, mediates the responses to environmental cues. Turbot (*Scophthalmus maximus*), a representative species of Pleuronectiformes, is one of the most commercially important fish species cultured in Europe and North China. However, the mechanisms by which the Kiss/Kissr system regulates the reproductive axis of turbot according to seasonal changes, especially photoperiod, have not been clearly characterized. In the current study, the cDNA sequences of *kiss2*/*kissr2*, along with *kiss1*/*kissr3* which was thought to be lost in flatfish species, were cloned and functionally characterized. The *kiss1*, *kiss2*, and *kissr3* transcripts were highly detected in the brain and gonad, while *kissr2* mRNA was only abundantly expressed in the brain. Moreover, *kiss*/*kissr* mRNAs were further examined in various brain areas of both sexes. The *kiss1*, *kissr2*, *kissr3* mRNAs were highly expressed in the mesencephalon, while a substantial degree of *kiss2* transcripts were observed in the hypothalamus. During annual reproductive cycle, both *kiss* and *kissr* transcript levels declined significantly from the immature to mature stages and increased at the degeneration stage in the brains of both sexes, especially in the mesencephalon and hypothalamus. The ovarian *kiss1*, *kiss2*, and *kissr2* mRNA levels were highest at the vitellogenic stage (mature stage), while expression of *kissr3* was highest at the immature stage. The testicular *kiss* and *kissr* transcripts were highest in the immature and degeneration stages, and lowest at the mature stage. In addition, intraperitoneal injection of Kiss1-10 and Kiss2-10 significantly stimulated mRNA levels of pituitary *lhβ*, *fhsβ*, and *gthα*. In summary, two Kiss/Kissr systems were firstly proven in a flatfish species

of turbot, and it has a positive involvement in controlling the reproduction of the Kiss/Kissr system in turbot. The results will provide preliminary information regarding how the Kiss/Kissr system controls seasonal reproduction in turbot broodstock.

Keywords: turbot, kisspeptin, kisspeptin receptor, reproduction, gonadal development

INTRODUCTION

In vertebrates, seasonal reproduction, as the most important biological rhythm, is classically controlled by the hypothalamus-pituitary-gonadal (HPG) axis. Hormones secreted from the HPG axis are regulated by seasonally changing patterns, such as photoperiod, which is an environmental signal that can trigger organism to sexually mature. The discovery of the kisspeptin/GPR54 signaling system has greatly improved our understanding of reproductive endocrinology (1–3). There is an abundance of evidence from mammals demonstrating that the kisspeptin/GPR54 system has a critical role in reproduction, especially in mediating the responses to environmental cues (4, 5). Previous studies in mammals have demonstrated that kisspeptin/GPR54 signaling, as a regulator of the HPG axis, regulates the secretion of gonadotropin-releasing hormone (GnRH) (2, 6). The influence of kisspeptin/GPR54 signaling on reproductive endocrine function has attracted increasing attention from reproductive physiologists.

The kisspeptin/GPR54 system is composed of the ligand, kisspeptin (Kiss), and its receptor, G protein-coupled receptor 54 (GPR54), now renamed kisspeptin receptor (Kissr) (7). The first kisspeptin transcript was isolated from human malignant melanoma cells and its cDNA was designated as Kiss1 (1). Subsequently, the Kiss1 cognate receptor gene (*kissr1*) was characterized in rats (8). Further studies on hypogonadism showed that mutation of *kissr1* with the idiopathic hypothalamic hypogonadism (IHH) syndrome could cause sterile in mice (6, 9). In mammals, the role of the Kiss/Kissr system in the reproductive system was investigated, and has led to significant breakthroughs. It has been demonstrated that the Kiss/Kissr system controls the HPG axis by directly influencing GnRH neurons and regulating the secretion of the GnRH, resulting in the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) (10, 11).

Following the investigations in mammals, the Kiss/Kissr system has also been characterized in fish species. Parhar and coworkers first reported that another kisspeptin receptor *kissr2* was co-expressed in GnRH neurons in Nile tilapia (*Oreochromis niloticus*), providing valuable insights into the kisspeptin system during normal sexual and reproductive development (12). Subsequently, up to two *kiss* genes (*kiss1*, *kiss2*) and three *kissr* genes (*kissr1*, *kissr2*, *kissr3*) have been identified in various teleosts, including zebrafish (*Danio rerio*) (13–15), medaka (*Oryzias latipes*) (16, 17), goldfish (*Carassius auratus*) (18, 19), European sea bass (*Dicentrarchus labrax*) (20, 21), orange-spotted grouper (*Epinephelus coioides*) (22), chub mackerel (*Scomber japonicas*) (23, 24), yellowtail kingfish (*Seriola lalandi*) (25, 26), European eel (*Anguilla anguilla*) (27, 28), pejerrey (*Odontesthes bonariensis*) (29, 30), lined seahorse (*Hippocampus erectus*) (31), yellowtail clownfish (*Amphiprion clarkia*) (32) as well as four flatfish species, Senegalese sole (*Solea senegalensis*) (33), Atlantic halibut (*Hippoglossus hippoglossus*) (34), Japanese flounder (*Paralichthys olivaceus*) (35), and half-smooth tongue sole (*Cynoglossus semilaevis*) (36, 37).

Kisspeptin has been proven as a key activator of the reproductive axis during the seasonal gonadal cycle in some fish species. For example, changes in expression of *kiss*/*kissr* mRNAs in golden mahseer (*Tor putitora*) and rohu (*Labeo rohita*) indicated that they have a role in gonadal development and annual reproductive cycle (38, 39). Increased transcription levels of the Kiss/Kissr system showed that it is related to the differentiation of the HPG axis during male development in pejerrey (30). Furthermore, kisspeptin neurons in the mediobasal hypothalamus indicated it is a site that is sensitive to feedback from the action of sex steroids in European sea bass (40). The administration of exogenous kisspeptin peptides has been shown to promote plasma levels of FSH and LH in several fish species (18, 31, 41, 42). However, Kiss knockout in zebrafish and medaka, the puberty onset and sexual differentiation are not affected (43–45). Kiss2 administration in recrudescent fish do not change the blood LH levels (43). It might be species-specific in the control of reproduction in teleosts. Of note, kisspeptin expression revealed specific patterns that coincided with seasonal environmental changes, more specifically photoperiod, and induced sexual development. Intracerebroventricular infusion of Kiss1 could override the inhibitory photoperiod and reactivate sexual activity in hamsters which are sexually active in long photoperiod and quiescent in short photoperiod (43, 46). Kiss1 in mice showed circadian patterns that peaked coincident with LH (46). Similarly, Kiss controlled of cyclic reproductive activity in teleost. Oocyte maturation, responding to day length, relied on the enhanced transcription of *kiss* and *gnrh3* in the brain of zebrafish (47). The rhythms of *kiss* and *gnrh/fshβ/lhβ* mRNA expression further suggested that the natural photoperiod is involved in activating the reproductive axis during maturation in Atlantic cod (*Gadus morhua*), European sea bass and grass puffer (*Takifugu niphobles*) (48–50).

Turbot (*Scophthalmus maximus*) is classified into the Pleuronectiformes, and is one of the most commercially important fish species cultured in Europe and North China. During turbot aquaculture, photoperiod manipulation is used to induce egg and sperm production on a year-round basis (51–53). Previous studies have shown that exposure to continuous light reduced the proportion of females that mature and egg production by as much as 90% (54). Similarly, the administration of a long-term photoperiod around the spring equinox significantly decreased and delayed maturation in adult

turbot (51, 55). Based on the previous studies, we hypothesized that there has a Kiss/Kissr system in turbot involved in the regulation of the entire reproductive axis in response to seasonal changes. Therefore, in this study, we aimed to identify the Kiss/Kissr system in turbot and investigate the expression profiles of kisspeptin genes in the brain and gonad of turbot within an annual reproductive axis under culture conditions exposed to artificial light. Moreover, the physiological role of Kiss1 and Kiss2 in regulating pituitary hormone gene expression was also examined. These data provide crucial information for controlling seasonal reproduction and breeding of turbot broodstock.

MATERIALS AND METHODS

Fish and Samples

The turbot individuals used in this study were obtained from a fish farm in Shandong Province, China. All of the broodstock were over 2 years old and had previously undergone gonadal maturation. The fish were cultured under a simulated natural photoperiod (12L:12D) and ambient temperature of 17 ± 0.8°C before the reproductive season. During the breeding season, from November 2018 to April 2019, the lighting regime progressed through three stages: an inhibitory photoperiod (8L:16D) for about 1.5 months, a natural photoperiod (12L:12D) for about 0.5 months, and a prolonged photoperiod (16L:8D) for about 4 months. The fish were fed with enhanced nutrition (small yellow croaker *Larimichthys polyactis* and Pacific sand lance *Ammodytes personatus*). Three males and three females were collected for sampling every month throughout the reproductive season. Before sampling, turbots were anesthetized with a 0.05% MS222 (Sigma-Aldrich). The gonad from each turbot was divided into two parts, one part was fixed in Bouin's fluid for histological analysis and the other was frozen in liquid nitrogen and then stored at -80°C. Besides gonads, brain and pituitary gland were also collected for qPCR analysis. In addition, various tissues, including brain, eye, skin, muscle, gill, liver, heart, spleen, kidney, stomach, intestine, liver, and gonad from immature male turbot were collected and stored at -80°C to examine the tissue distribution of the Kiss/Kissr system.

Histological Analysis

The fixed gonadal tissue samples were dehydrated through a graded series of alcohol concentrations and embedded in paraffin. Serial sections of 5 μm were sliced using a rotary microtome (Leica, Germany) and treated with HE (Haematoxylin and Eosin) stain. Stained sections were examined using a microscope Axio Scope A1 (Leica, Germany). The developmental stages of gonads were confirmed based on ovarian and testicular histology. All of the collected turbots were classified into II, III, IV, V, and VI stages according to the dominant gamete type and characteristics (56, 57).

Molecular Cloning

Total RNA was extracted from the brains of immature turbot using the RNA Isolation Kit (Catalog No. 220011) (Fastagen Biotech, China). Quality and concentration of RNA were measured using 1.0% agarose gels electrophoresis and assessed by spectrophotometry (ND-2000, Nanodrop, USA). The first-strand cDNA was synthesized using a TransScript First-Strand cDNA synthesis kit (AT301-02) (Transgen, China) following the manufacturer's instructions. Subsequently, cDNA fragments of turbot *kiss1*, *kiss2*, *kissr2*, and *kissr3* were amplified with primers (**Table 1**). Finally, 5'-RACE and 3'-RACE were performed for full-length cDNAs using the SMARTer RACE cDNA Amplification Kit (Catalog No.634923) (Clontech, USA) with gene-specific primers (GSPs) (**Table 1**). The diluted first-round PCR products were used as templates for nested PCR with corresponding primers of GSP-nest (**Table 1**). All PCR programs were performed using a PTC-100 thermal cycle (Bio-Rad, USA). PCR was performed in a 25 μl reaction volume containing 1 μl of diluted cDNA, 1 μl of each primer (10 μM), 12.5 μl of Premix buffer (with dNTPs), and 9.5 μl ddH_2O, with amplification procedure of denaturation at 94°C for 5minutes; 35 cycles of amplification at 94°C for 30 seconds, annealing with specific temperature of each primer (**Table 1**) for 45 seconds, elongation with 72°C for 30 seconds, an additional elongation at 72°C for 10 minutes.

Phylogenetic Analysis

Homology searches of the deduced turbot Kiss1, Kiss2, Kissr2, and Kissr3 sequences were performed using the National Center for Biotechnology Information website (http://www.ncbi.nlm.nih.gov/). The putative signal peptides were predicted by SignalP4.1. The putative transmembrane domain was predicted using the TMHMM server V2.0. The percentages of similarity and identity were calculated using LALIGN (http://www.ch.embnet.org/software/LALIGN-form.html). Multiple alignments of predicted amino acid sequences were conducted using BioEdit with the ClustalW alignment tool. A phylogenetic tree was constructed in the Mega7 software by the neighbor-joining method using bootstrapping with over 1,000 iterations.

Synteny Analyses of kiss1 and kiss2 Genes

The syntenic analysis of kiss1 and kiss2 genes among turbot, zebrafish, medaka and tilapia was conducted basing on the comparison the neighboring genes. Briefly, the protein sequences of neighbor genes of Kiss were predicted from the turbot chromosome sequences by FGENESH program. The identified neighboring protein sequences were annotated against NCBI by BLASTP. The conserved syntenic pattern of Kiss genes in other species were determined in Ensembl database (http://asia.ensembl.org/index.html) and Genomicus (https://www.genomicus.bio.ens.psl.eu/genomicus-83.01/cgi-bin/search.pl).

Administration of Kiss1 and Kiss2 to Turbot

According to the sequence analysis, turbot Kiss1-10 (YNLNSFGLRY-NH2) and Kiss2-10 (FNFNPFGLRF-NH2) peptides were synthetized by ChinaPeptides Co., Ltd. (Shanghai, China) with purities of 98% and 97%, respectively, as determined by HPLC. For *in vivo* experiments, juvenile turbot

TABLE 1 | List of primers used for molecular cloning of kiss and kissr cDNAs.

Primer name	Nucleotide sequence (5'-3')	Temperature of annealing (°C)	Purpose
kiss1-F	ACCTGCTGAYARRGTCCAKTCAG	58	Fragment PCR (138bp)
kiss1-R	TTTCCRTAACGKAGACCAAAGG		
kiss2-F	GCTCTGGTTGTTGTGTGCG	59	Fragment PCR (310bp)
kiss2-F	TCCTGGCTCTTTTAACGGCT		
Kissr3-F	CCACACTGTACCCTCTGCCCAGCTG	59	Fragment PCR (626bp)
Kissr3-R	TTGACGGAGGAGTTGGAGTA		
kissr2-F	GGMAACTCWCTGGTSATTTATGT	58	Fragment PCR (833bp)
kissr2-R	AYTTGGCGTAGGACATGCAGTT		
5'- kiss1-GSP1	TCCATAACGGAGACCAAAGGAGTTGAGGTT	65	5' RACE-PCR
5'- kiss1-GSP2	GATCCACCATCCTGATCTGGGAAACT	63	5' RACE-PCR(nested)
3'- kiss1-GSP1	CTGATAAGGTCCATTCAGCTGATGGAAAGT	62	3' RACE-PCR
3'- kiss1-GSP2	CATACAACCTCAACTCCTTTGGTCTCCG	63	3' RACE-PCR(nested)
5'- kiss2-GSP1	GCTCTCCTGTAGATGTAGCGTTTCCCGA	63	5' RACE-PCR
5'- kiss2-GSP2	TCCTCCTCCTGAGCGCGGAGAGGAC	60	5' RACE-PCR(nested)
3'- kiss2-GSP1	ATGAGGCTTGTGGCTCTGGTTGTTGTG	63	3' RACE-PCR
3'- kiss2-GSP2	GCTGTGCAACGACCGCAGGAGCAA	60	3' RACE-PCR(nested)
5'- kissr3 -GSP1	GGGCACACAGCAGACCAGGAACAAGAT	65	5' RACE-PCR
3'- kissr3 -GSP1	GTCTTACTCCAACTCCTCCGTCAACCC	65	3' RACE-PCR
5'- kissr2 -GSP1	CAGAAGGAGATAGTCAGGACGGGCAG	65	5' RACE-PCR
5'- kissr2 -GSP2	ATTTCAGAGGGTAGACGGTGACATAG	66	5' RACE-PCR(nested)
3'- kissr2 -GSP1	CCAAACACAGGCAGATGAGGACGGCG	66	3' RACE-PCR
3'- kissr2 -GSP2	ATAAGCATCAGAAGCAAAGTCTCCAA	65	3' RACE-PCR(nested)

(body weight (BW) = 9.575 ± 0.47 g) were used, at a rearing temperature of 16 ± 0.5°C with dissolved oxygen > 7.0 mg/L and a 12L:12D photoperiod.

The doses of Kiss peptides referred the administration experiments in some fish species, including goldfish and cinnamon clownfish (18, 31, 42). In these articles, it demonstrated that intraperitoneal injection of 0.01-1ug/g in 6h could stimulate expression of *lhβ* and *fshβ*. Therefore, doses of 100ng/g BW and 1000 ng/g BW with intraperitoneal injection were chosen. The synthetized peptides were dissolved in phosphate-buffered saline (PBS) and injected intraperitoneally into turbot anesthetized with 0.05% MS222. PBS alone acted as the negative control. At 3 and 6 h post-injection, sample fish were collected (n = 6) and their pituitary glands quickly dissected, frozen in liquid nitrogen, and stored at -80° C for qPCR analysis.

Quantitative Real-Time PCR (qPCR)

Quantitative real-time PCR was performed using the CFX96 Real-Time PCR Detection System (Bio-Rad, USA) with a SYBR Premix Ex Taq Kit (Takara, Japan) using the standard curve method with *β-actin* as the reference gene. Specific primers used for each target gene were designed by the Primer Premier 5.0 software (**Table 2**). The 20 μl reaction system contained 10 μl SYBR Premix Ex Taq, 0.2 μl forward primer (10 μM), 0.2 μl reverse primer (10 μM), 7.6 μl ddH_2O, and 2 μl cDNA template. The PCR procedure was programmed according to the manufacturer's protocol: 30 s at 95°C, 5 s at 95°C, and 30 s at 60°C for 40 cycles. A dissociation curve was performed at the end of each program to determine the amplification specificity. The relative gene expression levels were analyzed by the $2^{-\Delta\Delta CT}$ method.

Statistical Analysis

Statistical analysis was carried out using SPSS version 21.0. All results are presented as means ± SEM. Gene expression during gonadal stages were analyzed using one-way analysis of variance (ANOVA) followed by Duncan's multiple range tests. Differences were considered to be significant at $P < 0.05$. All assays were carried out independently in triplicate.

RESULTS

Molecular Cloning of *kiss*/*kissr* in Turbot

The full-length cDNA of turbot *kiss1* (GenBank accession no. MW057929) was 580 bp in size, with an open reading frame (ORF) of 312 bp encoding a preprohormone of 104 amino acids. The full-length cDNA of turbot *kiss2* (GenBank accession no. MW057930) was 622 bp, with an ORF of 369 bp encoding a precusor of 123 amino acids. Amino acid sequence analysis demonstrated that an obvious Kiss1 domain-YNLNSFGLRY (Kiss1-10) and Kiss2 domain-FNFNPFGLRF (Kiss2-10) were involved in turbot kisspeptin system (**Supplementary Figure 1**). However, the Kiss1-10 differed from Kiss2-10 by 4 amino acids in turbot. The sequence alignments of the deduced amino acid sequences of turbot Kiss1 and Kiss2 with other vertebrate species are presented in **Supplementary Figure 2**.

Notably, although the Kiss-10 regions are highly conserved across vertebrates, there were still 2 positions in Kiss1-10 and 3 positions in Kiss2-10 with different amino acids. For Kiss1-10, at the 3rd position from the N-terminus, most teleosts exhibit a Leucine and some exhibit phenylalanine, while mammals and amphibians exhibit a Tryptophan. For turbot Kiss2-10, the 3rd position from the N-terminus was identical to medaka, zebrafish, longtooth grouper (*Epinephelus bruneus)*, rare minnow (*Gobiocypris rarus)*, and Senegalese sole. However, turbot had Phenylalanine and Glycine at positions 6 and 7 from the N-terminus, which differed from the Leucine and Threonine in Japanese flounder.

TABLE 2 | List of primers used for qPCR expressions analysis of kiss and kissr mRNAs.

Primer name	Nucleotide sequence (5'-3')	Products (bp)	E	R^2
sm-kiss1-F	GCCTTGAGAGATTTAACCGATGC	143bp	0.93	0.991
sm-kiss1-R	ATAGAGTGAGGGAGGACCAAGTT			
sm-kiss2-F	GTGGATGAACGTTAACAGGCAC	142bp	0.97	0.996
sm-kiss2-R	GTCGAGTCATATCCTGGCAGAG			
sm-kissr3-F	GGTTTACGCCTTCATGGGCA	124bp	0.98	0.996
sm-kissr3-R	GTCCTTCCCTTCCTCCGCTT			
sm-kissr2-F	CAGCGTCTGCATTTGGATCG	164bp	1.03	0.998
sm-kissr2-R	AGGCGGCGATAAACTGGTAG			
sm-fshβ-F	TCGGCTGCAAACTGGC	185bp	1.02	0.997
sm-fshβ-R	ATCCGTTAATGTGCTTCG			
sm-lhβ-F	CAAGGAACCCTCCATCATCTTT	96bp	0.99	0.997
sm-lhβ-R	AGCTGCACCGTCCTGTAGTG			
sm-gthα-F	ACCCGACACCACTCAAGACA	148bp	0.97	0.998
sm-gthα-R	CTTTTATGCCGACACCCACA			
sm-β-actin-F	GCTGTGCTGTCCCTGTATGCC	187bp	0.92	0.997
sm-β-actin-R	AGGAGTAGCCACGCTCTGTCA			

E, reaction efficiencies; R2, Pearson's coefficients of determination.

Phylogenetic analysis revealed that the Kiss1 and Kiss2 sequences clustered in two separate clades (**Figure 1**). The synteny analysis of kiss showed that the *kiss1* gene was usually positioned in the genomic regions, including *plekha6*, *cntn2*, *psma5*, *foxp4*, *mapkapk2*, and *pik3c2b*, and *kiss2* gene was usually positioned including *gys2*, *spx*, *golt1b*, *ldhba*, *slc25a3a*, and *strap* (**Figure 2**).

The full-length cDNA of turbot *kissr3* (GenBank accession no. MT319113) was 2329 bp, with an ORF of 1092 bp encoding a peptide with 363 amino acids. The full-length cDNA of turbot *kissr2* (GenBank accession no. MW057931) was 1578 bp, with an ORF of 1128 bp encoding a peptide with 375 amino acids. The deduced amino acids of Kissr3 and Kissr2 all contained a seven transmembrane domain (**Supplementary Figure 3**). The sequence alignment of the deduced amino acid sequences of turbot Kissr2 and Kissr3 to those of other vertebrate species is presented in **Supplementary Figure 4**. In the phylogenetic analysis, the two turbot Kissr clustered into the Kissr2 and Kissr3 clades, respectively (**Figure 3**).

Tissue Distribution of *Kiss* and *Kissr* Transcripts

Expression patterns of turbot *kiss* (*kiss1*, *kiss2*) and *kissr* (*kissr2*, *kissr3*) genes in various tissues and different brain regions were detected by qPCR. Firstly, expression of tissues of brain, pituitary, gonad, eye, skin, muscle, gill, heart, spleen, kidney, stomach, intestine, and liver from adult male were shown in **Figure 4**. The *kiss1* mRNA was highly expressed in the brain and gonad, but had low expression in the skin, pituitary, and intestine. The *kiss2* mRNA was particularly highly expressed in brain and gonad, and barely expressed in other tissues. The *kissr3* mRNA was significantly expressed in brain, gonad, and eye. While the *kissr2* mRNA was abundantly expressed in brain and eye, but barely in gonad.

The expression patterns of turbot *kiss/kissr* mRNAs in central brain areas, including olfactory bulbs (Ob), telencephalon (Te) with preoptic area (POA), mesencephalon (Me) with thalamus, hypothalamus (Hy) with saccus vasculosus, cerebellum (Ce), medulla oblongata (Mo), and pituitary gland (Pi) were also analyzed (**Figure 5**). The expression in the eye was also investigated alongside the central brain areas. The *kiss/kissr* mRNAs were expressed in all central brain areas and eyes. The *kiss1* mRNA expression was high in Me, but low in Mo and Hy. However, the *kiss2* mRNA was highly detected in the Hy. The expressions of *kissr3* and *kissr2* mRNA were high in the Me and Hy. In addition, the expression patterns of *kiss/kissr* mRNAs were similar in the female and male turbot brains, however, expression levels were higher in the central brain areas of females than males.

Gonadal Morphology and Histology Assessment of Reproductive Stages

The morphology and histology of ovaries and testes during breeding season of turbot were examined. Five ovarian and five testicular developmental stages were identified from stage II to VI (**Figure 6**). First, the five ovarian stages are shown in **Figure 6A1–5**. At stage II, the ovary was present with light fleshy red color and the blood vessels on the ovarian membrane were not obvious. The germinal epithelium of the ovary was filled with oocytes (primary growth oocytes and perinucleolar oocytes) in stage II. At stage III, the appearance of the ovary was flesh-colored, and eggs could be seen in the ovary. The histological results showed the oocytes of stage III had developed into the previtellogenic stage and vitellogenesis had begun. At stage IV, the ovary exhibited significant growth and the oocytes were characterized as late vitellogenic. At stage V, matured oocytes were obvious in the ovary and the proportion of oocytes in the post vitellogenic stage increased. Stage VI was characterized by degenerated ovaries and there were only primary growth oocytes left.

Similarly, the appearance and major cell types in the five developmental stages of testis were observed (**Figure 6A6–10**). With development, the testis became bigger and wrinkles and blood vessels became clear. In addition, spermatogenesis was observed. At stage II, the testis contained a large number of spermatogonia. At stage III, spermatogenesis had begun and

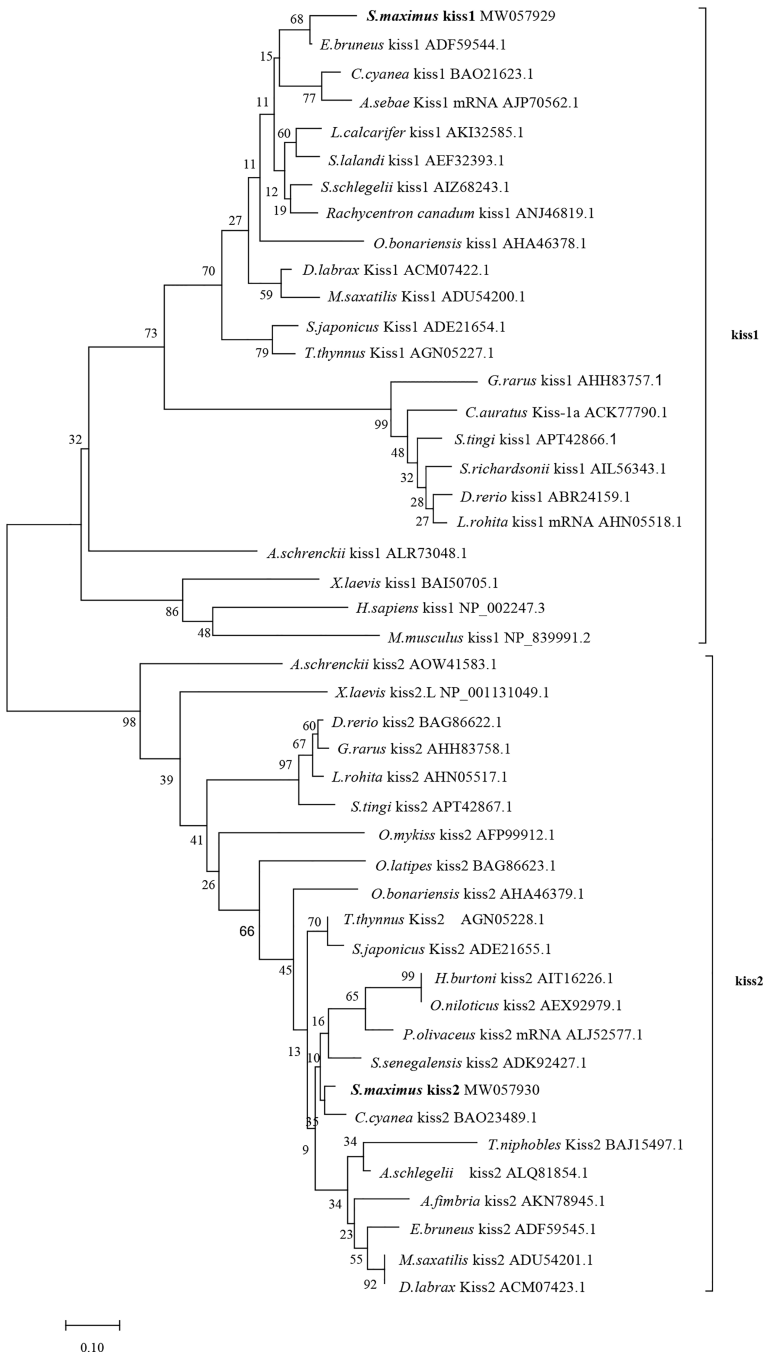


FIGURE 1 | Phylogenetic tree of deduced amino acid sequences of Kiss1 and Kiss2 from turbot and other vertebrates was constructed by Mega7 with neighbor-joining method. The bootstrap values at nodes indicate bootstrap percentage value for 1000 replicates (>80%). Relative branch lengths indicate the evolutionary rates of the lineages. The GenBank accession numbers of the sequences were presented after the species name.

most germ cells had become primary spermatocytes. At stage IV, secondary spermatocytes appeared. At stage V, the seminiferous tubules were filled with spermatozoa with tails, indicating that spermatogenesis was nearly complete. With the release of spermatozoa, the testis were classified as stage VI.

Meanwhile, the gonadosomatic index (GSI, gonad weight/body weight) and hepatosomatic index (HSI, liver mass/body weight) were calculated (**Figure 6B1–4**). In both male and female turbot, the GSI of ovaries and testes all increased from stage II to V, reaching their peak values at stage V, before decreasing at

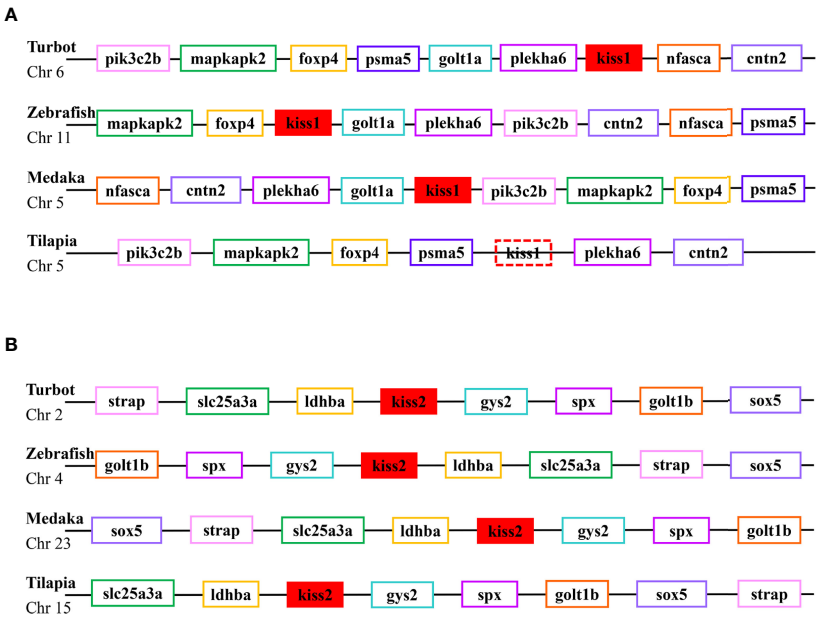


FIGURE 2 | Conserved synteny for the genomic region of *kiss1* **(A)** and *kiss2* **(B)** genes.

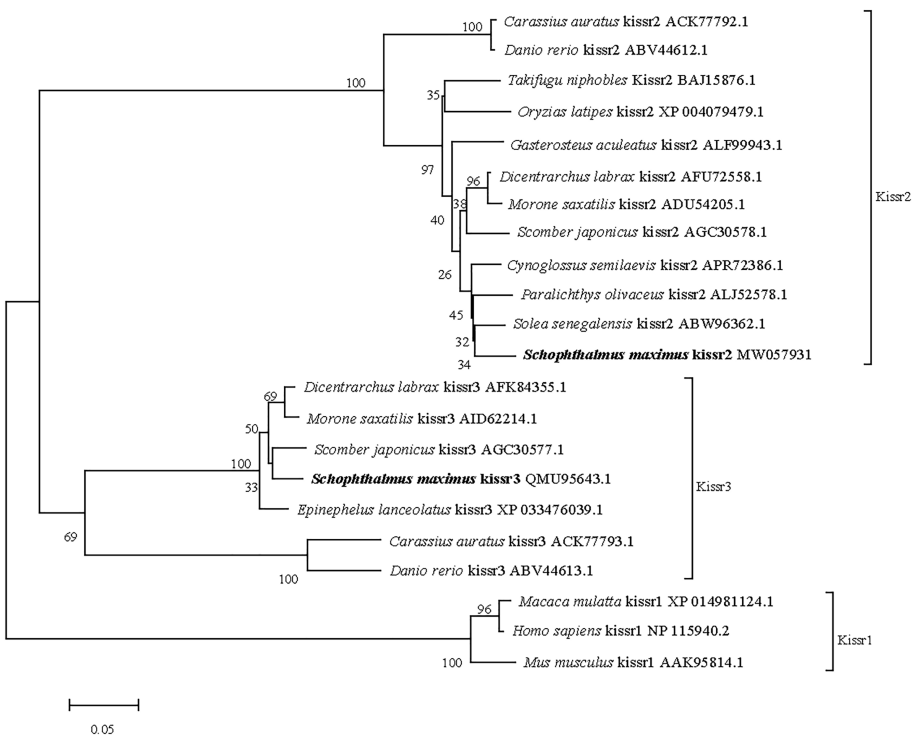


FIGURE 3 | Phylogenetic tree of deduced amino acid sequences of Kissr3 and Kissr2 from turbot and other vertebrates was constructed by Mega7 with neighbor-joining method. The bootstrap values at nodes indicate bootstrap percentage value for 1000 replicates (>80%). Relative branch lengths indicate the evolutionary rates of the lineages. The GenBank accession numbers of the sequences were presented after the species name.

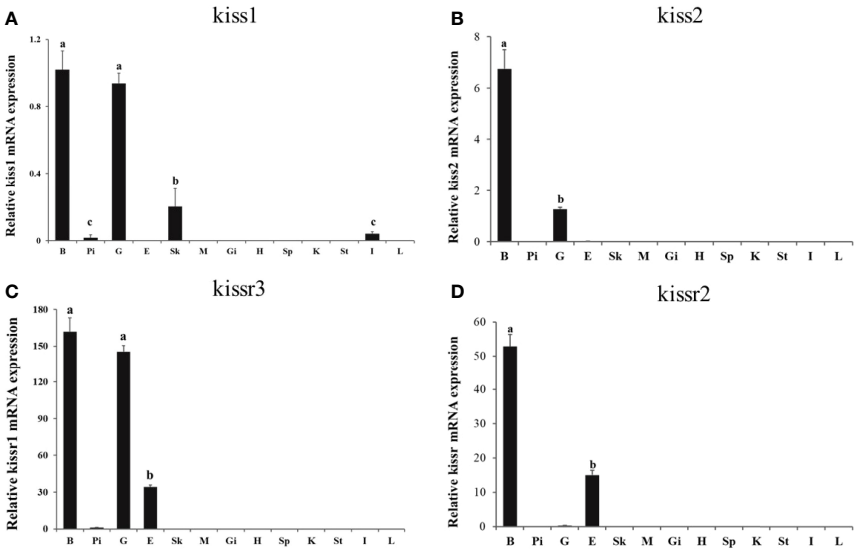


FIGURE 4 | Tissue distribution of *kiss/kissr* genes in turbot. **(A)** kiss1, **(B)** kiss2, **(C)** kissr3, **(D)** kissr2. The relative abundance of kiss/kissr genes were expressed as mean ± SEM (n=3). Tissue abbreviations: B, brain; Pi, pituitary; G, gonad; E, eye; Sk, skin; M, muscle; Gi, gill; H, heart; Sp, spleen; K, kidney; St, stomach; I, intestine; L, liver. Different letters above bars represent statistical significance ($p < 0.05$) between two different tissues.

stage VI. The HSI of ovaries at stage V was lower than all other stages. While in testes, the HSI was highest at stage V.

Expression Patterns of *Kiss* and *Kissr* Transcripts in the Brain During Reproductive Stages

In females, *kiss1* mRNA expression was very high during stage II, then gradually decreased from stage II to V, followed by a significant increase at stage VI (**Figure 7A**). The *kiss2* expression level declined significantly from stage II to III, and did not change significantly from stage III to VI (**Figure 7C**). Meanwhile, the *kissr3* and *kissr2* mRNA expression profiles showed a similar pattern to *kiss1* mRNA expression (**Figures 7E, G**). In males, the expression patterns of *kiss1* and *kiss2* mRNA were similar during the reproductive stages (**Figures 7B, D**). The highest expression levels appeared at stages II and VI. Their expression levels did not change significantly from stage III to V, but were significantly lower than stages II and VI. In addition, the expression patterns of *kissr3* and *kissr2* mRNA in males were similar to those in female turbot (**Figures 7F, H**).

Furthermore, to explore expression patterns in different brain regions, the expression levels of *kiss* and *kissr* transcripts in the Me and Hy of both sexes during three gonadal development stages, immature stage (II), mature stage (IV-V), and degeneration stage (VI), were analyzed (**Figure 8**). The results showed that the expression levels of *kiss* and *kissr* transcripts declined significantly from the immature to mature stages, and increased at the degeneration stage. However, *kiss2* expression in the Me of females was lowest at the degeneration stage. In males, *kiss2* expression in the Me showed no significant differences among the different gonad developmental stages.

Expression Patterns of *Kiss* and *Kissr* Transcripts in the Gonad During Reproductive Stages

In females, there was no a distinct trend of expression of kiss/kissr system. There was no difference of expression of *kiss1* in stage V and IV. However, the expression of *kiss1* in stage V was higher than that in stage II, III, and VI (**Figure 9A**). There was high expression of *kiss2* and *kissr2* mRNA at stage IV (**Figures 9C, G**). For *kiss2*, the lowest expression happened at stages III and VI. The lowest expression of *kissr2* happened at stage VI. The highest *kissr3* mRNA expression level appeared at the immature stage (II) (**Figure 9E**). In males, the highest *kiss* and *kissr* mRNA expression levels appeared at stages II and VI, and lowest at the mature stage (V) (**Figures 9B, D, F, H**).

3.6 Effects of Administration of Kiss1-10 and Kiss2-10 on the Pituitary of Turbot

The changes of *fshβ*, *lhβ*, and *gthα* transcription levels were analyzed to investigate the physiological effects of Kiss1-10 and Kiss2-10 on pituitary function. The *fshβ* (**Figure 10A**), *lhβ* (**Figure 10B**), and *gthα* (**Figure 10C**) transcription levels increased significantly at 3 h and 6 h post-injection of 100 ng/g and1000 ng/g Kiss1-10 and Kiss2-10. The transcription levels of *fshβ* peaked at 3 h under 100 ng/g of Kiss1-10 and Kiss2-10, and 1000 ng/g of Kiss2-10. The *lhβ* transcription reached the highest level at 6 h under 1000 ng/g of Kiss1-10 and Kiss2-10. *Gthα* transcription increased significantly from 3 h to 6 h under the administration of both doses. It was note that, after 3h injection, the *fshβ* and *Gthα* mRNA had a lower expression with 1000ng/g than that with 100ng/g.

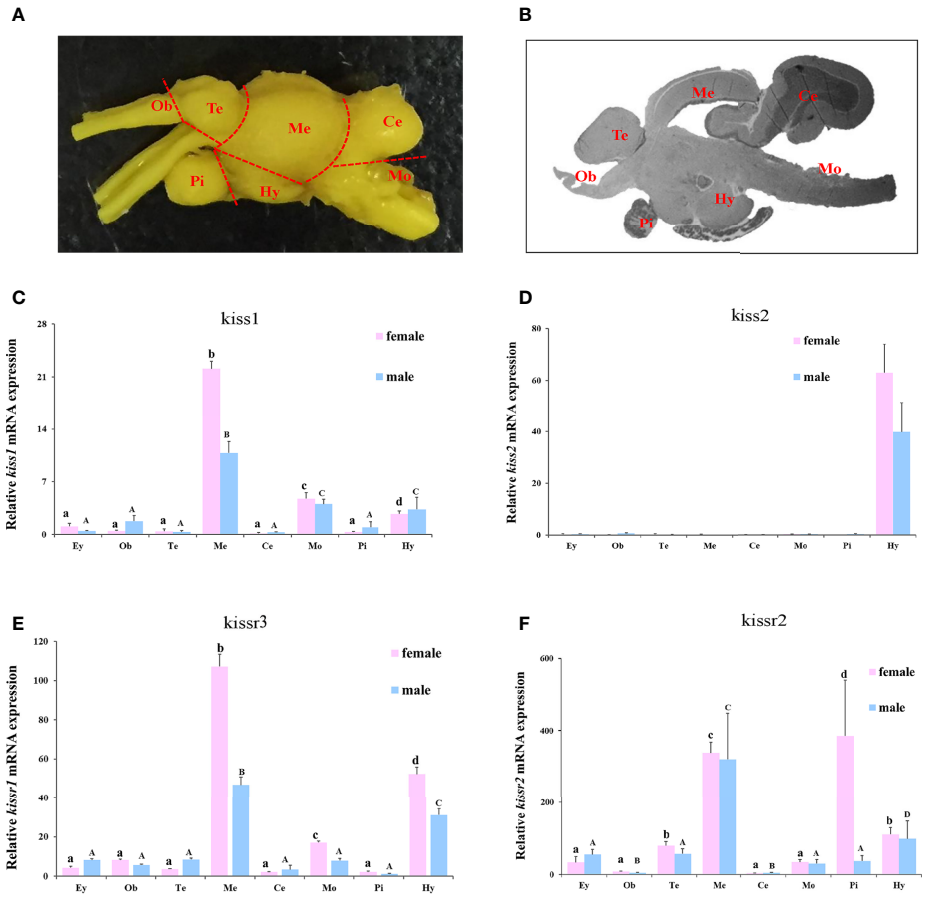


FIGURE 5 | The turbot brain areas and expression of *kiss/kissr* genes in different brain areas and eyes. **(A)** morphology of turbot brain areas. **(B)** histology of turbot brain areas. **(C)** *kiss1*. **(D)** *kiss2*. **(E)** *kissr3*. **(F)** *kissr2*. The relative abundance of *kiss/kissr* genes were expressed as mean ± SEM (n = 3). Ey, eye; Ob, olfactory bulbs; Te, telencephalon; Me, mesencephalon; Ce, cerebellum; Hy, hypothalamus; Pi, pituitary gland; Mo, medulla oblongata. The different uppercase letters in the figure represent statistical significance ($p < 0.05$) in male. The different lowercase letters in the figure represent statistical significance ($p < 0.05$) in female.

DISCUSSION

In the present study, we cloned and characterized the Kiss/Kissr system (*kiss1*, *kiss2*, *kissr2*, *and kissr3*) in turbot, and provided preliminary information on its roles throughout the seasonal reproductive period. To date, numerous studies have reported on the Kiss/Kissr system in vertebrates. In most mammals, there is only one kisspeptin gene, *kiss1*, except in the platypus which contains two forms, *kiss1* and *kiss2* (58). However, two forms of *kiss* genes have been identified in several fish species, such as zebrafish, medaka, goldfish, European seabass, among others (59). However, in almost 20 fish species, only *kiss2* can be found, such as the three-spined stickleback (*Gasterosteus aculeatus*), Nile tilapia (*Oreochromis niloticus*), and orange-spotted grouper (*Epinephelus coioides*) (20, 22, 60, 61).

Until now, four *kiss* and four *kissr* genes have been identified, indicating the Kiss/Kissr system has a high diversity in vertebrates (62). In this study, two *kiss* genes (*kiss1* and *kiss2*) and two *kissr* genes (*kissr3* and *kissr2*) were isolated in turbot. However, previous studies in other Pleuronectiformes only found *kiss2* and *kissr2*, including Japanese flounder (35), half-smooth tongue sole (36, 37), and Senegalese sole (63). Thus, a hypothesis that *kiss1* was probably lost in Pleuronectiformes was put forward. Many researchers have tried to explain the lack of *kiss1* and establish a clearer picture of their molecular diversity in evolutionary terms of whole genome duplication (35, 62). While teleost-specific whole genome duplication (also known as the third round,3R) should have generated additional *Kiss* paralogs, there is a loss of *Kiss1* in some teleosts (62). The reason might be a massive loss of 3R-Kiss paralogs shortly after the 3R event. However, according to synteny analysis and cognate neighboring genes, it is confirmed that turbot possesses *kiss1* gene. It's worth noting that Pleuronectiformes was split into 'real flatfish Pleuronectoidei' (RFP) and 'flatfish-like Psettodoidei' (FLP) lineages by genome analyses, and it was confirmed a polyphyletic origin for these two lineages in recent research (64). Meanwhile, there were morphological and genetic difference between the two lineages. Though it is clustered into RFP, the turbot seemed to be closer to FLP which forms one clade with Perciformes species according to the phylogenetic

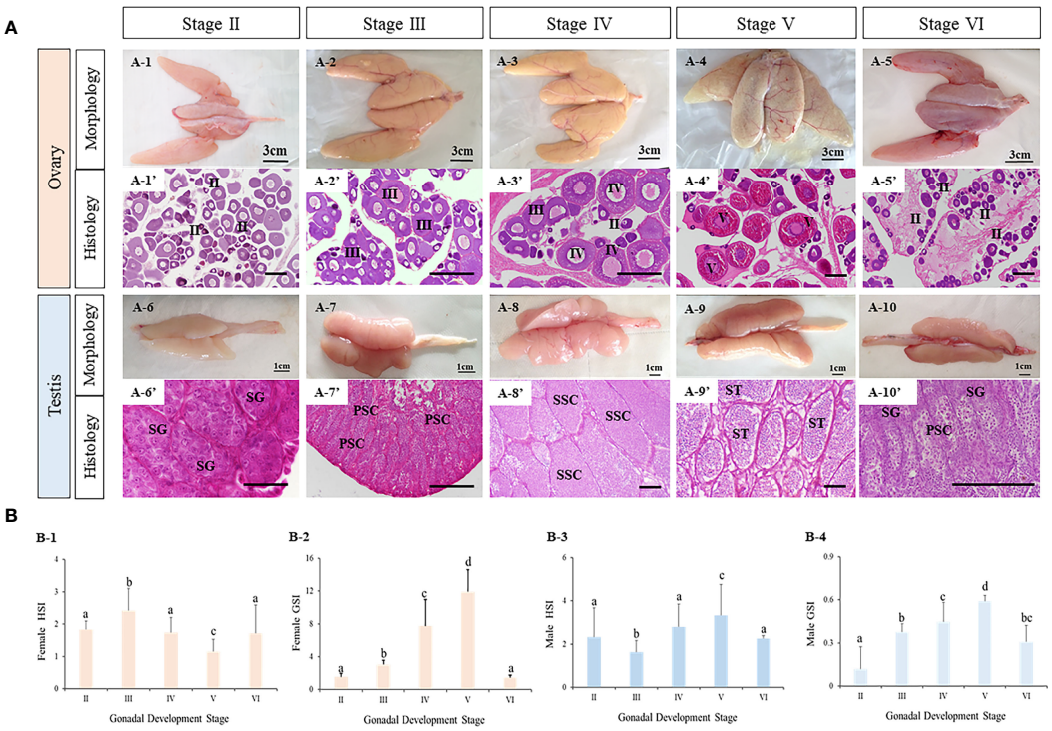


FIGURE 6 | The characteristics of tourbot testes and ovaries during reproductive cycle. **(A)** morphology and histology of turbot testes and ovaries. A1-5, morphology of turbot ovaries. A1'-5', histology of turbot ovaries. A6-10, morphology of turbot testes. A6'-10', histology of turbot testes. **(B)** GSI and HSI of turbot testes and ovaries. B1-2, GSI and HSI of female turbot. B3-4, GSI and HSI of male turbot. **(A)** PSC, primary spermatocytes; SG, spermatogonia; SSC, secondary spermatocytes; ST, spermatids; SZ, spermatozoa; **(B)** I, previtellogenic oocytes. II, primary vitellogenic oocytes; III-IV, large growth of vitellogenic oocytes. Scale: A1-5, 3 cm; A6-10, 1 cm; A1'-10', 50 µm. Different letters above bars represent statistical significance ($p < 0.05$) at different reproductive stages in **(B)**.

analysis. Therefore, it is supposed that turbot might manage to maintain *kiss1* as an exceptional case within Pleuronectiforms.

Generally, the *kiss* gene encodes a polypeptide precursor, which comprises the mature peptide kisspeptin. Matured kisspeptin peptides encompass a C-terminal 10- decapeptide, namely Kiss-10 (20, 58). Positions 1 and 10 correspond to aromatic amino acids that are fully conserved among vertebrates, and consists of the "Y-Y type" and "F-F type" forms (35). In this study, turbot Kiss1-10 was YNLNSFGLRY, belonging to the "Y-Y type", and Kiss2-10 was FNFNPFGLRF, belonging to the "F-F type". Positions 3 and 5 of Kiss1-10 and positions 3, 7, and 8 of Kiss2-10 always exhibited variations in this study. This feature may help in generating specific antibodies against distinct kisspeptins for functional investigations (61).

The receptors of Kiss ligands were GPR54, now known as Kissr, and were first discovered in the rat (8). Previous studies showed that Kissr have been highly conserved throughout evolutionary history. The amino acid sequence comprised seven transmembrane helices (TM1 to TM7) and has a high shared identity among different vertebrate species (58). To date, four Kissr paralogous have been described. In teleosts, all species investigated so far have possessed the *kissr2* gene, indicating the existence of one ancestral Kiss receptor (7). In this paper, phylogenetic analyses have revealed that turbot has two *kissr* paralogous, *kissr2* and *kissr3*, as in some fish species, including zebrafish, medaka, and chub mackerel (14, 58, 65). In addition, three different *kissr* were found in the European eel (*Anguilla anguilla*) (28), which suggested that teleost species also possess a diversity of *kissr* genes.

Tissue expression profiles of *kiss* and *kissr* genes have been reported in many fish species (9, 35, 59). Generally, the highest expression of *kiss*/*kissr* genes has been found in the brain and gonads of investigated species, as in turbot, demonstrating its conserved expression in the CNS along with its putative role in fish reproduction. It is worth mentioning that, in the present study, *kiss1* was more widely expressed in different tissues than *kiss2*. The *kissr* mRNA was also expressed in the eye, and *kiss1* mRNA was expressed in the intestine, which was in line with previous studies, suggesting that the Kiss/Kissr system might have additional roles in turbot beyond reproduction (14, 20). In addition, differences in expression levels of *kiss*/*kissr* among different brain regions were found in turbot. Expression levels of *kiss1* were highest in the Me and Hy, and *kiss2* was highest in the Hy, which was consistent with their expression patterns in adults of many other fish, including medaka, European sea bass, striped bass (*Morone saxatilis*), and Nile tilapia, among others (16, 60, 66). While turbot *kissr2* and *kissr3* had wide distributions, and *kissr3* had wider distribution than *kissr2*. A similarly wide distribution was observed in the medaka brain (17). However, the opposite was true in zebrafish and European

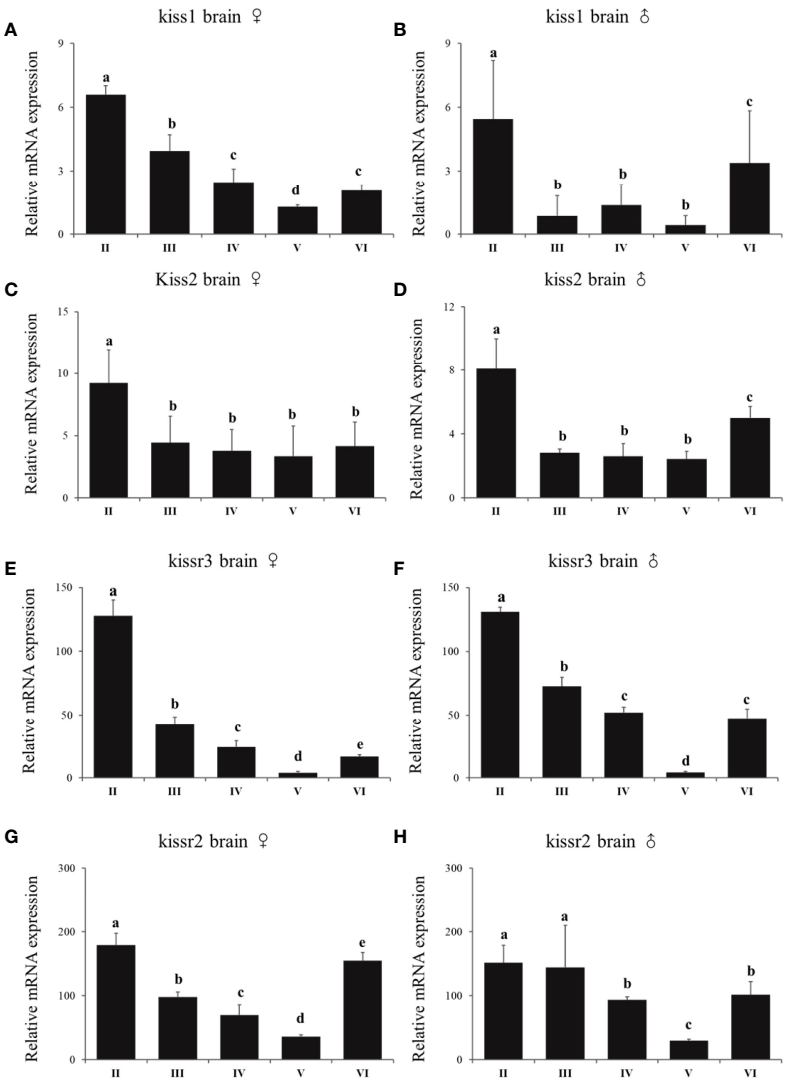


FIGURE 7 | Expression profiles of *kiss/kissr* genes throughout the reproductive cycle in the brains of female **(A, C, E, G)** and male **(B, D, F, H)**. Error bars are presented as the mean±SEM. Different letters above bars represent statistical significance ($p < 0.05$) between two reproductive stages.

seabass, with *kissr2* showing a wider distribution than *kissr3* (61, 67). These data suggest that the Kiss/Kissr system has diverse functions in the brain.

Numerous previous studies have used different approaches to help clarify the reproductive function of the Kiss/Kissr system, which has been established as an important regulator in the HPG axis of mammals and teleosts (10, 11, 68). The relative expression profiles of multiple genes in the Kiss/Kissr system in the brain and gonad were analyzed in different gonad developmental stages in the present study. First, the expression profiles of the four *kiss*/*kissr* genes were similar in the brains of both sexes, where the expression of the *kiss*/*kissr* genes gradually decreased from the immature stage to the mature stage, and increased again during the postspawning period, with the same result in the Me and Hy. The *kiss*/*kissr* genes can be different during the gonadal stages between males and females in different fish species (24, 30, 31, 68). For example, *kiss1* levels continuously declined from the immature to postspawning stages in male chub mackerel, but its level in females showed no significant difference during ovarian development (24). The expression of *kiss2* in the brain of seahorses significantly declined at the pregnancy stage. In fathead minnow (*Pimephales promelas*) the expression of *kissr2* mRNA was low in the advanced gonadal stages in males (69). In addition, the expression profiles of four *kiss*/*kissr* genes in the testis and ovaries of turbot showed different expression profiles. Studies in the Senegalese sole revealed the genes of the Kiss/Kissr system were expressed in all germ cell types during spermatogenesis (63). Moreover, in sea bass, expressions of *kissr2* and *kissr3* peaked at the initiation and completion of the spermiation period (21), which was consistent with the *kissr* expression patterns in male turbot. These results in the brain and gonad of both male and female turbot suggest the

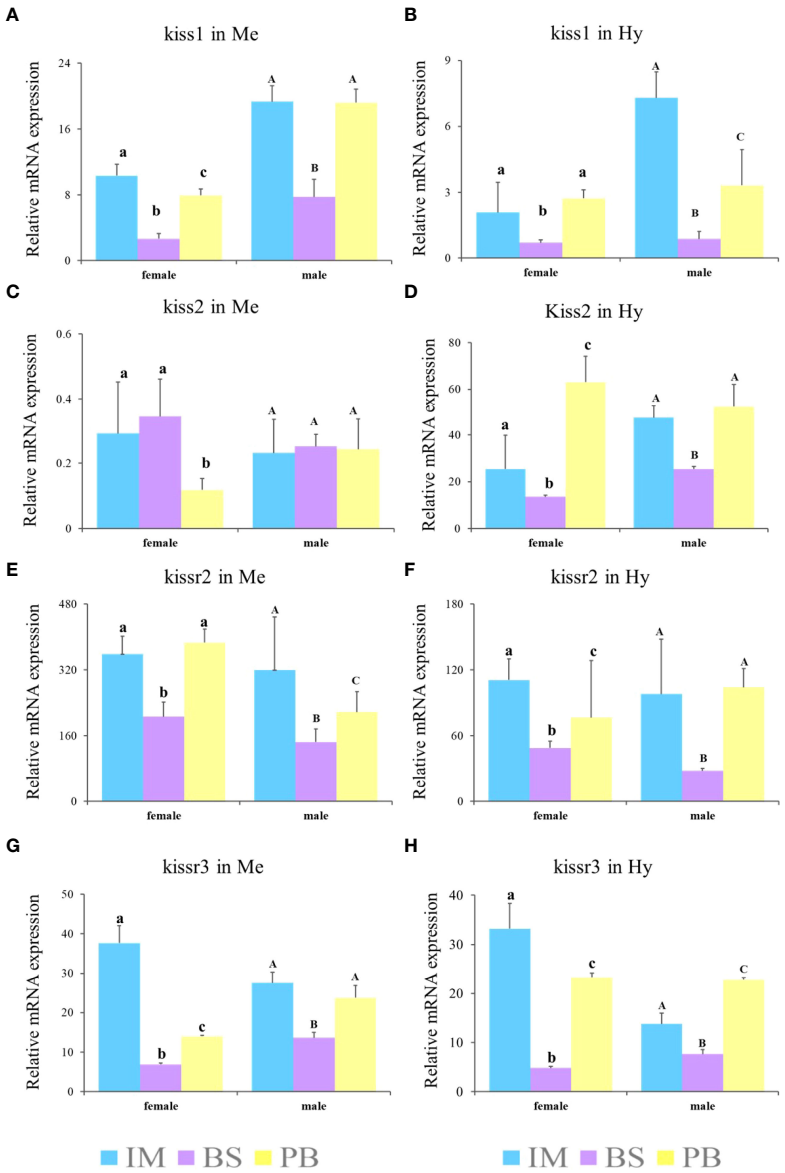


FIGURE 8 | Expression profiles of *kiss/kissr* genes in Me **(A, C, E, G)** and Hy **(B, D, F, H)** at immature stage (IM), the breeding season (BS) and post-breeding season (PB). Error bars are presented as the mean±SEM. The different lowercase letters in the figure represent statistical significance ($p < 0.05$) between two stages in female. The different uppercase letters in the figure represent statistical significance ($p < 0.05$) between two stages in male.

positive involvement of the Kiss/Kissr system in turbot reproduction cycle, as in other fishes and mammals. Moreover, the Kiss/Kissr system is essential in other reproductive activity. Kiss2 is involved in sex differentiation in chub mackerel and pejerrey (30, 65). Kiss1 and Kiss2 is essential for male spermiation in the striped bass (66).

It is well known that seasonal fish reproduction is modulated by a wide variety of environmental factors. Photoperiod is generally thought to synchronize sexual maturation and determine spawning time through the activation of neuroendocrine pathways in temperate regions (48, 49). The discovery of the Kiss/Kissr system has provided important insights into the relationship between seasonal reproduction and photoperiod. In mammals, studies have revealed that the Kiss/Kissr system is the most potent activator of GnRH neurons, and the kisspeptin neurons project directly to GnRH neurons and *kissr* is located at the key site of GnRH neurons (59, 67, 70). Evidence in sheep has suggested that *kiss* neurons in the arcuate nucleus could directly innervate GnRH neuron somata and dendrites to mediate the induction of *lhβ* and *fhsβ* gene expression (71). The *in vivo* action of Kiss also investigated in some fish species. In lined seahorse, the injection of Kiss2-10 significantly increased *fhsβ* and *lhβ* at 6h post-injection and it was higher than 3h in the expression of *fhsβ* (31). Significant increases in *fhsβ* and *lhβ* mRNA levels

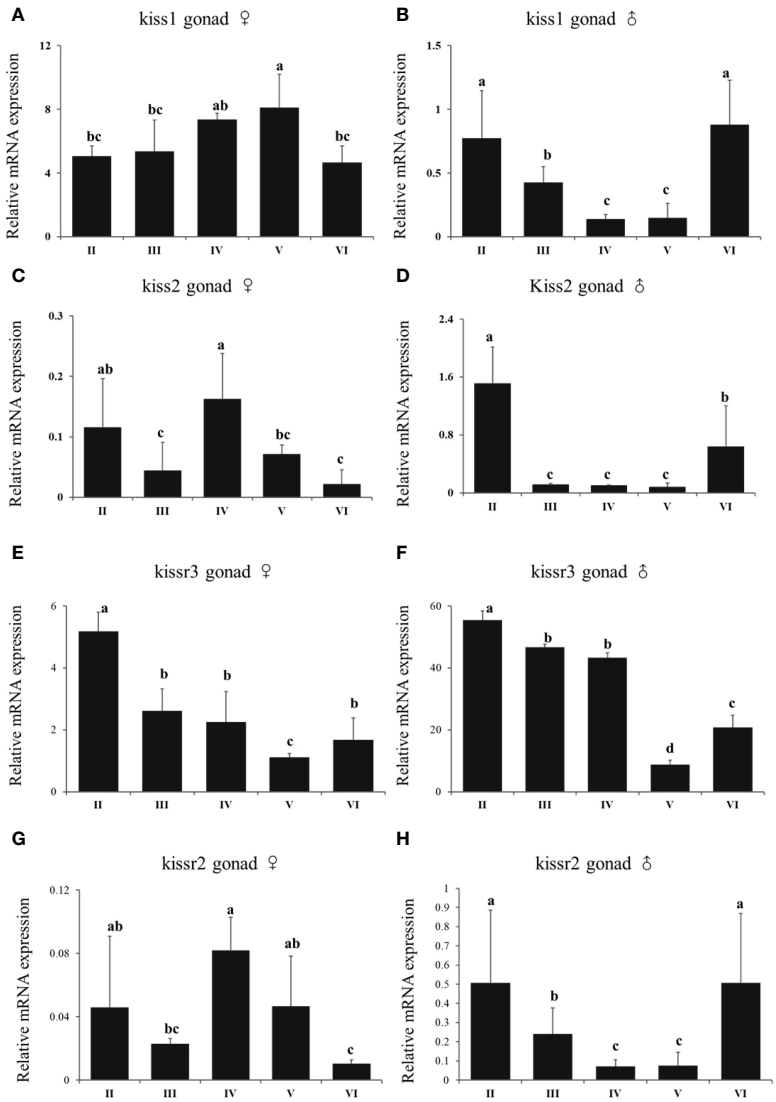


FIGURE 9 | Expression profiles of *kiss/kissr* genes throughout the reproductive cycle in the gonads of female **(A, C, E, G)** and male **(B, D, F, H)**. Error bars are presented as the mean±SEM. Different letters above bars represent statistical significance ($p < 0.05$) between two reproductive stages.

were seen in the pituitary of zebrafish injected with Kiss2 at 12h after injection (15, 47). Treatment with 0.1 and 0.5 μg/g of Kiss significantly increased the GTH mRNA levels in the pituitary of female and male cinnamon clownfish at 2, 4 and 6 weeks after injection (42). Moreover, in goldfish, compared to 0.01μg/g, the high doses of 0.1 and 1.0μg/g significantly increased serum LH levels after 6h, indicating a dose-dependent manner (19). Therefore, it is concluded that the high dose increased relative mRNA levels (*fhsβ*, *lhβ* and *GTH*) at detection time. In the present study, Kiss1-10 and Kiss2-10 stimulated the expression of *lhβ*, *fhsβ*, and *gthα* in the pituitary of turbot, indicating that the Kiss/Kissr system could elevate GTH release in turbot, which is conserved among vertebrates (31). Different from previous studies was that the expression of *fhsβ* and *gthα* mRNA at high dose of 1000ng/g after 3h injection was lower than 100ng/g after 3h injection. It was proposed that the Kiss directly increased the expression of *lhβ*, *fhsβ*, and *gthα* in pituitary cells, but the effects depended on the time course and dose. And the investigation of an extended period in the Kiss to genes in pituitary cells would be done in future.

On the other hand, GnRH neurons projecting to the pineal gland, which is a key gland in transducing light signals in the circadian production of melatonin, provide support for the role of GnRH in the transduction of seasonal photoperiod changes (49). Taken together, these observations show the importance of the Kiss/Kissr system in the mechanisms regulating seasonal reproduction. Furthermore, recent studies in mammals and in seasonal breeders of fish have demonstrated that reproduction can be controlled *via* the Kiss/Kissr system. In European sea bass, manipulating the photoperiod affected the expression of *kiss1* and

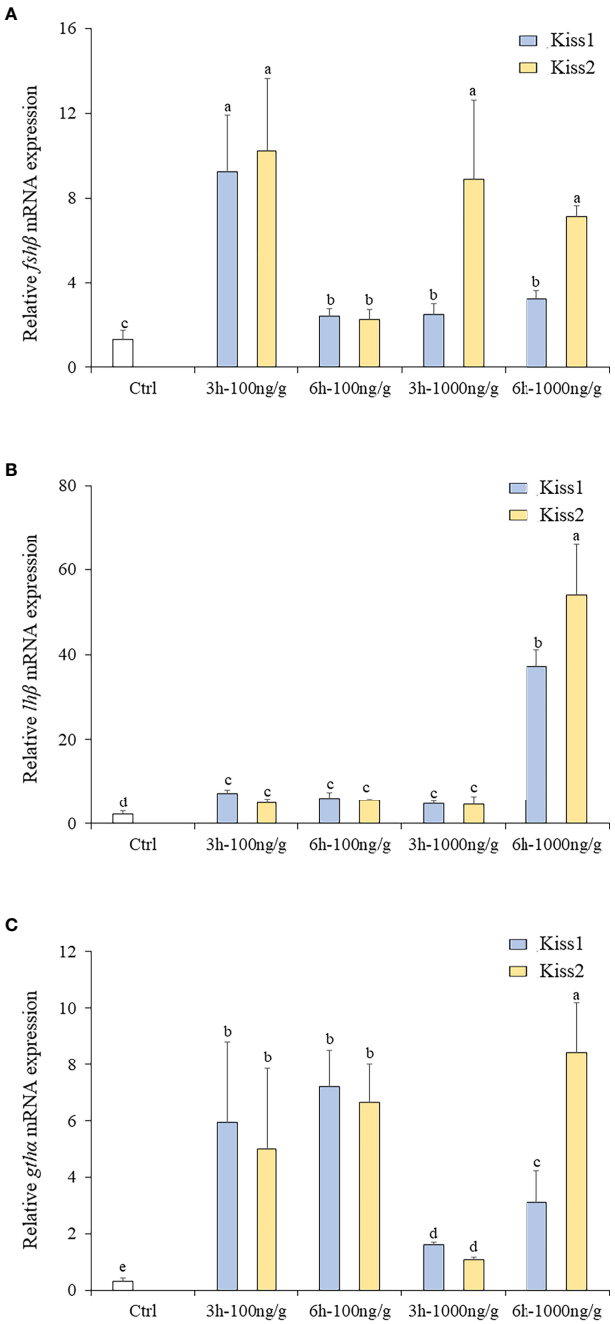


FIGURE 10 | Expression profiles of *fhsβ* **(A)**, *lhβ* **(B)** and *gthα* **(C)** in the pituitary of turbot by administration of Kiss1-10 and Kiss2-10 at 3 and 6 h post-injection. Error bars are presented as the mean ± SEM. Different letters above bars represent statistical significance ($p < 0.05$).

gnrh2 in the forebrain-midbrain to activate the reproductive axis (49). Increased *kiss2* and *kissr2* gene expression in Atlantic cod also showed the potential role of the Kiss/Kissr system in the entrainment of reproduction (49, 62). Similarly, changing the photoperiod in the present study directly regulated the seasonal gonadal development and modulated the expression of the *kiss/kissr* genes. However, further investigation is needed to better characterize the mechanism by which the Kiss/Kissr system affects reproduction in turbot.

In conclusion, the present study investigated the Kiss/Kissr system and the changes in the expression of *kiss* and *kissr* genes during the reproductive cycle in turbot. All four highly conserved kisspeptin genes (*kiss1*, *kiss2*, *kissr2*, and *kissr3*) of the Kiss/Kissr system were present in this species, which was different from other Pleuronectiformes species. The expression levels of *kiss* and *kissr* in HPG during the reproductive stages and in the pituitary gland after administration of synthetic Kiss1-10 and Kiss2-10 suggest the positive involvement of the Kiss/Kissr system in controlling the seasonal reproductive cycle in turbot.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**. Further inquiries can be directed to the corresponding author.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research and Ethics Committees of Qingdao Agricultural University.

AUTHOR CONTRIBUTIONS

CZ: Performed experiments, analyzed the data, wrote, and approved the manuscript; BW: Performed experiments, analyzed the data, edited and approved the manuscript; YL, CF, SX, WW, and QL: Performed experiments and reviewed the manuscript; JL: Designed research, edited and approved the manuscript. All authors contributed to the article and approved the submitted version.

FUNDING

This work was supported by National Natural Science Foundation of China (31802319, 32072949 and 31702321), Advanced Talents Foundation of QAU (6631119055), Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) (GML2019ZD0402), China Agriculture Research System (CARS-47).

SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.883608/full#supplementary-material

REFERENCES

1. Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, et al. Kiss-1, a Novel Human Malignant Melanoma Metastasis-Suppressor Gene. *J Natl Cancer Institute* (1996) 88(23):1731–7. doi: 10.1093/jnci/88.23.1731
2. De Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic Hypogonadism Due to Loss of Function of the Kiss1-Derived Peptide Receptor Gpr54. *Proc Natl Acad Sci USA* (2003) 100 (19):10972–6. doi: 10.1073/pnas.1834399100
3. Elizur A. The Kiss1/Gpr54 System in Fish. *Peptides* (2009) 30(1):164–70. doi: 10.1016/j.peptides.2008.08.018
4. Kauffman AS, Clifton DK, Steiner RA. Emerging Ideas About Kisspeptin–Gpr54 Signaling in the Neuroendocrine Regulation of Reproduction. *Trends Neurosci* (2007) 30(10):504–11. doi: 10.1016/j.tins.2007.08.001
5. Roa J, Tena-Sempere M. Kiss-1 System and Reproduction: Comparative Aspects and Roles in the Control of Female Gonadotropic Axis in Mammals. *Gen Comp Endocr* (2007) 153(1):132–40. doi: 10.1016/j.ygcen.2007.01.026
6. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS Jr., Shagoury JK, et al. The Gpr54 Gene as a Regulator of Puberty. *New Engl J Med* (2003) 349(17):1614–27. doi: 10.1056/NEJMoa035322
7. Pasquier J, Kamech N, Lafont A-G, Vaudry H, Rousseau K, Dufour S. Kisspeptin/Kisspeptin Receptors. *J Mol Endocrinol* (2014) 52(3):T101–T17. doi: 10.1530/jme-13-0224
8. Lee DK, Nguyen T, O'Neill GP, Cheng R, Liu Y, Howard AD, et al. Discovery of a Receptor Related to the Galanin Receptors. *FEBS Lett* (1999) 446(1):103–7. doi: 10.1016/S0014-5793(99)00009-5
9. Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, et al. The Kiss-1 Receptor Gpr54 Is Essential for the Development of the Murine Reproductive System. *Biochem Bioph Res Co* (2003) 312(4):1357–63. doi: 10.1016/j.bbrc.2003.11.066
10. Navarro VM, Castellano JM, Fernández-Fernández R, Tovar S, Roa J, Mayen A, et al. Effects of Kiss-1 Peptide, the Natural Ligand of Gpr54, on Follicle-Stimulating Hormone Secretion in the Rat. *Endocrinology* (2005) 146 (4):1689–97. doi: 10.1210/en.2004-1353
11. Roseweir AK, Millar RP. The Role of Kisspeptin in the Control of Gonadotrophin Secretion. *Hum Reprod Update* (2008) 15(2):203–12. doi: 10.1093/humupd/dmn058
12. Parhar IS, Ogawa S, Sakuma Y. Laser-Captured Single Digoxigenin-Labeled Neurons of Gonadotropin-Releasing Hormone Types Reveal a Novel G Protein-Coupled Receptor (Gpr54) During Maturation in Cichlid Fish. *Endocrinology* (2004) 145(8):3613–8. doi: 10.1210/en.2004-0395
13. van Aerle R, Kille P, Lange A, Tyler CR. Evidence for the Existence of a Functional Kiss1/Kiss1 Receptor Pathway in Fish. *Peptides* (2008) 29(1):57–64. doi: 10.1016/j.peptides.2007.10.018
14. Biran J, Ben-Dor S, Levavi-Sivan B. Molecular Identification and Functional Characterization of the Kisspeptin/Kisspeptin Receptor System in Lower Vertebrates. *Biol Reprod* (2008) 79(4):776–86. doi: 10.1095/biolreprod.107.066266
15. Kitahashi T, Ogawa S, Parhar IS. Cloning and Expression of Kiss2 in the Zebrafish and Medaka. *Endocrinology* (2009) 150(2):821–31. doi: 10.1210/en.2008-0940
16. Kanda S, Akazome Y, Matsunaga T, Yamamoto N, Yamada S, Tsukamura H, et al. Identification of Kiss-1 Product Kisspeptin and Steroid-Sensitive Sexually Dimorphic Kisspeptin Neurons in Medaka (*Oryzias Latipes*). *Endocrinology* (2008) 149(5):2467–76. doi: 10.1210/en.2007-1503
17. Mitani Y, Kanda S, Akazome Y, Zempo B, Oka Y. Hypothalamic Kiss1 But Not Kiss2 Neurons Are Involved in Estrogen Feedback in Medaka (*Oryzias Latipes*). *Endocrinology* (2010) 151(4):1751–9. doi: 10.1210/en.2009-1174
18. Li S, Zhang Y, Liu Y, Huang X, Huang W, Lu D, et al. Structural and Functional Multiplicity of the Kisspeptin/Gpr54 System in Goldfish (*Carassius Auratus*). *J Endocrinol* (2009) 201(3):407–18. doi: 10.1677/joe-09-0016
19. Yang B, Jiang Q, Chan T, Ko WK, Wong AO. Goldfish Kisspeptin: Molecular Cloning, Tissue Distribution of Transcript Expression, and Stimulatory Effects on Prolactin, Growth Hormone and Luteinizing Hormone Secretion and Gene Expression *Via* Direct Actions at the Pituitary Level. *Gen Comp Endocrinol* (2010) 165(1):60–71. doi: 10.1016/j.ygcen.2009.06.001
20. Felip A, Zanuy S, Pineda R, Pinilla L, Carrillo M, Tena-Sempere M, et al. Evidence for Two Distinct Kiss Genes in Non-Placental Vertebrates That Encode Kisspeptins With Different Gonadotropin-Releasing Activities in Fish and Mammals. *Mol Cell Endocrinol* (2009) 312(1-2):61–71. doi: 10.1016/j.mce.2008.11.017
21. Felip A, Espigares F, Zanuy S, Gomez A. Differential Activation of Kiss Receptors by Kiss1 and Kiss2 Peptides in the Sea Bass. *Reproduction* (2015) 150(3):227–43. doi: 10.1530/REP-15-0204
22. Shi Y, Zhang Y, Li S, Liu Q, Lu D, Liu M, et al. Molecular Identification of the Kiss2/Kiss1ra System and Its Potential Function During 17alpha-Methyltestosterone-Induced Sex Reversal in the Orange-Spotted Grouper. *Epinephelus Coioides*. *Biol Reprod* (2010) 83(1):63–74. doi: 10.1095/biolreprod.109.080044
23. Selvaraj S, Ohga H, Nyuji M, Kitano H, Nagano N, Yamaguchi A, et al. Subcutaneous Administration of Kiss1 Pentadecapeptide Accelerates Spermatogenesis in Prepubertal Male Chub Mackerel (*Scomber Japonicus*). *Comp Biochem Phys A* (2013) 166(2):228–36. doi: 10.1016/j.cbpa.2013.06.007
24. Selvaraj S, Kitano H, Fujinaga Y, Ohga H, Yoneda M, Yamaguchi A, et al. Molecular Characterization, Tissue Distribution, and Mrna Expression Profiles of Two Kiss Genes in the Adult Male and Female Chub Mackerel (Scomber Japonicus) During Different Gonadal Stages. *Gen Comp Endocrinol* (2010) 169(1):28–38. doi: 10.1016/j.ygcen.2010.07.011
25. Nocillado JN, Zohar Y, Biran J, Levavi-Sivan B, Elizur A. Chronic Kisspeptin Administration Stimulated Gonadal Development in Pre-Pubertal Male Yellowtail Kingfish (Seriola Lalandi; Perciformes) During the Breeding and Non-Breeding Season. *Gen Comp Endocrinol* (2013) 191:168–76. doi: 10.1016/j.ygcen.2013.06.005
26. Nocillado JN, Biran J, Lee YY, Levavi-Sivan B, Mechaly AS, Zohar Y, et al. The Kiss2 Receptor (Kiss2r) Gene in Southern Bluefin Tuna, Thunnus Maccoyii and in Yellowtail Kingfish, Seriola Lalandi - Functional Analysis and Isolation of Transcript Variants. *Mol Cell Endocrinol* (2012) 362(1-2):211–20. doi: 10.1016/j.mce.2012.06.024
27. Pasquier J, Lafont AG, Denis F, Lefranc B, Dubessy C, Moreno-Herrera A, et al. Eel Kisspeptins: Identification, Functional Activity, and Inhibition on Both Pituitary Lh and Gnrh Receptor Expression. *Front Endocrinol (Lausanne)* (2018) 8:3389/fendo.2017.00353. doi: 10.3389/fendo.2017.00353
28. Pasquier J, Lafont A-G, Jeng S-R, Morini M, Dirks R, van den Thillart G, et al. Multiple Kisspeptin Receptors in Early Osteichthyans Provide New Insights Into the Evolution of This Receptor Family. *PLoS One* (2012) 7(11):e48931. doi: 10.1371/journal.pone.0048931
29. Mechaly AS, Tovar Bohorquez MO, Mechaly AE, Suku E, Perez MR, Giorgetti A, et al. Evidence of Alternative Splicing as a Regulatory Mechanism for Kissr2 in Pejerrey Fish. *Front Endocrinol (Lausanne)* (2018) 9:3389/fendo.2018.00604. doi: 10.3389/fendo.2018.00604
30. Tovar Bohorquez MO, Mechaly AS, Hughes LC, Campanella D, Orti G, Canosa LF, et al. Kisspeptin System in Pejerrey Fish (*Odontesthes Bonariensis*). Characterization and Gene Expression Pattern During Early Developmental Stages. *Comp Biochem Physiol A Mol Integr Physiol* (2017) 204:146–56. doi: 10.1016/j.cbpa.2016.11.014
31. Zhang H, Zhang B, Qin G, Li S, Lin Q. The Roles of the Kisspeptin System in the Reproductive Physiology of the Lined Seahorse (*Hippocampus Erectus*), an Ovoviviparous Fish With Male Pregnancy. *Front Neurosci* (2018) 12:3389/fnins.2018.00940. doi: 10.3389/fnins.2018.00940
32. Zhang H, Zhang YY, Guo Y, Zhang X, Wang Q, Liu XC, et al. Kiss2 But Not Kiss1 Is Involved in the Regulation of Social Stress on the Gonad Development in Yellowtail Clownfish, *Amphiprion Clarkii*. *Gen Comp Endocr* (2020) 298:113551. doi: 10.1016/j.ygcen.2020.113551
33. Mechaly AS, Vinas J, Piferrer F. Identification of Two Isoforms of the Kisspeptin-1 Receptor (Kiss1r) Generated by Alternative Splicing in a Modern Teleost, the Senegalese Sole (*Solea Senegalensis*). *Biol Reprod* (2009) 80(1):60–9. doi: 10.1095/biolreprod.108.072173
34. Mechaly AS, Vinas J, Murphy C, Reith M, Piferrer F. Gene Structure of the Kiss1 Receptor-2 (Kiss1r-2) in the Atlantic Halibut: Insights Into the Evolution and Regulation of Kiss1r Genes. *Mol Cell Endocrinol* (2010) 317 (1-2):78–89. doi: 10.1016/j.mce.2009.11.005
35. Song H, Wang M, Wang Z, Yu H, Wang Z, Zhang Q. Identification and Characterization of Kiss2 and Kissr2 Homologs in Paralichthys Olivaceus. *Fish Physiol Biochem* (2016) 42(4):1073–92. doi: 10.1007/s10695-016-0199-1

36. Wang B, Liu Q, Liu X, Xu Y, Shi B. Molecular Characterization of Kiss2 Receptor and *In Vitro* Effects of Kiss2 on Reproduction-Related Gene Expression in the Hypothalamus of Half-Smooth Tongue Sole (*Cynoglossus Semilaevis*). *Gen Comp Endocr* (2017) 249:55–63. doi: 10.1016/j.ygcen.2017.04.006
37. Wang B, Liu Q, Liu X, Xu Y, Song X, Shi B. Molecular Characterization of Kiss2 and Differential Regulation of Reproduction-Related Genes by Sex Steroids in the Hypothalamus of Half-Smooth Tongue Sole (*Cynoglossus Semilaevis*). *Comp Biochem Physiol A Mol Integr Physiol* (2017) 213:46–55. doi: 10.1016/j.cbpa.2017.08.003
38. Shahi N, Singh AK, Sahoo M, Mallik SK, Thakuria D. Molecular Cloning, Characterization and Expression Profile of Kisspeptin1 and Kisspeptin1 Receptor at Brain-Pituitary-Gonad (Bpg) Axis of Golden Mahseer, Tor Putitora (*Hamilton*, 1822) During Gonadal Development. *Comp Biochem Phys B* (2017) 205:13–29. doi: 10.1016/j.cbpb.2016.11.010
39. Saha A, Pradhan A, Sengupta S, Nayak M, Samanta M, Sahoo L, et al. Molecular Characterization of Two Kiss Genes and Their Expression in Rohu (*Labeo Rohita*) During Annual Reproductive Cycle. *Comp Biochem Phys B* (2016) 191:135–45. doi: 10.1016/j.cbpb.2015.10.008
40. Alvarado MV, Servili A, Moles G, Gueguen MM, Carrillo M, Kah O, et al. Actions of Sex Steroids on Kisspeptin Expression and Other Reproduction-Related Genes in the Brain of the Teleost Fish European Sea Bass. *J Exp Biol* (2016) 219(21):3353–65. doi: 10.1242/jeb.137364
41. Zmora N, Stubblefield JD, Wong T-T, Levavi-Sivan B, Millar RP, Zohar Y. Kisspeptin Antagonists Reveal Kisspeptin 1 and Kisspeptin 2 Differential Regulation of Reproduction in the Teleost, *Morone Saxatilis*. *Biol Reprod* (2015) 93(3):76. doi: 10.1095/biolreprod.115.131870
42. Kim NN, Shin HS, Choi YJ, Choi CY. Kisspeptin Regulates the Hypothalamus-Pituitary-Gonad Axis Gene Expression During Sexual Maturation in the Cinnamon Clownfish, *Amphiprion Melanopus*. *Comp Biochem Phys B* (2014) 168:19–32. doi: 10.1016/j.cbpb.2013.11.002
43. Simonneaux V, Ansel L, Revel FG, Klosen P, Pévet P, Mikkelsen JD. Kisspeptin and the Seasonal Control of Reproduction in Hamsters. *Peptides* (2009) 30(1):146–53. doi: 10.1016/j.peptides.2008.06.006
44. Tang H, Liu Y, Luo D, Ogawa S, Yin Y, Li S, et al. The Kiss/Kissr Systems Are Dispensable for Zebrafish Reproduction: Evidence From Gene Knockout Studies. *Endocrinology* (2015) 156(2):589–99. doi: 10.1210/en.2014-1204
45. Nakajo M, Kanda S, Karigo T, Takahashi A, Akazome Y, Uenoyama Y, et al. Evolutionally Conserved Function of Kisspeptin Neuronal System Is Nonreproductive Regulation as Revealed by Nonmammalian Study. *Endocrinology* (2018) 159(1):163–83. doi: 10.1210/en.2017-00808
46. Robertson JL, Clifton DK, de la Iglesia HO, Steiner RA, Kauffman AS. Circadian Regulation of Kiss1 Neurons: Implications for Timing the Preovulatory Gonadotropin-Releasing Hormone/Luteinizing Hormone Surge. *Endocrinology* (2009) 150(8):3664–71. doi: 10.1210/en.2009-0247
47. Carnevali O, Gioacchini G, Maradonna F, Olivotto I, Migliarini B. Melatonin Induces Follicle Maturation in *Danio Rerio*. *PLoS One* (2011) 6(5):e19978. doi: 10.1371/journal.pone.0019978
48. Cowan M, Davie A, Migaud H. Photoperiod Effects on the Expression of Kisspeptin and Gonadotropin Genes in Atlantic Cod, *Gadus Morhua*, During First Maturation. *Comp Biochem Phys A* (2012) 163(1):82–94. doi: 10.1016/j.cbpa.2012.05.191
49. Espigares F, Rocha A, Gómez A, Carrillo M, Zanuy S. Photoperiod Modulates the Reproductive Axis of European Sea Bass Through Regulation of Kiss1 and Gnrh2 Neuronal Expression. *Gen Comp Endocr* (2017) 240:35–45. doi: 10.1016/j.ygcen.2016.09.007
50. Ando H, Ogawa S, Shahjahan M, Ikegami T, Doi H, Hattori A, et al. Diurnal and Circadian Oscillations in Expression of Kisspeptin, Kisspeptin Receptor and Gonadotrophin-Releasing Hormone 2 Genes in the Grass Puffer, a Semilunar-Synchronised Spawner. *J Neuroendocrinol* (2014) 26(7):459–67. doi: 10.1111/jne.12165
51. Imsland AK, Gunnarsson S, Roth B, Foss A, Le Deuff S, Norberg B, et al. Long-Term Effect of Photoperiod Manipulation on Growth, Maturation and Flesh Quality in Turbot. *Aquaculture* (2013) 416-417:152–60. doi: 10.1016/j.aquaculture.2013.09.005
52. Imsland AK, Folkvord A, Stefansson SO. Growth, Oxygen Consumption and Activity of Juvenile Turbot (*Scophthalmus Maximus* L.) Reared Under Different Temperatures and Photoperiods. *Netherlands J Sea Res* (1995) 34 (1):149–59. doi: 10.1016/0077-7579(95)90023-3
53. Foss A, Imsland AK, Roth B, Schram E, Stefansson SO. Effects of Chronic and Periodic Exposure to Ammonia on Growth and Blood Physiology in Juvenile Turbot (*Scophthalmus Maximus*). *Aquaculture* (2009) 296(1):45–50. doi: 10.1016/j.aquaculture.2009.07.013
54. Imsland AK, Dragsnes M, Stefansson SO. Exposure to Continuous Light Inhibits Maturation in Turbot (*Scophthalmus Maximus*). *Aquaculture* (2003) 219(1):911–9. doi: 10.1016/S0044-8486(03)00034-6
55. Imsland AK, Folkvord A, Jónsdóttir ÓDB, Stefansson SO. Effects of Exposure to Extended Photoperiods During the First Winter on Long-Term Growth and Age at First Maturity in Turbot (*Scophthalmus Maximus*). *Aquaculture* (1997) 159(1):125–41. doi: 10.1016/S0044-8486(97)00152-X
56. Xue R, Wang XY, Xu SH, Liu YF, Feng CC, Zhao CY, et al. Expression Profile and Localization of Vitellogenin Mrna and Protein During Ovarian Development in Turbot (*Scophthalmus Maximus*). *Comp Biochem Phys B* (2018) 226:53–63. doi: 10.1016/j.cbpb.2018.08.002
57. Zhao C, Xu S, Liu Y, Feng C, Xiao Y, Wang Y, et al. Changes of Melatonin and its Receptors in Synchronizing Turbot (*Scophthalmus Maximus*) Seasonal Reproduction and Maturation Rhythm. *Acta Oceanol. Sin* (2022) 41:84–98. doi: 10.1007/s13131-021-1923-y
58. Lee YR, Tsunekawa K, Moon MJ, Um HN, Hwang J-I, Osugi T, et al. Molecular Evolution of Multiple Forms of Kisspeptins and Gpr54 Receptors in Vertebrates. *Endocrinology* (2009) 150(6):2837–46. doi: 10.1210/en.2008-1679
59. Selvaraj S, Kitano H, Ohga H, Yamaguchi A, Matsuyama M. Expression Changes of Mrnas Encoding Kisspeptins and Their Receptors and Gonadotropin-Releasing Hormones During Early Development and Gonadal Sex Differentiation Periods in the Brain of Chub Mackerel (*Scomber Japonicus*). *Gen Comp Endocr* (2015) 222:20–32. doi: 10.1016/j.ygcen.2014.09.019
60. Ogawa S, Ng KW, Xue X, Ramadasan PN, Sivalingam M, Li S, et al. Thyroid Hormone Upregulates Hypothalamic Kiss2 Gene in the Male Nile Tilapia, *Oreochromis Niloticus*. *Front Endocrinol* (2013) 4:3389/fendo.2013.00184. doi: 10.3389/fendo.2013.00184
61. Escobar S, Felip A, Gueguen M-M, Zanuy S, Carrillo M, Kah O, et al. Expression of Kisspeptins in the Brain and Pituitary of the European Sea Bass (*Dicentrarchus Labrax*). *J Comp Neurol* (2013) 521(4):933–48. doi: 10.1002/cne.23211
62. Dufour S, Quérat B, Tostivint H, Pasqualini C, Vaudry H, Rousseau K. Origin and Evolution of the Neuroendocrine Control of Reproduction in Vertebrates, With Special Focus on Genome and Gene Duplications. *Physiol Rev* (2019) 100(2):869–943. doi: 10.1152/physrev.00009.2019
63. Mechaly AS, Viñas J, Piferrer F. Gene Structure Analysis of Kisspeptin-2 (Kiss2) in the Senegalese Sole (*Solea Senegalensis*): Characterization of Two Splice Variants of Kiss2, and Novel Evidence for Metabolic Regulation of Kisspeptin Signaling in Non-Mammalian Species. *Mol Cell Endocrinol* (2011) 339(1):14–24. doi: 10.1016/j.mce.2011.03.004
64. Lü Z, Gong L, Ren Y, Chen Y, Wang Z, Liu L, et al. Large-Scale Sequencing of Flatfish Genomes Provides Insights Into the Polyphyletic Origin of Their Specialized Body Plan. *Nat Genet* (2021) 53(5):742–51. doi: 10.1038/s41588-021-00836-9
65. Ohga H, Fujinaga Y, Selvaraj S, Kitano H, Nyuji M, Yamaguchi A, et al. Identification, Characterization, and Expression Profiles of Two Subtypes of Kisspeptin Receptors in a Scombroid Fish (*Chub Mackerel*). *Gen Comp Endocr* (2013) 193:130–40. doi: 10.1016/j.ygcen.2013.07.016
66. Zmora N, Stubblefield J, Zulperi Z, Biran J, Levavi-Sivan B, Muñoz-Cueto JA, et al. Differential and Gonad Stage-Dependent Roles of Kisspeptin1 and Kisspeptin2 in Reproduction in the Modern Teleosts, *Morone Species*. *Biol Reprod* (2012) 86(6):177. doi: 10.1095/biolreprod.111.097667
67. Ogawa S, Ng KW, Ramadasan PN, Nathan FM, Parhar IS. Habenular Kiss1 Neurons Modulate the Serotonergic System in the Brain of Zebrafish. *Endocrinology* (2012) 153(5):2398–407. doi: 10.1210/en.2012-1062
68. Ohga H, Selvaraj S, Matsuyama M. The Roles of Kisspeptin System in the Reproductive Physiology of Fish With Special Reference to Chub Mackerel Studies as Main Axis. *Front Endocrinol* (2018) 9:3389/fendo.2018.00147. doi: 10.3389/fendo.2018.00147
69. Filby AL, van Aerle R, Duitman J, Tyler CR. The Kisspeptin/Gonadotropin-Releasing Hormone Pathway and Molecular Signaling of Puberty in Fish. *Biol Reprod* (2008) 78(2):278–89. doi: 10.1095/biolreprod.107.063420
70. Piet R, de Croft S, Liu X, Herbison AE. Electrical Properties of Kisspeptin Neurons and Their Regulation of Gnrh Neurons. *Front Neuroendocrinol* (2015) 36:15–27. doi: 10.1016/j.yfrne.2014.05.006

71. Lehman MN, Coolen LM, Goodman RL. Minireview: Kisspeptin/Neurokinin B/Dynorphin (Kndy) Cells of the Arcuate Nucleus: A Central Node in the Control of Gonadotropin-Releasing Hormone Secretion. *Endocrinology* (2010) 151(8):3479–89. doi: 10.1210/en.2010-0022.

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Zhao, Wang, Liu, Feng, Xu, Wang, Liu and Li. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 15 July 2022
doi: 10.3389/fendo.2022.917258

Kisspeptin Exhibits Stimulatory Effects on Expression of the Genes for Kisspeptin Receptor, GnRH1 and GTH Subunits in a Gonadal Stage-Dependent Manner in the Grass Puffer, a Semilunar-Synchronized Spawner

Md. Mahiuddin Zahangir[1], *Md. Shahjahan*[2] *and Hironori Ando*[1]*

[1] Marine Biological Station, Sado Island Center for Ecological Sustainability, Niigata University, Niigata, Japan, [2] Department of Fisheries Management, Bangladesh Agricultural University, Mymensingh, Bangladesh

OPEN ACCESS

Edited by:
Bin Wang,
Chinese Academy of Fishery Sciences (CAFS), China

Reviewed by:
Karine Rousseau,
Muséum National d'Histoire Naturelle, France
Takayoshi Ubuka,
Cancer Medical Service, Japan
José Antonio Paullada Salmerón,
University of Cádiz, Spain

***Correspondence:**
Hironori Ando
hando311@cc.niigata-u.ac.jp

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

Received: 11 April 2022
Accepted: 22 June 2022
Published: 15 July 2022

Citation:
Zahangir MM, Shahjahan M and Ando H (2022) Kisspeptin Exhibits Stimulatory Effects on Expression of the Genes for Kisspeptin Receptor, GnRH1 and GTH Subunits in a Gonadal Stage-Dependent Manner in the Grass Puffer, a Semilunar-Synchronized Spawner. Front. Endocrinol. 13:917258. doi: 10.3389/fendo.2022.917258

Kisspeptin has an important role in the regulation of reproduction by directly stimulating the secretion of gonadotropin-releasing hormone (GnRH) in mammals. In non-mammalian vertebrates, there are multiple kisspeptins (Kiss1 and Kiss2) and kisspeptin receptor types, and the two kisspeptins in teleosts have different effects depending on fish species and reproductive stages, serving reproductive and non-reproductive functions. In the grass puffer, *Takifugu alboplumbeus*, which has only a single pair of *kiss2* and *kissr2*, both genes display seasonal, diurnal, and circadian oscillations in expression in association with the periodic changes in reproductive functions. To elucidate the role of kisspeptin in this species, homologous kisspeptin peptide (gpKiss2) was administered at different reproductive stages (immature, mature and regressed) and the expression levels of the genes that constitute hypothalamo-pituitary-gonadal axis were examined in male grass puffer. gpKiss2 significantly elevated the expression levels of *kissr2* and *gnrh1* in the brain and *kissr2*, *fshb* and *lhb* in the pituitary of the immature and mature fish. No noticeable effect was observed for *kiss2*, *gnih*, *gnihr*, *gnrh2* and *gnrh3* in the brain and *gpa* in the pituitary. In the regressed fish, gpKiss2 was ineffective in stimulating the expression of the *gnrh1* and GTH subunit genes, while it stimulated and downregulated the *kissr2* expression in the brain and pituitary, respectively. The present results indicate that Kiss2 has a stimulatory role in the expression of GnRH1/GTH subunit genes by upregulating the *kissr2* expression in the brain and pituitary at both immature and mature stages, but this role is mostly ineffective at regressed stage in the grass puffer.

Keywords: GnIH, GnRH, GPR54, hypothalamus, gonadotropin, kisspeptin, puffer fish, reproduction

INTRODUCTION

The reproduction in vertebrates is regulated by the complex interaction among multiple environmental factors and the reproductive neuroendocrine system, which is composed of kisspeptin, gonadotropin-inhibitory hormone (GnIH) and gonadotropin-releasing hormone (GnRH) in the hypothalamus and two pituitary gonadotropins (GTHs), namely follicle-stimulating hormone (FSH) and luteinizing hormone (LH) (1–5). Kisspeptin, a member of the RFamide peptide family, is encoded by the KISS1/Kiss1 gene in mammals. *KISS1* was originally identified as a metastasis suppressor gene (6) and its product was found to be the ligand for an orphan G-protein coupled receptor, GPR54, later named Kiss1r (7). Since mutations in *GPR54* were found to be responsible for idiopathic hypogonadotropic hypogonadism (8, 9), kisspeptin has received considerable attention as a potential key player in the neuroendocrine regulation of reproduction. It is well established in mammals that kisspeptin regulates reproductive events including puberty and ovulation through stimulating GnRH secretion (10).

Unlike most mammals that possess a single kisspeptin gene (*Kiss1*), most teleosts possess two paralogous genes for kisspeptin (*kiss1* and *kiss2*) and kisspeptin receptor (*kissr1*, *kissr2, kissr3* and *kissr4*) and this increases the complexity of the kisspeptin system in this group (11, 12). It has been shown that the role of the two kisspeptins in the regulation of reproduction varies among fish species. The administration of Kiss1 increased the plasma LH levels in goldfish (13) and stimulated gonadal development in chub mackerel (14). In striped bass, Kiss1 showed stimulatory effects on the expression of *fshb* and ovarian development, whereas Kiss2 exhibited no effect (5). In contrast, Kiss2 has been shown to have a stimulatory role in reproduction with higher potency when compared to Kiss1 in zebrafish, medaka, chub mackerel, European seabass, Nile tilapia and largemouth seabass (16–21). In addition, the actions of the two kisspeptins are different depending on the stage of gonadal development. In yellowtail kingfish, although only Kiss1 but not Kiss2 stimulated the expression of *fshb* and *lhb* during the breeding and non-breeding seasons, Kiss2 was more effective than Kiss1 in stimulating gonadal development during the non-breeding period (22). In hybrid bass, only Kiss2 was effective in stimulating LH secretion at puberty, whereas both Kiss1 and Kiss2 induced LH release at recrudescence stage (23). Moreover, Kiss2 upregulated *gnrh1* and *kiss2r* expressions in the brain at prepuberty, while it downregulated the expression of *gnrh1* and *kiss2r* at the recrudescence stage (23).

In contrast to this stimulatory role in most species depending on reproductive stage, it has recently been shown that the kisspeptin system is dispensable for reproduction in zebrafish (24–26) and medaka (27) using gene knockout models. Further studies suggest that this is because of physiological compensation that takes place in mutant fish to maintain reproductive processes (26, 28, 29). Moreover, co-expression of kisspeptin receptor in GnRH neurons has been controversial: co-expression has been demonstrated in Nile tilapia (30), African cichlid (31), striped bass (23), chub mackerel (32) and zebrafish (33), whereas the lack of co-expression has been shown in medaka (27, 34) and European sea bass (35). Taken together, the functional role and mode of action of kisspeptins in the control of reproduction in fish are controversial and varies depending on fish species and also reproductive stages.

The grass puffer, *Takifugu alboplumbeus*, shows unique reproductive physiology that is synchronized with the seasonal, lunar, and daily cycles (36, 37). During the spawning season from spring to early summer, spawning occurs on seashore only for several days around the new and full moon days every two weeks (36, 38). Mature fish usually aggregate for spawning at certain seashore locations 2–3.5 hours before high tide in the evening, and spawning occurs for 1.5–2 hours during the rising tidal phase. Therefore, the timing of spawning is tightly connected with seasonal, lunar, and tidal cycles as well as daily rhythm. Since the time and place of the spawning are known, spawning fish can be easily caught by dip net at the spawning bed. Thus, the grass puffer provides a unique animal model for studying the neuroendocrine mechanisms underlying the seasonal, lunar, and circadian controls of reproduction in wild animals.

Grass puffer has only a single pair of genes for kisspeptin (*kiss2*) and kisspeptin receptor (*kissr2*) and previous studies on their expression patterns with respect to seasonal, daily and circadian changes have indicated the possible importance of the kisspeptin system in the semilunar-synchronized spawning. The expression levels of both *kiss2* and *kissr2* show distinct changes during reproductive cycle with a significant increase from the early stage of gametogenesis to the pre-spawning and spawning stages (36, 39). This seasonal variations of *kiss2* and *kissr2* expressions are certainly important for the spawning in early summer and have recently been found to be regulated by water temperature: high water temperature conditions in summer (over 28°C) suppress the *kiss2* and *kissr2* expressions, leading to the termination of spawning period (40). Furthermore, *kiss2* and *kissr2* exhibit diurnal and circadian variations in expression during the spawning period (41). Therefore, the kisspeptin system is considered to be important in the stimulation, maintenance, and cyclicity of the reproductive function in the grass puffer.

In the present study, the effects of Kiss2 administration on the expressions of the genes for various hormones and receptors that are comprised in the hypothalamus-pituitary-gonadal (HPG) axis (*kiss2* and *kissr2*; *gnih* and *gnihr*; three GnRH genes, namely *gnrh1*, *gnrh2* and *gnrh3*; three GTH subunit genes, namely *gpa*, *fshb* and *lhb*) were examined to elucidate the functional significance of Kiss2 in the male grass puffer. For the possible different roles of Kiss2 during gonadal development, the fish were treated with Kiss2 at three reproductive stages, namely immature, mature and regressed stages.

MATERIALS AND METHODS

Animals

Male fish with fully matured testes were collected from the spawning ground in Kawana, Shizuoka, Japan in June. Male fish with regressed testes were collected from the spawning

ground in Minamiise, Mie, Japan at the end of July. The mature and regressed fish were transferred to the Marine Biological Station, Niigata University, Niigata, Japan, and reared in indoor tanks (500 L) with the flow of seawater under natural photoperiod (LD 14:10) for two weeks. The water temperature during the acclimatization period was similar to that of sampling ground, which was 20°C for the matured fish and 25°C for the regressed fish. The fish were fed daily with commercial pellets equivalent to 1% body weight (BW) until the experiment was conducted. Since immature grass puffer is unavailable from wild source, juvenile fish were artificially reared at the Fisheries Laboratory, University of Tokyo, Shizuoka, Japan, and they were transferred to the Marine Biological Station, Niigata University and reared in indoor conditions for one year. They were reared in indoor tanks (500 L) under natural photoperiod. Water temperature ranged from 16°C to 24°C depending on seasons. The experiment using the 1-year-old fish with immature testes was conducted in May.

Kiss2 Administration

Grass puffer Kiss2 (gpKiss2, SKFNLNPFGLRFamide) (AB548304) was synthesized and dissolved in 0.9% NaCl and stored at -80°C until use. The fish were anesthetized in 0.008% tricaine methanesulfonate (MS222, Sigma-Aldrich, Tokyo, Japan) for 30 sec. and were immobilized with their ventral side upward. The immature and mature fish were intraperitoneally (ip) injected with gpKiss2 (0.1 and 1.0 μg/4 μl/g BW, n = 6–8) using a fine needle (25G, Terumo Corporation, Tokyo, Japan). Control groups of fish were injected with 0.9% NaCl. For the regressed fish, a preliminary experiment was conducted to examine the effect of gpKiss2 administration (0.01 and 0.1 μg/4 μl/g BW, n = 4) because there had been few reports on the effect of kisspeptin on animals at recrudescence stage. Since there was a trend toward increased *kiss2* and *gnih* expressions in the forebrain sample (telencephalon and diencephalon) at 0.01 μg/g BW in this preliminary experiment, the regressed fish were ip injected with gpKiss2 at 0.01 μg/g BW (n = 6–7) and the control fish were injected with 0.9% NaCl. In all experiments, the fish were injected at Zeitgeber time 2:00 (7:00 a.m.) and left in indoor tanks (100 L, n = 7–9 per tank) for 12 hrs.

Sample Collection

The fish were anesthetized in 0.03% MS222 and total length and BW were recorded. Brains and pituitaries were removed after decapitation and soaked in RNAlater (Ambion, Austin, TX) at 4°C overnight. Gonads were removed and weighed for the calculation of gonadosomatic index (GSI = gonad weight/BW × 100). In the next day, brains were trimmed to prepare the forebrain sample that contained the telencephalon and diencephalon. The forebrain and pituitary samples were then stored at -80°C until RNA extraction. All the experimental procedures were carried out following the approved guidance by the Institutional Animal Care and Use Committee of the Niigata University, Niigata, Japan. Total length, BW, and GSI of the fish are shown in **Table 1**.

TABLE 1 | Total length, body weight and gonadosomatic index (GSI) of fish samples. Values are presented as mean ± SEM.

Gonadal condition	No. of fish	Total length (cm)	Body weight (g)	GSI (%)
Immature	26	7.9 ± 0.1	8.7 ± 0.3	0.6 ± 0.1
Mature	24	14.9 ± 0.3	63.7 ± 0.3	16.3 ± 1.6
Regressed	13	13.4 ± 0.5	43.5 ± 5.1	1.1 ± 0.1

Quantitative Real-Time PCR Assay

Real-time PCR assays for *kiss2*, *kissr2, gnih*, *gnihr*, *gnrh1*, *gnrh2*, *gnrh3*, *gpa*, *fshb* and *lhb* were carried out as described previously (39, 42, 43). Briefly, total RNA was extracted from the forebrain and pituitary samples and treated with DNase I (Takara, Ohtsu, Japan). Total RNA (200 or 500 ng) was used for synthesis of first strand cDNA using MultiScribe Reverse Transcriptase (Applied Biosystem, USA) and an oligo $d(T)_{12\text{-}18}$ primer (2.5 μM) as per manufacturer's instructions. The profile for reverse transcription reaction was 25°C for 10 min, 48°C for 30 min and 95°C for 5 min. The absolute amount of mRNA was determined using sense reference RNA, which was synthesized *in vitro* by a MAXIscript kit (Ambion) according to the manufacturer's instruction and were serially diluted to $1 \times 10^3 - 1 \times 10^8$ copies/μl. The standard sense RNAs were reverse transcribed and used as standard cDNAs to establish a standard curve. Real-time PCR was carried out with a Thermal Cycler Dice Real Time System III (TP970, TaKaRa Bio, Japan). PCR reaction mixture (10 μl) contained 1 μl of standard sample cDNA, 0.4 μl of forward and reverse primers (**Table 2**) and 5 μl of TB Green Premix DimerEraser (TaKaRa, Ohtsu, Japan). Amplification was carried out at 95°C for 30 sec, followed by 40 cycles at 95°C for 5 sec, 60°C for 30 sec and 72°C for 30 sec. Specific amplification of each cDNA was verified by melting curve analysis and gel electrophoresis of the product.

Statistical Analysis

To compare the effects of gpKiss2 administration on gene expression among various genes at three gonadal stages, the relative mRNA values with respect to control (0 μg/g BW) are expressed as mean ± standard error of the mean (SEM). Data were analyzed by ANOVA followed by Tukey's HSD *post hoc* test to assess the statistically significant difference among different groups of immature and mature fish. Student t-test were performed to compare significant difference between the gpKiss2-injected and control groups in the regressed fish. Statistical significance was set at $p < 0.05$ unless described anywhere in the text. All statistical analyses were performed using SPSS Version 23.0 for windows (SPSS Inc., Chicago, IL).

RESULTS

Effect of gpKiss2 on the Expression of *kiss2, kissr2, gnih, gnihr* and *three gnrhs* in the Brain of Immature and Mature Fish

The administration of gpKiss2 did not alter the expression levels of *kiss2* in the immature and mature fish (**Figure 1A**).

TABLE 2 | Primers used in the real-time PCR assays in this study.

Primers	Nucleotide sequences
GnRH1-qPCR-F1	5′-CGGGAGTCTGATGTCACAGCTC-3′
GnRH1-qPCR-R1	5′-AACACTGACGACGACCGTGTCC-3′
GnRH2-qPCR-F1	5′-CAGGAGCTCACCTGTCCAAC-3′
GnRH2-qPCR-R1	5′-CTGCATTCTCCTGCTTCACAG-3′
GnRH3-qPCR-F1	5′-AAGCAAACAGGGTGATGGTG-3′
GnRH3-qPCR-R1	5′-CTGATGGTTGCCTCCAACTC-3′
GnIH-qPCR-F1	5′-TGATTCGTCTGTGCGAGGAC-3′
GnIH-qPCR-R1	5′-TCAGCAGCTGTGCATTGACC-3′
GnIH-R-qPCR-F1	5′-AAGATGCTCATCCTGGTGGC-3′
GnIH-R-qPCR-R1	5′-AGATCCACCTGGTCACTGTCC-3′
Kiss2-qPCR-F1	5′-GACCTTCAGGGACAACGAGGAC-3′
Kiss2-qPCR-R1	5′-ATGAAGCGCTTGCCAAAGC-3′
Kissr2-qPCR-F1	5′-TCCCGTTTCTGTTCAAGCACAAG-3′
Kissr2-qPCR-R1	5′-ATTGTTGTTGCGCTCCTCTGC-3′
GPα-qPCR-F1	5′-AAGGTGAGGAACCACACCGAG-3′
GPα-qPCR-R1	5′-AGCTCAAGGCCAGGATGAAC-3′
FSHβ-qPCR-F1	5′-ACACATTGAGGGCTGTCCAGTGG-3′
FSHβ-qPCR-R1	5′-TCCCCATTGAAGCGACTGCAG-3′
LHβ-qPCR-F1	5′-CACTTGGTGCAAACAAGCATC-3′
LHβ-qPCR-R1	5′-CAACTTAGAGCCACGGGGTAG-3′

However, the expression of *kissr2* was significantly stimulated in the gpKiss2-injected fish at both immature and mature stages when compared to the control (**Figure 1B**). In contrast, gpKiss2 did not show any noticeable effect on the expression of *gnih* and *gnihr* in the brain of both immature and mature fish (**Figures 2A**, **B**). gpKiss2 significantly elevated the expression of *gnrh1* in the brain of immature and mature fish (**Figure 3A**). In the case of *gnrh2* and *gnrh3*, gpKiss2 did not show any effect at both immature and mature stages at any doses (**Figures 3B**, **C**).

Effect of gpKiss2 on the Expression of *kissr2* and GTH Subunit Genes in the Pituitary of Immature and Mature Fish

In the pituitary, the mRNA levels of *kissr2* were significantly increased by the gpKiss2 administration in both immature and mature fish and gpKiss2 showed higher potency to stimulate the *kissr2* expression in the immature fish compared to the mature fish (fold stimulation: immature 2.54 vs mature 1.14, $p = 0.047$ by t-test, **Figure 4**). Similarly, gpKiss2 significantly stimulated the expression of *fshb* and *lhb* in the immature and mature fish (**Figures 5B**, **C**), whereas no noticeable changes were observed for *gpa* (**Figure 5A**).

Effect of gpKiss2 on the Expression of *kiss2, kissr2, gnih, gnihr* and *three gnrhs* in the Brain of Regressed Fish

In the brain of regressed fish, gpKiss2 did not show any change in the *kiss2* expression but significantly stimulated the expression of *kissr2* (**Figure 6A**). The expression levels of *gnih* and *gnihr* tended to be increased by the gpKiss2 administration, though these changes were not statistically significant (**Figure 6B**). There were no significant changes in the expression levels of three *gnrhs* after gpKiss2 administration in the regressed fish (**Figures 6C–E**).

Effect of gpKiss2 on the Expression of *kissr2* and GTH Subunit Genes in the Pituitary of Regressed Fish

In the pituitary of regressed fish, gpKiss2 significantly decreased the *kissr2* expression (**Figure 7A**). There was a trend toward increased *gpa* and *fshb* expression by the gpKiss2 administration (**Figures 7B**, **C**) and no noticeable changes were observed in the *lhb* expression (**Figure 7D**).

DISCUSSION

The effect of gpKiss2 administration on the expression of the genes for the HPG axis was examined at three gonadal stages to elucidate the functional importance of the kisspeptin system in the male grass puffer. gpKiss2 significantly stimulated the expression of *kissr2* and *gnrh1* in the brain and *kissr2*, *fshb* and *lhb* in the pituitary of the immature and mature fish, showing a stimulatory role of gpKiss2 in reproduction by activating the *kissr2* expression in the brain and pituitary so as to stimulate the expression of GTHs. In the regressed fish, however, gpKiss2 was ineffective in stimulating the GnRH1 and GTH subunit gene expression, suggesting that the stimulatory role of gpKiss2 is dependent on the gonadal stage. Moreover, gpKiss2 did not alter the expression of *gnih*, *gnihr*, *gnrh2* and *gnrh3* as well as its own gene, *kiss2*.

In mammals, kisspeptin has a strong stimulatory effect on GTH secretion from the pituitary and this is mainly mediated through the stimulatory action on GnRH secretion (10, 29). Kiss1r is co-localized with GnRH neurons in the hypothalamus and the direct interaction between kisspeptin and GnRH is primarily important in the control of ovulatory cycle in mammals. In the present study, the effect of gpKiss2 was evaluated on the expression of three GnRH genes, *gnrh1*, *gnrh2* and *gnrh3* at three gonadal stages. In some teleost fish including the grass puffer, three GnRH neuronal groups are diversified in regard to localization and function (1, 5). GnRH1 neurons are mainly localized in the preoptic area (POA) and have a hypophysiotropic role through stimulating the GTH secretion from the pituitary. GnRH2 neurons are localized in the midbrain tegmentum and involved in appetite-related reproductive function (44–46). GnRH3 neurons are localized in the terminal nerve ganglion-POA region and have neuromodulatory action related to sexual behavior (47, 48). In the present study, gpKiss2 significantly stimulated the expression of *gnrh1* but not *gnrh2* nor *gnrh3*, with a concomitant increase in *kissr2* expression in the brain (**Figures 1B**, **3A–C**). Stimulatory action of kisspeptin on kisspeptin receptor gene expression has been reported in largemouth bass (21), yellowtail kingfish (22), and hybrid bass (23). In contrast, inhibitory action of Kiss2 on kisspeptin receptor gene expression was reported in the tongue sole (49). Although the co-localization of kisspeptin receptor with GnRH1

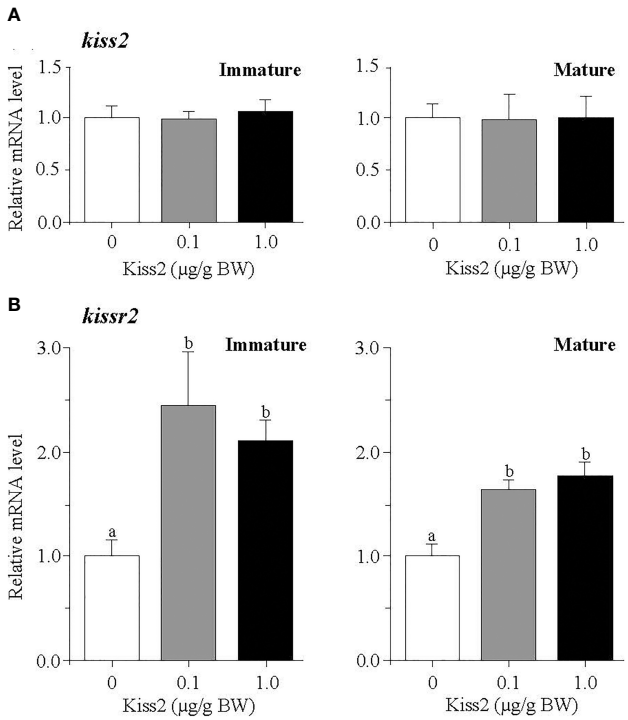


FIGURE 1 | Changes in the relative mRNA levels of *kiss2* **(A)** and *kissr2* **(B)** in the brain of grass puffer at immature and mature stages after intraperitoneal injection of gpKiss2 for the period of 12hrs. Values are presented as mean ± SEM (n = 6–8). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

neurons remain to be determined in the grass puffer, *kissr2* mRNA was previously localized in the magnocellular preoptic nucleus pars magnocellularis (PMm) in the POA (41), which is one of the major hypothalamic nuclei that consist of hypophysiotropic neurons including GnRH1 neurons in many teleost species [for reviews (1) and (5)]. Co-expression of kisspeptin receptor in GnRH1 neurons has been reported in many fishes such as Nile tilapia (30), African cichlid (31), striped bass (23), chub mackerel (32) and zebrafish (33). Moreover, Kiss2 was shown to stimulate *gnrh1* expression *via* kisspeptin receptor using brain slices in striped bass (50). These and the present results suggest that gpKiss2 may directly activate GnRH1 neurons and stimulate the secretion of FSH and LH from the pituitary. Nevertheless, it is also possible that gpKiss2 could act on GnRH1 neurons indirectly *via kissr2*-expressing cells that are adjacent to GnRH1 neurons. In zebrafish, Kiss2 neurons project widely in the brain including the POA, where GnRH3 neurons are contacted by Kiss2 fibers (51). The immunolocalization of Kiss2 receptor (Kiss2-R) further showed in this species that, in addition to a subset of preoptic GnRH3 neurons were Kiss2-R-immunoreactive (ir), Kiss2-R-ir processes were observed in proximity to some GnRH3 neurons that were negative to Kiss2-R, suggesting that there is a possibility of direct and indirect actions of Kiss2 (33). Such close apposition of kisspeptin receptor and preoptic GnRH neurons has been reported in striped bass (23), European sea bass (35) and medaka (34). Taken as a whole, it is of considerable interest and importance to determine the neuroanatomical structure of Kiss2-R and GnRH1 neurons in grass puffer, which is currently under investigation.

In the pituitary, the augmented expressions of *fshb*, *lhb* as well as *kissr2* by gpKiss2 administration in the immature and mature fish (**Figures 4**, **5B, C**) suggest that gpKiss2 could have direct action on the regulation of pituitary. Stimulatory action of peripherally administered Kiss2 on GTH synthesis and release in the pituitary has been shown in goldfish (13), zebrafish (16), sea bass (17), hybrid bass (23), Nile tilapia (20), orange-spotted grouper (52) and seahorse (53). In addition, stimulatory effects of centrally administered Kiss2 on the *fshb* and *lhb* expressions were reported in chub mackerel (18). On the other hand, direct effect of Kiss2 has been examined using primary pituitary cultures, and the stimulatory effects were observed in sea bass (19) and zebrafish (54), whereas an inhibitory effect on *lhb* expression was reported in the primary culture of eel pituitary cells (55). The expression of kisspeptin receptor gene in the pituitary has been demonstrated in goldfish (13), European seabass (19), largemouth bass (21), yellowtail kingfish (22), zebrafish (54), eel (55), tongue sole (49), including grass puffer (39). In zebrafish, however, Kiss2-R (Kiss1Ra)-immunoreactivity was seen in corticotropes and melanotropes but not in gonadotropes (33), and the innervation of Kiss2 fibers to the *pars distalis* and also *pars nervosa* has been shown in striped bass

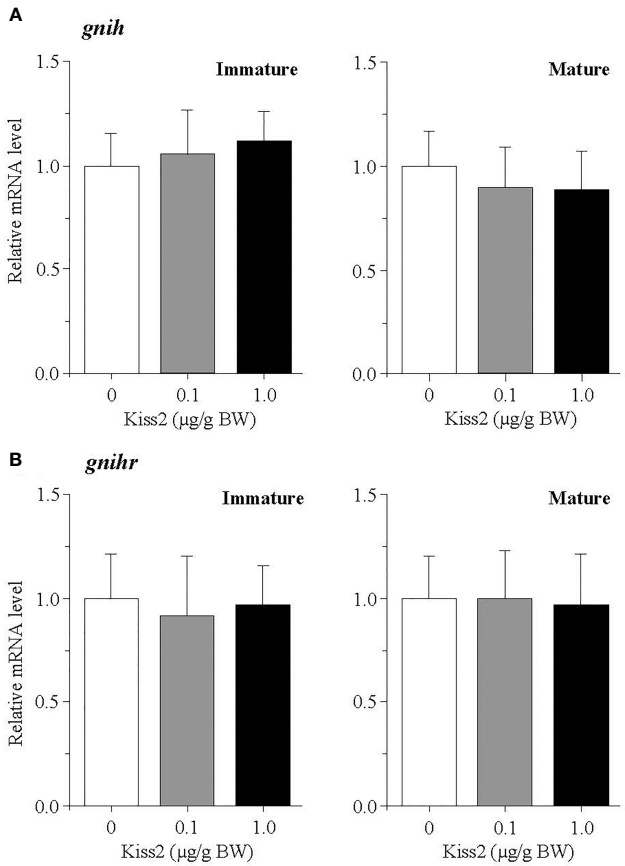


FIGURE 2 | Changes in the relative mRNA levels of *gnih* **(A)** and *gnihr* **(B)** in the brain of grass puffer at immature and mature stages after intraperitoneal injection of gpKiss2 for the period of 12hrs. Values are presented as mean ± SEM (n = 6–8).

(5), European sea bass (19, 35) and zebrafish (33, 54). These results suggest that Kiss2 may indirectly act on gonadotropes. Moreover, colocalization of Kiss2 cells with gonadotropes was observed in European sea bass (19). Taken together, these results indicate that Kiss2 has a local action on the pituitary in addition to the neuroendocrine action through preoptic GnRH neurons, and its action is most probably stimulatory in GTH secretion in many fishes including the grass puffer. Nevertheless, it should not be excluded in the present study that gonadal steroids are involved in the augmented expressions of *fshb* and *lhb* in response to the peripherally injected Kiss2 since kisspeptin and kisspeptin receptor are expressed in the gonads in many fishes including the grass puffer. The steroid feedback action on Kiss2 neurons and the pituitary gonadotropes needs be further examined.

The effect of gpKiss2 on the expression of *gnih* and *gnihr* was also examined in the present study. gpKiss2 did not show any significant changes in the expression of *gnih* and *gnihr* in the immature and mature fish (**Figure 2**). In the grass puffer, *gnih* and *gnihr* showed seasonal, daily and circadian variations like *kiss2* and *kissr2* (36, 37, 41, 42). GnIH administration experiments of grass puffer using a heterologous peptide (goldfish LPXRFamide) on the primary pituitary culture *in vitro* showed that goldfish LPXRFamide stimulated the expressions of *fshb* and *lhb* (42) as well as the genes for growth hormone and prolactin (56). These results suggest that GnIH is a multifunctional hypophysiotropic neurohormone, playing a stimulatory role in the control of reproduction in this species. Stimulatory and inhibitory effects of GnIH on reproduction have been reported in fish and the effects depend on the species and gonadal stages [for reviews (3) and (4)]. Although no changes in the *gnih* and *gnihr* expression in response to gpKiss2 administration were observed in the immature and mature fish, the *gnih* and *gnihr* mRNA levels tended to be higher in the gpKiss2-injected fish than the control fish at the regressed stage, suggesting that there may be a functional interaction between gpKiss2 and GnIH (**Figure 6B**). In the tongue sole, Kiss2 upregulated the expression of *gnih* and downregulated the expression of *gnihr* in the hypothalami in culture (49) and GnIH was shown to inhibit Kiss2-induced cAMP signaling in COS-7 cells transfected with both Kiss2-R and GnIHR expression vectors (57). It is tempting to speculate that Kiss2 and GnIH may have some functional interaction to stimulate GTH secretion in the grass puffer, in which GnIH positively

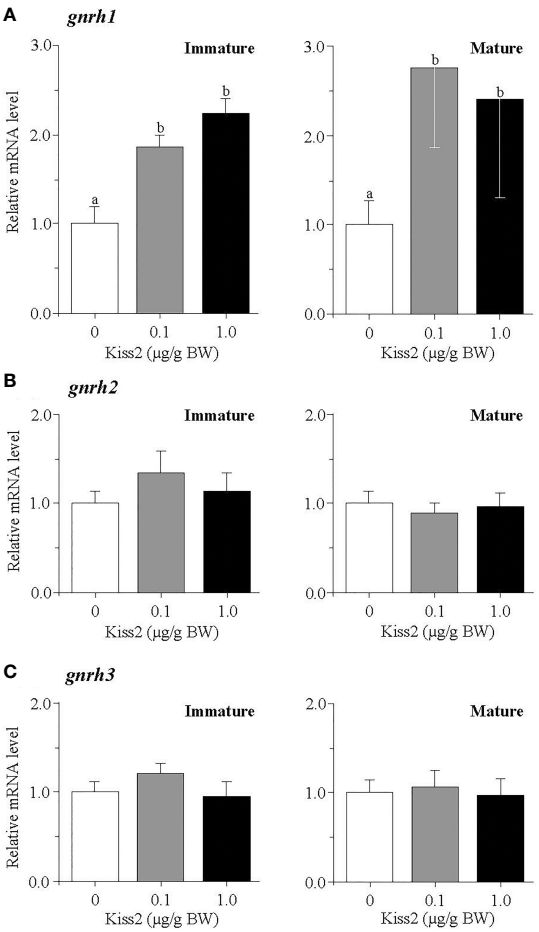


FIGURE 3 | Changes in the relative mRNA levels of *gnrh1* **(A)**, *gnrh2* **(B)** and *gnrh3* **(C)** in the brain of grass puffer at immature and mature stages after intraperitoneal injection of gpKiss2 for the period of 12hrs. Values are presented as mean ± SEM (n = 6–8). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

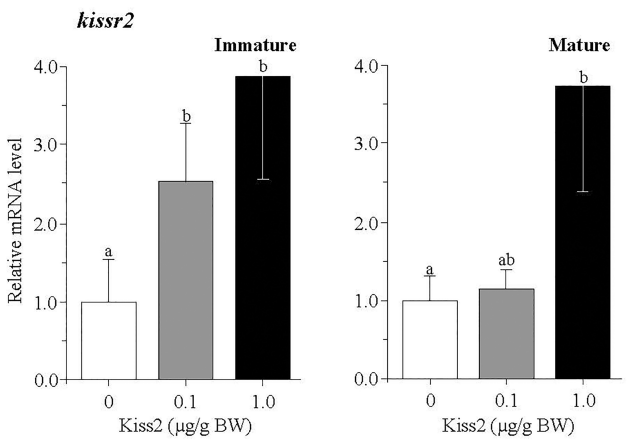


FIGURE 4 | Changes in the relative mRNA levels of *kissr2* in the pituitary of grass puffer at immature and mature stages after intraperitoneal injection of gpKiss2 for the period of 12hrs. Values are presented as mean ± SEM (n = 6–8). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

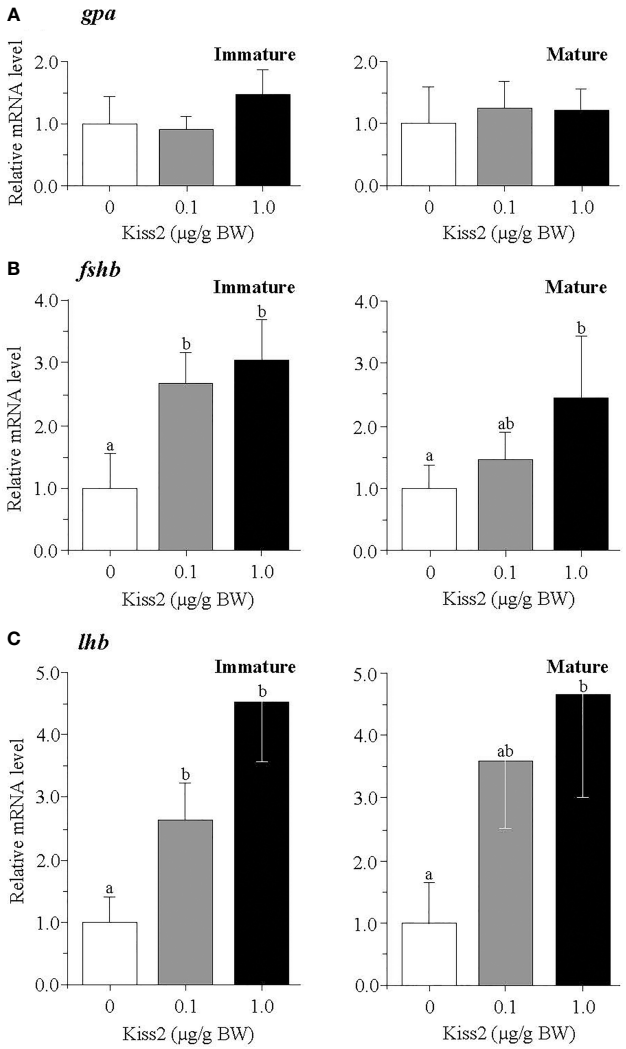


FIGURE 5 | Changes in the relative mRNA levels of *gpa* **(A)**, *fshb* **(B)** and *lhb* **(C)** in the pituitary of grass puffer at immature and mature stages after intraperitoneal injection of gpKiss2 for the period of 12hrs. Values are presented as mean ± SEM (n = 6–8). Values accompanied by different letters are statistically significantly different ($p < 0.05$).

regulates reproduction (37, 42, 56), and this needs to be further investigated.

In the regressed fish, there was no significant changes in the expression of *gnrh1*, *fshb* and *lhb* in the gpKiss2-injected fish (**Figures 6C**, **7C, D**), while *kissr2* expression was increased in the brain by the gpKiss2 injection but decreased in the pituitary (**Figures 6A**, **7A**). Although it is unclear why different response of *kissr2* expression was obtained in the brain and pituitary, the present results show that gpKiss2 is mostly ineffective in stimulating the expression of GnRH1/GTH subunit genes at the regressed stage. In yellowtail kingfish, Kiss1 administration significantly augmented the expression of pituitary *kiss2r* in the non-breeding season, whereas it was ineffective in increasing the *kiss2r* mRNA levels in the breeding season but was still effective in stimulating the *fshb* and *lhb* expressions (22). In hybrid bass, Kiss2 upregulated *gnrh1* and *kiss2r* expressions in the brain at prepuberty, while it downregulated the expression of *gnrh1* and *kiss2r* at the recrudescence stage (23). These results show that Kiss2 has a stimulatory role during gonadal maturation from prepubertal to mature stage in fish. In the grass puffer, the expression profiles of *kiss2* and *kissr2* during reproductive cycle show that both genes are activated at the early stage of gametogenesis and their expression levels are increased to the maximum levels at the breeding stages (36, 39). In chub mackerel, both *kiss1* and *kiss2* expressions were temporally increased at the onset of puberty and also during the breeding season (8). Increased expression of *kiss2* during the breeding season has also been reported in Senegalese sole (59) and European sea bass (60). Moreover, it has been reported that substantial increase in kisspeptin receptor gene expression occur

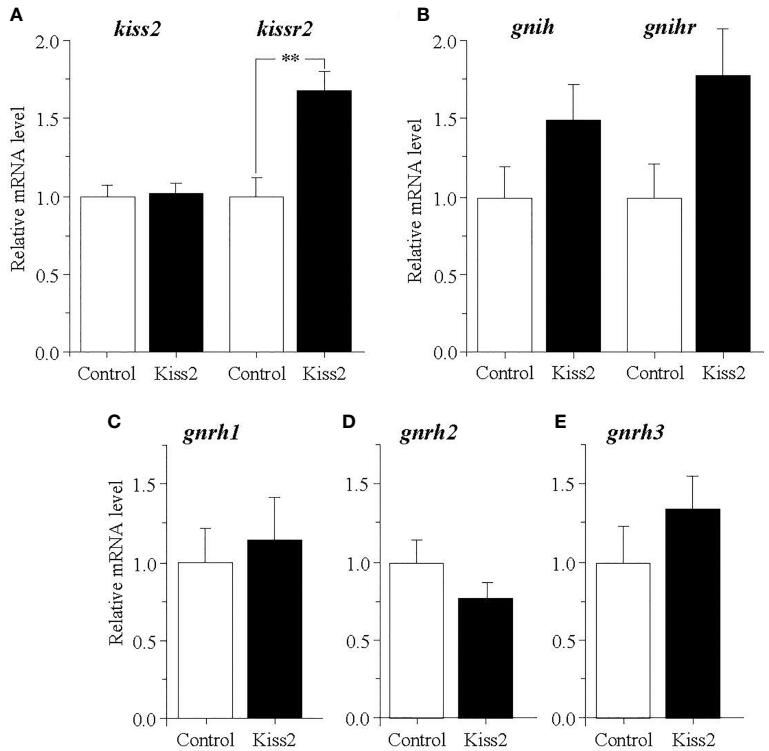


FIGURE 6 | Changes in the relative mRNA levels of *kiss2* and *kissr2* **(A)**, *gnih* and *gnihr* **(B)**, *gnrh1* **(C)**, *gnrh2* **(D)** and *gnrh3* **(E)** in the brain of grass puffer at regressed stage after intraperitoneal injection of gpKiss2 for the period of 12hrs. Values are presented as mean ± SEM (n = 6–7). Asterisks denotes a significant difference between the control and gpKiss2 injected fish (**, $p < 0.01$).

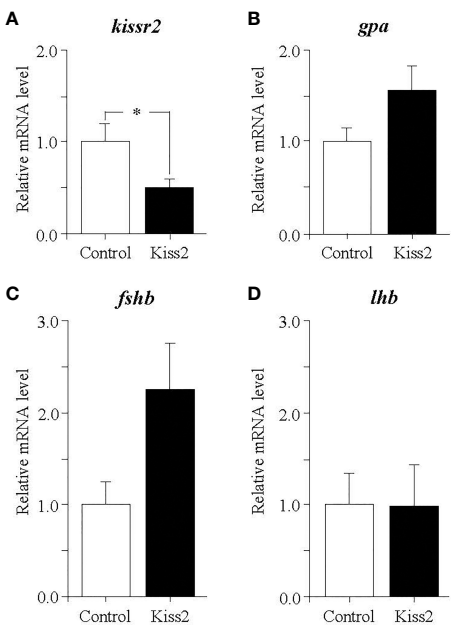


FIGURE 7 | Changes in the relative mRNA levels of *kissr2* **(A)**, *gpa* **(B)**, *fshb* **(C)** and *lhb* **(D)** in the pituitary of grass puffer at regressed stage after intraperitoneal injection of gpKiss2 for the period of 12hrs. Values are presented as mean ± SEM (n = 6–7). Asterisks denotes a significant difference between the control and gpKiss2 injected fish (*, $p < 0.05$).

before onset of puberty or during early puberty in zebrafish and medaka (16), Nile tilapia (20), cobia (61), grey mullet (62), flathead minnow (63), Atlantic halibut (64) and lined seahorse (53). Taken as a whole, these data show that the kisspeptin system is activated at the onset of puberty and also during the breeding stage in fish [see also review (2)]. The stimulatory effects of gpKiss2 on the expression of GnRH1/GTH subunit genes observed in the immature and mature fish in the present study are consistent with this notion.

In conclusion, gpKiss2 significantly stimulated the expression of *kissr2* and *gnrh1* in the brain and *kissr2*, *fshb* and *lhb* in the pituitary at immature and mature stages. The present results suggest that kisspeptin functions as a stimulatory neurohormone in the control of reproduction indirectly through preoptic GnRH1 neurons and directly by local action in the pituitary *via* the upregulation of *kissr2* expression. The stimulatory action of gpKiss2 on the expression of GnRH1/GTH genes depends on the reproductive stage, being mostly ineffective at the regressed stage in the grass puffer.

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 Institutional Animal Care and Use Committee of the Niigata University.

AUTHOR CONTRIBUTIONS

MMZ: design, experimentation, statistics, visualization, and writing. MS: design, experimentation, and statistics. HA: conception, design, writing, and supervision. All authors contributed to the article and approved the submitted version.

FUNDING

This study was supported by MEXT/JSPS KAKENHI grants (26·04068, 16H04812 and 20H03288 to HA).

ACKNOWLEDGMENTS

We are grateful to Mr. Tomonobu Uryu for his help in collecting wild grass puffer. We thank Dr. Kiyoshi Kikuchi and Mr. Naoki Mizuno (University of Tokyo) for providing juvenile grass puffer.

REFERENCES

1. Zohar Y, Muñoz-Cueto JA, Elizur A, Kah O. Neuroendocrinology of Reproduction in Teleost Fish. *Gen Comp Endocrinol* (2009) 165:438–55. doi: 10.1016/j.ygcen.2009.04.017
2. Mechaly AS, Viñas J, Piferrer F. The Kisspeptin System Genes in Teleost Fish, Their Structure and Regulation, With Particular Attention to the Situation in Pleuronectiformes. *Gen Comp Endocrinol* (2013) 188:258–68. doi: 10.1016/j.ygcen.2013.04.010
3. Muñoz-Cueto JA, Paullada- Salmerón JA, Aliaga-Guerrero M, Cowan ME, Parhar IS, Ubuka T. A Journey Through the Gonadotropin-Inhibitory Hormone System of Fish. *Front Endocrinol* (2017) 8:285. doi: 10.3389/fendo.2017.00285
4. Di Yorio MP, Muñoz-Cueto JA, Paullada-Salmerón JA, Somoza GM, Tsutsui K, Vissio PG. The Gonadotropin-Inhibitory Hormone: What We Know and What We Still Have to Learn From Fish. *Front Endocrinol* (2019) 10:78. doi: 10.3389/fendo.2019.00078
5. Muñoz-Cueto JA, Zmora N, Paullada-Salmeron JA, Marvel M, Mananos E, Zohar Y. The Gonadotropin-Releasing Hormones: Lessons From Fish. *Gen Comp Endocrinol* (2020) 291:113422. doi: 10.1016/j.ygcen.2020.113422
6. Lee JH, Miele ME, Hicks DJ, Phillips KK, Trent JM, Weissman BE, et al. KiSS-1, a Novel Human Malignant Melanoma Metastasis-Suppressor Gene. *J Natl Cancer Inst* (1996) 88:1731–7. doi: 10.1093/jnci/88.23.1731
7. Kotani M, Detheux M, Vandenbogaerde A, Communi D, Vanderwinden JM, Le Poul E, et al. The Metastasis Suppressor Gene KiSS-1 Encodes Kisspeptins, the Natural Ligands of the Orphan G Protein-Coupled Receptor GPR54. *J Biol Chem* (2001) 276:34631–6. doi: 10.1074/jbc.M104847200
8. de Roux N, Genin E, Carel JC, Matsuda F, Chaussain JL, Milgrom E. Hypogonadotropic Hypogonadism Due to Loss of Function of the KiSS1-Derived Peptide Receptor GPR54. *Proc Natl Acad Sci USA* (2003) 100:10972–6. doi: 10.1073/pnas.1834399100
9. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JSJr, Shagoury JK, et al. The GPR54 Gene as a Regulator of Puberty. *N Engl J Med* (2003) 349:1614–27. doi: 10.1056/NEJMoa035322
10. Oakley AE, Clifton DK, Steiner RA. Kisspeptin Signaling in the Brain. *Endocr Rev* (2009) 30:713–43. doi: 10.1210/er.2009-0005
11. Pasquier J, Kamech N, Lafont AG, Vaudry H, Rousseau K, Dufour S. Molecular Evolution of GPCRs: Kisspeptin/Kisspeptin Receptors. *J Mol Endocrinol* (2014) 52:T101–17. doi: 10.1530/JME-13-0224
12. Ogawa S, Parhar IS. Biological Significance of Kisspeptin–Kiss 1 Receptor Signaling in the Habenula of Teleost Species. *Front Endocrinol* (2018) 9:222. doi: 10.3389/fendo.2018.00222
13. Li S, Zhang Y, Liu Y, Huang X, Huang W, Lu D, et al. Structural and Functional Multiplicity of the Kisspeptin/GPR54 System in Goldfish (*Carassius Auratus*). *J Endocrinol* (2009) 201:407–18. doi: 10.1677/JOE-09-0016
14. Selvaraj S, Ohga H, Kitano H, Nyuji M, Yamaguchi A, Matsuyama M. Peripheral Administration of Kiss1 Pentadecapeptide Induces Gonadal Development in Sexually Immature Adult Scombroid Fish. *Zool Sci* (2013) 30:446–54. doi: 10.2108/zsj.30.446
15. Zmora N, Stubblefield J, Golan M, Servili A, Levavi-Sivan B, Zohar Y. The Medio-Basal Hypothalamus as a Dynamic and Plastic Reproduction-Related Kisspeptin-Gnrh-Pituitary Center in Fish. *Endocrinology* (2014) 155:1874–86. doi: 10.1210/en.2013-1894
16. Kitahashi T, Ogawa S, Parhar IS. Cloning and Expression of Kiss2 in the Zebrafish and Medaka. *Endocrinology* (2009) 150:821–31. doi: 10.1210/en.2008-0940
17. Felip A, Zanuy S, Pineda R, Pinilla L, Carrillo M, Tena-Sempere M, et al. Evidence for Two Distinct KiSS Genes in non-Placental Vertebrates That Encode Kisspeptins With Different Gonadotropin-Releasing Activities in Fish and Mammals. *Mol Cell Endocrinol* (2009) 312:61–71. doi: 10.1016/j.mce.2008.11.017
18. Ohga H, Selvaraj S, Adachi H, Imanaga Y, Nyuji M, Yamaguchi A, et al. Functional Analysis of Kisspeptin Peptides in Adult Immature Chub Mackerel (*Scomber Japonicus*) Using an Intracerebroventricular Administration Method. *Neurosci Lett* (2014) 561:203–7. doi: 10.1016/j.neulet.2013.12.072
19. Espigares F, Zanuy S, Gómez A. Kiss2 as a Regulator of Lh and Fsh Secretion *via* Paracrine/Autocrine Signaling in the Teleost Fish European Sea Bass (*Dicentrarchus Labrax*). *Biol Reprod* (2015) 93:1–12. doi: 10.1095/biolreprod.115.131029
20. Park JW, Jin YH, Oh S, Kwon JY. Kisspeptin2 Stimulates the HPG Axis in Immature Nile Tilapia (*Oreochromis Niloticus*). *Comp Biochem Physiol B* (2016) 202:31–8. doi: 10.1016/j.cbpb.2016.07.009

21. Li W, Hu J, Sun C, Dong J, Liu Z, Yuan J, et al. Characterization of *Kiss2*/*Kissr2* System in Largemouth Bass (*Micropterus Salmoides*) and Kiss2–10 Peptide Regulation of the Hypothalamic-Pituitary-Gonadal Axis. *Comp Biochem Physiol B* (2022) 257:110671. doi: 10.1016/j.cbpb.2021.110671
22. Nocillado JN,Zohar Y,Biran J, Levavi-Sivan B,Elizur A. Chronic Kisspeptin Administration Stimulated Gonadal Development in Pre-Pubertal Male Yellowtail Kingfish (*Seriola Lalandi*; Perciformes) During the Breeding and non-Breeding Season. *Gen Comp Endocrinol* (2013) 191:168–76. doi: 10.1016/j.ygcen.2013.06.005
23. Zmora N, Stubblefield J, Zulperi Z, Biran J, Levavi-Sivan B, Muñoz-Cueto JA, et al. Differential and Gonad Stage-Dependent Roles of Kisspeptin1 and Kisspeptin2 in Reproduction in the Modern Teleosts, Morone Species. *Biol Reprod* (2012) 86:177. doi: 10.1095/biolreprod.111.097667
24. Tang H, Liu Y, Luo D, Ogawa S, Yin Y, Li S, et al. The *Kiss*/*Kissr* Systems are Dispensable for Zebrafish Reproduction: Evidence From Gene Knockout Studies. *Endocrinology* (2015) 156:589–99. doi: 10.1210/en.2014-1204
25. Liu Y, Tang H, Xie R, Li S, Liu X, Lin H, et al. Genetic Evidence for Multifactorial Control of the Reproductive Axis in Zebrafish. *Endocrinology* (2017) 158:604–11. doi: 10.1210/en.2016-1540
26. Etzion T, Zmora N, Zohar Y, Levavi-Sivan B, Golan M, Gothilf Y. Ectopic Over Expression of *Kiss1* may Compensate for The Loss of *Kiss2*. *Gen Comp Endocrinol* (2020) 295:113523. doi: 10.1016/j.ygcen.2020.113523
27. Nakajo M, Kanda S, Karigo T, Takahashi A, Akazome Y, Uenoyama Y, et al. Evolutionally Conserved Function of Kisspeptin Neuronal System is non-Reproductive Regulation as Revealed by non-Mammalian Study. *Endocrinology* (2017) 159:163–83. doi: 10.1210/en.2017-00808
28. Somoza GM, Mechaly AS, Trudeau VL. Kisspeptin and GnRH Interactions in the Reproductive Brain of Teleosts. *Gen Comp Endocrinol* (2020) 298:113568. doi: 10.1016/j.ygcen.2020.113568
29. Sivalingam M, Parhar IS. Hypothalamic Kisspeptin and Kisspeptin Receptors: Species Variation in Reproduction and Reproductive Behaviours. *Front Neuroendocrinol* (2022) 64:100951. doi: 10.1016/j.yfrne.2021.100951
30. Parhar IS, Ogawa S, Sakuma Y. Laser-Captured Single Digoxigenin-Labeled Neurons of Gonadotropin-Releasing Hormone Types Reveal a Novel G Protein-Coupled Receptor (Gpr54) During Maturation in Cichlid Fish. *Endocrinology* (2004) 145:3613–8. doi: 10.1210/en.2004-0395
31. Grone BP, Maruska KP, Korzan WJ, Fernald RD. Social Status Regulates Kisspeptin Receptor mRNA in the Brain of *Astatotilapia Burtoni*. *Gen Comp Endocrinol* (2006) 169:98–107. doi: 10.1016/j.ygcen.2010.07.018
32. Ohga H, Adachi H, Kitano H, Yamaguchi A, Matsuyama M. Kiss1 Hexadecapeptide Directly Regulates Gonadotropin-Releasing Hormone 1 in the Scombroid Fish, Chub Mackerel. *Biol Reprod* (2017) 96:376–88. doi: 10.1095/biolreprod.116.142083
33. Ogawa S, Sivalingam M, Anthonysamy R, Parhar IS. Distribution of Kiss2 Receptor in the Brain and its Localization in Neuroendocrine Cells in the Zebrafish. *Cell Tissue Res* (2020) 379:349–72. doi: 10.1007/s00441-019-03089-5
34. Kanda S, Akazome Y, Mitani Y, Okubo K, Oka Y. Neuroanatomical Evidence That Kisspeptin Directly Regulates Isotocin and Vasotocin Neurons. *PloS One* (2013) 8:e62776. doi: 10.1371/journal.pone.0062776
35. Escobar S, Servili A, Espigares F, Gueguen MM, Brocal I, Felip A, et al. Expression of Kisspeptins and Kiss Receptors Suggests a Large Range of Functions for Kisspeptin Systems in the Brain of the European Sea Bass. *PloS One* (2013) 8:e70177. doi: 10.1371/journal.pone.0070177
36. Ando H, Shahjahan M, Hattori A. Molecular Neuroendocrine Basis of Lunar-Related Spawning in Grass Puffer. *Gen Comp Endocrinol* (2013) 181:211–4. doi: 10.1016/j.ygcen.2012.07.027
37. Ando H, Shahjahan M, Kitahashi T. Periodic Regulation of Expression of Genes for Kisspeptin, Gonadotropin-Inhibitory Hormone and Their Receptors in the Grass Puffer: Implications in Seasonal, Daily and Lunar Rhythms of Reproduction. *Gen Comp Endocrinol* (2018) 265:149–53. doi: 10.1016/j.ygcen.2018.04.006
38. Motohashi E, Yoshihara T, Doi H, Ando H. Aggregating Behavior of Grass Puffer, *Takifugu Niphobles*, Observed in Aquarium During the Spawning Period. *Zool Sci* (2010) 27:559–64. doi: 10.1016/j.ygcen.2007.07.009
39. Shahjahan M, Motohashi E, Doi H, Ando H. Elevation of Kiss2 and its Receptor Gene Expression in the Brain and Pituitary of Grass Puffer During the Spawning Season. *Gen Comp Endocrinol* (2010) 169:48–57. doi: 10.1016/j.ygcen.2010.07.008
40. Shahjahan M, Kitahashi T, Ando H. Temperature Affects Sexual Maturation Through the Control of Kisspeptin, Kisspeptin Receptor, GnRH and GTH Subunit Gene Expression in the Grass Puffer During the Spawning Season. *Gen Comp Endocrinol* (2017) 243:138–45. doi: 10.1016/j.ygcen.2016.11.012
41. Ando H, Ogawa S, Shahjahan M, Ikegami T, Doi H, Hattori A, et al. Diurnal and Circadian Oscillations in Expression of Kisspeptin, Kisspeptin Receptor and Gonadotrophin-Releasing Hormone 2 Genes in the Grass Puffer, a Semilunar-Synchronised Spawner. *J Neuroendocrinol* (2014) 26:459–67. doi: 10.1111/jne.12165
42. Shahjahan M, Ikegami T, Osugi T, Ukena K, Doi H, Hattori A, et al. Synchronised Expressions of LPXRFamide Peptide and its Receptor Genes: Seasonal, Diurnal and Circadian Changes During Spawning Period in Grass Puffer. *J Neuroendocrinol* (2011) 23:39–51. doi: 10.1111/j.1365-2826.2010.02081.x
43. Shahjahan Md, Hamabata T, Motohashi E, Doi H, Ando H. Differential Expression of Three Types of Gonadotropin-Releasing Hormone Genes During the Spawning Season in Grass Puffer, *Takifugu Niphobles*. *Gen Comp Endocrinol* (2010) 167:153–63. doi: 10.1016/j.ygcen.2010.01.018
44. Matsuda K, Nakamura K, Shimakura S, Miura T, Kageyama H, Uchiyama M, et al. Inhibitory Effect of Chicken Gonadotropin-Releasing Hormone II on Food Intake in the Goldfish, *Carassius Auratus*. *Horm Behav* (2008) 54:83–9. doi: 10.1016/j.yhbeh.2008.01.011
45. Nishiguchi R, Azuma M, Yokobori E, Uchiyama M, Matsuda K. Gonadotropin-Releasing Hormone 2 Suppresses Food Intake in the Zebrafish, *Danio Rerio*. *Front Endocrinol* (2012) 3:122. doi: 10.3389/fendo.2012.00122
46. Marvel MM, Spicer OS, Wong TT, Zmora N, Zohar Y. Knockout of Gnrh2 in Zebrafish (*Danio Rerio*) Reveals its Roles in Regulating Feeding Behavior and Oocyte Quality. *Gen Comp Endocrinol* (2019) 280:15–23. doi: 10.1016/j.ygcen.2019.04.002
47. Okuyama T, Yokoi S, Abe H, Isoe Y, Suehiro Y, Imada H, et al. A Neural Mechanism Underlying Mating Preferences for Familiar Individuals in Medaka Fish. *Science* (2014) 343:91–4. doi: 10.1126/science.1244724
48. Li L, Wojtowicz JL, Malin JH, Huang T, Lee EB, Chen Z. GnRH-Mediated Olfactory and Visual Inputs Promote Mating-Like Behaviors in Male Zebrafish. *PloS One* (2017) 12:e0174143. doi: 10.1371/journal.pone.0174143
49. Wang B, Liu Q, Liu X, Xu Y, Shi B. Molecular Characterization of Kiss2 Receptor and *In Vitro* Effects of Kiss2 on Reproduction-Related Gene Expression in the Hypothalamus of Half Smooth Tongue Sole (*Cynoglossus Semilaevis*). *Gen Comp Endocrinol* (2017) 249:55–63. doi: 10.1016/j.ygcen.2017.04.006
50. Zmora N, Stubblefield JD, Wong T-T, Levavi-Sivan B, Millar RP, Zohar Y. Kisspeptin Antagonists Reveal Kisspeptin 1 and Kisspeptin 2 Differential Regulation of Reproduction in the Teleost. *Morone saxatilis Biol Reprod* (2015) 93:76. doi: 10.1095/biolreprod.115.131870
51. Servili A, Le Page Y, Leprince J, Caraty A, Escobar S, Parhar IS, et al. Organization of Two Independent Kisspeptin Systems Derived From Evolutionary-Ancient Kiss Genes in the Brain of Zebrafish. *Endocrinology* (2011) 152:1527–40. doi: 10.1210/en.2010-0948
52. Shi Y, Zhang Y, Li S, Liu Q, Lu D, Liu M, et al. Molecular Identification of the Kiss2/Kiss1ra System and its Potential Function During 17-Alpha-Methyltestosterone-Induced Sex Reversal in the Orange-Spotted Grouper. *Epinephelus coioides Biol Reprod* (2010) 83:63–74. doi: 10.1095/biolreprod.109.080044
53. Zhang H, Zhang B, Qin G, Li S, Lin Q. The Roles of the Kisspeptin System in the Reproductive Physiology of the Lined Seahorse (*Hippocampus Erectus*), an Ovoviviparous Fish With Male Pregnancy. *Front Neurosci* (20182018) 12:. doi: 10.3389/fnins
54. Song Y, Chen J, Tao B, Luo D, Zhu Z, Hu W. Kisspeptin2 Regulates Hormone Expression in Female Zebrafish (*Danio Rerio*) Pituitary. *Mol Cell Endocrinol* (2020) 513:110858. doi: 10.1016/j.mce.2020.110858
55. Pasquier J, Lafont AG, Leprince J, Vaudry H, Rousseau K, Dufour S. First Evidence for a Direct Inhibitory Effect of Kisspeptins on LH Expression in the Eel, *Anguilla Anguilla*. *Gen Comp Endocrinol* (2011) 173:216–25. doi: 10.1016/j.ygcen.2011.05.019
56. Shahjahan M, Doi H, Ando H. LPXRFamide Peptide Stimulates Growth Hormone and Prolactin Gene Expression During the Spawning Period in the Grass Puffer, a Semi-Lunar Synchronized Spawner. *Gen Comp Endocrinol* (2016) 227:77–83. doi: 10.1016/j.ygcen.2015.09.008

57. Wang B, Yang G, Liu Q, Qin J, Xu Y, Li W, et al. Inhibitory Action of Tongue Sole LPXRFa, the Piscine Ortholog of Gonadotropin-Inhibitory Hormone, on the Signaling Pathway Induced by Tongue Sole Kisspeptin in COS-7 Cells Transfected With Their Cognate Receptors. *Peptides* (2017) 95:62–7. doi: 10.1016/j.peptides.2017.07.014
58. Ohga H, Adachi H, Matsumori K, Kodama R, Nyuji M, Selvaraj S, et al. mRNA Levels of Kisspeptins, Kisspeptin Receptors, and GnRH1 in the Brain of Chub Mackerel During Puberty. *Comp Biochem Physiol Part A: Mol Integr Physiol* (2015) 179:104–12. doi: 10.1016/j.cbpa.2014.09.012
59. Mechaly AS, Viñas J, Piferrer F. Sex-Specific Changes in the Expression of Kisspeptin, Kisspeptin Receptor, Gonadotropins and Gonadotropin Receptors in the Senegalese Sole (*Solea Senegalensis*) During a Full Reproductive Cycle. *Comp Biochem Physiol Part A: Mol Integr Physiol* (2012) 162:364–71. doi: 10.1016/j.cbpa.2012.04.003
60. Migaud H, Ismail R, Cowan M, Davie A. Kisspeptin and Seasonal Control of Reproduction in Male European Sea Bass (*Dicentrarchus Labrax*). *Gen Comp Endocrinol* (2012) 179:384–99. doi: 10.1016/j.ygcen.2012.07.033
61. Mohammed JS, Benninghoff AD, Holt GJ, Khan IA. Developmental Expression of the G Protein-Coupled Receptor 54 and Three GnRH mRNAs in the Teleost Fish Cobia. *J Mol Endocrinol* (2007) 38:235–44. doi: 10.1677/jme.1.02182
62. Nocillado JN, Levavi-Sivan B, Carrick F, Elizur A. Temporal Expression of G-Protein-Coupled Receptor 54 (GPR54), Gonadotropin-Releasing Hormones (GnRH), and Dopamine Receptor D2 (Drd2) in Pubertal Female Grey Mullet, *Mugil Cephalus*. *Gen Comp Endocrinol* (2007) 150:278–87. doi: 10.1016/j.ygcen.2006.09.008
63. Filby AL, Van Aerle R, Duitman J, Tyler CR. The Kisspeptin/Gonadotropin-Releasing Hormone Pathway and Molecular Signaling of Puberty in Fish. *Biol Reprod* (2008) 78:278–89. doi: 10.1095/biolreprod.107.063420
64. Mechaly AS, Vinas J, Murphy C, Reith M, Piferrer F. Gene Structure of the Kiss1 Receptor-2 (Kiss1r-2) in the Atlantic Halibut: Insights Into the Evolution and Regulation of Kiss1r Genes. *Mol Cell Endocrinol* (2010) 317:78–89. doi: 10.1016/j.mce.2009.11.005

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Zahangir, Shahjahan and Ando. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Endocrinology

ORIGINAL RESEARCH
published: 22 July 2022
doi: 10.3389/fendo.2022.848808

TAC3/TACR3 System Function in the Catadromous Migration Teleost, *Anguilla japonica*

Chenpeng Zuo[1†], *Likang Lyu*[1†], *Wenhui Zou*[2], *Haishen Wen*[1], *Yun Li*[1] *and Xin Qi*[1*]

[1] Key Laboratory of Mariculture, Ministry of Education, Ocean University of China, Qingdao, China, [2] College of Ocean, Key Laboratory of Tropical Biological Resources of Ministry of Education, Hainan University, Haikou, China

Neurokinin B (NKB), a member of the tachykinin (TAC) family, plays important roles in mammalian neuropeptide secretion in related to reproduction. However, its potential role in spawning migration teleost is less clear. In the present study, Japanese eel (*Anguilla japonica*) was employed to study the performance of NKB in regulating reproduction. Results showed that two *tac3* and one *tacr3* genes were identified in Japanese eel. Sequence analysis showed that two *tac3* transcripts, *tac3a* and *tac3b*, encode four NKBs: NKBa-13, NKBa-10, NKBb-13, and NKBb-10. However, compared with other species, a mutation caused early termination of TACR3 protein was confirmed, leading to the loss of the 35 amino acid (aa) C-terminal of the receptor. Expression analysis in different tissues showed that both *tac3a* and *tac3b* mRNAs were highly expressed in the brain. *In situ* hybridization localized both *tac3a* and *tac3b* mRNAs to several brain regions, mainly in the telencephalon and hypothalamus. Because of the mutation in TACR3 of Japanese eel, we further analyzed whether it could activate the downstream signaling pathway. Luciferase assay results showed the negative regulation of cAMP Response Element (CRE) and Sterol Response Element (SRE) signal pathways by Japanese eel NKBs. Intraperitoneal injection of four different NKB mature peptides at 100 ng/g had negative effect on either *gnrh* or *gth* gene expression. However, the high concentration of NKBa-10 and NKBb-13 (1,000 ng/g) upregulated *mgnrh* and *fshb* or *lhb* expression level significantly, which may be mediated by other receptors. In general, the NKBs/NK3Rs system has important functions in regulating eel puberty onset.

Keywords: tachykinins, neurokinin B, reproductive regulation, ovary development, Japanese eel

OPEN ACCESS

Edited by:
Bin Wang,
Yellow Sea Fisheries Research Institute (CAFS), China

Reviewed by:
Satoshi Ogawa,
Monash University Malaysia, Malaysia
Guangfu Hu,
Huazhong Agricultural University, China

****Correspondence:***
Xin Qi
qx@ouc.edu.cn

[†]These authors have contributed equally to this work

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

Received: *05 January 2022*
Accepted: *23 June 2022*
Published: *22 July 2022*

Citation:
Zuo C, Lyu L, Zou W, Wen H, Li Y and Qi X (2022) TAC3/TACR3 System Function in the Catadromous Migration Teleost, Anguilla japonica. Front. Endocrinol. 13:848808. doi: 10.3389/fendo.2022.848808

INTRODUCTION

Puberty refers to the developmental transition to a mature, reproductive state in mammals (1). In rodents, the first external sign of pubertal development is vaginal opening, although hormonal changes precede this (2). In fish, puberty is the developmental period during which an individual becomes capable of reproducing sexually for the first time and implies a functional competence of the brain–pituitary–gonad axis (3–5). It starts sometimes after sex differentiation and is associated with the initiation of germ cell maturation and full functional

differentiation of the germ cell–supporting somatic cells of the gonads and culminates in the first spermiation and sperm hydration or ovulation (6).

Japanese eel, *Anguilla japonica*, is one of the most important species of the aquaculture industry of East Asia. However, because of the catadromous migration reproductive character, the aquaculture industry is limited by the natural resources of glass eels from the ocean. Artificial reproduction studies of elopomorphes started in early 1930s, while until now, although great process has been made (7), the maturation rate of parental eels, survival rate of fertilized eggs, growth speed of hatched larva are still far beyond industrial application, especially the extremely high cost in raring hatched larva, limited this industry to the new horizon. The elopomorphes have a complex migratory life cycle with the occurrence of two metamorphoses (8). The first one is when leptocephali larvae metamorphose into glass eels at the time of their arrival over the continental shelf, at the end of a long oceanic journey, after which, glass eels become "yellow" eels; the second metamorphosis is termed "silvering (transition from yellow to silver eel)", after years of growing in freshwater, the yellow eels stop growth and start the downstream migration toward the area for breeding in the ocean (9). The silvering of eels was suggested to be considered as a pubertal development step, according to hormonal profiles and experimental results on the gonadotropic axis (10). It is believed that, at the silver stage, eels are blocked at a prepubertal stage (11), but chronic administration of carp or salmon pituitary extract (gonadotropic treatment) is able to induce sexual maturation (12, 13).

The neurokinin B (NKB), coded by the *tac3* gene (*tac2* gene in rodents), is an important member of tachykinin family. NKB, kisspeptin, and dynorphin are co-expressed in the arcuate nucleus and play an important role in the regulation of GnRH secretion (14–17). The NKB signal was important in reproduction especially puberty of human. Mutations in the genes encoding NKB (*tac3*) or its receptor NK3R (*tacr3*) lead to hypogonadotropic hypogonadism—a disease characterized by the failure of sexual maturation, impaired gametogenesis, and infertility, indicating that the NKB/NK3R system is indispensable for human reproduction (18, 19). In monkeys (*Macaca mulatta*), transient activation of NK3R stimulates GnRH release, but repeated stimulation of this pathway does not induce GnRH pulsatile release (20). In addition, injecting antagonists of NK3R could inhibit LH secretion levels in ovariectomized rats (*Rattus norvegicus*) (21). Taken together, in addition to its role in the downstream part of the pubertal mechanism (GnRH pulse generator), NKB signal may also be involved in the upstream part of the central pubertal system.

In teleost, NKB/NK3R signaling pathway has been characterized in zebrafish (*Danio rerio*) (22), goldfish (*Carassius auratus*) (23), tilapia (*Oreochromis mossambicus*) (24), spotted sea bass (*Lateolabrax maculatus*) (25) and carp (*Ctenopharyngodon idella*) (26). Previously, using goldfish as a modal, we have confirmed the NKB function in regulating GnRH and GtH expression both *in vivo* and *in vitro* (23). Furthermore, we have also demonstrated that NKB could act directly on the ovary to enhance the 17β-estradiol (E_2) synthesis and release and, therefore, the growth of oocytes (27). In the present study, we used Japanese eel, whose ovary development is blocked at the prepubertal stage, as a model to further investigate the role of NKBs in fish puberty onset. cDNA for *tac3a*, *tac3b*, and *tacr3s* were characterized and cloned, and the distribution of *tac3s* in different tissues was determined. Because *tac3s* mRNA was highly expressed in the brain, we further detected the location of both genes in brain regions of the eel. Interestingly, mutation caused early termination of NK3R protein translation was found in all tested samples. To confirm whether the mutated NK3R could active the downstream signaling, using luciferase assay, we confirmed that NKB of Japanese eel could not activate neither CRE nor SRE signaling pathway. Finally, to evaluate whether NKB can still regulate the reproductive functions by receptors of other genes, we measured two Japanese eel GnRHs that were homologous to mammalian or chicken GnRH (namely, *mgnrh* and *cgnrh*, respectively), *fshb* and *lhb* gene expression by real-time PCR in eels injected with NKBs. These novel findings demonstrated that the NKBs/NK3Rs system has important functions in regulating eel puberty onset.

MATERIALS AND METHODS

Animals

Female Japanese eels with body weight (BW) of 1.0 ± 0.2kg were obtained from Guangzhou, Zhuhai (Guangdong), and Qingdao (Shandong), China, and allowed to acclimatize for 7 days in tanks with a photoperiod of 14L:10D and temperature of 25°C. Zebrafish were obtained from an ornamental fish market in Qingdao, China. The animals were fed with commercial diet without any supplemental hormones. All animal experiments were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committees of Yat-Sen University and the Ocean University of China.

Cloning and Sequence Analysis of *tac3s*/*tacr3s* Genes

Total RNA from more than 80 Japanese eel brains and ovaries and three zebrafish brains were prepared by using TRIzol (Invitrogen, USA). One microgram of isolated RNA was used to synthesize cDNA using the ReverTra Ace-a First-Strand cDNA Synthesis Kit (TOYOBO, Japan). To amplify the cDNA fragments of the eel *tac3a*, *tac3b*, and *tacr3*, PCR primers were designed on the basis of the nucleotide sequences of searched from the genome data. Full-length cDNA sequences encoding these genes were obtained by the 5′ and 3′ rapid amplification of cDNA ends (RACE) with several gene-specific primers (**Table 1**). The *tacr3a1* sequence of zebrafish was obtained from National Center for Biotechnology Information (NCBI) and cloned. The primers used were listed in **Table 1**. For all PCR reactions, amplifications were performed as follows: denaturation at 94°C

TABLE 1 | Primers for RACE, gene clone, qPCR, and ISH.

Primers	Sequence (5′-3′)
TAC3a-race-F1	AACGGTATAGATTATGACAGC
TAC3a-race-F2	GCCTTATGGGTAGAAGAAGCA
TAC3a-race-R1	GAATAGGTGGACCTGCCTTG
TAC3a-race-R2	ACTCTGAACTCCTCCGACCC
TAC3b-race-F1	ATGACACCTTTGTAGGGCTGAT
TAC3b-race-F2	CTGATGGGCAGAAGAAGT
TAC3b-race-R1	TGGATTCTGAACTCCTCC
TAC3b-race-R2	CCATTAGTCCCACGAAGAT
TAC3R-race-F1	GAAGCCCAGACTGTCAGCCACG
TAC3R-race-F2	CGAAGGACCCTCTGCTATGTG
TAC3R-race-R1	GTCCATGGTAATTGTCTGAC
TAC3R-race-R2	ACACCAGCACAGTCACAA
NUP	AAGCAGTGGTATCAACGCAGAGT
UPM (long)	CTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
UPM (short)	CTAATACGACTCACTATAGGGC
TAC3a-ORF-F	CAGGAAAACAAAAGAGACGATG
TAC3a-ORF-R	GCTGCTGGGACTGGCCTA
TAC3b-ORF-F	CGGCCAGAAAGAGACGATG
TAC3b-ORF-R	AGGGCGAAAAGCTGGTCTTA
TAC3R-ORF-F	ATGGAAGCATCAAACAGCACAT
TAC3R-ORF-R	CTACCTGCTATTGAGGCAGCA
TAC3a-real-F	AAGAGCAGGAACCGCACAAGC
TAC3a-real-R	CCCATCAGGCCAACAAAGAAA
TAC3b-real-F	TATGACACCTTTGTAGGGCTGAT
TAC3b-real-R	AAATGGCTCCTCTTCCCTGTG
TAC3R-real-F	ATCGTCTGTATCTGGGCACTG
TAC3R-real-R	AATTGTCTGACGAATCTCCTGG
18S-real-F	CATTGGAGGGCAAGTCTGGTG
18S-real-R	GCGGGACACTCAGCTAAGAGC
EF1α-real-F	GGTATGGTGGTGACCTTTGCC
EF1α-real-R	CTACGTTGCCACGACGGATTT
β-actin-real-F	TGCGTGACATCAAGGAGAAGC
β-actin-real-R	ATTCCGCAGGACTCCATACCC
mGnRH-real-F	GCAATCCCTCTTCGTCACTC
mGnRH-real-R	CAGCCAGATTTGCCTGTAAG
cGnRH-real-F	CAGGTATTGGAAGAGATAAAGC
cGnRH-real-R	GCTGTATGCTATCCCCTCCT
LH-real-F	GTCCAAAATGTCTGGTGTTC
LH-real-R	GCACAGGTTACAGTCGCAGC
FSH-real-F	CGTGGAGAATGAAGAATGCG
FSH-real-R	CAGGGTAGGTGAAGTGGAGG
TAC3a-ISH-F	CGCATTTAGGTGACACTATAGAAGCGGTTGGCGATCCTGTCCCTTA
TAC3a-ISH-R	CCGTAATACGACTCACTATAGGGAGACAAAGAAGAATCCGCCTGTCCG
TAC3b-ISH-F	CGCATTTAGGTGACACTATAGAAGCGTACTTGGTGTTCGCGATCCT
TAC3b-ISH-R	CCGTAATACGACTCACTATAGGGAGACAAAATGGCTCCTCTTCCCTGTG
Zebrafish-F	TCCAGTTGTCACACAGAGCG
Zebrafish-R	GTGTTTGTCATGATCGCTTGC

for 5 min, followed by 40 cycles at 94°C for 20 s, 55°C–60°C for 20 s, and 72°C for 2 min. The reaction ended with a final extension at 72°C for 5 min. The amplification products were purified using the E.Z.N.A. Gel Extraction Kit (Omega BioTek, USA) and subcloned into the pGEM-T vector (Promega, USA). Three different positive clones were sequenced on an ABI 3700 sequencer (Applied Biosystems, USA). The putative amino acid (aa) sequences were predicted using the BioEdit software, and the putative seven-transmembrane domains were predicted using the TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM-2.0/). The NKBs aa sequences were aligned with Clustal W 1.81 (28). The protein phylogenetic analysis was conducted with MEGA 6.0 using the neighbor-joining method.

RNA Extraction, Reverse Transcription, and qPCR

Total RNA was extracted using TRIzol reagent (Life Technologies China, Inc.). The amount and purity of the RNA were determined on a NanoDrop 2000 spectrophotometer (ThermoFisher Scientific). One microgram of isolated RNA was used to synthesize first-strand cDNAs using the ReverTra Ace-a First-strand cDNA Synthesis Kit (TOYOBO, Japan). Real-time PCR was performed on a Roche LightCycler 480 using the SYBR Green I Kit (TOYOBO, Japan) according to the manufacturer's instructions. Elongation factor-1α, β-actin, and 18s were used as internal controls. Relative mRNA levels were determined using the standard ΔΔcycle threshold method.

In Situ Hybridization

The *in situ* hybridization was performed as we previously reported (23, 29). The brains of five female Japanese eels were removed and fixed in buffered 4% paraformaldehyde for 24 h and then embedded in paraffin. Seven-micron-thick sections were cut for ISH. The sections were pasted onto aminopropylsilane-treated glass slides and dried in an oven at 37°C. Sense and antisense digoxigenin (DIG)–labeled riboprobes about 300–400 bp in length were synthesized from the open reading frames (ORFs) of Japanese eels tac3s genes using a DIG RNA Labeling Kit (Roche Diagnostics, Mannheim, Germany). The sections were briefly rehydrated by a graded series of ethanol solutions (100% to 80%) after being cleared in xylene, permeabilized with 0.8 M HCl for 10 min followed by proteinase K (10 μg/ml) digestion for 2 min, washed in 1× Phosphate Buffered Saline (PBS) for 10 min, then washed in 2×Sodium Citrate Buffer (SSC) for 10 min, prehybridized at 55°C for 1 h, and hybridized with DIG-labeled riboprobes diluted in hybridization buffer at 55°C overnight in a wet box (25). After hybridization, the sections were washed in grade series of Sodium Citrate Buffer (SSC) and Phosphate Buffered Saline (PBS) solution and blocked with blocking reagent (Roche Diagnostics). DIG was detected with an alkaline phosphatase-conjugated anti-DIG antibody (Roche Diagnostics; diluted 1:3,000), and chromogenic development was conducted with an Nitro-Blue-Tetrazolium/5-bromo-4-chloro-3-inodlyl-phosphate (NBT/BCIP) stock solution (Roche Diagnostics).

Peptide Synthesis and Preparation

On the basis of the result that we got of the eel TAC3A and TAC3B, peptides corresponding to the eel NKB peptides (SGTGLSATLPQRF-NH2) were synthesized by GL Biochem (Shanghai, China). The purity of the synthesized peptides was >95% as determined by analytical HPLC. Eel peptides were dissolved into DMSO and diluted to the desired concentration in saline (0.7% NaCl) for *in vivo* injection experiments.

In vivo Injection of TAC in Japanese Eel

A total of 45 healthy Japanese eels with similar BW were selected and divided into nine groups. Five eels in each group were kept in different aquariums, and the aquariums were numbered. The control group was injected with 0.2 ml of saline with 0.1% Dimethyl sulfoxide (DMSO), whereas the treatment groups were injected intraperitoneally with high concentration (1,000 ng/g BW) and low concentration (100 ng/g BW).

Cell Culture and Co-transfection

The *tacr3* cDNAs of Japanese eel and zebrafish were subcloned into the pcDNA3.1 expression vector (Invitrogen, USA). The COS-7 cells and 293T cells were maintained at 37°C in Dulbecco's Modified Eagle Medium (DMEM) containing 10% fetal bovine serum (Gibco, USA). Twenty hours before transfection, 1×10^5 cells per well were seeded into 24-well tissue culture plates. A total of 500 ng of SRE-Luc or CRE-Luc reporter plasmid (30), 300 ng of pcDNA3.1-*tacr3* of Japanese eel, and 50 ng of pRL-CMV containing the Renilla luciferase reporter gene (31) were co-transfected into the COS-7 cells in 500 ml of serum-free medium using Lipofectamine reagent (Invitrogen, USA). Six hours after transfection, cells were incubated with vehicle or various (from 10^{-10} to 10^{-6} M) concentrations of NKBa-10, NKBa-13, NKBb-10, and NKBb-13 for a further 24 h (stimulated with 1×10^{-5} M FSK (Sigma, USA) for CRE promoter). A total of 250 ng of SRE-Luc or CRE-Luc reporter plasmid, 200 ng of pcDNA3.1-*tacr3a1* of zebrafish, and 50 ng of TK containing the Renilla luciferase reporter gene (31) were co-transfected into the 293T cells in 500 ml of serum-free medium using Lipofectamine 3000 reagent (Invitrogen, USA). Six hours after transfection, cells were incubated with vehicle or various (from 10^{-9} to 10^{-7} M) concentrations of NKBa-10 for a further 36 h. Cells were harvested, and luminescence was measured by the Dual Luciferase Kit (Promega, USA).

Statistical Analysis

All data were expressed as the mean ± S.E.M, and the number of samples is indicated in the figure legends. Statistical significance was determined using a one-way ANOVA followed by Dunnett's test for multiple rage comparison. A Student's t-test was used for comparison between two groups. Statistical significance was defined as $P < 0.05$.

RESULTS

Identification and Synteny Analysis of the *tac3* and *tacr3* Genes in Japanese Eel

Two *tac3* genes and one *tacr3* gene from the Japanese eel were cloned, named *tac3a* (OL804257), *tac3b* (OL804258), and *tacr3* (ON402757). The ORF of *tac3a* is 375 bp, coding a 125-aa precursor with a predicted signal peptide of 21 aa (**Figure 1A**). The ORF of *tac3b* is 297 bp, coding a 99-aa precursor with a predicted signal peptide of 21 aa (**Figure 1B**). In addition, the ORF of *tac3r* is 909 bp coding a 302-aa G protein–coupled receptor (GPCR) with seven transmembrane domains (**Figure 1C**). Sequence analysis showed that each precursor contains two putative peptides. The four Japanese eel tachykinin peptides were designated NKBa-13, NKBa-10, NKBb-13, and NKBb-10 according to their length. Sequence analysis showed that a common C-terminal sequence (FVGLM) was conversed in all NKB-10 and NKB-13 in teleost. Sequence alignment of TAC3 precursor in teleost showed that NKBa-10 and NKBa-13 were better conserved, whereas NKBb-10 and NKBb-13 had lower similarity in teleost (**Figures 2A, B**). Sequence comparison analysis revealed the TACR3 had a mutation of adenine at the 908th position, resulting an early termination of the GPCR translation (**Figure 2C**).

Phylogenetic trees of the TAC3s and TACRs were constructed. As shown in **Figure 3A**, TAC3s from teleost and other vertebrates formed two large clades, and the teleost clade was subdivided into TAC3a and TAC3b clades. The two TAC3 of the Japanese eel are clustered in the TAC3b clade, which is clustered together with the TAC3b of Atlantic salmon (*Salmo salar*), rainbow trout

FIGURE 1 | Nucleotide sequences and deduced amino acid sequences of *tac3a* **(A)** and *tac3b* **(B)** in Japanese eel. The signal peptides and NKB peptides were shaded, and their names under the corresponding sequence. Nucleotide sequences and deduced amino acid sequences of *tacr3* **(C)** in Japanese eel. The transmembrane domains were numbered under the corresponding sequence, and their sequence compositions are shaded.

(*Oncorhynchus mykiss*), and European sea bass (*Dicentrarchus labrax*) TAC3b. Phylogenetic tree of TACRs showed three TACR clades, which Japanese eel TACR3a was clustered into TACR3 clade, indicating the evolutionary conservation (**Figure 3B**).

Tissue Expression of Japanese Eel *tac3*s

The expression patterns of the *tac3*s genes in different tissues and brain regions were shown in **Figure 4**. *Tac3a* mRNA was mainly expressed in the pituitary, followed by the hypothalamus and telencephalon, with low level expressed in the optic tectum, medulla, liver, intestine, and gonad. However, *tac3b* mRNA was only expressed in telencephalon, optic tectum, medulla, and hypothalamus, and the expression was relatively high in the telencephalon.

Localization of the mRNA of *tac3s* in the Brain

Sections of brain tissue of Japanese eel were taken to detect the mRNA distribution of *tac3a* and *tac3b* by *in situ* hybridization. Localization of both *tac3a* and *tac3b* in the brain is shown in **Figures 5**, **6**. *Tac3a* positive signals were detected in the postcommissural nucleus of the ventral telencephalon (VP),

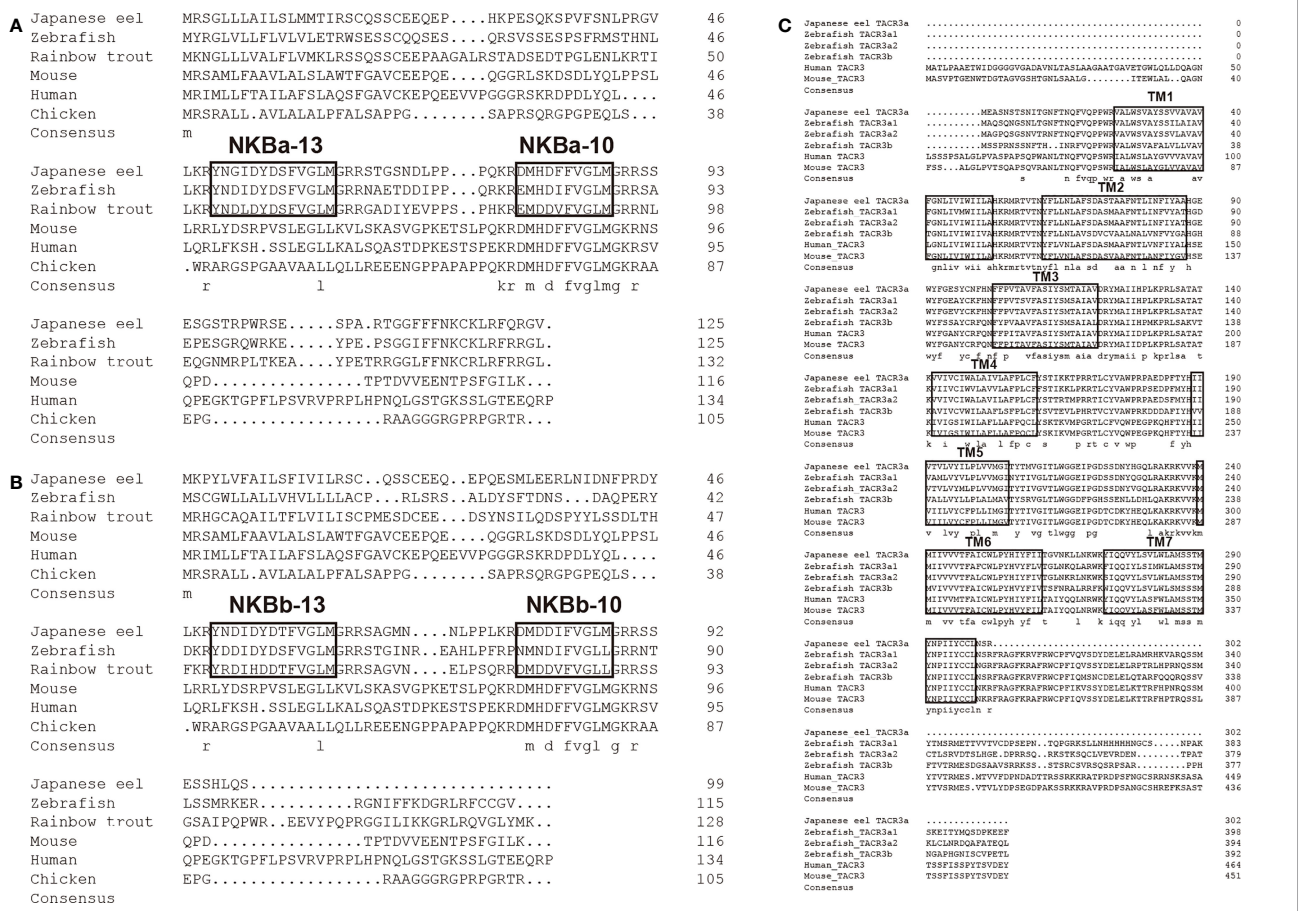


FIGURE 2 | Comparison of the amino acid sequences of Japanese eel *tac3a* **(A)** and *tac3b* **(B)** precursors from different species. The boxed letters indicated the sequence of mature NKB peptides in the detected species. Comparison of the amino acid sequences of Japanese eel *tacr3* **(C)** precursors from different species. The boxed letters indicated the sequence of transmembrane domains in the detected species.

the ventral hypothalamus (Hv), the nucleus of the posterior recess (NRP), the torus longitudinalis (TL), the reticular formation (RF), the molecular layer of corpus cerebelli (CCeM) (**Figures 5A–D**). The *tac3b* positive signals were detected in the lateral part of the dorsal telencephalon (Dl), the supracommissural nucleus of the ventral telencephalon (Vs), the Hv, the NRP, the TL, the RF, and the CCeM (**Figures 6A–D**).

Binding Analysis of NKB and TACR3 in Japanese Eel

As shown in **Figure 7**, NKBa-10 of Japanese eel could weakly activate the CRE pathway *via* TACR3, whereas NKBa-13, NKBb-10, and NKBb-13 had no effect under the same conditions. Moreover, none of the four peptides binding with the receptor TACR3 could activate the SRE pathway, which indicated that they could not cause the increase of transcriptional activity of SRE pathway. In **Figure 8**, NKBa-10 of Japanese eel binding with TACR3A1 receptor of zebrafish could effectively activate CRE and SRE signaling pathways, especially significantly increasing the transcriptional activity of SRE signaling pathway.

Regulation of mRNA Levels of *mgnrh*, *cgnrh*, *lhb*, and *fshb* genes in the Brain by NKB *in vivo*

The results showed that high concentration (1,000 ng/g BW) of NKBa-10 and NKBb-13 could increase the expression level of *mgnrh* significantly ($P < 0.05$), whereas NKBa-13 and NKBb-10 had no effect on the expression of *mgnrh*. However, neither of the four peptides in low concentration (100 ng/g BW) had effect on the expression of *mgnrh*. In addition, both high and low concentration of NKBa-10, NKBa-13, NKBb-10, and NKBb-13 were unable to activate *cgnrh* expression compared with control group (**Figure 9A**).

As shown in **Figure 9B**, high concentration (1,000 ng/g BW) of NKBa-10 and NKBb-13 could significantly upregulate the expression level of *fshb* ($P < 0.05$), whereas NKBa-13 and NKBb-10 had no effect on *fshb* in both peptide concentrations. In the regulation of *lhr*, only high concentration of NKBa-10 could increase *lhr* level significantly ($P < 0.05$). The other three peptides—NKBa-13, NKBb-10, and NKBb-13—could not regulate *lhr* level in both concentrations compared with control group.

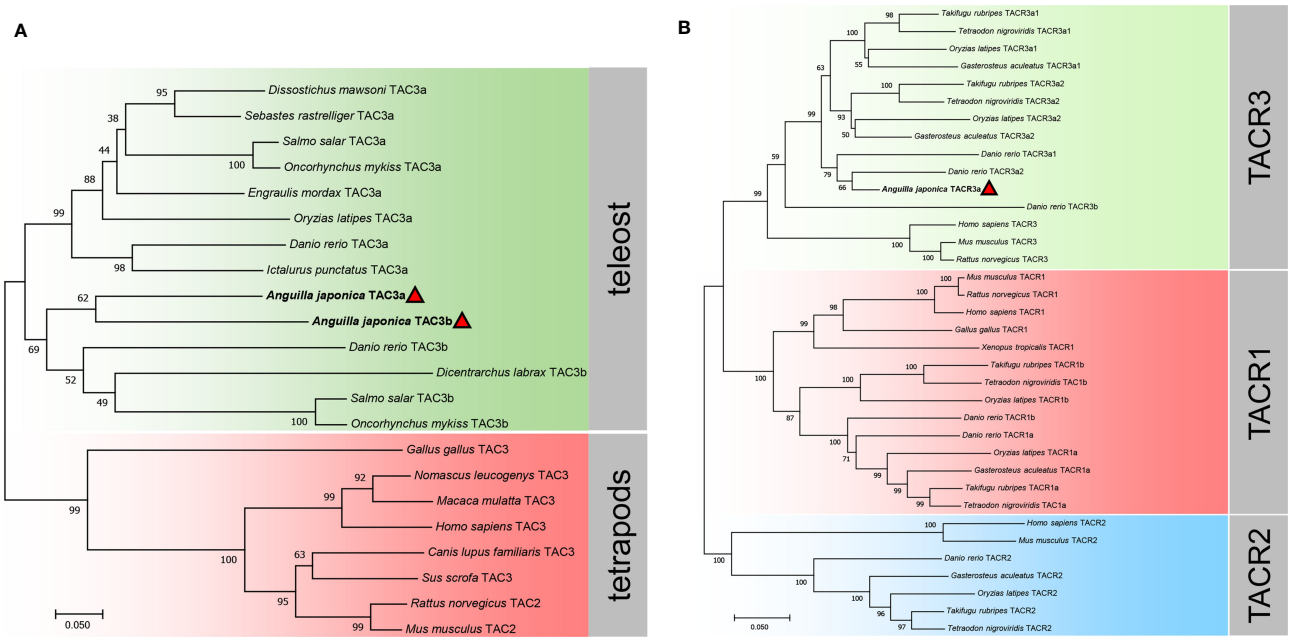


FIGURE 3 | Phylogenetic analysis of the tac3 **(A)** and tacr **(B)** gene family in vertebrates. The phylogenetic trees were constructed by MEGA 7 using the neighbor-joining method. Data were resampled with 1,000 bootstrap replicates. The accession numbers of each sequence are *Dissostichus mawsoni* tac3a (DAA35156.1), *Sebastes rastrelliger* tac3a (XP_037630002.1), *Salmo salar* tac3a (XP_013991334.1), *Oncorhynchus mykiss* tac3a (XP_021421533.1), *Engraulis mordax* tac3a (DAA35163.1), *Oryzias latipes* tac3a (NP_001265832.1), *Danio rerio* tac3a (NP_001243318.1), *Ictalurus punctatus* tac3a (XP_017343311.1), *Danio rerio* tac3b (NP_001243319.1), *Dicentrarchus labrax* tac3b (QYB17058.1), *Salmo salar* tac3b (XP_014022643.1), *Oncorhynchus mykiss* tac3b (CDQ91399.1), *Gallus gallus* tac3 (XP_015155892.2), *Nomascus leucogenys* tac3 (XP_003252831.1), *Macaca mulatta* tac3 (XP_001115535.2), *Homo sapiens* tac3 (AAQ10783.1), *Canis lupus familiaris* tac3 (NP_001279988.1), *Sus scrofa* tac3 (NP_001007197.1), *Rattus norvegicus* tac2 (NP_062035.1), *Mus musculus* tac2 (BAA03316.1), *Takifugu rubripes* tacr3a1 (NP_001267026.1), *Tetraodon nigroviridis* tacr3a1 (DAA35148.1), *Oryzias latipes* tacr3a1 (NP_001265839.1), *Gasterosteus aculeatus* tacr3a1 (XP_040043470.1), *Takifugu rubripes* tacr3a2 (NP_001267041.1), *Tetraodon nigroviridis* tacr3a2 (DAA35149.1), *Oryzias latipes* tacr3a2 (NP_001265803.1), *Gasterosteus aculeatus tacr3a2* (XP_040042202.1), *Danio rerio* tacr3a1 (NP_001243567.1), *Danio rerio* tacr3a2 (NP_001243564.1), *Danio rerio* tacr3b (XP_002666594.1), *Homo sapiens* tacr3 (NP_001050.1), *Mus musculus* tacr3 (AAH66845.1), *Rattus norvegicus* tacr3 (NP_058749.1), *Mus musculus* tacr1 (NP_033339.2), *Rattus norvegicus* tacr1 (NP_036799.1), *Homo sapiens* tacr1 (NP_001049.1), *Gallus gallus* tacr1 (NP_990199.1),*Xenopus tropicalis* tacr1 (NP_001106489.1), *Takifugu rubripes* tacr1b (XP_029693280.1), *Tetraodon nigroviridis* tacr1b (CAG05392.1), *Oryzias latipes* tacr1b (XP_011479687.1), *Danio rerio* tacr1b (NP_001268728.1), *Danio rerio* tacr1a (NP_001257407.1), *Oryzias latipes* tacr1a (NP_001265826.1), *Gasterosteus aculeatus* tacr1a (XP_040052094.1), *Takifugu rubripes* tacr1a (NP_001267036.1), *Tetraodon nigroviridis* tacr1a (CAG12579.1), *Homo sapiens* tacr2 (NP_001048.2), *Mus musculus* tacr2 (NP_033340.3), *Danio rerio* tacr2 (NP_001314788.1), *Gasterosteus aculeatus* tacr2 (XP_040059362.1), *Oryzias latipes* tacr2 (XP_011489426.1), *Takifugu rubripes* tacr2 (NP_001267009.1), and *Tetraodon nigroviridis* tacr2 (DAA35150.1).

DISCUSSION

Tachykinin, encoded by *tac1*, *tac3*, and *tac4*, is considered as one of the largest families of neuropeptides. It is named due to the ability to rapidly induce intestine contraction (32). More and more evidence showed that *tac1* gene produces substance P (SP) and neurokinin A (NKA), *tac3* encodes NKB and NKF, and *tac4* encodes hemokinin-1 (HK-1) and endokinins (33). Among them, NKB showed its essential role in the onset of puberty and gonadotropin secretion (22, 34). Different from mammals, with only one *tac* gene to code NKBs, teleosts undergo a teleost-specific genome duplication event (35), which, in the present, causes two *tac3* genes. In recent years, an increasing number of studies have reported that the NKB/NKR system plays a critical role in teleost, especially in reproduction (23, 36, 37). Previous studies also have reported two *tac3* genes (*tac3a* and *tac3b*) in zebrafish, goldfish, salmon, and spotted sea bass (22, 29, 36, 37). In the present study, we have identified two *tac3* and one *tacr3* genes in Japanese eel. There were two tachykinin peptides in each of Japanese eel TAC3 precursor including NKB-13 (also known as NKB-related peptide) and NKB-10, which suggested conserved functions in teleost.

Studies in mammals and other vertebrates showed that tachykinin shared conservative carboxyl-terminal sequence, -FXGLM-NH_2 (38, 39). The four NKBs of Japanese eel generated from two *tac3* genes presented the common C-terminal motif (-FVGLM-NH_2), which is also found in zebrafish (22), goldfish (23), and spotted sea bass (29). From a chemical point of view, two distinct moieties were presented in tachykinins: a N-terminal sequence with variability in all tachykinins and a C-terminal sequence, which is responsible for receptor activation (38). The terminal Met is regard as an essential factor for tachykinin function because the tachykinin-like peptides from invertebrates with a C-terminal Arg are unable to activate mammalian tachykinin receptors (40, 41). In the present study, three out of four NKBs in Japanese eel—NKBa-13, NKBa-10, and NKBb-13—had identical C-terminal Met, which is supported by the results

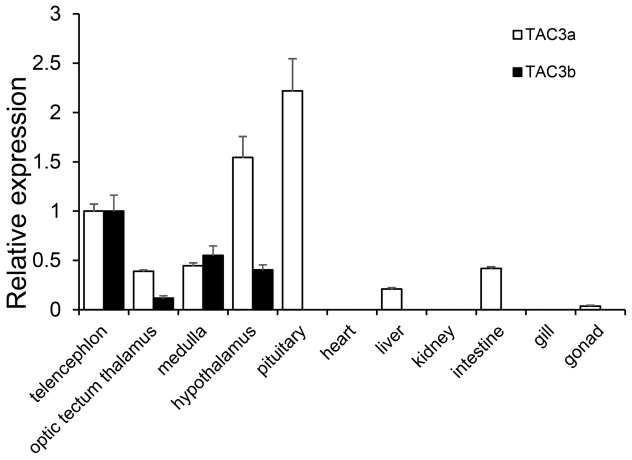


FIGURE 4 | Expression of *tac3s* mRNAs in various tissues (telencephalon, optic tectum thalamus, medulla, hypothalamus, pituitary, heart, liver, kidney, intestine, gill, and gonad) of Japanese eel. The X-axis indicates different tissues. The results are normalized to 18s rRNA. The data are shown as the mean ± S.E.M (n = 3), and the mRNA levels of *tac3a* and *tac3b* were calculated as fold change relative to the mRNA levels of the heart. The different lowercased letters indicated significant differences ($P < 0.05$).

in zebrafish (22), goldfish (23), spotted sea bass (29), and European eel (*Anguilla anguilla*) (42).

In the present study, we found mutation in *tacr3*. To confirm whether the mutation is ubiquitous or from an individual difference, we have collected more than 80 Japanese eels both cultured and wild caught from different locations, including Guangzhou (113°E, 23.5°N), Zhuhai (Guangdong) (113.5°E, 22°N), and Qingdao (Shandong) (120.4°E, 36°N), China. The DNA was purified for PCR with specific primers to amplified the mutation site. The PCR products were then subcloned into *E. coli*, and three clones were sent for Sanger's sequencing to confirm the accuracy. As a result, all the sequences confirmed the mutation. In addition, we have noticed the European eel (*Anguilla Anguilla*) TAC3R sequence (XM_035417067.1), when the mutation in Japanese eel was confirmed. However, the sequence is predicted on the basis of the whole genome sequence. Hence, we have further checked the whole genome sequences in both European eel and American eel (*Anguilla rostrata*). Interestingly, both genome sequences showed the same results with the Japanese eel, which ended at the mutation site. Unfortunately, we are not able to get the sample or DNA of these eels, so we can not 100% sure about the mutation in other eel species. However, we can be sure about the mutation in the Japanese eel.

Tissue distribution results showed that both *tac3a* and *tac3b* were highly expressed in the brain of Japanese eel, consistent with the results in other fishes including zebrafish, goldfish, orange-spotted grouper (*Epinephelus coioides*), and spotted sea bass (22, 23, 26, 29, 37, 43). Furthermore, *tac3a* was found mainly expressed in the hypothalamus and pituitary. It is proverbial that the hypothalamus plays an important role in reproductive regulation, which suggests that *tac3s* genes may be involved in reproductive regulation of Japanese eel as well. In zebrafish and spotted sea bass, *tac3a* was mainly expressed in the hypothalamus but not detected in the pituitary (22, 29, 37). Whereas, in goldfish, tac3a was strongly expressed in the pituitary (23), consistent with the result in Japanese eel. These results indicated that the expression pattern may be variable in

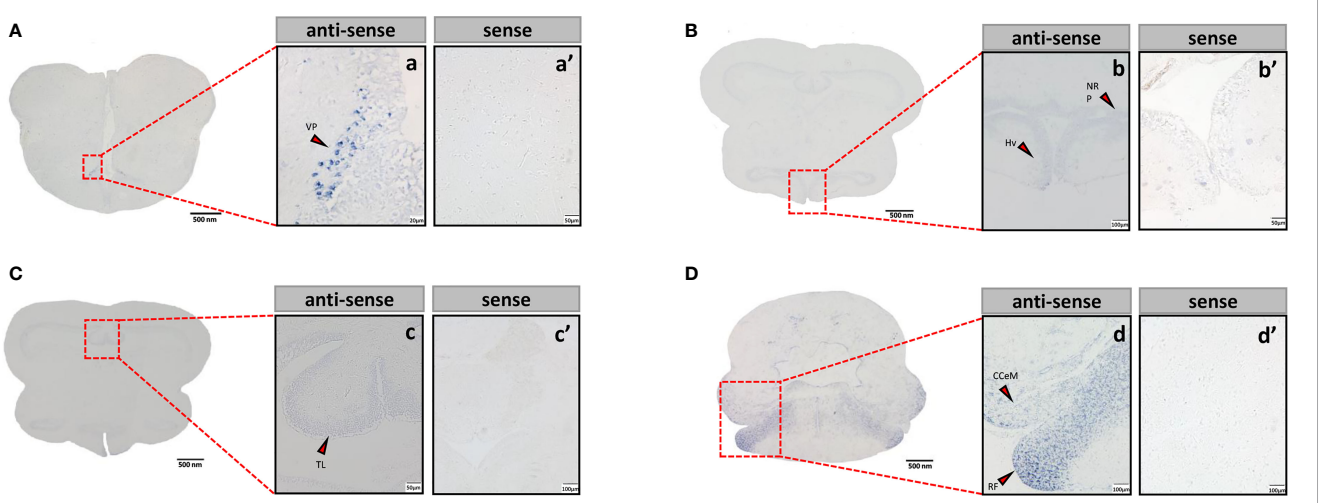


FIGURE 5 | *Tac3a* mRNA in the brain of Japanese eel was detected by *in situ* hybridization (ISH). *Tac3a* localization in the telencephalon **(A)**, thalamus **(B)**, midbrain **(C)**, and cerebellum **(D)**. Scale bar = 500 μm. Positive signal of *tac3a* in the telencephalon **(a)**, thalamus **(b)**, midbrain **(c)**, and cerebellum **(d)**. Negative signals of *tac3a* with sense probe results in the telencephalon **(a')**, thalamus **(b')**, midbrain **(c')**, and cerebellum **(d')**. b, c', d, d': Scale bar = 100 μm. a', b', c: scale bar = 50 μm. a: scale bar = 20 μm. VP, the postcommissural nucleus of the ventral telencephalon; Hv, the ventral hypothalamus; NRP, the nucleus of the posterior recess; TL, the torus longitudinalis; RF, the reticular formation; CCeM, the molecular layer of corpus cerebelli.

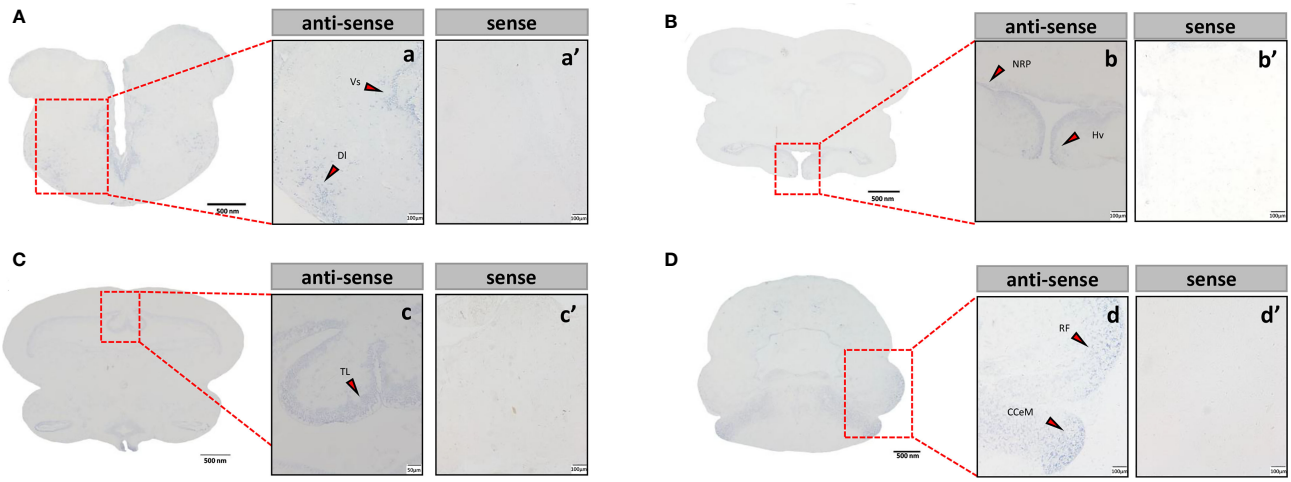


FIGURE 6 | *Tac3b* mRNA in the brain of Japanese eel was detected by *in situ* hybridization (ISH). *Tac3b* localization in the telencephalon. *Tac3b* localization in the telencephalon **(A)**, thalamus **(B)**, midbrain **(C)**, and cerebellum **(D)**. Scale bar = 500 μm. Positive signal of *tac3b* in the telencephalon **(a)**, thalamus **(b)**, midbrain **(c)**, and cerebellum **(d)**. Negative signals of *tac3a* with sense probe results in the telencephalon **(a')**, thalamus **(b')**, midbrain **(c')**, and cerebellum **(d')**. a, a', b, b', c', d, d': Scale bar = 100 μm. c: scale bar = 50 μm. Dl, the lateral part of the dorsal telencephalon; Vs, the supracommissural nucleus of the ventral telencephalon; Hv, the ventral hypothalamus; NRP, the nucleus of the posterior recess; TL, the torus longitudinalis; RF, the reticular formation; CCeM, the molecular layer of corpus cerebelli.

different species. The expression of *tac3b* in Japanese eel was detected only in the brain but not in pituitary, which is coincident with the result in goldfish (13). In gonad, *tac3b* was not detected in Japanese eel, which is different from zebrafish or spotted sea bass (29, 37), which may be caused by the different developing stages of gonad.

In situ hybridization results showed that *tac3*s positive signals were observed in multiple brain regions including telencephalon, mesencephalon, and hypothalamus, which were similar to the results in spotted sea bass (29) and orange-spotted grouper (36). Although it has been reported that *tac3*s expression is different in brain regions among different species, which is speculated to be caused by interspecies difference or physiological state difference (29). However, *tac3s* expression was still highest in the brains of teleosts. In addition, it has been established that NKBs is a key regulator of GnRH, regulating the onset of puberty and reproductive physiology in mammals (44), and is involved in the regulation of the reproductive axis in some teleosts, which suggests that NKBs may be involved in the reproductive process and have regulatory effect.

To test whether the mutation in *tacr3* cause intracellular structural incompletion, we synthesized four NKBs and used

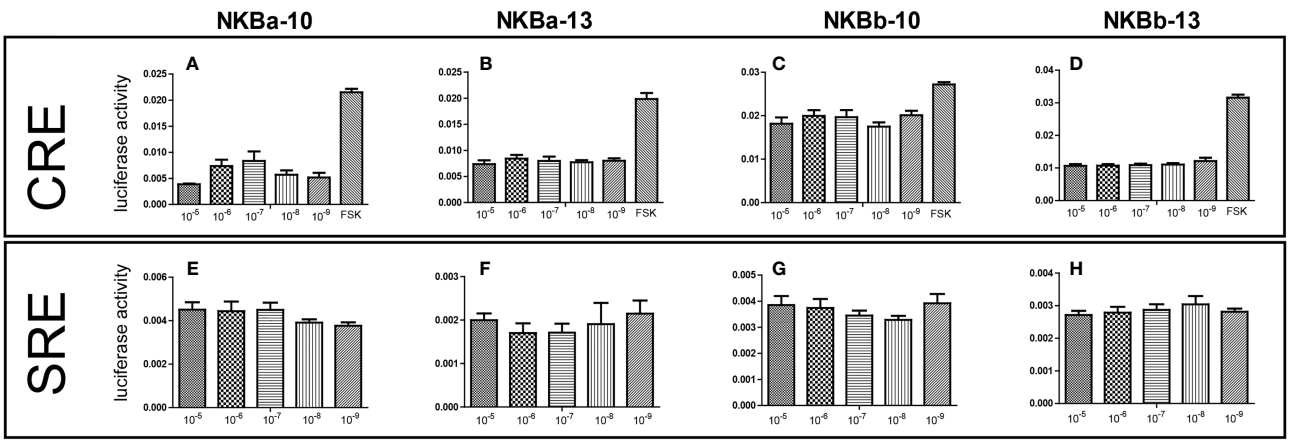


FIGURE 7 | **(A–D)** NKB peptides induced CRE driven promoter activities co-transfected with Japanese eel *tacr3*. **(E–H)** NKB peptides induced SRE driven promoter activities co-transfected with Japanese eel *tacr3*.

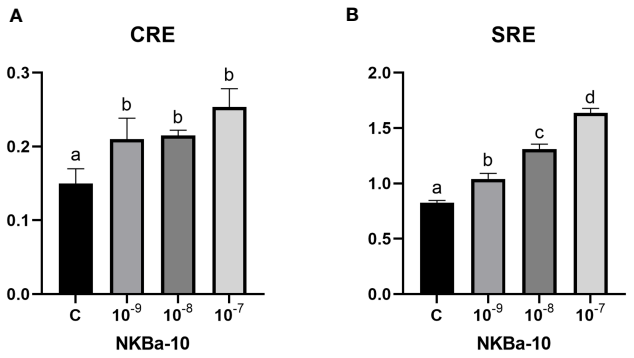


FIGURE 8 | **(A)** NKBa-10 peptides induced CRE driven promoter activities co-transfected with zebrafish *tacr3a1*. **(B)** NKBa-10 peptides induced SRE driven promoter activities co-transfected with zebrafish *tacr3a1*. The different lowercased letters indicated significant differences ($P < 0.05$).

luciferase assay to detect the activation of CRE and SRE signaling pathways. Our results showed that none of the NKBs could activate neither CRE nor SRE signaling pathway *via* Japanese eel TACR3. In addition, we conducted experiments using NKBa-10 through zebrafish receptor and found that it could activate both two signaling pathways effectively. As a result, the Japanese eel receptor is dysfunctional in further signal transduction (45, 46). By blasting the genome sequence, we figured out another potential tac3r; however, limited to the yellow eel samples, the cloning of the gene failed. These results indicate a possibility that the transcription of the other tac3r was started when the yellow eel turned to the silver eel. A large number of studies proved that mutations in the *tac3* or *tacr3* genes lead function decline at the hypothalamic level (47). In different species, NKB has different regulatory effects on the release of LH, and the similar condition occurs in different developmental stages of the same species. For example, NKB has no effect on the release of LH in luteinized ewes, but it can promote the release of LH in follicular phase

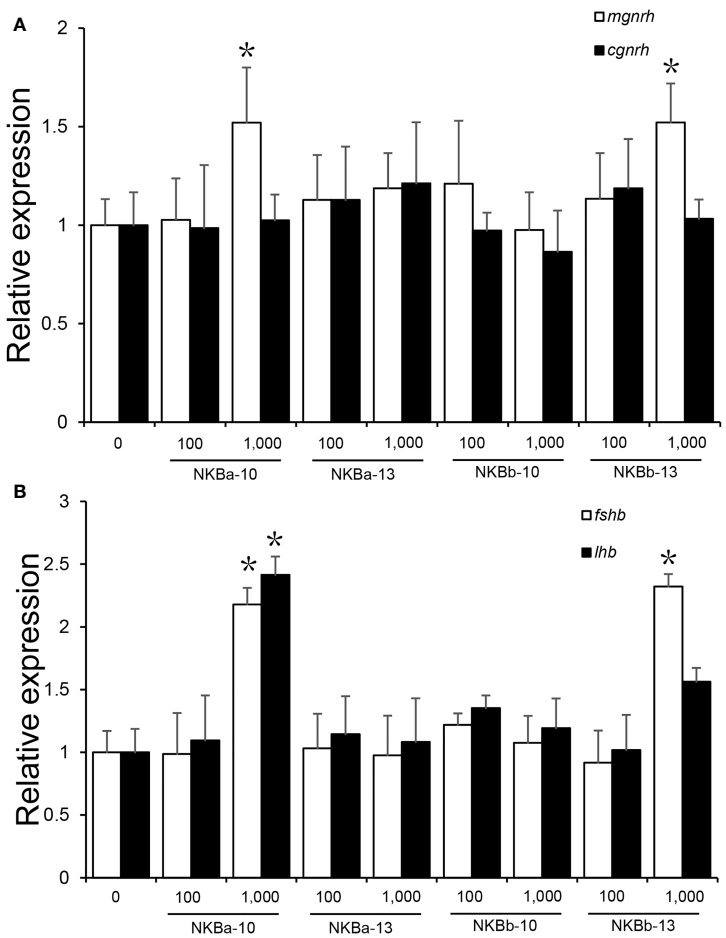


FIGURE 9 | **(A)** Effects of NKBa-10, NKBa-13, NKBb-10, and NKBb-13 on *mgnrh* and *cgnrh* expression of Japanese eel after intraperitoneal injection for 6 h. The X-axis indicates the different NKB peptides and their corresponding concentrations. **(B)** Effects of NKBa-10, NKBa-13, NKBb-10, and NKBb-13 on *fshb* and *lhb* of Japanese eel after intraperitoneal injection for 6 h. The X-axis indicates the different NKB peptides and their corresponding concentrations. The asterisk indicated significant difference of one gene among different stimulate concentration ($P < 0.05$).

(48). We investigated the reproductive regulation of NKB in Japanese eel. Quantitative Real-time (qPCR) results showed that NKBa-10 and NKBb-13 in high concentration can upregulate the mRNA levels of *mgnrh* and *fshb* of Japanese eel, whereas only high concentration of NKBa-10 can increase the mRNA levels of *lhb*; taking the luciferase assay and the *in vivo* injection results together, the NKBs may bind to other receptors to increase the *gnrh* or *gth* expression level.

In conclusion, our present study provides the novel insights into the reproductive function of NKB in Japanese eel. We identified and characterized the NKB/NK3R system in Japanese eel. In addition, we demonstrated dysfunction of the TAC3R and the stimulation of *gnrh* and *gth* by high dose of NKBs. These results present the first demonstrates of NKB/NK3R system in reproduction of Japanese eel.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found below: NCBI; OL804257.1, OL804258.1.

ETHICS STATEMENT

The animal study was reviewed and approved by Animal Research and Ethics Committees of Sun Yat-sen University and Ocean University of China.

AUTHOR CONTRIBUTIONS

XQ, HW, and YL designed the study. WZ performed the RNA extraction and cDNA preparation. WZ performed the sequence analysis and the tissue expression analysis. CZ performed the *in situ* hybridization (ISH) experiment. WZ performed cell culture and co-transfection. WZ performed the *in vivo* injection. CZ and LL wrote the manuscript. XQ provided manuscript editing and feedback. All authors read and approved the final manuscript.

FUNDING

This study was supported by grants from the National Natural Science Foundation of China (41976089).

REFERENCES

1. Waylen A, Wolke D. Sex 'N' Drugs 'N' Rock 'N' Roll: The Meaning and Social Consequences of Pubertal Timing. *Eur J Endocrinol* (2004) 151 Suppl 3: U151–9. doi: 10.1530/eje.0.151u151
2. Vandenbergh JG. Acceleration and Inhibition of Puberty in Female Mice by Pheromones. *J Reprod Fertil Suppl* (1973) 19:411–9.
3. Schulz RW, Henk J. Puberty in Male Fish: Concepts and Recent Developments With Special Reference to the African Catfish (Clarias Gariepinus). *Aquaculture* (1999) 177(1-4):5–12. doi: 10.1016/S0044-8486(99)00064-2
4. Weltzien FA, Andersson E, Andersen Ø, Shalchian-Tabrizi K, Norberg B. The Brain-Pituitary-Gonad Axis in Male Teleosts, With Special Emphasis on Flatfish (Pleuronectiformes). *Comp Biochem Physiol A Mol Integr Physiol* (2004) 137(3):447–77. doi: 10.1016/j.cbpb.2003.11.007
5. Jalabert B. Particularities of Reproduction and Oogenesis in Teleost Fish Compared to Mammals. *Reprod Nutr Dev* (2005) 45(3):261–79. doi: 10.1051/rnd:2005019
6. Okuzawa K, Kumakura N, Mori A, Gen K, Yamaguchi S, Kagawa H. Regulation of GnRH and its Receptor in a Teleost, Red Seabream. *Prog Brain Res* (2002) 141:95–110. doi: 10.1016/S0079-6123(02)41087-4
7. IjIRI S, Tsukamoto K, Chow S, Kurogi H, Adachi S, Tanaka H. Controlled Reproduction in the Japanese Eel (Anguilla Japonica), Past and Present. *Aquac Eur* (2011) 36(2):13–7.
8. Wald G. Metamorphosis: An Overview. In: *Metamorphosis*. Boston, MA: Springer (1981). p. 1–39. doi: 10.1007/978-1-4613-3246-6_1
9. Waters JM, McDowall RM. Phylogenetics of the Australasian Mudfishes: Evolution of an Eel-Like Body Plan. *Mol Phylogenet Evol* (2005) 37(2):417–25. doi: 10.1016/j.ympev.2005.07.003
10. Aroua S, Schmitz M, Baloche S, Vidal B, Rousseau K, Dufour S. Endocrine Evidence That Silvering, a Secondary Metamorphosis in the Eel, is a Pubertal Rather Than a Metamorphic Event. *Neuroendocrinology* (2005) 82(3-4):221–32. doi: 10.1159/000092642
11. Dufour S, Weltzien FA, Sebert ME, Le Belle N, Vidal B, Vernier P, et al. Dopaminergic Inhibition of Reproduction in Teleost Fishes: Ecophysiological and Evolutionary Implications. *Ann N Y Acad Sci* (2005) 1040:9–21. doi: 10.1196/annals.1327.002
12. Fontaine M, Bertrand E, Lopez E, Callamand O. On the Maturation of Genital Organs in the Female Eel (Anguilla Anguilla L.) and Spontaneous Emmision of Eggs in the Aquarium. *C R Hebd Seances Acad Sci* (1964) 259:2907–10. doi: 10.1002/pssb.2220690209
13. Laurent P, Kirsch R, Fontaine MM. Structural Modifications of the Esophagus Linked to Osmoregulation in Eels. *C R Hebd Seances Acad Sci Ser D: Sci Nat* (1975) 280(19):2227–9.
14. Dahl SK, Amstalden M, Coolen L, Fitzgerald M, Lehman M. Dynorphin Immunoreactive Fibers Contact GnRH Neurons in the Human Hypothalamus. *Reprod Sci (Thousand Oaks Calif)* (2009) 16(8):781–7. doi: 10.1177/1933719109336619
15. Ciofi P, Leroy D, Tramu G. Sexual Dimorphism in the Organization of the Rat Hypothalamic Infundibular Area. *Neuroscience* (2006) 141(4):1731–45. doi: 10.1016/j.neuroscience.2006.05.041
16. Kinoshita M, Tsukamura H, Adachi S, Matsui H, Uenoyama Y, Iwata K, et al. Involvement of Central Metastin in the Regulation of Preovulatory Luteinizing Hormone Surge and Estrous Cyclicity in Female Rats. *Endocrinology* (2005) 146(10):4431–6. doi: 10.1210/en.2005-0195
17. Clarkson J, Herbison AE. Postnatal Development of Kisspeptin Neurons in Mouse Hypothalamus; Sexual Dimorphism and Projections to Gonadotropin-Releasing Hormone Neurons. *Endocrinology* (2006) 147(12):5817–25. doi: 10.1210/en.2006-0787
18. Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, et al. TAC3 and TACR3 Mutations in Familial Hypogonadotropic Hypogonadism Reveal a Key Role for Neurokinin B in the Central Control of Reproduction. *Nat Genet* (2009) 41(3):354–8. doi: 10.1038/ng.306
19. Bhangoo A, Jacobson-Dickman E. The Genetics of Idiopathic Hypogonadotropic Hypogonadism:Unraveling the Biology of Human Sexual Development. *Pediatr Endocrinol Rev* (2009) 6(3):395–404.
20. Ramaswamy S, Seminara SB, Ali B, Ciofi P, Amin NA, Plant TM. Neurokinin B Stimulates GnRH Release in the Male Monkey (Macaca Mulatta) and is Colocalized With Kisspeptin in the Arcuate Nucleus. *Endocrinology* (2010) 151(9):4494–503. doi: 10.1210/en.2010-0223
21. Corander MP, Challis BG, Thompson EL, Jovanovic Z, Loraine Tung YC, Rimmington D, et al. The Effects of Neurokinin B Upon Gonadotrophin Release in Male Rodents. *J Neuroendocrinol* (2010) 22(3):181–7. doi: 10.1111/j.1365-2826.2009.01951.x

22. Biran J, Palevitch O, Ben-Dor S, Levavi-Sivan B. Neurokinin Bs and Neurokinin B Receptors in Zebrafish-Potential Role in Controlling Fish Reproduction. *Proc Natl Acad Sci USA* (2012) 109(26):10269–74. doi: 10.1073/pnas.1119165109
23. Qi X, Zhou W, Li S, Liu Y, Ye G, Liu X, et al. Goldfish Neurokinin B: Cloning, Tissue Distribution, and Potential Role in Regulating Reproduction. *Gen Comp Endocrinol* (2015) 221:267–77. doi: 10.1016/j.ygcen.2014.10.017
24. Biran J, Golan M, Mizrahi N, Ogawa S, Parhar IS, Levavi-Sivan B. Direct Regulation of Gonadotropin Release by Neurokinin B in Tilapia (Oreochromis Niloticus). *Endocrinology* (2014) 155(12):4831–42. doi: 10.1210/en.2013-2114
25. Zhou Y, Qi X, Wen H, Zhang K, Zhang X, Li J, et al. Identification, Expression Analysis, and Functional Characterization of Motilin and Its Receptor in Spotted Sea Bass (Lateolabrax Maculatus). *Gen Comp Endocrinol* (2019) 277:38–48. doi: 10.1016/j.ygcen.2019.02.013
26. Hu G, He M, Ko WK, Lin C, Wong AO. Novel Pituitary Actions of TAC3 Gene Products in Fish Model: Receptor Specificity and Signal Transduction for Prolactin and Somatolactin α Regulation by Neurokinin B (NKB) and NKB-Related Peptide in Carp Pituitary Cells. *Endocrinology* (2014) 155 (9):3582–96. doi: 10.1210/en.2014-1105
27. Qi X, Salem M, Zhou W, Sato-Shimizu M, Ye G, Smitz J, et al. Neurokinin B Exerts Direct Effects on the Ovary to Stimulate Estradiol Production. *Endocrinology* (2016) 157(9):3355–65. doi: 10.1210/en.2016-1354
28. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: Improving the Sensitivity of Progressive Multiple Sequence Alignment Through Sequence Weighting, Position-Specific Gap Penalties and Weight Matrix Choice. *Nucleic Acids Res* (1994) 22(22):4673–80. doi: 10.1093/nar/22.22.4673
29. Zhang Z, Wen H, Li Y, Li Q, Li W, Zhou Y, et al. TAC3 Gene Products Regulate Brain and Digestive System Gene Expression in the Spotted Sea Bass (Lateolabrax Maculatus). *Front Endocrinol* (2019) 10. doi: 10.3389/fendo.2019.00556
30. Liu J, Jones KL, Sumer H, Verma PJ. Stable Transgene Expression in Human Embryonic Stem Cells After Simple Chemical Transfection. *Mol Reprod Dev* (2009) 76(6):580–6. doi: 10.1002/mrd.20983
31. Huang YS, Richter JD. Analysis of mRNA Translation in Cultured Hippocampal Neurons. *Methods Enzymol* (2007) 431:143–62. doi: 10.1016/S0076-6879(07)31008-2
32. Carter MS, Krause JE. Structure, Expression, and Some Regulatory Mechanisms of the Rat Preprotachykinin Gene Encoding Substance P, Neurokinin A, Neuropeptide K, and Neuropeptide Gamma. *J Neurosci* (1990) 10(7):2203–14. doi: 10.1523/JNEUROSCI.10-07-02203.1990
33. Zhang Y, Lu L, Furlonger C, Wu GE, Paige CJ. Hemokinin is a Hematopoietic-Specific Tachykinin That Regulates B Lymphopoiesis. *Nat Immunol* (2000) 1(5):392–7. doi: 10.1038/80826
34. Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA. Neurokinin B and the Hypothalamic Regulation of Reproduction. *Brain Res* (2010) 1364:116–28. doi: 10.1016/j.brainres.2010.08.059
35. Christoffels A, Koh EG, Chia JM, Brenner S, Aparicio S, Venkatesh B. Fugu Genome Analysis Provides Evidence for a Whole-Genome Duplication Early During the Evolution of Ray-Finned Fishes. *Mol Biol Evol* (2004) 21(6):1146–51. doi: 10.1093/molbev/msh114
36. Chen H, Xiao L, Liu Y, Li S, Li G, Zhang Y, et al. Neurokinin B Signaling in Hermaphroditic Species, a Study of the Orange-Spotted Grouper (Epinephelus Coioides). *Gen Comp Endocrinol* (2018) 260:125–35. doi: 10.1016/j.ygcen.2018.01.009
37. Zhou W, Li S, Liu Y, Qi X, Chen H, Cheng CH, et al. The Evolution of Tachykinin/Tachykinin Receptor (TAC/TACR) in Vertebrates and Molecular Identification of the TAC3/TACR3 System in Zebrafish (Danio Rerio). *Mol Cell Endocrinol* (2012) 361(1-2):202–12. doi: 10.1016/j.mce.2012.04.007
38. Almeida TA, Rojo J, Nieto PM, Pinto FM, Hernandez M, Martín JD, et al. Tachykinins and Tachykinin Receptors: Structure and Activity Relationships. *Curr Med Chem* (2004) 11(15):2045–81. doi: 10.2174/0929867043364748
39. Hashemian P, Javid H, Tadayyon Tabrizi A, Hashemy SI. The Role of Tachykinins in the Initiation and Progression of Gastrointestinal Cancers: A Review. *Int J Cancer Manag* (2020) 13(5):e100717. doi: 10.5812/ijcm.100717
40. Siviter RJ, Coast GM, Winther AM, Nachman RJ, Taylor CA, Shirras AD, et al. Expression and Functional Characterization of a Drosophila Neuropeptide Precursor With Homology to Mammalian Preprotachykinin a. *J Biol Chem* (2000) 275(30):23273–80. doi: 10.1074/jbc.M002875200
41. Nässel DR. Tachykinin-Related Peptides in Invertebrates: A Review. *Peptides* (1999) 20(1):141–58. doi: 10.1016/S0196-9781(98)00142-9
42. Campo A, Lafont AG, Lefranc B, Leprince J, Tostivint H, Kamech N, et al. Tachykinin-3 Genes and Peptides Characterized in a Basal Teleost, the European Eel: Evolutionary Perspective and Pituitary Role. *Front Endocrinol* (2018) 9. doi: 10.3389/fendo.2018.00304
43. Ogawa S, Ramadasan PN, Goschorska M, Anantharajah A, Ng KW, Parhar IS. Cloning and Expression of Tachykinins and Their Association With Kisspeptins in the Brains of Zebrafish. *J Comp Neurol* (2012) 520(13):2991–3012. doi: 10.1002/cne.23103
44. Lehman MN, Coolen LM, Goodman RL. Minireview: Kisspeptin/Neurokinin B/dynorphin (KNDy) Cells of the Arcuate Nucleus: A Central Node in the Control of Gonadotropin-Releasing Hormone Secretion. *Endocrinology* (2010) 151(8):3479–89. doi: 10.1210/en.2010-0022
45. DeFea KA, Zalevsky J, Thoma MS, Déry O, Mullins RD, Bunnett NW. Beta-Arrestin-Dependent Endocytosis of Proteinase-Activated Receptor 2 is Required for Intracellular Targeting of Activated ERK1/2. *J Cell Biol* (2000) 148(6):1267–81. doi: 10.1083/jcb.148.6.1267
46. Li H, Leeman SE, Slack BE, Hauser G, Saltsman WS, Krause JE, et al. A Substance P (Neurokinin-1) Receptor Mutant Carboxyl-Terminally Truncated to Resemble a Naturally Occurring Receptor Isoform Displays Enhanced Responsiveness and Resistance to Desensitization. *Proc Natl Acad Sci USA* (1997) 94(17):9475–80. doi: 10.1073/pnas.94.17.9475
47. Young J, Bouligand J, Francou B, Raffin-Sanson ML, Gaillez S, Jeanpierre M, et al. TAC3 and TACR3 Defects Cause Hypothalamic Congenital Hypogonadotropic Hypogonadism in Humans. *J Clin Endocrinol Metab* (2010) 95(5):2287–95. doi: 10.1210/jc.2009-2600
48. Billings HJ, Connors JM, Altman SN, Hileman SM, Holaskova I, Lehman MN, et al. Neurokinin B Acts *via* the Neurokinin-3 Receptor in the Retrochiasmatic Area to Stimulate Luteinizing Hormone Secretion in Sheep. *Endocrinology* (2010) 151(8):3836–46. doi: 10.1210/en.2010-0174

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.

Publisher's Note: All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Copyright © 2022 Zuo, Lyu, Zou, Wen, Li and Qi. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 29 July 2022
DOI 10.3389/fendo.2022.923234

Check for updates

OPEN ACCESS

EDITED BY
Bin Wang,
Yellow Sea Fisheries Research Institute (CAFS), China

REVIEWED BY
Paula Gabriela Vissio,
University of Buenos Aires, Argentina
Azadeh Hatef,
University of Saskatchewan, Canada

*CORRESPONDENCE
Qiang Lin
linqiang@scsio.ac.cn

SPECIALTY SECTION
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 19 April 2022
ACCEPTED 06 July 2022
PUBLISHED 29 July 2022

CITATION
Zhang H, Liu Y, Qin G and Lin Q (2022) Identification of neurohypophysial hormones and the role of VT in the parturition of pregnant seahorses (*Hippocampus erectus*). *Front. Endocrinol.* 13:923234. doi: 10.3389/fendo.2022.923234

COPYRIGHT
© 2022 Zhang, Liu, Qin and Lin. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Identification of neurohypophysial hormones and the role of VT in the parturition of pregnant seahorses (*Hippocampus erectus*)

Huixian Zhang[1,2,3], Yali Liu[1,2], Geng Qin[1,2] and Qiang Lin[1,2,3]*

[1]Chinese Academy of Science (CAS) Key Laboratory of Tropical Marine Bio-resources and Ecology (LMB), Guangdong Provincial Key Laboratory of Applied Marine Biology, South China Sea Institute of Oceanology, Chinese Academy of Sciences, Guangzhou, China, [2]Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou), Guangzhou, China, [3]University of Chinese Academy of Sciences, Beijing, China

Neurohypophysial hormones regulate the reproductive behavior of teleosts; however, their role in the gestation and parturition of ovoviviparous fishes with male pregnancy (syngnathids) remains to be demonstrated. In the present study, the complementary DNA (cDNA) sequences of arginine vasotocin (VT) and isotocin (IT) from the lined seahorse (*Hippocampus erectus*) were cloned and identified. We observed that the mature core peptides of seahorse VT and IT were conserved among teleosts. In the phylogenic tree, seahorse VT and IT were clustered independently with teleost VT and IT. The tissue distribution patterns of *VT* and *IT* were similar, and both were highly expressed in the brain, gills, and gonads. Interestingly, they were also expressed to some extent in the brood pouch. *In situ* hybridization revealed that *VT* and *IT* messenger RNA (mRNA) signals in the brain were mainly located in the preoptic area region of the hypothalamus. Intraperitoneal administration of the VT core peptide to pregnant seahorses induced premature parturition, stimulated gonadotropin release, increased serum estrogen levels, and decreased prolactin secretion. Moreover, VT injection upregulated the mRNA expression of the membrane estrogen receptor in the brood pouch. In summary, neurohypophysial hormones promote premature parturition by regulating estrogen synthesis through the hypothalamus–pituitary–gonad axis.

KEYWORDS

seahorse, vasotocin, male pregnancy, parturition, reproductive behavior

Introduction

Neurohypophysial hormones are hypothalamic neuropeptides transported to the posterior pituitary and function as the regulators of vertebrate reproduction. In mammals, neurohypophysial hormones, including oxytocin (OT) and arginine vasopressin (AVP), are primarily involved in uterine contraction, parturition, and lactation (1). OT and AVP only differ in two amino acids, which arose from a gene duplication event during evolution (2). In teleosts, isotocin (IT) is homologous to the mammalian OT whereas arginine vasotocin (VT) is homologous to AVP (3, 4). VT and IT are nonapeptide neurohormones that are primarily synthesized in the neurons of the hypothalamus. They play central roles in fish physiology and behavior, including reproduction, metabolism, osmoregulation, and social behavior (5, 6).

The administration of nonapeptide hormones affects the sexual behaviors and parturition of teleosts (7, 8). IT affects the ovulation of oviparous fishes, such as killifish (*Fundulus heteroclitus*), seurukan fish (*Osteochilus vittatus*), and Hoven's carp (*Leptobarbus hoevenii*) (9–11). Similarly, VT injections stimulate the courtship behavior of the male white perch (*Morone americana*) (12) and influence the aggressive behavior of the rainbow trout (13). Moreover, VT can modulate steroidogenic shift and control serum E_2 levels to stimulate oocyte maturation in female catfish (*Heteropneustes fossilis*) (14). In ovoviviparous fishes, the administration of IT and VT induces premature parturition in the guppy (*Poecilia reticulata*) and topminnow (*Gambusia affinis*) (15).

Syngnathids (pipefish and seahorse) are ovoviviparous fishes with male pregnancy and complex reproductive behavior (16). Syngnathid females transfer eggs into the brood pouch, which is a special structure on the ventral surface of males. Then, the eggs are fertilized and developed in the brood pouch until hatching. The brood pouch is similar to the mammalian placenta, and it can provide nutrition and oxygen to the embryos (17, 18). However, the endocrinological mechanism underlying the seahorse reproductive behavior, including male pregnancy and parturition, is still unknown.

In this study, we characterized the neurohypophysial hormones (VT and IT) of the lined seahorse (*Hippocampus erectus*) and detected their expression patterns in adult tissues. We also analyzed the locations of *VT* and *IT* genes in the brain. Finally, VT was injected *in vivo* to evaluate its functional role in the parturition of pregnant seahorses, its effects on serum sex steroid hormones, and the expression profiles of regulatory genes in the hypothalamus–pituitary–gonad (HPG) axis.

Materials and methods

Experimental fish and tissue sampling

Cultured adult lined seahorses (*H. erectus*) (6 months old) were collected from the Zhangzhou Fisheries Center, Fujian Province, China. Seahorses were maintained in seawater tanks at 26 ± 0.5°C, 26 ± 2% salinity, and a 12:12-h day-light cycle. The seahorses were fed twice daily with frozen Mysis shrimp and reared for 2 weeks before the experiments. The lined seahorses were anesthetized with 0.05% MS-222 (Sigma, St. Louis, MO, USA) and dissected. Tissues were collected, frozen in liquid nitrogen, and stored at an -80°C freezer until RNA extraction. All animal experiments were conducted in accordance with the guidelines and approval of the Animal Research and Ethics Committee of the Chinese Academy of Sciences.

Cloning of the seahorse *VT* and *IT* genes

Total RNA was isolated from the brain using a TRIzol reagent (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's instructions. Total RNA (1 μg) was reverse-transcribed using the ReverTra Ace-α highly efficient reverse transcription kit (Toyobo, Osaka, Japan). Based on the brain transcriptome and genome data of the lined seahorse (19, 20), the full-length cDNA sequences of seahorse *VT* and *IT* were obtained using the rapid amplification of cDNA ends (RACE). The 5′-UTR and 3′-UTR ends were amplified using a SMARTer RACE 5′/3′ kit (Clontech, Palo Alto, CA, USA). The PCR primers used are listed in Table 1. Target fragments were subcloned into a PMD18-T vector (Takara, Dalian, China). The positive clones of target fragments were sequenced by Tsingke BioTech (Beijing, China).

Phylogenetic and synteny analysis of seahorse vasotocin and isotocin

The cDNA sequences of seahorse *VT* and *IT* were translated into amino acid sequences by using Expasy (http://web.expasy.org/protparam). Multiple sequence alignments were performed using the DNAMAN software. The signal peptide was predicted using the SignalP 5.0 server (http://www.cbs.dtu.dk/services/SignalP/). A phylogenetic tree was constructed with Mega 6.0 software (21) using the neighbor-joining method (bootstrap = 1,000). A synteny analysis of *VT* and *IT* genes was performed by comparing the gene arrangements of the *VT* and *IT* gene loci in zebrafish, platyfish, tilapia, medaka, fugu, tiger-tailed seahorse, and lined seahorse. The gene loci of these teleosts were identified using the genome data from Ensembl (http://www.ensembl.org).

Tissue distribution of seahorse vasotocin and isotocin

Total RNA was isolated from various tissues of male seahorses (n=3), including the brain regions (telencephalon, optic tectum, cerebellum, hypothalamus, and pituitary), liver, gill, intestine, kidney, muscle, heart, gonad, and brood pouch.

TABLE 1 Information of primers used in this study.

Primer sequence

Gene	Purpose	Primer	5'-3' sequence
VT	Partial cDNA	VT_F1	GCTGATGCTCCTCCTGCTTGG
		VT_R1	AAGGCCGTTCTTCCGTAGTGC
	5'RACE	VT_R2 (first)	GTACGGGACGGTCACATAGCC
		VT_R3 (nest)	CTTCCACGAGGACCTCGTCTG
	3'RACE	VT_F2 (first)	GCTACTCGGCTCTGGGAAAGC
		VT_F3 (nest)	GTGACCGCCACGTCAGGATC
	Real-time PCR	VT_qF	CCTCCGCCTGCTACATCCAG
		VT_qR	CAGGGAGTCAGCAGGTAGTTC
IT	Partial cDNA	IT_F1	GCTGCAGCTCGGCACCTTCTGC
		IT_R1	GACGATTGATCCGAAGAACATC
	5'RACE	IT_R2 (first)	CACAGGCAGGACTGTGCGTTC
		IT_R3 (nest)	CATCTTCAGGATTGTCTCAGTG
	3'RACE	IT_F2 (first)	GGCTTCTACCACGAGCACAC
		IT_F3 (nest)	TCCACTGGGAGAACATTCAGG
	Real-time PCR	IT_qF	CCTTCCGCTTGCTGGTCT
		IT_qR	CAATGGGACAGTTGGAGATG
FSHβ	Real-time PCR	FSHβ_qF	GCAATGGGAACTGGACCTAC
		FSHβ_qR	TGATTGATACGAGCAGCACA
LHβ	Real-time PCR	LHβ_qF	CCAATAAGGTGCCAGGATGT
		LHβ_qR	ACCTGGAAGGCAGTTAGACA
PRL	Real-time PCR	PRL_qF	TGACGTCGGTCAGGACAAGC
		PRL_qR	CTTCAGCACAAGTGAGGTTGC
ERα	Real-time PCR	ERα_qF	TTACTCACCAGCATGGCTGAC
		ERα_qR	CCTCGGACTTGAGTCTGAGC
ERβ	Real-time PCR	ERβ_qF	GTCCTCACACAGCAAGACTC
		ERβ_qR	TCCAGAAGACTGAGCTCCAC
GPER	Real-time PCR	GPER_qF	CGTCTTCATCAGCATCCAGC
		GPER_qR	ACCGAAGGTCCCAAATGGAG
β-actin	Real-time PCR	β-actin_qF	TTCACCACCACAGCCGAGA
		β-actin_qR	TGGTCTCGTGGATTCCGCAG

Reverse transcription PCR was performed using 1 μg of RNA isolated from each tissue. PCR conditions were as follows: predenaturation at 94°C for 3 min; 30 cycles at 94°C for 15 s, 55°C for 15 s, 72°C for 30 s, and extension at 72°C for 10 min; and cooling to 4°C. The housekeeping gene (β-actin) was used as a positive control.

RNA *in situ* hybridization

The fragments of *VT* and *IT* genes (528 and 589 bp, respectively) were subcloned into the pGEM-T easy vector. Plasmid DNA containing the *VT* and *IT* inserts was extracted using the E.Z.N.A Plasmid DNA Mini kit (Omega, Norcross, GA, USA). Sense and antisense riboprobes were synthesized from SalI- and NcoI-linearized pGEM-T easy plasmids using the DIG RNA Labeling kit (Roche, Mannheim, Germany). The brain samples of lined seahorse were fixed in 4% paraformaldehyde overnight at 4°C. The fixed brains were then dehydrated in 20% sucrose and embedded in an optimal cutting temperature (OCT) compound (Sakura, Tokyo, Japan). Cross-sections (10 μm), including those of the hypothalamus area, were collected on 3-aminopropylysilane-coated slides. Collected sections were digested with proteinase K (1 μg/ml) at 37°C for 20 min. The slices were prehybridized with hybridization buffer without probe at 58°C for 1 h and hybridized with DIG-labeled probes (0.5 μg/ml) in hybridization buffer at 58°C overnight in a sealed humidity chamber. After hybridization, slides were washed in 2× saline-sodium citrate (SSC) buffer and 0.1× SSC buffer for 30 min at 58°C. The slides were then blocked with 2% normal sheep serum and incubated with an alkaline phosphatase–conjugated anti-DIG antibody (Roche; 1:1,000 dilution). The color change reaction was conducted using an nitroblue tetrazolium (NBT)/5-bromo-4-chloro-3-indolye phosphate

(BCIP) stock solution (Roche, Basel, Switzerland), and the reaction was stopped using tap water.

In vivo effects of vasotocin on the parturition of pregnant seahorses

To evaluate the functional role of VT in parturition, pregnant seahorses were injected with [Arg^8] vasotocin acetate salt (VT, V7009; Sigma). The pregnant seahorses (6 months old) were anesthetized and injected intraperitoneally with VT (high dose: 500 ng/g; low dose: 50 ng/g) and saline (n = 6) as the sham control. Seahorses from each group were sampled at 6-h postinjection. The pituitary and brood pouch were dissected and frozen in liquid nitrogen until RNA extraction. Blood samples were collected from the tail vessels at 6-h postinjection. Serum samples were separated by centrifugation at 3,000 × *g* for 30 min at 4°C and stored at -20°C. The serum levels of estrogen and testosterone were determined *via* an enzyme-linked immunosorbent assay (ELISA) kit (Cayman Chemical Company, Ann Arbor, MI, USA) (Estradiol ELISA kit no. 501890 and Testosterone ELISA kit no. 582701) according to the manufacturer's protocol (22). Approximately 5 µl of serum sample was diluted in 45 µl of ELISA buffer to detect the concentration of estrogen and testosterone using the standard samples as a reference. The mRNA expression profiles of gonadotropins, follicle-stimulating hormone (FSHβ), luteinizing hormone (LHβ), and prolactin (PRL) in the pituitary were analyzed.

Quantitative real-time PCR

The expression levels of all the tested genes were measured using the qRT-PCR methodology described previously (22). The primers used for the qRT-PCR assays are listed in Table 1. The template was amplified at 94°C for 1 min, followed by 40 cycles at 94°C for 15 s, 50–55°C for 15 s, and extension at 72°C for 20 s. The housekeeping gene β-actin was used as the reference gene. The relative abundance of each target gene was evaluated using the $2^{-\Delta\Delta Ct}$ method (23).

Statistical analysis

Data were shown as the mean ± standard error of the mean (SEM). Significant differences were evaluated by one-way analysis of variance (ANOVA), followed by Duncan's multiple range tests using Prism 6.0 (GraphPad Software, San Diego, CA, USA). Differences between groups with $p < 0.05$ were deemed significant.

Results

Characterization of seahorse *VT* and *IT* genes

The lined seahorse *VT* cDNA sequence (GenBank Accession No. MW490038) had an Open Reading Frame (ORF) of 477 bp, encoding for a protein of 158 amino acids (aa) (Figure 1A). The VT precursor had a signal peptide of 24 aa, a 9-aa hormone moiety (CYIQNCPRG), an enzymatic processing site [Gly-Lys-Arg (GKR)], and an 88-aa neurophysin. The *IT* precursor cDNA (GenBank Accession No. MW490039) had an ORF of 471 bp, encoding for a protein of 156 aa (Figure 1B). The IT precursor had a signal peptide of 19 aa, a hormone moiety of 9 aa (CYIQNCPLG), an enzymatic processing site, a 95-aa neurophysin, and a 30-aa copeptin region with a leucine-rich core segment. Homology analysis showed that seahorse VT had the highest sequence identity with the VT sequences of medaka (77.8%) and clownfish (77.8%) and that the IT sequence was highly similar to that of clownfish (80.1%) and flounder (78.8%) (Table 2).

Phylogeny and synteny analyses of seahorse vasotocin and isotocin

The phylogenetic tree revealed that the neurohypophysial hormones were clustered into two separate clades: VT and IT clades. Seahorse VT were clustered together with other teleost VT precursors, while seahorse IT were clustered with other teleost IT precursors (Figure 2). Human vasopressin (VP) and OT were used as outgroups. A synteny analysis of *VT* and *IT* showed that the organization and orientation of these genes in the loci were highly conserved among teleost species (Figure S1). The synteny of *VT* and *IT* in seahorse was the same as that in other teleosts, except for zebrafish, in which *VT* and *IT* were located on two separate chromosomes.

Tissue distribution of seahorse vasotocin and isotocin

The tissue distribution of seahorse *VT* and *IT* was determined using semiquantitative reverse transcription PCR (Figure 3). Both *VT* and *IT* mRNAs were widely expressed in the central nervous system and peripheral tissues and were highly expressed in the hypothalamus, gills, and gonads. Interestingly, these genes were also expressed in the brood pouch to some extent.

10.3389/fendo.2022.923234

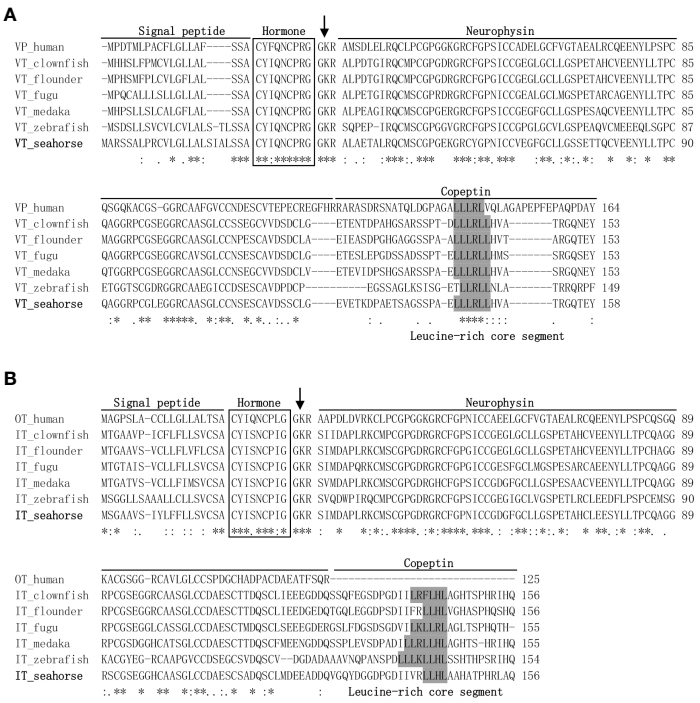


FIGURE 1
Genetic structure and multiple sequence alignments of vasotocin (VT) **(A)** and isotocin (IT) **(B)**. The mature hormone peptides are boxed, and the GKR cleavage site was marked with an arrow. Genetic structures, including a signal peptide, neurophysin, and copeptin parts, were lined out. GenBank accession numbers for teleost VT and IT proteins are listed in Table 2. Symbol asterisks means that the amino acid residues in that column are identical in the alignment.

TABLE 2 Similarities of amino acid sequences of the neurohypophysial hormone between the lined seahorse and other teleosts.

Species	Accession no.	Amino acid sequence identity (%)	
		VT	IT
VT_seahorse	MW490038	100.0	56.2
VT_clownfish	AEB00559.1	77.8	58.2
VT_flounder	BAA98140.1	75.8	58.2
VT_fugu	AAC60293.1	73.9	54.9
VT_medaka	BAM15897.1	77.8	56.2
VT_zebrafish	NP_840078.1	59.1	50.7
VP_human	NP_000481.2	52.0	45.2
IT_seahorse	MW490039	56.2	100.0
IT_clownfish	AEB00560.1	60.8	80.1
IT_flounder	BAA98141.1	58.2	78.8
IT_fugu	O42493.1	54.9	72.9
IT_medaka	BAM15898.1	55.9	75.5
IT_zebrafish	AAL50209.1	49.3	59.5
OT_human	NP_000906.1	54.4	50.4

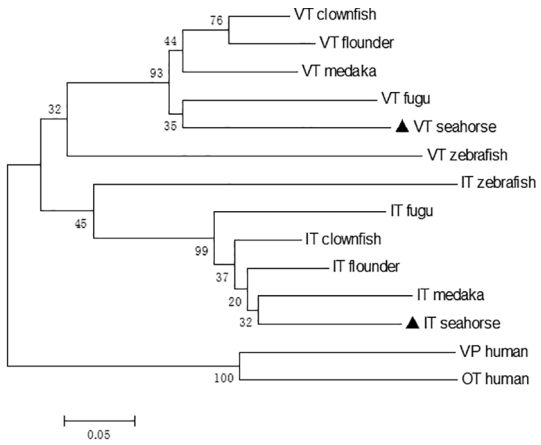


FIGURE 2
Neighbor-joining phylogenetic tree of VT and IT and their homologous sequences in vertebrates. The numbers indicate the bootstrap values of 1,000 replicates.

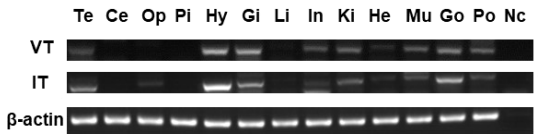


FIGURE 3
Analysis of *VT* and *IT* expression patterns in various tissues of male seahorses by reverse transcription PCR. The brain regions: Te, telencephalon; Ce, cerebellum; Op, optic tectum; Pi, pituitary; and Hy, hypothalamus. The peripheral tissues: Gi, gill; Li, liver; In, intestine; Ki, kidney; He, heart; Mu, muscle; Go, gonad; Po, brood pouch. Nc, negative control.

Localization of vasotocin and isotocin in the brain

The functions of lined seahorse VT and IT were evaluated based on their relative expression patterns. Therefore, the localization of the cells expressing *VT* and *IT* mRNAs was detected in the lined seahorse brain (hypothalamus) *via in situ* hybridization. Both *VT* and *IT* mRNAs were located in the preoptic area (POA) region of the hypothalamus (Figure 4). *VT* mRNA was mainly expressed in the central area of the POA region, whereas *IT* mRNA was expressed in the edge area of the POA region.

Effects of vasotocin administration on the parturition of pregnant seahorses

After VT injection, serum estrogen levels increased remarkably, whereas serum testosterone levels did not change significantly (Figure 5A). The pregnant seahorses continued swinging their body to perform parturition behavior to let the seahorse larvae hatch out from the brood pouch. In addition, premature seahorse larvae (induced by high and low doses of VT) still had large yolk sacs and were shorter than the larvae that hatched normally (Figure 5B, Table 3). *FSHβ* and *LHβ* mRNA levels increased significantly, whereas *PRL* mRNA levels decreased significantly in the pituitary of pregnant seahorses.

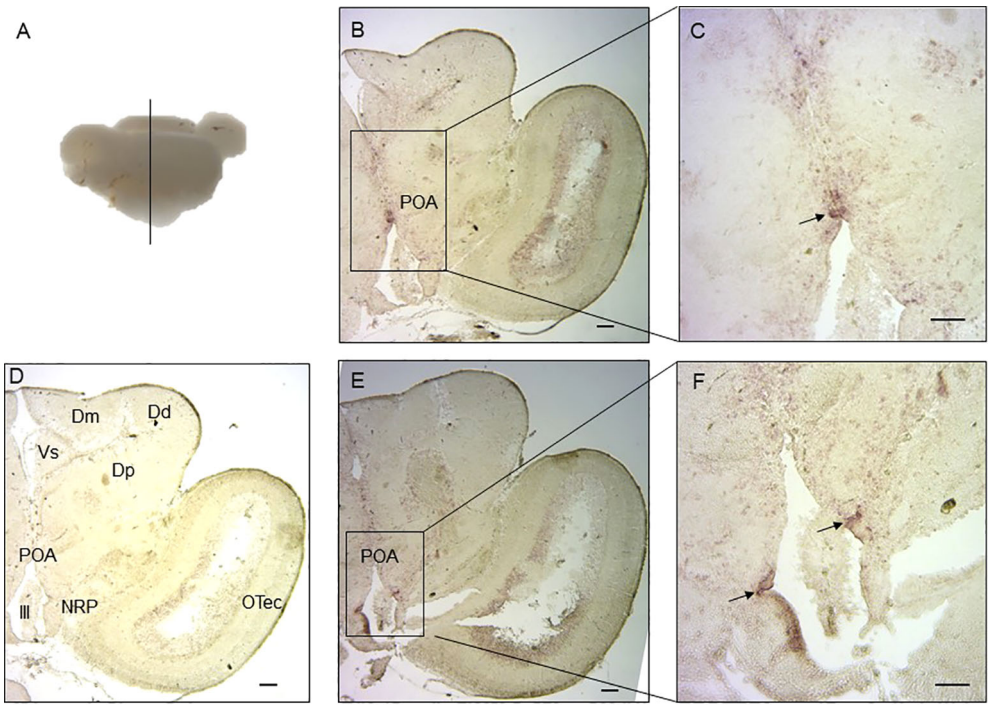


FIGURE 4
Localization of cells expressing *VT* and *IT* mRNAs in the lined seahorse brain. **(A)** Schematic diagram indicating the position of the coronal section and brain slides selected in the following. **(B)** *In situ* hybridization with an antisense *VT* mRNA probe showing the midbrain coronal section. **(C)** Enlarged part of the hypothalamus region using a *VT* antisense probe. **(D)** *In situ* hybridization with a sense *VT* probe within hypothalamus and midbrain regions of the lined seahorse. The brain regions are marked out in the micrograph. **(E)**. *In situ* hybridization with an antisense *IT* mRNA probe showing the midbrain coronal section. **(F)** Enlarged part of the hypothalamus region using and *IT* antisense probe. OTec, optic tectum; Dd, dorsal part of dorsal telencephalon; Dp, posterior part of dorsal telencephalon; Dm, medial part of dorsal telencephalon; Vs, supracommissural nucleus of ventral telencephalon; POA, preoptic area; NRP, the nucleus of posterior recess; III, the third ventricle. Scale bars = 200 μm.

10.3389/fendo.2022.923234

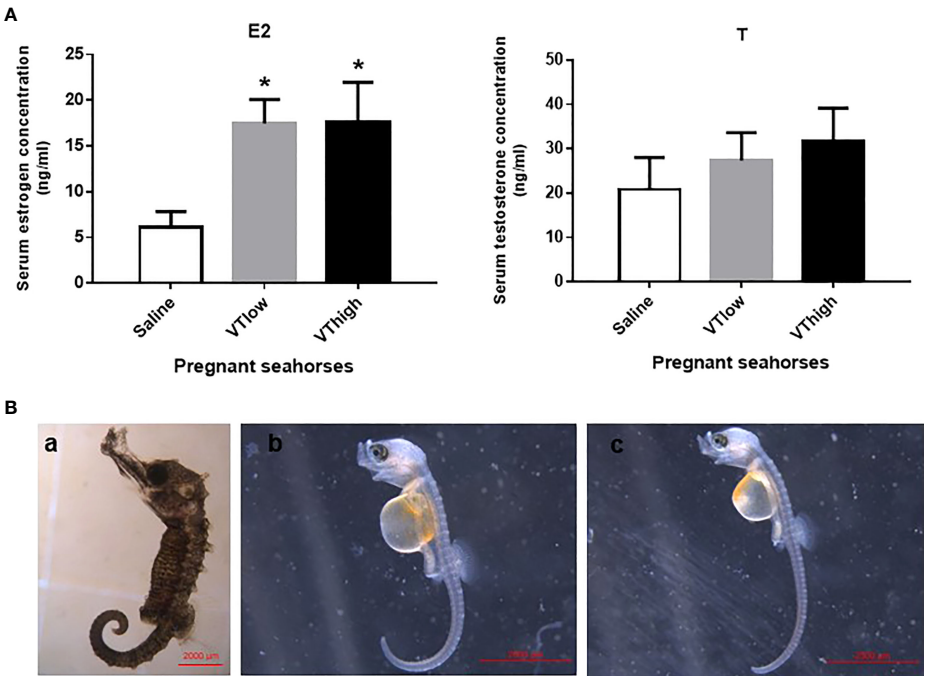


FIGURE 5
Serum estrogen and testosterone levels after VT injection **(A)**. Seahorse larvae injected with low **(B-b)** and high doses **(B-c)** of VT compared to normal hatched seahorse larvae **(B-a)**. Data are presented as mean ± SEM. Asterisks indicate significant differences between different treatments ($p < 0.05$).

Moreover, the mRNA expression of the membrane estrogen receptor [G protein-coupled estrogen receptor (*GPER*)] in the brood pouch significantly increased after a high-dose VT injection, whereas *ERα* and *ERβ* mRNAs showed no significant changes (Figure 6).

Discussion

In the present study, the cDNAs of the neurohypophysial hormones *VT* and *IT* were cloned and characterized in the lined seahorse. Sequence analysis revealed that the mature peptides of VT and IT were highly conserved among the teleost species. In addition to the signal and mature peptides, the neurohypophysial nonapeptide precursor had three domains: GKR, neurophysin, and copeptin (2, 24). The GKR domain, which is involved in the cleavage and amidation of the hormone, was conserved. However, the N-terminus of neurophysin was divergent whereas the rest was conserved. The domains of neurohypophysial hormones in teleosts are identical (3). Phylogeny analysis revealed that seahorse VT was clustered with other teleost VTs, and seahorse IT was clustered with other teleost ITs independently. Synteny analysis showed that *VT* and *IT* gene loci were conserved among teleosts. These results showed that the cloned seahorse *VT* and *IT* genes are orthologous to those of other teleosts.

TABLE 3 Effect of the AVT hormone on parturition in the lined seahorses.

Dose (ng/g body weight)	Total no. of seahorses	Standard length of larvae (mm)	Yolk sac diameter (mm)
0 (Saline)	6	7.82 ± 0.02	<0.05
50 (Vasotocin)	6	6.73 ± 0.05*	0.94 ± 0.03*
500 (Vasotocin)	6	6.24 ± 0.03*	0.85 ± 0.02*

Values shown are means ± standard errors. *Significantly ($P < 0.05$) different from control.

Reverse transcription PCR showed that both *VT* and *IT* mRNAs are highly expressed in the hypothalamus, gills, and gonads of the lined seahorse, similar to the findings in other teleosts (25, 26). Seahorse *VT* mRNA was expressed much more widely than *IT* mRNA, suggesting that VT plays more diverse roles in various tissues than IT. The high expression in sexual tissues suggests that VT and IT play important roles in regulating reproductive behavior. In addition, the expression in the gills suggests that VT and IT are involved in osmoregulation (5).

In situ hybridization showed that seahorse *VT* and *IT* were mainly expressed in the cells of the POA region in the hypothalamus. This result is consistent with the findings in the orange-spotted grouper (27), bluehead wrasse (7), and weakly

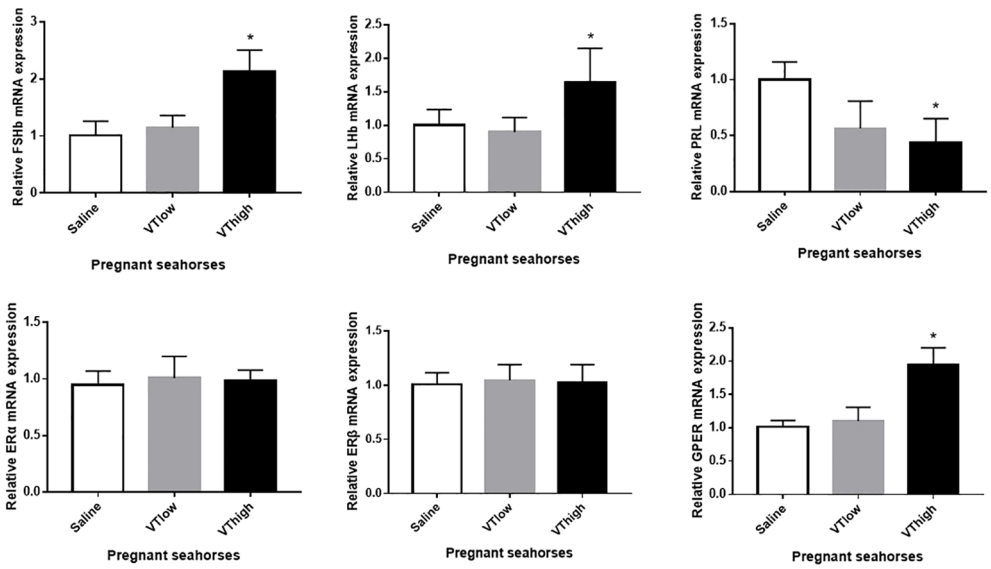


FIGURE 6
Effects of the VT core peptide injection on the expression of *FSHβ*, *LHβ*, and *PRL* genes in the pituitary and *ERα*, *ERβ*, and *GPER* genes in the brood pouch of pregnant lined seahorses. The mRNA expression levels were determined using qRT-PCR. Data are presented as mean ± SEM. Asterisks indicate significant differences between different treatments ($p < 0.05$).

electric fish (28). Most studies in teleosts have suggested that *VT* and *IT* expression in the brain is limited to the hypothalamus, especially in the POA region, which is the center of sex-related genes. Surprisingly, *VT* mRNA is expressed in the dorsal and medial telencephalic regions of the cichlid fish (*Astatotilapia burtoni*) (29). These results indicate that the neurohypophysial hormones regulate sexual behavior through interactions with other key genes involved in reproductive behavior.

VT plays a role in regulating the sexual behavior of both male and female fishes. VT controls oocyte maturation and ovulation in female catfish by stimulating estrogen secretion and inhibiting progestin steroids (14). In male zebrafish, VT stimulates courtship behavior and reproductive physiology (30). In addition, VT plays a role in regulating aggressive behavior in mudskippers (31). In ovoviviparous fishes, VT can induce premature parturition in the topminnow (*G. affinis*) and guppy (*P. reticulata*) (15). VT can also induce premature parturition in ovoviviparous fishes with male pregnancy, including in lined seahorses. We observed that after VT injection, pregnant seahorses performed parturition and continuously shook their bodies to let the seahorse fries hatch out from the brood pouch. Sex steroids, including estrogen, play an important role in regulating sex-role reversal behavior (32). In lined seahorses, serum estrogen levels increase during pregnancy and decrease during the post-pregnancy stage (33). In the present study, the mRNA levels of *GPER* increased significantly after VT injection. This finding is in agreement with the postparturition upregulation of *GPER* in big-belly seahorses (34). These findings demonstrate that sex steroids, such as estrogen, play important roles in the parturition of pregnant seahorses. Moreover, VT induces parturition in pregnant seahorses and brood pouch compression, a process that may be mediated by GPER.

In the lined seahorse, VT induces estrogen secretion by stimulating gonadotropin (FSHβ and LHβ) synthesis during the parturition stage. This finding suggests that VT regulates reproductive physiology through the HPG axis in lined seahorses. VT also downregulated the *PRL* mRNA expression during parturition in lined seahorses. PRL maintains the brood pouch in a proper condition to support embryonic development in seahorses. In addition, PRL hormone levels are higher in the mating stage than in the parturition stage (16). These results indicate that VT is involved in the premature parturition of seahorses by upregulating estrogen secretion and downregulating PRL synthesis. Taken together, VT regulates the fish reproductive behavior *via* the HPG axis, in which the VT-stimulated gonadotropin synthesis and inhibited prolactin secretion in the pituitary regulate the estrogen secretion.

The premature release of embryos might be a stress response in pregnant seahorses. Insufficient nutrition, characterized by limited food consumption for a relatively long period, might promote premature parturition to release the embryos and keep them alive through VT stimulation. Studies have shown that VT is involved in the stress response; VT neurons innervate the corticotropic cells of the pituitary and influence adrenocorticotropin, which is secreted from the interrenal (5,

35). Pregnant seahorses may upregulate VT hormones in response to stress when suffering from nutritional deficiencies.

In conclusion, the present study reported the physiological role of neurohypophysial hormones in the reproductive behavior of ovoviviparous fishes with male pregnancy. Moreover, we demonstrated their roles in regulating parturition through the HPG axis. These findings suggest that VT regulates parturition behavior by upregulating serum estrogen levels. In addition, VT induces parturition through the membrane estrogen receptor (GPER). Our results shed light on the endocrinological mechanism of neurohypophysial hormones in regulating the reproductive behavior of teleosts.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Animal research and Ethics Committees of the Chinese Academy of Sciences.

Author contributions

HZ and QL designed the research. HZ and YL carried out the experiment. HZ, YL, and GQ analyzed the experiment data. QL provided lab space and equipment. All authors contributed to the article and approved the submitted version.

Funding

The present study was supported by the Key Special Project for Introduced Talents Team of Southern Marine Science and Engineering Guangdong Laboratory (Guangzhou) (GML2019ZD0401, GML2019ZD0407), the Strategic Priority Research Program of the Chinese Academy of Sciences (No. XDA19060301), the National Science Foundation of China (No. 41976116, 41825013), the Youth Innovation Promotion Association CAS (2019337), and Guangdong Basic and Applied Basic Research Foundation (2021A1515011393) the Special Foundation for National Science and Technology Basic Research Program of China (2018FY100100), Science and Technology Planning Project of Guangdong Province, China (2020B1212060058).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.923234/full#supplementary-material

SUPPLEMENTARY FIGURE 1
Conserved synteny for the genomic region comprising *VT* and *IT* gene loci in teleosts. The gene organization and orientation in the genomic region containing the *VT* and *IT* genes were obtained from the Ensembl genome browser (http://www.ensembl.org).

References

1. Baribeau DA, Anagnostou E. Oxytocin and vasopressin: Linking pituitary neuropeptides and their receptors to social neurocircuits. *Front Neurosci* (2015) 9:335. doi: 10.3389/fnins.2015.00335

2. Gwee PC, Tay BH, Brenner S, Venkatesh B. Characterization of the neurohypophysial hormone gene loci in elephant shark and the Japanese lamprey: Origin of the vertebrate neurohypophysial hormone genes. *BMC Evol Biol* (2009) 9:47. doi: 10.1186/1471-2148-9-47

3. Banerjee P, Chaube R, Joy KP. Molecular cloning, sequencing and tissue expression of vasotocin and isotocin precursor genes from ostariophysian catfishes: Phylogeny and evolutionary considerations in teleosts. *Front Neurosci* (2015) 9:166. doi: 10.3389/fnins.2015.00166

4. Urano A, Ando H. Diversity of the hypothalamo-neurohypophysial system and its hormonal genes. *Gen Comp Endocrinol* (2011) 170:41–56. doi: 10.1016/j.ygcen.2010.09.016

5. Balment RJ, Lu W, Weybourne E, Warne JM. Arginine vasotocin a key hormone in fish physiology and behaviour: A review with insights from mammalian models. *Gen Comp Endocrinol* (2006) 147:9–16. doi: 10.1016/j.ygcen.2005.12.022

6. Thompson RR, Walton JC. Peptide effects on social behavior: Effects of vasotocin and isotocin on social approach behavior in Male goldfish (*Carassius auratus*). *Behav Neurosci* (2004) 118:620–6. doi: 10.1037/0735-7044.118.3.620

7. Godwin J, Thompson R. Nonapeptides and social behavior in fishes. *Hormones Behav* (2012) 61:230–8. doi: 10.1016/j.yhbeh.2011.12.016

8. Munakata A, Kobayashi M. Endocrine control of sexual behavior in teleost fish. *Gen Comp Endocrinol* (2010) 165:456–68. doi: 10.1016/j.ygcen.2009.04.011

9. Lyu LK, Li JS, Wang XJ, Yao YJ, Li JF, Li Y, et al. Arg-vasotocin directly activates isotocin receptors and induces COX2 expression in ovoviviparous guppies. *Front Endocrinol (Lausanne)* (2021) 12:617580. doi: 10.3389/fendo.2021.617580

10. Muchlisin ZA, Arfandi G, Adlim M, Fadli N, Sugianto S. Induced spawning of seurukan fish, *Osteochilus vittatus* (Pisces: Cyprinidae) using ovaprim, oxytocin and chicken pituitary gland extracts. *Aacl Bioflux* (2014) 7:412–8.

11. Pickford GE, Strecker EL. The spawning reflex response of the killifish, *Fundulus heteroclitus*: Isotocin is relatively inactive in comparison with arginine vasotocin. *Gen Comp Endocrinol* (1977) 32:132–7. doi: 10.1016/0016-6480(77)90143-5

12. Salek SJ, Sullivan CV, Godwin J. Arginine vasotocin effects on courtship behavior in Male white perch (*Morone americana*). *Behav Brain Res* (2002) 133:177–83. doi: 10.1016/S0166-4328(02)00003-7

13. Backstrom T, Winberg S. Arginine-vasotocin influence on aggressive behavior and dominance in rainbow trout. *Physiol Behav* (2009) 96:470–5. doi: 10.1016/j.physbeh.2008.11.013

14. Joy KP, Chaube R. Vasotocin–a new player in the control of oocyte maturation and ovulation in fish. *Gen Comp Endocrinol* (2015) 221:54–63. doi: 10.1016/j.ygcen.2015.02.013

15. Venkatesh B, Tan CH, Lam TJ. Prostaglandins and teleost neurohypophyseal hormones induce premature parturition in the guppy, *Poecilia reticulata*. *Gen Comp Endocrinol* (1992) 87:28–32. doi: 10.1016/0016-6480(92)90146-B

16. Scobell SK, MacKenzie DS. Reproductive endocrinology of syngnathidae. *J Fish Biol* (2011) 78:1662–80. doi: 10.1111/j.1095-8649.2011.02994.x

17. Stolting KN, Wilson AB. Male Pregnancy in seahorses and pipefish: Beyond the mammalian model. *BioEssays News Rev molecular Cell Dev Biol* (2007) 29:884–96. doi: 10.1002/bies.20626

18. Whittington CM, Friesen CR. The evolution and physiology of Male pregnancy in syngnathid fishes. *Biol Rev Cambridge Philos Soc* (2020) 95:1252–72. doi: 10.1111/brv.12607

19. Lin Q, Fan S, Zhang Y, Xu M, Zhang H, Yang Y, et al. The seahorse genome and the evolution of its specialized morphology. *Nature* (2016) 540:395–9. doi: 10.1038/nature20595

20. Lin Q, Qiu Y, Gu R, Xu M, Li J, Bian C, et al. Draft genome of the lined seahorse, *Hippocampus erectus*. *GigaScience* (2017) 6:1–6. doi: 10.1093/gigascience/gix030

21. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: Molecular evolutionary genetics analysis version 6.0. *Mol Biol Evol* (2013) 30:2725–9. doi: 10.1093/molbev/mst197

22. Zhang H, Zhang B, Qin G, Li S, Lin Q. The roles of the kisspeptin system in the reproductive physiology of the lined seahorse (*Hippocampus erectus*), an ovoviviparous fish with Male pregnancy. *Front Neurosci* (2018) 12:940. doi: 10.3389/fnins.2018.00940

23. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2(T)(-delta delta c) method. *Methods* (2001) 25:402–8. doi: 10.1006/meth.2001.1262

24. Banerjee P, Chaube R, Joy KP. Molecular cloning and characterisation of an isotocin paralogue ([V8] isotocin) in catfishes (Superorder ostariophysi): Origin traced likely to the fish-specific whole genome duplication. *J Neuroendocrinol* (2018) 30:e12647. doi: 10.1111/jne.12647

25. Godwin J, Sawby R, Warner RR, Crews D, Grober MS. Hypothalamic arginine vasotocin mRNA abundance variation across sexes and with sex change in a coral reef fish. *Brain Behav Evol* (2000) 55:77–84. doi: 10.1159/000006643

26. Ramallo MR, Grober M, Canepa MM, Morandini L, Pandolfi M. Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish *Cichlasoma dimerus*. *Gen Comp Endocrinol* (2012) 179:221–31. doi: 10.1016/j.ygcen.2012.08.015

27. Nagarajan G, Aruna A, Chang CF. Neuropeptide arginine vasotocin positively affects neurosteroidogenesis in the early brain of grouper, *Epinephelus coioides*. *J Neuroendocrinol* (2015) 27:718–36. doi: 10.1111/jne.12298

28. Pouso P, Cabana A, Goodson JL, Silva A. Preoptic area activation and vasotocin involvement in the reproductive behavior of a weakly pulse-type electric fish, *Brachyhypopomus gauderio*. *Front Integr Neurosci* (2019) 13:37. doi: 10.3389/fnint.2019.00037

29. Rodriguez-Santiago M, Nguyen J, Winton LS, Weitekamp CA, Hofmann HA. Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. *Front Endocrinol* (2017) 8. doi: 10.3389/fendo.2017.00195

30. Altmieme Z, Jubouri M, Touma K, Cote G, Fonseca M, Julian T, et al. A reproductive role for the nonapeptides vasotocin and isotocin in Male zebrafish (*Danio rerio*). *Comp Biochem Phys B* (2019) 238:110333. doi: 10.1016/j.cbpb.2019.110333

31. Kagawa N, Nishiyama Y, Kato K, Takahashi H, Kobayashi Y, Sakamoto H, et al. Potential roles of arginine-vasotocin in the regulation of aggressive behavior in the mudskipper (*Periophthalmus modestus*). *Gen Comp Endocrinol* (2013) 194:257–63. doi: 10.1016/j.ygcen.2013.09.023

32. Eens M, Pinxten R. Sex-role reversal in vertebrates: Behavioural and endocrinological accounts. *Behav Processes* (2000) 51:135–47. doi: 10.1016/S0376-6357(00)00124-8

33. Lin T, Liu X, Xiao D, Zhang D. Plasma levels of immune factors and sex steroids in the Male seahorse *Hippocampus erectus* during a breeding cycle. *Fish Physiol Biochem* (2017) 43:889–99. doi: 10.1007/s10695-017-0343-6

34. Whittington CM, Griffith OW, Qi W, Thompson MB, Wilson AB. Seahorse brood pouch transcriptome reveals common genes associated with vertebrate pregnancy. *Mol Biol Evol* (2015) 32:3114–31. doi: 10.1093/molbev/msv177

35. Batten TFC, Cambre ML, Moons L, Vandesande F. Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, poecilia-latipinna. *J Comp Neurol* (1990) 302:893–919. doi: 10.1002/cne.903020416

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 01 August 2022
DOI 10.3389/fendo.2022.984797

Check for updates

OPEN ACCESS

EDITED BY
Takashi Yazawa,
Asahikawa Medical University, Japan

REVIEWED BY
Guokun Yang,
Henan Normal University, China
Claudia Ladisa,
University of Calgary, Canada

*CORRESPONDENCE
Yongjiang Xu
xuyj@ysfri.ac.cn

SPECIALTY SECTION
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 02 July 2022
ACCEPTED 11 July 2022
PUBLISHED 01 August 2022

CITATION
Wang B, Wang K, Tian Z, Cui A, Liu X, Jin Z, Liu X, Jiang Y and Xu Y (2022) New evidence for SPX2 in regulating the brain-pituitary reproductive axis of half-smooth tongue sole (*Cynoglossus semilaevis*). *Front. Endocrinol.* 13:984797. doi: 10.3389/fendo.2022.984797

COPYRIGHT
© 2022 Wang, Wang, Tian, Cui, Liu, Jin, Liu, Jiang and Xu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

New evidence for SPX2 in regulating the brain-pituitary reproductive axis of half-smooth tongue sole (*Cynoglossus semilaevis*)

Bin Wang[1,2], Kaijie Wang[1,3], Zhenfang Tian[1,4], Aijun Cui[1,2], Xin Liu[1,5], Zhixin Jin[1,3], Xuezhou Liu[1,2], Yan Jiang[1,2] and Yongjiang Xu[1,2]*

[1]Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture and Rural Affairs, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China, [2]Joint Laboratory for Deep Blue Fishery Engineering, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao, China, [3]National Engineering Research Center For Marine Aquaculture, Zhejiang Ocean University, Zhoushan, China, [4]College of Fisheries, Tianjin Agricultural University, Tianjin, China, [5]College of Fisheries and Life Science , Dalian Ocean University, Dalian, China

Spexin (SPX) is an evolutionarily conserved neuropeptide, which was first identified in human proteome by data mining. Two orthologs (SPX1 and SPX2) are present in some non-mammalian species, including teleosts. It has been demonstrated that SPX1 is involved in reproduction and food intake, whereas the functional role of SPX2 is still absent in any vertebrate. The aim of the current study was to evaluate the actions of intraperitoneal injection of endogenous SPX2 peptide on the expression levels of some key reproductive genes of the brain-pituitary axis in half-smooth tongue sole. Our data showed an inhibitory action of SPX2 on brain *gnih*, *spx1*, *tac3* and pituitary *gthα*, *lhβ* mRNA levels. However, SPX2 had no significant effect on brain *gnihr*, *gnrh2*, *gnrh3*, *kiss2*, *kiss2r*, *spx2* expression or pituitary *gh* expression. On the other hand, SPX2 induced an increase in pituitary *fshβ* expression. Taken together, our results provide initial evidence for the involvement of SPX2 in the regulation of reproduction in vertebrates, which is in accordance with previous studies on SPX1.

KEYWORDS

spexin, GnIH, GnRH, kisspeptin, gonadotropin, reproduction

Introduction

Spexin (SPX), also termed neuropeptide Q (NPQ) or C12ORF39, is a novel hypothalamic neuropeptide that was first identified by bioinformatics approach (1, 2) , and subsequently its orthologs have been found from fish to mammals (3–5). The SPX mature peptide is a tetradecapeptide that is flanked by two dibasic protein cleavage sites (RR and GRR), and its amino acid sequence is highly conserved in various vertebrates, with only one amino acid substitution at position 13 between tetrapods and teleosts (3–5). Consistent with its widespread distribution in different tissues of teleosts and other vertebrates, SPX participates in a variety of physiological functions, such as glucose homeostasis, lipid metabolism, feeding, digestion, reproduction, among others (6–10). Preliminary evidence in teleosts and other vertebrates have indicated that SPX binds to the membrane galanin receptor 2 (Galr2) and Galr3, but not Galr1, to exert its functions (11–13).

Based on data acquisition and comparative synteny analysis, a novel SPX form, namely SPX2, has been identified in a few non-mammalian species, such as chicken, anole lizard, *Xenopus tropicalis*, zebrafish, medaka, and coelacanth (11). However, SPX2 is absent in mammals and the initial SPX is designated as SPX1 now (11). Interestingly, Nile tilapia and other cichlid fish species have two SPX1 paralogs (SPX1a and SPX1b) but have no SPX2 (12). In teleosts, the physiological functions of SPX are just emerging, and mainly focus on the control of reproduction and appetite (3–5). For instance, *in vivo* and *in vitro* administration of SPX1 suppress LH secretion in goldfish (14), and both LH and FSH plasma levels are significantly reduced after a single intraperitoneal injection of SPX1a or SPX1b in Nile tilapia (12). However, the reproductive capability is not impaired in SPX1 mutant zebrafish, suggesting that SPX1 is not essential for reproduction in this species (15).

SPX1 expression can be altered by nutritional status in several fish species (12, 16–22), and SPX1 has been shown to act as a satiety factor in goldfish (16) and zebrafish (15). Moreover, overexpression of SPX1 in the dorsal habenula reduces anxiety in zebrafish (23). On the other hand, no information is available regarding the biological role of SPX2 in any vertebrate, other than two studies on the SPX2-Galr2/3 interaction and detailed brain distribution of SPX2 in zebrafish (3–5). The serum response element-driven luciferase (SRE-luc) activity is significantly elevated by zebrafish SPX2 in HEK293 cells expressing zebrafish, *Xenopus*, and human Galr2 or Galr3, suggesting that SPX2 is an endogenous ligand for Galr2/3 (11). Recent data in zebrafish have revealed that SPX2 expression is restricted in the preoptic area of the hypothalamus by whole-mount *in situ* RNA hybridization, implying that SPX2 is implicated in reproduction and feeding control in this species (24). Accordingly, further investigation is urgently needed to clarify the potential role of SPX2 in vertebrates.

In all vertebrates, reproduction is mainly regulated by the brain-pituitary-gonadal (BPG) axis. A plethora of neuropeptides are involved in the control of reproduction, including gonadotropin-releasing hormone (GnRH), kisspeptin (Kiss), gonadotropin-inhibitory hormone (GnIH), neurokinin B (NKB), among others (25–35). Half-smooth tongue sole (*Cynoglossus semilaevis*) is an economically important marine flatfish that is widely cultured in China, and this species needs approximately 3 years of sexual maturation. In nature, the body length of mature females is twice larger and the body weight is over six times greater than those of mature males, exhibiting a sexual dimorphism of growth (36). Genes encoding these key factors have been cloned in half-smooth tongue sole, namely *gnrh2* (37), *gnrh3* (38), *kiss2* (39), Kiss2 receptor (*kiss2r*) (36), *gnih* (40), GnIH receptor (*gnihr*) (41), and *tac3* (42). Furthermore, growth hormone (*gh*) and three gonadotropin subunits (*gthα*, *lhβ*, and *fshβ*) are also available in this species (43, 44). Previous studies have indicated the existence of SPX1 and SPX2 in half-smooth tongue sole, and SPX1 exerts an action on the expression levels of brain and pituitary reproductive genes (20, 45). Herein, this study aimed to further clarify the possible role of SPX2 in the regulation of reproduction in this flatfish species.

Materials and methods

Animals

Approximately 2-year-old immature female tongue sole (body weight (BW), total length (TL) and gonadosomatic index (GSI) of 772.61 ± 25.69 g, 49.97 ± 0.51 cm and 2.66 ± 0.25%, respectively) were purchased from Haiyang Yellow Sea Aquatic Product Co., Ltd. (Haiyang, China), and maintained in an indoor concrete tank with recirculating seawater (water temperature 21–23°C and dissolved oxygen > 6 mg/L). Fish specimens were acclimatized for one week under a cyclical light–dark photoperiod (12 h: 12 h) and fed to satiation twice daily with commercial dry pellets. The animal study protocol was approved by the Animal Care and Use Committee of Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences (ID Number: YSFRI-2021025).

Peptide synthesis

The tongue sole SPX2 mature peptide (45) with amidation at the C-terminus (LNIHWGPQSMMYLKGKY-NH2) was synthesized by ChinaPeptides Co., Ltd. (Shanghai, China) with a purity of 95%, as determined by HPLC. The SPX2 peptide was dissolved in phosphate-buffered saline (PBS) just before the intraperitoneal injection experiments.

In vivo effects of SPX2 on the brain-pituitary reproductive axis

SPX2 *in vivo* treatment experiments were generally performed as the previous study on tongue sole SPX1 (20). After acclimatization for one week as mentioned above, the fish were divided into three groups, anesthetized with MS222 (Sigma, 200 mg/L), weighed, and injected intraperitoneally with SPX2 peptide at two doses (100 ng/g BW and 1000 ng/g BW) or PBS alone (n = 8 fish/group). The injection volume of each dosage varied depending on the body weight of each fish. The whole brain and pituitary tissues were collected 6 h after the injection, frozen in liquid nitrogen, and stored at −80°C until use.

RNA extraction, reverse transcription, and real-time quantitative PCR

All experiments were performed as described previously (46). Total RNA was isolated using the RNAiso Plus reagent (Takara), and 1 μg of total RNA was used as a template for the first-strand cDNA synthesis using the PrimeScript™ RT reagent Kit with gDNA Eraser (Takara). Real-time quantitative PCR analysis was performed on the LightCycler® 96 PCR Instrument (Roche) using TB Green® *Premix Ex Taq*™ II (Takara) and the specific primers (Table 1). The thermal cycling profiles were as follows: 95°C for 30 s, and 40 cycles of 95°C for 5 s, 60°C for 20 s, and 72°C for 10 s. Melting curve analysis was performed in order to confirm the specificity of each product. *18s* ribosomal RNA was used as the internal reference for data normalization. The relative expression levels of each gene were normalized against those of the housekeeping gene and calculated by the comparative Ct method.

Statistical analysis

Data were analyzed by one-way ANOVA followed by Duncan's multiple comparison test using SPSS17.0, and are presented as mean ± SEM. Differences were considered statistically significant at $p < 0.05$.

TABLE 1 List of primers for real-time quantitative PCR.

Name	Primer sequence (5'-3')	Amplicon size (bp)	GenBank accession NO.
gnih-F	GGAAATCAGCCTACAGTGACAAAA	120	KU612223
gnih-R	GCCTCTCCAAGTCCAAACTCC		
gnihr-F	GCTTTTCATGTTGTCCTGGTTG	147	KX839491
gnihr-R	GGGTTGATGCTTGAGTTGGAG		
gnrh2 -F	GGAATCTGAACTGGAGAACTGCT	121	KX090947
gnrh2 -R	TGGCTGCTCACAACTTTATCAC		
gnrh3 -F	AGGCAGCAGAGTGATCGTG	92	JQ028869
gnrh3 -R	CACCTGGTAGCCATCCATAAGAC		
kiss2-F	GGCAACTGCTGTGCAACGA	133	KX090946
kiss2-R	AAGACAGAAAGCGGGGAGAAC		
kiss2r-F	AGTTGTGATCGTCCTCCTCTTTG	92	KX685668
kiss2r-R	AGTTGGGTTGGTATTTGGGATG		
spx1 -F	GCTCCTAAGGGTTCGTTCCA	185	MG775238
spx1 -R	AGTATGGTGGCTGCCTGGTC		
spx2 -F	TCGTTAATCGCCTCCCTGTT	137	MH782165
spx2 -R	AGTGGTGCCTTGTTGTTCTCCT		
tac3-F	TCTGGTCCTCGTCGTCAAAC	175	MK336423
tac3-F	CGTGTTCCTTCTGCCCATC		
gh-F	TTATAGACCAGCGGCGTTTC	179	HQ334196
gh-R	ATGCTTGTTGTCGGGGATG		
gthα-F	TTCCCCACTCCTCTAACGACA	116	JQ364953
gthα-R	ACCACAATACCAGCCACCACTAC		
lhβ-F	TCCACCTGACACTAACGCTG	191	JQ277934
lhβ-R	GTTTGGTTCCTTTGTTCTGC		
fshβ-F	TGATGGGTGTCCAGAGGAAG	*95*	JQ277933
fshβ-R	CAACAAACCGTCCACAGTCC		
18s-F	GGTCTGTGATGCCCTTAGATGTC	107	GQ426786
18s-R	AGTGGGGTTCAGCGGGTTAC		

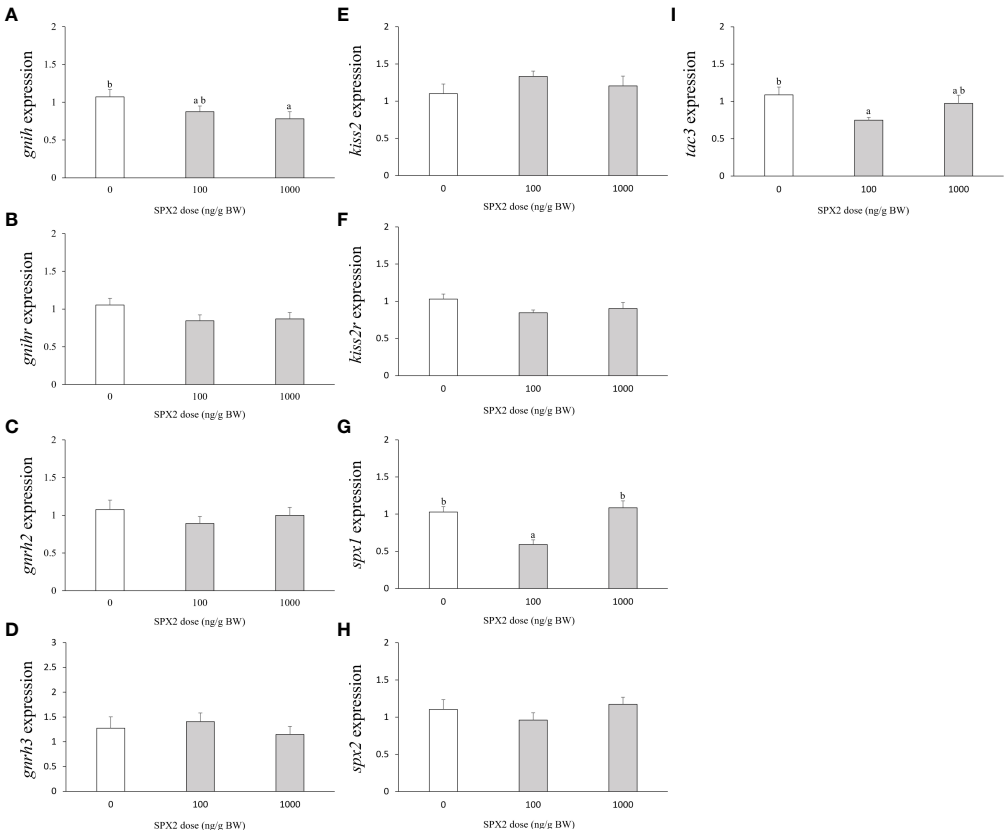


FIGURE 1
Effects of intraperitoneal injection of SXP2 peptide on brain *gnih* **(A)**, *gnihr* **(B)**, *gnrh2* **(C)**, *gnrh3* **(D)**, *kiss2* **(E)**, *kiss2r* **(F)**, *spx1* **(G)**, *spx2* **(H)** and *tac3* **(I)** transcript levels in tongue sole. Data are presented as mean ± SEM (n = 7-8). Different letters indicate significant differences between groups ($p < 0.05$).

Results

Effects of SPX2 peptide on the brain gene expression

First of all, we studied the *in vivo* effects of tongue sole SPX2 peptide on the expression levels of *gnih* and its cognate receptor *gnihr* genes in the brain (Figures 1A, B). Intraperitoneal injection of SPX2 at 1000 ng/g BW significantly inhibited *gnih* mRNA levels when compared to the control group (Figure 1A). However, no apparent variation in *gnihr* expression was noticed after administration of SPX2 at any of the two doses (Figure 1B).

Second, to investigate whether the GnRH system is a target of SPX2 action, brain expression levels of *gnrh2* and *gnrh3* were examined after treatment with SPX2 peptide (Figures 1C, D). Neither *gnrh2* nor *gnrh3* mRNA transcripts were altered by administration of SPX2 at the two doses tested (Figures 1C, D).

Third, we further evaluated the central action of SPX2 on the kisspeptin system (*kiss2* and its cognate receptor *kiss2r*). Similarly, SPX2 had no significant effects on brain *kiss2* and *kiss2r* mRNA levels compared to the control group (Figures 1E, F).

Fourth, to analyze the autocrine regulation of the spexin system, we examined the brain expression levels of *spx1* and *spx2* after administration of SPX2 peptide (Figures 1G, H). Only the fish treated with SPX2 at 100 ng/g BW showed an evident reduction in *spx1* mRNA levels (Figure 1G). However, *spx2* mRNA levels were not modified by the SPX2 peptide at the two doses tested (Figure 1H).

Finally, we detected the effects of SPX2 on the expression levels of *tac3* expressed in the brain (Figure 1I). Only SPX2 at the dose of 100 ng/g BW exerted an inhibitory action on *tac3* expression levels (Figure 1I).

Effects of SPX2 peptide on the pituitary gene expression

As shown in Figure 2A, *gh* mRNA levels were not significantly altered by SPX2 at the two doses tested when compared to the control group. For *gthα* and *lhβ*, a significant suppression in their mRNA levels was observed by SPX2 at 1000 ng/g BW (Figures 2B, C). In contrast, SPX2 markedly stimulated *fshβ* mRNA levels with the lower dose of 100 ng/g BW when compared to the control group (Figure 2D).

10.3389/fendo.2022.984797

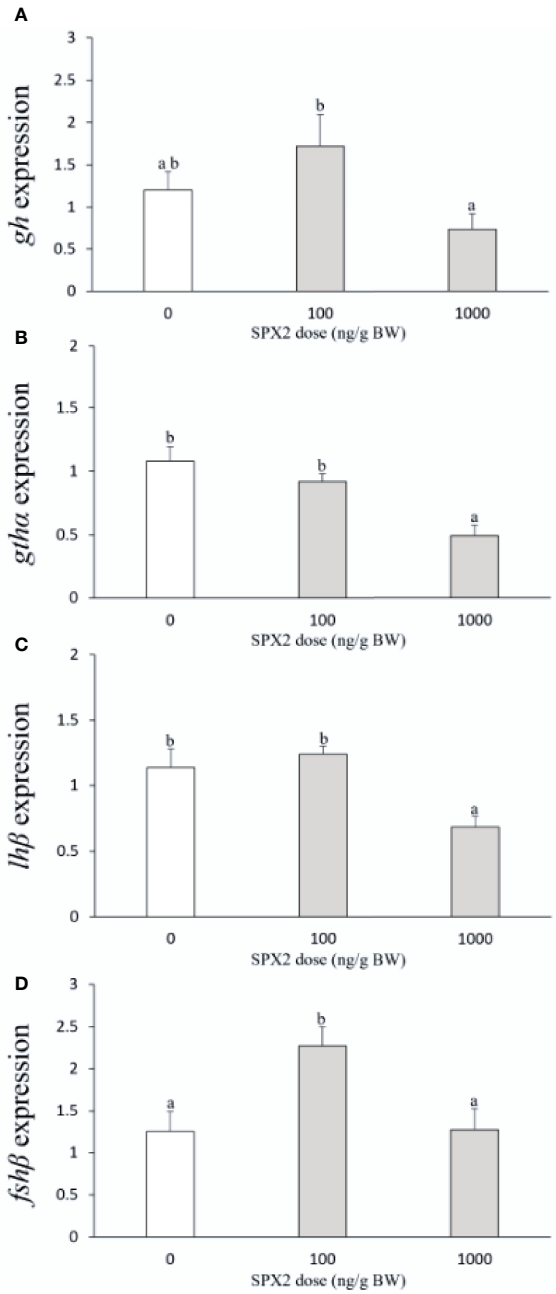


FIGURE 2
Effects of intraperitoneal injection of SXP2 peptide on pituitary *gh* **(A)**, *gthα* **(B)**, *lhβ* **(C)** and *fshβ* **(D)** transcript levels in tongue sole. Data are presented as mean ± SEM (n = 6-8). Different letters indicate significant differences between groups ($p < 0.05$).

Discussion

SPX, which was first discovered by bioinformatics tools, is a newly described neuropeptide with pleiotropic functions in mammals (3, 4). Two SPX orthologs (SPX1 and SPX2) have been reported in some non-mammalian species, while the physiological functions of SPX are still largely unknown and remain to be investigated in this group of vertebrates. In bony fish, SPX1 exerts an inhibitory effect on reproduction (12, 14) and food intake (15, 16). However, no information exists about the potential biological functions of SPX2 in any vertebrate (3, 4). In the current study, therefore, half-smooth tongue sole was used as a model to investigate the *in vivo* actions of SPX2 on expression levels of reproductive genes in the brain-pituitary axis.

There is compelling evidence supporting that GnIH plays a critical role in the regulation of reproduction by acting at three levels of the BPG axis from fish to humans *via* its cognate receptor GnIHR (25, 27, 47). Our previous studies also have revealed that GnIH1 and GnIH2 peptides encoded by the same precursor exert a direct action on mRNA levels of pituitary hormones through the PKA and PKC signaling pathways in half-smooth tongue sole (41, 46). Results obtained in the present study indicated that intraperitoneal injection of SPX2 reduced *gnih* mRNA levels, without any effect on *gnihr* expression. In contrast, administration of SPX1 provoked an increase of *gnih* mRNA levels in immature females of the same species (20). Overall, these data suggest that SPX2 is implicated in the control of reproduction, while SPX1 and SPX2 may have different biological roles in half-smooth tongue sole. It is worth mentioning that Galr2 is an alternative endogenous receptor for SPX in zebrafish and Nile tilapia (11, 12). However, the morphological relationship between SPX and GnIH neurons is very limited in fish, thus much more studies need to be done to unveil whether Galr2 exists in GnIH neurons of half-smooth tongue sole and other species.

GnRH has been well demonstrated to be a master stimulator of the reproductive axis in vertebrates, and two or three distinct GnRH isoforms (GnRH1, GnRH2, and GnRH3) exist in all teleosts investigated so far. The brain distribution and physiological functions of these three GnRH variants are quite different. In a teleost species possessing all three GnRH types, GnRH1 is the main hypophysiotropic hormone regulating the BPG axis. However, GnRH3 takes over the role of GnRH1 in other teleost species that have GnRH2 and GnRH3 only (28, 29). One of the other key hypothalamic neuropeptides established in the control of reproduction is kisspeptin, which can exhibit potent action on pituitary directly or on GnRH neurons indirectly to regulate LH and FSH synthesis and secretion (30, 32, 33). In this study, none of *gnrh2*, *gnrh3*, *kiss2* or *kiss2r* expression levels were altered by SPX2 injection, indicating that the GnRH and kisspeptin systems may be not the central targets of SPX2 action on reproduction. Similarly, there is no significant effect of SPX1 on *gnrh1* and *gnrh2* mRNA levels in orange-spotted grouper (17) and immature females of half-smooth tongue sole (20), respectively. However, *gnrh3* expression is evidently elevated after SPX1 administration in the latter (20). Therefore, SPX1 and SPX2 might regulate different aspects of fish physiology.

In the present study, we evaluated the effects of SPX2 on the autocrine and paracrine regulation of spexin system. Peripheral injection of SPX2 suppressed *spx1* expression, without any effect on *spx2* mRNA levels. It has been demonstrated that SPX1 is involved in feeding, reproduction, and other functions in fish (3, 4), and these data indicate that SPX2 may participate in these physiological processes *via* SPX1 indirectly. Whether SPX1 has any effect on *spx2* expression is still unknown, which warrants further studies in various vertebrates. On the other hand, NKB encoded by the *tac3* gene has emerged as a key regulator of reproduction in mammals (34) and several teleost species, including zebrafish (48, 49), Nile tilapia (50), goldfish (51, 52), striped bass (53), European eel (54), and half-smooth tongue sole (42). Results obtained in this study indicated that SPX2 can reduce brain *tac3* mRNA levels, suggesting the regulation of reproduction by SPX2 *via* NKB indirectly.

In addition to its effects on brain functions, SPX can also modulate the synthesis and release of pituitary hormones. On one hand, both *gthα* and *lhβ* expression were down-regulated after intraperitoneal injection of SPX2, whereas *fshβ* mRNA levels were up-regulated in half-smooth tongue sole. On the other hand, SPX1 suppressed the expression levels of *gthα* and *fshβ*, without affecting *lhβ* expression in immature females of the same species (20). Neither *lhβ* nor *fshβ* transcripts were modified after SPX1 treatment in orange-spotted grouper (17). For hormone secretion, an inhibitory action of SPX1 on the plasma LH level was observed in goldfish and Nile tilapia (12, 14) along with a reduction of plasma FSH level in the latter. Interestingly, SPX1 evoked a decrease in the serum LH level, but an increase in the serum FSH level in mature female rats (55). Of note, SPX2 had no effect on *gh* expression in this study. However, SPX1 reduced *gh* mRNA levels in orange-spotted grouper and half-smooth tongue sole immature females (17, 20). It is worth mentioning that sexually immature female specimens were used in this study, and sexual maturity could be a contributing factor influencing the obtained results. Accordingly, further studies in sexually mature females during the seasonal reproductive cycle will contribute to a more complete picture of these two SPX peptides in this species. Taken together, SPX2 can modulate the reproduction of half-smooth tongue sole through actions on the expression of the components of brain-pituitary reproductive axis, and SPX1 and SPX2 seem to have divergent roles in the same species.

Despite its functional significance, the molecular mechanisms of SPX actions are incipient in vertebrates. A ligand-receptor interaction study has revealed that both SPX1 and SPX2 could increase SRE-luc activity in HEK293 cells expressing zebrafish Galr2a and Galr2b (11). Both SRE-luc and cAMP-response element luciferase (CRE-luc) activities are significantly elevated after SPX1a or SPX1b treatment in COS-7 cells expressing tilapia Galr2b (12). These data indicate that SPXs are a functional agonist for Galr2, and both PKC and PKA pathways mediate SPX functions. It is worth mentioning that clarifying the intricate web of intracellular pathways in response to SPX and its interaction with GnRH (28, 56), GnIH (57, 58), and kisspeptin (32, 35), is a promising area for future research not only in fish but also in other vertebrates.

Conclusions

In summary, this study provides preliminary evidence for the involvement of SPX2 in the regulation of reproduction in

vertebrates by acting at the brain and pituitary levels. Combined with previous studies on SPX1, it appears that some functional divergences exist between SPX1 and SPX2 peptides, perhaps due to the differences in their structures and binding affinity to their cognate receptors. Further studies on the molecular mechanisms involved in SPX actions on the target cells would contribute to the knowledge of the functional significance and divergence of this emerging neuropeptide in vertebrate species.

Data availability statement

The original contributions presented in the study are included in the article/supplementary material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by the Animal Care and Use Committee of Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences.

Author contributions

BW: conceptualization, validation, investigation, writing—original draft preparation, funding acquisition; KW: validation, investigation; ZT: investigation; AC: investigation; XiL: investigation; ZJ: investigation; XuL: resources, writing—review and editing; YJ: formal analysis; YX: validation, writing—review and editing, supervision, funding acquisition.

Funding

This research was funded by the National Natural Science Foundation of China (32072949, 32072993), Central Public-interest Scientific Institution Basal Research Fund, YSFRI, CAFS (NO. 20603022022018), Central Public-interest Scientific Institution Basal Research Fund, CAFS (NO. TD202047), and China Agriculture Research System of MOF and MARA (CARS-47).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Mirabeau O, Perlas E, Severini C, Audero E, Gascuel O, Possenti R, et al. Identification of novel peptide hormones in the human proteome by hidden Markov model screening. *Genome Res* (2007) 17(3):320–7. doi: 10.1101/gr.5755407

2. Sonmez K, Zaveri NT, Kerman IA, Burke S, Neal CR, Xie X, et al. Evolutionary sequence modeling for discovery of peptide hormones. *PloS Comput Biol* (2009) 5(1):e1000258. doi: 10.1371/journal.pcbi.1000258

3. Lim CH, Lee MYM, Soga T, Parhar I. Evolution of structural and functional diversity of spexin in mammalian and non-mammalian vertebrate species. *Front Endocrinol (Lausanne)* (2019) 10:379. doi: 10.3389/fendo.2019.00379

4. Ma A, Bai J, He M, Wong AOL. Spexin as a neuroendocrine signal with emerging functions. *Gen Comp Endocrinol* (2018) 265:90–6. doi: 10.1016/j.ygcen.2018.01.015

5. Mohd Zahir I, Ogawa S, Dominic NA, Soga T, Parhar IS. Spexin and galanin in metabolic functions and social behaviors with a focus on non-mammalian vertebrates. *Front Endocrinol (Lausanne)* (2022) 13:882772. doi: 10.3389/fendo.2022.882772

6. Mills EG, Izzi-Engbeaya C, Abbara A, Comninos AN, Dhillo WS. Functions of galanin, spexin and kisspeptin in metabolism, mood and behaviour. *Nat Rev Endocrinol* (2021) 17(2):97–113. doi: 10.1038/s41574-020-00438-1

7. Behrooz M, Vaghef-Mehrabany E, Maleki V, Pourmoradian S, Fathifar Z, Ostadrahimi A. Spexin status in relation to obesity and its related comorbidities: a systematic review. *J Diabetes Metab Disord* (2020) 19(2):1943–57. doi: 10.1007/s40200-020-00636-8

8. Kolodziejski PA, Pruszynska-Oszmalek E, Wojciechowicz T, Sassek M, Leciejewska N, Jasaszwili M, et al. The role of peptide hormones discovered in the 21st century in the regulation of adipose tissue functions. *Genes (Basel)* (2021) 12(5):756. doi: 10.3390/genes12050756

9. Tran A, He W, Chen JTC, Belsham DD. Spexin: Its role, regulation, and therapeutic potential in the hypothalamus. *Pharmacol Ther* (2021) p:108033. doi: 10.1016/j.pharmthera.2021.108033

10. Lv SY, Zhou YC, Zhang XM, Chen WD, Wang YD. Emerging roles of NPQ/Spexin in physiology and pathology. *Front Pharmacol* (2019) 10:457. doi: 10.3389/fphar.2019.00457

11. Kim DK, Yun S, Son GH, Hwang JI, Park CR, Kim JI, et al. Coevolution of the spexin/galanin/kisspeptin family: Spexin activates galanin receptor type II and III. *Endocrinology* (2014) 155(5):1864–73. doi: 10.1210/en.2013-2106

12. Cohen Y, Hausken K, Bonfil Y, Gutnick M, Levavi-Sivan B. Spexin and a novel cichlid-specific spexin paralog both inhibit FSH and LH through a specific galanin receptor (Galr2b) in tilapia. *Front Endocrinol (Lausanne)* (2020) 11:71. doi: 10.3389/fendo.2020.00071

13. Lin CY, Zhang M, Huang T, Yang LL, Fu HB, Zhao L, et al. Spexin enhances bowel movement through activating l-type voltage-dependent calcium channel *via* galanin receptor 2 in mice. *Sci Rep* (2015) 5:12095. doi: 10.1038/srep12095

14. Liu Y, Li S, Qi X, Zhou W, Liu X, Lin H, et al. A novel neuropeptide in suppressing luteinizing hormone release in goldfish, carassius auratus. *Mol Cell Endocrinol* (2013) 374(1-2):65–72. doi: 10.1016/j.mce.2013.04.008

15. Zheng B, Li S, Liu Y, Li Y, Chen H, Tang H, et al. Spexin suppress food intake in zebrafish: Evidence from gene knockout study. *Sci Rep* (2017) 7(1):14643. doi: 10.1038/s41598-017-15138-6

16. Wong MK, Sze KH, Chen T, Cho CK, Law HC, Chu IK, et al. Goldfish spexin: solution structure and novel function as a satiety factor in feeding control.

Am J Physiol Endocrinol Metab (2013) 305(3):E348–66. doi: 10.1152/ajpendo.00141.2013

17. Li S, Liu Q, Xiao L, Chen H, Li G, Zhang Y, et al. Molecular cloning and functional characterization of spexin in orange-spotted grouper (*Epinephelus coioides*). *Comp Biochem Physiol B Biochem Mol Biol* (2016) 196-197:85–91. doi: 10.1016/j.cbpb.2016.02.009

18. Wu H, Lin F, Chen H, Liu J, Gao Y, Zhang X, et al. Ya-fish (*Schizothorax prenanti*) spexin: identification, tissue distribution and mRNA expression responses to periprandial and fasting. *Fish Physiol Biochem* (2016) 42(1):39–49. doi: 10.1007/s10695-015-0115-0

19. Ma A, He M, Bai J, Wong MK, Ko WK, Wong AO, et al. Dual role of insulin in spexin regulation: Functional link between food intake and spexin expression in a fish model. *Endocrinology* (2017) 158(3):560–77. doi: 10.1210/en.2016-1534

20. Wang S, Wang B, Chen S. Spexin in the half-smooth tongue sole (*Cynoglossus semilaevis*): molecular cloning, expression profiles, and physiological effects. *Fish Physiol Biochem* (2018) 44(3):829–39. doi: 10.1007/s10695-018-0472-6

21. Deng SP, Chen HP, Zhai Y, Jia LY, Liu JY, Wang M, et al. Molecular cloning, characterization and expression analysis of spexin in spotted scat (*Scatophagus argus*). *Gen Comp Endocrinol* (2018) 266:60–6. doi: 10.1016/j.ygcen.2018.04.018

22. Tian Z, Xu S, Wang M, Li Y, Chen H, Tang N, et al. Identification, tissue distribution, periprandial expression, and anorexigenic effect of spexin in Siberian sturgeon, acipenser baeri. *Fish Physiol Biochem* (2020) 46(6):2073–84. doi: 10.1007/s10695-020-00856-y

23. Jeong I, Kim E, Seong JY, Park HC. Overexpression of spexin 1 in the dorsal habenula reduces anxiety in zebrafish. *Front Neural Circuits* (2019) 13:53. doi: 10.3389/fncir.2019.00053

24. Kim E, Jeong I, Chung AY, Kim S, Kwon SH, Seong JY, et al. Distribution and neuronal circuit of spexin 1/2 neurons in the zebrafish CNS. *Sci Rep* (2019) 9 (1):5025. doi: 10.1038/s41598-019-41431-7

25. Trudeau VL. Neuroendocrine control of reproduction in teleost fish: Concepts and controversies. *Annu Rev Anim Biosci* (2022) 10:18.1–18.24. doi: 10.1146/annurev-animal-020420-042015

26. Zohar Y, Zmora N, Trudeau VL, Munoz-Cueto JA, Golan M. A half century of fish gonadotropin-releasing hormones: Breaking paradigms. *J Neuroendocrinol* (2021) 34:e13069. doi: 10.1111/jne.13069

27. Munoz-Cueto JA, Paullada-Salmeron JA, Aliaga-Guerrero M, Cowan ME, Parhar IS, Ubuka T, et al. A journey through the gonadotropin-inhibitory hormone system of fish. *Front Endocrinol (Lausanne)* (2017) 8:285. doi: 10.3389/fendo.2017.00285

28. Munoz-Cueto JA, Zmora N, Paullada-Salmeron JA, Marvel M, Mananos E, Zohar Y, et al. The gonadotropin-releasing hormones: Lessons from fish. *Gen Comp Endocrinol* (2020) 291:113422. doi: 10.1016/j.ygcen.2020.113422

29. Duan C, Allard J. Gonadotropin-releasing hormone neuron development in vertebrates. *Gen Comp Endocrinol* (2020) 292:113465. doi: 10.1016/j.ygcen.2020.113465

30. Somoza GM, Mechaly AS, Trudeau VL. Kisspeptin and GnRH interactions in the reproductive brain of teleosts. *Gen Comp Endocrinol* (2020) 298:113568. doi: 10.1016/j.ygcen.2020.113568

31. Di Yorio MP, Munoz-Cueto JA, Paullada-Salmeron JA, Somoza GM, Tsutsui K, Vissio PG, et al. The gonadotropin-inhibitory hormone: What we know and what we still have to learn from fish. *Front Endocrinol (Lausanne)* (2019) 10:78. doi: 10.3389/fendo.2019.00078

32. Ohga H, Selvaraj S, Matsuyama M. The roles of kisspeptin system in the reproductive physiology of fish with special reference to chub mackerel studies as main axis. *Front Endocrinol (Lausanne)* (2018) 9:147. doi: 10.3389/fendo.2018.00147

33. Sivalingam M, Ogawa S, Trudeau VL, Parhar IS. Conserved functions of hypothalamic kisspeptin in vertebrates. *Gen Comp Endocrinol* (2022) 317:113973. doi: 10.1016/j.ygcen.2021.113973

34. Hu G, Lin C, He M, Wong AO. Neurokinin b and reproductive functions: "KNDy neuron" model in mammals and the emerging story in fish. *Gen Comp Endocrinol* (2014) 208:94–108. doi: 10.1016/j.ygcen.2014.08.009

35. Wang B, Mechaly AS, Somoza GM. Overview and new insights into the diversity, evolution, role, and regulation of kisspeptins and their receptors in teleost fish. *Front Endocrinol (Lausanne)* (2022) 13:862614. doi: 10.3389/fendo.2022.862614

36. Wang B, Liu Q, Liu X, Xu Y, Shi B. Molecular characterization of Kiss2 receptor and *in vitro* effects of Kiss2 on reproduction-related gene expression in the hypothalamus of half-smooth tongue sole (*Cynoglossus semilaevis*). *Gen Comp Endocrinol* (2017) 249:55–63. doi: 10.1016/j.ygcen.2017.04.006

37. Wang B, Liu X, Liu Q, Zhao M, Xu Y, Shi B. Molecular cloning, localization, and expression analysis of gnrh2 in different tissues of half-smooth tongue sole (*Cynoglossus semilaevis*) during ovarian maturation. *Prog Fish Sci* (2017) 38(1):63–72. doi: 10.11758/yykxjz.20160816002

38. Zhou X, Yi Q, Zhong Q, Li C, Muhammad S, Wang X, et al. Molecular cloning, tissue distribution, and ontogeny of gonadotropin-releasing hormone III gene (GnRH-III) in half-smooth tongue sole (*Cynoglossus semilaevis*). *Comp Biochem Physiol B Biochem Mol Biol* (2012) 163(1):59–64. doi: 10.1016/j.cbpb.2012.04.010

39. Wang B, Liu Q, Liu X, Xu Y, Song X, Shi B, et al. Molecular characterization of *kiss2* and differential regulation of reproduction-related genes by sex steroids in the hypothalamus of half-smooth tongue sole (*Cynoglossus semilaevis*). *Comp Biochem Physiol A Mol Integr Physiol* (2017) 213:46–55. doi: 10.1016/j.cbpa.2017.08.003

40. Wang B, Liu Q, Liu X, Xu Y, Shi B. Molecular characterization and expression profiles of LPXRFa at the brain-pituitary-gonad axis of half-smooth tongue sole (*Cynoglossus semilaevis*) during ovarian maturation. *Comp Biochem Physiol B Biochem Mol Biol* (2018) 216:59–68. doi: 10.1016/j.cbpb.2017.11.016

41. Wang B, Yang G, Liu Q, Qin J, Xu Y, Li W, et al. Characterization of LPXRFa receptor in the half-smooth tongue sole (*Cynoglossus semilaevis*): Molecular cloning, expression profiles, and differential activation of signaling pathways by LPXRFa peptides. *Comp Biochem Physiol A Mol Integr Physiol* (2018) 223:23–32. doi: 10.1016/j.cbpa.2018.05.008

42. Wang B, Cui A, Zhang Y, Xu Y, Wang W, Jiang Y, et al. Neurokinin b in a flatfish species, the half-smooth tongue sole (*Cynoglossus semilaevis*), and its potential role in reproductive functions. *Aquac Rep* (2021) 20:100651. doi: 10.1016/j.aqrep.2021.100651

43. Ji XS, Liu HW, Chen SL, Jiang YL, Tian YS. Growth differences and dimorphic expression of growth hormone (GH) in female and male *Cynoglossus semilaevis* after male sexual maturation. *Mar Genomics* (2011) 4(1):9–16. doi: 10.1016/j.margen.2010.11.002

44. Shi B, Liu X, Xu Y, Wang S. Molecular characterization of three gonadotropin subunits and their expression patterns during ovarian maturation in *Cynoglossus semilaevis*. *Int J Mol Sci* (2015) 16(2):2767–93. doi: 10.3390/ijms16022767

45. Wang B, Cui A, Tian J, Zhang Y, Jiang Y, Xu Y, et al. Characterization of a novel spexin gene (*spx2*) in the half-smooth tongue sole and regulation of its expression by nutritional status. *Aquac Rep* (2020) 18:100544. doi: 10.1016/j.aqrep.2020.100544

46. Wang B, Yang G, Xu Y, Zhang Y, Liu X. In vitro effects of tongue sole LPXRFa and kisspeptin on relative abundance of pituitary hormone mRNA and inhibitory action of LPXRFa on kisspeptin activation in the PKC pathway. *Anim Reprod Sci* (2019) 203:1–9. doi: 10.1016/j.anireprosci.2019.01.009

47. Tsutsui K, Ubuka T. Gonadotropin-inhibitory hormone (GnIH): A new key neurohormone controlling reproductive physiology and behavior. *Front Neuroendocrinol* (2021) 61:100900. doi: 10.1016/j.yfrne.2021.100900

48. Biran J, Palevitch O, Ben-Dor S, Levavi-Sivan B. Neurokinin bs and neurokinin b receptors in zebrafish-potential role in controlling fish reproduction. *Proc Natl Acad Sci U.S.A.* (2012) 109(26):10269–74. doi: 10.1073/pnas.1119165109

49. Qi X, Salem M, Zhou W, Sato-Shimizu M, Ye G, Smitz J, et al. Neurokinin b exerts direct effects on the ovary to stimulate estradiol production. *Endocrinology* (2016) 157(9):3355–65. doi: 10.1210/en.2016-1354

50. Biran J, Golan M, Mizrahi N, Ogawa S, Parhar IS, Levavi-Sivan B. Direct regulation of gonadotropin release by neurokinin b in tilapia (*Oreochromis niloticus*). *Endocrinology* (2014) 155(12):4831–42. doi: 10.1210/en.2013-2114

51. Qi X, Zhou W, Li S, Liu Y, Ye G, Liu X, et al. Goldfish neurokinin b: Cloning, tissue distribution, and potential role in regulating reproduction. *Gen Comp Endocrinol* (2015) 221:267–77. doi: 10.1016/j.ygcen.2014.10.017

52. Liu Y, Wang Q, Wang X, Meng Z, Liu Y, Li S, et al. NKB/NK3 system negatively regulates the reproductive axis in sexually immature goldfish (*Carassius auratus*). *Gen Comp Endocrinol* (2019) 281:126–36. doi: 10.1016/j.ygcen.2019.05.020

53. Zmora N, Wong TT, Stubblefield J, Levavi-Sivan B, Zohar Y. Neurokinin b regulates reproduction *via* inhibition of kisspeptin in a teleost, the striped bass. *J Endocrinol* (2017) 233(2):159–74. doi: 10.1530/JOE-16-0575

54. Campo A, Lafont AG, Lefranc B, Leprince J, Tostivint H, Kamech N, et al. Tachykinin-3 genes and peptides characterized in a basal teleost, the European eel: Evolutionary perspective and pituitary role. *Front Endocrinol (Lausanne)* (2018) 9:304. doi: 10.3389/fendo.2018.00304

55. Golyszny M, Obuchowicz E, Zielinski M. Neuropeptides as regulators of the hypothalamus-pituitary-gonadal (HPG) axis activity and their putative roles in stress-induced fertility disorders. *Neuropeptides* (2022) 91:102216. doi: 10.1016/j.npep.2021.102216

56. Chang JP, Pemberton JG. Comparative aspects of GnRH-stimulated signal transduction in the vertebrate pituitary - contributions from teleost model systems. *Mol Cell Endocrinol* (2018) 463:142–67. doi: 10.1016/j.mce.2017.06.002

57. Son YL, Ubuka T, Tsutsui K. Molecular mechanisms of gonadotropin-inhibitory hormone (GnIH) actions in target cells and regulation of GnIH expression. *Front Endocrinol (Lausanne)* (2019) 10:110. doi: 10.3389/fendo.2019.00110

58. Wang B, Yang G, Xu Y, Li W, Liu X. Recent studies of LPXRFa receptor signaling in fish and other vertebrates. *Gen Comp Endocrinol* (2019) 277:3–8. doi: 10.1016/j.ygcen.2018.11.011

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 03 August 2022
DOI 10.3389/fendo.2022.902737

Check for updates

OPEN ACCESS

EDITED BY
Bin Wang,
Chinese Academy of Fishery Sciences (CAFS), China

REVIEWED BY
Alejandro S. Mechaly,
CONICET Instituto de Investigaciones en Biodiversidad y Biotecnología (INBIOTEC), Argentina
Alejandro Lomniczi,
Oregon Health &Science University, United States

*CORRESPONDENCE
Qian Wang
wangqian170@163.com

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

RECEIVED 23 March 2022
ACCEPTED 04 July 2022
PUBLISHED 03 August 2022

CITATION
Bu S-Y, Zhang Y-Y, Zhang X, Li T-X, Zheng D-C, Huang Z-X and Wang Q (2022) Regulation of the *kiss2* promoter in yellowtail clownfish (*Amphiprion clarkii*) by cortisol *via* GRE-dependent GR pathway. *Front. Endocrinol.* 13:902737. doi: 10.3389/fendo.2022.902737

COPYRIGHT
© 2022 Bu, Zhang, Zhang, Li, Zheng, Huang and Wang. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Regulation of the *kiss2* promoter in yellowtail clownfish (*Amphiprion clarkii*) by cortisol *via* GRE-dependent GR pathway

Shao-Yang Bu†, Yan-Yu Zhang†, Xian Zhang, Tian-Xiu Li, De-Cai Zheng, Ze-Xiang Huang and Qian Wang*

Department of Aquaculture, College of Marine Sciences, Hainan University, Haikou, China

Kisspeptin plays a vital role in mediating the stress-induced reproductive regulation. Cortisol, known as a stress-related hormone, is involved in gonadal development and sexual differentiation by binding with glucocorticoid receptor (GR) to regulate the expression of *kiss* gene. In the present study, cortisol treatment in yellowtail clownfish (*Amphiprion clarkii*) showed that the expression of *kiss* (*kiss1* and *kiss2*) and *gr* (*gr1* and *gr2*) genes were increased significantly. We demonstrated that the yellowtail clownfish Kiss neurons co-express the glucocorticoid receptors in the telencephalon, mesencephalon, cerebellum, and hypothalamus. We further cloned the promoter of *kiss2* gene in yellowtail clownfish and identified the presence of putative binding sites for glucocorticoid receptors, estrogen receptors, androgen receptors, progesterone receptors, AP1, and C/EBP. Applying transient transfection in HEK293T cells of the yellowtail clownfish *kiss2* promoter, cortisol (dexamethasone) treatment was shown to enhance the promoter activities of the yellowtail clownfish *kiss2* gene in the presence of GRs. Deletion analysis of *kiss2* promoter indicated that cortisol-induced promoter activities were located between position −660 and −433 with GR1, and −912 and −775 with GR2, respectively. Finally, point mutation studies on the *kiss2* promoter showed that cortisol-stimulated promoter activity was mediated by one GRE site located at position −573 in the presence of GR1 and by each GRE site located at position −883, −860, −851, and −843 in the presence of GR2. Results of the present study provide novel evidence that cortisol could regulate the transcription of *kiss2* gene in the yellowtail clownfish *via* GRE-dependent GR pathway.

KEYWORDS

cortisol, stress, *kiss2* promoter, glucocorticoid receptor, *Amphiprion clarkii*

Introduction

Kisspeptin is regarded as the key factor of reproduction and plays a role in the hypothalamus–pituitary–gonad axis (HPG axis) in vertebrates (1, 2). Kisspeptin binds to its receptor (G protein-coupled receptor 54, GPR54) and releases gonadotropin-releasing hormone (GnRH) from the hypothalamus, thereby stimulating the secretion of the gonadotropic hormone (GtH). Knockout of *Kiss1/Gpr54* in mice has been shown to prevent sexual maturity, cause gonad hypoplasia, hypogonadotropic hypogonadism, and infertility (3, 4). Moreover, kisspeptin has been reported to be involved in a variety of physiological activities, such as glucose homeostasis and light signal regulation (5, 6). Especially, kisspeptin is supposed to be involved regulation of various hormones on the hypothalamus–pituitary–adrenal axis (HPA axis) and the HPG axis under stress induction (7, 8).

Stress can lead to the dysfunction of the HPG axis and reproductive behavior through the HPA axis in vertebrates (9). There is a close relationship between stress and reproductive disorders (10). In patients with depression under stress, an excessive corticotropin-releasing hormone (CRH) level leads to the inhibition of the HPG axis, and increased cortisol level further inhibits the action of GnRH neurons, luteinizing hormone (LH) amplitude, follicle-stimulating hormone (FSH) levels, and LH pulse frequency (11–13). In mice, both psychosocial stress and unpredictable chronic stress reduce the expression of hypothalamic *kiss1* and the activity of kisspeptin neuron (14, 15). Moreover, corticotropin-releasing hormone or corticosterone treatment suppress *kiss1* expression and kisspeptin neuron activity in the brain of female rats and mice (16, 17). Glucocorticoid, a steroid regulated by stress, can bind with glucocorticoid receptor (GR) to regulate gene expression by associating with specific genomic glucocorticoid response elements (GREs) (18). In female rats, GR protein is detected in the kisspeptin neurons of periventricular nucleus continuum (AVPV/PeN) and arcuate nucleus (ARC), demonstrating that kisspeptin neurons can be modulated directly by glucocorticoid *via* GR (19). Additionally, GRE is found in the promoter regions of *kiss* genes in goldfish (*Carassius auratus*) (20). However, the molecular mechanism of glucocorticoid-regulated *kiss* genes in vertebrate species is still unknown.

Kisspeptin could be encoded by multiple genes in non-mammalian, and two paralogous *kiss* genes, known as *kiss1* and *kiss2*, are found in some teleosts, such as zebrafish and medaka (2). The yellowtail clownfish (*Amphiprion clarkii*) is a protandrous teleost whose sex is associated with social status within a group, including a male–female breeding pair and some non-breeders (21, 22). In one social unit, females occupy the first dominant status and inhibited sexual development of subdominant male and non-breeders, and subdominant male could undergo sexual development to female after female disappeared or the largest non-breeder change sex to male after the disappearance of male (22, 23). The level of cortisol, the main component of glucocorticoid in teleosts, depends on their social status in the population. In the protogynous orange-spotted grouper (*Epinephelus coioides*), the female treated with cortisol will change to male (24). In our previous research, the higher levels of hypothalamic *kiss2*/*gr2* expression and gonadal hormone were found in the subordinate of yellowtail clownfish (25, 26). Moreover, there is the sexually dimorphic distribution of *kiss1* and *kiss2* in the brain of yellowtail clownfish, especially in dorsal habenular nucleus (NHd) and dorsal part of the nucleus of the lateral recess (NRLd), which are involved in the regulation of reproductive function and environmental cues (27).

In order to better understand how cortisol exerts its action on the expression of *kiss* gene *via* the GR and thereafter regulates the reproduction in yellowtail clownfish, the expression of *kiss* and *gr* genes were examined after cortisol treatment. Moreover, the co-localization of *gr1*, *gr2*, and *kiss1*/*kiss2* mRNA were also studied in the brain by RNAscope. Then, the promoter region of *kiss2* was cloned by genome walking and predicted with the online tool for potential GR binding sites. In addition, after cortisol treatment, the kisspeptin promoter activities were detected in HEK-293T cells expressing yellowtail clownfish GR1 or GR2. Finally, the regulatory regions and binding sites of GR were identified by deletion analysis and site-directed mutagenesis analysis.

Materials and methods

Animals

Sexually mature and 3-month-old immature yellowtail clownfish were purchased from a local aquarium market (Dongfang city, Hainan, China) in June of 2021. The fish, nine per group, were reared in glass tank (length, 45 cm; width, 35 cm; and height, 60 cm) with continuously flowing aerated seawater at 27 ± 1°C. The photoperiod was a 12:12-h light–dark cycle, with lights turn on at 07:00 and off at 19:00. The fish were fed with commercial feed twice a day (09:00 and 18:00) and reared for a period of 1 week before experiment.

All animals used in this study were conducted in accordance with the guidelines of the animal welfare of the National Committee and approval of the Institutional Animal Care and Use Committee of Hainan University (HNUAUCC-2021-00014).

Experimental design and sampling

The cortisol (hydrocortisone 21-hemisuccinate; MCE, NJ, USA) was dissolved in dimethyl sulfoxide (DMSO) and then diluted in the ratio of 10% cortisol, 40% polyethylene glycol 300 (MCE, NJ, USA), and 50% saline (0.9%). The immature fish

(length, 4 ± 0.5 cm; weight, 2 ± 0.5 g) were anesthetized with MS-222 (Sigma, MO, USA) and then given an intraperitoneal injection with cortisol (10 or 50 mg/g body mass) at a volume of 10 µl/g body mass. The control group was injected the same liquid but without cortisol. We collected the whole brain at 6, 12, 24, and 48 h after injection, respectively.

The gonads of sexually mature yellowtail clownfish (length, 11 ± 1 cm; weight, 21 ± 5 g) were isolated and fixed in Bouin's solution (Sigma, MO, USA) after anesthesia. The gonadal tissues were embedded in paraffin and cut into 5-µm paraffin sections for indentation of gonadal development. The brain of fish was fixed in 4% paraformaldehyde fix solution (Sigma, MO, USA) for *in situ* hybridization.

RNA extraction, reverse transcription, and quantitative real-time PCR

Total RNA from the whole brain were extracted by the TRIzol method and then was reversed transcribed into cDNA using the HiScript II 1st Strand cDNA Synthesis Kit (Vazyme, Nanjing, China). Primer sequences and primer efficiency for quantitative PCR (qPCR) are listed in Supplementary Table S1. The quantitative real-time PCR was performed by Roche Light Cycler 96 real-time PCR System using ChamQ Universal SYBR qPCR Master Mix (Vazyme, Nanjing, China) according to the manufacturer's protocol. The qPCR program was as follows: denaturation at 95°C for 30 s, then followed by 40 cycles at 95°C for 5 s and 55–58°C for 30 s and 72°C for 30 s. Each sample was used in triplicate. The relative mRNA levels of *kiss1*, *kiss2*, *gr1*, and *gr2* were evaluated using comparative threshold cycle (Ct) method with β-actin as internal reference gene and then calculated with the formula $2^{-\Delta\Delta Ct}$ (28).

RNAscope *in situ* hybridization

Brain samples of female yellowtail clownfish were fixed in 4% paraformaldehyde at 4°C overnight. Samples successively were immersed in 10%, 20%, and 30% sucrose containing phosphate-buffered saline (PBS) for dehydration. Cross-sections (10 µm) were generated after being frozen on dry ice in optimal cutting temperature (OCT) compound. RNAscope *in situ* hybridization was performed following the manufacturer's protocol from Advanced Cell Diagnostics (ACD). All steps demanding incubation at 40°C were achieved in the HybEZ Oven (ACD, Hayward USA). Binding of the specific probes against *kiss1* (1044931), *kiss2* (1044941), *gr1* (1088191), and *gr2* (1088201) were detected with RNAscope® Multiplex Fluorescent Reagent Kit v2 (ACD, Hayward, USA). Probes *actb2* (1045881) and *dapB* (310043) were used as positive and negative controls, respectively. Images were taken by Nikon ECLIPSE Ti2 (Nikon, NY, USA).

Cloning *kiss2* promotor

Genomic DNA was extracted from yellowtail clownfish muscle using phenol-chloroform methods. The 5′-flanking region of the *kiss2* was isolated in the reference to Universal Genome Walker Kit (Takara, Tokyo, Japan). The gene-specific primers were designed in the exon 1 based on the sequences of yellowtail clownfish *kiss2* (GenBank: MK368702.1) and are shown in Supplementary Table S1. Products of primary PCR were diluted 100 times, and then, secondary PCR was performed with the diluted products as the template. The secondary PCR products were purified using FastPure Plasmid Mini Kit (Vazyme, Nanjing, China) and were subcloned into the pMD 19-T Vector (Takara, Tokyo, Japan) for sequencing. The transcriptional start site (TSS) of *kiss2* was determined by our previous result of 5′-rapid amplification of cDNA ends. Transcription factor binding sites were predicted using the online PROMO (http://alggen.lsi.upc.es/recerca/menu_recerca.html) and gene-regulation tool (http://gene-regulation.com/cgi-bin/pub/programs/alibaba2/webbaba2.cgi).

Construction of recombinant vector

A 1,442-bp 5′-flanking region and 48-bp exon 1 of *kiss2* was obtained from a pair of primers containing two different restriction enzyme sites, respectively, namely, KpnI and XhoI. PCR was performed with PrimeSTAR HS DNA Polymerase (Takara, Tokyo, Japan). The PCR products and pGL4.10 vector (Promega, WI, USA) were digested by KpnI and XhoI restriction endonucleases (NEB, MA, USA). After purification, the digested products were ligated using T4 DNA Ligase Kit (NEB, MA, USA). The construction of recombinant vector above, namely, pkiss2-1442, was used as template to construct a series of deletion vectors, namely, pkiss2-912, pkiss2-775, pkiss2-660, pkiss2-433, and pkiss2-335. All constructs were sequenced ensuring accuracy. The recombinant vectors were extracted with Omega Endo-Free plasmid DNA mini kit II (OMEGA, GA, USA). Primers used in here are presented in Supplementary Table S1.

Cell culture, transient transfections, and luciferase assays

HEK-293T cells (Bosterbio, CA, USA) were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum at 37°C with 5% CO_2 and passed at least two generations prior to transfection. The healthy cells were seeded into 48-well plates, and approximately 1.5×10^5 cells/well were cultured for 12 h. Cells were then co-transfected with 0.5 µg pkiss2-1442/pkiss2-912/pkiss2-775/pkiss2-660/pkiss2-433/

pkiss2-335, 0.05 μg pcDNA3.1-GR1/pcDNA3.1-GR2 (yellowtail clownfish glucocorticoid receptor expression plasmid) and 0.025 μg pRL-CMV in 250 μl Opti-MEM using EZ Trans (Liji, Shanghai, China). After transfection of 6 h, cells were treated with 10^{-7} M dexamethasone sodium (DXMS, exogenous cortisol). Luciferase activities were detected 24 h later with Dual Luciferase Kit (Promega, WI, USA) in GloMax Discover (Promega, WI, USA).

Site-directed mutagenesis

Mutations of putative GRE sites in the *kiss2* promoter were carried out using a series of specific primers (Supplementary Table S1) by two rounds of PCR amplification. Briefly, primers containing restriction enzyme site and mutation point were used to amplify the mutated fragments of upstream and downstream from the mutation site. The first-round PCR conditions were as follows: denaturation at 95°C for 30 s, followed by 40 cycles at 95°C for 5 s and 67–69°C for 30 s and 72°C for 1 min, with the final extension at 72°C for 10 min. The two PCR products were purified and mixed together for the second-round PCR. The conditions were as follows: denaturation at 95°C for 30 s, then followed by 9 cycles at 95°C for 5 s and 67–69°C for 30 s and 72°C for 1 min and 30 s. Then, primers for full-length promoter amplification of *kiss2*-1442-F and *kiss2*-1442-R were added and continued for an additional 20 cycles at 95°C for 5 s and 69°C for 30 s and 72°C for 1 min 30 s, with the final extension at 72°C for 10 min. PCR products were digested by KpnI and XhoI restriction endonucleases and were subcloned into pGL4.10 vector. All constructs were sequenced ensuring accuracy, and plasmid DNAs were extracted with Omega Endo-Free plasmid DNA mini kit II.

Statistical analysis

All data are shown as mean ± standard error of the mean (SEM). Statistical analysis was performed using one-way ANOVA followed by Tukey's multiple comparisons test in GraphPad Prism 7.0 (GraphPad Software, SD, USA). Results were considered significantly different when *p*-value was <0.05 ($p < 0.05$).

Results

Effects of cortisol on yellowtail clownfish *kiss1*, *kiss2*, *gr1*, and *gr2* expression profiles

After cortisol injection, real-time PCR was performed to investigate the expression profiles of *kiss1*, *kiss2*, *gr1*, and *gr2* in the brain of immature yellowtail clownfish. The highest *kiss1* levels were detected at cortisol treatment with concentrations of 10 and 50 μg/g after 6 h (Figure 1A). For *kiss2*, transcripts were elevated 2-fold at 6 h and 3.5-fold at 12 h in the 50 μg/g cortisol-treated group relative to the other groups and were highest at 48 h in the 10 μg/g cortisol treatment (Figure 1B). The *gr1* mRNA levels were significantly higher at 24 and 48 h cortisol treatment with a dose of 10 μg/g (Figure 1C), but the most abundant *gr2* transcripts were at 6 and 12 h cortisol treatment with 50 μg/g (Figure 1D). These results indicated that cortisol treatment enhanced the transcription of *kiss1* and *kiss2* and *gr1* and *gr2* in the brain of yellowtail clownfish.

Co-expression of *gr1* and *gr2* and *kiss1*/*kiss2* genes

According to the distribution of *kiss1* and *kiss2* genes in the brain of yellowtail clownfish (unpublished), RNAscope *in situ* hybridization for *gr1* and *gr2* and *kiss1*/*kiss2* genes were performed in areas of the telencephalon (Te), mesencephalon (Me), cerebellum (Ce), and hypothalamus (Hy). The co-expression of *gr1*, *gr2*, and *kiss1* was found in the in the dorsal habenular nucleus (NHd), subdivision 2 of the medial dorsal telencephalic area (Dm2), subdivision 3 of the medial dorsal telencephalic area (Dm3), lateral posterior part of the dorsal telencephalic area (DIP), posterior portion of the dorsal telencephalon (DP), corpus of the cerebellum (CCe), and lateral part of the diffuse nucleus of the inferior lobe (NDLIl) (Figures 2A–E, F–K). The *gr2* signal was more abundantly distributed than *gr1* in NHd, Dm2, and CCe (Figures 2A, F, J) and weakly expressed in the DIP and DP (Figures 2H, I). The *gr1*, *gr2*, and *kiss2* were simultaneously detected in the dorsal part of the nucleus of the lateral recess (NRLd), Dm2, Dm3, DIP, DP, CCe, optic tectum (OT), NDLIl, posterior part of glomerular nucleus (NGP), tegmentum (TEG), and periventricular nucleus of the posterior tuberculum (TPp) (Figures 3A–E, F–O). Compared with *gr1*, the stronger *gr2* signals were detected in the NRLd, Dm2, Dm3, OT, NGP, and TPp (Figures 3A, F, G, K, M, O), but the weaker signaling molecules were examined in the DIP and NDLIl (Figures 3H, L).

In silico analysis of 5′-flanking region for the yellowtail clownfish *kiss2* gene

To further investigate the transcriptional regulatory mechanism of *kiss2* by cortisol in yellowtail clownfish, we isolated the 5′-flanking region of *kiss2* gene. The putative *kiss2* promoter sequence includes a 1,442-bp upstream of the transcription start site and a 48-bp first exon fragment (Figure 4). *In silico* analysis revealed that *kiss2* promoter sequence possessed 13 potential glucocorticoid response elements (GREs). In addition, several motifs for other steroid receptors were identified on the *kiss2* promoter, such as five

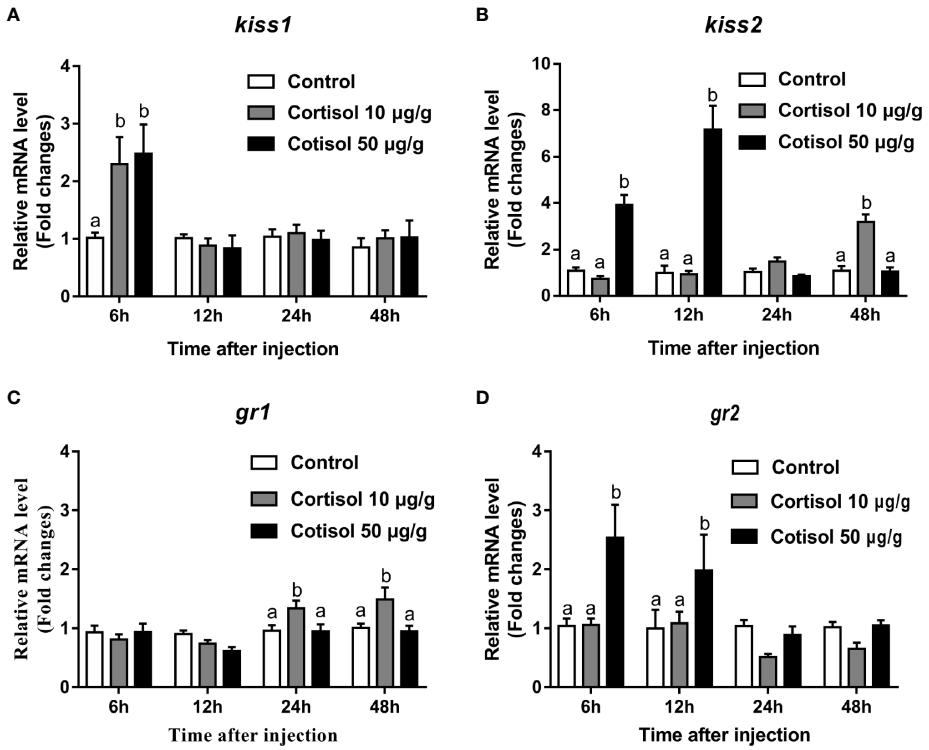


FIGURE 1
Effects of cortisol on *kiss1*, *kiss2*, *gr1*, and *gr2* mRNA levels in the brain of yellowtail clownfish. Expression of *kiss1* **(A)**, *kiss2* **(B)**, *gr1* **(C)**, and *gr2* **(D)** after injection of cortisol with both concentrations at 10 and 50 μg/g during 48 h. The data are expressed as mean ± SEM (n = 5–8). Bars with different letters indicate significant differences between treatments at the same sampling time ($p < 0.05$).

androgen response elements, three estrogen response elements, and two progesterone response elements (Figure 4).

Effects of cortisol on *kiss2* promoter activity

GR-negative HEK293T cells were transfected with the recombinant vector for *kiss2* promoter (pkiss2-1442), in combination with expression plasmids for yellowtail clownfish glucocorticoid receptor (GR1 or GR2), to analyze the transcriptional regulation of *kiss2* by cortisol. Basal promoter activity was examined for the *kiss2* promoter, indicating that functional promoter activity existed in the 5′-flanking region (Figure 5A). DXMS significantly increased *kiss2* promoter activity in the presence of GR, being more efficient in GR1 than GR2 (Figure 5B). The *kiss2* promoter activities were detected after treatment with different DXMS concentrations and significantly upregulated at 10^{-7} M DXMS in the presence of GR1 or GR2 (Figures 6A, B). Therefore, this concentration of DXMS was chosen for the subsequent investigation.

Identification of glucocorticoid-responsive region and functional GRE site on the yellowtail clownfish *kiss2* promoter

Using the full length of the *kiss2* promoter vector as a template, a series of deletion constructs were established as shown in the left panel of Figures 7, 8. In the presence of GR1, the promoter activity of *kiss2* was significantly higher after 10^{-7} M DXMS treatment (Figure 7A). Deletions of *kiss2* promoter to position −433 (pkiss2-433) abolished cortisol-induced promoter activity, indicating that the region from −660 to −433 is relevant with cortisol-induced promoter activity by GR1 (Figure 7A). Site-directed mutagenesis was conducted to determine whether the GRE at −573 ($^{-573}$ TGTAC^{-569}) was the key regulatory site on *kiss2* promoter. Mutation of a GRE at −573 eradicated cortisol-induced promoter activity (Figure 8A). However, we found that mutation in other GRE sites at −1,236 ($^{-1,236}$AGTTCT$^{-1,231}$) or −1,188 ($^{-1,188}$AGGAT$^{-1,184}$) did not change cortisol-induced promoter activity (Figure 8B). Thus, the GRE at −573 is critical for cortisol/GR1-induced *kiss2* promoter activity.

10.3389/fendo.2022.902737

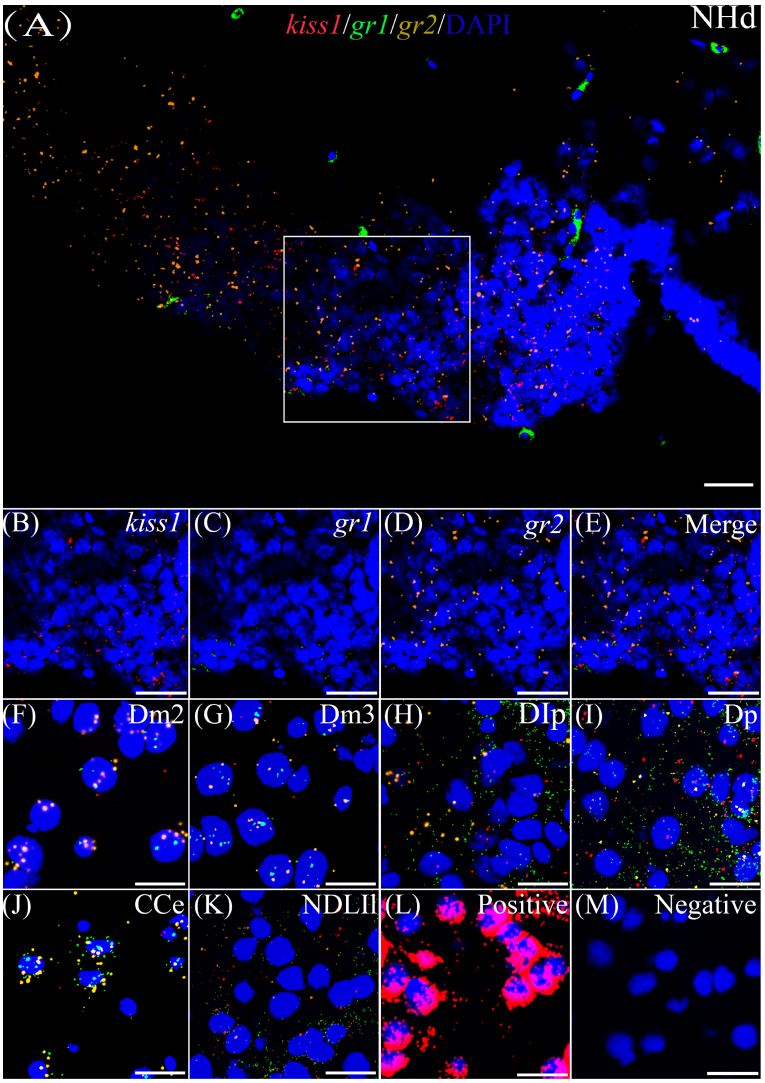


FIGURE 2
Co-expression of *gr1*, *gr2*, and *kiss1*. The view of NHd **(A)** shows *kiss1* (red), *gr1* (green), and *gr2* (yellow) with DAPI cell nuclear staining (blue). **(B–E)** The view of boxed region in panel **(A)**, showing *kiss1* (red), *gr1* (green), and *gr2* (yellow) with DAPI cell nuclear staining (blue) in panels **(B–D)**, respectively, and the "merge" in panel **(E)** shows *kiss1* (red), *gr1* (green), and *gr2* (yellow) with DAPI cell nuclear staining (blue). Representative images display *gr1* (green) and *gr2* (yellow) co-expression of *kiss1* (red) with DAPI cell nuclear staining (blue) in Dm2 **(F)**, Dm3 **(G)**, DIp **(H)**, Dp **(I)**, CCe **(J)**, and NDLII **(K)**. Positive and negative controls are shown in panels **(L, M)**, respectively. NHd, dorsal habenular nucleus; Dm2, subdivision 2 of the medial dorsal telencephalic area; Dm3, subdivision 3 of the medial dorsal telencephalic area; DIp, lateral posterior part of the dorsal telencephalic area; Dp, posterior portion of the dorsal telencephalon; CCe, corpus of the cerebellum; NDLII, lateral part of the diffuse nucleus of the inferior lobe. Bars = 20 μm.

In the presence of GR2, 10^{-7} M DXMS treatment significantly upregulated the wild-type *kiss2* promoter activity (Figure 7B).Truncation of the *kiss2* promoter to −775 bp abolished by cortisol-induced promoter activity (Figure 7B). Mutations of two GRE at −1,236 or −1,188 of the *kiss2* promoter still responded to the cortisol treatment (Figure 8D). However, cortisol-induced promoter activities of *kiss2* were removed in mutations of the following four GRE at −883 ($^{-883}$AGGAT^{-879}), −860 ($^{-860}$TGTAC^{-856}), −851 ($^{-851}$AGTTCT^{-846}), and −843 ($^{-843}$ATCCT^{-839}), indicating that these GRE binding sites contribute to cortisol-/GR2-induced *kiss2* promoter activity (Figure 8C).

Discussion

Previous studies in vertebrates have demonstrated that kisspeptin plays a vital role in mediating the stress-induced

10.3389/fendo.2022.902737

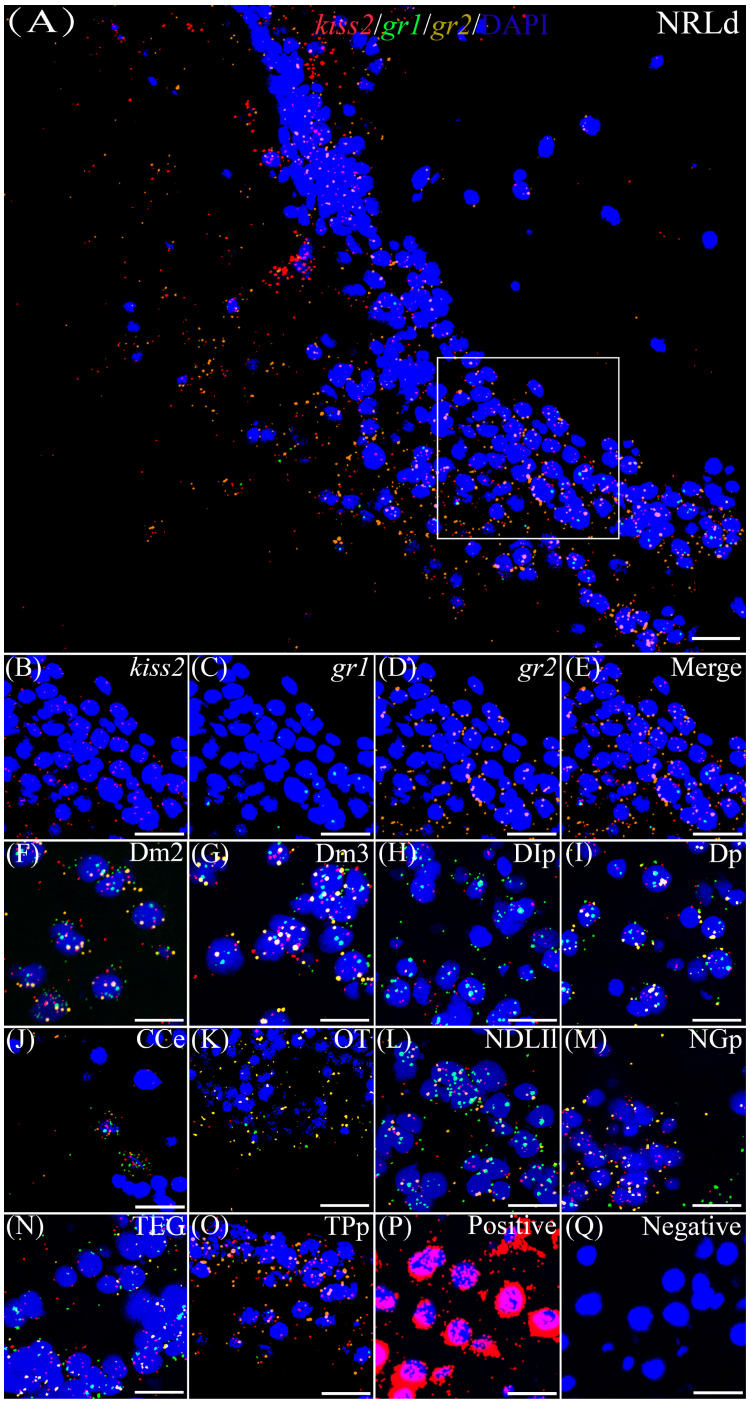


FIGURE 3
Co-expression of *gr1*, *gr2*, and *kiss2*. The view of NRLd **(A)** shows *kiss2* (red), *gr1* (green), and *gr2* (yellow) with DAPI cell nuclear staining (blue). **(B–E)** The view of boxed region in panel **(A)**, showing *kiss2* (red), *gr1* (green), and *gr2* (yellow) with DAPI cell nuclear staining (blue) in panels **(B–D)**, respectively; and the "merge" in panel **(E)** shows *kiss2* (red), *gr1* (green), and *gr2* (yellow) with DAPI cell nuclear staining (blue). Representative images display *gr1* (green) and *gr2* (yellow) co-expression of *kiss2* (red) with DAPI cell nuclear staining (blue) in Dm2 **(F)**, Dm3 **(G)**, DIp **(H)**, Dp **(I)**, CCe **(J)**, OT **(K)**, NDLII **(L)**, NGp **(M)**, TEG **(N)**, and TPp **(O)**. Positive and negative controls are shown in panels **(P, Q)**, respectively. NRLd, dorsal part of the nucleus of the lateral recess; Dm2, subdivision 2 of the medial dorsal telencephalic area; Dm3, subdivision 3 of the medial dorsal telencephalic area; DIp, lateral posterior part of the dorsal telencephalic area; Dp, posterior portion of the dorsal telencephalon; CCe, corpus of the cerebellum; OT, optic tectum; NDLII, lateral part of the diffuse nucleus of the inferior lobe; NGp, posterior part of glomerular nucleus; TEG, tegmentum; TPp, periventricular nucleus of the posterior tuberculum. Bars = 20 μm.

10.3389/fendo.2022.902737

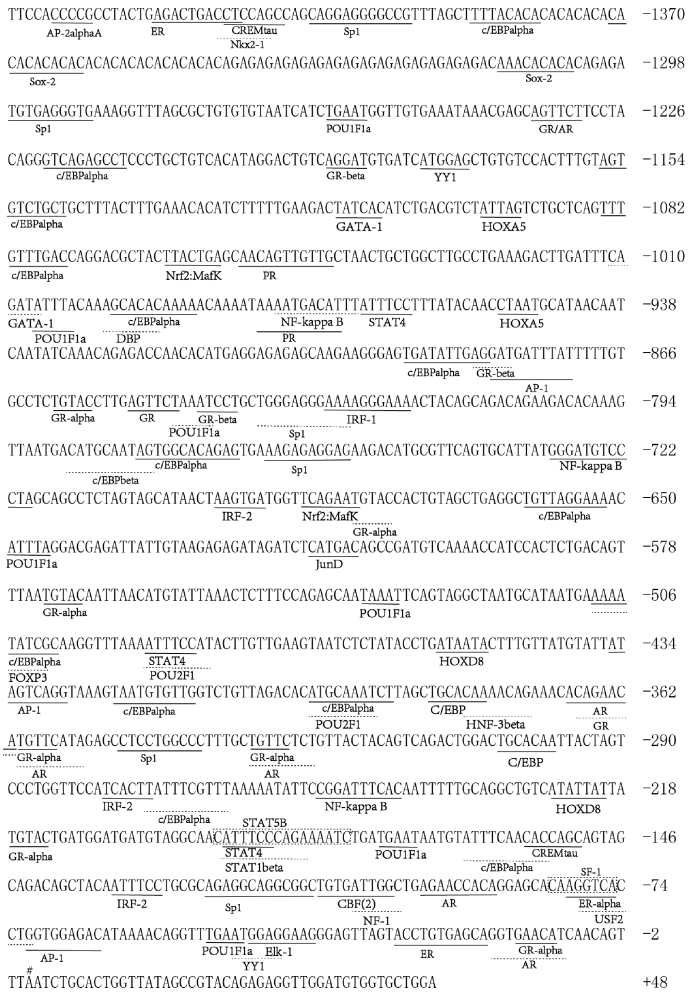


FIGURE 4
Sequence analysis of 5′-flanking region for *kiss2*. The numbering of the sequence is relative to the transcription start site marked on # and designated as +1. Putative binding sites for transcription factors are underlined and labeled. Transcription factor binding sites were predicted using the online PROMO and gene-regulation tool.

reproductive regulation (7). Cortisol, the main steroid hormone for stress response, is involved in gonadal development and sexual differentiation by regulating *kiss* genes (26). This study aims to observe the molecular mechanism of glucocorticoid regulation of the *kiss* genes in teleosts. In rodents, the hypothalamic *kiss1* mRNA level and kisspeptin neuron activity are reduced after the administration of cortisol (16, 17). There is only one *kiss* gene in rodents, but there are two *kiss* genes (*kiss1* and *kiss2*) in several teleost fish participating in the reproductive regulation (2). Cortisol treatment showed that the expression of *kiss* (*kiss1* and *kiss2*) and *gr* (*gr1* and *gr2*) genes were increased significantly in the brain of yellowtail clownfish. In addition, elevation of two *kiss* genes expression by cortisol has been reported in zebrafish (29). Moreover, our previous study revealed that *kiss2*, E_2, and testosterone (T) levels are higher in the subordinate than in the dominant yellowtail clownfish (25). A study in protandrous false clown anemonefish (*Amphiprion ocellaris*) showed that the social rank reflects the blood cortisol value (30).

GR signals are detected in the Kiss neurons of periventricular nucleus continuum (AVPV/PeN) and arcuate nucleus (ARC) by double-labeling immunohistochemistry in female rats (19). In the present study, we have demonstrated that Kiss neurons co-expressed the glucocorticoid receptors in the Te, Me, Ce, and Hy, suggesting that cortisol could directly affect kisspeptin neurons *via* GR in yellowtail clownfish. Habenular *kiss1* and serotonin-related genes are downregulated after exposure to alarm substance (AS), and Kiss1 antagonist injection can reduce AS-evoked fish fear

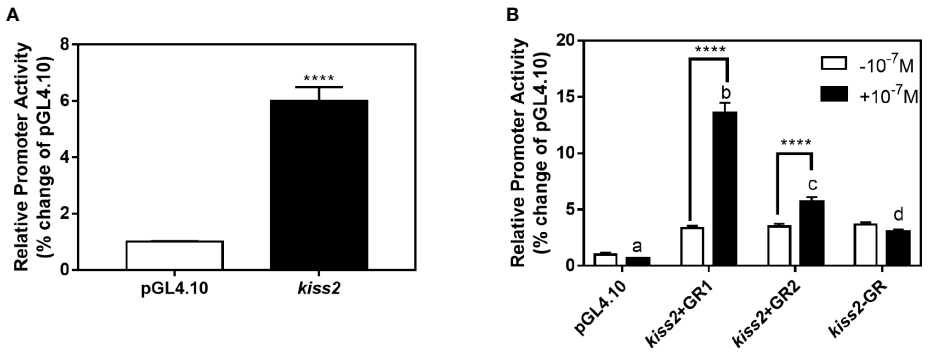


FIGURE 5
Effects of cortisol on *kiss2* promoter activity. **(A)** Basic activity of *kiss2* gene promoter in HEK-293T cell lines. The cells were transfected with 0.5 μg pkiss2-1442 and 0.025 μg pRL-CMV, or 0.5 μg pGL4.10 and 0.025 μg pRL-CMV as the control. Luciferase activity was measured after 24 h. Relative promoter activities are expressed as percentage of pGL4.10. **(B)** The activities of *kiss2* promoter in the presence of cortisol in HEK293T cell lines. Cells were co-transfected with 0.5 μg pkiss2-1442, and 0.025 μg pRL-CMV with or without 0.05 μg yellowtail clownfish glucocorticoid receptor (GR1 or GR2) expression plasmid. The transfected cells were treated with or without 10^{-7} M cortisol. The luciferase activity was measured 24 h later. Relative promoter activities are expressed as percentage of pGL4.10 in the absence of cortisol. Data are represented as mean ± SEM (n = 4). **** ($p < 0.0001$) indicates that significant difference compared with the corresponding control. The different letters mean significant differences between groups with cortisol treatment ($p < 0.05$).

response, indicating that habenular kisspeptin modulates fear in zebrafish (31). The yellowtail clownfish non-breeders are always attacked by both female and male, and hypothalamic *gr2* levels of non-breeders are significantly higher than that of the others in one social group (21, 26). From our results showing *gr* genes co-expression with *kiss1* in NHd, we raise the possibility that habenular *kiss1* is involved in the regulation of fear response in the yellowtail clownfish. Moreover, *kiss2* levels in the NRLd are decreased under testosterone (T) treatment in sea bass (32). In yellowtail clownfish, *gr1* and *gr2* were found to be co-expressed with *kiss2* in the NRLd, indicating that this region may participate in stress-induced reproductive functions *via kiss2*.

Multiple GR binding sites were found in *kiss2* promoter in the yellowtail clownfish by silicon analysis, implying that *kiss2* could be regulated by cortisol *via* GR through binding with GRE. In the present study, cortisol injection also enhanced the *gr1* and *gr2* mRNA levels in the brain. Other binding sites such as AP1, Sp1, and C/EBP were also predicted in the *kiss2* promoter region. AP1 could interact with GR for GR-regulated transcription and recruitment to co-occupied AP1 binding site by DNaseI accessibility and chromatin immunoprecipitation with high-throughput sequencing (33). In addition, a series of binding sites for sex steroid receptors existed in the *kiss2* promoter of yellowtail clownfish, which is similar to the results of goldfish

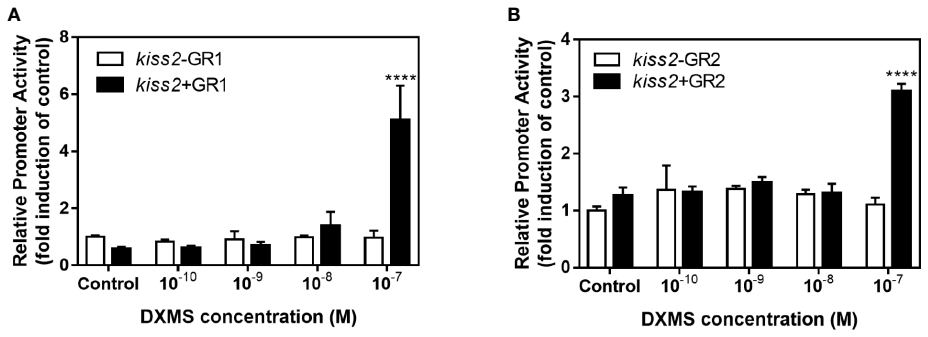


FIGURE 6
Effects of cortisol with different concentrations on the activities of *kiss2* gene promoter. HEK-293T cells were transfected with 0.5 μg pkiss2-1442, 0.05 μg GR1 **(A)** or GR2 **(B)** and 0.025 μg pRL-CMV; 0.5 μg pGL4.10 co-transfected with 0.025 μg pRL-CMV as the control. Cells were treated with or without cortisol. Luciferase activity was detected after 24 h. Data are represented as mean ± SEM (n = 4). **** ($p < 0.0001$) indicates significant differences between groups with cortisol treatment.

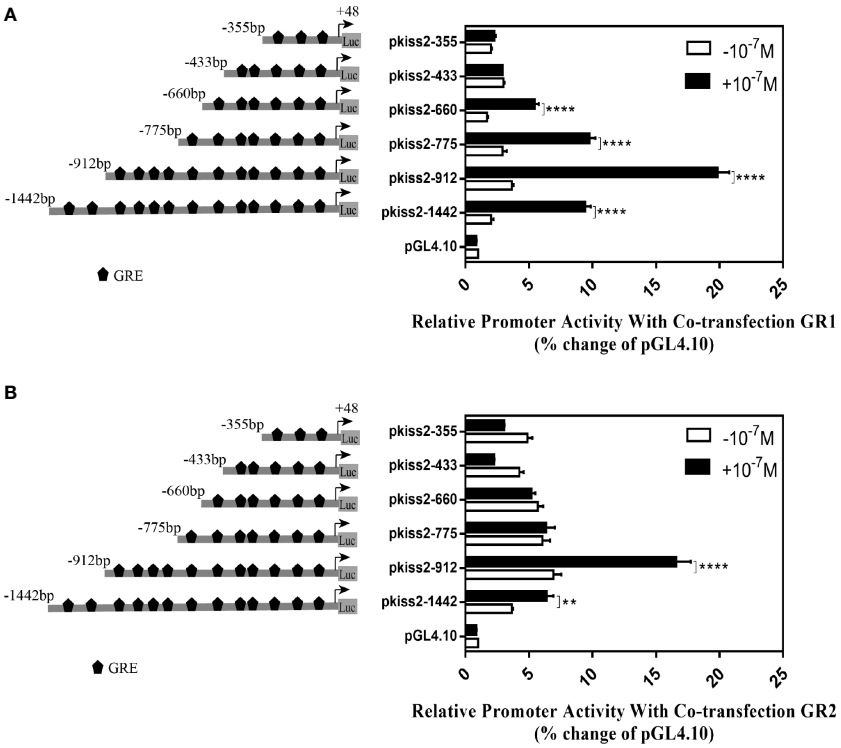


FIGURE 7
Deletion analysis of the *kiss2* promoter. Schematic of the putative GRE sites and deletion constructs of *kiss2* promoter is shown on the left. HEK-293T cells were transfected with deletion constructs and pRL-CMV with GR1 **(A)** or GR2 **(B)** expression plasmid. Cells were incubated with or without 10^{-7} M cortisol treatment. Relative promoter activities are expressed as percentage of pGL4.10 in the absence of cortisol. Data are represented as mean ± SEM (n = 4). **($p < 0.01$) and **** ($p < 0.0001$) indicate the significant differences compared with the corresponding control.

and zebrafish (20, 34), indicating that the potential ability of *kiss2* is involved in the regulation of reproduction. Our previous study found that hypothalamic *kiss2* had higher expression in non-breeders than females and males, which may contribute to the regulation of gonad development under social stress in the yellowtail clownfish (25).

The cortisol treatment could enhance yellowtail clownfish *kiss2* promoter activities in HEK293T cells in the presence of GR, whereby GR1 was more effective than GR2. In yellowtail clownfish, GR1 contains conserved nine amino acids, which are present in the most known teleostean GR1 proteins but absent in other vertebrates (26, 35, 36). A previous study revealed that the additional nine amino acids made GR1a to better bind with single GRE than GR1b in rainbow trout (*Oncorhynchus mykiss*) (37). Thus, we speculate that GR1 has a better binding affinity for GRE than GR2 in the yellowtail clownfish. Altogether, GRs play a vital role in the mediation of cortisol effect on *kiss2* promoter.

GR can activate or repress gene expression by binding with GRE directly or interacting with other transcription factors (38). Using a series of deletion constructs, we have demonstrated that cortisol-induced promoter activities of *kiss2* gene were located between position −660 and −433 with GR1, and −912 and −775 with GR2, respectively. Point mutations in the *kiss2* promoter were generated by site-directed mutagenesis. Our results showed that in the presence of GR1, cortisol-stimulated promoter activity was only mediated by one GRE site located at the position of −573, whereas in the presence of GR2, promoter activity could be modulated by all four GRE sites located at positions −883, −860, −851, and −843. Therefore, the *kiss2* gene is regulated by cortisol through the GRE-dependent mechanism in yellowtail clownfish. It has also been reported that there is a synergistic action between the enhancer binding protein (C/EBP) and GR in the regulation of thymidine kinase promoter activity (39).

In conclusion, the present study demonstrated for the first time the molecular mechanism of glucocorticoid regulation of the *kiss* genes in teleosts. It was found that cortisol treatment could upregulate the expression levels of *kiss* and *gr* genes in the yellowtail clownfish. The Kiss neurons coexpressed the glucocorticoid receptors in Te, Me, Ce, and Hy. Cortisol could enhance *kiss2* promoter activity in the presence of GRs and was

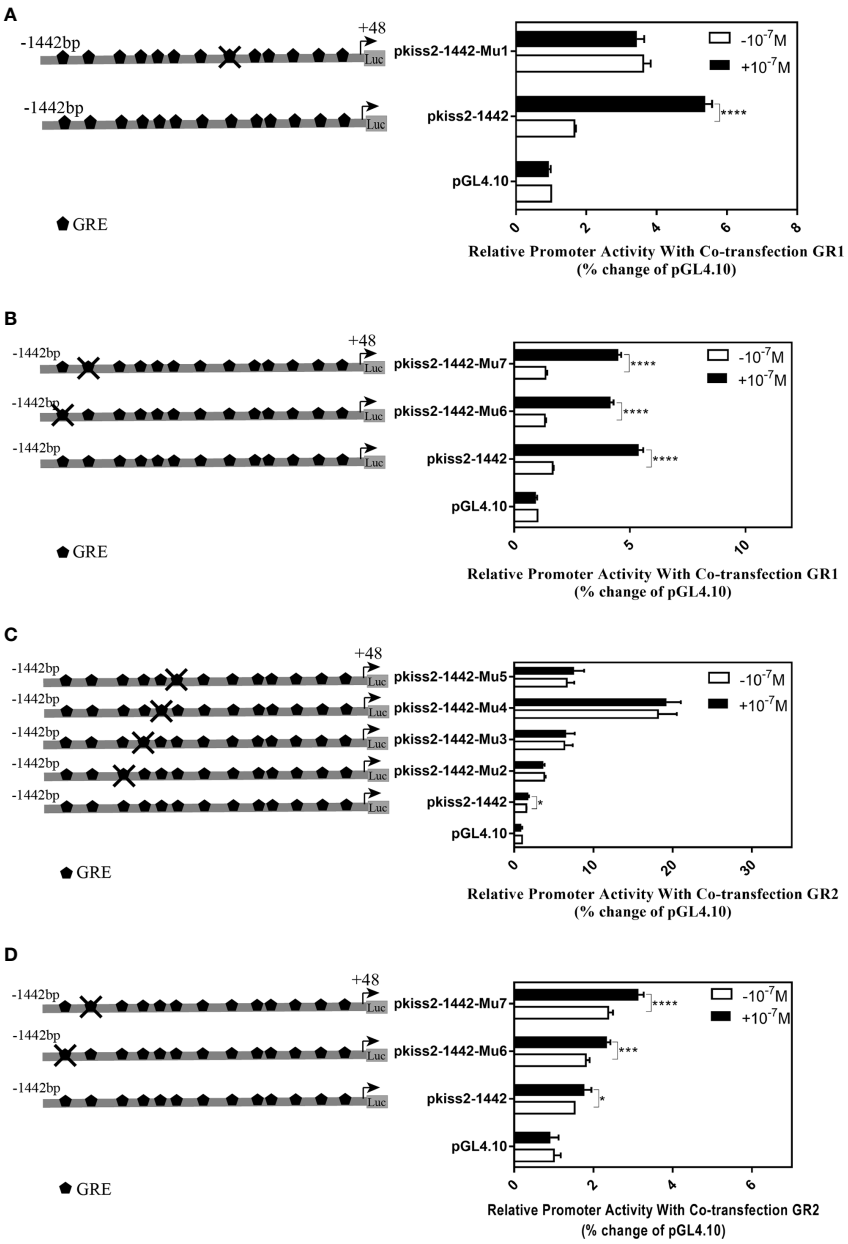


FIGURE 8
Mutations of the putative glucocorticoid receptor binding sites in *kiss2* promoter. Schematic of mutated GRE sites is shown on the left. HEK-293T cells were transfected with GRE-mutated promoter constructs and pRL-CMV with GR1 **(A, B)** or GR2 **(C, D)** expression plasmid. Cells were incubated with or without 10^{-7} M cortisol treatment. Relative promoter activities are expressed as percentage of pGL4.10 in the absence of cortisol. Data are represented as mean ± SEM (n = 4). * ($p < 0.05$), *** ($p < 0.001$), and **** ($p < 0.0001$) indicate significant differences compared with the corresponding control.

more effective with GR1 than GR2. Moreover, cortisol was shown to regulate *kiss2* promoter activity by one GRE site through GR1 and four GRE sites *via* GR2. Our findings demonstrate that cortisol could directly regulate the expression of *kiss2* gene *via* the GRE-dependent GR pathway in the yellowtail clownfish.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding author.

Ethics statement

The animal study was reviewed and approved by Committee of Hainan University (HNUAUCC-2021-00014).

Author contributions

S-YB planned and wrote the manuscript, participated in experiments and composed the figures. Y-YZ planned, edited, and drafted the manuscript. XZ, T-XL, D-CZ, and Z-XH participated in experiments. QW planned, edited, supervised, and reviewed the manuscript. All authors read the final article and approved its submission.

Funding

This work was supported by National Natural Science Foundation of China (NSFC Grant No. 31760759).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.902737/full#supplementary-material

References

1. Castellano J, Roa J, Luque R, Dieguez C, Aguilar E, Pinilla L, et al. KiSS-1/kisspeptins and the metabolic control of reproduction: physiologic roles and putative physiopathological implications. *Peptides* (2009) 30:139–45. doi: 10.1016/j.peptides.2008.06.007

2. Ogawa S, Parhar IS. Anatomy of the kisspeptin systems in teleosts. *Gen Comp Endocrinol* (2013) 181:169–74. doi: 10.1016/j.ygcen.2012.08.023

3. De Roux N, Genin E, Carel J-C, Matsuda F, Chaussain J-L, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci* (2003) 100:10972–6. doi: 10.1073/pnas.1834399100

4. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JSJr, Shagoury JK, et al. The GPR54 gene as a regulator of puberty. *Obstetrical Gynecol Survey* (2004) 59:351–3. doi: 10.1056/NEJMoa035322

5. Hussain MA, Song W-J, Wolfe A. There is kisspeptin–and then there is kisspeptin. *Trends Endocrinol Metab* (2015) 26:564–72. doi: 10.1016/j.tem.2015.07.008

6. Espigares F, Rocha A, Gómez A, Carrillo M, Zanuy S. Photoperiod modulates the reproductive axis of European sea bass through regulation of kiss1 and gnrh2 neuronal expression. *Gen Comp Endocrinol* (2017) 240:35–45. doi: 10.1016/j.ygcen.2016.09.007

7. Iwasa T, Matsuzaki T, Yano K, Mayila Y, Irahara M. The roles of kisspeptin and gonadotropin inhibitory hormone in stress-induced reproductive disorders. *Endocrine J* (2018) 65:133–40. doi: 10.1507/endocrj.EJ18-0026

8. Wang B-Q, Chen Y-Y, Lan X-X, Zhou Z-Y, Xu X-X, Wu X-Q. The effect of neonatal immune challenge on reproduction by altering intraovarian kisspeptin/GPR54 system in the rat. *Reprod Toxicol* (2017) 74:40–7. doi: 10.1016/j.reprotox.2017.08.021

9. Ralph C, Lehman M, Goodman R, Tilbrook A. Impact of psychosocial stress on gonadotrophins and sexual behaviour in females: role for cortisol. *Reproduction* (2016) 152:R1–R14. doi: 10.1530/REP-15-0604

10. Padda J, Khalid K, Hitawala G, Batra N, Pokhriyal S, Mohan A, et al. Depression and its effect on the menstrual cycle. *Cureus* (2021) 13. doi: 10.7759/cureus.16532

11. Claes S. Corticotropin-releasing hormone (CRH) in psychiatry: from stress to psychopathology. *Ann Med* (2004) 36:50–61. doi: 10.1080/07853890310017044

12. Joseph DN, Whirledge S. Stress and the HPA axis: balancing homeostasis and fertility. *Int J Mol Sci* (2017) 18:2224. doi: 10.3390/ijms18102224

13. Raftogianni A, Roth LC, García-González D, Bus T, Kühne C, Monyer H. Deciphering the contributions of CRH receptors in the brain and pituitary to stress-induced inhibition of the reproductive axis. *Front Mol Neurosci* (2018) 11:305. doi: 10.3389/fnmol.2018.00305

14. Hirano T, Kobayashi Y, Omotehara T, Tatsumi A, Hashimoto R, Umemura Y, et al. Unpredictable chronic stress-induced reproductive suppression associated with the decrease of kisspeptin immunoreactivity in male mice. *J Vet Med Sci* (2014) 76, 1201–8. doi: 10.1292/jvms.14-0177

15. Yang JA, Song CI, Hughes JK, Kreisman MJ, Parra RA, Haisenleder DJ. Acute psychosocial stress inhibits LH pulsatility and Kiss1 neuronal activation in female mice. *Endocrinology* (2017) 158:3716–23. doi: 10.1210/en.2017-00301

16. Kinsey-Jones J, Li X, Knox A, Wilkinson E, Zhu X, Chaudhary A. Down-regulation of hypothalamic kisspeptin and its receptor, Kiss1r, mRNA expression is associated with stress-induced suppression of luteinising hormone secretion in the female rat. *J Neuroendocrinol* (2009) 21:20–9. doi: 10.1111/j.1365-2826.2008.01807.x

17. Luo E, Stephens SB, Chaing S, Munaganuru N, Kauffman AS, Breen KM. Corticosterone blocks ovarian cyclicity and the LH surge *via* decreased kisspeptin neuron activation in female mice. *Endocrinology* (2016) 157:1187–99. doi: 10.1210/en.2015-1711

18. Yamamoto K, Darimont B, Wagner R, Iniguez-Lluhi J. Building transcriptional regulatory complexes: signals and surfaces. *Cold Spring Harbor Symp quant Biol* (1998), 63, 587–98. doi: 10.1101/sqb.1998.63.587

19. Takumi K, Iijima N, Higo S, Ozawa H. Immunohistochemical analysis of the colocalization of corticotropin-releasing hormone receptor and glucocorticoid receptor in kisspeptin neurons in the hypothalamus of female rats. *Neurosci Lett* (2012) 531:40–5. doi: 10.1016/j.neulet.2012.10.010

20. Wang Q, Sham KW, Ogawa S, Li S, Parhar IS, Cheng CH. Regulation of the two kiss promoters in goldfish (Carassius auratus) by estrogen *via* different ERα pathways. *Mol Cell Endocrinol* (2013) 375:130–9. doi: 10.1016/j.mce.2013.04.023

21. Fricke H, Fricke S. Monogamy and sex change by aggressive dominance in coral reef fish. *Nature* (1977) 266:830–2. doi: 10.1038/266830a0

22. Fricke HW. Social control of sex: field experiments with the anemonefish amphiprion bicinctus. *Z für Tierpsychologie* (1983) 61:71–7. doi: 10.1111/j.1439-0310.1983.tb01327.x

23. Hattori A, Yanagisawa Y. Life-history pathways in relation to gonadal sex differentiation in the anemonefish, amphiprion clarkii, in temperate waters of Japan. *Environ Biol Fishes* (1991) 31:139–55. doi: 10.1007/BF00001015

24. Chen J, Peng C, Yu Z, Xiao L, Yu Q, Li S. The administration of cortisol induces female-to-male sex change in the protogynous orange-spotted grouper, epinephelus coioides. *Front Endocrinol* (2020) 11:12. doi: 10.3389/fendo.2020.00012

25. Zhang H, Zhang Y, Guo Y, Zhang X, Wang Q, Liu X. Kiss2 but not kiss1 is involved in the regulation of social stress on the gonad development in yellowtail clownfish, amphiprion clarkii. *Gen Comp Endocrinol* (2020) 298:12. doi: 10.3389/fendo.2020.00012

26. Zhang Y, Zhang H, Wang J, Zhang X, Bu S, Liu X. Molecular characterization and expression patterns of glucocorticoid receptor (GR) genes in protandrous hermaphroditic yellowtail clownfish, amphiprion clarkii. *Gene* (2020) 745:144651. doi: 10.1016/j.gene.2020.144651

27. Zhang Y, Zhang X, Bu S, Zhang W, Li T, Zheng D, et al. Sexually dimorphic distribution of kiss1 and kiss2 in the brain of yellowtail clownfish, Amphiprion clarkii. *Endocr Connect* (2022) in press. doi: 10.1530/EC-22-0136

28. Livak KJ, Schmittgen TD. Analysis of relative gene expression data using real-time quantitative PCR and the 2– ΔΔCT method. *methods* (2001) 25:402–8. doi: 10.1006/meth.2001.1262

29. Khor YM, Soga T, Parhar IS. Early-life stress changes expression of GnRH and kisspeptin genes and DNA methylation of GnRH3 promoter in the adult zebrafish brain. *Gen Comp Endocrinol* (2016) 227:84–93. doi: 10.1016/j.ygcen.2015.12.004

30. Iwata E, Mikami K, Manbo J, Moriya-Ito K, Sasaki H. Social interaction influences blood cortisol values and brain aromatase genes in the protandrous false clown anemonefish, amphiprion ocellaris. *Zoological Sci* (2012) 29:849–55. doi: 10.2108/zsj.29.849

31. Ogawa S, Nathan FM, Parhar IS. Habenular kisspeptin modulates fear in the zebrafish. *Proc Natl Acad Sci* (2014) 111:3841–6. doi: 10.1073/pnas.1314184111

32. Alvarado M, Servili A, Molés G, Gueguen M-M, Carrillo M, Kah O. Actions of sex steroids on kisspeptin expression and other reproduction-related genes in the brain of the teleost fish European sea bass. *J Exp Biol* (2016) 219:3353–65. doi: 10.1242/jeb.137364

33. Biddie SC, John S, Sabo PJ, Thurman RE, Johnson TA, Schiltz RL. Transcription factor AP1 potentiates chromatin accessibility and glucocorticoid receptor binding. *Mol Cell* (2011) 43:145–55. doi: 10.1016/j.molcel.2011.06.016

34. Nocillado JN, Mechaly AS, Elizur A. In silico analysis of the regulatory region of the yellowtail kingfish and zebrafish kiss and kiss receptor genes. *Fish Physiol Biochem* (2013) 39:59–63. doi: 10.1007/s10695-012-9642-0

35. Ducouret B, Tujague M, Ashraf J, Mouchel N, Servel N, Valotaire Y. Cloning of a teleost fish glucocorticoid receptor shows that it contains a deoxyribonucleic acid-binding domain different from that of mammals. *Endocrinology* (1995) 136:3774–83. doi: 10.1210/en.136.9.3774

36. Stolte EH, van Kemenade BLV, Savelkoul HF, Flik G. Evolution of glucocorticoid receptors with different glucocorticoid sensitivity. *J Endocrinol* (2006) 190:17–28. doi: 10.1677/joe.1.06703

37. Lethimonier C, Tujague M, Kern L, Ducouret B. Peptide insertion in the DNA-binding domain of fish glucocorticoid receptor is encoded by an additional exon and confers particular functional properties. *Mol Cell Endocrinol* (2002) 194:107–16. doi: 10.1016/S0303-7207(02)00181-8

38. Bamberger CM, Schulte HM, Chrousos GP. Molecular determinants of glucocorticoid receptor function and tissue sensitivity to glucocorticoids. *Endocrine Rev* (1996) 17:245–61. doi: 10.1210/edrv-17-3-245

39. Strähle U, Schmid W, Schütz G. Synergistic action of the glucocorticoid receptor with transcription factors. *EMBO J* (1988) 7:3389–95. doi: 10.1002/j.1460-2075.1988.tb03212.x

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 16 August 2022
DOI 10.3389/fendo.2022.982246

Check for updates

OPEN ACCESS

EDITED BY
Oliana Carnevali,
Marche Polytechnic University, Italy

REVIEWED BY
Takayoshi Ubuka,
Cancer Medical Service, Japan
Tomohiro Osugi,
Suntory Foundation for Life Sciences, Japan

*CORRESPONDENCE
José A. Muñoz-Cueto
munoz.cueto@uca.es

SPECIALTY SECTION
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 30 June 2022
ACCEPTED 22 July 2022
PUBLISHED 16 August 2022

CITATION
Wang B, Paullada-Salmerón JA, Vergès-Castillo A, Gómez A and Muñoz-Cueto JA (2022) Signaling pathways activated by sea bass gonadotropin-inhibitory hormone peptides in COS-7 cells transfected with their cognate receptor. *Front. Endocrinol.* 13:982246. doi: 10.3389/fendo.2022.982246

COPYRIGHT
© 2022 Wang, Paullada-Salmerón, Vergès-Castillo, Gómez and Muñoz-Cueto. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author (s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Signaling pathways activated by sea bass gonadotropin-inhibitory hormone peptides in COS-7 cells transfected with their cognate receptor

Bin Wang[1,2,3], José A. Paullada-Salmerón[1,4,5], Alba Vergès-Castillo[1,4,5], Ana Gómez[6] and José A. Muñoz-Cueto[1,4,5]*

[1]Department of Biology, Faculty of Marine and Environmental Sciences, University of Cádiz, Cádiz, Spain, [2]Key Laboratory of Sustainable Development of Marine Fisheries, Ministry of Agriculture and Rural Affairs, Yellow Sea Fisheries Research Institute, Chinese Academy of Fishery Sciences, Qingdao, China, [3]Laboratory for Marine Fisheries and Food Production Processes, Pilot National Laboratory for Marine Science and Technology (Qingdao), Qingdao, China, [4]Marine Research Institute (INMAR), Marine Campus of International Excellence (CEIMAR) and Agrifood Campus of International Excellence (ceiA3), Cádiz, Spain, [5]The European University of the Seas (SEA-EU), Cádiz, Spain, [6]Institute of Aquaculture of Torre de la Sal, CSIC, Castellón, Spain

Results of previous studies provided evidence for the existence of a functional gonadotropin-inhibitory hormone (GnIH) system in the European sea bass, *Dicentrarchus labrax*, which exerted an inhibitory action on the brain-pituitary-gonadal axis of this species. Herein, we further elucidated the intracellular signaling pathways mediating in sea bass GnIH actions and the potential interactions with sea bass kisspeptin (Kiss) signaling. Although GnIH1 and GnIH2 had no effect on basal CRE-luc activity, they significantly decreased forskolin-elicited CRE-luc activity in COS-7 cells transfected with their cognate receptor GnIHR. Moreover, an evident increase in SRE-luc activity was noticed when COS-7 cells expressing GnIHR were challenged with both GnIH peptides, and this stimulatory action was significantly reduced by two inhibitors of the PKC pathway. Notably, GnIH2 antagonized Kiss2-evoked CRE-luc activity in COS-7 cells expressing GnIHR and Kiss2 receptor (Kiss2R). However, GnIH peptides did not alter NFAT-RE-luc activity and ERK phosphorylation levels. These data indicate that sea bass GnIHR signals can be transduced through the PKA and PKC pathways, and GnIH can interfere with kisspeptin actions by reducing its signaling. Our results provide additional evidence for the understanding of signaling pathways activated by GnIH peptides in teleosts, and represent a starting point for the study of interactions with multiple neuroendocrine factors on cell signaling.

KEYWORDS

GnIH, GnIH receptor, kisspeptin, kisspeptin receptor, signaling pathway

Introduction

Since the first discovery of gonadotropin-inhibitory hormone (GnIH) in the quail, the presence of GnIH orthologs has been reported in a variety of vertebrate species, including fish (1, 2). Phylogenetic, synteny and functional analysis revealed that the GnIH and NPFF genes, both of which belong to the family of the RFamide peptides, may have diverged from a common ancestral gene by whole-genome duplication during vertebrate evolution (2, 3). Two paralogous G protein-coupled receptors (GPCRs), GPR147 and GPR74, have been identified as the common receptors for GnIH (GnIHRs) and NPFF (NPFFRs) (2). However, GPR147 is regarded as the primary receptor for GnIH based on the higher binding affinity of GnIH to GPR147 compared to GPR74 (4, 5). In turn, the NPFF precursor encodes NPFF and NPAF mature peptides, and these two peptides preferentially activate GPR74 (2). Multiple lines of evidence indicated that GnIH not only suppresses reproduction in vertebrates through its inhibitory actions on the brain-pituitary-gonadal axis, but also participates in stress response, feeding and reproductive behaviors (1, 2, 6). Despite its functional significance, the detailed signaling pathways mediating the actions of GnIH on target cells have not been fully elucidated (7, 8).

Luciferase (luc) transactivation assays have been validated to discriminate different GPCR pathways, such as cAMP response element (CRE-luc), serum response element (SRE-luc), and nuclear factor of activated T-cells response element (NFAT-RE-luc) for adenylate cyclase (AC)/cAMP/protein kinase A (PKA), extracellular signal regulated kinase (ERK)/mitogen-activated protein kinase (MAPK) (principally considered protein kinase C [PKC]-mediated activation), and intracellular Ca^{2+} mobilization, respectively (9, 10). Until now, the mechanisms underlying in the signaling pathways of GnIH actions have been extensively elucidated in mammals (7) and birds (11), but only in a few fish species using mammalian cell lines transfected with the corresponding cognate receptors combined with the response element luciferase assays (8). There is an evident increase of CRE-luc activity and SRE-luc activity induced by tilapia and chub mackerel GnIH peptides in COS-7 cells transfected with their GnIHRs, indicating that their GnIHR signals are transduced through PKA and PKC pathways (12, 13). However, the three orange-spotted grouper GnIH peptides markedly decreased forskolin-induced CRE-luc activity in COS-7 cells expressing their cognate receptor, and SRE-luc activity was also reduced by GnIH1 (14). Activation of half-smooth tongue sole GnIHR by GnIH2 also significantly inhibited forskolin-induced CRE-luc activity, whereas both GnIH1 and GnIH2 evoked SRE-luc activity in COS-7 cells expressing tongue sole GnIHR (15). The three zebrafish GnIH peptides activated GnIHR2 and GnIHR3 through the PKA pathway, whereas the PKC pathway cannot be activated by any of the three GnIH peptides *via* any of the three GnIHRs (16). Interestingly, medaka GnIH exerted a dual action on CRE-luc activity depending on the doses used and the presence/absence of forskolin stimulation, indicating a possible switch of coupling of GnIHR to Gαi and Gαs proteins in this species (17). In mammals, ovine GnIH3 potently reduced gonadotropin-releasing hormone (GnRH)-induced intracellular Ca^{2+} mobilization and ERK phosphorylation in primary pituitary cell cultures (18, 19). Moreover, mouse GnIH peptides exerted a suppressive effect on GnRH-elicited mRNA levels of gonadotropin subunit genes by inhibiting AC/cAMP/PKA-dependent ERK pathway in LβT2 cells (20). Whether and how Ca^{2+} and ERK pathways participate in GnIH actions remains unknown in fish, and merits further studies.

Using the European sea bass (*Dicentrarchus labrax*) as a model, we cloned the full-length cDNA encoding the GnIH precursor polypeptide that contained two putative mature peptides (GnIH1 and GnIH2), developed a specific antibody against GnIH2, and characterized its central and pituitary GnIH projections (21). Subsequently, we investigated the effects of intracerebroventricularly-administered GnIH1 and GnIH2 on gene expression of brain-pituitary reproductive hormones and their receptors along with plasma levels of Fsh and Lh, and found that GnIH peptides played a suppressive action on the reproductive axis of this species (22). We further demonstrated that chronic peripheral implants of GnIH1 and GnIH2 peptides delayed gonadal development and steroidogenesis during the reproductive cycle of male sea bass (23). On the other hand, two distinct forms of kisspeptins (Kiss1 and Kiss2) and kisspeptin receptors (Kiss1R or Kissr2 and Kiss2R or Kissr3) have been identified in sea bass, with Kiss2 being more potent in eliciting gonadotropin secretion (24–26). *In vitro* functional analysis showed that the two sea bass KissR signals are transduced through the PKA and PKC pathways (25). Because little information is available in teleosts regarding the signaling pathway mechanisms of GnIH actions and the interactions with cell signaling evoked by other neuroendocrine factors (8), the aims of the current study, therefore, were (1) to examine the potential intracellular signaling pathways (e.g. PKA, PKC, Ca^{2+} and ERK) evoked by the GPR147 GnIHR in response to sea bass GnIH peptides, and (2) to investigate the possible interactions with sea bass kisspeptin signaling.

Materials and methods

Peptides

Synthetic peptides (23, 24, 27) corresponding to European sea bass GnIH1 (PLHLHANMPMRF-NH_2), GnIH2 (SPNSTPNMPQRF-NH_2), NPFF (NSVLHQPQRF-NH_2), NPAF (DWEAAPGQIWSMAVPQRF-NH_2), Kiss1 ([pGLU]

DVSSYNLNSFGLRY-NH_2) and Kiss2 (SKFNFNPFGLRF-NH_2) were purchased from ChinaPeptides Co., Ltd. (Shanghai, China) with a purity of 98.09%, 96.18%, 96.18%, 96.54%, 96.10% and 96.12%, respectively, as determined by HPLC. All peptides were amidated at the C-termini, and Kiss1 contained a pyroglutamylated N-terminus. These neuropeptides were prepared with distilled water and aliquots were stored at -20°C.

Plasmids

Both CRE-luc and SRE-luc plasmids (BD Biosciences Clontech, CA, USA) contained the firefly luciferase gene and have been validated in a previous study (25). The NFAT-RE-luc plasmid also included the firefly luciferase gene and was purchased from Promega (Madison, WI, USA). The pRL-TK plasmid, which constitutively expresses the *Renilla reniformis* luciferase gene, was provided by Promega and used for normalization of the transfection efficiency. The entire open reading frames of sea bass *gnihr* (GPR147-type), *kiss1r* and *kiss2r* genes were obtained by PCR amplification using Q5® High-Fidelity DNA Polymerase (New England Biolabs, Ipswich, MA, USA) and the specific primers (Table 1), and then subcloned into the *HindIII* and *EcoRI* sites of the expression vector pcDNA3.1/Zeo(+) (Invitrogen, Waltham, MA, USA), respectively. All receptor constructs (pcDNA3.1-GnIHR, pcDNA3.1-Kiss1R and pcDNA3.1-Kiss2R) were extracted with Endo-free Plasmid DNA Mini Kit (Omega Bio-tek, Norcross, GA, USA) and verified by sequencing.

Reagents for cell culture, transfection and signaling pathways

COS-7 cells (ATCC, Manassas, VA, USA), Dulbecco's Modified Eagle Medium (DMEM) containing high glucose (4.5 g/L, Gibco, Waltham, MA, USA), fetal bovine serum (FBS, Gibco), 100×penicillin/streptomycin antibiotics (Gibco), Opti-MEM (Gibco), Lipofectamine 3000 (Invitrogen), 5×Passive Lysis Buffer (Promega), Dual-Glo® Luciferase Assay System (Promega), forskolin (FSK, Calbiochem), U73122 (Calbiochem), and GF109203X (Calbiochem) were purchased from the manufacturers. FSK, U73122, and GF109203X were dissolved in dimethyl sulfoxide and aliquots were stored at -80°C as described elsewhere previously (28).

Transient transfection and luciferase reporter gene assays

All experimental protocols were followed as described previously (15, 29) with some modifications. One day before transfection, COS-7 cells were seeded in 24-well plates at a density of 1×10^5 cells/well/mL of DMEM supplemented with 10% FBS and 1% penicillin/streptomycin and maintained in a humidified 5% CO_2 atmosphere at 37°C. For each well, cells were co-transfected with CRE-luc/SRE-luc/NFAT-RE-luc (200 ng), pcDNA3.1-GnIHR (200 ng), and pRL-TK (20 ng) using Lipofectamine 3000 in 500 µL Opti-MEM. After starvation overnight, (1) cells were then treated with GnIH peptides (10, 100, 1000 nM), NPFF (1000 nM), and NPAF (1000 nM) for 6 h; (2) cells were challenged for 6 h with 10 µM FSK alone or co-treated with 1000 nM GnIH1, GnIH2, NPFF, and NPAF; (3) cells were incubated for 6 h with 1000 nM GnIH peptides alone or in the presence of U73122 (phospholipase C [PLC] inhibitor, 10 µM) and GF109203X (PKC inhibitor, 10 µM). Finally, cells were harvested using 1×Passive Lysis Buffer (100 µL/well) and luminescence was determined with Dual-Glo® Luciferase Assay System on the LB963 luminometer (Berthold Technologies GmbH & Co.KG, Bad Wildbad, Germany). Luciferase activity values were calculated by dividing the firefly luciferase units by the *Renilla* luciferase values for each sample. The values obtained for the controls were set as 1 for each experiment, and the experimental values which were divided by those of the controls are presented as fold increase. Each transfection experiment was performed in triplicate and repeated at least twice. A parallel control transfection experiment was performed with the empty pcDNA3.1 vector, CRE-luc, SRE-luc, or NFAT-RE-luc and the internal reference pRL-TK.

In addition, we further evaluated the possible interactions between sea bass GnIH and kisspeptin signaling involved in the PKA pathway. First, to determine if GnIH peptides are capable of activating Kiss1R and Kiss2R through the CRE-luc pathway and vice versa, cells were co-transfected with pcDNA3.1-GnIHR/pcDNA3.1-Kiss1R/pcDNA3.1-Kiss2R (200 ng/well), CRE-luc (200 ng/well), and pRL-TK (20 ng/well). After

TABLE 1 Primer list for construction of pcDNA3.1-receptors.

Gene	Primer sequence (5'-3')	GenBank accession no.
gnihr	Forward: CCCAAGCTTATGGAGGTACTAGACAAC	LN681206
	Reverse: CGGAATTCTCAGTTATCCCACGCCTG	
kiss1r	Forward: CCCAAGCTTATGGTGGAATCAGCAGCC	JN202446
	Reverse: CGGAATTCTTAGGATCCAGATGAAAG	
kiss2r	Forward: CCCAAGCTTATGTACTCCTCCGAGGAG	JN202447
	Reverse: CGGAATTCTCAATTCATTGCATTATT	

starvation overnight, cells were treated with GnIH and kisspeptin peptides (1 μM) for 6 h, and luciferase activity in cell extracts was measured. Second, to investigate the potential interactions among GnIHR, Kiss1R and Kiss2R signaling, cells were co-transfected with pcDNA3.1-GnIHR, pcDNA3.1-Kiss1R/pcDNA3.1-Kiss2R, CRE-luc, and pRL-TK, challenged with GnIH and kisspeptin alone or a combination of the two peptides for 6 h, and then harvested for assays.

Western blot analysis

Whether the ERK pathway is activated by GnIH peptides was investigated by Western blot analysis (30). As mentioned above, COS-7 cells were seeded in 24-well plates (2×10^5 cells/well/mL of DMEM), transfected with pcDNA3.1-GnIHR (200 ng/well), starved overnight, and then challenged with 1 μM GnIH1, GnIH2, NPFF, and NPAF for 10 min. The dose and treatment time were chosen based on previous reports (20, 31). Cells were harvested using 1×Cell Lysis Buffer (100 μL/well, Cell Signaling Technology, Danvers, MA, USA) supplemented with Pierce Protease and Phosphatase Inhibitor Mini Tablets (ThermoFisher Scientific, Waltham, MA, USA), and protein concentrations were measured with Pierce™ BCA Protein Assay Kit (ThermoFisher Scientific). Equal amounts of total proteins (14 μg/lane) were separated by 12% SDS-PAGE, and then electrotransferred onto nitrocellulose membranes, which was blocked with 5% bovine serum albumin in TBST at room temperature for 1 h. The membranes were washed three times (10 min each time) with TBST and incubated with Phospho-p44/42 MAPK (Erk1/2) (Thr202/Tyr204) antibody (1:1000, Cell Signaling Technology) overnight at 4°C. After another three washes, the membranes were incubated with HRP-linked anti-rabbit IgG antibody (1:2000, Cell Signaling Technology) at room temperature for 1 h, washed, and visualized with Pierce™ ECL Plus Western Blotting Substrate (ThermoFisher Scientific). The protein bands were quantified using a densitometry software (Bio-Rad, Hercules, CA, USA). Subsequently, the membranes were incubated with Restore™ Western Blot Stripping Buffer (ThermoFisher Scientific) and reused for another immunodection with p44/42 MAPK (Erk1/2) antibody (Cell Signaling Technology) to normalize the blots.

Statistical analysis

Data are presented as the mean ± SEM and were analyzed by one-way ANOVA followed by Duncan's multiple range test using SPSS17.0 software. Normality and homoscedasticity assumptions were tested prior to the analysis. Differences were considered to be statistically significant when $p < 0.05$.

Results

Absence of sea bass GnIH receptor and NPFF receptor in COS-7 cells

As depicted in Figure 1A, there was no response in CRE-luc activity when COS-7 cells transfected with the empty expression vector pcDNA3.1 were challenged with 1 μM GnIH1, GnIH2, NPFF and NPAF peptides. Parallel treatment with 10 μM FSK acted as a positive control (Figure 1A). Similarly, neither SRE-luc activity nor NFAT-RE-luc activity were altered by the four peptides tested (1 μM, Figures 1B, C). These data indicated that COS-7 cells do not naturally express endogenous receptors for sea bass GnIH and NPFF peptides.

Coupling of sea bass GnIH receptor to $G_{\alpha i}$ protein

As shown in Figure 2A, COS-7 cells transfected with sea bass GnIHR did not respond to GnIH1 and GnIH2 at doses ranging from 10 to 1000 nM in CRE-luc activity. As a comparative control, 1 μM NPFF and NPAF also did not modify CRE-luc activity (Figure 2A). However, these four peptides (1 μM) significantly reduced FSK-stimulated CRE-luc activity (Figure 2B), suggesting that sea bass GnIHR is coupled to $G_{\alpha i}$ protein and can be activated by both GnIH and NPFF peptides.

Coupling of sea bass GnIH receptor to $G_{\alpha q}$ protein

SRE-luc was employed as a reporter gene for activation of the PLC/PKC pathway. Both GnIH1 and GnIH2 increased SRE-luc activity in COS-7 cells transfected with sea bass GnIHR in a dose-dependent manner (Figure 3A). Similarly, a significant induction of SRE-luc activity was observed by 1 μM NPFF and NPAF (Figure 3A). These results indicated that sea bass GnIHR is coupled to $G_{\alpha q}$ protein. To further confirm the involvement of the PLC/PKC pathway, two specific inhibitors (U73122 and GF109203X) were employed. As observed in Figure 3B, the stimulatory effects of GnIH peptides (1 μM) on SRE-luc activity were attenuated by 10 μM U73122 (PLC inhibitor) and totally abolished by 10 μM GF109203X (PKC inhibitor).

Absence of GnIH and NPFF effects on Ca^{2+} and ERK activation

NFAT-RE-luc was used to examine the possible participation of intracellular Ca^{2+} mobilization in activation of sea bass GnIHR. None of the peptides assayed (GnIH1, GnIH2,

10.3389/fendo.2022.982246

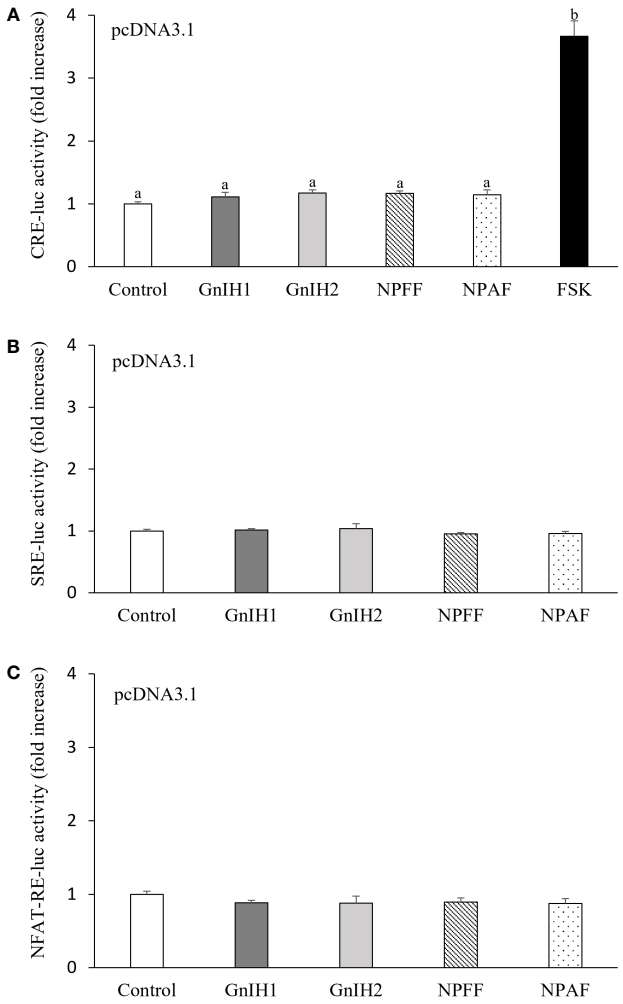


FIGURE 1
Effects of GnIH and NPFF peptides on CRE-luc **(A)**, SRE-luc **(B)**, and NFAT-RE-luc **(C)** activity in COS-7 cells transfected with the empty pcDNA3.1 vector. Cells were challenged with each peptide (1 μM) for 6 h and then harvested for assays. FSK (10 μM) acted as a positive control. Data are presented as the mean ± SEM (n = 6). Different letters indicate statistically significant differences between mean values (ANOVA one-way $p < 0.05$).

NPFF, and NPAF) had any effect on NFAT-RE-luc activity (Figure 4A). On the other hand, ERK phosphorylation levels were also unaffected by these four peptides (1 μM), either (Figure 4B).

Activation of GnIH receptor reduces kisspeptin receptor signaling

Subsequently, we investigated the potential interactions between GnIH and kisspeptin on PKA pathway signaling. There was no response in CRE-luc activity when COS-7 cells expressing sea bass GnIHR were stimulated with 1 μM Kiss1 or Kiss2 (Figure 5A). Similarly, there was no activation of Kiss1R and Kiss2R after treatment with 1 μM GnIH1 and GnIH2 (Figures 5B, C). FSK (10 μM, Figure 5A), Kiss1 and Kiss2 (1 μM, Figures 5B, C) acted as positive controls. These results evidenced that each peptide functions *via* its own receptor.

Both Kiss1 and Kiss2 induced a significant increase in CRE-luc activity in COS-7 cells co-transfected with sea bass GnIHR and Kiss1R, while neither GnIH1 nor GnIH2 affected the stimulatory effects evoked by kisspeptin peptides (Figure 5D). Similar results were observed in COS-7 cells expressing sea bass GnIHR and Kiss2R as a result of treatment with Kiss1 alone as well as co-administration of Kiss1 and GnIH1/GnIH2 (Figure 5E). However, GnIH2 elicited a significant reduction of CRE-luc activity when co-administered with Kiss2 compared to the stimulation provoked by Kiss2 alone (Figure 5E). Although not significant, there was also a slight reduction of

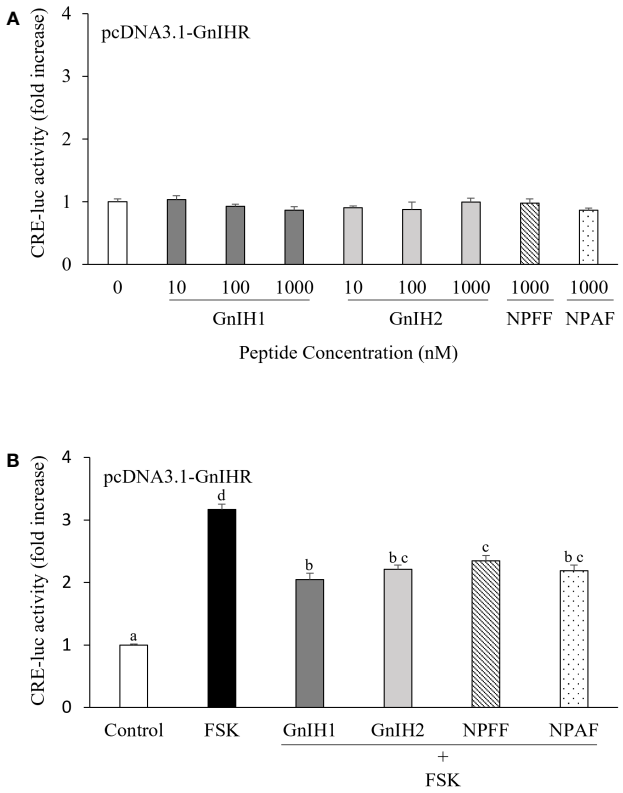


FIGURE 2
Effects of GnIH and NPFF peptides on CRE-luc activity in COS-7 cells transfected with sea bass GnIHR. Cells were challenged with GnIH and NPFF peptides alone **(A)** or co-treated with FSK (10 μM, **B**) for 6 h and then harvested for assays. Data are presented as the mean ± SEM (n=6). Different letters indicate statistically significant differences between mean values (ANOVA one-way $p < 0.05$).

CRE-luc activity when cells were co-treated with Kiss2 and GnIH1 (Figure 5E).

Discussion

So far, physiological functions of the GnIH/GnIHR system have been investigated in different vertebrate groups, including fish, but the intricate web of intracellular signaling pathways mediating GnIH actions is still far from being fully understood (1, 8, 32). Results of our previous studies have revealed the existence of a functional GnIH system in sea bass, and provided evidence for the inhibitory role of GnIH in the reproductive axis of male sea bass, by acting at the brain, pituitary and gonadal levels (33). In the current study, the potential involvement of the PKA, PKC, Ca^{2+}, and ERK pathways in the actions of sea bass GnIH peptides was evaluated using COS-7 cells expressing their cognate receptor. Neither GnIH1 nor GnIH2 had effects on basal CRE-luc activity in COS-7 cells expressing sea bass GnIHR, but efficiently reduced FSK-induced CRE-luc activity. These data indicate that sea bass GnIHR couples to $G_{\alpha i}$ protein, which is consistent with previous studies in orange-spotted grouper (14), half-smooth tongue sole (15), and chicken (11). On the contrary, tilapia GnIHR (12), chub mackerel GnIHR (13), and zebrafish GnIHR2 and GnIHR3 (16) are coupled to $G_{\alpha s}$ protein. Interestingly, a switch between $G_{\alpha i}$ and $G_{\alpha s}$ proteins is observed for medaka GnIHR (17). Taken together, these results show that GnIHRs in various species seem to couple to different heterotrimeric G proteins, which may underlie the functional diversity of the GnIH system reported in fish. For example, tilapia GnIH2 positively regulated both Lh and Fsh release *in vivo* and *in vitro* (12), whereas sea bass GnIH1 and GnIH2 down-regulated plasma Lh levels *in vivo* (22). It is worth mentioning that NPFF and NPAF can also suppress FSK-stimulated CRE-luc activity in COS-7 cells expressing sea bass GnIHR, indicating that GnIHR is a candidate receptor for these two peptides (5). Further studies are being directed in the laboratory to investigate NPFFR (GPR74) signaling pathways and how they are regulated by NPFF, NPAF and GnIH peptides, in order to determine the potency of each peptide in eliciting their responses through both paralogous receptors (GPR147 and GPR74).

In this study, both GnIH1 and GnIH2 increased SRE-luc activity in COS-7 cells expressing sea bass GnIHR, indicating

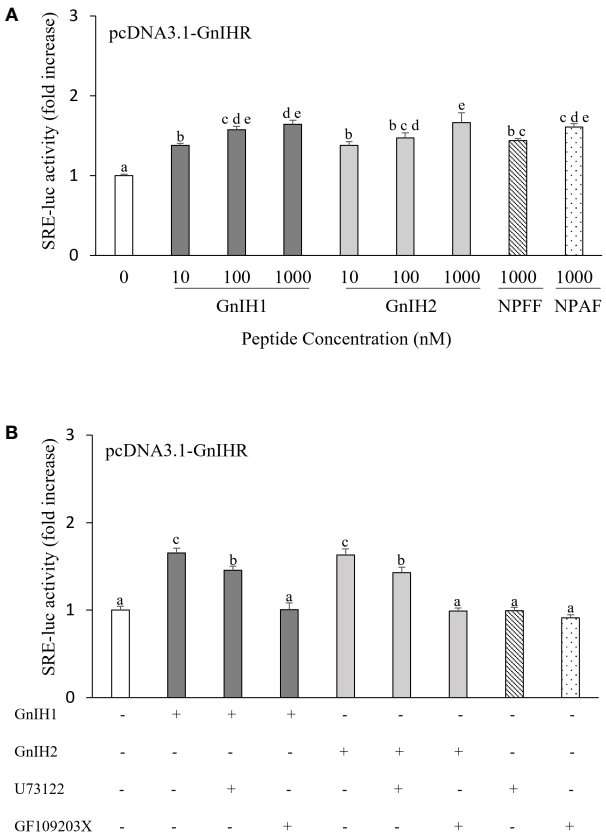


FIGURE 3
Effects of GnIH and NPFF peptides on SRE-luc activity in COS-7 cells transfected with sea bass GnIHR. Cells were challenged with GnIH and NPFF peptides alone **(A)** or co-incubated with GnIH peptides (1 μM) in the absence/presence of 10 μM PLC inhibitor U73122 **(B)** and 10 μM PKC inhibitor GF109203X **(B)** for 6 h and then harvested for assays. Data are presented as the mean ± SEM (n=6-9). Different letters indicate statistically significant differences between mean values (ANOVA one-way $p < 0.05$).

that this receptor may couple to $G_{\alpha q}$ protein and convey its signaling *via* the PKC pathway, which is in line with previous reports in tilapia (12), and tongue sole (15). However, orange-spotted grouper GnIH1 reduced SRE-luc activity in COS-7 cells transfected with its cognate receptor (14). No response in SRE-luc activity was observed by any of the three GnIH peptides with any of the three GnIHRs identified in zebrafish (16). Moreover, the stimulatory effect of sea bass GnIH on SRE-luc activity was inhibited by the PLC inhibitor U73122 and specially by the PKC inhibitor GF109203X, as observed in tongue sole (15), further confirming the involvement of the PLC/PKC pathway in sea bass GnIH actions.

Very limited information is available with respect to Ca^{2+} and ERK pathways mediating GnIH actions on target cells. Neither sea bass GnIH1 nor GnIH2 altered NFAT-RE-luc activity and ERK phosphorylation levels in the present study. Likewise, the three mouse GnIH peptides tested had no direct inhibitory effect on basal or kisspeptin-induced NFAT-RE-luc activity and ERK phosphorylation levels in GT1-7 cells (10). In contrast, sheep GnIH3 potently reduced GnRH-stimulated mobilization of intracellular calcium and phosphorylation of ERK in pituitary gonadotropes (18, 19). Previous results showed that goldfish Kiss1 can directly stimulate Lh and Gh release from primary cultures of pituitary cells in a Ca^{2+}-dependent manner (34), and zebrafish Kiss2 can also enhance the ERK and Akt phosphorylation levels in the female pituitary explants *in vitro* (35). Considering the opposite actions of GnIH and kisspeptin on gonadotropin secretion in sea bass (22, 24), we hypothesize that GnIH could antagonize kisspeptin signaling involved in Ca^{2+} and ERK routes, which is a promising topic of future research not only in sea bass but also in other fish species.

As mentioned above, sea bass GnIHR is coupled to Gαi protein, while sea bass Kiss1R and Kiss2R are coupled to Gαs protein (25). This implies that activation of GnIHR could interfere with signaling of Kiss1R and Kiss2R in this species, as reported in half-smooth tongue sole, in which GnIH2 reduced Kiss2-elicited CRE-luc activity in a dose-dependent manner when COS-7 cells were co-transfected with half-smooth tongue sole GnIHR and Kiss2R and co-stimulated with both Kiss2 and GnIH2 (29). Indeed, in the present study, an

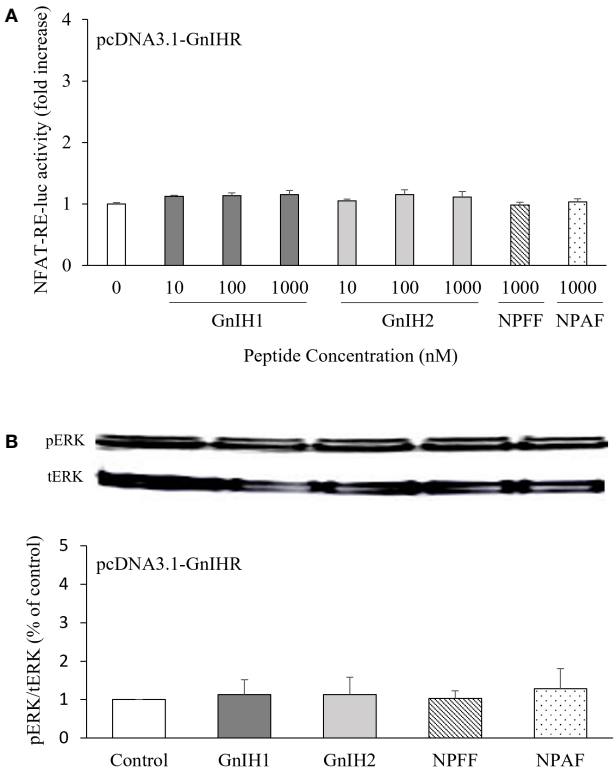


FIGURE 4
Effects of GnIH and NPFF peptides on NFAT-RE-luc activity **(A)** and ERK phosphorylation levels **(B)** in COS-7 cells transfected with sea bass GnIHR. **(A)** Cells were challenged with GnIH and NPFF peptides for 6 h and then harvested for assays. **(B)** Cells were challenged with 1 μM GnIH and NPFF peptides for 10 min and then harvested for Western blot analysis. Data are presented as the mean ± SEM (n=3-6).

inhibitory action of sea bass GnIH2 on Kiss2-induced CRE-luc activity was observed in COS-7 cells expressing both GnIHR and Kiss2R, which is in accordance with the fact that GnIH2 and Kiss2 are more potent regulators in the control of sea bass reproduction than GnIH1 and Kiss1, respectively (22, 24). It should be noted that GnIH2 (but not GnIH1) inhibited the synthesis of Kiss1, Kiss1R, and notably Kiss2, in sea bass (22). Reasons for the lack of effects of GnIH peptides on Kiss1R signaling are not known, but could perhaps be due to a low ratio of GnIHR to Kiss1R (1:1), which may cause less responsiveness to the ligand. For instance, chicken GnIH inhibited GnRH receptor (GnRHR) signaling more effectively as the ratio of GnIHR to GnRHR increased (11). Thus, it seems necessary to further investigate the temporal expression patterns of *gnihr*, *kiss1r* and *kiss2r* mRNAs along the reproductive axis of sea bass during a reproductive cycle. Another possibility is that GnIH may exert more potent inhibitory actions partially through GPR74 which also couples to Gαi protein (36). Further investigation is warranted to clarify whether a synergistic effect can be detected for GPR147 and GPR74 combined.

To the best of our knowledge, neuroanatomical co-localisation of GnIHR with Kiss1R or Kiss2R in the same cell has never been shown in sea bass or other fish species. However, the presence of GnIHR (12, 37, 38) and/or kisspeptin receptors (39–42) has been reported in the pituitary of several teleost species, including sea bass, suggesting that some endocrine cells of the adenohypophysis (e.g., gonadotropes, corticotropes, melanotropes) could exhibit both receptor types. Interestingly, the distribution of GnIH-immunoreactive fibres (21) overlaps with Kiss2 projections and Kiss1R- and Kiss2R-expressing cells (39) in many central areas of the sea bass, suggesting that GnIH and Kiss receptors could also co-localise in brain cells of this species. Therefore, future studies should also be directed to elucidate which pituitary and brain cells co-express GnIHR and Kiss1R/Kiss2R in sea bass.

In summary, we have investigated the possible signaling pathways involved in the actions of sea bass GnIH peptides, and revealed that sea bass GnIHR signals can be transduced *via* both PKA and PKC pathways. In addition, our results support the consideration that sea bass GnIH can interfere with kisspeptin

10.3389/fendo.2022.982246

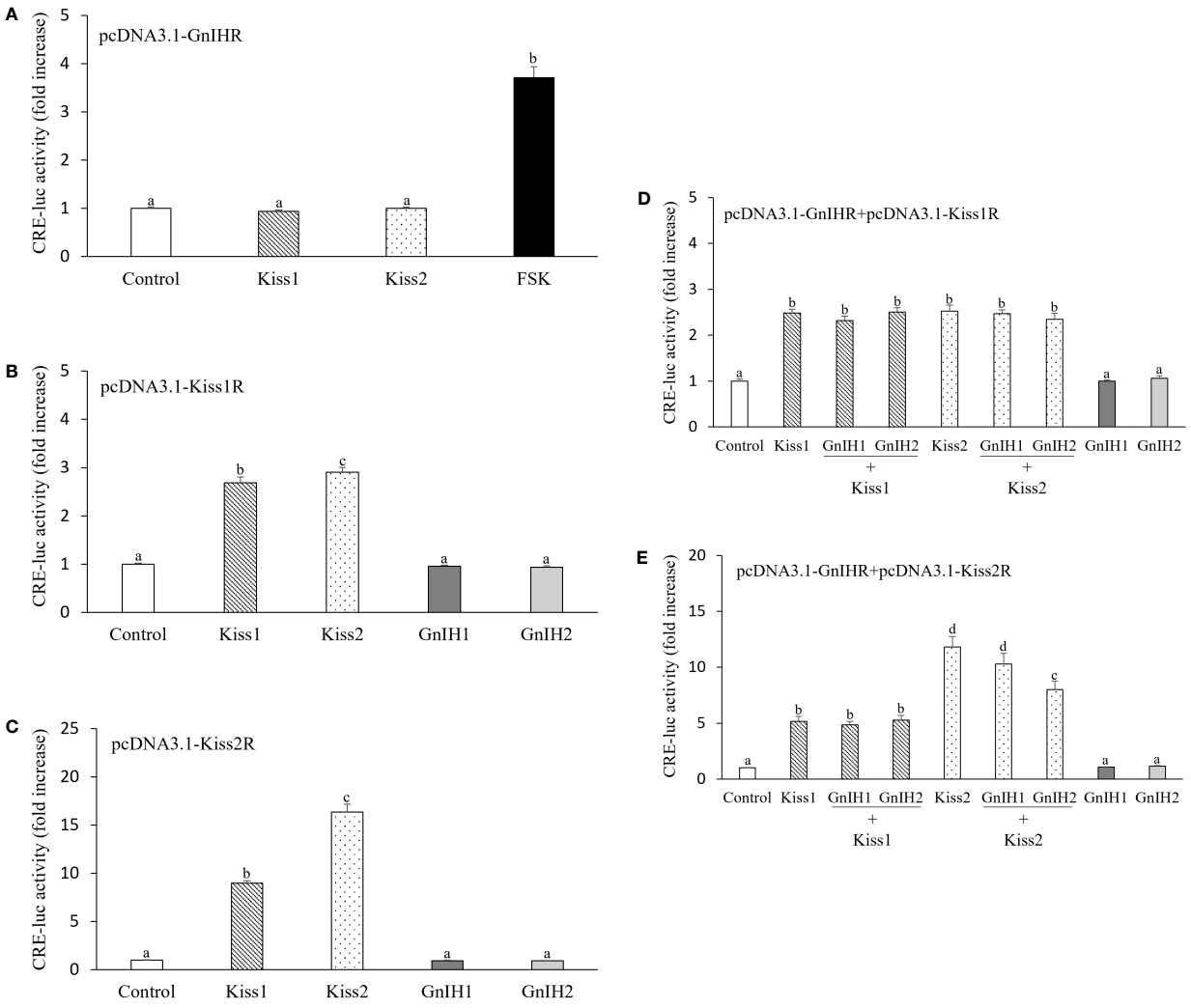


FIGURE 5
Interactions of GnIH on kisspeptin-elicited CRE-luc activity in COS-7 cells co-transfected with their cognate receptors. **(A)** CRE-luc activity in COS-7 cells expressing sea bass GnIHR and stimulated with 1 µM Kiss1 and Kiss2 or 10 µM FSK alone. **(B, C)** CRE-luc activity in COS-7 cells transfected with sea bass Kiss1R **(B)** or Kiss2R **(C)** and stimulated with 1 µM Kiss1, Kiss2, GnIH1 and GnIH2 alone. **(D, E)** CRE-luc activity in COS-7 cells co-transfected with sea bass GnIHR and Kiss1R **(D)** or Kiss2R **(E)** and stimulated with 1 µM Kiss1 and Kiss2 alone or in the presence of 1 µM GnIH peptides. Data are presented as the mean ± SEM (n=6). Different letters indicate statistically significant differences between mean values (ANOVA one-way $p < 0.05$).

signaling involving the PKA pathway. The results obtained in the present study enlarge our knowledge on GnIH signaling pathways in teleosts and represent a starting point to further examine the interactions of GnIH with other neuroendocrine factors (e.g., GnRH, Npy, Spexin) on cell signaling.

Data availability statement

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

Author contributions

BW and JAM-C designed research. BW, JAP-S, and AV-C performed experiments. BW analyzed data and wrote the paper. AG and JAM-C edited the manuscript. AG provided some plasmids, and JAM-C provided funding. All authors contributed to the article and approved the submitted version.

Funding

This work was supported by a grant from PAIDI2020 (Consejería de Economía, Conocimiento, Empresas y Universidad. Junta de Andalucía. Grant no P18-RT-5152) to

JAM-C. BW was awarded a scholarship sponsored by the China Scholarship Council (CSC, File No. 201903260004).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Muñoz-Cueto JA, Paullada-Salmerón JA, Aliaga-Guerrero M, Cowan ME, Parhar IS, Ubuka T. A journey through the gonadotropin-inhibitory hormone system of fish. *Front Endocrinol (Lausanne)* (2017) 8:285. doi: 10.3389/fendo.2017.00285

2. Tsutsui K, Ubuka T. Gonadotropin-inhibitory hormone (GnIH): A new key neurohormone controlling reproductive physiology and behavior. *Front Neuroendocrinol* (2021) 61:100900. doi: 10.1016/j.yfrne.2021.100900

3. Osugi T, Okamura T, Son YL, Ohkubo M, Ubuka T, Henmi Y, et al. Evolutionary origin of GnIH and NPFF in chordates: insights from novel amphioxus RFamide peptides. *PloS One* (2014) 9:e100962. doi: 10.1371/journal.pone.0100962

4. Bonini JA, Jones KA, Adham N, Forray C, Artymyshyn R, Durkin MM, et al. Identification and characterization of two G protein-coupled receptors for neuropeptide FF. *J Biol Chem* (2000) 275:39324–31. doi: 10.1074/jbc.M004385200

5. Ikemoto T, Park MK. Chicken RFamide-related peptide (GnIH) and two distinct receptor subtypes: identification, molecular characterization, and evolutionary considerations. *J Reprod Dev* (2005) 51:359–77. doi: 10.1262/jrd.16087

6. Ubuka T, Parhar IS, Tsutsui K. Gonadotropin-inhibitory hormone mediates behavioral stress responses. *Gen Comp Endocrinol* (2018) 265:202–6. doi: 10.1016/j.ygcen.2018.03.004

7. Son YL, Ubuka T, Tsutsui K. Molecular mechanisms of gonadotropin-inhibitory hormone (GnIH) actions in target cells and regulation of GnIH expression. *Front Endocrinol (Lausanne)* (2019) 10:110. doi: 10.3389/fendo.2019.00110

8. Wang B, Yang G, Xu Y, Li W, Liu X. Recent studies of LPXRFa receptor signaling in fish and other vertebrates. *Gen Comp Endocrinol* (2019) 277:3–8. doi: 10.1016/j.ygcen.2018.11.011

9. Cheng Z, Garvin D, Paguio A, Stecha P, Wood K, Fan F. Luciferase reporter assay system for deciphering GPCR pathways. *Curr Chem Genomics* (2010) 4:84–91. doi: 10.2174/1875397301004010084

10. Son YL, Ubuka T, Soga T, Yamamoto K, Bentley GE, Tsutsui K. Inhibitory action of gonadotropin-inhibitory hormone on the signaling pathways induced by kisspeptin and vasoactive intestinal polypeptide in GnRH neuronal cell line, GT1-7. *FASEB J* (2016) 30:2198–210. doi: 10.1096/fj.201500055

11. Shimizu M, Bedecarrats GY. Activation of the chicken gonadotropin-inhibitory hormone receptor reduces gonadotropin releasing hormone receptor signaling. *Gen Comp Endocrinol* (2010) 167:331–7. doi: 10.1016/j.ygcen.2010.03.029

12. Biran J, Golan M, Mizrahi N, Ogawa S, Parhar IS, Levavi-Sivan B. LPXRFa, the piscine ortholog of GnIH, and LPXRF receptor positively regulate gonadotropin secretion in tilapia (Oreochromis niloticus). *Endocrinology* (2014) 155:4391–401. doi: 10.1210/en.2013-2047

13. Ohga H, Matsuyama M. Effects of LPXRFamide peptides on chub mackerel gonadotropin secretion. *Biol Reprod* (2021) 105:1179–88. doi: 10.1093/biolre/ioab130

14. Wang Q, Qi X, Guo Y, Li S, Zhang Y, Liu X, et al. Molecular identification of GnIH/GnIHR signal and its reproductive function in protogynous hermaphroditic orange-spotted grouper (Epinephelus coioides). *Gen Comp Endocrinol* (2015) 216:9–23. doi: 10.1016/j.ygcen.2015.04.016

15. Wang B, Yang G, Liu Q, Qin J, Xu Y, Li W, et al. Characterization of LPXRFa receptor in the half-smooth tongue sole (Cynoglossus semilaevis): Molecular cloning, expression profiles, and differential activation of signaling pathways by LPXRFa peptides. *Comp Biochem Physiol A Mol Integr Physiol* (2018) 223:23–32. doi: 10.1016/j.cbpa.2018.05.008

16. Spicer OS, Zmora N, Wong TT, Golan M, Levavi-Sivan B, Gothilf Y, et al. The gonadotropin-inhibitory hormone (Lpxrfa) system's regulation of reproduction in the brain-pituitary axis of the zebrafish (Danio rerio). *Biol Reprod* (2017) 96:1031–42. doi: 10.1093/biolre/iox032

17. Akazome Y, Yamamoto E, Oka Y. (2015). Ligand dose-dependent switch in G-protein coupling (Gi and gs) of medaka (Oryzias latipes) neuropeptide FF receptors, NPFFR1 (GPR147) and NPFFR2 (GPR74). *Endocrine Society's 97th Annual Meeting and Expo March 7*, San Diego, California. Available at: https://endo.confex.com/endo/2015endo/webprogram/Paper20838.html.

18. Clarke IJ, Sari IP, Qi Y, Smith JT, Parkington HC, Ubuka T, et al. Potent action of RFamide-related peptide-3 on pituitary gonadotropes indicative of a hypophysiotropic role in the negative regulation of gonadotropin secretion. *Endocrinology* (2008) 149:5811–21. doi: 10.1210/en.2008-0575

19. Sari IP, Rao A, Smith JT, Tilbrook AJ, Clarke IJ. Effect of RF-amide-related peptide-3 on luteinizing hormone and follicle-stimulating hormone synthesis and secretion in ovine pituitary gonadotropes. *Endocrinology* (2009) 150:5549–56. doi: 10.1210/en.2009-0775

20. Son YL, Ubuka T, Millar RP, Kanasaki H, Tsutsui K. Gonadotropin-inhibitory hormone inhibits GnRH-induced gonadotropin subunit gene transcriptions by inhibiting AC/cAMP/PKA-dependent ERK pathway in LbetaT2 cells. *Endocrinology* (2012) 153:2332–43. doi: 10.1210/en.2011-1904

21. Paullada-Salmerón JA, Cowan M, Aliaga-Guerrero M, Gómez A, Zanuy S, Mañanós E, et al. LPXRFa peptide system in the European sea bass: A molecular and immunohistochemical approach. *J Comp Neurol* (2016) 524:176–98. doi: 10.1002/cne.23833

22. Paullada-Salmerón JA, Cowan M, Aliaga-Guerrero M, Morano F, Zanuy S, Muñoz-Cueto JA. Gonadotropin inhibitory hormone down-regulates the brain-pituitary reproductive axis of Male European Sea bass (Dicentrarchus labrax). *Biol Reprod* (2016) 94:121. doi: 10.1095/biolreprod.116.139022

23. Paullada-Salmerón JA, Cowan M, Aliaga-Guerrero M, López-Olmeda JF, Mañanós EL, Zanuy S, et al. Testicular steroidogenesis and locomotor activity are regulated by gonadotropin-inhibitory hormone in Male European Sea bass. *PloS One* (2016) 11:e0165494. doi: 10.1371/journal.pone.0165494

24. Espigares F, Zanuy S, Gómez A. Kiss2 as a regulator of lh and fsh secretion *via* Paracrine/Autocrine signaling in the teleost fish European Sea bass (Dicentrarchus labrax). *Biol Reprod* (2015) 93:114. doi: 10.1095/biolreprod.115.131029

25. Felip A, Espigares F, Zanuy S, Gómez A. Differential activation of kiss receptors by Kiss1 and Kiss2 peptides in the sea bass. *Reproduction* (2015) 150:227–43. doi: 10.1530/REP-15-0204

26. Felip A, Zanuy S, Pineda R, Pinilla L, Carrillo M, Tena-Sempere M, et al. Evidence for two distinct KiSS genes in non-placental vertebrates that encode kisspeptins with different gonadotropin-releasing activities in fish and mammals. *Mol Cell Endocrinol* (2009) 312:61–71. doi: 10.1016/j.mce.2008.11.017

27. Li Q, Wen H, Li Y, Zhang Z, Zhou Y, Qi X. Evidence for the direct effect of the NPFF peptide on the expression of feeding-related factors in spotted Sea bass (Lateolabrax maculatus). *Front Endocrinol (Lausanne)* (2019) 10:545. doi: 10.3389/fendo.2019.00545

28. Wang B, Qin C, Zhang C, Jia J, Sun C, Li W. Differential involvement of signaling pathways in the regulation of growth hormone release by somatostatin and growth hormone-releasing hormone in orange-spotted grouper (Epinephelus coioides). *Mol Cell Endocrinol* (2014) 382:851–9. doi: 10.1016/j.mce.2013.10.025

29. Wang B, Yang G, Liu Q, Qin J, Xu Y, Li W, et al. Inhibitory action of tongue sole LPXRFa, the piscine ortholog of gonadotropin-inhibitory hormone, on the signaling pathway induced by tongue sole kisspeptin in COS-7 cells transfected with their cognate receptors. *Peptides* (2017) 95:62–7. doi: 10.1016/j.peptides.2017.07.014

30. Zhang C, Sun C, Wang B, Yan P, Wu A, Yang G, et al. Orexin-a stimulates the expression of GLUT4 in a glucose dependent manner in the liver of orange-

spotted grouper (Epinephelus coioides). *Comp Biochem Physiol A Mol Integr Physiol* (2016) 199:95–104. doi: 10.1016/j.cbpa.2016.05.027

31. Chen J, Huang S, Zhang J, Li J, Wang Y. Characterization of the neuropeptide FF (NPFF) gene in chickens: evidence for a single bioactive NPAF peptide encoded by the NPFF gene in birds. *Domest Anim Endocrinol* (2020) 72:106435. doi: 10.1016/j.domaniend.2020.106435

32. Ubuka T, Son YL, Tsutsui K. Molecular, cellular, morphological, physiological and behavioral aspects of gonadotropin-inhibitory hormone. *Gen Comp Endocrinol* (2016) 227:27–50. doi: 10.1016/j.ygcen.2015.09.009

33. Paullada-Salmerón JA, Cowan ME, Loentgen GH, Aliaga-Guerrero M, Zanuy S, Mañanós EL, et al. The gonadotropin-inhibitory hormone system of fish: The case of sea bass (Dicentrarchus labrax). *Gen Comp Endocrinol* (2019) 279:184–95. doi: 10.1016/j.ygcen.2019.03.015

34. Chang JP, Mar A, Wlasichuk M, Wong AO. Kisspeptin-1 directly stimulates LH and GH secretion from goldfish pituitary cells in a Ca2+-dependent manner. *Gen Comp Endocrinol* (2012) 179:38–46. doi: 10.1016/j.ygcen.2012.07.028

35. Song Y, Chen J, Tao B, Luo D, Zhu Z, Hu W. Kisspeptin2 regulates hormone expression in female zebrafish (Danio rerio) pituitary. *Mol Cell Endocrinol* (2020) 513:110858. doi: 10.1016/j.mce.2020.110858

36. Gouarderes C, Mazarguil H, Mollereau C, Chartrel N, Leprince J, Vaudry H, et al. Functional differences between NPFF1 and NPFF2 receptor coupling: high intrinsic activities of RFamide-related peptides on stimulation of [35S] GTPgammaS binding. *Neuropharmacology* (2007) 52:376–86. doi: 10.1016/j.neuropharm.2006.07.034

37. Ogawa S, Sivalingam M, Biran J, Golan M, Anthonysamy R, Levavi-Sivan B, et al. Distribution of LPXRFa, a gonadotropin-inhibitory hormone (GnIH) ortholog peptide and LPXRFa receptor in the brain and pituitary of the tilapia. *J Comp Neurol* (2016) 524:2753–75. doi: 10.1002/cne.23990

38. Zhang Y, Li S, Liu Y, Lu D, Chen H, Huang X, et al. Structural diversity of the GnIH/GnIH receptor system in teleost: its involvement in early development and the negative control of LH release. *Peptides* (2010) 31:1034–43. doi: 10.1016/j.peptides.2010.03.003

39. Escobar S, Servili A, Espigares F, Gueguen MM, Brocal I, Felip A, et al. Expression of kisspeptins and kiss receptors suggests a large range of functions for kisspeptin systems in the brain of the European sea bass. *PloS One* (2013) 8:e70177. doi: 10.1371/journal.pone.0070177

40. Li S, Zhang Y, Liu Y, Huang X, Huang W, Lu D, et al. Structural and functional multiplicity of the kisspeptin/GPR54 system in goldfish (Carassius auratus). *J Endocrinol* (2009) 201:407–18. doi: 10.1677/JOE-09-0016

41. Martinez-Chavez CC, Minghetti M, Migaud H. GPR54 and rGnRH I gene expression during the onset of puberty in Nile tilapia. *Gen Comp Endocrinol* (2008) 156:224–33. doi: 10.1016/j.ygcen.2008.01.019

42. Ogawa S, Sivalingam M, Anthonysamy R, Parhar IS. Distribution of Kiss2 receptor in the brain and its localization in neuroendocrine cells in the zebrafish. *Cell Tissue Res* (2020) 379:349–72. doi: 10.1007/s00441-019-03089-5

frontiers | Frontiers in Endocrinology

TYPE Original Research
PUBLISHED 11 October 2022
DOI 10.3389/fendo.2022.982297

Check for updates

OPEN ACCESS

EDITED BY
Bin Wang,
Yellow Sea Fisheries Research Institute (CAFS), China

REVIEWED BY
Gustavo M. Somoza,
Instituto Tecnológico de Chascomús (CONICET-UNSAM), Argentina
María Paula Di Yorio,
Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET), Argentina

*CORRESPONDENCE
Zhan Yin
zyin@ihb.ac.cn
Guangfu Hu
huguangfu@mail.hzau.edu.cn

†These authors have contributed equally to this work

SPECIALTY SECTION
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 30 June 2022
ACCEPTED 29 August 2022
PUBLISHED 11 October 2022

CITATION
Li W, Du R, Xia C, Zhang H, Xie Y, Gao X, Ouyang Y, Yin Z and Hu G (2022) Novel pituitary actions of GnRH in teleost: The link between reproduction and feeding regulation. *Front. Endocrinol.* 13:982297. doi: 10.3389/fendo.2022.982297

COPYRIGHT
© 2022 Li, Du, Xia, Zhang, Xie, Gao, Ouyang, Yin and Hu. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Novel pituitary actions of GnRH in teleost: The link between reproduction and feeding regulation

Wei Li[1†], Ruixin Du[1†], Chuanhui Xia[1†], Huiying Zhang[1], Yunyi Xie[1], Xiaowen Gao[1], Yu Ouyang[1], Zhan Yin[2*] and Guangfu Hu[1*]

[1]Hubei Province Engineering Laboratory for Pond Aquaculture, College of Fisheries, Huazhong Agricultural University, Wuhan, China, [2]State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan, China

Gonadotropin-releasing hormone (GnRH), as a vital hypothalamic neuropeptide, was a key regulator for pituitary luteinizing hormone (LH) and follicle-stimulating hormone (FSH) in the vertebrate. However, little is known about the other pituitary actions of GnRH in teleost. In the present study, two GnRH variants (namely, GnRH2 and GnRH3) and four GnRH receptors (namely, GnRHR1, GnRHR2, GnRHR3, and GnRHR4) had been isolated from grass carp. Tissue distribution displayed that GnRHR4 was more highly detected in the pituitary than the other three GnRHRs. Interestingly, ligand–receptor selectivity showed that GnRHR4 displayed a similar and high binding affinity for grass carp GnRH2 and GnRH3. Using primary culture grass carp pituitary cells as model, we found that both GnRH2 and GnRH3 could not only significantly induce pituitary reproductive hormone gene (GtHα, LHβ, FSHβ, INHBa, secretogranin-2) mRNA expression mediated by AC/PKA, PLC/IP_3/PKC, and Ca^{2+}/CaM/CaMK-II pathways but also reduce dopamine receptor 2 (DRD2) mRNA expression *via* the Ca^{2+}/CaM/CaMK-II pathway. Interestingly, GnRH2 and GnRH3 could also stimulate anorexigenic peptide (POMCb, CART2, UTS1, NMBa, and NMBb) mRNA expression *via* AC/PKA, PLC/IP_3/PKC, and Ca^{2+}/CaM/CaMK-II pathways in grass carp pituitary cells. In addition, food intake could significantly induce brain GnRH2 mRNA expression. These results indicated that GnRH should be the coupling factor to integrate the feeding metabolism and reproduction in teleost.

KEYWORDS

GnRH, reproduction, feeding, grass carp, pituitary

Abbreviations: GnRH, gonadotropin-releasing hormone; LH, luteinizing hormone; FSH, follicle stimulating hormone; GnRHR, gonadotropin-releasing hormone receptor; GtHα, gonadotropic hormone alpha; INHBA, inhibin-βA; SgII, secretogranin II; POMCb, proopiomelanocortin B; CART2, cocaine and amphetamine-regulated transcript II; UTS1, urotensin 1; NMB, neuromedin-B; LEPR, leptin receptor; AC, adenylate cyclase; PKA, protein kinase A; PLC, phospholipase C; IP3, inositol trisphosphate PKC, protein kinase C; CaM, calmodulin; CaMK, calmodulin kinase; DRD2, dopamine D2 receptor; NFAT, nuclear factor of activated T cells; ORF, open reading frame; RT-PCR, real-time PCR; TMD, transmembrane domain.

Introduction

As an extremely important hypothalamus neuroendocrine peptide, gonadotropin releasing hormone 1 (GnRH1) was initially isolated from the mammalian hypothalamus (1, 2). Subsequently, GnRH2 had been firstly found in chicken so that this variant was also named as chicken GnRH (cGnRH) (3). In addition, the third GnRH variant (GnRH3) was an ubiquitous and unique subtype existing in fish (3), which was primitively discovered in salmon so that it was named as salmon GnRH (sGnRH) (4). Multiple GnRH variants (two or three forms) are present in all teleosts (5), but the receptor selectivity and functions of different GnRH variants are still unclear in teleost. Similar to mammals, GnRH could also participate in the reproductive regulation in the teleost, including spawning activity (6) and oocyte development (7). A recent study further found that GnRH3 could regulate primordial germ cell (PGC) proliferation and sex differentiation in zebrafish (8). In addition, GnRH2 knockout zebrafish females display decreased oocyte quality (9). In teleost, several studies reported that the two GnRH variants (namely, GnRH2 and GnRH3) could stimulate luteinizing hormone (LH) and follicle-stimulating hormone (FSH) secretion in the pituitary (5, 10). However, besides LH and FSH, could GnRH regulate other reproductive genes in the pituitary?

As we know, energy metabolism was associated with reproductive behavior in vertebrates (11), and adequate energy reserve was essential for breeding (12). Previous studies suggested that several hypothalamus neuropeptides could regulate both reproduction and feeding in teleost (13). Our recent study also found that neurokinin B (NKB) could regulate not only reproduction but also feeding in grass carp (14). In addition, prolactin-releasing peptide (PRRP), a typical anorexigenic peptide in hypothalamus, could significantly induce LH secretion and synthesis in grass carp pituitary (15). Similarly, GnRH, the typical reproductive peptide in hypothalamus, could also regulate food intake in mammals (16) and teleosts (17). Recent studies further confirmed that knockout of gnrh2 in zebrafish could increase food intake (9). However, little is known about the regulatory mechanism of GnRH in feeding regulation.

In this study, grass carp were used as a model to examine the pituitary actions of GnRH in reproduction and feeding. Firstly, two GnRHs and four GnRH receptors (GnRHRs) were isolated from grass carp, and the tissue distribution of these genes was examined by using specific primers. Then, ligand–receptor selectivity was performed by the established pGL3-nuclear factor of activated T cell (NFAT)-RE-luciferase reporters in HEK293-T cells. Besides, using primary culture grass carp pituitary cells as a model, direct pituitary actions of GnRH3 were examined by RNA-seq technique. Afterward, we further confirmed that GnRHs could significantly regulate five pituitary reproductive hormone genes (GtHα, LHβ, FSHβ, INHBa, secretogranin-2) and five anorexigenic peptides (POMCb, CART2, UTS1, NMBa, and NMBb) in grass carp pituitary cells. Finally, we further examined the signal pathways of GnRH-regulated reproductive and feeding genes in the pituitary. Our findings demonstrated the functional roles of GnRH in the regulation of reproduction and feeding in the teleost.

Materials and methods

Animals and chemicals

In the present study, 2-year-old grass carp (*Ctenopharyngodon idellus*) with a body weight of 1.5–2.5 kg were acquired from local markets and maintained in 250-l aquaria under a 12-h light, 12-h dark photoperiod at 20°C. Because sexual dimorphism was not apparent in these fish, grass carps of mixed sexes were used for pituitary cell preparation according to the protocol approved by the committee for animal use at Huazhong Agricultural University. Grass carp GnRH2 (QHWSHGWYPG-NH_2), GnRH3 (QHWSYGWLPG-NH_2), and human GnRH1 (QHWSYGLRPG-NH_2) were synthesized by GenScript (Piscataway, NJ) and dissolved in double-distilled water at 1 mM which were sub-packaged and stored at lower than -80°C. The full-length open reading frame (ORF) of grass carp GnRH receptors (GnRHR1, GnRHR2, GnRHR3, GnRHR4) were cloned and then inserted into pcDNA3.1(+) vector (Invitrogen) used for transfection, while human GnRHR (GenBank No: L07949.1) was synthesized by BT Lab (Wuhan, China). All the signal pathway inhibitors, such as H89, MDL12330A, U73122, GF109203X, 2-APB, nifedipine, KN62, and calmidazolium (CMZ), were purchased from Calbiochem (San Diego, CA) (for details, please refer to Supplementary Table S1) and dissolved using dimethyl sulfoxide (DMSO) at a concentration of 10 mM. Once being used in *in vitro* tests, these drugs were diluted to working concentration by testing medium beforehand.

Molecular cloning and tissue distribution of grass carp Gnrhs and Gnrhrs

Total RNA was extracted from grass carp pituitary and hypothalamus and reverse transcribed into cDNA with Hifair™ III 1st Strand cDNA Synthesis Kit (Yeasen, Shanghai, China). The full-length ORF regions of grass carp GnRHR1, GnRHR2, GnRHR3, and GnRHR4 were cloned using specific primers designed based on grass carp genomes, respectively (for the conditions of primers, please refer to Supplementary Table S2). The sequence alignment based on the corresponding cDNA or mature peptide sequences which were reported in other species was conducted with BioEdit 7.2, and phylogenetic analysis of target sequences was conducted with MEGA7.0 and ClustalX 2.1

using the neighbor-joining method. The three-dimensional protein models of grass carp GnRH2, GnRH3, GnRHR1, GnRHR2, GnRHR3, and GnRHR4 were predicted and constructed by using SWISS-MODEL and I-TASSER based on the deduced amino acid sequence. For tissue distribution analysis, the total RNA of various brain subregions and several selected peripheral tissues were isolated and reverse transcribed to cDNA to detect the transcript level using primers specific for gene targets by real-time PCR (RT-PCR), respectively (for the conditions of primers, please refer to Supplementary Table S3). In these studies, RT-PCR for β-actin was performed as an internal control.

Transfection and luciferase reporter assay

According to our previous study, the pGL3-NFAT-RE-luciferase reporter system was used to verify the ligand–receptor selectivity of the newly cloned GnRHRs in HEK-293T cells (18). Briefly, the ORFs of grass carp GnRHR1, GnRHR2, GnRHR3, GnRHR4, and hGnRHR were isolated and subcloned into eukaryotic expression vector pcDNA3.1(+) to generate corresponding expression vectors. For transient transfection experiments, HEK-293T cell lines were seeded at a density of 0.05×10^6 cells/0.5 ml/well in 24-well plates. After overnight incubation for recovery, transfection was carried out in 400 μf OPTI-MEM for 6 h with 200 ng NFAT-Luc reporter or CRE-Luc reporter, 10 ng pTK-RL, 20 ng pEGFP-N1, 10 ng pcDNA3.1(+)-GnRHR, and 0.99 μl Lipofectamine 3000 (Thermo Fisher). pTK-RL (the Renilla luciferase-expressing reporter) and GFP-expressing vector pEGFP-N1 were both used as the internal control. Parallel transfection with the blank vector pcDNA3.1(+) without GnRHR insert was used as the negative control. After transfection, the cells were allowed to incubate for 18–24 h at 37°C in Dulbecco's modified Eagle medium (DMEM) supplemented without fetal bovine serum (FBS, Gibco) before drug treatment. Based on our validation, the duration of drug treatment has been optimized for 24 h for luciferin expression. After a 24-h drug treatment, the cells were washed with ice-cold PBS and dissolved in passive lysis buffer (Yeasen, Shanghai, China). The prepared cellular lysate was then used for the measurement of firefly luciferase activities using Luciferase Assay Reagent (Yeasen, Shanghai, China) by a dual luciferase reporter system. Furthermore, transfection experiments were performed in quadruplicate with cells cultured in separate wells.

RNA-seq and bioinformatics

The grass carp pituitaries were obtained and dispersed by the trypsin/DNase II/EDTA digestion method (19). Grass carp pituitary cells were seeded in 24-well plates and initially cultured in a plating medium at the density of 2.5×10^6 cells/0.8 ml per well under the condition of 28°C with 5% CO_2. After adding 5% FBS to each well and incubating for 18 h, GnRH3 (final concentration of 1 μM) was used to incubate the pituitary cells for another 24 h. Then, total RNA was extracted from each well by TRIzol reagent (Yeasen) and DNase II was used to eliminate the interference of genomic DNA. The concentration and purity of each RNA sample were detected by a NanoDrop 2000 spectrophotometer, while the quality of RNA was identified on an Agilent 2100 Bioanalyzer using the RNA 6000 Nano Kit (Agilent Technologies, Santa Clara, CA, USA). After that, the RNA (RIN >8.0) including the control group and GnRH-treated group (both three replicates) were sent to Majorbio Genome Center (Shanghai, China) for subsequent library preparation and sequencing on HiSeq 4000 (Illumina). In this study, a read depth of 0.6 billion 150-bp single-end reads was used and about 90% of reads were mapped to the genome. Gene expression levels were assigned individual values by being normalized to the number of transcripts per kilobase of exon model per million (TPM). The fold changes (FC) were calculated using RSEM software v 1.2.7 (20), and different gene expressions (DEGs) were analyzed by using the R Bioconductor package. The P-value indicated the credibility of each differential gene expression and was corrected by the false discovery rate (FDR) (21). We set up conditions of TPM >5, FDR <0.05, and FC >1.5 to select satisfactory DEGs. Finally, both Gene Ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) enrichment analyses were performed using Goatools software (22).

Quantitative real-time PCR in pituitary cells

The preparation of grass carp pituitary cells was performed as mentioned above. After drug treatment, the total RNA of these cells was isolated by TRIzol reagent and reverse transcribed into cDNA with Hifair™ III 1st Strand cDNA Synthesis Kit. The transcript levels of several anorectic peptides (POMCb, CART2, UTS1, NMBa, NMBb, and Lepr) and reproductive genes (GtHα, LHβ, FSHβ, INHBa, SgII, DRD2) were detected by using an ABI 7500 quantitative real-time PCR (qRT-PCR) system (Biosystems, USA) (information of primers for target genes is listed in Supplementary Table S3). In this process, a serial gradient dilution of plasmid DNA of these genes was used as a standard for data calibration. The conditions of qRT-PCR were set to 10 min, 95°C, for pre-degeneration; 15 s, 95°C, for degeneration; 30 s, 55°C–60°C, for annealing; 30 s, 72°C, for extending; and 20 s, 82°C, for signal detection with 40 cycles. Finally, the melt curve obtained through each test was used to verify and check the reliability and specificity of the corresponding qRT-PCR.

Postprandial changes in GnRH expression

To further confirm the potential functional role of GnRH on feeding, we detected the GnRH2 and GnRH3 mRNA expression in grass carp brain after feeding. Grass carp were temporarily raised in a well-aerated 250-l tank and fed one meal per day for at least 7 days at fixed times (9:00 a.m.). The grass carp were divided into equal portions as feed group and unfed group (as a control group). On the experiment day, the food supply point (9:00 a.m.) was considered as 0 h. Therefore, the brains were harvested at 0, 1, 3, and 6 h after food administration from these two groups, respectively. Then, the total RNA was extracted by TRIzol method and transcribed into cDNA to detect the GnRH2 and GnRH3 mRNA expression by the qRT-PCR system.

Data transformation and statistical analysis

The transcript level was detected using qRT-PCR by ABI 7500 software, while the data calibration of each reaction was performed through standard curves with the dynamic range of 10^5 and correlation coefficient >0.95. The transcript level of β-actin was used as an internal control, and target gene mRNA expression was normalized and calculated as a percentage of the mean value (as "% Ctrl"). Based on merging four to eight replicates (as mean ± SEM), the data were analyzed with a one-way ANOVA to differentiate the significant differences from other treatment experiments. The SPSS Statistics 26.0 software was used to do a Dunnett's *post-hoc* test. Finally, $p < 0.05$ ("*") or $p < 0.01$ ("**") was used to present significant differences among each group. The different letters represent a significant difference at $p < 0.05$ between groups.

Results

Molecular cloning and sequence analysis of GnRHs and GnRHRs in grass carp

In grass carp, the full lengths of GnRH2 and GnRH3 were cloned by using specific primers. According to sequence alignment, we found that the ORF of GnRH2 contained 261 bp in size as well as an encoded 86-amino acid protein precursor, while GnRH3 possessed 285 bp in size and an encoded 94-amino acid protein precursor (Supplementary Figure S1). Besides, GnRH2 and GnRH3 encoded one 10-aa mature peptide (QHWSHGWYPG-NH2 and QHWSYGWLPG-NH2, respectively), which were both with the common motif (PG-NH2) in the C terminus (Figure 1A). At the protein level, we compared the mature peptides of GnRH in different species; the results indicated that grass carp GnRH2 showed 100% identity to all the contrastive counterparts including zebrafish, goldfish, medaka, and chicken. Similar to GnRH2, grass carp GnRH3 revealed 100% identity to the counterparts in teleost (Figure 1A). The three-dimensional protein structures for human GnRH1, grass carp GnRH2, and grass carp GnRH3 were predicted by using I-TASSER (Figure 1A). The phylogenetic analysis revealed that the two different subtypes of GnRHs were clustered into separate branches, and GnRH2 showed a closer relationship to GnRH1 compared with GnRH3 which was a unique isoform for teleost (Figure 1B). GnRHR1, GnRHR2, GnRHR3, and GnRHR4 had been cloned from grass carp pituitary, which encoded 381, 414, 373, and 406 aa proteins, respectively. The amino acid sequence of the four receptors, as members of the GPCR group, could be structured into seven transmembrane domains (TMD 1 to 7) with three extracellular loops and three intracellular loops, together with an endocellular C-terminal and an extracellular N-terminal tail (Supplementary Figures S2-5). Similarly, phylogenetic analysis showed that grass carp GnRHR1 and GnRHR3 were clustered in the same brand, which was close to mammalian GnRHR. Besides, GnRHR2 and GnRHR4 were clustered into a distinct brand (Supplementary Figure S6).

The tissue distribution showed that GnRH2 was mainly distributed in the brain, heart, and gonad, whereas GnRH3 primarily existed in the brain and liver (Figure 1C). At the brain level, GnRH2 was highly detected in the olfactory bulb, olfactory tract, and optic tectum. However, high transcript levels of GnRH3 were detected in the olfactory bulb, olfactory tract, hypothalamus, and pituitary (Figure 1C). The four GnRHRs were mainly detected in the brain and gonad (Figure 1C). In various brain subregions, the transcript signals of all the four receptors were detected in the telencephalon, optic tectum, and hypothalamus (Figure 1C).

Ligand–receptor selectivity of GnRHs for GnRHRs in HEK-293T cells

It had been reported that the GnRH-induced rapid increase in intracellular calcium was essential for gonadotropin secretion (17). Therefore, a pGL3-NFAT-RE-luciferase reporter system, which could monitor the changes in intracellular calcium concentration, was used in the present study. As shown in Figure 2A for grass carp GnRHR, the GnRH variants (GnRH1, GnRH2, and GnRH3) were all effective in stimulating luciferase activity expression in a dose-dependent manner *via* the Ca^{2+} pathway. GnRH3 (EC_{50}: 3.343 nM) was found to be the most effective in activating GnRHR1 compared to GnRH1 (EC_{50}: 923 nM) and GnRH2 (EC_{50}: 104.4 nM). In addition, GnRH2 (EC_{50}: 0.61 nM) showed higher potency for GnRHR3 than GnRH1 (EC_{50}: 2283 nM) and GnRH3 (EC_{50}: 205.2 nM) (Figure 2A). Interestingly, GnRH1 (EC_{50}: 267.4 nM), GnRH2 (EC_{50}: 2.2 nM), and GnRH3 (EC_{50}: 9.9 nM) all displayed high potency for GnRHR4, which suggested that GnRHR4 acted as a universal receptor for GnRHs in grass carp (Figure 2A). Furthermore, human GnRHR had been used to examine the potency for

10.3389/fendo.2022.982297

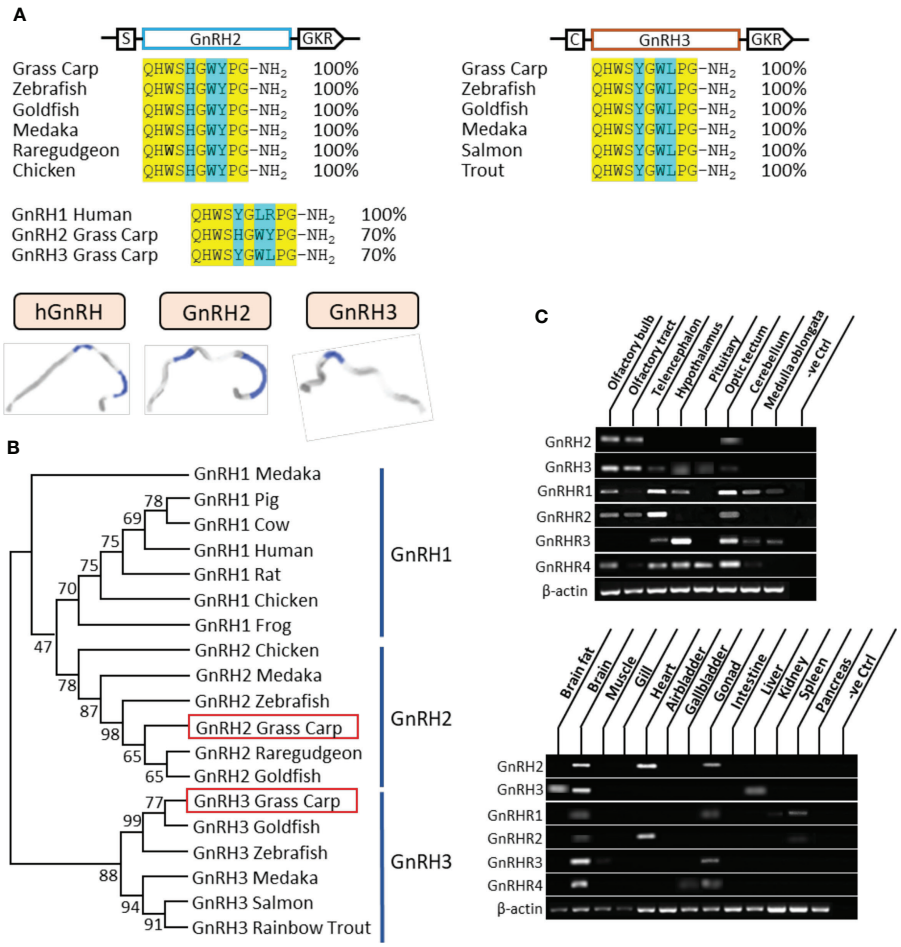


FIGURE 1
Sequence analysis and tissue distribution of grass carp GnRHs/GnRHRs. **(A)** The mature peptide sequence alignment of GnRHs. The conserved amino acid sequences are processed into a yellow background, whereas the different amino acid residues compared with human GnRH1, grass carp GnRH2, and GnRH3 are marked in blue background. **(B)** Phylogenetic analysis of GnRHs from mammal or non-mammal vertebrates are generated with the neighbor-joining method (MEGA 6.0); grass carp GnRH2 and GnRH3 are highlighted into the red frame. **(C)** Tissue distribution of GnRHs and GnRHRs was detected in grass carp peripheral tissues (on the bottom) and various brain subregions (on the top). Total RNA was extracted, reverse transcribed, and underwent RT-PCR using specific primers; the results have been intercepted and spliced according to corresponding PCR product size. Besides, the transcript level of β-actin was considered as an internal control.

GnRHs. The result revealed that human GnRH1 could highly activate hGnRHR with EC EC_{50}: 14.2 nM for NFAT-Luc and 10 nM for CRE-Luc, respectively. In addition, grass carp GnRH2 (EC_{50}: 994.9 nM for NFAT-Luc and 54 nM for CRE-Luc) and GnRH3 (EC_{50}: 54 nM for NFAT-Luc and 24.7 nM for CRE-Luc) also had a high activating potency for hGnRHR *via* the Ca^{2+} and PKA pathway (Figure 2B).

Transcriptomic analysis of the pituitary actions of GnRH3 in grass carp

In the present study, using grass carp pituitary cells as model, high-throughput RNA-seq was used to examine the pituitary actions of GnRH3 in teleost. According to the transcripts per kilobase of exon model per million mapped (TPM) reads method, a total of 820 different expression genes (DEGs) were screened under the condition of TPM >5, FDR <0.05. Subsequently, 245 upregulated genes (FC >1.5) and 575 downregulated genes (FC <0.7) were filtrated and used to perform GO analysis. The results showed that these DEGs were divided into three main ontologies, namely, cellular component, biological process, and molecular function (Figure 3A). The most abundant GO terms among the cellular component category were 'intracellular part', 'membrane-bounded organelle', 'intracellular membrane-bounded organelle', 'organelle part', and 'intracellular organelle part'. Furthermore, the plentiful groups of biological processes were

10.3389/fendo.2022.982297

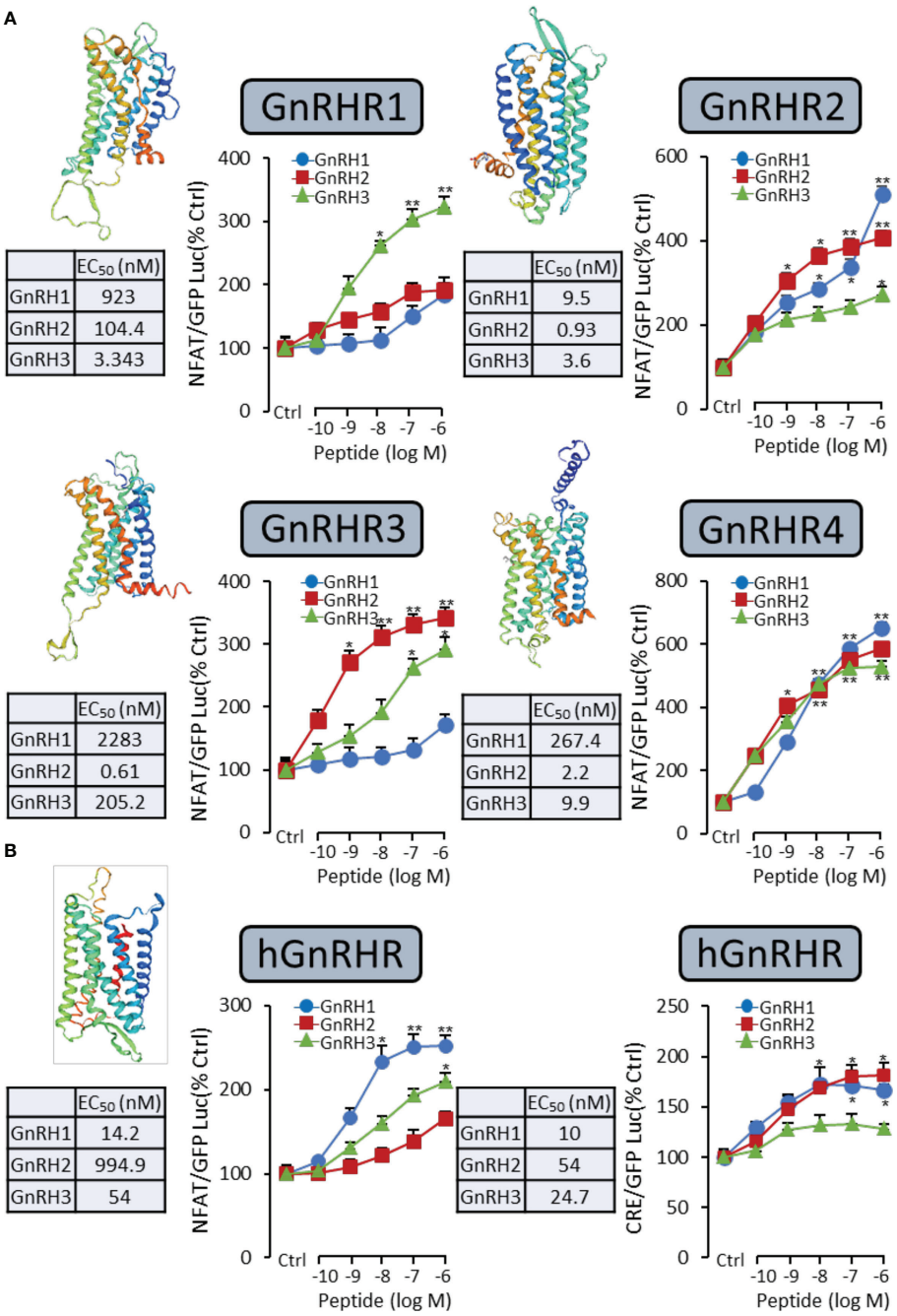


FIGURE 2
Functional analysis of grass carp GnRHRs and human GnRHR in HEK-293T cells. To examine the ligand–receptor selectivity of the GnRHs for GnRHRs, the NFAT-Luc or CRE-Luc reporter system was used in HEK-293T cells which were treated with various concentrations of GnRHs for 24 h, to detect the luciferase activity of GnRHs for grass carp GnRHR1, GnRHR2, GnRHR3, GnRHR4 **(A)**, and hGnRHR **(B)**. Each point was determined in quadruplicate, and data presented were expressed as mean ± SEM. $p < 0.05$ ("*") or $p < 0.01$ ("**") was used to present significant differences among each group.

'metabolic process', 'organic substance metabolic process', 'primary metabolic process', 'cellular metabolic process', and 'biological regulation (Figure 3A). In addition, the GO enrichment analysis of molecular function was divided into two main contents, namely, 41 pivotal upregulated DEGs (Table 1) and 39 pivotal downregulated DEGs (Table 2). The Kyoto Encyclopedia of Genes and Genomes (KEGG) analysis showed that a total of 162 DEGs were enriched in the top 10

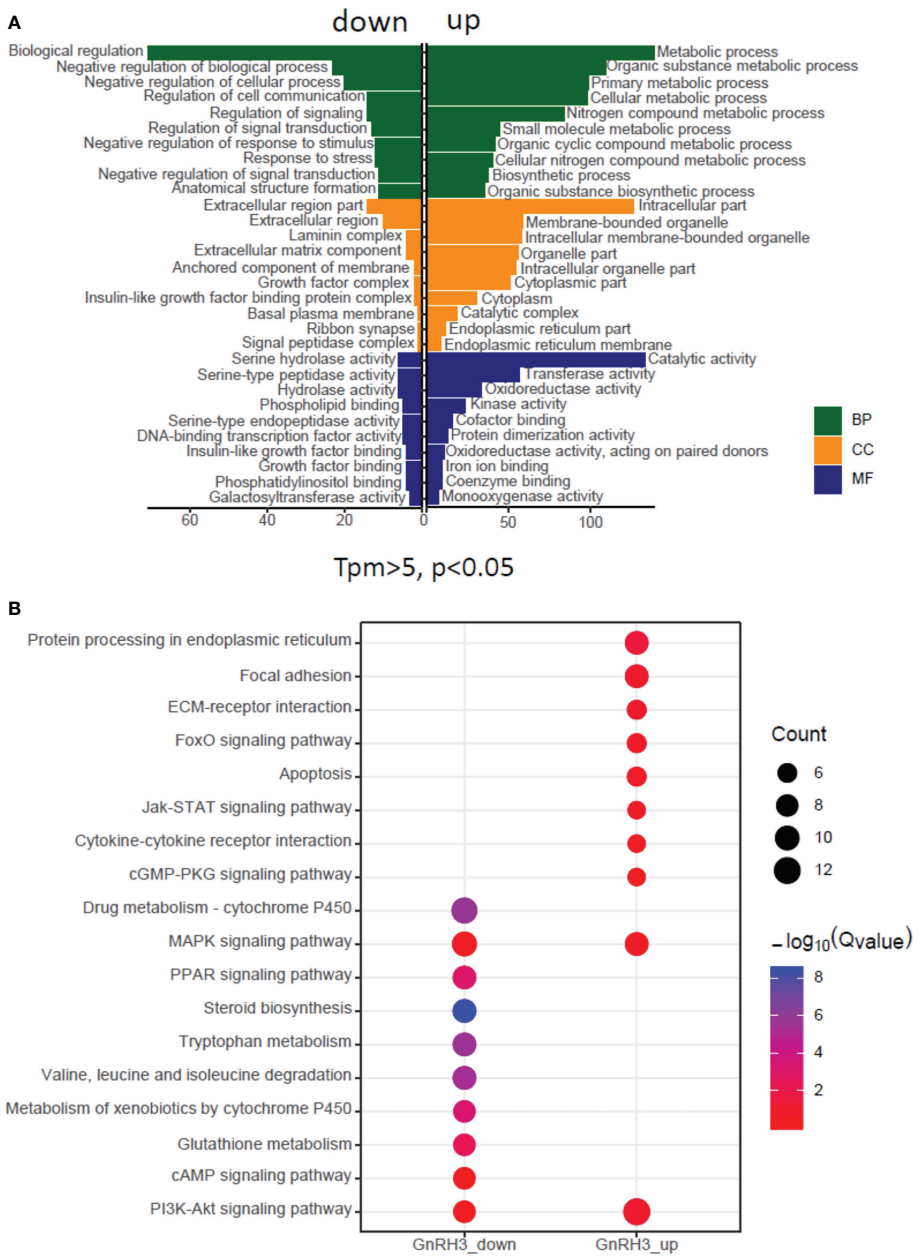


FIGURE 3
Gene Ontology (GO) analysis and Kyoto Encyclopedia of Genes and Genomes **(KEGG)** analysis based on transcriptome. **(A)** GO analysis revealed that the selected DEGs which satisfied the conditions of TPM >5, FDR <0.05, FC >1.5, or FC <0.7 were made up of three parts, namely, cellular component (CC), biological process (BP), and molecular function (MF). **(B)** One hundred sixty-two DEGs were classified into the top 10 enriched pathways using KEGG analysis which included upregulation and downregulation DEGs. Qvalue, Recalibration of P value. Count, the number of DEGs.

pathways. Among them, the upregulated DEGs were mostly enriched in 'PI3K–Akt signaling pathway' and 'Focal adhesion' and the downregulated DEGs were mainly enriched in 'Drug metabolism-cytochrome P450', 'MAPK signaling pathway', and 'PPAR signaling pathway' (Figure 3B). Finally, several key DEGs were selected to display the regulation of GnRH3 in signal transduction, feeding regulation, hormone activity, and metabolic process (Figure 4). In feeding regulation, several key genes such as proopiomelanocortin B (POMCb), cocaine and amphetamine-regulated transcript 2 (CART2), urotensin 1 (UTS1), neuromedin-B a (NMBa), neuromedin-B b (NMBb), and leptin receptor (LEPR) were selected and used as target genes on the detection of primary pituitary cells. In addition, several reproductive hormone genes, such as LHβ, FSHβ,

TABLE 1 Upregulated genes by GnRH3 in grass carp pituitary cells.

Gene	FC	FDR	Description	Molecular Function
tph1	17.70	0	Tryptophan 5-hydroxylase 1	Amino acid binding
bip	1.78	5.39E-31	GRP78	ATP binding
pim1	3.25	0	threonine-protein kinase pim-1	ATP binding
plk2	5.78	0	Serine/threonine-protein kinase PLK2	ATP binding
mcfd2	1.65	3.57E-236	Multiple coagulation factor deficiency protein	Calcium ion binding
nucb1	1.56	1.72E-149	Nucleobindin-1 precursor	Calcium ion binding
anx11	3.17	0	Annexin 11a	Calcium ion binding
nfasc	10.71	5.61E-52	Neurofascin	Cell–cell adhesion mediator activity
npr3	11.53	0	Atrial natriuretic peptide receptor 3 isoform X1	Chloride ion binding
e2.7.3.2	3.88	0	Brain creatine kinase b	Creatine kinase activity
ctsl	2.55	0	Cathepsin La isoform X1	Cysteine-type peptidase activity
scg2	1.51	0	Secretogranin II precursor	Cytokine activity
lepr	2.60	6.73E-61	Leptin receptor long form	Cytokine binding
grp	1.52	1.46E-176	Gastrin-releasing peptide-like	Enzyme binding
fth1	1.90	0	Ferritin heavy chain	Ferric iron binding
gpr61	7.06	2.17E-78	Probable G-protein coupled receptor-like	G-protein-coupled receptor activity
manf	1.71	6.58E-124	Astrocyte-derived neurotrophic factor	Growth factor activity
rergl	6.29	2.35E-26	Estrogen-regulated inhibitor-like protein	GTP binding
nmb	3.29	4.39E-07	Neuromedin-B-like	Hormone activity
crh	1.74	6.72E-06	Urotensin 1	Hormone activity
tsg6	5.12	1.99E-06	Tumor necrosis factor-inducible gene 6 protein	Hyaluronic acid binding
il10ra	27.19	0.009871	Interleukin-10 receptor 1	Interleukin-10 binding
tt39b	6.76	6.74E-06	Tetratricopeptide repeat protein 39B-like	Lipid metabolic process
agrb1	8.87	0	Brain-specific angiogenesis inhibitor 1-like	Lipopolysaccharide binding
zbtb8	1.69	1.08E-73	Zinc finger domain-containing protein 8A	Metal ion binding
ctro	5.24	3.29E-164	Citron Rho-interacting kinase	Metal ion binding
sdf2l	1.55	1.30E-57	Stromal cell-derived factor 2-like 1	Misfolded protein binding
cart2	19.16	1.73E-22	Amphetamine-regulated transcript II precursor	Neuropeptide hormone activity
ggh	1.51	1.06E-186	Gamma-glutamyl hydrolase	Omega peptidase activity
pdyn	5.14	7.38E-17	Proenkephalin-B-like isoform X1	Opioid receptor binding
spcs3	1.77	2.74E-239	Signal peptidase complex subunit 3	Peptidase activity
fkbp11	1.94	0	Peptidyl-prolyl cis-trans isomerase	cis-trans isomerase activity
r4rl2	93.68	6.85E-299	Reticulon 4 receptor-like 2b precursor	Protein binding
bha15	1.66	0	Class A basic helix-loop-helix protein 15	Protein dimerization activity
junb	1.89	2.36E-96	Transcription factor jun-B	Sequence-specific DNA binding
nec1	1.54	0	Neuroendocrine convertase 1	Serine-type endopeptidase activity
hpt	5.16	4.16E-131	Haptoglobin-like	Serine-type endopeptidase activity
sec11	1.55	1.55E-131	Signal peptidase complex catalytic subunit	Serine-type peptidase activity
nab1	5.88	3.65E-131	NGFI-A-binding protein 1	Transcription factor binding
rdl	1.57	3.51E-111	Thiosulfate sulfurtransferase	Transferase activity
sqstm1	1.69	0	Sequestosome-1 isoform X1	Zinc ion binding
fsh	1.35	6.77E-14	Follicle stimulating hormone	Hormone activity
lhβ	1.32	0	Luteinizing hormone	Hormone activity
gthα	1.28	0	Glycoprotein hormones alpha chain	Hormone activity
inhba	1.90	1.08E-11	Inhibin-beta A	Growth factor activity
SgII	1.31	2.73E-241	Secretogranin II	Calcium ion binding
prl	1.22	0	Prolactin	Hormone activity
cckar	1.41	0.40	Cholecystokinin receptor-like isoform	G protein-coupled receptor activity

(Continued)

TABLE 1 Continued

Gene	FC	FDR	Description	Molecular Function
creb2	1.56	1.69E-07	Activating transcription factor 4b	cAMP response element binding protein binding
smad7	1.78	7.44E-31	Mothers against decapentaplegic homolog 7	Beta-catenin binding

FC, fold change; FDR, false discovery rate.

TABLE 2 Downregulated genes by GnRH3 in grass carp pituitary cells.

Gene	FC	FDR	Description	Molecular function
arpc4	0.66	1.86E-42	Actin-related protein 2/3 subunit 4-like	Actin binding
nlrp3	0.26	0.000497	PYD domains-containing protein 3-like	ATP binding
chd4	0.64	3.30E-172	Chromodomain-DNA-binding protein 4	Binding
calm	0.68	2.37E-221	Calmodulin	Calcium ion binding
marcksl1b	0.67	2.63E-101	Myristoylated alanine-rich C substrate 2	Calmodulin binding
hint1	0.66	7.80E-16	Histidine triad nucleotide-binding protein 1	Catalytic activity
dgkz	0.41	0.003213	Diacylglycerol kinase zeta-like isoform X1	Diacylglycerol kinase activity
ppp1r10	0.42	2.43E-39	Threonine-protein phosphatase 1 regulatory	DNA binding
hmgb1	0.69	7.95E-46	High-mobility group box 1	DNA binding
rfx1	0.28	0	MHC class II regulatory factor RFX1	DNA-binding factor activity
fabp7	0.50	5.12E-107	Fatty acid binding protein 7	Fatty acid binding
gst	0.67	8.59E-06	Glutathione S-transferase	Glutathione transferase activity
gmpr1	0.61	2.64E-126	GMP reductase 1	GMP reductase activity
rhoa	0.32	0.042946	Transforming protein rhoa	GTP binding
mx1	0.29	0.004203	Antiviral effector Mx3	GTPase activity
h4	0.68	0.002615	Histone H4	Histone demethylase activity
gphb5	0.69	2.13E-64	Glycoprotein hormone beta-5	Hormone activity
atp5me	0.69	5.40E-07	ATP synthase subunit e	Hydrolase activity
erg7	0.43	1.00E-62	Lanosterol synthase	Intramolecular activity
mdh1	0.68	4.31E-77	Cytosolic malate dehydrogenase	L-malate dehydrogenase activity
m3k20	0.31	0.00526	Mitogen-activated protein kinase kinase 20	MAP kinase kinase activity
s100b	0.31	3.01E-240	Protein S100-B	Metal ion binding
cdc42bp	0.37	0.004184	Serine/threonine-protein kinase MRCK beta	Metal ion binding
zfp36l	0.66	1.92E-125	Zinc finger protein 36, C3H type-like 1b	Metal ion binding
ppp1r17	0.68	2.87E-10	Protein phosphatase 1 regulatory 17-like	Phosphatase inhibitor activity
pa2g3	0.29	6.47E-212	Group 3 secretory phospholipase A2-like	Phospholipase A2 activity
snf8	0.44	1.18E-06	Vacuolar-sorting protein SNF8-like	Protein binding
h2b3	0.53	4.58E-23	Histone H2B 3	Protein heterodimerization activity
h2a	0.63	1.56E-20	Histone H2A	Protein heterodimerization activity
mvp	0.64	2.14E-255	Major vault protein	Ribonucleoprotein
estd	0.37	4.80E-36	S-formylglutathione hydrolase	S-Formylglutathione activity
sstr3	0.42	3.09E-126	Somatostatin receptor type 3	Somatostatin receptor activity
tubb	0.40	3.79E-49	Tubulin beta-4B chain	Structural constituent of cytoskeleton
suhb	0.44	2.86E-22	3-Beta-hydroxysteroid sulfotransferase	Sulfotransferase activity
sod1	0.69	1.09E-31	Copper/zinc superoxide dismutase	Superoxide dismutase activity
nltp	0.41	2.86E-12	Non-specific lipid-transfer protein	Transferase activity
fabp3	0.63	1.56E-177	Fatty-acid binding protein 3b	Transporter activity
tpisb	0.68	8.61E-53	Triosephosphate isomerase B	Triose-phosphate isomerase activity
vdac3	0.69	5.87E-44	Voltage-dependent anion-selective protein 3	Voltage-gated anion channel activity
drd2	0.35	2.63E-37	Dopamine D2 receptor	G-protein coupled receptor activity

(Continued)

10.3389/fendo.2022.982297

TABLE 2 Continued

Gene	FC	FDR	Description	Molecular function
pomca	0.90	0	Pro-opiomelanocortin I	Neuropeptide signaling pathway
zak	0.31	0.00526009	Mitogen-activated protein kinase kinase 20	Catalytic Activity
npy2r	0.44	0.07342027	Europeptide Y receptor type 2-like	Peptide YY receptor activity
mshr1	0.43	0	Melanocyte-stimulating hormone receptor	Hormone receptor activity
stat5a	0.89	0.04174356	Stat5.2 protein	DNA-binding factor activity
stat4	0.86	9.86E-21	Stat 4-like	Binding to a chemokine receptor

FC, fold change; FDR, false discovery rate.

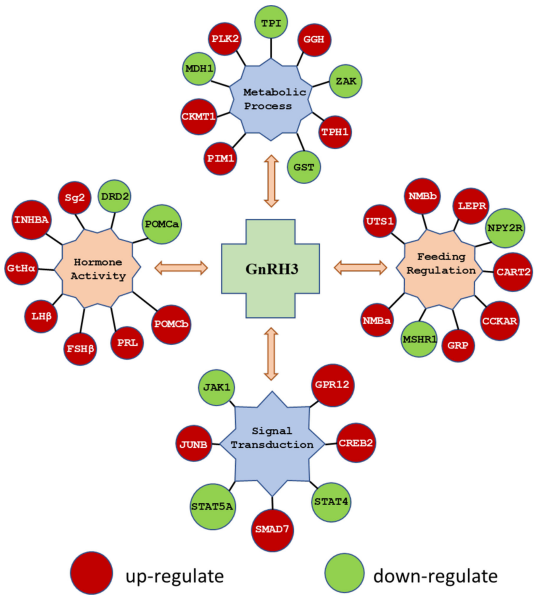


FIGURE 4
The key DEGs were selected to display the regulation of GnRH3 in signal transduction, feeding regulation, hormone activity, and metabolic process. The DEGs in red bubbles indicated the upregulated genes, and the DEGs in green bubbles indicated the downregulated genes by GnRH3 in grass carp pituitary cells.

glycoprotein hormones alpha (GtHα), inhibin-beta A (INHBA), secretogranin II, and dopamine D2 receptor (DRD2), were all regulated by GnRH3 and SgII (Figure 4).

Regulation of reproductive hormones genes by different GnRH variants in grass carp pituitary cells

To further verify the reproductive function of GnRHs, time- and dose-dependent tests were performed to detect GnRH2- or GnRH3-regulated GtHα, LHβ, FSHβ, INHBa, SgII, and DRD2 mRNA expression in grass carp pituitary cells. The time-course test showed that both GnRH2 (1 μM) and GnRH3 (1 μM) could significantly induce pituitary GtHα, LHβ, FSHβ, INHBa, and SgII mRNA expression and inhibit pituitary DRD2 mRNA expression in a time-dependent manner (Figure 5A). In the dose-dependent test, a continuous gradient dilution of GnRH2 (0.1–1,000 nM) or GnRH3 (0.1–1,000 nM) was incubated with grass carp pituitary cells for 24 h. The results revealed that both GnRH2 and GnRH3 could significantly induce GtHα, LHβ, FSHβ, INHBa, and SgII mRNA expression in a dose-dependent manner (Figure 5B). In addition, all treated doses of GnRH2 and GnRH3 could significantly inhibit DRD2 mRNA expression (Figure 5B).

10.3389/fendo.2022.982297

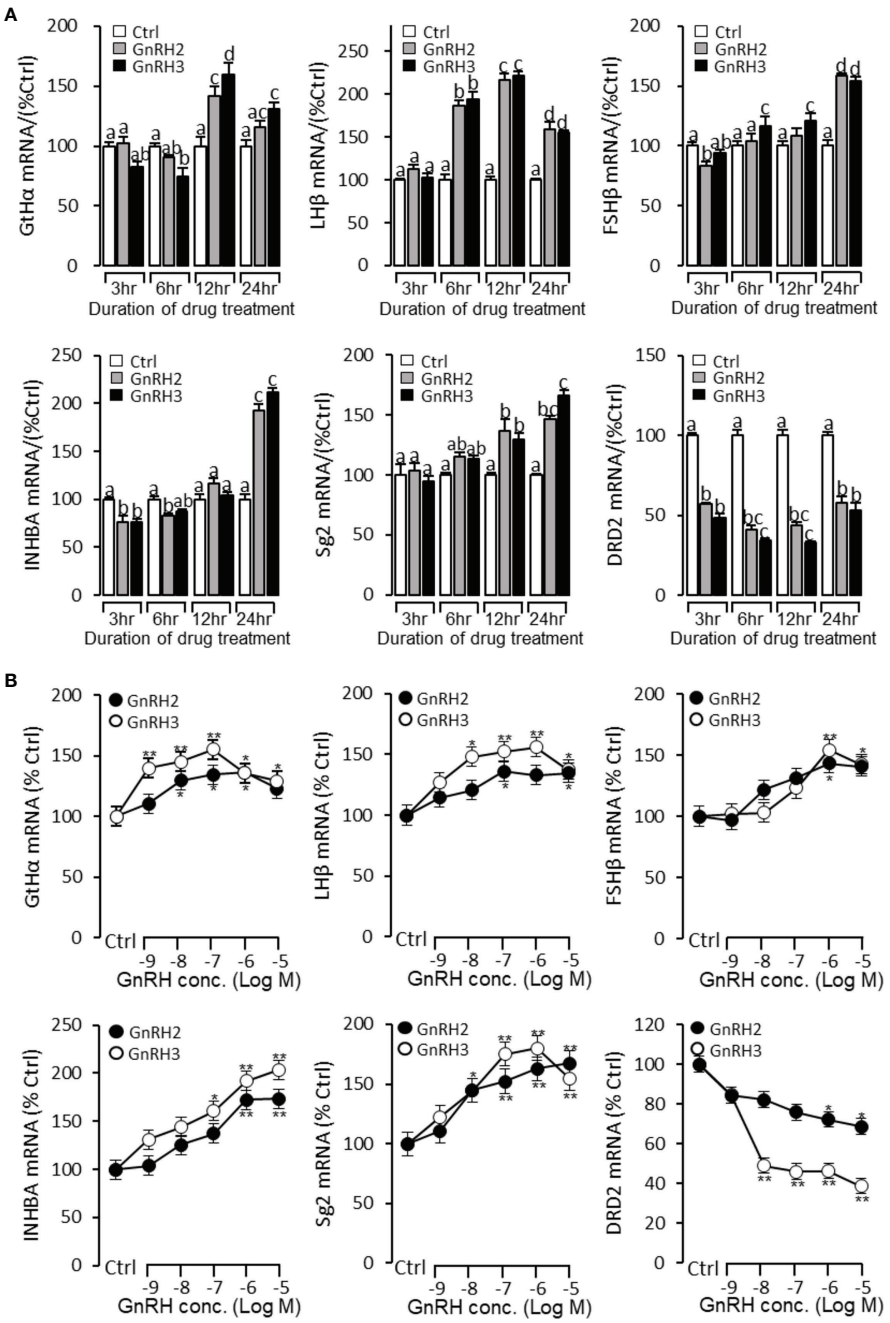


FIGURE 5
Regulation of reproductive hormone gene mRNA expression by GnRH2 and GnRH3 in grass carp pituitary cells. **(A)** Time-course experiment of grass carp GnRH2 (1 μM) and GnRH3 (1 μM) on GtHα, LHβ, FSHβ, INHBa, SgII, and DRD2 mRNA expression in grass carp pituitary cells. **(B)** Dose dependence of a 24-h treatment with increasing levels of GnRH2 and GnRH3 (0.1–1,000 nM) on GtHα, LHβ, FSHβ, INHBa, SgII, and DRD2 mRNA expression in grass carp pituitary cells. After drug treatment, the pituitary cells were extracted to total RNA, reversed transcribed, and used for RT-PCR to detect the target genes' mRNA expression. Data presented are expressed as mean ± SEM. $p < 0.05$ ("*") or $p < 0.01$ ("**") was used to present significant differences among each group. The different letters represent a significant difference at $p < 0.05$ between groups (ANOVA followed by a Dunnett test).

Signal transduction for GnRH-regulated reproductive hormone gene mRNA expression in grass carp pituitary cells

To explore the signal transduction for GnRH-regulated reproductive hormone gene mRNA expression, the inhibitors of AC/PKA, PLC/PKC, and IP_3/Ca^{2+} signal pathways were cotreated with GnRH2 or GnRH3 in grass carp pituitary cells. The results revealed that PLC inhibitor U73122 (10 μM), PKC inhibitor GF109203X (20 μM), and IP3 receptor blocker 2-APB (100 μM) could block GnRH2- (Figure 6A) or GnRH3- (Figure 6B) induced GtHα, LHβ, FSHβ, INHBa, and SgII mRNA expression in grass carp pituitary cells, respectively. In addition, the stimulatory effects of GnRH2 (Figure 7A) or GnRH3 (Figure 7B) on GtHα, LHβ, FSHβ, INHBa, and SgII mRNA expression could also be blocked by AC inhibitor MDL12330A (20 μM) or PKA inhibitor H89 (20 μM) in grass carp pituitary cells. Furthermore, the VSCC inhibitor nifedipine (10 μM), CaM antagonist calmidazolium (1 μM), or CaMk-II blocker KN62 (5 μM) could also block GnRH2- (Figure 8A) or

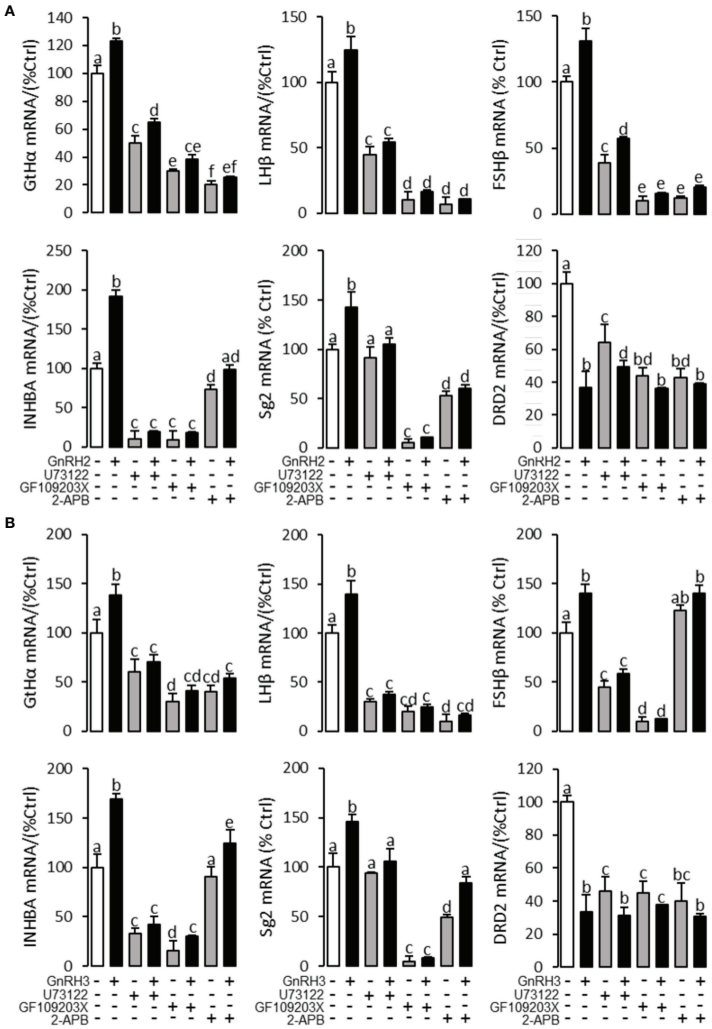


FIGURE 6
Signal transduction for GnRH2- and GnRH3-regulated GtHα, LHβ, FSHβ, INHBa, SgII, and DRD2 mRNA expression in grass carp pituitary cells. In this experiment, GnRH2 **(A)** or GnRH3 **(B)** combined with/without the PLC inhibitor U73122 (10 μM), PKC inhibitor GF109203X (20 μM), or IP3 receptor blocker 2-APB (100 μM) was used to incubate the grass carp pituitary cells for 24 (h) After drug treatment, the total RNA was extracted from the cells for real-time PCR of the respective genes. Data presented are expressed as mean ± SEM, and the different letters represent a significant difference at $p < 0.05$ between groups (ANOVA followed by a Dunnett test).

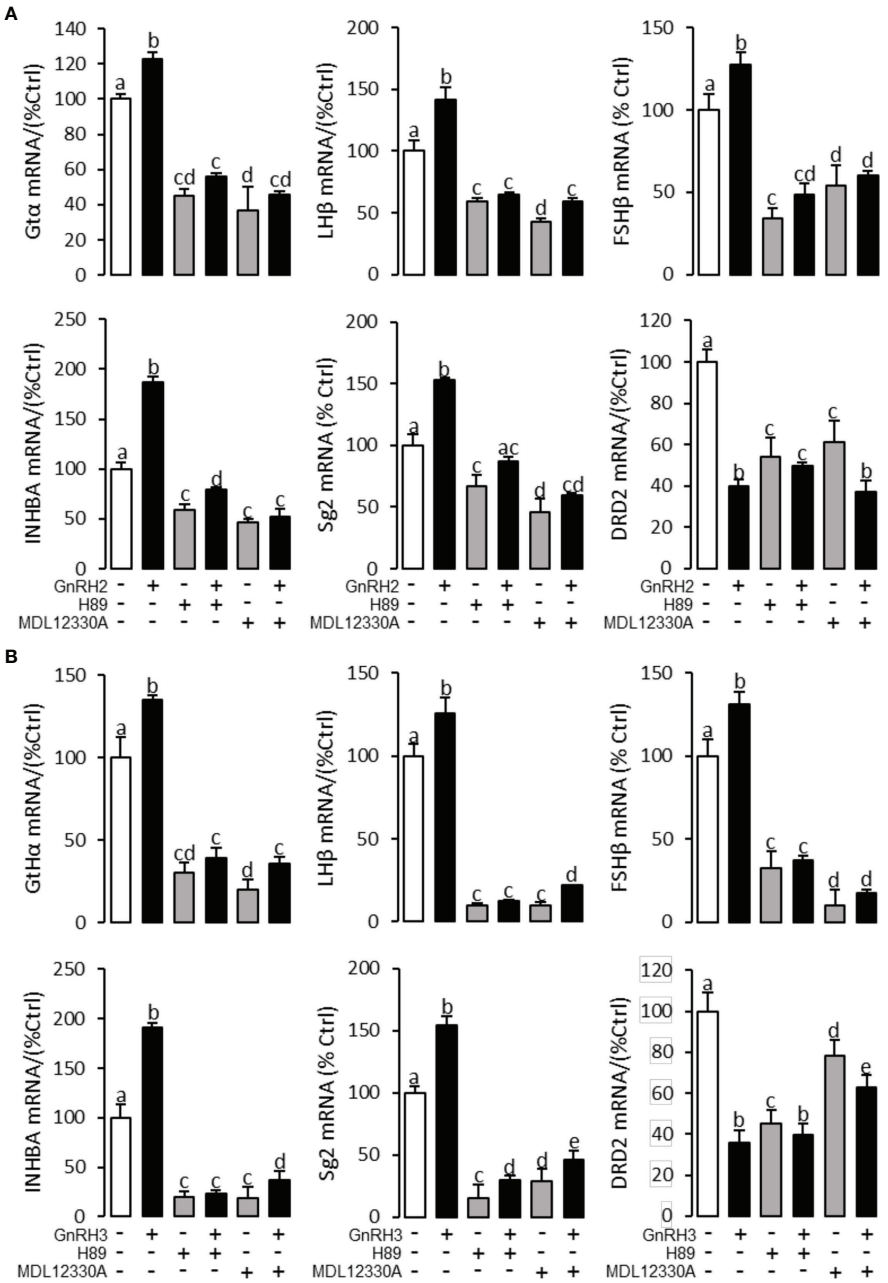


FIGURE 7
Signal transduction for GnRH2- and GnRH3-regulated GtHα, LHβ, FSHβ, INHBa, SgII, and DRD2 mRNA expression in grass carp pituitary cells. In this experiment, GnRH2 **(A)** or GnRH3 **(B)** combined with/without the AC inhibitor MDL12330A (20 μM) or PKA inhibitor H89 (20 μM) was used to incubate the grass carp pituitary cells for 24 (h) After drug treatment, the total RNA was extracted from the cells for real-time PCR of the respective genes. Data presented are expressed as mean ± SEM, and the different letters represent a significant difference at $p < 0.05$ between groups (ANOVA followed by a Dunnett test).

GnRH3-(Figure 8B) induced GtHα, LHβ, FSHβ, INHBa, and SgII mRNA expression. Interestingly, GnRH2- or GnRH3-reduced pituitary DRD2 mRNA expression could not be blocked by PLC/PKC inhibitors (Figure 6) or AC/PKA inhibitors (Figure 7). However, the inhibitors for the Ca^{2+}/CaM/CaM-II cascade could significantly block GnRH2-(Figure 8A) or GnRH3- (Figure 8B) reduced DRD2 mRNA expression in grass carp pituitary cells.

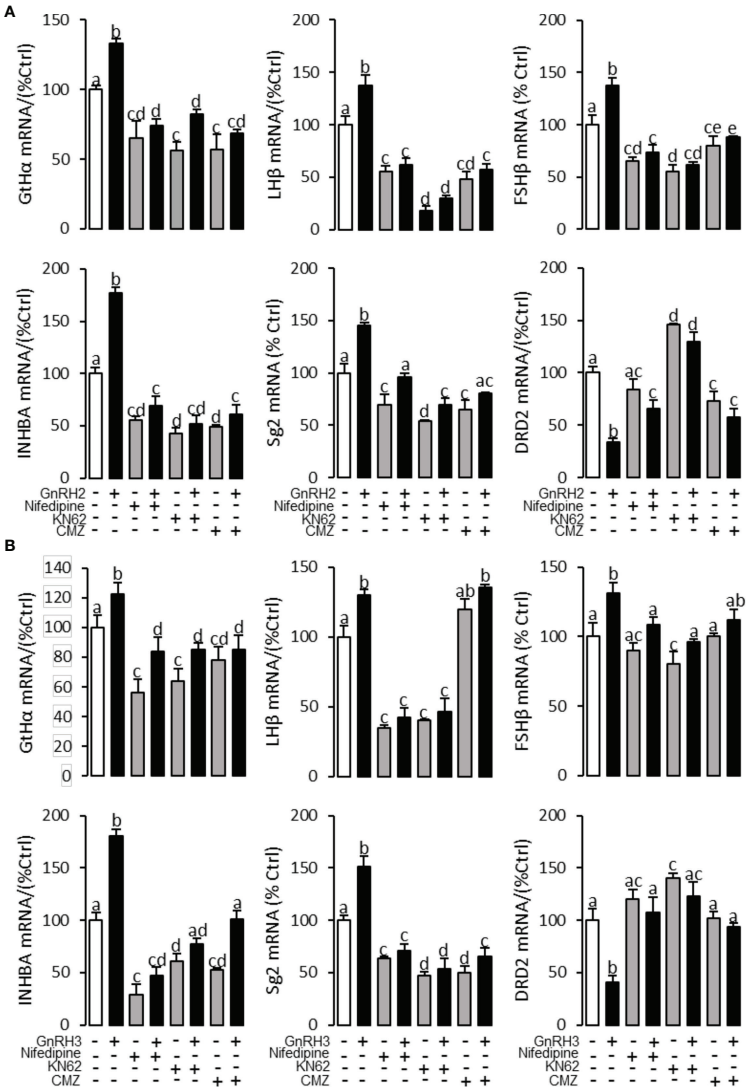


FIGURE 8
Signal transduction for GnRRH2- and GnRH3-induced GtHα, LHβ, FSHβ, INHBa, SgII, and DRD2 mRNA expression in grass carp pituitary cells. In this experiment, GnRH2 **(A)** or GnRH3 **(B)** combined with/without the VSCC inhibitor nifedipine (10 μM), CaM antagonist calmidazolium (1 μM), or CaMK-II blocker KN62 (5 μM) was used to incubate the grass carp pituitary cells for 24 (h) After drug treatment, the total RNA was extracted from the cells for real-time PCR of the respective genes. Data presented are expressed as mean ± SEM, and the different letters represent a significant difference at $p < 0.05$ between groups (ANOVA followed by a Dunnett test).

Regulation of six anorectic genes' (POMCb, CART2, UTS1, NMBa, NMBb, and LEPR) mRNA expression by GnRH2 and GnRH3 in grass carp pituitary cells

To further verify the functional role of GnRH2 and GnRH3 on feeding regulation, time- and dose-dependent tests were performed to detect the effect of GnRH2 and GnRH3 on anorectic gene (POMCb, CART2, UTS1, NMBa, NMBb, and LEPR) mRNA expression. In the time-course test, the grass carp pituitary cells were incubated by GnRH2 or GnRH3 (1 μM) for 3, 6, 12, and 24 h, respectively. The results showed that both GnRH2 and GnRH3 could significantly induce POMCb, CART2, UTS1, NMBa, NMBb, and LEPR mRNA expression in a time-dependent manner (Figure 9A). In the dose-dependent test, grass carp pituitary cells were incubated by the continuous gradient dilution of GnRH2 or GnRH3 (0.1–1,000 nM) for 24 h. The results revealed that both GnRH2 and GnRH3 could significantly induce POMCb, CART2, UTS1, NMBa, NMBb, and LEPR mRNA expression in a dose-dependent manner (Figure 9B).

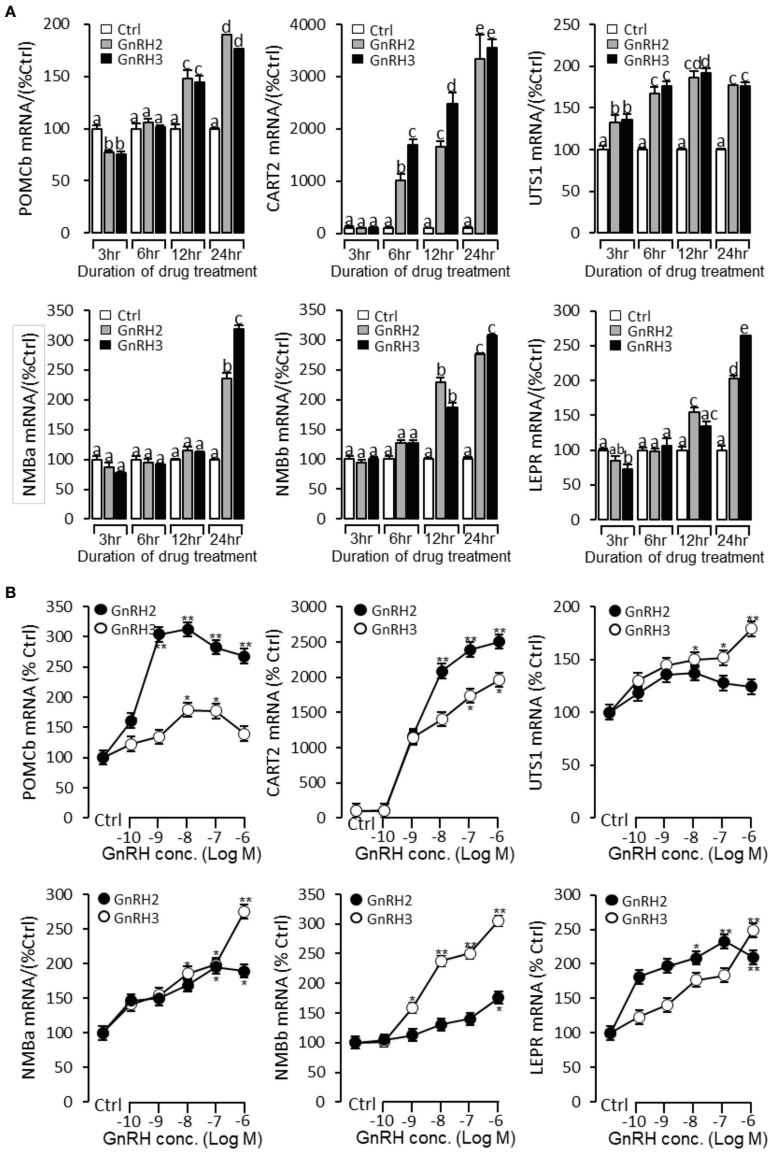


FIGURE 9
Regulation of feeding hormone gene mRNA expression by GnRH2 and GnRH3 in grass carp pituitary cells. **(A)** Time-course experiment of grass carp GnRH2 (1 μM) and GnRH3 (1 μM) on POMCb, CART2, UTS1, NMBa, NMBb, and LEPR mRNA expression in grass carp pituitary cells. **(B)** Dose dependence of a 24-h treatment with increasing levels of GnRH2 and GnRH3 (0.1–1,000 nM) on POMCb, CART2, UTS1, NMBa, NMBb, and LEPR mRNA expression in grass carp pituitary cells. After drug treatment, the pituitary cells were extracted to total RNA, reverse transcribed, and used for RT-PCR to detect the target genes' mRNA expression. Data presented are expressed as mean ± SEM. $p < 0.05$ ("*") or $p < 0.01$ ("**") was used to present significant differences among each group. The different letters represent a significant difference at $p < 0.05$ between groups (ANOVA followed by a Dunnett test).

Signal transduction for GnRH-induced POMCb, CART2, UTS1, NMBa, NMBb, and LEPR mRNA expression in grass carp pituitary cells

To explore the signal transduction for GnRH-induced target gene mRNA expression, the method mentioned above was performed. The results revealed that PLC inhibitor U73122 (10 μM), PKC inhibitor GF109203X (20 μM), IP3 receptor blocker 2-APB (100 μM) (Figure 10), AC inhibitor MDL12330A (20 μM), PKA inhibitor H89 (20 μM) (Figure 11), VSCC inhibitor nifedipine (10 μM), CaM antagonist calmidazolium (1 μM), and CaMk-II blocker KN62 (5 μM) (Figure 12) could block both GnRH2- or GnRH3-induced target gene mRNA expression in grass carp pituitary cells, respectively.

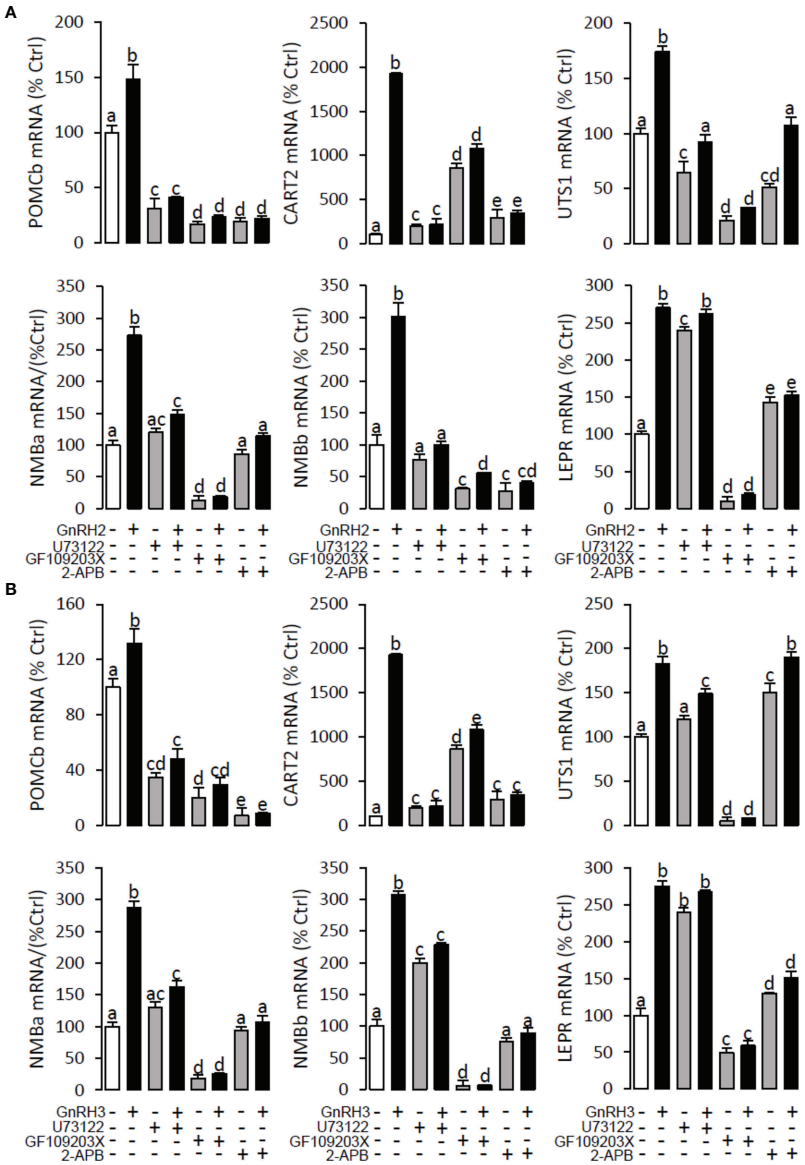


FIGURE 10
Signal transduction for GnRH2- and GnRH3-induced POMCb, CART2, UTS1, NMBa, NMBb, and LEPR mRNA expression in grass carp pituitary cells. In this experiment, GnRH2 **(A)** or GnRH3 **(B)** combined with/without the PLC inhibitor U73122 (10 μM), PKC inhibitor GF109203X (20 μM), or IP3 receptor blocker 2-APB (100 μM) was used to incubate the grass carp pituitary cells for 24 (h) After drug treatment, the total RNA was extracted from the cells for real-time PCR of the respective genes. Data presented are expressed as mean ± SEM, and the different letters represent a significant difference at $p < 0.05$ between groups (ANOVA followed by a Dunnett test).

Postprandial changes in brain GnRH2 and GnRH3 expression after food intake in grass carp

To further verify the potential functional role of GnRHs on feeding regulation, the expressions of GnRH2 and GnRH3 mRNA in grass carp brain were monitored after the meal. In the control group without feeding (unfed group), the transcript levels of GnRH2 and GnRH3 in the brain did not change from 1 to 6 h. In contrast, food intake could significantly induce brain GnRH2 and GnRH3 mRNA expression with a peak at 1 h (Figure 13).

Discussion

In mammal, only one or two GnRHR variants have been identified (23). However, five GnRHRs have been detected in teleosts, such as European seabass (24) and pufferfish (25).

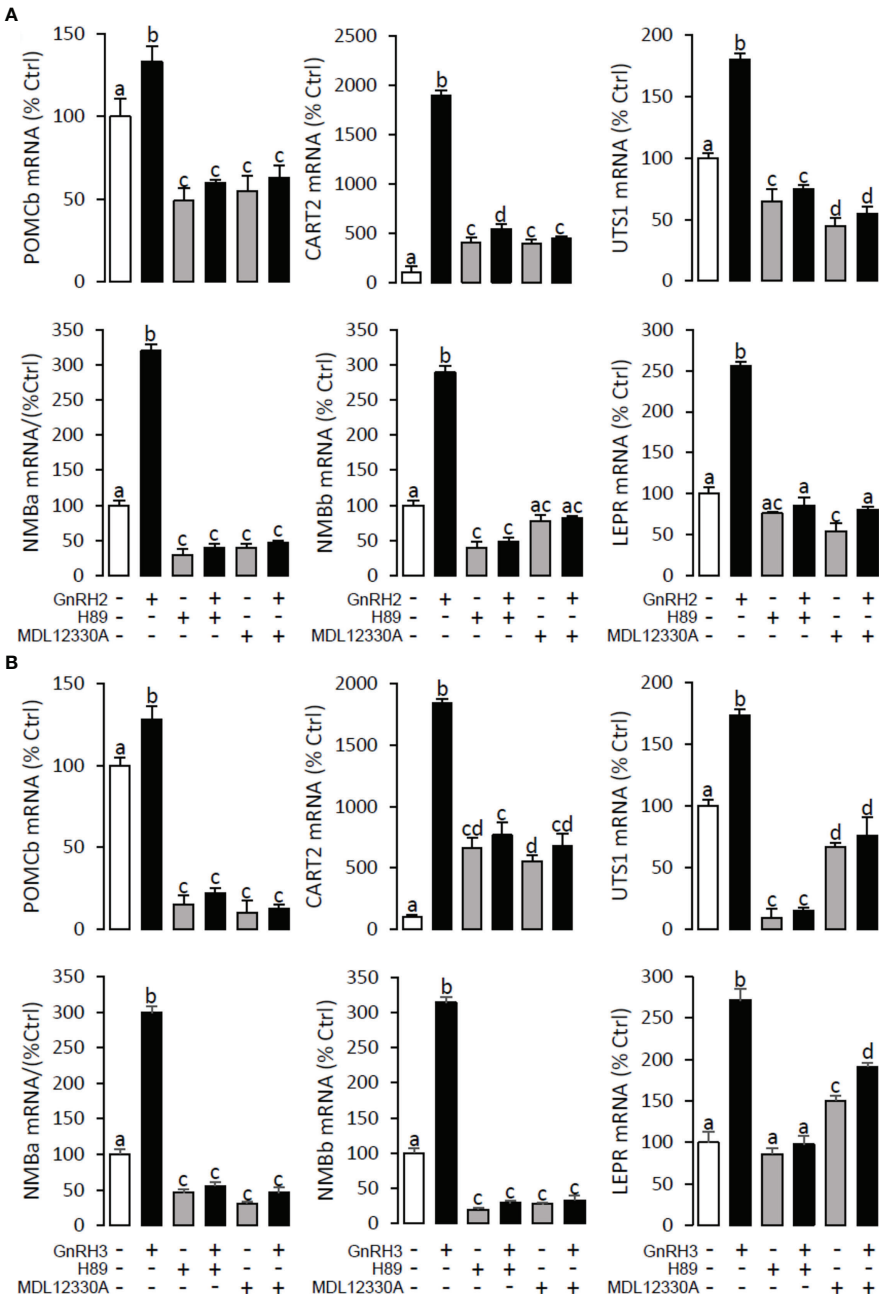


FIGURE 11
Signal transduction for GnRH2- and GnRH3-induced POMCb, CART2, UTS1, NMBa, NMBb, and LEPR mRNA expression in grass carp pituitary cells. In this experiment, GnRH2 **(A)** or GnRH3 **(B)** combined with/without the AC inhibitor MDL12330A (20 μM) or PKA inhibitor H89 (20 μM) was used to incubate the grass carp pituitary cells for 24 (h) After drug treatment, the total RNA was extracted from the cells for real-time PCR of the respective genes. Data presented are expressed as mean ± SEM, and the different letters represent a significant difference at $p < 0.05$ between groups (ANOVA followed by a Dunnett test).

Similar to zebrafish (26), four GnRH receptors (namely, GnRHR1, GnRHR2, GnRHR3, GnRHR4) have been cloned in grass carp. In the present study, by using transfection and luciferase assay, we found that individual subtypes of GnRHR exhibited differential selectivity for various members of GnRHs, with GnRHR3 preferring for GnRH2, and GnRHR1 preferring for GnRH3, respectively. Interestingly, grass carp GnRHR4 could display a rank order of GnRH1≈GnRH2≈GnRH3 for receptor activation, which suggested that GnRHR4 should be a multiligand receptor with promiscuity for three GnRHs. In grass carp, our previous

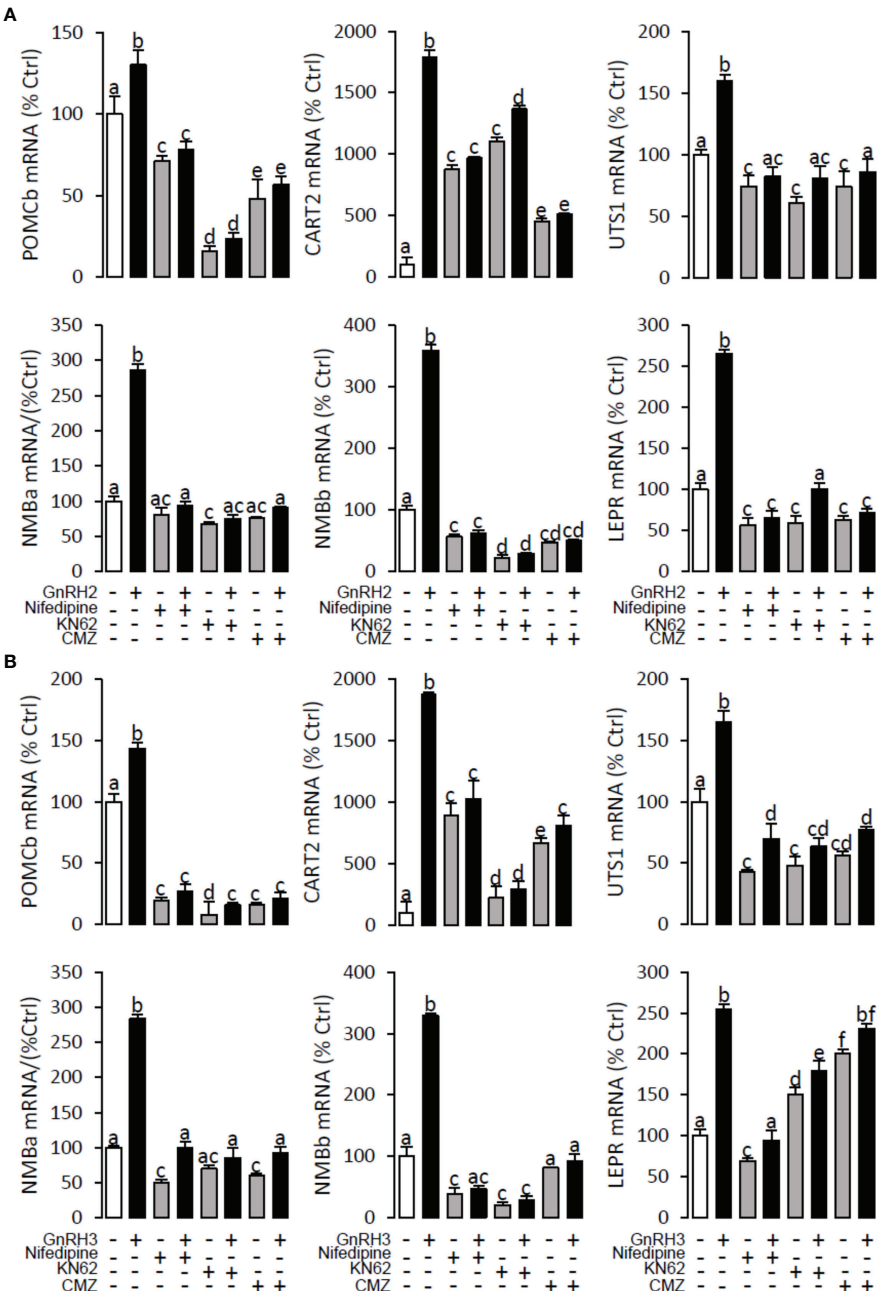


FIGURE 12
Signal transduction for GnRH2- and GnRH3-induced POMCb, CART2, UTS1, NMBa, NMBb, and LEPR mRNA expression in grass carp pituitary cells. In this experiment, GnRH2 **(A)** or GnRH3 **(B)** combined with/without the VSCC inhibitor nifedipine (10 μM), CaM antagonist calmidazolium (1 μM), or CaMK-II blocker KN62 (5 μM) were used to incubate the grass carp pituitary cells for 24 (h) After drug treatment, the total RNA was extracted from the cells for real-time PCR of the respective genes. Data presented are expressed as mean ± SEM, and the differences between groups were significant at $p < 0.05$ by labelling diverse letters (ANOVA followed by a Dunnett test).

study has found that NK2R was a multiligand receptor for various tachykinin peptides (15). In mammals, multiligand receptors with promiscuity for structurally related ligands (or even unrelated ligands) have been reported, for example, class A (27) and class B type I scavenger receptors (28), receptor for advanced glycation end product (29), and related protein for low-density lipoprotein receptor (30). The deviation from the "one ligand/one receptor" model for receptor activation is thought to have occurred during early evolution and allows for effective integration of extracellular signals mediated by ligands of the same family or even dissimilar

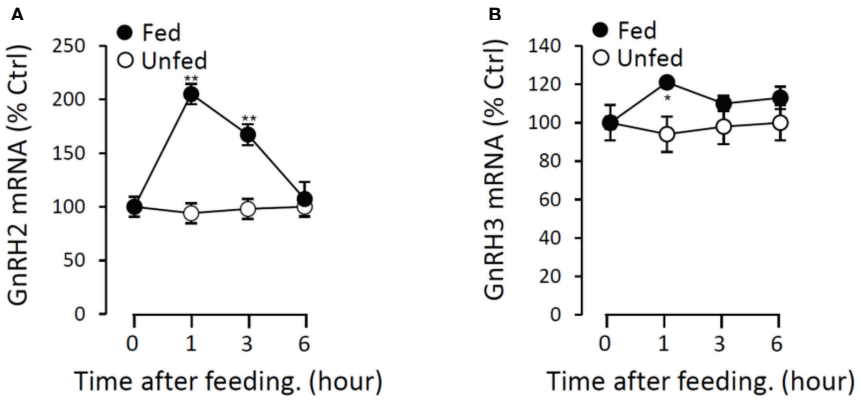


FIGURE 13
Postprandial changes of GnRH2 **(A)** and GnRH3 **(B)** mRNA expression in grass carp. Grass carp were temporarily raised in a well-aerated 250-l storage pond and fed one meal per day for at least 7 days on fixed times (9:00 a.m.). The brains were harvested at 0, 1, 3, and 6 h after food administration. Then, the total RNA were extracted to detect the GnRH mRNA level expression by the RT-PCR system. Data presented are expressed as mean ± SEM, and the differences between groups were significant at $p < 0.05$ by labeling symbol "*" ("**" means $p < 0.01$).

ligands with related functions (31). In addition, the ligand–receptor selectivity experiment also showed that grass carp GnRHRs could be activated by human GnRH1 with comparable efficacy and potency. This is the reason why hGnRH1 could be used in artificial reproduction of grass carp. In addition, we also found that human GnRHR could also be activated by grass carp GnRH2 and GnRH3, suggesting that the GnRH/GnRHR system was very conserved from teleost to mammal.

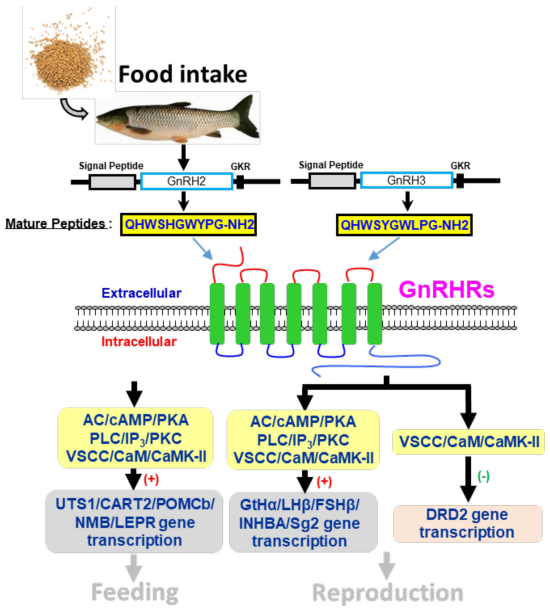


FIGURE 14
Working model of GnRH-mediated feeding and reproduction in grass carp. Two GnRH ligands and four GnRHRs were cloned from grass carp brain, and ligand–receptor selectivity showed that GnRHR3 was a specific receptor for GnRH2 when GnRH3 was preferentially chosen combined with GnRHR1, and GnRHR2 and GnRHR4 might be universal receptors to both GnRHs. Besides, GnRH2 and GnRH3 could both significantly induce pituitary reproductive hormone gene (GtHα, LHβ, FSHβ, INHBa, SgII) and feeding hormone gene (POMCb, CART2, UTS1, NMBa, NMBb, and LEPR) mRNA expression mediated by AC/PKA, PLC/IP3/PKC and Ca^{2+}/CaM/CaMK-II pathways *in vitro*. Besides, GnRH2 and GnRH3 could modulate the inhibition of LH by dopamine according to a restrained DRD2 expression *via* the Ca^{2+}/CaM/CaMK-II pathway. Finally, food intake could significantly induce brain GnRH2 and GnRH3 mRNA expression.

In the present study, although the transcript level of GnRH2 was not detected in immature grass carp pituitary by RT-PCR, GnRH2 could also significantly induce LHβ and FSHβ mRNA expression in grass carp pituitary cells. Similarly, recent studies in zebrafish, a two-GnRH model species exhibiting two GnRH variants into the brain (GnRH2 and GnRH3), with a dominant pituitary presence of the hypophysiotropic GnRH3 (32), showed that under fasting conditions, GnRH3 disappeared from the pituitary, while the levels of GnRH2 increased (9, 33). In the two-GnRH goldfish model, GnRH2 elicited a more robust LH secretion compared to GnRH3 in sexually mature, pre-spawning fish, while in sexually regressed animals, GnRH3 had potent LH-releasing activity and GnRH2 had no effect (34, 35). These results indicated that GnRH2 may serve as a "backup" system to ensure the integrity of reproduction under suboptimal or other specific physiological conditions. In addition, we found that GnRH2 and GnRH3 could not only induce pituitary GtHα, LHβ, and FSHβ mRNA expression but also stimulate SgII mRNA expression in grass carp pituitary cells. A recent study has reported that mutation of SgII in zebrafish could lead to disrupted sexual behaviors, reduced ovulation and egg laying, and suboptimal fertility and embryo survival (36). These results suggested that GnRH could also regulate the reproduction through inducing SgII expression in teleost. Interestingly, we found that GnRHs could significantly reduce dopamine D2 receptor expression in grass carp pituitary cells. As we know, SgII dopamine inhibited LH synthesis *via* activation of DRD2 in teleost (37), suggesting that GnRH2 and GnRH3 could block dopamine-reduced LH expression by inhibiting pituitary DRD2 mRNA expression. After using the inhibitors of AC/PKA, PLC/PKC, and IP_3/Ca^{2+} signal pathways to cotreat with GnRH2 or GnRH3 in grass carp pituitary cells, we found that these inhibitors could block GnRH2- or GnRH3-induced GtHα, LHβ, FSHβ, INHBa, and SgII mRNA expression.

For feeding regulation, it had been reported that intracerebroventricular (ICV) injections of GnRH2 could significantly decrease food intake in zebrafish (38) and goldfish (39). Besides, knockout of GnRH2 could observably increase the food intake in zebrafish (9). In the present study, postprandial GnRH*2* and GnRH3 mRNA expression increased in a short time after food intake, indicating that GnRHs could act as a transient anorexigenic peptide in grass carp. Subsequently, we found that POMCb (40), CART2 (41), UTS1 (42), NMBa, NMBb (43), and LEPR (44), which had been reported as anorexigenic peptides, could be significantly stimulated by GnRH2 and GnRH3 in grass carp pituitary cells. These results suggested that GnRHs should be the satiety factor and involved in the regulation of pituitary anorectic peptides in teleost.

In summary, the two GnRH ligands (GnRH2 and GnRH3) and four GnRHRs (namely, GnRHR1, GnRHR2, GnRHR3, GnRHR4) were cloned from grass carp brain and pituitary. Then, ligand–receptor selectivity showed that individual subtypes of GnRHR exhibited differential selectivity for various members of GnRHs, with GnRHR3 preferring for GnRH2 and GnRHR1 preferring for GnRH3, respectively. Interestingly, GnRHR4 should be a multiligand receptor for GnRH2 and GnRH3. Using grass carp pituitary cells as model, we found that GnRH2 and GnRH3 could not only directly induce LHβ and FFSHβ mRNA expression but also stimulate other reproductive genes' (INHBa and SgII) mRNA expression mediated by AC/PKA, PLC/IP3/PKC, and Ca^{2+}/CaM/CaMK-II pathways. In addition, GnRH2 and GnRH3 could inhibit DRD2 mRNA expression to block dopamine-reduced LH secretion and synthesis. Finally, food intake could significantly induce brain GnRH2 and GnRH3 mRNA expression, and both GnRH2 and GnRH3 could significantly induce pituitary anorexigenic peptides' (POMCb, CART2, UTS1, NMBa, and NMB*b*) mRNA expression *via* AC/PKA, PLC/IP3/PKC, and Ca^{2+}/CaM/CaMK-II pathways (Figure 14). These results indicated that GnRHs could be involved in the regulation of reproduction and feeding.

Data availability statement

The transcriptomic data presented in the study were deposited to NCBI repository https://www.ncbi.nlm.nih.gov/sra, accession number SRR21736247. In addition, the sequences of grass carp GnRHs and GnRHRs were also submitted to Genbank https://www.ncbi.nlm.nih.gov/genbank/, the accession numbers were BankIt2624165 GnRHR1 OP482096, BankIt2624665 GnRHR2 OP482097, BankIt2622642 GnRHR3 OP433498, BankIt2622651 GnRHR4 OP433499, BankIt2622656 GnRH2 OP433500, BankIt2622662 GnRH3 OP433501, respectively.

Ethics statement

The animal study was reviewed and approved by Huazhong Agricultural University.

Author contributions

Data curation, CX and WL; formal analysis, YX and HZ; funding acquisition, GH and ZY; investigation, YO, RD, and YX; methodology, WL and RD; resources, XG; software, WL; supervision, GH; writing—original draft, WL and CX; writing—review and editing, GH. All authors contributed to the article and approved the submitted version.

Funding

Funding support was provided by the National Key R&D Program of China (2018YFD0900404 to ZY), China Postdoctoral Science Foundation (2019M662747 to GH), and

Hubei Province Postdoctoral Science Foundation (237934 to GH).

Acknowledgments

This article is dedicated to Prof. Anderson O.L. Wong (University of Hong Kong, China) for his genuine interest in training young scientists in the field of comparative endocrinology.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fendo.2022.982297/full#supplementary-material

References

1. Choi D. Evolutionary viewpoint on GnRH (gonadotropin-releasing hormone) in Chordata - amino acid and nucleic acid sequences. *Dev Reprod* (2018) 22(2):119–32. doi: 10.12717/DR.2018.22.2.119

2. Baba Y, Matsuo H, Schally AV. Structure of the porcine LH- and FSH-releasing hormone. II. confirmation of the proposed structure by conventional sequential analyses. *Biochem Biophys Res Commun* (1971) 44(2):459–63. doi: 10.1016/0006-291x(71)90623-1

3. Sherwood NM. Evolution of a neuropeptide family: Gonadotropin releasing hormone. *Am Zool* (1986) 26(4):1041–54.

4. Tostivint H. Evolution of the gonadotropin-releasing hormone (GnRH) gene family in relation to vertebrate tetraploidizations. *Gen Comp Endocrinol* (2011) 170 (3):575–81. doi: 10.1016/j.ygcen.2010.11.017

5. Muñoz-Cueto JA, Zmora N, Paullada-Salmerón JA, Marvel M, Mañanos E, Zohar Y. The gonadotropin-releasing hormones: Lessons from fish. *Gen Comp Endocrinol* (2020) 15(291):113422. doi: 10.1016/j.ygcen.2020.113422

6. Kudo H, Hyodo S, Ueda H, Hiroi O, Aida K, Urano A, et al. Cytophysiology of gonadotropin-releasing-hormone neurons in chum salmon (Oncorhynchus keta) forebrain before and after upstream migration. *Cell Tissue Res* (1996) 284 (2):261–7. doi: 10.1007/s004410050586

7. Abraham E, Palevitch O, Gothilf Y, Zohar Y. Targeted gonadotropin-releasing hormone-3 neuron ablation in zebrafish: Effects on neurogenesis, neuronal migration, and reproduction. *Endocrinology* (2010) 151(1):332–40. doi: 10.1210/en.2009-0548

8. Feng K, Cui X, Song Y, Tao B, Chen J, Wang J, et al. Gnrh3 regulates PGC proliferation and sex differentiation in developing zebrafish. *Endocrinology* (2020) 161(1):bqz024. doi: 10.1210/endocr/bqz024

9. Marvel MM, Spicer OS, Wong TT, Zmora N, Zohar Y. Knockout of Gnrh2 in zebrafish (Danio rerio) reveals its roles in regulating feeding behavior and oocyte quality. *Gen Comp Endocrinol* (2019) 280:15–23. doi: 10.1016/j.ygcen.2019.04.002

10. Yaron Z, Gur G, Melamed P, Rosenfeld H, Elizur A, Levavi-Sivan B. Regulation of fish gonadotropins. *Int Rev Cytol* (2003) 225:131–85. doi: 10.1016/s0074-7696(05)25004-0

11. Wade GN, Schneider JE. Metabolic fuels and reproduction in female mammals. *Neurosci Biobehav Rev* (1992) 16(2):235–72. doi: 10.1016/s0149-7634(05)80183-6

12. Van der Spuy ZM. Nutrition and reproduction. *Clin Obstet Gynaecol* (1985) 12(3):579–604.

13. Parker CG, Cheung E. Metabolic control of teleost reproduction by leptin and its complements: Understanding current insights from mammals. *Gen Comp Endocrinol* (2020) 292:113467. doi: 10.1016/j.ygcen.2020.113467

14. Xu S, Zhou L, Hu Q, Shi X, Xia C, Zhang H, et al. Novel pituitary actions of NKB for anorectic peptides regulation in grass carp. *Aquaculture* (2021) 531:735857. doi: 10.1016/j.aquaculture.2020.735857

15. Xia C, Qin X, Zhou L, Shi X, Cai T, Xie Y, et al. Reproductive regulation of PrRPs in teleost: The link between feeding and reproduction. *Front Endocrinol (Lausanne)* (2021) 12:762826. doi: 10.3389/fendo.2021.762826

16. Kauffman AS, Wills A, Millar RP, Rissman EF. Evidence that the type-2 gonadotrophin-releasing hormone (GnRH) receptor mediates the behavioural effects of GnRH-II on feeding and reproduction in musk shrews. *J Neuroendocrinol* (2005) 17(8):489–97. doi: 10.1111/j.1365-2826.2005.01334.x

17. Zhao Y, Lin MC, Farajzadeh M, Wayne NL. Early development of the gonadotropin-releasing hormone neuronal network in transgenic zebrafish. *Front Endocrinol (Lausanne)* (2013) 4:107. doi: 10.3389/fendo.2013.00107

18. Hu G, He M, Ko WKW, Wong AOL. TAC1 gene products regulate pituitary hormone secretion and gene expression in prepubertal grass carp pituitary cells. *Endocrinology* (2017) 158(6):1776–97. doi: 10.1210/en.2016-1740

19. Hu G, He M, Ko WK, Lin C, Wong AO. Novel pituitary actions of TAC3 gene products in fish model: Receptor specificity and signal transduction for prolactin and somatolactin α regulation by neurokinin b (NKB) and NKB-related peptide in carp pituitary cells. *Endocrinology* (2014) 155(9):3582–96. doi: 10.1210/en.2014-1105

20. Li B, Dewey CN. RSEM: Accurate transcript quantification from RNA-seq data with or without a reference genome. *BMC Bioinf* (2011) 12:323. doi: 10.1186/1471-2105-12-323

21. Benjamini Y, Drai D, Elmer G, Kafkafi N, Golani I. Controlling the false discovery rate in behavior genetics research. *Behav Brain Res* (2001) 125(1-2):279–84. doi: 10.1016/s0166-4328(01)00297-2

22. Xie C, Mao X, Huang J, Ding Y, Wu J, Dong S, et al. KOBAS 2.0: A web server for annotation and identification of enriched pathways and diseases. *Nucleic Acids Res* (2011) 39(Web Server issue):W316–22. doi: 10.1093/nar/gkr483

23. Hapgood JP, Sadie H, van Biljon W, Ronacher K. Regulation of expression of mammalian gonadotrophin-releasing hormone receptor genes. *J Neuroendocrinol* (2005) 17(10):619–38. doi: 10.1111/j.1365-2826.2005.01353.x

24. Moncaut N, Somoza G, Power DM, Canário AV. Five gonadotrophin-releasing hormone receptors in a teleost fish: Isolation, tissue distribution and phylogenetic relationships. *J Mol Endocrinol* (2005) 34(3):767–79. doi: 10.1677/jme.1.01757

25. Ikemoto T, Park MK. Identification and molecular characterization of three GnRH ligands and five GnRH receptors in the spotted green pufferfish. *Mol Cell Endocrinol* (2005) 242(1-2):67–79. doi: 10.1016/j.mce.2005.07.004

26. Tello JA, Wu S, Rivier JE, Sherwood NM. Four functional GnRH receptors in zebrafish: Analysis of structure, signaling, synteny and phylogeny. *Integr Comp Biol* (2008) 48(5):570–87. doi: 10.1093/icb/icn070

27. Platt N, Gordon S. Is the class a macrophage scavenger receptor (SR-a) multifunctional?–the mouse's tale. *J Clin Invest* (2001) 108(5):649–54. doi: 10.1172/JCI200113903

28. Krieger M. Scavenger receptor class b type I is a multiligand HDL receptor that influences diverse physiologic systems. *J Clin Invest* (2001) 108(6):793–7. doi: 10.1172/JCI14011

29. Schmidt AM, Yan SD, Yan SF, Stern DM. The multiligand receptor RAGE as a progression factor amplifying immune and inflammatory responses. *J Clin Invest* (2001) 108(7):949–55. doi: 10.1172/JCI14002

30. Herz J, Strickland DK. LRP: A multifunctional scavenger and signaling receptor. *J Clin Invest* (2001) 108(6):779–84. doi: 10.1172/JCI13992

31. Krieger M, Stern DM. Series introduction: Multiligand receptors and human disease. *J Clin Invest* (2001) 108(5):645–7. doi: 10.1172/JCI13932

32. Steven C, Lehnen N, Kight K, Ijiri S, Klenke U, Harris WA, et al. Molecular characterization of the GnRH system in zebrafish (Danio rerio): Cloning of chicken GnRH-II, adult brain expression patterns and pituitary content of salmon GnRH and chicken GnRH-II. *Gen Comp Endocrinol* (2003) 133:27–37. doi: 10.1016/s0016-6480(03)00144-8

33. Xia W, Smith O, Zmora N, Xu S, Zohar Y. Comprehensive analysis of GnRH2 neuronal projections in zebrafish. *Sci Rep* (2014) 4:3676. doi: 10.1038/srep03676

34. Khakoo Z, Bhatia A, Gedamu L, Habibi HR. Functional specificity for salmon gonadotropin-releasing hormone (GnRH) and chicken GnRH-II coupled to the gonadotropin release and subunit messenger ribonucleic acid level in the goldfish pituitary. *Endocrinology* (1994) 134:838–47. doi: 10.1210/endo.134.2.7507838

35. Murthy CK, Peter RE. Functional evidence regarding receptor subtypes mediating the actions of native gonadotropin-releasing hormones (GnRH) in goldfish, carassius auratus. *Gen Comp Endocrinol* (1994) 94:78–91. doi: 10.1006/gcen.1994.1062

36. Mitchell K, Zhang WS, Lu C, Tao B, Chen L, Hu W, et al. Targeted mutation of secretogranin-2 disrupts sexual behavior and reproduction in zebrafish. *Proc Natl Acad Sci U.S.A.* (2020) 117(23):12772–83. doi: 10.1073/pnas.2002004117

37. Wang X, Zhao T, Wei H, Zhou H. Regulation of dopamine D2 receptor expression in grass carp pituitary cells: A possible mechanism for dopaminergic modification of luteinizing hormone synthesis. *Gen Comp Endocrinol* (2011) 173 (1):48–55. doi: 10.1016/j.ygcen.2011.04.024

38. Nishiguchi R, Azuma M, Yokobori E, Uchiyama M, Matsuda K. Gonadotropin-releasing hormone 2 suppresses food intake in the zebrafish, danio rerio. *Front Endocrinol (Lausanne)* (2012) 3:122. doi: 10.3389/fendo.2012.00122

39. Matsuda K, Nakamura K, Shimakura S, Miura T, Kageyama H, Uchiyama M, et al. Inhibitory effect of chicken gonadotropin-releasing hormone II on food intake in the goldfish, carassius auratus. *Horm Behav* (2008) 54(1):83–9. doi: 10.1016/j.yhbeh.2008.01.011

40. Valen R, Jordal AE, Murashita K, Rønnestad I. Postprandial effects on appetite-related neuropeptide expression in the brain of Atlantic salmon, salmo salar. *Gen Comp Endocrinol* (2011) 171(3):359–66. doi: 10.1016/j.ygcen.2011.02.027

41. Yuan D, Wei R, Wang T, Wu Y, Lin F, Chen H, et al. Appetite regulation in schizothorax prenanti by three CART genes. *Gen Comp Endocrinol* (2015) 224:194–204. doi: 10.1016/j.ygcen.2015.08.015

42. Negri L, Noviello L, Noviello V. Effects of sauvagine, urotensin I and CRF on food intake in rats. *Peptides Prog Neurobiol* (1985) 6 Suppl 3:53–7. doi: 10.1016/0196-9781(85)90350-x

43. Ohki-Hamazaki H, Neuromedin B. *Prog neurobiol.* (2000) 62(3):297–312. doi: 10.1016/s0301-0082(00)00004-6

44. de Vrind VAJ, Rozeboom A, Wolterink-Donselaar IG, Luijendijk-Berg MCM, Adan RAH. Effects of GABA and leptin receptor-expressing neurons in the lateral hypothalamus on feeding, locomotion, and thermogenesis. *Obes (Silver Spring)* (2019) 27(7):1123–32. doi: 10.1002/oby.22495

frontiers | Frontiers in Endocrinology

TYPE Review
PUBLISHED 13 October 2022
DOI 10.3389/fendo.2022.1005863

Check for updates

OPEN ACCESS

EDITED BY
Shan He,
Huazhong Agricultural University, China

REVIEWED BY
Christian W. Gruber,
Medical University of Vienna, Austria
Paula Pouso,
University of the Republic, Uruguay

*CORRESPONDENCE
Jan A. Mennigen
jan.mennigen@uottawa.ca

SPECIALTY SECTION
This article was submitted to Experimental Endocrinology, a section of the journal Frontiers in Endocrinology

RECEIVED 28 July 2022
ACCEPTED 23 September 2022
PUBLISHED 13 October 2022

CITATION
Mennigen JA, Ramachandran D, Shaw K, Chaube R, Joy KP and Trudeau VL (2022) Reproductive roles of the vasopressin/oxytocin neuropeptide family in teleost fishes. *Front. Endocrinol.* 13:1005863. doi: 10.3389/fendo.2022.1005863

COPYRIGHT
© 2022 Mennigen, Ramachandran, Shaw, Chaube, Joy and Trudeau. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Reproductive roles of the vasopressin/oxytocin neuropeptide family in teleost fishes

Jan A. Mennigen[1]*, Divya Ramachandran[1], Katherine Shaw[1], Radha Chaube[2], Keerikkattil P. Joy[3] and Vance L. Trudeau[1]

[1]Department of Biology, Faculty of Science, University of Ottawa, ON, Canada, [2]Department of Zoology, Institute of Science, Banaras Hindu University, Varanasi, India, [3]Department of Biotechnology, Cochin University of Science and Technology, Kochi, India

The vertebrate nonapeptide families arginine vasopressin (AVP) and oxytocin (OXT) are considered to have evolved from a single vasopressin-like peptide present in invertebrates and termed arginine vasotocin in early vertebrate evolution. Unprecedented genome sequence availability has more recently allowed new insight into the evolution of nonapeptides and especially their receptor families in the context of whole genome duplications. In bony fish, nonapeptide homologues of AVP termed arginine vasotocin (Avp) and an OXT family peptide (Oxt) originally termed isotocin have been characterized. While reproductive roles of both nonapeptide families have historically been studied in several vertebrates, their roles in teleost reproduction remain much less understood. Taking advantage of novel genome resources and associated technological advances such as genetic modifications in fish models, we here critically review the current state of knowledge regarding the roles of nonapeptide systems in teleost reproduction. We further discuss sources of plasticity of the conserved nonapeptide systems in the context of diverse reproductive phenotypes observed in teleost fishes. Given the dual roles of preoptic area (POA) synthesized Avp and Oxt as neuromodulators and endocrine/paracrine factors, we focus on known roles of both peptides on reproductive behaviour and the regulation of the hypothalamic-pituitary-gonadal axis. Emphasis is placed on the identification of a gonadal nonapeptide system that plays critical roles in both steroidogenesis and gamete maturation. We conclude by highlighting key research gaps including a call for translational studies linking new mechanistic understanding of nonapeptide regulated physiology in the context of aquaculture, conservation biology and ecotoxicology.

KEYWORDS

endocrine system, hormone, hypothalamic–pituitary–gonadal axis, isotocin, neuromodulator, courtship behaviour, paracrine, vasotocin

1 Introduction

Members of the arginine vasopressin (AVP)/oxytocin (OXT) molecular family and their receptors are phylogenetically ancient, originating in a common ancestor evolved from a common ancestor of the Bilateria (1). As conserved cyclic nonapeptides, they exert pleiotropic functions in reproduction, sociosexual behavior, energy balance, osmoregulation, and the cardiovascular system, among others. In this review we take a focussed approach and examine their importance in the reproductive physiology of teleost fishes.

1.1 The teleost nonapeptide system

1.1.1 Nonapeptide structure and evolution

In basal cephalochordates, such as the Florida lancelet, *Branchiostoma floridae*, and urochordates such as the sea vase, *Ciona intestinalis*, single nonapeptides, [Ile^4]-VP and Ciona (ciVP) have been reported (2–4). The presence of a single AVP family peptide extends to basal vertebrate agnathans, including lampreys such as the Japanese lamprey, *Lethenteron japonicum*, and hagfishes such as *Eptatretus burgeri* (5, 6). It is believed that two rounds of whole genome duplication (2R WGD), one before and one after the separation of agnathans and gnathostomes (7), led to a duplication of the ancestral arginine vasotocin (*avp)* gene. This duplication gave rise to two nonapeptide families conserved in vertebrates; the AVP and OXT family peptides (8). In vertebrates, both genes code for precursor proteins which include a 5' signal sequence, a highly conserved nonapeptide and neurophysin, and, in the case of the AVP family, a C-terminal glycoprotein termed copeptin without known biological function (9). Processing into the mature nonapeptides occurs *via* prohormone convertases, and the acidic neurophysins I (OXT family) and neurophysin II (AVP family) associate with mature nonapeptides acting as carrier molecules within the neurosecretory system (9).

In teleost fishes, vasotocin (Avp) was originally isolated and characterized in pout, *Gadus luscus* (10), pollock, *Polacchius virens* (11), and European hake, *Mercluccius mercluccius* (12), and has since been identified in genomes of all teleost fishes studied to date (3, 6). Avp occurs in all verebrates except for mammals, where it appears to have mutated to give rise to vasopressin (AVP) in which ^{3}Ile was substituted for ^{3}Phe (3, 5, 6). All AVP family members are basic nonapeptides due to a basic amino acid (AA) at position 8.

In contrast to the AVP family peptides, the duplicated nonapeptide gene appears to have accumulated more mutations giving rise to the OXT family (3, 5, 6). The OXT nonapeptides are characterized by a neutral AA at position 8 (Leu, Ile, Gln or Val). In fishes alone, as many as 12 Oxt family peptides are known and likely arose due to lineage-specific duplications followed by substitutions in AA positions 3, 4 and 5 in the ring structure and position 8 in the tail structure (Table 1). With reference to mammalian OXT, the substitutions are Tyr^2 by Phe^2, Ile^3 by Phe^3, Gln^4 by Ser^4 or Asp^4, and Leu^8 by Ile^8 or Val^8. In teleost fishes, a single OXT family peptide (Oxt; [Ser^4-Ile^8]-OXT) was first isolated in pout, pollock, and the European hake and originally termed isotocin due to the presence of Ile in position 8 (18).

While position 8 mutations are very common in cartilaginous fishes (19) specific Oxt family peptides have also been reported in Sarcopterygii, such as the Australian lungfish, *Neoceratodus forsteri*, which expresses [Phe^2-Ile^3] OXT (20). Based on more recent genomic data, it has become evident that non-teleost Actinopterygians also express specific Oxt peptides (6). Indeed, in the spotted gar, *Lepisosteus oculatus*, which possess a pre-3R genome, two gene paralogues coding for Oxt were identified - one coding for a pro-[Ser^4-Ile^8]-OXT with a long C-terminal (NCBI Accession No. XM_006626499.1) and the other coding for a novel pro-[Phe^2, Ser^4]-OXT, which has a short C-terminal like other vertebrate neutral OXT family peptide precursors (NCBI Accession no. XM_006626523.1). An analysis of tissue expression profiles based on a fish RNA-seq expression database (21) does not reveal clear-cut differential expression of both *oxt* paralogues in spotted gar (Figure 1A).

In teleosts, characterized by a 3R genome condition, occurrence of two copies each of pro-*avp* (pro-a*vp*1 and pro-*avp2*) and pro-*oxt* (pro-*oxt*1 and pro-*oxt*2) have been reported in the blind cave fish, *Astyanax mexicanus*, based on genomic information and in salmonids and catastomids based on cloning studies (6, 22–24). In the blind cave fish, a diploid fish, synteny analysis suggests that the gene duplications may be due to 3R without subsequent gene loci losses (6). In salmonids and catostomids, the multiple *oxt* copies may be due to tetraploidization (4R WGD) and/or gene conversion (22–24). Regarding tissue expression profiles of paralogous *oxt* genes in salmonids, analysis of RNA-seq data (21) suggests similar expression profiles, at least in, *rainbow trout, Oncorhynchus mykiss* (Figure 1B), and brook trout, *Salvelinus fontinalis* (Figure 1C).

More recently however, the paradigm that teleost fishes, while encoding multiple gene loci for neutral nonapeptides in some cases, nevertheless exclusively express Oxt ([Ser^4-Ile^8]-OXT) was challenged by the cloning and discovery of two different neutral Oxt family peptides in the Asian stinging catfish, *Heteropneustes fossilis*, and the walking catfish, *Clarias batrachus* (6). In both species, a conventional *oxt* gene coding for a [Ser^4-Ile^8]-OXT and a second *oxt* gene coding for the novel [Ser^4-Val^8]-OXT, coined sevatocin, are expressed in addition to a single *avt* gene (6). Like pro-[Ser^4-Ile^8]-OXT, the peptide precursor encoding for [Ser^4-Val^8]-OXT has a similar organization (Table 2), with an extended C-terminal and a Leu-rich region. The functional implications of the *oxt* genes in these catfish species deserve special mention. The substitutions in the hormone moiety (Ile^8/Val^8) may lead to

TABLE 1 Oxt nonapeptide family orthologues in different fish groups.

Species/Groups	Historical name	AA sequence	Reference
Holocephali: *Callorhinchus milli* *Hydrolagus colliei*	Oxytocin	CYISNCPQG	(3)
Skates: *Raja miraletus*	Glumitocin	CYISNCPQG	(8)
Sharks: *Squalus acanthias*	Aspargtocin Valitocin	CYINNCPLG CYIQNCPVG	(13)
Scyliorhinus caniculus	Asavatocin Phasavatocin	CYINNCPVG CYFNNCPVG	(14)
Triakis scyllium	Asavatocin Phasitocin	CYINNCPVG CYFNNCPIG	(15)
Rays: *Torpedo marmorata*	Isotocin b	CYISNCPIG	(16)
Spotted gar: *Lepisosteus oculatus*	[Phe^2, Ser^4]-Oxytocin (a variant of ITb) Isotocin b	CFISNCPIG CYISNCPIG	Genomic sources (6)
Most teleost fishes	Isotocin b	CYISNCPIG	(8)
Catfishes: *Heteropneustes fossilis, Clarias batrachus*	Isotocin a (Sevatocin) Isotocin b	CYISNCPVG CYISNCPIG	(6)
Some lungfish, Coelacanth, *Latimeria chalumnae*	Mesotocin	CYIQNCPIG	(17)
Australian lungfish, *Neoceratodus forsteri*	[Phe^2]-Mesotocin	CFIQNCPG	(8)
Human *Homo sapiens*	Oxytocin	CYIQNCPLG	

The AA sequences of the mature hormones are given. Coloured and underlined AA are modifications from the mammalian OXT AA sequence.

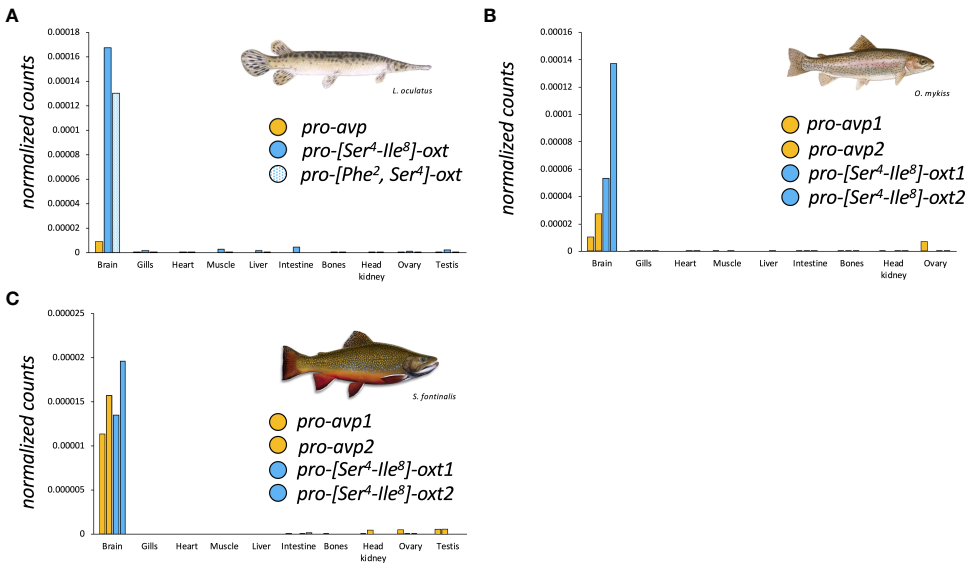


FIGURE 1
Tissue-specific expression profiles of nonapeptide precursor coding genes in spotted gar **(A)**, rainbow trout, *Oncorhynchus mykiss* **(B)**, and brook trout, *Salvelinus fontinalis* **(C)** derived from the Phylofish RNA-seq database (www.phylofish.signae.org) queried on June 1st, 2022 (20).

TABLE 2 General features of the cDNAs of an encoded precursor proteins of Oxta, Oxtb and Avp in the catfish Heteropneustes fossilis.

Nonapeptide	cDNA (bp)	Coding sequence (bp)	Precursor protein (AAs)	Signal peptide (AAs)	Neurophysin peptide (NP)(AAs)	Cys in NP (AA)	Leucine-rich core
Oxta	619	1-462	153	19	122	14	LLRKLLHL
Oxtb	708	56-508	151	29	118	14	LLKLLHL
Avp	618	60-524	155	20	122	14	LLLRILH

The mature nonapeptide hormone moiety (9 AA), cleavage site (GKR), and the domain between signal peptide and neurophysin are not indicated.

altered receptor-ligand interaction with possible sub- or neo-functionalization. Both *oxt*-like genes are similarly expressed in the preoptic area (POA), and functional studies show that both synthetic [Ser^4-Ile^8]-OXT and [Ser^4-Val^8]-OXT similarly regulate *fshb, lhb* and *gpa* expression in the catfish pituitary (6, 24). It is possible that both Oxt peptides bind to the same receptors, but receptor characterization has not yet been undertaken.

For the purpose of this review, we follow the recent Zebrafish Information Network (ZFIN, www.zfin.org) nomenclature, which uses a*vp*/Avp and *oxt*/Oxt to designate teleost genes and their protein products. The reasoning for this recent change in the literature is to highlight the homologous nature of *avp* and *oxt* family genes and their products in vertebrates. Consequently, this nomenclature no longer uses the historical distinction between teleost and mammalian nonapeptides (arginine vasotocin/arginine vasopressin and isotocin/oxytocin) which is reflective of their AA composition. Thus, while the historically widely used nomenclature is not used in this review, it is implicitly understood that the teleost *avp*/Avp and *oxt*/Oxt differ from mammalian and other vertebrate nonapeptide homologues in their AA residues as described.

1.1.2 Anatomy of the nonapeptide system in the context of reproduction

The distribution of Avp and Oxt has been examined in many teleosts and will not be covered in detail here. Controversies arise largely because of differing sensitivities of the neuroanatomical methods used, variable control experiments and other technical challenges (25, 26). Nevertheless, the use of transgenic approaches is helping to firmly establish the key locations of neuronal soma, and a new appreciation for their wide projection fields (27–30). In teleosts, Avp and Oxt neurons are intermingled and have been classified into three populations in the POA based on soma size. These are the giganto-, magno- and parvocellular neurons. Preoptic Avp and Oxt neurons send their fibers into diverse regions of the brain such as the hypothalamus, ventral telencephalon, mesencephalon and diencephalon, as well as the hindbrain and the spinal cord (20–30, amongst many others). Through the latter two systems, a role for POA-derived nonapeptides in modulating motor output related to reproductive behaviour and gamete release has been postulated in some, but not all teleost species (31–33).

In fish species in which specific sensory modalities have been shown to play a key role in reproductive behaviours, nonapeptide innervation has been described for distinct brain regions involved in sending and receiving sensory information. For example, in male zebrafish, *Danio rerio*, known to respond to female sex pheromones (34), fibers positive for Oxt have been identified in the olfactory bulb (35). In the plainfin midshipman, *Porichtys notatus*, which relies on vocalization as part of their courtship behaviour (36), especially Avp but also Oxt innervation was found in fore- and mid-brain regions involved in vocalization, and diencephalic regions of the ascending auditory pathway (37, 38). In the weakly electric gymnotiform bluntnose knifefish, *Brachyhypopomus gauderio*, which uses electric organ discharge (EOD) signals for mate selection (39), Avp innervation in the medulla was found to be in proximity of the pacemaker nucleus that controls EOD (40). Together, neuroanatomical evidence thus suggests a role for nonapeptides in modulating both emitting and receiving pathways of diverse sensory signals linked to reproductive behaviours in various teleost fishes.

In all teleost species studied to date, prominent preoptic and ventral hypothalamic projections terminating in the posterior pituitary for release to the pituitary vasculature have been reported (41, 42). In some species, such as the dwarf gourami, *Colisa lalia*, a colocalization of parvocellular Oxt with gonadotropin releasing hormone (Gnrh) has been reported (43). In the goldfish, *Carassius auratus*, Oxt colocalizes with secretoneurin a (44), an important hypophysiotropic stimulator of the hypothalamo-pituitary-gonadal (HPG) axis (45, 46). Such neuroanatomical data suggest that nonapeptides may regulate the HPG axis by co-release with other neuropeptides.

Detailed neuroanatomical studies of the pituitary in the sailfin molly, *Poecilia lattipinna*, the European bass, *Dicentrarchus labrax*, and the African sharptooth catfish, *Clarias gariepinus*, revealed that nonapeptidergic innervation principally forms contact with pituitary vasculature in the form of terminal release buttons which highlights an endocrine role of the nonapeptides (41, 42, 47, 48). Central and circulating Avp and Oxt concentrations in the nM range have been reported to be sex-specific, correlated, and reproductive stage-dependent in at least some species, such as the round goby, *Neogobius melanostomus* (49), and the air-breathing catfish, *Heteropneustes fossilis* (50). In addition to pituitary release,

nonapeptide fibers innervating gonadotrophs in the proximal pars distalis through gaps in the basement lamina have been demonstrated in the sailfin molly, *Poecilia latipinna* (41, 48). While there are far fewer contact sites of nonapeptide innervation of gonadotrophs compared to pituitary blood vessels, this evidence does nevertheless suggest that the neuronal organization of nonapeptide fibers in the pituitary also provides a basis for paracrine effects on gonadotrophs. Whether a single nonapeptide neuron originating from the teleost POA can be both encephalotropic and hypophysiotropic, and thus simultaneously regulate brain function as a neuromodulator and peripheral function as a paracrine factor or hormone, remains an open question. Evidence to-date suggests that this may be species-dependent. While single nonapeptide neurons originating in the POA have been shown to extend to both extrahypothalamic brain regions and the pituitary in Atlantic salmon, *Salmo salar* (51), clearly distinct Avp and Oxt neurons originating in the POA have been shown to innervate either extrahypothalamic brain regions or the pituitary, but not both, in zebrafish, *Danio rerio* (28, 29).

Transcript and/or protein abundance of nonapeptides in female and, to a lesser extent, male gonads has also been reported in several species. In rainbow trout, *Onchorhynchus mykiss*, ovarian expression of both *avp* and *oxt* has been reported (52). High *oxt* expression is also noted for whole zebrafish ovaries (53). In the air-breathing catfish, high-performance liquid chromatography (HPLC) analysis indicated the presence of Avp in ovaries and, albeit to a much lower extent, in testes (50). Immunohistochemistry approaches localized Avp to the ovarian follicular cell layer, with positive staining in both theca and granulosa cells, but failed to locate Avp in testes (50). Conversely, Avp has been located to interstitial cells in the testes of the chanchita, *Cichlasoma dimerus* (54). The expression of gonadal nonapeptide systems, and especially *avp*, appears to be a more widespread feature in teleosts, as suggested by RNA-seq data mined from the Phylofish database (20) and presented here (Figures 1A-C). Together, evidence of expression of a gonadal nonapeptide system in female and male gonads provides the anatomical basis for an additional reproductive role of nonapeptides thorough paracrine modulation of processes such as gametogenesis, steroidogenesis, and gamete release.

1.1.3 The teleost nonapeptide receptor repertoire

Nonapeptide systems can regulate reproductive physiology *via* central neuromodulatory and/or peripheral endocrine and/or paracrine pathways *via* G-protein coupled membrane-bound AVP and OXT peptide family receptors (AVPRs and OXTRs). Since newly available genome sequences have recently allowed for a comprehensive description of the vertebrate nonapeptide receptor repertoires and their evolution resulting in proposed nomenclature changes (55–59), we here review the repertoire of teleost receptors considering these findings in detail. For the purpose of this review, we once again follow the ZFIN nomenclature, which is largely reflective of these changes. This focused review provides the basis to critically review relevant information regarding specific teleost Avpr and Oxtr function in mediating central and peripheral nonapeptide effects on teleost reproduction.

The teleost nonapeptide receptor repertoire consists of five distinct gene family members, although not all are present in every teleost species. These receptors include *avpr1a, avpr2, avprl* as well as *oxtr* and their paralogues. A fifth vertebrate nonapeptide receptor family member, *avpr1b* is present in basal ray-finned fishes as well as tetrapods but is not present in elasmobranchs and teleost fishes (55–59) (Figures 2A-C). With regard to intracellular signaling mechanisms, Avprs and Oxtrs use diacylglycerol (DAG), inositol triphosphate (IP_3) and calcium (Ca^{2+}) as second-messengers with the exception of Avpr2l, which uses cyclic adenosine monophosphate (cAMP) (60). Avpr2b signaling has not directly been assessed in teleost fishes but may be similar to Avpr2aa, based on intracellular domain AA sequence similarities (57). For *avpr1*, all teleost genomes analyzed to date possess two paralogues, termed *avpr1aa* and *avpr1ab* (Figure 2A). Additionally, all teleost fishes appear to possess *avpr2aa* and *avpr2ab* paralogues (Figure 2B). The presence and retention of these paralogues is in line with the teleost-specific genome duplication, as single *avpr1a* and *avpr2a* genes exist in early non-teleost fishes (Figures 2A, B). Conversely, the situation appears to be more complex with regard to *avpr2b* and *avpr2l* genes which are retained in different paralogue numbers and lost in some but not all teleost fishes in no obvious evolutionary pattern (Figures 2A, B). The *oxtra* and *oxtrb* paralogues are found in all teleosts assessed to date, again indicative of a retention of paralogues following genome duplication at the base of teleost evolution (Figure 2C). Teleost-specific changes in the nonapeptide receptor repertoires have been reported, especially regarding possible intracellular AA sequences involved in cell signalling. For example, the teleost Avpr2aa AA sequence harbours extensions of the intracellular loop 3 region, which include relatively well-conserved Tyr, Ser and Thr residues which can be phosphorylated by a variety of intracellular kinases to alter intracellular signaling cascades as well as G-protein coupled receptor attenuation/desensitization, endocytosis, and intracellular trafficking (59). Lineage-specific changes in the nonapeptide repertoires have also been reported even within the infraclass of teleost fishes. For example, otocephalans lack an *avpr2bb* paralogue found in early teleosts as well as euteleosts (59), as shown in Figure 2B. In our current microsynteny analysis, we found additional otocephalan-specific changes for the *oxtrb* nonapeptide receptor locus (Figure 3A). These microsynteny changes translate into C-terminal AA sequence changes in an intracellular domain which, based on annotation from functional mutation studies in the human OXTR (61), is

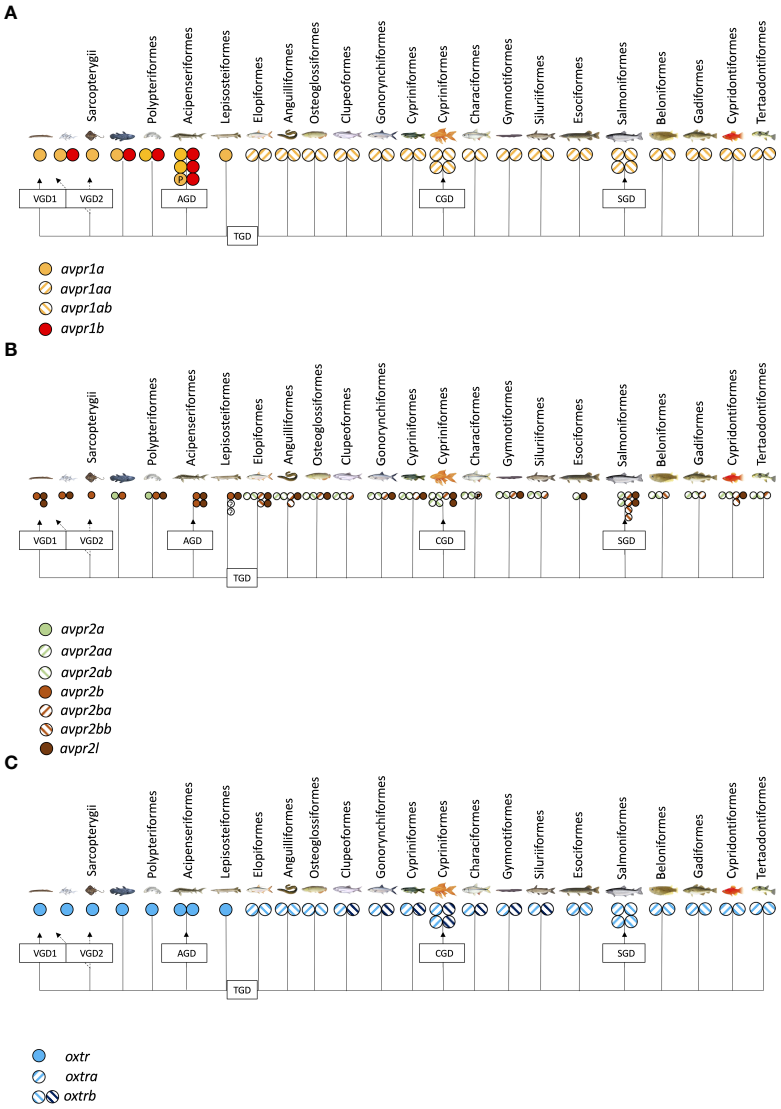


FIGURE 2
Presence of *avpr1* **(A)**, *avpr2/avpr2l* **(B)** and *oxtr* **(C)** genes in published fish genomes accessed on NCBI (www.pubmed.com) on June 1st, 2022.

linked to G_q-protein recruitment and cell signaling (Figure 3B). Otocephala, which diverged from Euteleostei during the Jurassic ~220 million years ago, are characterized by innovations in communication and sensing, notably through Schreckstoff, the alarm substance released through skin injury from conspecifics, and auditory capacity through a link between the swim bladder and inner ear (otophysic link) (62). It is thus tempting to speculate that such rearrangements, with possible functional implications, represent changes which facilitated sub- or neo-functionalization linked to increased social cues relevant to reproduction in otocephalans. In the case of otocephalan Oxtrb, a loss of highly conserved intracellular domain AA residues was observed (Figure 3B). These are specifically located in the C-terminal sequence and have been shown to be required for $G_{q/11}$ and subsequent IP_3, DAG and Ca^{2+} signaling (61). They also include consecutive Cys residues, which through palmitoylation, have been shown to anchor the C-terminal region to the membrane (61). Finally, it includes consecutive Ser residues believed to be involved in receptor retention for receptors following ligand-dependent internalization (61). Investigation of *oxtra* and *oxtrb* expression using an RNA-seq-based fish tissue database (21) reveal that in the otocephalans Allis shad, *Alosa alosa*, and Mexican cavefish, expression pattern for both *oxtra* and *oxtrb* receptor paralogues are similar in most tissues, apart from the brain where *oxtra* expression exceeded *oxtrb* expression (Figure 3C). In zebrafish ovary, *oxtrb* appears to be more strongly expressed compared to *oxtra* (53), suggesting possible differential roles in regulating ovarian function (53).

10.3389/fendo.2022.1005863

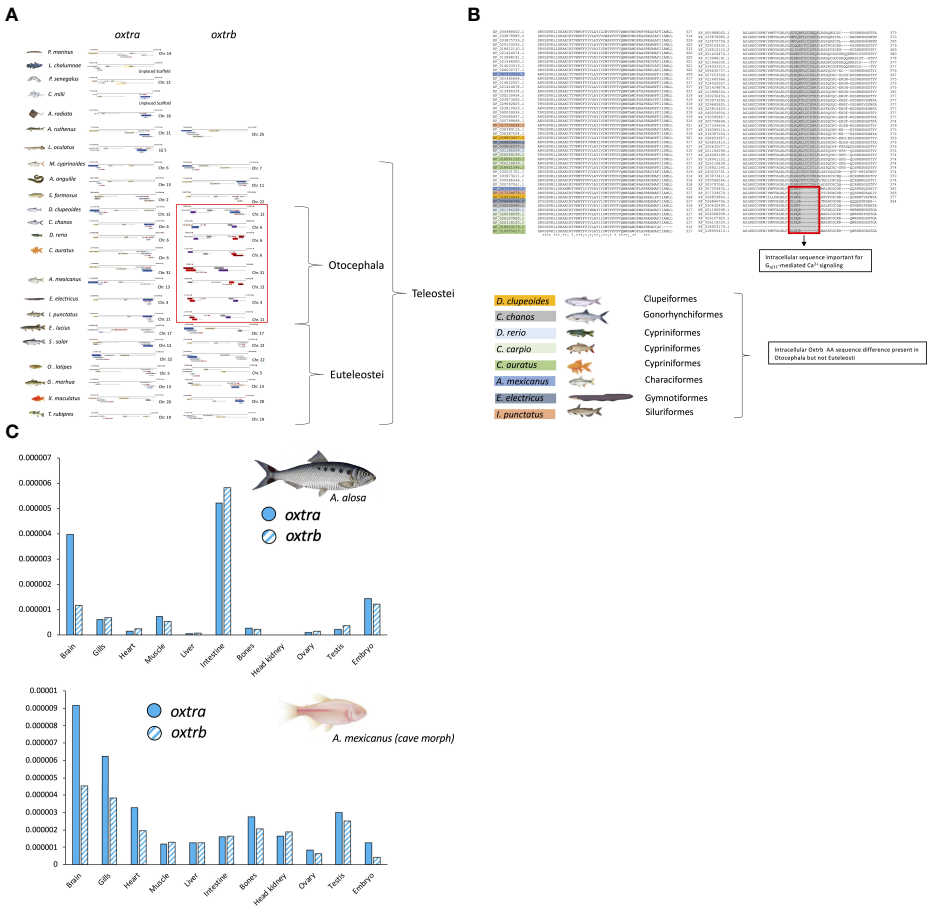


FIGURE 3
Microsynteny analysis **(A)**, predicted AA sequence alignment **(B)** and RNA-seq based tissue expression profiles **(C)** of *oxtra* and *oxtrb* paralogues in select teleost fishes. Micro-synteny analysis of *oxtr* paralogue gene loci was manually conducted on NCBI (www.pubmed.com) derived genome sequences retrieved on June 1st, 2022, while *oxtr* gene paralogue sequence-predicted AA sequences were aligned using Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/). RNA-seq based tissue expression profiles of *oxtr* paralogues was created derived from the Phylofish RNA-seq database queried on June 1st, 2022 (20).

In addition to *oxtr* paralogues, the expression of other teleost nonapeptide receptors and in some instances specific paralogues have been localized to tissues relevant to teleost reproduction (63–73) and are summarized in Table 3. Much of our current information on reproductive roles of nonapeptides has been derived from studies of otocephalan species including the air-breathing catfish (4, 5, 24, 84), goldfish (85–87), and zebrafish (35). Given that the nonapeptide repertoire is characterized by genomic rearrangements and potentially important AA sequence changes in this clade, caution is warranted when attempting to generalize functionality across all teleosts.

In contrast to the detailed evolutionary history, the functional characterization of nonapeptide receptors remains limited, and in some instances, such as for *avpr2l*, virtually unexplored in teleost fishes. Early studies in a cyprinid, the white sucker, *Catostomus commersonii*, investigated binding kinetics and specificity of both Avpr (88, 89) and Oxtr (90). Based on teleost genome sequences available today, these can retroactively be classified as Avpr1ab and Oxtra, respectively. These studies revealed that the Avpr1ab is highly selective for Avp (EC_{50} 13 nM ± 6 nM) over Oxt. Both nonapeptides bound to and activated cell signaling of a heterologously expressed Oxtra receptor with 3- to 4-fold higher affinity for Oxt (80 ± 30 nM) compared to Avp (300 ± 90 nM). More recent studies in zebrafish assessed specificity of nonapeptides and a mammalian OXT receptor antagonist (L-368,899) in heterologous expression assays (91). It was reported that Oxt exhibits similar affinities for both Oxtr parlogues with EC_{50} values of 2.99 ± 0.93 nM for Oxtra and 3.14 ± 1.10 nM for Oxtrb, respectively. In comparison, Avp exhibited slightly lower affinities to Oxtrs with EC_{50} values of 11.0 ± 3.0 nM for Oxtra and 27.0 ± 9.5 nM for Oxtrb, respectively. Both Avp and Oxt had low affinities for Avpr1aa (EC_{50} 727 ± 338 nM and 317 ± nM, respectively) and high affinities for Avpr1ab (2.79 ± 1.4 nM and

TABLE 3 Nonapeptide receptor expression in central and peripheral teleost tissues relevant to reproduction.

Tissue	Receptor type	Species	Description	Reference
Brain	*avpr1aa avpr1ab avpr2ab*	Air-breathing catfish *Heteropneustes fossilis*	*avpr1aa* type receptor mRNA is expressed in major neuroendocrine hypothalamic and telencephalic nuclei including the POA and sensorimotor centres; *avpr2ab* type receptor mRNA is largely confined to subependymal telencephalon	(74, 75)
	avpr1aa avpr1ab avpr2aa avpr2ab oxtrb	Pupfish, *Cyprinodon nevadensis amargosae*	*avpr1aa*, *avpr1ab*, *avpr2ab* and *oxtr* mRNA is expressed in telencehalon, hypothalamus and hindbrain	(76)
	avpr1aa; avpr1ab	Zebrafish, *Danio rerio*	mRNAs are expressed in forebrain, midbrain, and hindbrain. *avpr1aa* positive hindbrain neurons are contacted by *avp* neurons originating from POA and lateral longitudinal fasciculus and extending to sensorimotor areas such as the medial longitudinal fasciculus	(77)
	avpr1ab	Atlantic Croaker	mRNA and protein localized to hypothalamic GnRH neurons	(78)
	avpr1ab	Rock hind, *Epinephelus adscensionis*	mRNA is widely distributed in brain areas linked to reproductive and sensorimotor control including hypothalamic GnRH neurons, POA and olfactory bulb	(63, 79)
	avpr1aa; oxtra	*Astatotilapia burtoni*	mRNA and protein expressed in telencephalon and hypothalamus	(80)
Pituitary	*avpr1aa; avpr1ab; avpr2ab*	Air-breathing catfish *Heteropneustes fossilis*	mRNA expressed in male and female rostral pars distalis and pars nervosa	(74, 75)
	oxtra	Rice-field eel *Monopterus albus*	mRNA located to Lh but not Fsh cells	(81)
Gonad	*avpr1aa; avpr1ab; avpr2ab*	Air-breathing catfish *Heteropneustes fossilis*	In testes, *avpr1ab* and *avpr2ab* receptor mRNA are localized to interstitial tissue seminiferous epithelium. In ovaries, *avpr1aa* and *avpr1ab* receptors are localized to the follicular layer and an *avpr2ab* receptor to the oocyte membrane	(74, 75)
	oxtra	Guppy	Ovaries, expressed in follicular layer	(82)
	avpr1aa; avpr1ab	Bluehead wrasse	Ovaries, Testes	(83)

3.52 ± 0.94 nM, respectively). The guppy Oxtra was shown to be activated by both Oxt and Avp (82). While Oxt induced a strong dose-response (10 nM-1 µM) induction of co-transfected luciferase CRE-element, Avp induced a weaker, yet significant response at all concentrations tested (82). Together these data show that at physiological concentrations, it is likely that some degree of cross activation between Avp and Oxt ligands and the nonapeptides receptors occurs. It is therefore important to consider that reproductive roles of Avp and Oxt may, at least in part, be dependent on receptor cross-activation.

2 External and internal reproductive cues regulate teleost central nonapeptide systems

In several seasonal teleost fishes, nonapeptide expression and/or protein abundance in the POA is positively correlated with mature reproductive status (49, 50), which in turn, is dependent on environmental cues such as photoperiod and temperature (92). In female goldfish, hypothalamic *oxt* mRNA abundance peaks in the seasonal breeding period when fish have maximal gonadosomatic index (GSI) and is likely photoregulated (93). In male goldfish, exposure to the female releaser pheromone $PGF_{2\alpha}$, an important olfactory reproductive cue for males, increases *oxt* and *avp* mRNA levels in the telencephalon while stimulating circulating testosterone (T) concentrations and strippable milt volumes (94). Together, such association of nonapeptide expression in the neuroendocrine brain and enhanced pituitary and gonadal activity during seasonal cycles and after pheromone exposure is supportive of a physiological role in reproduction. Exposure to androstenedione, another pheromone known to play an important role in male-male competition in a reproductive context in goldfish (95), increases parvocellular *avp* (96). Thus, it appears that reproductive pheromones represent important environmental cues to regulate male goldfish nonapeptide expression. In male and female round goby, *Neogobius melanostomus*, maximal seasonal brain Avp concentration was observed just before spawning in March-April, whereas that of Oxt peaked during spawning in May-June (97). The lowest brain Avp level was noted in the non-breeding season from November

to January, while the level of Oxt decreased immediately at the end of the spawning. The results show that high Avp levels correlate with pre-spawning period whereas the highest Oxt levels correspond to spawning. In female round gobies, these increases appear, at least in part, to be dependent on estrogens acting *via* genomic (nuclear receptors), and genomic and non-genomic mechanisms in the case of Avp and Oxt, respectively (98). This suggests that season-dependent gonadally-derived positive sex steroid feedback mechanisms may reinforce the seasonal activation of hypothalamic nonapeptide systems. In the half-spotted goby, *Asterropteryx semipunctata*, Avp and Gnrh protein abundances were positively correlated and exhibited significant peaks in their abundances in sexually mature females (99). In male sticklebacks, the highest brain concentrations of Avp were observed in the most aggressive males that cared for eggs and nuptial-colored subordinates that fought to change their social status. Oxt was significantly higher in brains of aggressive dominant males (100). In the stickleback, the highest Avp levels were found in brains of females that did not deposit eggs, regardless of whether they were kept with courting or non-courting males and whether they had a nest or not. The highest Oxt levels were observed in females that did not deposit eggs but were kept with a courting male. The presence of courting or non-courting males that somehow activate Oxt- or/and Avp-producing neurones may be decisive for both behaviour and/or final oocyte maturation or egg deposition, because brain levels of both nonapeptides decreased sharply after egg deposition (101).

In the anadromous chum salmon, *Oncorhynchus keta*, dynamic changes in nonapeptide transcript and protein abundance have been reported during the reproductive migration period (102, 103). Lower transcript but higher immunoreactivity of both Oxt and Avp in the POA of fish collected upstream in a freshwater system compared to those caught in the marine bay, which serves as an entry point of the reproductive migration (102, 103). Based on these results it is tempting to speculate that due to their well-described role in osmoregulation, nonapeptide changes may act to integrate relevant environmental signals, such as the change in salinity, to subsequently change aspects of reproductive physiology in these anadromous fish. It has, however, been shown that salinity changes are not consistently the principal factor mediating changes in the nonapeptide systems of migrating salmonids and temperature and endogenous sex steroid concentrations also act as important regulators of these systems (104). Nevertheless, these data demonstrate a reproductive phase-dependent regulation of the nonapeptide system in chum salmon. Within the context of salmonid reproduction and migration, it is important to note that alternative reproductive tactics exist among individuals. In addition to males migrating to the ocean to grow and mature, some precocious parr achieve sexual maturity quickly before migrating to the ocean and can thus fertilize female eggs as 'sneaker males' (105, 106). Two transcriptomic and targeted gene expression studies investigating differential gene expression in the whole brain of maturing males and precocious parr of Atlantic salmon identified nonapeptides as being differentially regulated (107). The functional relevance of the differential expression with regard to reproduction remains, however, unknown.

In some group-living cichlid species in which HPG axis activity and reproductive behaviour is linked to social dominance, *avp* and Avp abundance has been investigated (108). The results show differential effects on *avp* mRNA levels and Avp neuron size in different POA subpopulations, with higher levels in gigantocellular neurons and, conversely, lower levels in parvocellular neurons of dominant fish. While underlining the responsiveness of the nonapeptide system to social status, a determinant of reproductive status in at least some cichlids, these findings also highlight the importance of considering potentially differential effects on nonapeptide subpopulations in the POA. However, the finding that *avp* expression in the POA of dominant and reproductively active African cichlid fish, *Astatotilapia burtoni*, is significantly reduced compared to subordinates who are reproductively supressed (109), clearly suggests caution is warranted to avoid simplified and global paradigms regarding the role of nonapeptides in species in which social dominance is linked to increased reproductive capacity.

Teleost fish exhibit significant plasticity in terms of their reproductive biology and life history traits. Some species are sequential hermaphrodites, beginning life as one sex, and changing sometime later to the other. Such species are capable of protandrous (male-to-female), protogynous (female-to-male), or serial (bidirectional) sex change (110). Changes in nonapeptide systems have been observed in response to social context-induced sexual plasticity. For example, the bluehead wrasse, *Thalassoma bifasciatum*, exhibits specific increases in Avp immunoreactivity in the magnocellular POA when undergoing behavioural female-to-male sex change that occurs rapidly following the removal of a large terminal colour male and is independent of the gonads (111). The increase in magnocellular *avp* expression coincides with a rapid increase in dominant and male courting behaviour, suggesting a functional link (111, 112). Serial adult sex change in the marine goby, *Trimma okinawae*, is associated with significant and reversible changes in the size of Avp-producing forebrain cells, which are higher in males and coincide with increased male mating behaviour (113). In overcrowded single sex groups of female black mollies, *Poecilia sphenops*, which do not form social hierarchies, masculinization of reproductive behaviour occurs and is linked to a decrease of higher, female-typical Avp concentrations in this species to male typical concentrations (114). In a transcriptomic analysis of the bluehead wrasse forebrain, increased *oxt* transcript abundance was one of the few statistically significant changes detected across female-to-male sex change (115). The opposite trend was observed in the bluebanded goby, *Lythrypnus dalli*, where lower Oxt

immunoreactivity was observed in the POA of males and late-stage female-to-male sex-changers compared to females (116).

Overall, these studies reveal context-dependent regulation of central nonapeptide systems across several species with diverse reproductive strategies. This strongly implicates nonapeptides in the regulation of teleost reproductive physiology. However, caution is clearly warranted to avoid oversimplified paradigms for specific roles of nonapeptides across all teleost species (117), as at least some species-specific roles are likely to have evolved among teleosts with such varied reproductive strategies.

3 Reproductive function of nonapeptides in teleost fishes

3.1 Nonapeptide-dependent regulation of reproductive behaviour

The plainfin midshipman fish, *Porychthys notatus*, displays sex- and morph-specific vocalization during mating. Oxt and Avp regulate these sex- and morph-specific effects on the vocal circuitry (37). Type I males mating call are stimulated by Avp, whereas female and type II males' grunting sounds are stimulated by Oxt. Using homozygous Japanese medaka, *Oryzias latipes*, knockout mutants, an essential role for Avp in male mate-guarding behaviours in this non-monogamous species has been demonstrated (118). For example, under natural conditions, two medaka males kept in triads with a female are in competition and the dominant medaka male that is 'guarding' the female exhibits increased reproductive success measured as increased paternity in offspring. Males harbouring mutations in a*vp* and *avpr1aa* exhibit significantly reduced male guarding behaviour indicating a key role for *avp* in dominant status-dependent reproductive success (118). Similarly, *oxt* and *oxtra*, exert sex-specific effects in Japanese medaka: mutant female fish exhibit a lack of mate preference for familiar males and mutant male fish have reduced courtships displays to unfamiliar females, but exhibit increased mate-guarding behaviour towards familiar females (64). Since the potential effects on the HPG axis were not quantified in these studies, it is not clear whether these effects are entirely mediated by the nonapeptides, or whether altered HPG axis regulation also contributes to the behavioural observations.

In male bluehead wrasse, *Thalassoma bifasciatum*, a species with alternate male reproductive tactics (territorial and non-territorial), Avp intraperitoneal injection increased courtship behaviour in the field irrespective of male reproductive tactic and promoted a territorial-like phenotype in non-territorial males (65). An opposite effect was observed following administration of Manning's compound, a mammalian AVPR1 receptor antagonist, suggesting that this effect is mediated *via* this nonapeptide receptor subtype (65). In male white perch, *Morone americana*, intracerebroventricular but not intraperitoneal administration of Avp significantly stimulated an important courtship behavior termed 'attending' without affecting whole body or circulating androgens (119). These data suggest that central rather than peripheral HPG axis actions are involved in mediating the effects of Avp on the male white perch courtship behaviour. In male beaugregory damselfish, *Stegastes leucostictus*, Manning's compound significantly lowered male courtship behaviour, while exogenous Avp administration did not affect male courtship behaviour (120). Similarly, administration of Manning's compound significantly reduced male reproductive courtship behaviour and reproductive success in mating assays with female zebrafish, *Danio rerio* without affecting whole body androgen (T and 11-keto-testosterone; 11-KT) levels, suggesting Avp acutely regulates male zebrafish courtship behaviour including chasing, nudging, and circling *via* central Avpr1a receptors and independently of HPG axis regulation (35). In the weakly electric fish, *Brachyhypopomus gauderio*, male courtship behaviour observed in male-female dyads resulted in a higher degree of Avp neuron activation in the nucleus preopticus ventricularis anterior compared to isolated males (121). In the same species, Avp increases dominance in part *via* direct modulation of the EOD rate (122). Together, these findings raise the possibility that male reproductive behaviour *via* electric signaling may be under Avp control in this species. Despite the reviewed evidence, a universal Avp-dependent stimulation of male reproductive behaviour in teleost fishes is unlikely, as the reproductive phenotype of females and sneaker males, but not dominant males, is sensitive to Avp in the peacock blenny, *Salaria pavo* (123). There is a need for detailed comparative studies of the roles of nonapeptides in teleosts which exhibit the most diverse reproductive strategies and behaviours amongst the vertebrates (117).

Several studies have provided evidence for regulatory roles of nonapeptides in teleost species with parental care. In the primarily paternal teleost the common clownfish, *Amphiprion ocellaris* for example, administration of an OXTR antagonist abolished paternal behaviours such as nips, fanning the eggs, and proportion of time in the nest, without affecting aggressive behaviours in paired non-reproductive fish (124). This suggests a specific action of Oxt in controlling male common clownfish parental care behaviours (124); however, whether the high selectivity of antagonist (desGly-NH2-d(CH2)5[D-Tyr2, Thr4]OVT) for mammalian OXTR also applies to teleosts has not been formally investigated. When introducing domino damselfish, *Dascyllus trimaculatus*, as non-conspecific intruders, administration of the OXTR antagonist reduced paternal care behaviour in clownfish, but increased aggression towards the non-conspecific intruder, demonstrating the importance of social context in behavioural responses (125). Conversely, administration of an AVTR1 receptor antagonist increased male parental behaviours while reducing aggression towards intruders (125), thus demonstrating antagonistic roles of the nonapeptide systems in common clownfish (124, 125). These data suggest a role for nonapeptides in paternal care. In

the monogamous convict cichlid, *Amatitlania nigrofasciata*, single fathers increase paternal care behaviours quickly after removal of the female partner, and this increase coincides with increased activation of parvocellular Oxt neurons in the POA (126). Administration of a mammalian OXTR antagonist in biparental males inhibited paternal care behaviour, indicating a functional role for Oxt neurons (126).

3.2 A role for central nonapeptides in regulating saliency to reproductive cues in teleost fishes

In addition to the direct modulation of teleost reproductive behaviours, central roles for nonapeptides may not be restricted to the role of transducing and integrating reproductive cues, but also to act as a filter and/or amplifier of exogenous or endogenous cues. This latter concept has recently gained more traction as the 'salience hypothesis', especially in higher vertebrate species, including humans. The salience hypothesis is based on the notion that an individual is being constantly inundated with sensory information in its environment and therefore needs to be able to filter the information to identify what is relevant and important (i.e., signal) from that which is unimportant (i.e., background noise). The nonapeptides OXT and AVP have been proposed to serve as two important neuromodulators in the central nervous system that can help to increase the salience of sensory information, such as conspecific olfactory cues, to induce effects on endocrine and behavioural responses (127). In fish, however, this concept remains comparatively poorly explored, especially in the context of the diverse reproductive strategies in different environmental conditions and cues. Olfactory stimuli in particular play important roles in at least some groups of teleost fish such as cyprinids (35, 94, 95). Future studies exploring sensitizing roles of nonapeptides to reproductive pheromonal and visual cue detection are thus clearly warranted. A recent study in zebrafish identified Oxt-like innervation in the male olfactory bulb (35), providing a neuroanatomical basis for functional studies. Similarly, the widely studied electrical and vocal communication systems in some teleost fishes (128) should provide excellent models to investigate possible modulatory roles of nonapeptides in the reception and integration rather than production of reproductive signals.

3.3 Nonapeptide-dependent regulation of the HPG axis

3.3.1 Nonapeptides are an integral part in the hypothalamic circuitry controlling the HPG axis

Both stimulatory and inhibitory factors regulating the teleost HPG axis have been well described (92). Nonapeptides may affect the HPG axis *via* modulation of stimulatory and/or inhibitory hypophysiotropic systems. To date, little evidence exists for potential roles of nonapeptides in directly affecting Gnrh in teleost fish. In rock hind, *Epinephelus adscensionis*, and in Atlantic Croaker, *Micropogonias undulatus*, Avpr1ab receptors are co-localized with Gnrh1 in preoptic anterior hypothalamic neurons. The functional relevance of this crosstalk other than reported concordant regulation of gene expression between *avpr1ab* and *gnrh1* (78, 79, 99) must be investigated. Studies in female goldfish show that serotonin neurons, known to stimulate pituitary Lh release in this species (129), are found in proximity to Oxt neurons in the POA and Oxt fibres in the pars nervosa of the pituitary gland, suggesting a possible interaction between them (87). However, the functional relevance of possible Oxt dependent modulation of serotonin-dependent gonadotropin release has not been investigated.

Nonapeptides may ostensibly also act to promote pituitary gonadotropin release *via* reduction of potent dopaminergic inputs on Lh release (92). This is supported by work in the walking catfish, *Clarias batrachus*, which suggests that Oxt may stimulate Lh release *via* the inhibition of dopaminergic blockage (66). In walking catfish Oxt immunoreactivity was greatly enhanced in the POA in female pre-spawning and spawning fish, and superfusion of brain slices with Oxt resulted in a ~50% reduction of tyrosine hydroxylase staining, suggesting rapid inhibitory effects on dopamine or other catecholaminergic neurons (66).

In addition to hypothalamic interaction between nonapeptide systems and the neuronal circuitry involved in HPG axis regulation in several teleost models (66, 86, 87), a few lines of evidence also demonstrated co-expression of nonapeptides with neuropeptides with known stimulatory function on gonadotropin release. For example, Oxt and secretoneurin were found to be colocalized in the POA and fibers innervating the pituitary in goldfish, *Carassius auratus* (44), while colocalization of Oxt and GnRH was reported in the dwarf gourami, *Colisa lalia* (43). Together, these studies suggest that potential co-release of nonapeptides with other neuropeptides known to stimulate gonadotropin release represent an understudied aspect of HPG axis regulation.

In addition to targeting neuronal circuitry involved in the regulation of the HPG axis, POA nonapeptide neurons in teleost fish themselves receive neuronal input from reproductive neuropeptides. These include Gnrh, which contact Oxt neurons in rainbow trout, *Oncorhynchus mykiss* (130), and kisspeptin, which contact Oxt and Avp neurons in Japanese medaka (131) and striped bass, *Morone saxatillis* (132). Together, this neuroanatomical evidence points to potential neuromodulatory roles for nonapeptides in HPG stimulation in the context of multimodal signaling systems regulating gonadotrophs in teleosts (133). Given the reported roles of nonapeptides in teleost sociosexual and courtship behaviour, neuroanatomical evidence may also reflect synchronization of

HPG axis activation stimulation with nonapeptidergic behavioural pathways to maximize reproductive success.

Several studies have also demonstrated the sensitivity of both Oxt and Avp neuronal populations to sex steroids. Administration of both low (0.1 µg/g body weight) and high (0.5 µg/g body weight) estradiol (E_2) doses (0.5 µg/g body weight) normalizes ovariectomy-induced decreases in brain and plasma Avp concentrations in Asian stinging catfish (134). This effect appears to be, at least in part, indirectly mediated *via* the modulation of dopaminergic control, as treatment with α-methyl-para-tyrosine, a tyrosine hydroxylase inhibitor, partially abolished the restorative effect of the low E_2 dose on Avp abundance in the ovariectomized fish (134). A male-specific stimulatory effect of androgens on parvocellular Oxt neurons in the medaka POA has also been reported (135). This regulation also appears to be indirect, as androgen receptor expression was not found in Oxt neurons but on kisspeptin neurons known to stimulate Oxt neurons in this species (131, 135). In female round gobies, circulating E_2 levels are higher in the spawning phase compared to non-spawning phase and coincide with high circulating Avp and Oxt concentrations (98). Brain explant exposure to E_2 in spawning and non-spawning phases stimulated Avp and Oxt release in this species; however, pharmacological studies using the estrogen receptor (ER) antagonist fulvestrant and the transcription inhibitor actinomycin D showed that the effect of E_2 on Avp and Oxt release was mediated by different signaling pathways (98). E_2-dependent Avp release was mediated by ERs *via* both genomic and non-genomic pathways, while Oxt release was mediated through ERs *via* a genomic pathway only (98). Whether E_2 acted directly on nonapeptide neurons in female gobies was not resolved, as the study did not investigate whether nonapeptide neurons express ERs. Oxt neurons in the POA of goldfish, *Carassius auratus*, are, at least based on immunohistochemical evidence, direct targets for estrogen actions, as they express the membrane estrogen receptor Gper1 (96) and are surrounded by radial glia, the only cells in the teleost brain expressing *cyp19a1b* and capable of producing neuroestrogens (136). While these studies provide evidence for effects of steroids on nonapeptide systems, future studies are warranted to delineate direct and indirect mechanisms of action, and whether effects are mediated by gonadal steroids and feedback regulation and/or local neurosteroids.

In sum, POA nonapeptide systems have been shown to be integrated into neuronal circuits involved in the regulation of the HPG axis in several teleosts. Additionally, recent evidence suggests that nonapeptide neurons are also responsive to sex steroids, suggesting the potential for endocrine feedback and/or modulation *via* neurosteroids. Additional studies investigating nonapeptide crosstalk with other hypothalamic regulators of the HPG axis are clearly warranted, as are careful studies investigating the direct or indirect regulation of hypothalamic nonapeptide systems to sex steroids, the critical endogenous reproductive signals.

3.3.2 Nonapeptides exert direct hypophysiotropic effects on gonadotrophs in several species

In the goldfish, hypothalamic expression of *oxt* was found to peak seasonally in reproductively mature females (93). Pharmacological investigations demonstrate that hypothalamic induction of goldsfish *oxt* is dependent on GABAergic and dopaminergic signaling (137), in line with an integration of this nonapeptide system with seasonally-regulated HPG neurocircuitry in this species (92). Intraperitoneal injection of 1 µg/g body weight Oxt in sexually recrudescent female goldfish significantly increased circulating Lh by 167% 5h post-injection (86) with subsequent increases in circulating E_2 12h post injection (137). Unfortunately, Oxt-dependent stimulation of Lh release in sexually mature female goldfish was not investigated. Subsequent examination of potential direct effects of Oxt on gonadotrophs were investigated using primary goldfish dispersed pituitary cell cultures (87). In these preparations, Oxt significantly stimulated Lh release without affecting *lhb* or *fshb* subunit mRNAs, suggesting direct, transcription-independent stimulation of Lh release (87). These findings are in line with reported neuroanatomical evidence in other teleosts, such as the sailfin molly, *Poecilia latipinna*, the European bass, *Dicentrarchus labrax*, and the African sharptooth catfish, *Clarias gariepinus*, in which nonapeptidergic innervation of gonadotrophs has been reported (41, 48). Similar to Oxt, a stimulatory effect of Avp on Lh release has been demonstrated in at least two teleosts: in the sailfin molly 18 h pituitary incubation with Avp stimulated Lh synthesis and release, with lower dose- responsiveness and more consistent effects in male compared to female pituitaries (41). In the Asian stinging catfish, Avp, and to a much lesser extent Oxt and servatocin, stimulated *gpa*, *fshb* and *lhb* in pituitary cultures in a sex- and reproductive stage-dependent manner (24). The less potent effects of Oxt and servatocin where largely limited to *lhb* stimulation in pre-spawning females, with no effect on *fshb*. In female walking catfish, *Clarias batrachus*, pituitaries superfused with 20 nM Oxt for 1h displayed a significant decrease in Lhb staining reflective of increased Lh release was reported (138). In the ricefield eel, *Monopterus albus*, Oxt-stimulated Lh release from dispersed pituitaries *via* an Oxtra-activated IP_3/Ca^{2+} pathway (81). In males of the Chanchita, *Cichlasoma dimerus*, Avp-stimulated gonadotropin secretion in single pituitary culture, with biphasic stimulation of Lh release at the lowest (0.1 µM) and highest (10 µM) concentration of Avp tested (54) and a stimulation of Fsh release at the highest Avp concentration tested.

These data establish a stimulatory role of nonapeptides on gonadotrophs, with sex-, reproductive stage- and species-dependent differences in potency and gonadotropin specificity. While it is important to keep in mind that observations are limited to only a few teleost species, data to date suggest that hypophysiotropic nonapeptide systems stimulate Lh release in

teleost fishes similar to the situation reported in rodents and humans (139, 140).

3.3.3 Endocrine and paracrine roles of nonapeptides in gonads

Both nonapeptides and their receptors have also been identified in ovaries and testes, and roles for nonapeptides in the regulation key gonadal functions *via* endocrine and paracrine signaling have been reported.

3.3.3.1 Steroidogenesis

Effects of nonapeptides on male steroidogenesis have been reported in testicular cultures of the rainbow trout (141). Testosterone production stimulated by Avp and Oxt has been observed in immature but not mature testes *in vitro*. Exposure to Avp elicited a stronger maximal response in T production compared baseline production (6-fold) than Oxt (4-fold). The maximally active concentration of 100 nM Avp was furthermore found to augment dose-dependent Lh-stimulated T production, suggesting a synergistic role. In a similar study in chanchita, Avp-stimulated T production in testes incubated *in vitro* dose-dependently, reaching a significant, 2-fold increase at 50 nM (54). A limitation of these two studies is that the authors measured T, which is the prohormone for the more potent teleost sex steroids: 11-KT and E_2. Unfortunately, nonapeptide receptor antagonists were not used in these experiments to probe specific receptor involvement. Since recent gene expression and *in-situ* hybridization data suggests a role for *avpr1aa* and *avpr2aa* receptors (Table 3), additional studies with teleost-validated antagonists are warranted to probe the molecular mechanistic basis involved in Avp-induced T production in testes.

Several studies have investigated the role of nonapeptides on ovarian steroidogenesis. While a stimulatory role for Oxt on circulating E_2 has been reported in female goldfish *in vivo* (87), it was not investigated whether these effects are linked to prior increases in Lh (86) or mediated *via* direct action at the ovary. In contrast, a comprehensive study determining the role of Avp and Oxt on *in vitro* ovarian steroidogenesis at different seasonal reproductive developmental stages was conducted in the air-breathing catfish (142). Dose-dependent, biphasic stimulatory effects of Avp on E_2 production for pre-vitellogenic ovarian tissue were reported. In contrast, Avp produced a dose- and time-dependent inhibition of E_2 production in early postvitellogenic ovaries (142). In comparison, Oxt produced a low, yet significant, stimulation of E_2 production without any dose effect in the previtellogenic ovaries, and a dose- and time-dependent inhibition like Avp in the early postvitellogenic ovary (142). The inhibitory effect of Avp on the E_2 synthesis in the postvitellogenic ovary may be part of a trigger for the steroidogenic shift to decreased E_2 in favour of synthesis of the maturation-inducing steroid (MIS) 17, 20β-dihydroxy-4-pregnen-3, 20-dione (17, 20β-DP) in air-breathing catfish (143). The MIS reinitiates oocyte meiosis up to the second metaphase (144).

Concurrent with the modulation of E_2 synthesis, Avp and, to a lesser extent, Oxt also stimulated progesterone (P_4) production (142). The Avp-stimulated increase in P_4 was generally dose-dependent in pre- and early post-vitellogenic ovaries, reaching approximately a 30% increase in production compared to baseline at high doses (142). In late postvitellogenic ovaries, concentrations as low as 1 nM Avp induced a 60% increase over baseline concentrations (142). This effect was similar to that of hCG, and combined administration of 20 IU hCG and Oxt was found to be additive at least after 16 h incubation. Avp stimulated 17-hydroxyprogesterone (17-OHP_4) synthesis in the previtellogenic phase ovaries following both 8 h and 16 h incubation (142). Avp stimulated the production of 17,20β-Dihydroxy-4-pregnen-3-one (17,20β-DP), which acts as maturation induced steroid in this species, about 2-fold more in the spawning phase than pre-spawning phase, similar to hCG. The stimulatory effect of Oxt was several-fold lower compared to Avp and occurred at higher concentrations (142). The combination of Avp and hCG elicited a cumulative effect on the 17,20β-DP level especially after 16 h of incubation in the spawning phase (142). The authors concluded that Avp was more potent than Oxt to stimulate the progestin pathway, and that Avp paralleled the actions of hCG. The finding that hCG and steroid hormones (E_2, P_4 and 17, 20β-DP) stimulate ovarian Avp production suggests a positive feedback loop (145) underscoring the functional significance of Avp in follicular growth, maturation, and ovulation. The stimulatory effect of Avp on ovarian P_4 secretion is conserved as similar actions for AVP family peptides have been reported for chicken, mouse, and cow *in vitro* (146).

3.3.3.2 Gametogenesis, gamete release and parturition

In the air-breathing catfish ovaries, Avp induces germinal vesicle breakdown (GVBD) and ovulation in a dose- and time-dependent manner (145). In this experiment, postvitellogenic follicles were co-incubated with Avp and an AVPR1 antagonist (deamino-Pen^1, O-Me-Try^2, Arg^8 vasopressin), an AVPR2 antagonist (1-adamantane acetyl O-Et-D-Try^2Val^4, Abu^6, $Arg^{8,9}$ vasopressin), or both. GVBD, ovulation and 17,20β-DP concentration were inhibited or reduced by 92-94% after 24h co-incubation with both antagonists. The AVPR1 antagonist inhibited GVBD and ovulation by 82-83%, and the MIS concentration by 70%. The AVPR2 antagonist inhibited GVBD, ovulation and MIS concentration by 29%, 26% and 15%, respectively. The results show that the effects of Avp are mediated mainly by Avpr1 receptors with a minor role for Avpr2 receptors.

Prostaglandins (PGs) have a critical role in diverse aspects of reproduction in vertebrates (147). The cyclooxygenase inhibitor

indomethacin can block MIS-induced final oocycte maturation and ovulation in yellow perch, *Perca flavescens*, and Atlantic croaker, indicating dependence on PGs (148, 149) The functional relationship between Avp and PGs was investigated in air-breathing catfish (150): Avp stimulated $PGF_{2\alpha}$ and PGE_2 levels in a dose- and time-dependent manner *in vitro* and the effects were similar to that produced by hCG. Both Avp and hCG-induced stimulation of PG levels were inhibited by indomethacin, supporting involvement of cyclooxygenase. The Avp stimulation of PG levels was strongly inhibited by the AVPR1 receptor antagonist but not by the AVPR2 receptor antagonist. Indomethacin inhibited the Avp and hCG-induced GVBD and ovulation. Both $PGF_{2\alpha}$ and PGE_2 stimulated GVBD and ovulation in a dose- and time-dependent manner and $PGF_{2\alpha}$ was more effective than PGE_2. Taken together, these observations highlight a relationship between Avp and PGs, and their interaction in the control of oocyte maturation and ovulation.

Follicular or oocyte hydration is a phenomenon conspicuous and widespread in marine and catadromous fish eggs associated with follicular and oocyte maturation (FOM) and ovulation (151), and this process is retained to some extent in freshwater and anadromous fishes. Singh and Joy (152) reported a 23% rise in oocyte water content during the FOM and ovulation in *H. fossilis* with Avp eliciting a significant effect on oocyte water content, diameter, volume, osmolality, Na^+K^+ ATPase activity, Na^+, K+, Mg^{2+}, Ca^{2+} concentration, GVBD and ovulation, similar to hCG. The combination of Avp and hCG produced a higher effect. In a further study, Acharjee et al. reported that Avp regulates *aqp1ab*, (ovary-specific aquaporin 1ab) expression through an Avpr2 receptor (153, 154), which is linked to the cAMP-PKA pathway, similar to AVP in mammalian kidney tubules.

The involvement of nonapeptides in sperm release was first demonstrated in the killifish, *Fundulus heteroclitus*, in which fish and mammalian neurohypophyseal preparations as well as synthetic OXT initiated a spawning reflex response (155). The relative effectiveness of the nonapeptides to induce spawning reflex in the killifish was estimated to be the highest and equipotent for AVP and Avp, followed by Oxt and OXT (155). However, concentrations used in these original studies were high compared to physiological concentrations. In male African sharptooth catfish testes slices 30-min incubation with OXT (at 10 IU), but not Oxt, AVP, epinephrine, $PGF_{2\alpha}$, LH and pituitary extracts increased milt release (156). In male walking catfish, nonapeptides and their nanotube composites designed for slower release were tested for their efficacy to promote stripping of milt by abdominal massage (157). Both naked or nano-conjugated nonapeptides increased strippable milt concentrations without altering reproductive success of fertilized eggs and increased the expression of the steroidogenesis pathway enzymes *star*, *3bhsd*, *17bhsd*, *cyp17a1a*, ands *cyp11a1a* (157).

Regarding parturition in teleosts, nonapeptides have been reported to stimulate premature parturition in the guppy, *Poecilia reticulata*, an ovoviviparous teleost (82, 158). The injection of Avp, Oxt and PGs to guppy, *Poecilia reticulata*, a live-bearing teleost, induced premature parturition (157). Both Avp and Oxt stimulated *cox2* mRNA expression in guppy ovaries *in vitro*, which in the case of Avp, but not Oxt, translated into increased PG concentrations (82). Together this data suggests that the nonapeptide-dependent stimulation of premature parturition in guppies is mediated by PG. Interestingly, both Avp and Oxt exposure upregulated a guppy *oxtr* paralogue, suggesting interaction between ovarian nonapeptide systems in the guppy ovary (82). In zebrafish, a recent study investigating downstream effectors linked to a reduced ovulation phenotype observed in female chromosome 23 miR-200 cluster KO mutants showed that co-injection of hCG, Avp and Oxt, but not injection of synthetic human GnRH and LH analogues were able to partially rescue the phenotype (159). Together, this data suggests a Gnrh-independent role for Avp and Oxt in zebrafish ovulation. Nevertheless, early comparative evidence from teleost fishes has demonstrated that roles for nonapeptides on male and female spawning cannot be generalized in teleost fish and are possibly indirect following application of supraphysiological concentrations (160).

While we acknowledge a generally high degree of evolutionary conservation of nonapeptide genes and the gross (neuro)anatomical distribution of their expression, differences in reproductive function of nonapeptides between teleost species certainly exist. We have integrated the current state of knowledge of nonapeptides on central and HPG axis components of teleost reproduction in Figure 4. It is anticipated, however, that additional detailed comparative studies will uncover diversity of nonapeptide-dependent regulation of teleost reproductive physiology.

4 Translational aspects

As evolutionarily conserved systems regulating reproduction (3), research investigating roles of nonpeptides in teleost fish reproduction in detail have translational relevance in the areas of aquaculture and species conservation; both of which rely on methods informed by mechanistic understanding of reproductive physiology. As such, we anticipate that modulation of nonapeptide function in teleost species has significant potential to stimulate and possibly coordinate behavioural and endocrine processes necessary to promote reproduction for species in captivity (161). A third area of translational relevance is ecotoxicology. We review emerging evidence suggesting that neuroendocrine disruption of teleost nonapeptides may be linked to decreased

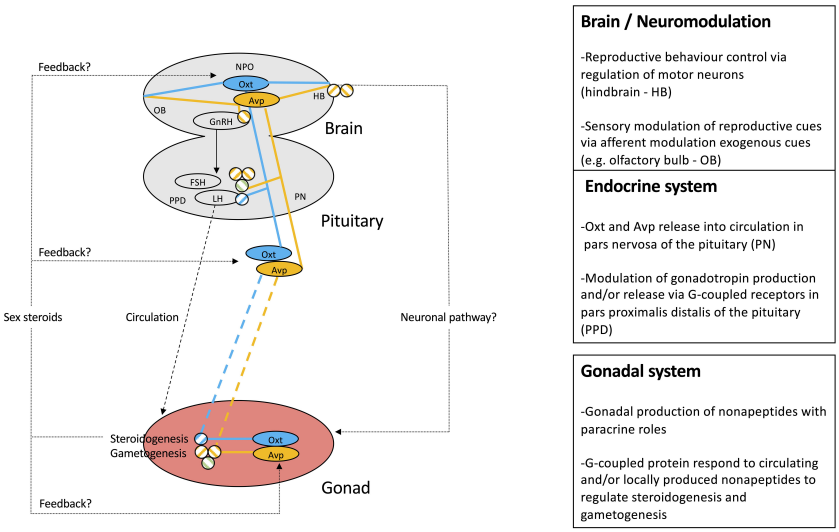


FIGURE 4
Schematic representation of mechanistic knowledge of nonapeptide roles in teleost reproduction. The Avp system is highlighted in yellow, while the Oxt system is represented in blue. Circled nonapeptides indicate sites of synthesis, while connecting lines represent neuronal, endocrine, and paracrine pathways. Specific receptor involvement in regulatory function of HPG axis components is symbolized as defined in Figure 2.

reproductive success in teleost fishes, an ecologically meaningful endpoint (162).

4.1 Neuroendocrine disruption

In teleost fish, relatively recent studies have investigated Avp and Oxt nonapeptide systems as targets of different groups of aquatic contaminants. Histological studies of POA magnocellular neurons revealed that a six-month exposure of channel catfish, *Channa punctatus*, to inorganic mercury at a concentration of 10 µg/L resulted in smaller and less active Avp-secreting neurons (163). Persistent organic pollutants have also been shown to affect nonapeptide systems in teleost fishes: in Atlantic Croaker, a four week daily dietary exposure (2 and 8 µg/g body weight) to the planar polychlorinated biphenyl congener 3,3′,4,4′-tetrachlorobiphenyl (PCB-77) significantly reduced hypothalamic expression of *avpr1a* mRNA and Avpr1a protein levels, as well as (co-localized) *gnrh1* mRNA levels in the brain (78).

Pharmaceuticals and plasticizers are other major environmental contaminants with reported effects on nonapeptide systems. Repeated intraperitoneal injections of pharmacological doses (5 µg/g) of the selective serotonin reuptake inhibitor and aquatic contaminant fluoxetine (FLX) significantly reduced *oxt* mRNA levels in female goldfish telencephalon and hypothalamus, an effect that was linked to reduced circulating E_2 concentrations (85). Subsequent waterborne FLX exposure studies in both female and male goldfish revealed that *oxt* transcript abundance in the same tissues was not affected by a two- week exposure to FLX at 540 ng/L and 54 µg/L concentrations, but that the same two-week exposure to waterborne fluoxetine significantly diminished releaser pheromone $PGF_{2\alpha}$-induced increases in *oxt* mRNA (94, 164). Similarly, targeted gene expression analysis of zebrafish larvae acutely exposed to 50 and 500 ng/L FLX for 96 h (165) as well as transcriptomic screens of whole brains collected from a wild zebrafish population exposed to 100 µg/L FLX at a concentration for two weeks (166) identified *oxt* transcripts as being differentially expressed in FLX-exposed fish compared to unexposed control fish. Together, these studies reveal that the Oxt system is responsive to FLX at both early developmental and adult life-stages, raising the possibility of mediating organisational as well as activational effects. Repeated injection of pharmacological concentrations of 6 µg/g body weight FLX over a period of two weeks significantly reduced *avt* transcript abundance in giganto-, magno- and parvocellular neurons of the POA in male bluehead wrasses, an effect that correlated with decreased territorial aggression (167). The responsiveness of teleost nonapeptide systems to FLX corresponds to several observations in mammalian models (168, 169) and suggests an evolutionarily conserved serotonin-dependent regulation of these systems (87, 170, 171).

In Japanese medaka chronically exposed to environmentally relevant and high concentrations of waterborne metamphetamine for a period of 90 days, a dose-dependent, significant increase in whole brain *oxtr* mRNA and Oxt peptide were observed (172). Because both FLX and metamphetamine affect neurotransmitter

systems and neuroactive contaminants may exert reproductive effects, at least in part, *via* nonapeptidergic systems, these findings support the concept of neuroendocrine disruption (173). In line with this interpretation, a meta-analysis of transcriptomic screens of the goldfish hypothalamus identified *oxt* as the single transcript affected by drugs modulating serotonergic, dopaminergic, and GABAergic systems, all of which have established roles in goldfish reproduction (137). Concurrent with previously described responsiveness of teleost POA nonapeptide systems to sex steroids, zebrafish chronically exposed to 1,10 and 30 µg/L Bisphenol A (BPA), a weakly estrogenic compound, as well as 1 µg/L E_2, exhibited complex dose-dependent and sex-specific effects on whole brain nonapeptide and nonapeptide receptor gene expression levels, which corresponded with alterations in social but not overall locomotor behaviour (174, 175). Similarly, developmental (2-5dpf) exposure to BPA and its replacement compound Bisphenol S (BPS) in the low µM range revealed non-linear alteration of *oxt* and Oxt protein abundance in association with quantifiable behavioural disruptions at 21dpf (176, 177). There is a need to study the involvement of nonapeptide systems in mediating organizational and/or activational effects of endocrine disrupting chemicals. This is an area understudied in teleost fish (178) compared to rodent models (179, 180), and will inform the possible development of teleost nonapeptides as functional biomarkers relevant to reproductive function in teleost fishes.

5 The state of the art and current limitations

Having critically reviewed the current state of knowledge regarding reproductive roles of teleost nonapeptides, we conclude by briefly discussing key insights and current limitations in the field. We furthermore suggest conceptual and technical approaches in the hope of stimulating collaborative research in the field.

1. It is well-appreciated that teleosts are champions of reproductive plasticity and diversity. There is a high degree of evolutionary conservation of nonapeptides and their gross (neuro)anatomical distribution. However, novel resources such as genomic sequences and detailed comparative investigation increasingly reveal areas of plasticity of teleost nonapeptide systems. A point in case is the nonapeptide receptor inventory, which exhibits differences between teleost fishes and other vertebrates, but, importantly, also within teleost fish lineages such as the otocephala and euteleosti (59). It will thus be important to address functional differences in nonapeptide receptor paralogue expression, regulation, and function to assess their potential roles in mediating reproductive functions in a reproductively diverse fishes.
2. While increasing comparative studies to delineate plasticity of nonapeptides in regulating reproduction in the diverse group of teleost fishes will be important, it will be equally crucial to comprehensively study the reproductive roles of nonapeptides within single teleost fish species. In reviewing the current knowledge of the role of nonapeptides in teleost reproduction, it has become clear that, apart from detailed studies in the Asian stinging catfish (50, 84) and, to a lesser degree, the canchita cichlid (54), few if any studies have investigated the role of nonapeptides across the different levels of the HPG axis within a single teleost species. The need for detailed studies within single fish species becomes even more clear when considering that research investigating behavioural and endocrine roles of nonpeptides in teleost reproduction has largely co-existed in isolation focussing on separate fish species. However, there is a need to consider (nonapeptide-regulated) courtship behaviour in the context of HPG axis function (165). Investigation of the salience hypothesis and possible roles for nonapeptides in the integration of reproductive cues are also warranted.
3. The recent generation of nonapeptide and/or nonapeptide receptor knock-out models in genetically-tractable model systems such as zebrafish (67) and medaka (64, 118) may hold particular promise. However, neither of these models have, to-date, been used to explore nonapeptide effects on the HPG axis. Receptor-specific knock-outs may furthermore prove fruitful to probe the role of virtually uncharacterized nonapeptide receptors such as *avtr2l* and generally circumvent possible specificity issues linked to the use of mammalian nonapeptide agonists and antagonists. Furthermore, GFP-reporter lines have been described in zebrafish, at least for Oxt (68, 69). While such lines represent powerful tools to investigate regulation and activation of central and peripheral nonapeptide systems in response to environmental and endogenous cues relevant to reproduction (35), few studies have been conducted in this area. Transgenic reporter lines may also allow endocrine disrupting) chemical screening for effects on nonapeptide systems. Adult-specific transgenic ablation studies and optogenetic approaches may in the future permit specific investigation of organisational and activational reproductive effects of the nonapeptides.
4. Previous co-localization experiments (43, 44) and more recent single-cell techniques (28) have demonstrated co-localization of nonapeptides with other neuropeptide regulators of reproduction in neuroendocrine neurons. Thus, future functional studies should investigate co-release and interactions between nonapeptides and co-expressed reproductive regulators to quantify potential

combinatorial effects on courtship behaviour and HPG axis function.

Author contributions

JM, DR, KS,RC, KJ, and VLT contributed to conception and design of the review. DR conducted in silico analyses. JM wrote the first draft of the manuscript. DR, KS, RC, KJ and VLT wrote sections of the manuscript. All authors contributed to manuscript revision, read, and approved the submitted version.

Funding

The authors gratefully acknowledge the following funding sources: NSERC-Discovery Grants (Grant no. 147476 to JM, and to VLT), an NSERC-CGS-D (to KS), and partial funding from the Institute of Eminence of the Banaras Hindu University (to RC).

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Odekunle EA, Elphick MR. Comparative and evolutionary physiology of Vasopressin/Oxytocin type neuropeptide signaling in invertebrates. *Front Endocrinol* (2020) 11:225. doi: 10.3389/fendo.2020.00225

2. Kawada T, Sekiguchi T, Itoh Y, Ogasawara M, Satake H. Characterization of a novel vasopressin/oxytocin superfamily peptide and its receptor from an ascidian, ciona intestinalis. *Peptides* (2008) 29:1672–8. doi: 10.1016/j.peptides.2008.05.030

3. Gwee P-C, Tay B-H, Brenner S, Venkatesh B. Characterization of the neurohypophysial hormone gene loci in elephant shark and the Japanese lamprey: origin of the vertebrate neurohypophysial hormone genes. *BMC Evol Biol* (2009) 9:47. doi: 10.1186/1471-2148-9-47

4. Matsubara S, Shiraishi A, Osugi T, Kawada T, Satake H. The regulation of oocyte maturation and ovulation in the closest sister group of vertebrates. *Elife* (2019) 8:e49062. doi: 10.7554/eLife.49062

5. Banerjee P, Chaube R, Joy KP. Molecular cloning, sequencing, and tissue expression of vasotocin and isotocin precursor genes from ostariophysian catfishes: phylogeny and evolutionary considerations in teleosts. *Front Neurosci* (2015) 9:166. doi: 10.3389/fnins.2015.00166

6. Banerjee P, Chaube R, Joy KP. Molecular cloning and characterisation of an isotocin paralogue ([V8] isotocin) in catfishes (superorder ostariophysi): Origin traced likely to the fish-specific whole genome duplication. *J Neuroendocrinol* (2018) 30:e12647. doi: 10.1111/jne.12647

7. Panopoulou G, Poustka AJ. Timing and mechanism of ancient vertebrate genome duplications – the adventure of a hypothesis. *Trends Genet* (2005) 21:559–67. doi: 10.1016/j.tig.2005.08.004

8. Acher R. Molecular evolution of fish neurohypophysial hormones: Neutral and selective evolutionary mechanisms. *Gen Comp Endocrinol* (1996) 102:157–72. doi: 10.1006/gcen.1996.0057

9. Acher R, Chauvet J. Structure, processing, and evolution of the neurohypophysial hormone-neurophysin precursors. *Biochimie* (1988) 70:1197–207. doi: 10.1016/0300-9084(88)90185

10. Acher R, Chauvet J, Chauvet MT, Crepy D. Neurohypophysial hormones from fish: isolation of a vasotocin from the whiting-pour (Gadus luscus l.). *Biochim Biophys Acta* (1961) 51:419–20. doi: 10.1016/0006-3002(61)90198-6

11. Heller H, Pickering BT. Neurohypophysial hormones of non-mammalian vertebrates. *J Physiol* (1961) 155:98–114. doi: 10.1113/jphysiol.1961.sp006616

12. Chauvet J, Chauvet-Lenci MT, Acher R. Some observations on the neurohypophysial hormones of a teleostfish, the hake (Merluccius merluccius l.). *C R Seances Soc Biol Fil* (1961) 252:2145–7.

13. Acher R, Chauvet J, Chauvet MT. Phylogeny of the neurohypophysial hormones. *Two New active pept isolated cartilaginous fish Squalus acanthias. Eur J Biochem* (1972) 29:12–9. doi: 10.1111/j.1432-1033.1972.tb01951.x

14. Chauvet J, Rouille Y, Chauveau C, Chauvet MT, Acher R. Special evolution of neurohypophysial hormones in cartilaginous fishes: asvatocin and phasvatocin, two oxytocin-like peptides isolated from the spotted dogfish (Scyliorhinus caniculus). *PNAS* (1994) 91:11266–70. doi: 10.1073/pnas.91.23.11266

15. Hyodo S, Tsukada T, Takei Y. Neurohypophysial hormones of dogfish, triakis scyllium: structures and salinity-dependent secretion. *Gen Comp Endocrinol* (2004) 138:97–104. doi: 10.1016/j.ygcen.2004.05.009

16. Buchholz H, Schönrock C, Fehr S, Richter D. Sequence analysis of a cDNA encoding an isotocin precursor and localization of the corresponding mRNA in the brain of the cartilaginous fish torpedo marmorata. *Mol Mar Biol Biotechnol* (1995) 4:179–84.

17. Gwee P-C, Amemiya CT, Brenner S, Venkatesh B. Sequence and organization of coelacanth neurohypophysial hormone genes: evolutionary history of the vertebrate neurohypophysial hormone gene locus. *BMC Evol Biol* (2008) 8:93. doi: 10.1186/1471-2148-8-93

18. Acher R, Chauvet J, Chauvet MT, Crepy D. Isolation of a new neurohypophysial hormone, isotocin, present in bony fish. *Biochim Biophys Acta* (1962) 58:624–5. doi: 10.1016/0006-3002(62)90085-9

19. Acher R, Chauvet J, Chauvet MT, Rouille Y. Unique evolution of neurohypophysial hormones in cartilaginous fishes: possible implications for urea-based osmoregulation. *J Exp Zool* (1999) 284:475–84. doi: 10.1002/(sici)1097-010x

20. Hyodo S, Ishii S, Joss JMP. Australian Lungfish neurohypophysial hormone genes encode vasotocin and [Phe^2] mesotocin precursors homologous to tetrapod-type precursors. *Proc Natl Acad Sci USA* (1997) 94:13339–44. doi: 10.1073/pnas.94.24.13339

21. Pasquier J, Cabau C, Nguyen T, Jouanno E, Severac D, Braasch I, et al. Gene evolution and gene expression after whole genome duplication in fish: the PhyloFish database. *BMC Genomics* (2016) 17:368. doi: 10.1186/s12864-016-2709-z

22. Suzuki M, Hyodo S, Urano A. Cloning and sequence analyses of vasotocin and isotocin precursor cDNAs in the masu salmon, oncorhynchus masou: evolution of neurohypophysial hormone precursors. *Zoolog Sci* (1992) 9:157–67. doi: 10.1006/gcen.1993.1165

23. Heierhorst J, Morley SD, Figueroa J, Krentler C, Lederis K, Richter D. Vasotocin and isotocin precursors from the white sucker, catostomus commersoni: cloning and sequence analysis of the cDNAs. *Proc Natl Acad Sci USA* (1989) 86:5242–6. doi: 10.1073/pnas.86.14.5242

24. Acharjee A, Chaube R, Joy KP. Reproductive stage- and sex-dependant effects of neurohypophyseal nonapeptides on gonadotropin subunit mRNA expression in the catfish heteropneustes fossilis: An *in vitro* study. *Gen Comp Endocrinol* (2018) 260:80–9. doi: 10.1016/j.ygcen.2018.01.001

25. Rodriguez-Santiago M, Nguyen J, Winton LS, Weitekamp CA, Hofmann HA. Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. *Front Endocrinol (Lausanne)* (2017) 8:195. doi: 10.3389/fendo.2017.00195

26. Loveland JL, Hu CK. Commentary: Arginine vasotocin preprohormone is expressed in surprising regions of the teleost forebrain. *Front Endocrinol (Lausanne)* (2018) 9:63. doi: 10.3389/fendo.2018.00063

27. Gutnick A, Blechman J, Kaslin J, Herwig L, Belting H-G, Affolter M, et al. The hypothalamic neuropeptide oxytocin is required for formation of the neurovascular interface of the pituitary. *Dev Cell* (2011) 21:642–54. doi: 10.1016/j.devcel.2011.09.004

28. Herget U, Ryu S. Coexpression analysis of nine neuropeptides in the neurosecretory preoptic area of larval zebrafish. *Front Neuroanat* (2015) 9:2. doi: 10.3389/fnana.2015.00002

29. Herget U, Gutierrez-Triana JA, Salazar Thula O, Knerr B, Ryu S. Single-cell reconstruction of oxytocinergic neurons reveals separate hypophysiotropic and encephalotropic subtypes in larval zebrafish. *eNeuro* (2017) 4:1–16. doi: 10.1523/ENEURO.0278-16.2016

30. Kagawa N, Honda A, Zenno A, Omoto R, Imanaka S, Takehana Y, et al. Arginine vasotocin neuronal development and its projection in the adult brain of the medaka. *Neurosci Lett* (2016) 613:47–53. doi: 10.1016/j.neulet.2015.12.049

31. Demski LS, Sloan HE. A direct magnocellular-preopticospinal pathway in goldfish: Implications for control of sex behavior. *Neurosci Lett* (1985) 55:283–8. doi: 10.1016/0304-3940(85)90449-5

32. Walton JC, Waxman B, Hoffbuhr K, Kennedy M, Beth E, Scangos J, et al. Behavioral effects of hindbrain vasotocin in goldfish are seasonally variable but not sexually dimorphic. *Neuropharmacology* (2010) 58:126–34. doi: 10.1016/j.neuropharm.2009.07.018

33. Macey MJ, Pickford GE, Peter RE. Forebrain localization of the spawning reflex response to exogenous neurohypophysial hormones in the killifish. *Fundulus heteroclitus. J Exp Zool* (1974) 190:269–79. doi: 10.1002/jez.1401900303

34. Yabuki Y, Koide T, Miyasaka N, Wakisaka N, Masuda M, Ohkura M, et al. Olfactory receptor for prostaglandin F2α mediates male fish courtship behavior. *Nat Neurosci* (2016) 19:897–904. doi: 10.1038/nn.4314

35. Altmieme Z, Jubouri M, Touma K, Coté G, Fonseca M, Julian T, et al. A reproductive role for the nonapeptides vasotocin and isotocin in male zebrafish (Danio rerio). *Comp Biochem Physiol B Biochem Mol Biol* (2019) 238:110333. doi: 10.1016/j.cbpb.2019.110333

36. Forlano PM, Bass AH. Neural and hormonal mechanisms of reproductive-related arousal in fishes. *Horm Behav* (2011) 59:616–29. doi: 10.1016/j.yhbeh.2010.10.006

37. Goodson JL, Bass AH. Forebrain peptides modulate sexually polymorphic vocal circuitry. *Nature* (2000) 403:769–72. doi: 10.1038/35001581

38. Goodson JL, Evans AK, Bass AH. Putative isotocin distributions in sonic fish: Relation to vasotocin and vocal-acoustic circuitry. *J Comp Neurol* (2003) 462:1–14. doi: 10.1002/cne.10679

39. Silva A, Perrone R, Macadar O. Environmental, seasonal, and social modulations of basal activity in a weakly electric fish. *Physiol Behav* (2007) 90:525–36. doi: 10.1016/j.physbeh.2006.11.003

40. Perrone R, Migliaro A, Comas V, Quintana L, Borde M, Silva A. Local vasotocin modulation of the pacemaker nucleus resembles distinct electric behaviors in two species of weakly electric fish. *J Physiol Paris* (2014) 108:203–12. doi: 10.1016/j.jphysparis.2014.07.007

41. Batten Trevor FC. Ultrastructural characterization of neurosecretory fibres immunoreactive for vasotocin, isotocin, somatostatin, LHRH and CRF in the pituitary of a teleost fish, poecilia latipinna. *Cell Tissue Res* (1986) 244: 661–72. doi: 10.1007/BF00212547

42. Groves DJ, Batten TFC. Direct control of the gonadotroph in a teleost, poecilia latipinna. *Gen Comp Endocrinol* (1986) 62:315–26. doi: 10.1016/0016-6480(86)90122-X

43. Maejima K, Oka Y, Park MK, Kawashima S. Immunohistochemical double-labeling study of gonadotropin-releasing hormone (GnRH)-immunoreactive cells and oxytocin-immunoreactive cells in the preoptic area of the dwarf gourami, colisa lalia. *J Neurosci Res* (1994) 20:189–93. doi: 10.1016/0168-0102(94)90037-X

44. Canosa LF, Lopez GC, Scharrig E, Lesaux-Farmer K, Somoza GM, Kah O, et al. Forebrain mapping of secretoneurin-like immunoreactivity and its colocalization with isotocin in the preoptic nucleus and pituitary gland of goldfish. *J Comp Neurol* (2011) 519:3748–65. doi: 10.1002/cne.22688

45. Zhao E, Basak A, Wong AOL, Ko W, Chen A, López GC, et al. The secretogranin II-derived peptide secretoneurin stimulates luteinizing hormone secretion from gonadotrophs. *Endocrinology* (2009) 150:2273–82. doi: 10.1210/en.2008-1060

46. Mitchell K, Zhang WS, Lu C, Tao B, Chen L, Hu W, et al. Targeted mutation of secretogranin-2 disrupts sexual behavior and reproduction in zebrafish. *Proc Natl Acad Sci USA* (2020) 117:12772–83. doi: 10.1073/pnas.2002004117

47. Batten TFC, Cambre ML, Moons L, Vandesande F. Comparative distribution of neuropeptide-immunoreactive systems in the brain of the green molly, poecilia latipinna. *J Comp Neurol* (1990) 302:893–919. doi: 10.1002/cne.903020416

48. Batten TFC, Moons L, Vandesande F. Innervation and control of the adenohypophysis by hypothalamic peptidergic neurons in teleost fishes: EM immunohistochemical evidence. *Microsc Res Tech* (1999) 44:19–35. doi: 10.1002/(SICI)1097-0029(19990101)44:1<19::AID-JEMT4>3.0.CO;2-L

49. Sokołowska E, Gozdowska M, Kulczykowska E. Nonapeptides arginine vasotocin and isotocin in fishes: Advantage of bioactive molecules measurement. *Front Mar Sci* (2020) 7:610. doi: 10.3389/fmars.2020.00610

50. Singh V, Joy KP. Immunocytochemical localization, HPLC characterization, and seasonal dynamics of vasotocin in the brain, blood plasma and gonads of the catfish heteropneustes fossilis. *Gen Comp Endocrinol* (2008) 159:214–25. doi: 10.1016/j.ygcen.2008.09.003

51. Saito D, Komatsuda M, Urano A. Functional organization of preoptic vasotocin and isotocin neurons in the brain of rainbow trout: central and neurohypophysial projections of single neurons. *Neuroscience* (2004) 124:973–84. doi: 10.1016/j.neuroscience.2003.12.038

52. Bobe J, Montfort J, Nguyen T, Fostier A. Identification of new participants in the rainbow trout (Oncorhynchus mykiss) oocyte maturation and ovulation processes using cDNA microarrays. *Reprod Biol Endocrinol* (2006) 4:39. doi: 10.1186/1477-7827-4-39

53. Chou M-Y, Hung J-C, Wu L-C, Hwang S-PL, Hwang P-P. Isotocin controls ion regulation through regulating ionocyte progenitor differentiation and proliferation. *Cell Mol Life Sci* (2011) 68:2797–809. doi: 10.1007/s00018-010-0593-2

54. Ramallo MR, Grober M, Cánepa MM, Morandini L, Pandolfi M. Arginine-vasotocin expression and participation in reproduction and social behavior in males of the cichlid fish cichlasoma dimerus. *Gen Comp Endocrinol* (2012) 179:221–31. doi: 10.1016/j.ygcen.2012.08.015

55. Banerjee P, Joy KP, Chaube R. Structural and functional diversity of nonapeptide hormones from an evolutionary perspective: A review. *Gen Comp Endocrinol* (2017) 241:4–23. doi: 10.1016/j.ygcen.2016.04.025

56. Mayasich SA, Clarke BL. Vasotocin and the origins of the vasopressin/oxytocin receptor gene family. *Vitam Horm* (2020) 113:1–12. doi: 10.1016/bs.vh.2019.08.018

57. Theofanopoulou C. Reconstructing the evolutionary history of the oxytocin and vasotocin receptor gene family: Insights on whole genome duplication scenarios. *Dev Biol* (2021) 479:99–106. doi: 10.1016/j.ydbio.2021.07.012

58. Theofanopoulou C, Gedman G, Cahill JA, Boeckx C, Jarvis ED. Universal nomenclature for oxytocin–vasotocin ligand and receptor families. *Nature* (2021) 592:747–55. doi: 10.1038/s41586-020-03040-7

59. Ocampo Daza D, Bergqvist CA, Larhammar D. The evolution of oxytocin and vasotocin receptor genes in jawed vertebrates: A clear case for gene duplications through ancestral whole-genome duplications. *Front Endocrinol* (2022) 12:792644. doi: 10.3389/fendo.2021.792644

60. Yamaguchi Y, Kaiya H, Konno N, Iwata E, Miyazato M, Uchiyama M, et al. The fifth neurohypophysial hormone receptor is structurally related to the V2-type receptor but functionally similar to V1-type receptors. *Gen Comp Endocrinol* (2012) 178:519–28. doi: 10.1016/j.ygcen.2012.07.008

61. Gimpl G, Fahrenholz F. The oxytocin receptor system: Structure, function, and regulation. *Physiol Rev* (2001) 81:629–83. doi: 10.1152/physrev.2001.81.2.629

62. Helfman G, Collette B, Facey D, Bowen BW. *The diversity of fishes: biology, evolution, and ecology. 2nd ed*. Chichester: Blackwell (2009).

63. Kline RJ, O'Connell LA, Hofmann HA, Holt GJ, Khan IA. The distribution of an AVT V1a receptor in the brain of a sex changing fish, epinephelus adscensionis. *J Chem Neuroanat* (2011) 42:72–88. doi: 10.1016/j.jchemneu.2011.06.005

64. Yokoi S, Naruse K, Kamei Y, Ansai S, Kinoshita M, Mito M, et al. Sexually dimorphic role of oxytocin in medaka mate choice. *Proc Natl Acad Sci USA* (2020) 117:4802–8. doi: 10.1073/pnas.1921446117

65. Semsar K, Kandel FLM, Godwin J. Manipulations of the AVT system shift social status and related courtship and aggressive behavior in the bluehead wrasse. *Horm Behav* (2001) 40:21–31. doi: 10.1006/hbeh.2001.1663

66. Singh O, Kumar S, Singh U, Bhute Y, Singru PS. Role of isotocin in the regulation of the hypophysiotropic dopamine neurones in the preoptic area of the catfish, clarias batrachus. *J Neuroendocrinol* (2016) 28:1–11. doi: 10.1111/jne.12441

67. Wee CL, Song E, Nikitchenko M, Herrera KJ, Wong S, Engert F, et al. Social isolation modulates appetite and avoidance behavior *via* a common oxytocinergic circuit in larval zebrafish. *Nat Commun* (2022) 13:2573. doi: 10.1038/s41467-022-29765-9

68. Blechman J, Amir-Zilberstein L, Gutnick A, Ben-Dor S, Levkowitz G. The metabolic regulator PGC-1α directly controls the expression of the hypothalamic

neuropeptide oxytocin. *J Neurosci* (2011) 31:14835–40. doi: 10.1523/JNEUROSCI.1798-11.2011

69. Coffey CM, Solleveld PA, Fang J, Roberts AK, Hong S-K, Dawid IB, et al. Novel oxytocin gene expression in the hindbrain is induced by alcohol exposure: transgenic zebrafish enable visualization of sensitive neurons. *PloS One* (2013) 8: e53991. doi: 10.1371/journal.pone.0053991

70. Canosa LF, Chang JP, Peter RE. Neuroendocrine control of growth hormone in fish. *Gen Comp Endocrinol* (2007) 151:1–26. doi: 10.1016/j.ygcen.2006.12.010

71. Pouso P, Perrone R, Silva A. Immunohistochemical description of isotocin neurons and the anatomo-functional comparative analysis between isotocin and vasotocin systems in the weakly electric fish, gymnotus omaroum. *Gen Comp Endocrinol* (2021) 313:113886. doi: 10.1016/j.ygcen.2021.113886

72. Pouso P, Radmilovich M, Silva A. An immunohistochemical study on the distribution of vasotocin neurons in the brain of two weakly electric fish, gymnotus omarorum and brachyhypopomus gauderio. *Tissue Cell* (2017) 49:257–69. doi: 10.1016/j.tice.2017.02.003

73. Arias Padilla LF, Castañeda-Cortés DC, Rosa IF, Moreno Acosta OD, Hattori RS, Nóbrega RH, et al. Cystic proliferation of germline stem cells is necessary to reproductive success and normal mating behavior in medaka. *Elife* (2021) 10:e62757. doi: 10.7554/eLife.62757

74. Rawat A, Chaube R, Joy KP. *In situ* localization of vasotocin receptor gene transcripts in the brain-pituitary-gonadal axis of the catfish heteropneustes fossilis: a morpho-functional study. *Fish Physiol Biochem* (2019) 45:885–905. doi: 10.1007/s10695-018-0590-1

75. Rawat A, Chaube R, Joy KP. Molecular cloning, sequencing, and phylogeny of vasotocin receptor genes in the air-breathing catfish heteropneustes fossilis with sex dimorphic and seasonal variations in tissue expression. *Fish Physiol Biochem* (2015) 41:509–32. doi: 10.1007/s10695-015-0026-0

76. Lema SC, Sanders KE, Walti KA. Arginine vasotocin, isotocin and nonapeptide receptor gene expression link to social status and aggression in sex-dependent patterns. *J Neuroendocrinol* (2015) 27:142–57. doi: 10.1111/jne.12239

77. Iwasaki K, Taguchi M, Bonkowsky JL, Kuwada JY. Expression of arginine vasotocin receptors in the developing zebrafish CNS. *Gene Expr Patterns* (2013) 13:335–42. doi: 10.1016/j.gep.2013.06.005

78. Rahman MS, Kline RJ, Vázquez OA, Khan IA, Thomas P. Molecular characterization and expression of arginine vasotocin V1a2 receptor in Atlantic croaker brain: Potential mechanisms of its downregulation by PCB77. *J Biochem Mol Toxicol* (2020) 34: 1–12. doi: 10.1002/jbt.22500

79. Kline RJ, Holt GJ, Khan IA. Arginine vasotocin V1a2 receptor and GnRH-I co-localize in preoptic neurons of the sex changing grouper, epinephelus adscensionis. *Gen Comp Endocrinol* (2016) 225:33–44. doi: 10.1016/j.ygcen.2015.07.013

80. Huffman LS, O'Connell LA, Kenkel CD, Kline RJ, Khan IA, Hofmann HA. Distribution of nonapeptide systems in the forebrain of an African cichlid fish, astatotilapia burtoni. *J Chem Neuroanat* (2012) 44:86–97. doi: 10.1016/j.jchemneu.2012.05.002

81. Yang W, Zhang N, Wu Y, Zhang L, Zhang L, Zhang W. Oxytocin-like signal regulates lh cells directly but not fsh cells in the ricefield eel *Monopterus albus* †. *Biol Reprod* (2021) 104:399–409. doi: 10.1093/biolre/ioaa202

82. Lyu LK, Li JS, Wang XJ, Yao YJ, Li JF, Li Y, et al. Arg-vasotocin directly activates isotocin receptors and induces COX2 expression in ovoviviparous guppies. *Front Endocrinol* (2021) 12:617580. doi: 10.3389/fendo.2021.617580

83. Lema SC, Slane MA, Salvesen KE, Godwin J. Variation in gene transcript profiles of two V1a-type arginine vasotocin receptors among sexual phases of bluehead wrasse (Thalassoma bifasciatum). *Gen Comp Endocrinol* (2012) 179:451–64. doi: 10.1016/j.ygcen.2012.10.001

84. Joy KP, Chaube R. Vasotocin – a new player in the control of oocyte maturation and ovulation in fish. *Gen Comp Endocrinol* (2015) 221:54–63. doi: 10.1016/j.ygcen.2015.02.013

85. Mennigen JA, Martyniuk CJ, Crump K, Xiong H, Zhao E, Popesku J, et al. Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). *Physiol Genomics* (2008) 35:273–82. doi: 10.1152/physiolgenomics.90263.2008

86. Popesku JT, Mennigen JA, Chang JP, Trudeau VL. Dopamine d $_1$ receptor blockage potentiates AMPA-stimulated luteinising hormone release in the goldfish: D $_1$ r blockage potentiates AMPA-stimulated LH release. *J Neuroendocrinol* (2011) 23:302–9. doi: 10.1111/j.1365-2826.2011.02114.x

87. Mennigen JA, Volkoff H, Chang JP, Trudeau VL. The nonapeptide isotocin in goldfish: Evidence for serotonergic regulation and functional roles in the control of food intake and pituitary hormone release. *Gen Comp Endocrinol* (2017) 254:38–49. doi: 10.1016/j.ygcen.2017.09.008

88. Mahlmann S, Meyerhof W, Hausmann H, Heierhorst J, Schönrock C, Zwiers H, et al. Structure, function, and phylogeny of [Arg8] vasotocin receptors from teleost fish and toad. *PNAS* (1994) 91:1342–5. doi: 10.1073/pnas.91.4.1342

89. Hausmann H, Richters A, Kreienkamp HJ, Meyerhof W, Mattes H, Lederis K, et al. Mutational analysis and molecular modeling of the nonapeptide hormone binding domains of the [Arg8] vasotocin receptor. *Proc Natl Acad Sci USA* (1996) 93:6907–12. doi: 10.1073/pnas.93.14.6907

90. Hausmann H, Meyerhof W, Zwiers H, Lederis K, Richter D. Teleost isotocin receptor: structure, functional expression, mRNA distribution and phylogeny. *FEBS Lett* (1995) 370:227–30. doi: 10.1016/0014-5793(95)00832-T

91. Landin J, Hovey D, Xu B, Lagman D, Zettergren A, Larhammar D, et al. Oxytocin receptors regulate social preference in zebrafish. *Sci Rep* (2020) 10:5435. doi: 10.1038/s41598-020-61073-4

92. Trudeau V. Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, carassius auratus. *Rev Reprod* (1997) 2:55–68. doi: 10.1530/ror.0.0020055

93. Zhang D, Xiong H, Mennigen JA, Popesku JT, Marlatt VL, Martyniuk CJ, et al. Defining global neuroendocrine gene expression patterns associated with reproductive seasonality in fish. *PloS One* (2009) 4:e5816. doi: 10.1371/journal.pone.0005816

94. Mennigen JA, Lado WE, Zamora JM, Duarte-Guterman P, Langlois VS, Metcalfe CD, et al. Waterborne fluoxetine disrupts the reproductive axis in sexually mature male goldfish, carassius auratus. *Aquat Toxicol* (2010) 100:354–64. doi: 10.1016/j.aquatox.2010.08.016

95. Sorensen PW, Pinillos M, Scott AP. Sexually mature male goldfish release large quantities of androstenedione into the water where it functions as a pheromone. *Gen Comp Endocrinol* (2005) 140:164–75. doi: 10.1016/j.ygcen.2004.11.006

96. Mangiamele LA, Keeney ADT, D'Agostino EN, Thompson RR. Pheromone exposure influences preoptic arginine vasotocin gene expression and inhibits social approach behavior in response to rivals but not potential mates. *Brain Behav Evol* (2013) 81:194–202. doi: 10.1159/000350589

97. Sokołowska E, Kleszczyńska A, Nietrzeba M, Kulczykowska E. Annual changes in brain concentration of arginine vasotocin and isotocin correspond with phases of reproductive cycle in round goby, neogobius melanostomus. *Chronobiol Int* (2015) 32:917–24. doi: 10.3109/07420528.2015.1052142

98. Kalamarz-Kubiak H, Gozdowska M, Guellard T, Kulczykowska E. . doi: 10.1242/bio.024844

99. Maruska KP, Mizobe MH, Tricas TC. Sex and seasonal co-variation of arginine vasotocin (AVT) and gonadotropin-releasing hormone (GnRH) neurons in the brain of the halfspotted goby. *Comp Biochem Physiol Part A Mol Integr* (2007) 147:129–44. doi: 10.1016/j.cbpa.2006.12.019

100. Kleszczyńska A, Sokołowska E, Kulczykowska E. Variation in brain arginine vasotocin (AVT) and isotocin (IT) levels with reproductive stage and social status in males of three-spined stickleback (Gasterosteus aculeatus). *Gen Comp Endocrinol* (2012) 175:290–6. doi: 10.1016/j.ygcen.2011.11.022

101. Kulczykowska E, Kleszczyńska A. Brain arginine vasotocin and isotocin in breeding female three-spined sticklebacks (Gasterosteus aculeatus): The presence of male and egg deposition. *Gen Comp Endocrinol* (2014) 204:8–12. doi: 10.1016/j.ygcen.2014.04.039

102. Ota Y, Ando H, Ban M, Ueda H, Urano A. Sexually different expression of neurohypophysial hormone genes in the preoptic nucleus of pre-spawning chum salmon. *Zool Sci* (1996) 13:593–601. doi: 10.2108/zsj.13.593

103. Hiraoka S, Ando H, Ban M, Ueda H, Urano A. Changes in expression of neurohypophysial hormone genes during spawning migration in chum salmon, oncorhynchus keta. *J Mol Endocrinol* (1997) 18:49–55. doi: 10.1677/jme.0.0180049

104. Makino K, Onuma TA, Kitahashi T, Ando H, Ban M, Urano A. Expression of hormone genes and osmoregulation in homing chum salmon: A minireview. *Gen Comp Endocrinol* (2007) 152:304–9. doi: 10.1016/j.ygcen.2007.01.010

105. Aubin-Horth N, Letcher BH, Hofmann HA. Gene-expression signatures of Atlantic salmon's plastic life cycle. *Gen Comp Endocrinol* (2009) 163:278–84. doi: 10.1016/j.ygcen.2009.04.021

106. Guiry A, Flynn D, Hubert S, O'Keeffe AM, LeProvost O, White SL, et al. Testes and brain gene expression in precocious male and adult maturing Atlantic salmon (Salmo salar). *BMC Genomics* (2010) 11:211. doi: 10.1186/1471-2164-11-211

107. Ota Y, Ando H, Ueda H, Urano A. Differences in seasonal expression of neurohypophysial hormone genes in ordinary and precocious Male masu salmon. *Gen Comp Endocrinol* (1999) 116:40–8. doi: 10.1006/gcen.1999.7344

108. Maruska KP. Social regulation of reproduction in male cichlid fishes. *Gen Comp Endocrinol* (2014) 207:2–12. doi: 10.1016/j.ygcen.2014.04.038

109. Greenwood AK, Wark AR, Fernald RD, Hofmann HA. Expression of arginine vasotocin in distinct preoptic regions is associated with dominant and subordinate behaviour in an African cichlid fish. *Proc R Soc B* (2008) 275:2393–402. doi: 10.1098/rspb.2008.0622

110. Bass AH, Grober MS. Social and neural modulation of sexual plasticity in teleost fish. *Brain Behav Evol* (2001) 57:293–300. doi: 10.1159/000047247

111. Semsar K, Godwin J. Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish. *J Neurosci* (2003) 23:4386–93. doi: 10.1523/JNEUROSCI.23-10-04386.2003

112. Godwin J, Sawby R, Warner RR, Crews D, Grober MS. Hypothalamic arginine vasotocin mRNA abundance variation across sexes and with sex change in a coral reef fish. *Brain Behav Evol* (2000) 55:77–84. doi: 10.1159/000006643

113. Grober MS, Sunobe T. Serial adult sex change involves rapid and reversible changes in forebrain neurochemistry. *Neuroreport* (1996) 7:2945–9. doi: 10.1097/00001756-199611250-00029

114. Kulczykowska E, Kalamarz-Kubiak H, Nietrzeba M, Gozdowska M. Brain nonapeptide and gonadal steroid responses to deprivation of heterosexual contact in the black molly. *Biol Open* (2015) 4:69–78. doi: 10.1242/bio.20149597

115. Liu H, Lamm MS, Rutherford K, Black MA, Godwin JR, Gemmell NJ. Large-Scale transcriptome sequencing reveals novel expression patterns for key sex-related genes in a sex-changing fish. *Biol Sex Differ* (2015) 6:26. doi: 10.1186/s13293-015-0044-8

116. Black MP, Reavis RH, Grober MS. Socially induced sex change regulates forebrain isotocin in lythrypnus dalli. *NeuroReport* (2004) 15:185–9. doi: 10.1097/00001756-200401190-00036

117. Godwin J, Thompson R. Nonapeptides and social behavior in fishes. *Horm Behav* (2012) 61:230–8. doi: 10.1016/j.yhbeh.2011.12.016

118. Yokoi S, Okuyama T, Kamei Y, Naruse K, Taniguchi Y, Ansai S, et al. An essential role of the arginine vasotocin system in mate-guarding behaviors in triadic relationships of medaka fish (Oryzias latipes). *PloS Genet* (2015) 11:e1005009. doi: 10.1371/journal.pgen.1005009

119. Salek SJ, Sullivan CV, Godwin J. Arginine vasotocin effects on courtship behavior in male white perch (Morone americana). *Behav Brain Res* (2002) 133:177–83. doi: 10.1016/S0166-4328(02)00003-7

120. Santangelo N, Bass AH. Individual behavioral and neuronal phenotypes for arginine vasotocin mediated courtship and aggression in a territorial teleost. *Brain Behav Evol* (2010) 75:282–91. doi: 10.1159/000316867

121. Pouso P, Cabana Á, Goodson JL, Silva A. Preoptic area activation and vasotocin involvement in the reproductive behavior of a weakly pulse-type electric fish, brachyhypopomus gauderio. *Front Integr Neurosci* (2019) 13:37. doi: 10.3389/fnint.2019.00037

122. Perrone R, Silva A. Vasotocin increases dominance in the weakly electric fish brachyhypopomus gauderio. *J Physiol-Paris* (2016) 110:119–26. doi: 10.1016/j.jphysparis.2016.12.004

123. Carneiro LA, Oliveira RF, Canário AVM, Grober MS. The effect of arginine vasotocin on courtship behaviour in a blenniid fish with alternative reproductive tactics. *Fish Physiol Biochem* (2003) 28:241–3. doi: 10.1023/B:FISH.0000030542.31395.8a

124. DeAngelis R, Gogola J, Dodd L, Rhodes JS. Opposite effects of nonapeptide antagonists on paternal behavior in the teleost fish amphiprion ocellaris. *Horm Behav* (2017) 90:113–9. doi: 10.1016/j.yhbeh.2017.02.013

125. DeAngelis R, Dodd L, Rhodes J. Nonapeptides mediate trade-offs in parental care strategy. *Horm Behav* (2020) 121:104717. doi: 10.1016/j.yhbeh.2020.104717

126. O'Connell LA, Matthews BJ, Hofmann HA. Isotocin regulates paternal care in a monogamous cichlid fish. *Horm Behav* (2012) 61:725–33. doi: 10.1016/j.yhbeh.2012.03.009

127. Wacker DW, Ludwig M. Vasopressin, oxytocin, and social odor recognition. *Horm Behav* (2012) 61(3):259–65. doi: 10.1016/j.yhbeh.2011.08.014

128. Dunlap KD, Koukos HM, Chagnaud BP, Zakon HH, Bass AH. Vocal and electric fish: Revisiting a comparison of two teleost models in the neuroethology of social behavior. *Front Neural Circuits* (2021) 15:713105. doi: 10.3389/fncir.2021.713105

129. Somoza GM, Yu KL, Peter RE. Serotonin stimulates gonadotropin release in female and male goldfish, *Carassius auratus* l. *Gen Comp Endocrinol* (1988) 72:374–82. doi: 10.1016/0016-6480(88)90159-1

130. Saito D, Hasegawa Y, Urano A. Gonadotropin-releasing hormones modulate electrical activity of vasotocin and isotocin neurons in the brain of rainbow trout. *Neurosci Lett* (2003) 351:107–10. doi: 10.1016/j.neulet.2003.08.017

131. Kanda S, Akazome Y, Mitani Y, Okubo K, Oka Y. Neuroanatomical evidence that kisspeptin directly regulates isotocin and vasotocin neurons. *PloS One* (2013) 8:e62776. doi: 10.1371/journal.pone.0062776

132. Zmora N, Stubblefield JD, Wong T-T, Levavi-Sivan B, Millar RP, Zohar Y. Kisspeptin antagonists reveal kisspeptin 1 and kisspeptin 2 differential regulation of reproduction in the teleost, morone saxatilis. *Biol Reprod* (2015) 93:1–12. doi: 10.1095/biolreprod.115.131870

133. Trudeau VL, Somoza GM. Multimodal hypothalamo-hypophysial communication in the vertebrates. *Gen Comp Endocrinol* (2020) 293:113475. doi: 10.1016/j.ygcen.2020.113475

134. Chaube R, Singh RK, Joy KP. Estrogen regulation of brain vasotocin secretion in the catfish heteropneustes fossilis: An interaction with catecholaminergic system. *Gen Comp Endocrinol* (2012) 175:206–13. doi: 10.1016/j.ygcen.2011.11.012

135. Yamashita J, Kawabata Y, Okubo K. Expression of isotocin is male-specifically up-regulated by gonadal androgen in the medaka brain. *J Neuroendocrinol* (2017) 29:e12545. doi: 10.1111/jne.12545

136. Da Fonte DF, Xing L, Mikwar M, Trudeau VL. Secretoneurin-a inhibits aromatase b (cyp19a1b) expression in female goldfish (*Carassius auratus*) radial glial cells. *Gen Comp Endocrinol* (2018) 257:106–12. doi: 10.1016/j.ygcen.2017.04.014

137. Popesku JT, Martyniuk CJ, Mennigen J, Xiong H, Zhang D, Xia X, et al. The goldfish (Carassius auratus) as a model for neuroendocrine signaling. *Mol Cell Endocrinol* (2008) 293:43–56. doi: 10.1016/j.mce.2008.06.017

138. Singh U, Kumar S, Singru PS. Interaction between dopamine- and isotocin-containing neurones in the preoptic area of the catfish, clarias batrachus: role in the regulation of luteinising hormone cells. *J Neuroendocrinol* (2012) 24:1398–411. doi: 10.1111/j.1365-2826.2012.02350.x

139. Robinson G, Evans JJ, Catt KJ. Oxytocin stimulates LH production by the anterior pituitary gland of the rat. *J Endocrinol* (1992) 132:277–83. doi: 10.1677/joe.0.1320277

140. Evans JJ, Robinson G, Catt KJ. Luteinizing hormone response to oxytocin is steroid-dependent. *Neuroendocrinology* (1992) 55:538–43. doi: 10.1159/000126167

141. Rodríguez M, Specker JL. *In vitro* effects of arginine vasotocin on testosterone production by testes of rainbow trout (Oncorhynchus mykiss). *Gen Comp Endocrinol* (1991) 83:249–57. doi: 10.1016/0016-6480(91)90028-5

142. Singh V, Joy KP. Relative *in vitro* seasonal effects of vasotocin and isotocin on ovarian steroid hormone levels in the catfish heteropneustes fossilis. *Gen Comp Endocrinol* (2009) 162:257–64. doi: 10.1016/j.ygcen.2009.03.024

143. Mishra A, Joy KP. Effects of gonadotrophin *in vivo* and 2-hydroxyoestradiol-17β *in vitro* on follicular steroid hormone profile associated with oocyte maturation in the catfish heteropneustes fossilis. *J Endocrinol* (2006) 189:341–53. doi: 10.1677/joe.1.06686

144. Nagahama Y. Endocrine regulation of gametogenesis in fish. *Int J Dev Biol* (1994) 38:217–29.

145. Singh V, Joy KP. Vasotocin induces final oocyte maturation and ovulation through the production of a maturation-inducing steroid in the catfish heteropneustes fossilis. *Gen Comp Endocrinol* (2011) 174(1):15–21. doi: 10.1016/j.ygcen.2011.07.009

146. Sirotkin AV, Gerasimova GG, Golubev AK, Dmitriev VB. The effect of arginine-vasotocin on the production of steroid hormones by mouse, cow, and chicken ovarian tissues. *vitro. BioMed Sci* (1990) 1:308–10.

147. Lister AL, van der Kraak G. An investigation into the role of prostaglandins in zebrafish oocyte maturation and ovulation. *Gen Comp Endocrinol* (2008) 159:46–57. doi: 10.1016/j.ygcen.2008.07.017

148. Bradley JA, Goetz FW. The inhibitory effects of indomethacin, nordihydroguaiaretic acid, and pyrrolidinedithiocarbamate on ovulation and prostaglandin synthesis in yellow perch (Perca flavescens) follicle incubates. *Prostaglandins* (1994) 48:11–20. doi: 10.1016/0090-6980(94)90092-2

149. Patiño R, Yoshizaki G, Bolamba D, Thomas P. Role of arachidonic acid and protein kinase c during maturation-inducing hormone-dependent meiotic resumption and ovulation in ovarian follicles of Atlantic croaker. *Biol Reprod* (2003) 68:516–23. doi: 10.1095/biolreprod.102.009662

150. Joy KP, Singh V. Functional interactions between vasotocin and prostaglandins during final oocyte maturation and ovulation in the catfish heteropneustes fossilis. *Gen Comp Endocrinol* (2013) 186:126–35. doi: 10.1016/j.ygcen.2013.02.043

151. Cerdà J. Molecular pathways during marine fish egg hydration: the role of aquaporins. *J Fish Biol* (2009) 75:2175–96. doi: 10.1111/j.1095-8649.2009.02397.x

152. Singh V, Joy KP. An involvement of vasotocin in oocyte hydration in the catfish heteropneustes fossilis: A comparison with effects of isotocin and hCG. *Gen Comp Endocrinol* (2010) 166:504–12. doi: 10.1016/j.ygcen.2010.02.020

153. Chaube R, Chauvigné F, Tingaud-Sequeira A, Joy KP, Acharjee A, Singh V, et al. Molecular and functional characterization of catfish (Heteropneustes fossilis) aquaporin-1b: changes in expression during ovarian development and hormone-induced follicular maturation. *Gen Comp Endocrinol* (2011) 170:162–71. doi: 10.1016/j.ygcen.2010.10.002

154. Acharjee A, Chaube R, Joy KP, Cerda J. Hormonal regulation of aquaporin-1ab in heteropneustes fossilis oocytes *in vitro*. *Indian J Sci Technol* (2011) 4: 165–166.

155. Wilhelmi AE, GE P, Sawyer WH. Initiation of the spawning reflex response in fundulus by the administration of fish and mammalian neurohypophysial

preparations and synthetic oxytocin. *Endocrinology* (1955) 57:243–52. doi: 10.1210/endo-57-2-243

156. Viveiros ATM, Jatzkowski A, Komen J. Effects of oxytocin on semen release response in African catfish (Clarias gariepinus). *Theriogenology* (2003) 59:1905–17. doi: 10.1016/s0093-691x(02)01290-6

157. Wisdom KS, Bhat IA, Pathan MA, CT I, Kumar P, Babu PG, et al. Teleost nonapeptides, isotocin and vasotocin administration released the milt by abdominal massage in Male catfish, clarias magur. *Front Endocrinol* (2022) 13:899463. doi: 10.3389/fendo.2022.899463

158. Venkatesh B, Tan CH, Lam TJ. Prostaglandins and teleost neurohypophyseal hormones induce premature parturition in the guppy, poecilia reticulata. *Gen Comp Endocrinol* (1992) 87:28–32. doi: 10.1016/0016-6480(92)90146-b

159. Xiong S, Tian J, Ge S, Li Z, Long Z, Guo W, et al. The microRNA-200 cluster on chromosome 23 is required for oocyte maturation and ovulation in zebrafish. *Biol Reprod* (2020) 103:769–78. doi: 10.1093/biolre/ioaa125

160. Peter RE. The preoptic nucleus in fishes: A comparative discussion of function-activity relationships. *Amer Zool* (1977) . 17:775–85. doi: 10.1093/icb/17.4.775

161. Zohar Y. Fish reproductive biology - reflecting on five decades of fundamental and translational research. *Gen Comp Endocrinol* (2021) 300:113544. doi: 10.1016/j.ygcen.2020.113544

162. Arcand-Hoy LD, Benson WH. Fish reproduction an ecologically relevant indicator of fish endocrine disruption. *Env Toxicol Chem* (1998) 17:49–57. doi: 10.1002/etc.5620170108

163. Ram RN, Joy KP. Mercurial induced changes in the hypothalamo-neurohypophysical complex in relation to reproduction in the teleostean fish, channa punctatus (Bloch). *Bull Environ Contam Toxicol* (1988) 41:329–36. doi: 10.1007/BF01688875

164. Mennigen JA, Zamora JM, Chang JP, Trudeau VL. Endocrine disrupting effects of waterborne fluoxetine exposure on the reproductive axis of female goldfish, carassius auratus. *Comp Biochem Physiol Part-C: Toxicol Pharmacol* (2017) 202:70–8. doi: 10.1016/j.cbpc.2017.08.003

165. Parolini M, Ghilardi A, De Felice B, Del Giacco L. Environmental concentration of fluoxetine disturbs larvae behavior and increases the defense response at molecular level in zebrafish (Danio rerio). *Environ Sci pollut Res Int* (2019) 26:34943–52. doi: 10.1007/s11356-019-06619-4

166. Wong RY, Oxendine SE, Godwin J. Behavioral and neurogenomic transcriptome changes in wild-derived zebrafish with fluoxetine treatment. *BMC Genomics* (2013) 14:348. doi: 10.1186/1471-2164-14-348

167. Semsar K, Perreault HAN, Godwin J. Fluoxetine-treated male wrasses exhibit low AVT expression. *Brain Res* (2004) 1029:141–7. doi: 10.1016/j.brainres.2004.09.030

168. Cantor JM, Binik YM, Pfaus JG. Chronic fluoxetine inhibits sexual behavior in the male rat: reversal with oxytocin. *Psychopharmacol (Berl)* (1999) 144:355–62. doi: 10.1007/s002130051018

169. de Jong TR, Veening JG, Olivier B, Waldinger MD. Oxytocin involvement in SSRI-induced delayed ejaculation: a review of animal studies. *J Sex Med* (2007) 4:14–28. doi: 10.1111/j.1743-6109.2006.00394.x

170. Jørgensen HS. Studies on the neuroendocrine role of serotonin. *Dan Med Bull* (2007) 54:266–88.

171. Mennigen JA, Stroud P, Zamora JM, Moon TW, Trudeau VL. Pharmaceuticals as neuroendocrine disruptors: lessons learned from fish on Prozac. *J Toxicol Environ Health B Crit Rev* (2011) 14:387–412. doi: 10.1080/10937404.2011.578559

172. Wang Z, Mao K, Du W, Cai M, Zhang Z, Li X. Diluted concentrations of methamphetamine in surface water induce behavior disorder, transgenerational toxicity, and ecosystem-level consequences of fish. *Water Res* (2020) 184:116164. doi: 10.1016/j.watres.2020.116164

173. León-Olea M, Martyniuk CJ, Orlando EF, Ottinger MA, Rosenfeld C, Wolstenholme J, et al. Current concepts in neuroendocrine disruption. *Gen Comp Endocrinol* (2014) 203:158–73. doi: 10.1016/j.ygcen.2014.02.005

174. Salahinejad A, Naderi M, Attaran A, Meuthen D, Niyogi S, Chivers DP. Effects of chronic exposure to bisphenol-s on social behaviors in adult zebrafish: Disruption of the neuropeptide signaling pathways in the brain. *Environ pollut* (2020) 262:113992. doi: 10.1016/j.envpol.2020.113992

175. Salahinejad A, Attaran A, Meuthen D, Rachamalla M, Chivers DP, Niyogi S. Maternal exposure to bisphenol s induces neuropeptide signaling dysfunction and oxidative stress in the brain, and abnormal social behaviors in zebrafish (Danio rerio) offspring. *Sci Total Environ* (2022) 830:154794. doi: 10.1016/j.scitotenv.2022.154794

176. Naderi M, Puar P, JavadiEsfahani R, Kwong RWM. Early developmental exposure to bisphenol a and bisphenol s disrupts socio-cognitive function, isotocin equilibrium, and excitation-inhibition balance in developing zebrafish. *Neurotoxicology* (2022) 88:144–54. doi: 10.1016/j.neuro.2021.11.009

177. Naderi F, Miguez JM, Soengas JL, López-Patiño MA. SIRT1 mediates the effect of stress on hypothalamic clock genes and food intake regulators in rainbow trout, oncorhynchus mykiss. *Comp Biochem Physiol A Mol Integr Physiol* (2019) 235:102–11. doi: 10.1016/j.cbpa.2019.05.021

178. Kalamarz-Kubiak H. Endocrine-disrupting compounds in fish physiology, with emphasis on their effects on the arginine Vasotocin/Isotocin system. *Endocr Metab Immune Disord Drug Targets* (2021) 22(7):738–47. doi: 10.2174/1871530321666210202150947

179. Patisaul HB. Endocrine disruption of vasopressin systems and related behaviors. *Front Endocrinol (Lausanne)* (2017) 8:134. doi: 10.3389/fendo.2017.00134

180. Reilly MP, Kunkel MN, Thompson LM, Zentay A, Weeks CD, Crews D, et al. Effects of endocrine-disrupting chemicals on hypothalamic oxytocin and vasopressin systems. *J Exp Zool A Ecol Integr Physiol* (2022) 337:75–87. doi: 10.1002/jez.2475

frontiers | Frontiers in Endocrinology

TYPE Review
PUBLISHED 16 December 2022
DOI 10.3389/fendo.2022.1056939

Check for updates

OPEN ACCESS

EDITED BY
Bin Wang,
Yellow Sea Fisheries Research Institute (CAFS), China

REVIEWED BY
Guangfu Hu,
Huazhong Agricultural University, China
Honoo Satake,
Suntory Foundation for Life Sciences, Japan
Li Shuisheng,
Sun Yat-sen University, China

*CORRESPONDENCE
Karine Rousseau
karine.rousseau@mnhn.fr

SPECIALTY SECTION
This article was submitted to Neuroendocrine Science, a section of the journal Frontiers in Endocrinology

RECEIVED 29 September 2022
ACCEPTED 07 November 2022
PUBLISHED 16 December 2022

CITATION
Campo A, Dufour S and Rousseau K (2022) Tachykinins, new players in the control of reproduction and food intake: A comparative review in mammals and teleosts.
Front. Endocrinol. 13:1056939.
doi: 10.3389/fendo.2022.1056939

COPYRIGHT
© 2022 Campo, Dufour and Rousseau. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) and the copyright owner(s) are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.

Tachykinins, new players in the control of reproduction and food intake: A comparative review in mammals and teleosts

Aurora Campo[1,2], Sylvie Dufour[1] and Karine Rousseau[1,3]*

[1]Muséum National d'Histoire Naturelle, Research Unit Unité Mixte de Recherche Biologie des Organsimes et Ecosystèmes Aquatiques (UMR BOREA), Biology of Aquatic Organisms and Ecosystems, Centre National pour la Recherche Scientifique (CNRS), Institut de Recherche pour le Développemen (IRD), Sorbonne Université, Paris, France, [2]Volcani Institute, Agricultural Research Organization, Rishon LeTsion, Israel, [3]Muséum National d'Histoire Naturelle, Research Unit PhyMA Physiologie Moléculaire et Adaptation CNRS, Paris, France

In vertebrates, the tachykinin system includes tachykinin genes, which encode one or two peptides each, and tachykinin receptors. The complexity of this system is reinforced by the massive conservation of gene duplicates after the whole-genome duplication events that occurred in vertebrates and furthermore in teleosts. Added to this, the expression of the tachykinin system is more widespread than first thought, being found beyond the brain and gut. The discovery of the co-expression of neurokinin B, encoded by the tachykinin 3 gene, and kisspeptin/dynorphin in neurons involved in the generation of GnRH pulse, in mammals, put a spotlight on the tachykinin system in vertebrate reproductive physiology. As food intake and reproduction are linked processes, and considering that hypothalamic hormones classically involved in the control of reproduction are reported to regulate also appetite and energy homeostasis, it is of interest to look at the potential involvement of tachykinins in these two major physiological functions. The purpose of this review is thus to provide first a general overview of the tachykinin system in mammals and teleosts, before giving a state of the art on the different levels of action of tachykinins in the control of reproduction and food intake. This work has been conducted with a comparative point of view, highlighting the major similarities and differences of tachykinin systems and actions between mammals and teleosts.

KEYWORDS

tachykinins, neurokinins, substance P, endokinin/hemokinin, reproduction, food intake, mammals, teleosts

1 Introduction

Tachykinins (TAC) are members of a large family of peptides present from cnidaria [for reviews (1, 2)]: to bilateria [for reviews (1–8):. Tachykinins are usually considered as brain and gut peptides, as they are mainly expressed in neurons from the central nervous system and from the gastrointestinal tract. However, they are also present in non-neuronal cells, such as the immune and inflammatory cells of mammals, and various tissues like the skin of amphibians, as well as the salivary gland of mosquito and octopus, where they serve for exocrine secretion [for reviews (1, 4, 6, 8)]. In addition, in sea squirt, they are found in endostyle and gonad (8), where they act as neurotransmitters of endocrine and local autocrine/paracrine regulations [for reviews (4, 8)].

Since the discovery of the co-expression of neurokinin B, encoded by tachykinin 3 gene (*tac3*), and kisspeptin/dynorphin in neurons involved in the generation of GnRH pulses in mammals, rekindled attention has emerged for studying tachykinins in vertebrate reproductive physiology. Reproduction is classically controlled by the hypothalamus–pituitary–gonad (HPG) neuroendocrine axis in vertebrates [for review (9)]. The gonadotropin-releasing hormone (GnRH), produced and released by hypothalamic neurons, acts on the pituitary to stimulate the synthesis and release of gonadotropins, luteinizing hormone (LH), and follicle-stimulating hormone (FSH). These pituitary hormones act themselves on the gonads to control gametogenesis and the production of sex steroids, mainly estrogens in females and androgens in males. These peripheral hormones exert feedbacks at brain and pituitary levels to regulate GnRH and gonadotropin production. The hypothalamus is also the cerebral center involved in the control of food intake, integrating both external and internal factors and producing neuropeptides stimulating (orexigenic) or inhibiting (anorexigenic) appetite [for reviews (10–13)].

In vertebrates, feeding and reproduction are linked processes, as the presence of sufficient energy reserves is critical to achieve successful reproduction [for reviews (14, 15)]. Any state of negative energy balance thus affects not only central appetite-regulating systems but, often, also reproductive pathways and reproductive performance. Hypothalamic hormones classically involved in the control of reproduction, such as kisspeptin, are reported to regulate appetite and energy homeostasis as well, in mammals [for reviews (16–18)] and teleosts [for review (19)].

As teleosts represent the most diversified group of vertebrates, with nearly 30,000 species, species-specific regulating mechanisms are often encountered inside this lineage. In addition, some physiological differences exist between fish and mammalian regulatory mechanisms, even if major regulatory features are conserved. Some differences may be due to anatomical specificities of teleost neuroendocrine systems such as direct neuronal innervation and cell regionalization of the pituitary [for reviews (9, 20)]. Major breakthroughs in the studies of neuropeptide actions in teleosts have been allowed thanks to recently available published genomes and novel genome editing technics. These new tools are of particular interest and necessity in this group of vertebrates, as due to the teleost-specific whole-genome duplication (3R), teleosts possess an expanded number of genes encoding hormones/peptides that will share initial pleiotropic functions (subfunctionalization) or get new functions (neofunctionalization) [for review (9)].

With a comparative perspective, the purpose of this review is to provide a general overview of the tachykinins and their receptors in mammals and teleosts and then focus on the state-of-the-art literature on the different levels of action of tachykinins in the control of reproduction and food intake in these two groups of vertebrates.

2 Tachykinin system

Some of the first peptides of the tachykinin (TAC) family were discovered in neurons in mammals and therefore named neurokinins (NK). However, many subsequent data showed their production by non-neuronal cells. Especially the discovery in 2000 by Zhang and collaborators of a third tachykinin gene, PPT-C (21), renamed *tac4* and encoding several new tachykinin peptides, with widespread peripheral distributions and with a preferred receptor NK1 receptor, led to debates on their nomenclature [for reviews (22, 23)]. A revised nomenclature was proposed, with the preferred term "tachykinin" compared with "neurokinin," which then appeared inappropriate. Similarly, for tachykinin receptors, for example, the NK1 receptor can no longer be defined only as a substance P (SP) receptor [for reviews (23, 24)]. More recently, the Human Genome Organization (HUGO) Gene Nomenclature Committee approved the names TACR1, TACR2, and TACR3 for the three TAC receptors (25). This nomenclature will be adopted in our review. In the following text, we will use TAC for tachykinin peptides and *tac* for tachykinin genes and transcripts, and likewise TACR and *tacr* for the receptors.

A recent review highlights the widespread distribution and the functional pleiotropy of TACs and their receptors with a special focus on invertebrates (2) and is complementary to our present comparative review in vertebrates.

2.1 Tachykinins

The evolutionary scenario of tachykinins in chordates suggests that an ancestral *tac* gene in proto-chordates generated four paralogs [(26), for reviews (1, 27)] after the two

whole-genome duplication rounds (1R/2R) which occurred in early vertebrates (28, 29). A possible loss of one of the four paralogs occurred before the split of the ray-finned fish, actinopterygians (leading to teleost fish), and the lobe-finned fish, sarcopterygians (leading to mammals) [(26), for review (1)]. Among teleosts, a specific third-genome duplication (3R) produced a tetraploidization, followed by gene loss or conservation of duplicated paralogs (30, 31). Our recent study shows a wide conservation of the duplicated tachykinin genes in the teleost fish lineage (Campo et al. in preparation), thus increasing the scope of previous research (26).

Vertebrate *tac* genes consist of five to seven exons that encode a pre-pro-tachykinin (PPT) peptide, named PPT-A or PPT-I for *tac1*, PPT-B or PPT-II for *tac3*, and PPT-C or PPT-III for *tac4* [for reviews (4, 32–34)]. One or two peptides are cleaved from each of the three PPT [for reviews (1)]. The TAC peptide placed close to the N terminal of PPT is called TAC-related peptide or TACRP, while the other tachykinin peptide closer to the C terminal of PPT is named TAC. For the *tac1* gene, TACRP is substance P (SP) and TAC are neurokinin A (NKA), neuropeptide K (NPK), and neuropeptide γ (NPγ), these last two being NH2-terminally extended forms of NKA. For the *tac3* gene, TACRP (only found in teleosts) is TAC3RP or neurokinin B-related peptide (NKBRP) and TAC is NKB. For the *tac4* gene, TACRP are hemokinin-1 (HK1), endokinin A (EKA), and endokinin B (EKB), while TAC are endokinin C (EKC) and endokinin D (EKD), depending on the splicing variant (Figure 1). Indeed in mammals, differential alternative mRNA splicing and precursor processing are observed for each of the three tachykinin genes, and as the different transcripts are regulated in a tissue-specific manner [*tac1* (35–37); *tac3* (38); *tac4* (39); for reviews (40, 41)], these mechanisms are likely to

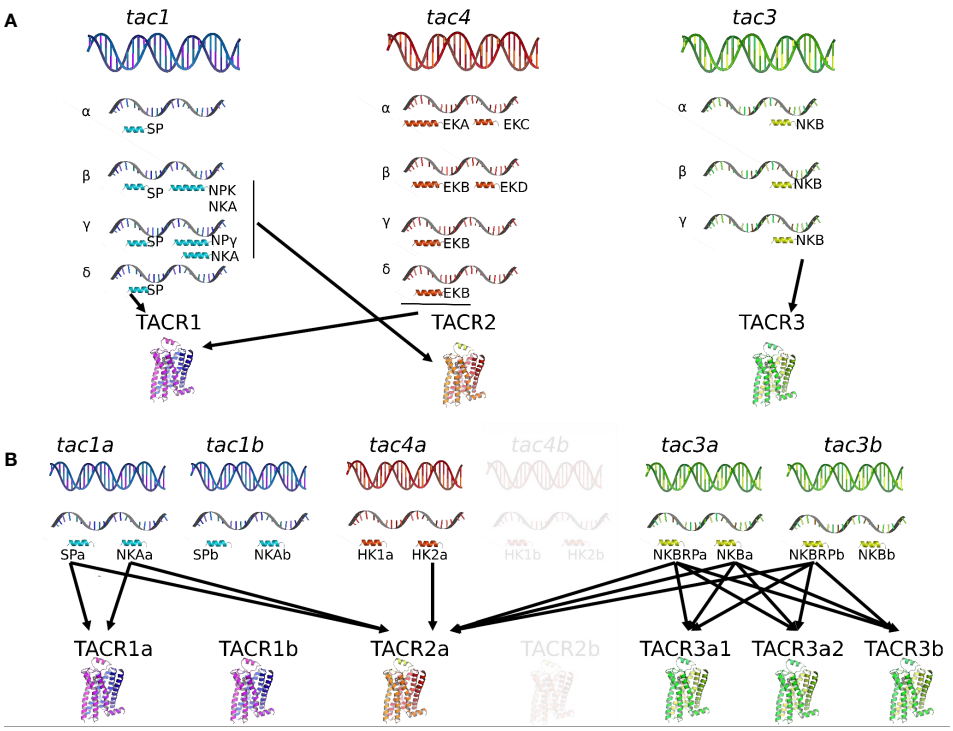


FIGURE 1
Comparison of the tachykinin system in a mammal, the human, and in a teleost, the grass carp. In human **(A)**, the tachykinin system comprises three *tac* genes (*tac1*, *tac2*, and *tac3*) encoding up to 10 different TAC peptides (SP, NKA, NPK, and NPγ for the *tac1* gene; HK1, EKA, EKB, EKC, and EKC for the *tac4* gene; NKB for the *tac3* gene) due to the existence of various spliced variants (α, β, γ, and δ for the tac1 and tac4 genes; (α, β, and γ for the tac3 gene). These human TAC peptides bind to three TACR (TACR1, TAC2, and TACR3) with different affinities: SP, HK1, EKA, and EKB for TACR1; NKA, NPK, and NPγ for TACR2; NKB for TACR3. In teleosts **(B)**, due to the teleost-specific whole-genome duplication (3R), duplicates for *tac1* (*tac1a* and *tac1b*), *tac3* (*tac3a* and *tac3b*), and *tac4* (*tac4a* and *tac4b*) exist and up to 12 different TAC peptides have been identified up to now. Up to six TACR have been yet characterized: two TACR1 (TACR1a and TACR1b), one TACR2, and three TACR3 (TACR3a1, TACR3a2, and TACR3b). One of the 3R-duplicated *tacr2* paralogs (*tacr2b*) was lost in the teleost lineage but conserved in the eels (elopomorphs) (Campo et al. in preparation). Binding studies with the complete available tachykinin system have only been performed in the grass carp, the unique teleost species for now in which the *tac4* gene has been identified and published. In this species, *tac4b* and *tacr2b* have not yet been identified and appear in transparency in the figure. For more information, please refer to part 1 of this review. EKA, endokinin A; EKB, endokinin B; EKC, endokinin C; EKD, endokinin D; HK1, hemokinin 1; HK2, hemokinin 2; NKA, neurokinin A; NKB, neurokinin B; NKBRP, neurokinin B-related peptide; NPK, neuropeptide K; NPγ, neuropeptide gamma; SP, substance P; TAC, tachykinin peptide; *tac*, tachykinin gene; TACR, tachykinin receptor (protein); *tacr*, tachykinin receptor gene.

play an important role in the pleiotropic actions of the various tachykinins.

TAC are characterized by a FxGLMamide carboxy-terminus, where x is a variable, aromatic or aliphatic, amino acid [for review (5)]. Some exceptions are found: for example, the human EKC and EKD present a substitution of the final M by L (42, 43). For NKA and NKB, as well as the extended forms of NKA (NPK and NPγ), x is always a valine, leading to a FVGLM C-terminal motif (44).

2.1.1 Tachykinin 1

2.1.1.1 Mammals

Four peptides can be translated from the gene *tac1* (Figure 1): substance P (SP) or TAC1RP from exon 3; neurokinin A (NKA) or TAC1 from exon 6; neuropeptide K (NPK) or TAC1-NPK from exons 4, 5, and 6; and neuropeptide gamma (NPγ) or TAC1-NPγ from exons 3, 5, and 6 [for reviews (5, 34)]. The *tac1* gene produces four different splicing variants (α-, β-, γ-, and δ-*tac1*): *α*- and *δ*-*tac1* generate only SP; *β*-*tac1* encodes SP, NKA, and NPK; *γ*-*tac1* generates SP, NKA, and NPγ [for reviews (5, 34, 45)]. Substance P/TAC1RP was the first neuropeptide ever to be extracted in 1931 [(46); for review (47)]. In their pioneer study, von Euler and Gaddum found in extracts from horse brain and intestine an atropine-resistant factor, which induced contraction of the isolated rabbit jejunum and transient hypotension in anesthetized rabbits (46). They named this new factor substance P, with P for powder. It was only in 1971 that SP was purified and sequenced from bovine hypothalamus (48) and synthetized (49). Neurokinin A/TAC1 was discovered later in extracts of porcine spinal cord by different research groups and named differently at that time: neurokinin α (50), substance K (51), or neuromedin L (52). As SP, it was involved in ileum contraction of guinea pig (50, 52). Further analyses of the pre-pro-peptide structure revealed that peptides other than SP and NKA were encoded by the precursor and that tissue-specific alternative splicing occurred (35, 51): neuropeptide K (NPK) with 36 amino acids and neuropeptide gamma (NPγ) with 21 amino acids. Both sequences share the last 10 amino acids in the C terminal with a NKA/TAC1 sequence. NPK, isolated from porcine brain, stimulates guinea pig gallbladder contraction, plasma extravasation, hypotension, and bronchial smooth muscle spasm (53). NPγ was isolated from rabbit intestine and found to derive from γ-pre-pro-tachykinin, hence its name (54).

2.1.1.2 Teleosts

The first TAC peptide to be characterized in teleosts was substance P. In 1956, a factor purified from cod brain and intestine extracts was found to have the same properties as those of mammalian substance P (55). Later, a tachykinin of 21 amino acid residues, which possesses mammalian NPγ characteristics and was named carassin, was isolated from the brain of the goldfish *Carassius auratus* (56). Then, SP and NKA were measured by radioimmunoassay in the brain of rainbow trout (57). In goldfish, Lin and Peter described two cDNAs encoding γ-PPT that may represent different transcripts resulting from the alternative transcriptional start sites and that contains the sequences of SP, carassin, and NKA (58). The *Tac1* gene was first characterized in zebrafish and found in the genomes of goldfish, medaka, and stickleback; it encodes SP and NKA (59, 60). One *tac1* gene was then found in many other teleost species, including grass carp (61). It was only recently that a second *tac1* gene, likely the result of the 3R, was identified in the grass carp; the duplicated genes were named *tac1a* and *tac1b* and shown to encode SPa and NKAa, and SPb and NKAb, respectively (62) (Figure 1). Before that study, it was thought that one of the duplicated *tac1* paralogs obtained by 3R was lost in teleosts [for review (1)]. Our recent bioinformatic studies revealed a wide conservation of the 3R-duplicated *tac1* genes, even those obtained by the further whole-genome duplication of the salmonids (4R) (Campo et al. in preparation).

2.1.2 Tachykinin 3

2.1.2.1 Mammals

The *Tac3* gene has been named *tac2* in rodents, while it is in fact an ortholog of human *tac3* (5, 63–66); so, for easier reading, rodent *tac2* will be replaced by *tac3* throughout this review. One peptide is encoded in the *tac3* gene: neurokinin B (NKB) or TAC3 (5, 32, 38, 41). NKB was purified from the extract of porcine spinal cord simultaneously by two research groups and given different names at that time: neurokinin β (50) or neuromedin K (67). As SP and NKA, it induces contraction of the guinea pig ileum (50, 67). The structure and gene organization of the neuromedin K/NKB precursor (or pre-pro-tachykinin B) was first determined in bovine (68), then in rat (69). In human, a single gene transcript encoding a single precursor and a single TAC was first revealed (70), but then three TAC3 precursors (α, β, and γ) were shown to exist (Figure 1) (38).

2.1.2.2 Teleosts

The *tac3* gene has been characterized in a number of teleost species: zebrafish *Danio rerio* (26, 60, 71), Nile tilapia *Oreochromis niloticus* (72), goldfish *Carassius auratus* (73), striped bass *Morone saxatilis* (74), grass carp *Ctenopharyngodon idella* (62, 75), European eel *Anguilla* (76), orange-spotted grouper *Epinephelus coioides* (77), spotted sea bass *Lateolabrax maculatus* (78), and half-smooth tongue sole *Cynoglossus semilaevis* (79). While the *tac3* gene codes for only one TAC3 peptide in mammals, its ortholog in teleosts codes for two putative tachykinin peptides, TAC3 and a TAC3-related peptide [TAC3RP or NKBRP (76, 77)], earlier named neurokinin F (NKF) (71) with "F" for "fish" as it was thought to be present only in fish species and preserved along the whole teleost radiation (71, 72). As the whole-genome

duplication event specific to the teleost lineage (3R) led to the duplication of the *tac3* gene into *tac3a* and *tac3b*, up to four neurokinin B peptides may exist in teleosts, namely, NKBRPa, NKBa, NKBRPb, and NKBb (Figure 1). Loss of *tac3b* is observed in orange-spotted grouper (77), tongue sole (79), striped bass *Morone saxatilis* (74), olive flounder *Paralichthys olivaceus*, tiger puffer *Tetraodon nigroviridis*, medaka *Oryzias latipes*, Atlantic herring *Clupea harengus*, alosa, rainbow smelt *Osmerus mordax*, and sheepshead minnow *Cyprinodon variegatus* (76), leading to the presence of only two peptides in these species (Campo et al. in preparation).

2.1.3 Tachykinin 4

2.1.3.1 Mammals

The molecular cloning of a mouse third PPT gene, PPT-C (later renamed *tac4*), was reported in 2000 (21). PPT-C mRNA was primarily detected in hematopoietic cells, and its derived peptide was shown to be a crucial factor for the survival of B-cell precursors and thus named hemokinin 1 [HK1 (21);]. Rat HK1 is identical to mouse HK1 (mHK1) (80). In human, the *tac4* transcript predicts two tachykinin-like peptides: one, at the N terminus, a homolog of mouse and rat HK1, was named endokinin A (HK1/EKA), and the second at the C terminus was named endokinin C (EKC), in line with their proposed peripheral endocrine roles in contrast to the neuroendocrine/neuronal role of neurokinins (39). Apart from this *tac4* transcript that was named α-*tac4*, three other splicing variants exist in human (β-, γ-, and δ-*tac4*) (Figure 1): β-*tac4* codes for EKB and EKD, while γ-*tac4* and δ-*tac4* encode only EKB [(39); for review (66)]. TAC4RP-EKA and TAC4RP-EKB are N-terminal extended versions of TAC4RP-HK1, with different lengths; EKB is a truncated form of EKA and EKD an N-terminally modified version of EKC (39). Thus, the *tac4* gene encodes up to five peptides in mammals: HK1, EKA, EKB, EKC, and EKD. Three of them can be translated from the TAC4-RP site and two from the TAC4 site: HK1 or TAC4RP-HK1 from exon 2; EKA or TAC4RP-EKA from exons 1 and 2; EKB or TAC4RP-EKB from exons 1 and 2; EKC or TAC4-EKC from exons 3 and 4; and EKD or TAC4-EKD from exon 4. EKC and EKD are designated tachykinin gene-related peptides [for reviews (5, 34, 40, 66)]. Interestingly, TAC4-EKC and TAC4-EKD that correspond to the TAC4 peptide have substituted the C-terminal methionine by a lysine, thus reducing or suppressing the affinity for all TAC receptors, and they differ by the length of the N terminus (39).

2.1.3.2 Teleosts

It was not clear whether homologs of HK and EK were present in non-mammalian vertebrates until the *tac4* gene was identified in the genomes of various teleosts (71) and recently characterized in brain grass carp (62). The grass carp *tac4* gene encodes two mature peptides, hemokinin 1 (HK1) and hemokinin 2 (HK2) (Figure 1), as the mammalian *tac4*. However, the mammalian *tac4* can produce up to four different peptides depending on alternative splicing events that have not been observed in teleosts until now. HK-1 displays very weak activation for neurokinin receptors compared with HK2, likely due to a phenylalanine-to-valine substitution in the C-terminal FXGLM signature motif, leading to an inefficiency on pituitary hormone expression in grass carp pituitary cells (62). Shi and colleagues proposed that the fact that only one TAC4 isoform was isolated up to now in teleosts "might be the result of the non-functionalization by forming pseudogenes or deletion/mutations leading to the loss of redundant genes" (62). Our recent study demonstrates that the *tac4* gene has been duplicated during the 3R and two copies of the gene were conserved in most studied species (Campo et al. in preparation) (Figure 1).

2.2 Tachykinin receptors

In vertebrates, TAC peptides bind to three receptors (TACR), belonging to the first-class rhodopsin-like G-protein-coupled receptors (GPCR) (also named as family A GPCRs): TACR1, TACR2, and TACR3. These receptors are normally encoded by five exons that include the seven transmembrane domains, an extracellular N terminus enrolled in peptide recognition, and an intracellular C-terminal end in charge of the cellular response after activation of the receptor [for reviews (34, 44)].

The evolutionary scenario of tachykinin receptors in chordates (26) suggests that an ancestral tac receptor (*tacr*) gene in protochordates generated four paralogs after 1R/2R in early vertebrates (28, 29). One *tacr*, the *tacr4* gene, would have been lost before the split of the actinopterygians (ray-finned fish) and the sarcopterygians (lobe-finned fish). Further duplication of the *tacr* genes occurred during the teleost 3R. The 3R-duplicated *tacr1* and *tacr3* genes are conserved in most teleosts (Campo et al. in preparation), while one of the 3R-duplicated *tacr2* paralogs (*tacr2b*) was subsequently lost in the teleost lineage but conserved in the eels (elopomorphs) (Campo et al. in preparation). A local duplication of *tacr3a* might have occurred to give rise to *tacr3a1* and *tacr3a2* genes in the teleost lineage (26) Our recent gene search and phylogenetic study confirms this local duplication, but only in Clupeocephala, not in elopomorphs (*Anguilla* species) or osteoglossomorphs (bony tongue) (Campo et al. in preparation).

2.2.1 Tachykinin receptors in mammals

Nakanishi's group first demonstrated, by electrophysiological measurements of *Xenopus* oocytes injected with brain and stomach mRNAs, the expression of the receptors for mammalian SP (named NK1 receptor, here TACR1) and NKA (named NK2 receptor, here TACR2), respectively (81). The same

year, the receptor for bovine NKA (TACR2) was cloned from bovine stomach (82). The receptors for substance P, TACR1 (83), and those for NKB, TACR3 (84), were then cloned from rat brain. When expressed in *Xenopus* oocytes and in COS cells, they can produce an electrophysiological response as follows: SP>NKA>NKB for TACR1; NKA>NKB>SP for TACR2, and NKB>NKA>SP for TACR3 (Figure 1) [for review (34)]. Thus, the three tachykinin receptors can bind all TAC peptides (except EKC and EKD) but with differential selectivity (44, 85–87). HK1 and EKs (EKA and EKB) exhibit the highest affinity to TACR1 (Figure 1) [for reviews (8, 34)].

2.2.2 Tachykinin receptors in teleosts

Up to six tachykinin receptors have been characterized in teleosts (Figure 1), results of both whole-genome duplication and local gene duplication. In zebrafish, two 3R-duplicated *tacr1* (*tacr1a* and *tacr1b*), one *tacr2*, and three *tacr3* (*tacr3a1*, *tacr3a2*, and *tacr3b*) are identified, with *tacr3a2* arising from a local duplication of *tacr3a1* (26). In the grass carp, the same receptors are found: duplicated *tacr1* (NK1Ra and NK1Rb in the article), single *tacr2* (NK2R in the article), and three *tacr3* (NK3Ra1, NK3Ra2, and NK3Rb in the article) (62).

Using COS-7 cells that expressed zebrafish TACR3a1 (Tac3ra in the article) or zebrafish TACR3a2 (Tac3rb in the article), Biran and collaborators reported that both zebrafish NKBa and NKBRP (NKF in the article) were endogenous ligands of TACR3, while zebrafish NKBb was less effective (71). The same year, a local duplication of *tacr3a* was reported in zebrafish and binding studies of the three zebrafish TACR3 (TACR3a1, TACR3a2, and TACR3b) were investigated. NKBRPa (NKBa-13 in the article) and NKBRPb (NKBb-13 in the article) have higher potencies for inducing promoter activity of TACR3a1 and TACR3a2 in both CRE and SRE transactivation assays than NKBa-10 (26). For TACR3b, the same three NKB peptides have an inducing effect only using the SRE promoter (26). Zebrafish NKBb (NKBb-11 in the article) cannot activate any of the three TACR3s (26). In the same system, tilapia NKBRP (NKF in the article) was more effective than tilapia NKB in inducing the activity of tilapia TACR3a (Tac3ra in the article) and tilapia TACR3b (Tac3rb in the article) (72). In transfected 293-T cells, goldfish NKBa (NKBa-10 in the article), NKBRPa (NKBa-13 in the article), NKBb (NKBb-11 in the article), and NKBRPb (NKBb-13 in the article) can activate TACR3a1 (Tac3ra in the article), while TACR3b (Tac3rb in the article) can be slightly activated only by NKBa-10 (88).

In the grass carp, three studies investigated the receptor selectivity of TAC peptides, using HEK293T cells transfected with each of the six TACRs identified in this species (61, 62, 89) (Figure 1). The first two articles were reported before the cloning of the *tac4* gene and thus did not include TAC4 peptides (61, 89). The authors found that for TACR1 activation, the potencies were grass carp SP>NKA>NKBa>NKBRPa>NKBRPb>NKBb, and for TACR2 activation, grass carp SP, NKA, NKBa, NKBRPa, and NKBRPb had similar potency, except for NKBb which showed a low potency (61). This reveals that carp TACR2 is a multiligand receptor, which could be activated by various TACs with comparable efficacy and potency. Concerning TACR3 activation, for both TACR3a2 (NK3Ra in the article) and TACR3b (NK3Rb in the article), grass carp NKBRPb, NKBa, and NKBRPa were found to be the most effective compared with NKA, SP, and NKBb (89). Interestingly, in the third publication, HK2, product of the *tac4* gene, was shown to be able to activate all six TACRs, but with the highest activity for TACR2 (TACR2>TACR3b>TACR1a≈TACR3a1≈TACR1B>TACR3a2), while HK1 displays a very weak activation for each of TACR isoforms (62). These results suggest that while mammalian hemokinin HK1 exhibits the highest affinity for TACR1, teleost HK2 preferentially stimulates the multiligand receptor TACR2 and may thus have a similar function as other tachykinins through its activation (62).

3 The physiological role of tachykinins in the regulation of reproduction

Activation of the gonadotropic axis at puberty onset and maintenance of the reproductive state (with generation of GnRH pulse in mammals) are under a complex regulatory network. A major breakthrough occurred in 2003 for reproductive neuroendocrinology with the description of hypogonadotropic hypogonadism in human and mice bearing mutation in either kisspeptin gene (*kiss*) or its receptor (*kissr*) (90–92) and the following multiple studies stating that kisspeptins were the most potent secretagogues of GnRH in all mammals [for reviews (93–95)]. In addition, the involved kisspeptin neurons of the arcuate nucleus (ARC) in the hypothalamus were shown to co-express neurokinin B and dynorphin and were thus referred to as KNDy neurons, first in sheep (96) then in a variety of mammals [for review (97)]. A model was proposed in mammals for the GnRH pulse generator with NKB stimulating kisspeptin release and dynorphin inhibiting it [for reviews (98–101)]. All these data rekindle attention to other tachykinins, namely, SP and NKA, in reproductive physiology. In contrast, little is known concerning a potential involvement of tachykinin peptides derived from the *tac4* gene, hemokinin, and endokinins, in the control of reproduction, but potential actions at peripheral level are observed (Figure 2).

Some redundancies among the three TACR signaling pathways in the control of reproduction may occur (at least in rodents), as blockade of all three receptors (by the use of an antagonist for all three receptors) is required to inhibit LH secretion [ovariectomized (OVX) rat (104)] and the *in vitro* stimulatory effect of NKB on KNDy neurons is blocked only in

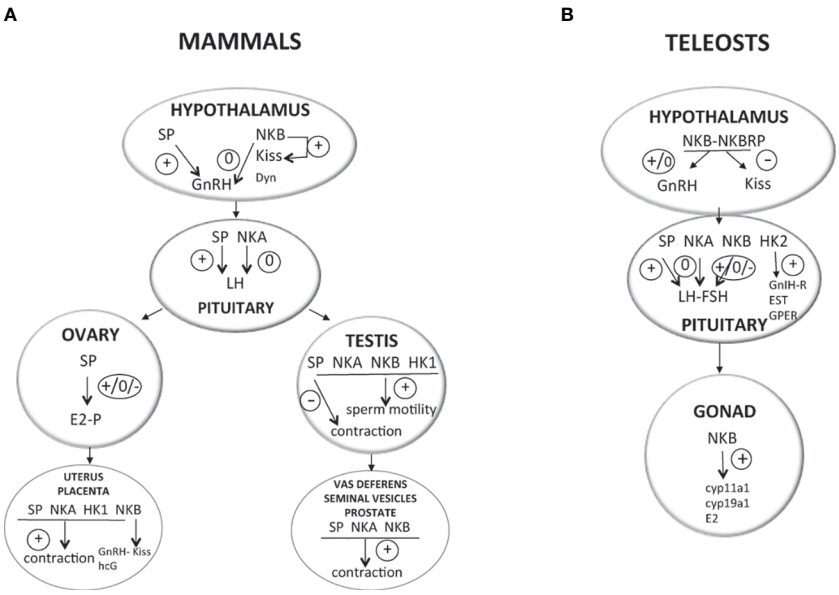


FIGURE 2
Direct effects of TAC peptides in the control of reproductive function in mammals and teleosts. TAC peptides can act directly at all the levels of the HPG axis (hypothalamus, pituitary, gonads in both mammals **(A)** and teleosts **(B)**, and other peripheral reproductive organs in mammals). At the brain level (hypothalamus), the effect of TAC on GnRH is likely kisspeptin-dependent in both mammals (KNDy neurons) and teleosts. At the pituitary level, while TAC action is only stimulatory on gonadotropins in mammals, a species specificity is observed in teleosts with either no, stimulatory, or inhibitory effects. At the peripheral level, TAC can act on ovarian steroidogenesis in mammals (either no, positive, or negative effects) and teleosts (positive effects). In mammals, a positive effect is also noted on the contraction of secondary sex organs (uterus in females; vas deferens, seminal vesicles, and prostate gland in male), as well as on sperm motility in males. For more details and for data from *in vivo* experiments, please refer to part 2 of this review and to Table 1. +, direct stimulatory effect; -, direct inhibitory effect; 0, no direct effect; cyp11a1, gene encoding cholesterol side-chain cleavage enzyme P450scc; cyp19a1, gene encoding aromatase; Dyn, dynorphin; E2, estradiol; ESR, nuclear estrogen receptor; FSH, follicle-stimulating hormone; GnRH, gonadotropin-releasing hormone; GnIH-R, gonadotropin-inhibitory hormone receptor; GPER, G-protein-coupled (membrane) estrogen receptor; hCG, human chorionic gonadotropin; HK1, hemokinin 1; HK2, hemokinin 2; kiss, kisspeptin; LH, luteinizing hormone; NKA, neurokinin A; NKB, neurokinin B; NPK, neuropeptide K; NPγ, neuropeptide gamma; P, progesterone; SP, substance P; T, testosterone.

the presence of a cocktail of all three receptor antagonists [male mouse (105)]. In both studies, the use of specific receptor antagonists individually has no effect (104, 105).

3.1 At the central level of the HPG axis

The main central targets of tachykinins on the HPG axis are the brain gonadotropin-releasing hormone (GnRH) and pituitary gonadotropins (LH and FSH). A recent review deals with the latest advances in our understanding of the biology of tachykinins in the control of GnRH release in mammals (106). However, as the HPG axis is also controlled by dopamine (DA) in teleosts, amphibians, and seasonal mammals, future studies should aim at investigating the potential regulation of dopaminergic neurons by the products of tachykinin genes. Indeed, in mammals, reports have shown interactions between tachykinins and DA at the hypothalamic level [for review (107)]. For example, Billings and colleagues demonstrated the presence of TACR3 in DA neurons in the ewe, abundantly during anestrous when DA mediates the suppression of GnRH and LH release and considerably less during the breeding season (108).

3.1.1 TAC1 peptides

3.1.1.1 Inactivation of TAC1 system and reproduction

To date, in humans, hypogonadotropic hypogonadism has never been associated with mutation of either the *tac1* gene or *tacr1*/*tacr2* genes. However, in mice, inactivation of these different genes leads to different degrees of reproductive impairments, suggesting the need for the whole tachykinin system to get full reproduction. Knockout of the *tac1* gene was obtained in female (109) and male (110) mice, which induced a delay in the onset of puberty in both sexes plus a subfertility in females (109). In contrast, mutant mice for *tacr1* are fertile (111). More recently, the characterization of a novel mouse line with congenital ablation of *tacr2* has allowed to show partially suppressed basal and stimulated LH secretion, with moderate reproductive impact (normal puberty onset and fertility), in these null mice (112). However, in the same species, impairment

of TAC1 peptide action does not seem to impact reproduction, as when TACR1 or TACR2 antagonists are ip injected to 8-week-old or subcutaneously (sc) to 6-month-old female mice, no effect is observed on reproductive success or litter size (113).

3.1.1.2 Effects of TAC1 peptides on GnRH expression, synthesis, and release

3.1.1.2.1 Mammals

Before the description of KNDy neuron involvement in GnRH pulse generation and the renewed interest in SP and NKA as regulators of the HPG, few studies were available for a potential direct effect of TAC1 peptides on GnRH [for review (97)]. Using a perifusion system, Ohtsuka and collaborators (114) were the first to show that SP stimulated the *in vitro* release of GnRH by rat medio-basal hypothalamus (Figure 2). Consistent with this direct action of SP on GnRH in rodents, its receptor, TACR1, was shown to be expressed in a fourth of GnRH neurons in the mice (115), and binding sites for SP detected in the rat hypothalamus (116). In addition, SP neurons establish inputs to GnRH neurons in the rat septopreoptic area (117) as well as in human diencephalon (118) and eminence median (119). In contrast, in another mammal, the ewe, no co-expression of SP or TACR1 was detected in GnRH neurons, implying no possible direct effect of SP on GnRH in this species (120). None of GnRH neurons express TACR2 in mice (115) and no detection of NKA binding sites was observed in the hypothalamus of rats (116), suggesting a lack of potential direct effect of NKA on GnRH in both species. However, Sahu and Kalra reported in female rat that the TACR2 agonist, but not the TACR1 one, could suppress GnRH release by fragments of median eminence and arcuate nucleus in culture (121).

Apart from a potential direct action of SP and NKA on GnRH neurons *via* their respective receptors, data in mammals report effects on kisspeptin neurons, as for NKB in the KNDy network. A stimulatory action of TAC1 peptides on kisspeptin has been demonstrated in rodents. In mice, SP and NKA modulate Kiss1 neurons and kisspeptin release [SP and NKA, males (105); SP and NKA, males and ovariectomized and supplemented with E2 (OVX+E2) females (115); SP, females (109); NKA, females (122)]. SP and NKA are also able to depolarize Kiss1 neurons in male mice (105). In addition, the induction of GnRH release by icv infusion of an SP agonist in adult male and OVX+E2 mice is not observed in Kiss1r−/− mice (115). These results on the kisspeptin-dependent effect of SP on GnRH are consistent with the fact that half of kiss1 neurons express *tacr1* in this species (115). In rat, icv injection of SP elevates both *gnrh* and *kiss1* mRNA levels (123).

In other mammalian species, anatomical data suggest a possible action of TAC1 peptides on kisspeptin or GnRH, but *in vivo* studies are often lacking or report only limited action. In postmenopausal women, SP immunoreactivity is detected within Kiss1 neurons in infundibular nucleus (124) and Kiss-SP occasionally contact with GnRH in the postinfundibular eminence (119). However, to our knowledge, no *in vivo* data for SP or NKA action on GnRH *via* kisspeptin are yet available in humans. In the goat, SP fibers are also observed in close apposition within ARC KNDy (125), and high doses of TACR1 and TACR2 agonists are needed, and not effective in all individuals, to induce GnRH pulse generator activity in OVX goats (103). In the male rhesus monkey, an absence of expression of SP in kisspeptin neurons has been reported but SP fibers are observed in close apposition on ARC kisspeptin perikarya (126). In the ewe, only a small proportion of ARC kiss neurons contain *tacr1* or SP expression (120).

Recently, results in rat showed that icv injection of SP antagonizes the inhibitory effect of the mammalian ortholog of gonadotropin-inhibitory hormone (GnIH), RFRP-3, on the expression of hypothalamic *gnrh* and *kiss1* (123). These first data suggest that SP may act at different levels of the central control of HPG (GnRH, kisspeptin, or GnIH). More studies in mammals and in other vertebrates should be performed to see whether this effect of SP on GnIH action is conserved throughout evolution.

3.1.1.2.2 Teleosts

In zebrafish, direct actions of TAC1 peptides are more likely on GnRH than on Kiss neurons, as associations between TAC1-immunoreactive processes and neurons for GnRH3 (the hypophysiotropic GnRH form in this species) in the ventral telencephalic area are observed, while there is no apparent proximity of TAC1 processes to kiss2 mRNA-expressing neurons in the hypothalamus (60). Recently, expression of *tacr1a* mRNA was reported in several brain regions containing GnRH3, as well as Kiss2, cells such as olfactory bulb, preoptic area, and hypothalamus (127), leading the authors to suggest that TAC1 peptides may act on both neurons. To the best of our knowledge, no functional study has been yet performed to investigate the action of TAC1 peptides on GnRH production in teleosts.

3.1.1.3 Effects of TAC1 peptides on LH and FSH expression, synthesis, and release

3.1.1.3.1 Mammals

A direct action of SP and NKA at the pituitary level is possible as expression of their receptors has been detected in pituitary cells, and sometimes specifically on gonadotrophs, in mammals [tacr1 (128–130); tacr2 (131)]. Moreover, SP and NKA fibers have been reported surrounding hypophyseal blood capillary vessels in the median eminence and these peptides to be present in the pituitary of mammals [NKA, rat (132); SP, rat (133–136); SP, NKA, rat (137); SP, rhesus monkey (126, 138)]. The first reported study on the *in vitro* effect of tachykinin on gonadotropins dates back to 1974. Fisher and colleagues observed that SP induced release of LH and FSH by pituitaries, from intact rats, cultured *in vitro* (139). These preliminary data using few pituitaries and a high dose of SP

were followed by a contradictory one, which reported no effect of SP on LH and FSH release by hemi-pituitaries of OVX rats (140). These first data already point out the potential importance of sex steroid on SP action in the control of gonadotropins. A stimulatory effect of SP on LH release from anterior pituitary cells in culture is reported during the peripubertal period in male and female rats but not at the prepubertal age and long after maturation (141). *In vitro* perifusion of anterior pituitaries from female rats allowed to show an inhibition of GnRH-induced LH release by SP, an effect abolished by the use of the TACR1 antagonist (142). All these results obtained in rat highlight the sex steroid dependence of the *in vitro* effect of SP on gonadotropins in this species. Data are also available in another mammal, the pig. In cultured porcine gonadotrophs, SP was reported to stimulate LH release without affecting intracellular LH content (143). It also potentiated GnRH-stimulated LH release and reversed the GnRH-induced decrease of gonadotroph LH stores, these effects not being blocked by the use of the GnRH-receptor antagonist. A few years later, the same group demonstrated that the SP direct effect on pig gonadotrophs and LH release was extracellular Ca^{2+}-dependent and did not involve an effect on *lhβ* transcript levels (144). Altogether, these *in vitro* data in mammals converge toward a predominantly stimulatory effect of SP on LH release (Figure 2). Concerning NKA, few data are available. Incubation of hemi-pituitaries with NPK and NPγ stimulates LH release in intact male rats but is not significant in castrated animals, while no significant effect is seen with NKA in both situations (145).

In vivo data concerning SP and NKA action on gonadotropins in mammals have been reviewed by Fergani and Navarro (97), and the following text will give a summary of them, complemented with one more recent publication (122). These *in vivo* studies, which cannot discriminate between direct and indirect effects, report diverse effects of TAC1 peptides on LH and FSH release, mostly depending on the presence or absence of sex steroids and also likely due to species differences [for review (97)]. Most of these studies reported a stimulatory effect of SP on LH release [OVX+E2 female rat (146); prepubertal female and male rats (147); normal men (148); intact female rabbit (149); OVX+E2 female mouse and intact male mouse (115); prepubertal female mouse (109); intact ewe (120)]. However, an absence of effect was sometimes observed [intact adult male rat (145); intact adult female rat (147); OVX female rat (145, 146); castrated male monkey (126); OVX or OVX+E2 female goat (103)]. Even inhibition was reported in castrated male rats (145, 150). Intravenous (iv) injections of SP failed to induce LH release in the rhesus monkey (126). However, in the cynomolgus monkey, there is a reduction in the duration and amplitude of LH surge after intragastric administrations of the TACR1 antagonist (151). In the ewe, much higher doses of SP compared with NKB are needed to stimulate LH release (120). Similarly, for NKA, various effects were obtained depending on the "sex steroid status" of the animals. Stimulation of LH release was observed in intact mature animals [male rat (145); OVX+E2 female mouse and intact male mouse (115, 147)] and in prepubertal intact male and female rats (147), while inhibition was reported in castrated animals [male rat (145); female rat (145); female mouse (115)]. An absence of effect was also sometimes seen by some authors in OVX adult female rats (121). Recently, the stimulatory action of NKA in the presence of sex steroids during adulthood in female mice was reported to be NKB-independent, as NKA was able to induce LH release in NKB-deficient mice (*tac3*KO mice) or after blockade of TACR3 by a specific antagonist (122). In addition, the stimulatory effect of NKA was kisspeptin-dependent, as it was absent in *Kiss1*KO mice (122). Interestingly, the inhibitory action of NKA on LH release in the absence of sex steroids during adulthood in female mice was found to be NKB- and dynorphin-dependent (122). In the ewe, much higher doses of NKA are needed to stimulate LH release, compared with NKB (120). In the female goat, the NKA agonist was inefficient to induce LH release either in OVX or OVX+E2 animals (103). NPK was also shown to modulate gonadotropin as icv injection of the peptide produced a suppression of LH release in ovariectomized rats (145).

A few studies addressed the effects of TAC1 peptides on FSH release in rodents. A sex difference was obtained in prepubertal rats: acute administration of the TACR1 agonist stimulated it only in females, while it was the TACR2 agonist that was able to induce it in males (147). In intact adult rats, the TACR1 agonist had no effect on FSH release in both sexes and the TACR2 one could elevate it in females (147). SP was ineffective in stimulating FSH release in OVX+E2 rats (146). In the intact male mice, central injection of TACR1 or TACR2 agonists induced an elevation of FSH secretion (115).

3.1.1.3.2 Teleosts

A direct action of tachykinins at the pituitary level is also possible in teleosts as SP fibers directly innervate the pituitary (58, 152, 153). Moreover, TACR1 expression has been detected in the pituitary [zebrafish (71):; grass carp (62, 75)] and specially in LH cells [grass carp (61)]. In contrast, in grass carp pituitary, spatial distribution of TACR2 is only overlapping with prolactin cells (61, 75), and not LH cells (61). FSH cells were not investigated in these studies (61, 75).

To date, only one study investigated the direct effects of peptides encoded by the *tac1* gene on teleost pituitary hormone expression and release [Figure 2 (61)]. Using primary culture of prepubertal grass carp pituitary cells, Hu and collaborators showed that grass carp SP and NKA could elevate prolactin (*prl*) and somatolactin-α (*slα*) mRNAs and hormone secretion, without any effect on proopiomelanocortin (*pomc*), *fshβ*, thyrotropin β (*tshβ*), glycoprotein α-subunit (*gp-α*), growth hormone (*gh*), and *slβ* expression. For LH, SP but not NKA could induce a dose-dependent inhibition of *lhβ* mRNA levels

(after 24 h of treatment), while both peptides induced a dose-dependent stimulation of LH release with a lower potency and efficacy for NKA (after 3 h). This induction of LH release by SP and NKA and the inhibition of *lhβ* mRNA by SP were blocked by the use of the TACR1 antagonist but not of TACR2 or TACR3 antagonists, in agreement with the fact that TACR1 was the only form of TACRs detected in grass carp gonadotrophs. Moreover, SP was able to partially suppress GnRH induction of *lhβ* mRNA, while co-treatment with the TACR1 antagonist enhanced this induction. TAC1 peptides in grass carp can thus have differential effects on LH release and *lhβ* mRNA levels *via* activation of TACR1 in gonadotrophs. More studies in other teleost species are needed to decipher whether these actions are species-specific or common to all teleosts.

3.1.2 TAC3 peptides

3.1.2.1 Inactivation of the TAC3 system and reproduction

In 2003, Pintado and collaborators injected intraperitoneally an antagonist of TACR3, the preferential receptor for TAC3 peptides, to 8-week-old female rats and showed no effect on reproductive success or litter size, while a 6-month-old subcutaneous injection of the same antagonist resulted in a reduction in the litter size (113). Later, mutations in the *tac3* or *tacr3* genes were characterized which lead to hypogonadotropic hypogonadism in human [(154–159); for review (160)], which could be reversed in adulthood (157). Similarly, in mice, *tac3* (161) or *tacr3* (162) null females show delayed sexual maturation and abnormal estrous cyclicity, which recover in adulthood leading to fertility, although they produced fewer pups per liter. In contrast, timing of sexual maturation and fertility are preserved in *tac3* (161) or *tacr3* (162) null males. In a teleost, the zebrafish, the knockout of either *tac3a*, *tac3b*, or both does not disrupt the reproduction (spermatogenesis and folliculogenesis are not impaired) (163). The impact of *tac3* gene mutation should be now studied in other teleost species, as knockout studies of reproductive genes such as the different types of *gnrh* [*gnrh3* (164); *gnrh2* (165)] and *kiss* [*kiss1*, *kiss2*, *kissr1*, and *kissr2* (166)] system genes all generate zebrafish with normal gametogenesis, suggesting that this species may have a high compensatory mechanism [for reviews (167, 168)] and may not reflect the situation observed in all teleosts.

3.1.2.2 Effects of TAC3 peptides on GnRH expression, synthesis, and release

3.1.2.2.1 Mammals

In mammals, the first analysis of *tacr3* expression was performed in rodents and showed its presence in GnRH neurons, indicating a possible direct effect of TAC3 on GnRH [rat (169); mouse (170)]. However, an absence of direct regulation of GnRH release by NKB was demonstrated using hypothalamic explants from adult male mice (171). In addition, in the same *in vitro* system, NKB was able to completely abolish the stimulation of GnRH release induced by kisspeptin (171). These results in mice suggested that NKB could regulate GnRH only *via* an action on kisspeptin (Figure 2), which is in agreement with the more recent demonstration of a minimal expression of *tacr3* in GnRH neurons, but its expression on virtually all KNDy neurons, in this species (115, 172). In female sheep, no TACR3 immunoreactivity was revealed in GnRH neurons, but GnRH neurons and fibers were in proximity to NK3R-containing ones (173). Use of the immortalized GT1-7 cell line, which represents mature post-migratory GnRH neurons with expression of TACR3, allowed to show differential effects on GnRH release depending on the length of exposure: acute treatment with NKB increases GnRH secretion, while long-term treatment decreases it by repressing transcription (174). In the arcuate nucleus of the hypothalamus, TAC3 is co-expressed with kisspeptin and dynorphin in the so-called KNDy neurons [for review (98)]. This was first demonstrated in sheep (96). KNDy neurons project to GnRH neurons and positively regulate their activity, being responsible for the generation of GnRH pulsatility in the hypothalamus of mammals [for reviews (97, 100, 175)]. Ablation of these neurons in female rats induces hypogonadotropic hypogonadism (176). Most of the studies show that neurons of ARC and particularly KNDy neurons project to the axonic terminals of GnRH neurons (98). Therefore, it is possible to assume that KNDy neurons might act on those terminals in a direct manner or using intermediate neurons to regulate the GnRH release (175). The most accepted hypothesis for mammalian KNDy neurons proposes that TAC3 acts in a positive manner and that Dyn acts in a negative way on the pulsatile release of kisspeptin by KNDy neurons (172, 177). In the ewe also, a high percentage of kisspeptin neurons produces dynorphin and NKB (96). A recent review addresses the question whether the KNDy model for the control of GnRH pulses applies to humans and other primates, compiling data showing that colocalization of kisspeptin and NKB is also observed in rhesus monkeys and humans (178). In addition, the ability of kisspeptin to induce LH release in patients with mutations in TAC or TACR3 tends also toward a proximal action of NKB to kisspeptin in stimulating GnRH secretion (179).

In vivo studies in different mammals have shown the stimulatory effect of NKB on GnRH secretion [prepubertal and pubertal rhesus monkeys: female (180) and male (181); ewe (182); goat (103, 168)]. Electrophysiological studies showed that icv administration of the TACR3 agonist (senktide) suppressed the GnRH pulse generator in OVX rats (183), while it induced GnRH release in intact male mice (184).

3.1.2.2.2 Teleosts

In tilapia, Mizrahi and colleagues investigated the co-expression of the three different forms of GnRH present in this species with TACR3s. They show that GnRH3 neurons expressed *tac3ra*, but not *tac3rb*, while the contrary was observed

for GnRH2 neurons, and GnRH1 ones expressed both *tacr3* (185). In the striped bass, TAC3 peptides, NKB, and NKBRP (NKF in the article) have an inconsistent effect (no or stimulatory only at the highest dose) on *gnrh1* expression by brain slices in culture, while persistently downregulating *kiss2* expression (74) (Figure 2).

In contrast to human and rodents, the expressions of tachykinins and kisspeptins are not always expressed in the same neurons in teleosts. In the zebrafish, NKB/TAC3 and NKF/NKBRP/TAC3RP are expressed in the *nuclear lateralis tuberis* (NLT), which is the teleost homologous structure to the ARC (71), but *kiss2* expression has not been found in this area (186). This finding in zebrafish, however, does not exclude that TAC neurons project on kisspeptin ones. In the striped bass, NKB neurons innervate the largest kiss2 neuronal population in the hypothalamus, which also expresses TACR3, while no expression of TACR3 or no NKB neuronal projection is detected for GnRH1 soma (74). In addition, in this species, TAC3 peptides, NKB and NKBRP (NKF in the article), are able to downregulate *kiss2* gene expression *in vivo*, while having no effect on *gnrh1* expression (74). In addition, cotreatment with a NK3R antagonist abolishes the negative effect of TAC3 peptides on *kiss2* mRNA levels (74). These results in the striped bass suggest that tachykinin peptides may act preferentially on the kisspeptin system, as in mammals. When injected to goldfish females in mid-vitellogenesis and males in late-spermatogenesis, three NKB peptides (NKBa-13, NKBa-11, and NKBb-13), but not the fourth one (NKBb-11), increase hypothalamic *gnrh3* mRNA levels (73). NKBa-10 and NKBa-13 ip injected to goldfish females at the early vitellogenic oocyte stage and males at the early spermatogenesis stage decrease mRNA levels of both hypothalamic *kiss2* and *gnrh3* (except NKBa-13 on *gnrh3*) (88). In tilapia, NKBRP injected to mature male tilapia inhibits the expression of brain *gnrh-I* and *kiss2*, while NKB has no effect (187). A recent study in the Japanese eel, *Anguilla japonica*, reports that ip injection of each of the four mature peptides found in this species gives different effects depending on the peptides and the doses used: a low dose of the four peptides had no effect on neither *gnrh1* (*mgnrh*) and *gnrh2* (*cgnrh*) expression, while a high dose of NKBa-10 and NKBb-13 (and not NKBa-13 and NKBb-10) stimulates *gnrh1* expression (188). All these data demonstrate that different regulations of *gnrh* and *kiss* expression by TAC3 peptides (from none to stimulatory or inhibitory effects) may be encountered among teleosts, depending on the species, the maturity stage, the doses, and the peptides tested.

The non-systematic action of TAC3 peptides on GnRH and kisspeptin in teleosts, compared with the situation observed in mammals, is likely due to the surprisingly non-essential character of these two neuropeptides for reproduction in some teleost species. Indeed, recent knockout studies demonstrated that *gnrh3* and *gnrh2* in zebrafish (164, 189), *gnrh1* in male medaka (190), and *kiss1* and *kiss2* in zebrafish (166) and medaka (191) were dispensable for normal reproductive function. In zebrafish, even triple mutants for *gnrh3*, *kiss1*, and *kiss2* undergo normal puberty and gonad maturation (192). This lack of effect on reproduction of GnRH and kisspeptin gene editing led many scientists to make assumptions on the possibility of physiological compensatory phenomena in teleosts (for reviews: 167, 193–196). In addition to these knockout results, many data are available, stating reproductive actions of GnRH (for review: 194) and kisspeptins at various HPG levels in teleosts (for reviews: 195, 196).

3.1.2.3 Effects of TAC3 peptides on LH and FSH expression, synthesis, and release

3.1.2.3.1 Mammals

A direct action of tachykinins at the pituitary level is possible as *tacr3* expression has been detected in this gland in mammals [ewes (197); pigs (198), and gilts (199)]. Moreover, NKB fibers have been reported surrounding hypophyseal blood capillary vessels in the median eminence [monkeys (200)]. To the best of our knowledge, only one *in vitro* study has investigated the potential direct effect of NKB on gonadotropins in mammals, by using a gonadotroph cell line (201). The authors reported no effect of NKB on *lhβ* and *fshβ* mRNA expression, even if TACR3 was detected in this cell line.

Comparing various tachykinins *in vivo*, Sahu and Kalra (121) were the first to report that NKB-containing implants, in the third ventricle of OVX rat brain, did not induce any change in LH release. Later, this absence of NKB effect on LH was also shown after either ip or icv administration to intact adult male mice (171). However, evidence for stimulatory effects of NKB on LH has since been documented in many mammalian species [for reviews (97, 106, 178)], as for example in prepubertal female rats (16, 202). In some studies, the stimulatory effect of NKB on LH is only observed under physiological sex steroid levels (i.e., intact or OVX+E2 adult animals) [adult male and female mice (115, 203); adult male and female rats (203, 204); lactating female cattle (205)]. In contrast, in monkeys, NKB is able to stimulate LH release in castrated juvenile (200, 206) and adult (207) males. In the sheep, castration does not prevent the stimulatory action of NKB in adult females (207, 208), as compared with intact females [adult (108, 209); prepubertal (210)]. A similar situation is observed in the female goat with a stimulatory effect of the iv administered NKB agonist (senktide) (103) or no effect of the icv injected NKB (177), regardless of the gonadal status. Recently, iv administration of senktide has even been shown to be efficient in stimulating LH release in fetal male and female sheep (211). In humans, early studies report no gonadotropin-stimulating effect of NKB iv administered in adult men and women (212, 213), but a series of data obtained by Skorupskaite and collaborators using the TACR3 antagonist given orally show a decrease in overall circulating LH levels and LH pulsatility in adult men (214) and women (215–217). Few studies report an inhibitory action of senktide on LH release, regardless of the steroid milieu in female

rat (183, 218) or only in the absence of sex steroids in female mice (172).

Concerning FSH, either stimulatory [mouse (115, 147, 219); monkey (207); man (214, 220)] or no effect [mouse (171); rat (147); woman (212, 214, 220); man (212, 213)] of NKB has been reported.

These various effects on LH and FSH in mammals could be due to species, physiological status, or mode of peptide administration. Table 1 gives details on all these *in vivo* studies of NKB action on gonadotropins.

3.1.2.3.2 Teleosts

TACR3s (*tac3ra1*, *tac3ra2*, and/or *tac3rb*) are expressed at the pituitary level in various teleosts [zebrafish (26, 71); spotted sea bass (78); grass carp (62, 89, 221, 222)] and in both LH and FSH cells in tilapia (72), making a direct effect of NKB and NKBRP possible on gonadotropin synthesis and release. Interestingly, in the grass carp, Xu and collaborators reported that *tacr3a* is expressed in both LH and somatolactin α (SLα) cells, while *tacr3b* expression is only found in SLα cells (89). FSH cells were not investigated in this study (89). In tilapia, when NKB and NKBRP are applied to mature male pituitary cells, they both increase FSH and LH release (72). In culture of pituitaries from mixed sexed juveniles of this species, NKBRP downregulates *fshβ* and *lhβ* mRNAs, while NKB has no effect (187). Still in tilapia, Mun and colleagues recently compared responses of pituitary cells and pituitaries to NKB and NKF in males and females. They reported that expressions of *fshβ* and *lhβ* mRNAs did not show any change after treatment of whole pituitaries with NKB or NKF in both sexes (223). In contrast, the use of primary culture of pituitary cells allowed them to find that NKB could stimulate *fshβ* and *lhβ* mRNAs in female and inhibit them in male, while the contrary was observed with NKF (223). These results highlight major differences according to the maturation stage, to the protein or mRNA, or to the type of culture (primary cell culture versus organotypic culture) concerning the effects TAC3 peptides have on gonadotropin in tilapia. The other studies available suggest in addition to species differences, using the same method, primary cultures of pituitary cells (Figure 2). In the striped bass, the effects of NKB and NKBRP were stimulatory on LH and FSH release but absent on their mRNAs (74). In the European eel, the four peptides encoded by the *tac3* gene were able to inhibit *lhβ* mRNAs by pituitary cells in culture but had no effect on *fshβ* mRNAs (76). Some other studies, using this cell culture system, reported no effect of NKB and NKBRP on *fshβ* and *lhβ* mRNAs [grass carp (75); orange-spotted grouper (77)].

In teleosts, most of the *in vivo* data showed an increase of gonadotropin release and expression after treatment with TAC3 peptides. In zebrafish, homologous (zebrafish) NKBa and NKBRP (NKF in the article) induce LH release when injected to mature females (71). Among the four neurokinin B peptides characterized in zebrafish, NKBb presents a modified C-terminal motif from the typical tachykinin FVGLM to FVGLL, thus losing the final methionine. This change leads to a decreased affinity of this peptide for the two TACR3 and a highly reduced *in vivo* effect, compared with other neurokinin peptides (71, 73). However, the finding of a third TACR3 in the zebrafish genome (26) increases the chances that the NKBb with a different C-terminal motif may be active. In tilapia, ip injections of homologous NKB to mature males increase both FSH and LH plasma levels, while homologous NKBRP induces only LH release (72). In mature female tilapia, ip injection of tilapia NKB and NKBRP has no effect on pituitary *lhβ* and *fshβ* mRNA levels (185). More recently, Mizrahi and colleagues developed specific NKB and NKBRP (NKF in the article) analogs based on the structure of the mammalian NKB analog, senktide (185). When ip injected to mature female tilapia, these analogs increase plasma LH levels as native (tilapia) NKB and NKBRP do, and they are even able to increase FSH release while native ones have no effect (185). Concerning mRNA levels, native NKB and NKB analogs are efficient in stimulating *lhβ* and *fshβ*, whereas native NKBRP has no effect and NKBRP (NKF in the article) analog stimulates only *lhβ* (185). When injected to goldfish females at mid-vitellogenesis and males at late spermatogenesis, homologous NKBa-10, NKBa-13, and NKBb-10, but not NKBb-11, increase pituitary *lhβ* mRNA levels (73). An increase in *fshβ* mRNA levels is observed in females only after administration of goldfish NKBa-13 and NKBb-13, while in males all peptides, except NKBb-11, induce these levels (73). In sexually immature goldfish, NKBa-10 and NKBa-13 ip injected to females at the early vitellogenic oocyte stage and males at the early spermatogenesis stage decrease mRNA levels of both pituitary *lhβ* and *fshβ* (88). In the female orange-spotted grouper *Epinephelus coioides* at early vitellogenic stages, ip injection of NKB increases pituitary *lhβ*, but not *fshβ*, mRNA levels, while administration of NKBRP has no effect on these expressions (77). A recent study in the Japanese eel reports that ip injection of each of the four mature peptides found in this species gives different effects depending on the peptides and the doses used: a high dose of the four peptides inhibits *lhβ* and *fshβ* expression, while a low dose of NKBa-10 and NKBb-13 stimulates them (188). All these data show various effects of TAC3 peptides on gonadotropin release and expression among teleosts, depending on the species, the maturity stage, the doses, and the peptides tested. Table 1 compiles all these *in vivo* studies of NKB action on gonadotropins.

3.1.3 TAC4 peptides

Little is known on the effects of TAC4 peptides, HK and EK, on the central reproductive brain–pituitary axis. To the best of our knowledge, the only available study was realized in a teleost, the grass carp. Using transcriptomic analysis of TAC4 peptide effects on pituitary cells, Shi and colleagues have recently demonstrated that HK2 downregulates the pituitary expression

TABLE 1 *In vivo* studies concerning NKB action on gonadotropins in mammals and teleosts.

Species	Sex	Age	Gonadal status	Treatment	Route	Effect	References
Mammals							
Human	Man	Adult		NKB	iv	None on LH-FSH release	Jayasena et al (34)
	Woman	Adult		NKB	iv	None on LH-FSH release	Jayasena et al (34)
	Man	Adult		NKB	iv	None on LH-FSH release	Narayanaswamy et al (35)
	Women	Adult		TACR3 antagonist	Orally	Inhibitory on LH release	Skorupskaite et al (36)
	Man	Adult		TACR3 antagonist	Orally	Inhibitory on LH-FSH release	Skorupskaite et al (37)
	Woman	Adult		TACR3 antagonist	Orally	Inhibitory on LH release	Skorupskaite et al (38)
	Woman	Adult	Postmenopausal	TACR3 antagonist	Orally	Inhibitory on LH release None on FSH release	Skorupskaite et al (39)
Rhesus monkey *Macaca mulatta*	Male	Juvenile	Agonadal	NKB Senktide	iv	Stimulatory on LH release	Ramaswamy et al (40)
Rhesus monkey	Male	Juvenile	Agonadal	Senktide	iv	Stimulatory on LH release	Ramaswamy et al (41)
Cynomolgus monkey *Macaca fascicularis*	Male	Adult	ORX	TACR3 antagonist	Orally	Inhibitory on LH-FSH release	Fraser et al (42)
Rat	Female	Adult	OVX	NKB	icv	None of LH release	Sahu and Kalra (43)
	Female	Adult	OVX+E2	Senktide	icv	Inhibitory LH release	Sandoval-Guzman et al (44)
	Female	Adult	Intact	Senktide	icv	Stimulatory on LH release	Navarro et al (45)
			OVX+E2	Senktide	icv	Stimulatory on LH release	Navarro et al (45)
			OVX+Sham	Senktide	icv	Inhibitory on LH release	Navarro et al (45)
	Female	Prepubertal	Intact	Senktide	icv	Stimulatory on LH release	Navarro et al (45)
	Female	Adult	Intact	Senktide	icv	Stimulatory on LH release	Navarro et al (45)
	Female	Prepubertal	Intact	TACR3 antagonist	icv	Decreasing trend on LH release	Navarro et al (45)
	Female	Adult	OVX	Senktide	icv	Inhibitory LH release	Kinsey-Jones et al (46)
		Adult	OVX+E2	Senktide	icv	Inhibitory LH release	Kinsey-Jones et al (46)
	Female	10-days	Intact	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)
		25-days	Intact	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)
		30-days	Intact	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)
		36-days (Pubertal)	Intact	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)
		Adult	Intact	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)
		Adult	OVX+T	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)
		Adult	OVX+Sham	Senktide	icv	None on LH release	Ruiz-Pino et al (47)
	Male	10-days	Intact	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)
		25-days	Intact	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)

(Continued)

TABLE 1 Continued

Species	Sex	Age	Gonadal status	Treatment	Route	Effect	References
		30-days	Intact	Senktide	icv	Stimulatory on LH release	Ruiz-Pino et al (47)
		45-days (Pubertal)	Intact	Senktide	icv	None on LH release	Ruiz-Pino et al (47)
		Adult	Intact	Senktide	icv	None on LH release	Ruiz-Pino et al (47)
		Adult	ORX+E2	Senktide	icv	None on LH release	Ruiz-Pino et al (47)
		Adult	ORX+Sham	Senktide	icv	Inhibitory on LH release	Ruiz-Pino et al (47)
	Female	Prepubertal	Intact	Senktide	icv	Stimulatory on LH release	Grachev et al (48)
	Female	Adult	OVX+E2	Senktide	icv	Inhibitory on LH release	Grachev et al (48)
		Adult	OVX+E2	Senktide +Antagonist	icv	Blockade of inhibitory on LH release	Grachev et al (48)
	Female	10-days	Intact	Senktide	icv	Stimulatory on FSH release	Ruiz-Pino et al (49)
		25-days	Intact	Senktide	icv	Stimulatory on FSH release	Ruiz-Pino et al (49)
		36-days (Pubertal)	Intact	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
		Diestrus 1	Intact	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
		Proestrus	Intact	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
		Adult	OVX	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
	Male	10-days	Intact	Senktide	icv	Stimulatory on FSH release	Ruiz-Pino et al (49)
		25-days	Intact	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
		30-days	Intact	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
		45-days (Pubertal)	Intact	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
		Adult	Intact	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
		Adult	ORX	Senktide	icv	None on FSH release	Ruiz-Pino et al (49)
Mouse	Female	Adult	OVX+E2	Senktide	icv	None on LH release	Navarro et al (50)
		Adult	OVX+Sham	Senktide	icv	Inhibitory on LH release	Navarro et al (50)
	Male	Adult	Intact	NKB	ip	None on LH release	Corander et al (51)
	Male	Adult	Intact	Senktide	icv	Stimulatory on LH-FSH release	Navarro et al (52)
	Male	Adult	Intact	Senktide	icv	Stimulatory on LH-FSH release	Navarro et al (53)
	Female	Adult	OVX+E2	Senktide	icv	Stimulatory on LH-FSH release	Navarro et al (53)
		Adult	OVX+Sham	Senktide	icv	Inhibitory on LH release	Navarro et al (53)
Sheep	Female	Adult	Anestrous	Senktide	icv	Stimulatory on LH release	Billings et al (54)
		Adult	Follicular phase	Senktide	icv	Stimulatory on LH release	Billings et al (54)
		Adult	Luteal phase	Senktide	icv	None on LH release	Billings et al (54)
	Female	Prepubertal		Senktide	iv	Stimulatory on LH release	Nestor et al (55)
	Female	Adult	Anestrous	NKB	icv	Stimulatory on LH release	Sakamoto et al (56)

(Continued)

TABLE 1 Continued

Species	Sex	Age	Gonadal status	Treatment	Route	Effect	References
	Female	Adult	OVX	TACR3 antagonist	Microimplants	Inhibitory on LH release	Goodman et al (57)
	Female	Adult	OVX	TACR3 antagonist	iv	Inhibitory on LH release = prolongs LH interpulse interval	Fraser et al 5 (42)
	Female	Adult	Luteal phase	Senktide	icv	Stimulatory on LH release	Li et al (102)
			OVX	TACR3 antagonist	icv	Inhibitory on LH release	Li et al (102)
	Female	Fetal		Senktide	iv	Stimulatory on LH release	Amodei et al (58)
	Male	Fetal		Senktide	iv	Stimulatory on LH release	Amodei et al (58)
Cattle	Female		Lactating	Senktide	iv	Stimulatory on LH release	Nakamura et al (59)
Goat	Female	Adult	OVX	NKB	icv	None on LH release	Wakabayashi et al (60)
			OVX+E2	NKB	icv	None on LH release	Wakabayashi et al (60)
			OVX	Senktide	iv	Stimulatory on LH release	(103)
			OVX+E2	Senktide	iv	Stimulatory on LH release	(103)
Teleosts							
Zebrafish	Female	Adult	Mature	zfNKBa zfNKBb zfNKBRP (NKF)	ip	Stimulatory on LH release Stimulatory on LH release Stimulatory on LH release	Biran et al (61)
Tilapia	Male	Adult	Mature	tiNKB tiNKBRP (NKF)	ip	Stimulatory on LH-FSH release Stimulatory on LH release	Biran et al (62)
	Female	Adult	Mature	tiNKB tiNKBRP	ip	None on *lh-fsh* mRNAs None on *lh-fsh* mRNAs	Jin et al (63)
	Female	Adult	Mature	ti NKB tiNKBRP (NKF) NKB analog NKBRP analog	ip	Stimulatory on LH release Stimulatory on *lh-fsh* mRNAs Stimulatory on LH release None on *lh-fsh* mRNAs Stimulatory on LH-FSH release Stimulatory on *lh-fsh* mRNAs Stimulatory on LH-FSH release Stimulatory on *lh* mRNAs None on *fsh* mRNAs	Mizrahi et al (64)
Goldfish	Female	Adult Sexually immature	Early vitellogenesis	gfNKBa-10 gfNKBa-13	ip	Inhibitory on *lh-fsh* mRNAs Inhibitory on *lh-fsh* mRNAs	Liu et al (65)
			Mid-vitellogenesis	gfNKBa-10 gfNKBa-13 gfNKBb-11 gfNKBb-13	ip	Stimulatory on *lh* mRNAs None on *fsh* mRNAs Stimulatory on *lh-fsh* mRNAs None on *lh-fsh* mRNAs Stimulatory on *lh-fsh* mRNAs	Qi et al (66)
	Male	Adult Sexually immature	Early spermatogenesis	gfNKBa-10 gfNKBa-13	ip	Inhibitory on *lh-fsh* mRNAs Inhibitory on *lh* mRNAs None on *fsh* mRNAs	Liu et al (65)
			Late spermatogenesis	gfNKBa-10 gfNKBa-13 gfNKBb-11 gfNKBb-13	ip	Stimulatory on *lh-fsh* mRNAs Stimulatory on *lh-fsh* mRNAs None on *lh-fsh* mRNAs Stimulatory on *lh-fsh* mRNAs	Qi et al (66)
Orange-spotted grouper	Female	Adult	Early vitellogenesis	grouperNKB grouperNKBRP	ip ip	Stimulatory on *lh* mRNAs None on *fsh* mRNAs None on *lh-fsh* mRNAs	Chen et al (67)

(Continued)

TABLE 1 Continued

Species	Sex	Age	Gonadal status	Treatment	Route	Effect	References
Japanese eel	Female	Silver stage (prepubertal stage)	Immature	eelNKBa-10 eelNKBa-13 eelNKBb-10 eelNKBb-13	ip	Stimulatory on *lh* and *fsh* mRNAs None on *lh-fsh* mRNAs None on *lh-fsh* mRNAs Stimulatory *fsh* mRNAs None on *lh* mRNAs	Zuo et al (68)

of one GnIH receptor (GnIHR3) and five estrogen receptors (nuclear: ESR1, ESR2a, ESR2b, and ESRRβ; membrane: GPER1), while upregulating the expression of another nuclear estrogen receptor (ESRRγ) (62) (Figure 2). In mammals, the known reproductive role of HK1 takes place at the peripheral level (33), while up to now, none is attributed to EKs.

3.2 At the peripheral level

Tachykinins can also act at the levels of the gonads and the secondary sex organs, *via* paracrine and autocrine effects, in both females and males (Figure 2).

3.2.1 Effects on the female reproductive system

3.2.1.1 Mammals

A review has already been dedicated to tachykinin involvement in mammalian ovarian function (224), and the text below is only a summary. Expression of *tac1* and *tac3* (mRNA), as well as their receptors, is detected in the mammalian ovary, oocytes, and granulosa cells (113, 224), indicating potential autocrine/paracrine effects. Isolated cumulus granulosa cells in mice express *tac1*, *tac3*, and *tac4* (113). The control of ovarian steroid secretion by tachykinins in mammals has been previously reviewed (224). Briefly, data were obtained in various mammalian species with different results. In rats, exposure of granulosa cell culture or ovarian fragment in culture with SP and SP analog was unable to modify estrogen or progesterone release (225). In hamster, depending on the age of the animals, treatment of ovaries in culture with SP could stimulate (15-day-old hamsters) or inhibit (adult hamsters) or have no effect (neonatal hamsters) on estradiol release (226). Concerning progesterone release, in the same culture system, SP has a stimulatory effect (neonatal and adult hamsters) or no (15-day-old hamsters) effects (226). Using luteal cells in culture and exposure to SP, opposite results were obtained on progesterone release in basal conditions or under stimulation with LH in two different artiodactyla/ungulata species: stimulation in bovine (227) and inhibition in pig (228). In pig, SP treatment did not change estradiol release by granulosa cells in culture but stimulated it by luteal cells (228). These various effects of SP on *in vitro* ovarian sex steroid release in mammals thus likely depend on species, type of cells, and age of animal.

Genes encoding tachykinin peptides (*tac1*, *tac3*, and *tac4*) and receptors *(tacr1*, *tacr2*, and *tacr3*) are all expressed in the uterus of mice (45, 113, 229), rats (230), and humans (231, 232), suggesting potential autocrine/paracrine effects. Their expressions change during the estrous cycle and during pregnancy [mouse (45):; rat (233, 234)]. TACR2 is involved in human uterine contraction and is regulated during pregnancy (33). An altered expression of SP, NKA, and HK1 and their receptors is observed in uterine leiomyomata in human (235). When applied to isolated myometrium from non-pregnant women, SP, NKA, and NKB produce contractions, while the TACR2 receptor-selective antagonist abolishes the uterotonic effect of the NKA agonist (231). These TAC peptides also produce a direct contractile effect on uterine smooth muscle in mice (5, 236), rats (229, 234, 237), and pregnant women (231, 238). Human HK1 is also a uterine stimulant in humans (33).

In the rat placenta, downregulation of *tac3* and *tacr3* expression is associated with pregnancy (41). NKB placental levels are increased at term labor in women (239). In the placenta of preeclampsia women, elevated circulating NKB and increased *tac3* expression are reported as compared with placenta of normal pregnant women (41, 70, 240). TAC3 and TACR3 may contribute to preeclampsia during late pregnancy (41, 241). NKB stimulates the expression of *gnrh*, *kiss*, and human chorionic gonadotropin (hCG) by primary cultures of rat placental cells (242).

3.2.1.2 Teleosts

An autocrine/paracrine action is also possible in teleosts as both NKB and NK3R are expressed in the ovary [zebrafish (26, 71); tilapia (72)]. In the zebrafish, a direct effect of neurokinin B on the ovary is reported, as it stimulates estradiol production and increases the expression of cyp11a1 and cyp19a1 in primary cultures of follicular cells (243).

3.2.2 Effects on the male reproductive system

3.2.2.1 Mammals

The involvement of tachykinins in the regulation of mammalian testicular function has already been reviewed (236, 244, 245), and the text below is a brief summary.

SP inhibits testosterone production and release by isolated Leydig cells in hamster (246, 247). *Tac1*, *tac3*, and *tac4* genes are expressed in the human sperm (248). Tachykinins are likely to enhance the sperm motility by TACR1- and TACR2-dependent mechanisms, as TACR1- and TACR2- (but not TACR3-) selective antagonists can reduce the stimulating effect of phosphoramidon in human (248). Human HK1 also promotes progressive sperm motility (Figure 2) with a potency similar than that of NKA (lower than that of SP and higher than that of NKB) (249). All classical TACRs seem to be involved in these actions, but the role of TACR1 was predominant (249).

Tachykinins also stimulate contractility of the *vas deferens* and of seminal vesicles [Figure 2, for review (236)]. SP (TAC1RP) and NKA (TAC1) are present in the prostate of guinea pig and rat at low levels, of dog abundantly and absent in human prostate [for review (236)], while *tac1, tac3*, and *tac4* mRNA expressions have been detected in human prostate (39, 232). Prostate contraction by tachykinins in human involves TACR2 (250).

3.2.2.2 Teleosts

An autocrine/paracrine action is also possible in teleosts as both NKB and NK3R are expressed in the testis [zebrafish (71); tilapia (72)]. In tilapia, recent use of NKB antagonists by ip injections on adult males reduced the number of spermatozoa, leading to lower fertility (251), an effect which can be direct as male tilapia have significant amounts of TACR3 in the testis (72).

4 The physiological role of tachykinins in the regulation of food intake

As described previously in this review, TAC peptides (SP and NKA) were discovered for their contractile role on the gastrointestinal tract (GIT) in mammals. However, far less direct evidence is available concerning the regulation of food intake by tachykinin peptides when compared with their role in reproduction. Nevertheless, recent data, notably in two teleosts, the sea bass *Dicentrarchus labrax*, and the grass carp, highlight a potential major regulatory role of TAC3 and TAC4 peptides in the regulation of genes involved in feeding and gut motility.

The control of food intake involves two major populations of ARC hypothalamic neurons, in mammals as well as in other vertebrates: neurons producing neuropeptide Y (NPY) and agouti-related peptide (AgRP), which are orexigenic (appetite stimulator) peptides, and neurons producing proopiomelanocortin (POMC) and cocaine-and-amphetamine-regulated transcript (CART), which are anorexigenic (appetite inhibitor) peptides [Figure 3; for reviews (12, 252–256)]. These hypothalamic neurons integrate information from peripheral hormones such as leptin, an anorexigenic hormone produced by adipose tissue in mammals and by liver in teleosts, and ghrelin, an orexigenic stomachal hormone [Figure 3; for reviews (12, 252–256)].

As gut peptides, tachykinins also have a potential direct role on the GIT in vertebrates. A well-described one is the stimulation of its motility, the first necessary step of food digestion after its intake [for review (257)].

4.1 Mammals

4.1.1 Energy state and the tachykinin system

TAC3 neurons, mainly as part of KNDy neurons, have been involved in the regulation of both negative and positive energy balance. For example, they mediate the anorexigenic effect of estradiol in young female rats, as selective ablation of KNDy neurons suppresses the post-ovariectomy weight gain (258). In addition, many studies report the regulation of the tachykinin system by a change of energy status. Fasting and caloric restriction (CR) induce a decrease in hypothalamic ARC *tac3* and/or *tacr3* expression in rodents [pubertal female rat (259); OVX female mice (260); adult female rat (261)]. Nevertheless, in adult male mice, fast increases hypothalamic ARC *tac3* and *tacr3* (171) while CR and fast (261, 262) have no effect in adult female rats. In sheep, chronic food restriction downregulates *kiss* and *tac3* mRNA levels [castrated male sheep (263); OVX ewe lambs (264); for review (265)]. Feeding a high-fat diet does not change ARC mRNA levels for *tac3* in pubertal female mice (266) while it has a stimulatory effect in pubertal female rats (267). All these data suggest differential regulation of the tachykinin system (and of its involvement in the control of metabolism) according to species, sexual maturation stage, and/or degree of negative energy balance.

4.1.2 Effects of tachykinins on food intake and GIT motility

Abundant distribution of TAC1, TAC2, and TAC3 receptors is found in the hypothalamic nuclei involved in the control of food intake such as ARC, paraventricular nucleus (PVN), and lateral hypothalamus (LHA) [for reviews (4, 97, 106)]. The full tachykinin system is also detected in the neurons and nerve fibers of the mammalian gut with remarkable diversity between species [for reviews (268–270)]. These distributions point out toward a potential involvement of TAC peptides in the control of feeding and GIT motility.

Administration of NPK to food-deprived rats for 24 h delays the onset of (re)feeding and decreases the cumulative food intake [(271); for review (272)]. Achapu and coworkers show that the inhibition of food intake induced by centrally injected NPK may be due to the intense grooming induced by the injection (273). Similarly, icv injection of SP to food-deprived male rats suppresses

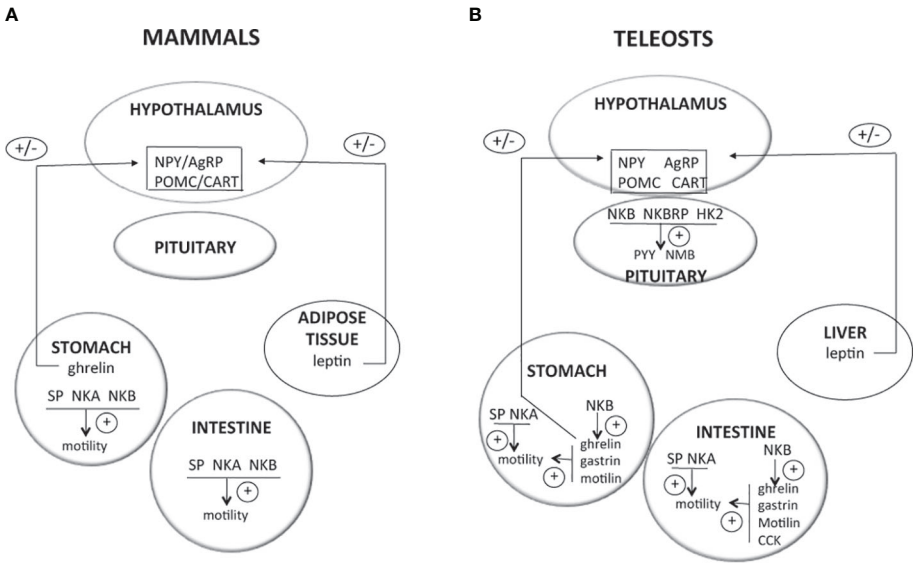


FIGURE 3
Direct effects of TAC peptides in the control of food intake and gut motility in mammals and teleosts. TAC peptides can act directly at different levels (hypothalamus, pituitary, and gastrointestinal tract) to influence food intake and gut motility. In mammals **(A)**, most of the available studies report the stimulatory effects of tachykinins on gut motility. In teleosts **(B)**, recent *in vitro* studies are emerging, showing direct effects of TAC3 and TAC4 peptides on the expression of neuropeptides highly expressed in the pituitary and that are involved in the central control of food intake. These TAC peptides can also influence the expression of genes from the gut that control its motility. For more details and for data from *in vivo* experiments, please refer to part 3 of this review. +, direct stimulatory effect; -, direct inhibitory effect; 0, no direct effect; AgRP, agouti related peptide; CART, cocaine and amphetamine regulated transcript; CCK, cholescystokinin; HK2, hemokinin 2; NKA, neurokinin A; NKB, neurokinin B; NMB, neuromedin B; NPY, neuropeptide Y; POMC, proopiomelanocortin; PYY, peptide YY; SP, substance P.

food intake, but an increase of locomotor activity is also observed (274). The fact that icv injections of NKA induce an increase in *pomc* mRNA levels in the rat ARC (275) also argues toward such anorexigenic action of tachykinins in mammals or at least in rodents. However, later, Karagiannides and collaborators consider SP as a novel antiobesity target after showing that the blockade of SP signaling by mean of an TACR1 antagonist leads to a decrease of food intake and body weight in two obese mouse models, an HFD-induced one and a leptin-deficient (*ob*/*ob*) one (276). They also report that peripheral injection of SP increases food intake and induces upregulation of hypothalamic *npy* as well as downregulation of *pomc* and mRNA levels (276). In male rats, ghrelin negatively regulates the *tac1* gene in the hypothalamus and acute icv injection of NPK and NPγ (but not SP nor NKA) reduces food intake (277). In addition, in male mice, the hyperphagic effect of peripheral injection of ghrelin disappears in *tac1*KO animals, suggesting the *tac1* requirement in the control of food intake by ghrelin in rodents (277). The first study using *tac1*-null mice does not show any difference in body size compared with controls (276), while a more recent one reports that these animals have a significantly lower body weight during adulthood and also show increased hypothalamic *pomc* expression and reduced food intake (278). All these data indicate that, in rodents, TAC1 peptides may function as either endogenous anorexigenic or orexigenic peptides.

The capacity of SP, NKA, and NKB to induce intestine contraction was one of the actions that led to their discoveries (refer to part 1.1. of this review). Their action on motility is observed in all parts of the gut through the tachykinin receptors and has been previously reviewed (279, 280).

4.2 Teleosts

4.2.1 Energy state and the tachykinin system

In goldfish, a short-term postprandial increase in *tac1* mRNA levels (γ-PPT in the article) has been reported in both the hypothalamus and the olfactory bulbs (281). In zebrafish, fasting increases the brain expression of *tac3* in females (282). In grass carp, food intake can significantly induce hypothalamic *tac3a* and *tac3b* mRNA expression (222). Thus, in teleosts, change in energy state may have positive and negative effects on the TAC system.

4.2.2 Effects of tachykinins on food intake and GIT motility

Immunohistochemical studies report high concentrations of tachykinins and their receptors in teleost hypothalamic areas involved in the control of food intake [SP in goldfish (283); SP in sea bass (152); carassin in goldfish (284); TACR1 and TACR3 in

electric fish *Apteronotus leptorhynchus* (285)]. In goldfish, the *tac1* mRNA (γ-PPT in the article) encoding SP, carassin, and NKA presents a higher expression in olfactory bulbs and hypothalamus, while being present throughout the brain (58, 281). More recently, RT-PCR, qPCR, and ISH data confirmed the expression in the hypothalamus of the different *tac* and *tacr* genes [zebrafish (26, 71); tilapia (72); grass carp (61, 62, 75, 89); goldfish (73, 88); orange-spotted grouper (77); spotted sea bass (78); tongue sole (79)].

While tachykinins and their receptors are expressed in the teleost hypothalamus and its nuclei involved in the control of food intake, to our knowledge, no data have yet shown their direct effect on the expression of neuropeptides such as *pomc*, *npy*, or *agrp* at the brain level. Due to the direct innervation of pituitary cells by hypophysiotropic neurons in teleosts, Hu and colleagues demonstrated a high expression, in the brain and pituitary, of neuropeptides involved in the regulation of feeding (221). They subsequently reported that TAC3 and TAC4 peptides could change the expression of some of these genes, using transcriptomic analysis of TAC peptide effects on grass carp pituitary cells (62, 222). NKB can induce *in vitro* the expression levels of urotensin 1 (UTS1), cocaine-and-amphetamine-regulated transcript 2 precursor (CART2), proopiomelanocortin b (pomcb), and neuromedin B1 (NMB1) mRNA, all four anorexigenic peptides, an effect that is also reported *in vivo* after ip injection (222). HK2, a peptide encoded by the *tac4* gene in grass carp, upregulates CART2, CART3, and CART5, peptide YY2 (PYY2), UTS1, and NMB1 expression, while downregulating type 2 neuropeptide Y receptor (NPY2R) expression (62). Thus, NKB and HK2 inhibit the expression of the orexigenic pathway (such as NPY one) and stimulate anorexigenic peptides, playing roles of satiety factors (Figure 3). In grass carp, TACR3b mediates this role, while TACR3a modulates NKB action on reproduction (222), indicating a typical case of subfunctionalization where paralogs share initial pleiotropic functions. Tachykinins have also been involved in live prey food preference in hybrid *Siniperca chuatsi* × *Siniperca scherzeri* mandarin fish as *tac 1* expression is higher in feeders compared with non-feeders in this species (286).

Tachykinins and their receptors are also expressed in the stomach and intestine of many teleosts, suggesting potential autocrine or paracrine actions [zebrafish (26, 71); tilapia (72); goldfish (73, 88); grass carp (61, 75, 211); orange-spotted grouper (77); spotted sea bass (78); tongue sole (79)].

Peripheral action with direct contraction of the smooth gut muscle has also been described in teleosts [for reviews (10, 287)]. Substance P stimulates the motility of isolated intestine or stomach in a variety of fish [*Pleuronectes platessa*, *Labrus bergylta*, *Gadus* species, *Lophius* species, *Anguilla* species (55); rainbow trout (288, 289); cod *Gadus morhua* (290, 291); common carp *Cyprinus carpio* (292); bichir *Polypterus senegalensis* (293)]. In many of these species, it is demonstrated that the effect of SP is in part direct (cotreatment with tetrodotoxin, a sodium channel blocker) and in part *via* stimulation of cholinergic and serotonergic neurons (cotreatment with cholinergic or serotonergic antagonists, atropine and methysergide) [Figure 3, common carp (294):; rainbow trout (288, 289, 293)]. NKA is also able to stimulate the motility of isolated trout intestinal muscle and the vascularly perfused trout stomach, but with less efficiency than SP (289). This stimulatory control of gut motility by the tachykinin system takes place at an early stage in development as NKA modulates zebrafish larval gut before or around the time for the onset of feeding (295). More recently, an increase in the expression of *tac1* has been detected by RNA-seq in the giant grouper *Epinephelus lanceolatus* at the onset of feeding (296). Using *in vitro* stomach and intestine incubation assays in the sea bass, Zhang and collaborators showed that NKB peptides may modulate the expression of hormones (78) (Figure 3), known to have stimulatory activity on GIT motility in vertebrates, such as motilin and ghrelin [for review (297)]. In the stomach, NKBa-13 and NKBb-13 stimulate gastrin mRNA levels, while NKB-10 peptides have no effect (78). NKBb-13 can stimulate stomachal motilin and ghrelin expression, while the other three NKB peptides have no effect (78). In the intestine, NKBa-13, NKBa-10, and NKBb-13 stimulate cholecystokinin mRNA levels, while NKBb-10 has no effect (78). Only NKBb-10 can stimulate intestinal gastrin expression and NKBa-10 motilin expression. None of the four NKB peptides can change ghrelin mRNA levels in the intestine (78).

5 Conclusions and perspectives

Cumulating evidence places the tachykinin system with not only NKB (TAC3) but also other tachykinin peptides, SP (TAC1) and NKA (TAC1), as major stimulatory actors in the control of reproductive function, in mammals. In teleosts, the two TAC3 peptides NKB and NKBRP and their 3R duplicates can have various effects (stimulatory, inhibitory, or none) mainly according to the species, the maturity stage, and the peptide tested. These sometimes opposite effects of TAC3 peptides on reproductive genes among teleost species are also reported for other neuropeptides involved in the control of the HPG axis, such as kisspeptin and gonadotropin-inhibitory factor (GnIH) (for review: 9). One may take into consideration the variety of reproductive strategies and life cycles among these more than 25,000 different species to try to find explanations, as well as the physiological compensations between neuropeptides that are likely to exist in teleosts, perhaps due to the anatomical direct innervation of pituitary cells and the existence of various 3R paralogs. Concerning potential involvement of TAC1 and TAC4 peptides in teleost reproduction, too few data are available to draw any conclusion. Past studies in mammals and recent ones in teleosts suggest that the tachykinin system may also be involved in the regulation of food intake and metabolism. Even if more studies are still needed, especially concerning the role of TAC4 peptides, it looks like the control of food intake may be taken over by TAC1 peptides in mammals but also by

TAC3 and 4 peptides in teleosts. Tachykinins and their receptors thus seem to be part of networks linking metabolism and reproduction and involving central and peripheral hormones, such as kisspeptin, leptin, and ghrelin.

Due to the multiple whole-genome duplication events that occurred in vertebrates, phenomena of divergence and subfunctionalization or neofunctionalization of the ancestral functions are expected and observed, especially in teleosts. Therefore, an analysis of the tachykinin system is recommended for each organism of interest in order to obtain a clear view of the function of this family of peptides and receptors according to vertebrate species.

Future studies should aim at stating whether or not KNDy neurons exist in some teleost species. Too few species have been considered so far. Surprisingly, up to now, no study has yet investigated the possible direct effects of TAC3 peptides on pituitary LH and FSH cells in mammals; this should be performed in the future using primary cultures of pituitary cells from different mammalian species. It would also be interesting to study whether TAC peptides could directly modify the expression of central actors involved in the control of food intake, such as *pomc* and *npy*, in both mammals and teleosts. Future directions on the study of the tachykinin system should also include investigations on Mas-related GPCRs (Mrgprs), as TACR1 antagonists have off-target activity on them (298) and substance P recruits these receptors in immune cells to release cytokine contributing to inflammatory pain in mice (299, 300). For example, characterizing Mrgprs in teleosts and knowing whether they are present in the HPG tissues will help to decipher their potential involvement in the reproductive role of the tachykinin system.

Author contributions

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

Funding

This work was supported by grants from the National Research Agency NEMO No. ANR 14-CE02-0020 and from the European Community, Innovative Training Network, ITN IMPRESS MSCA-ITN-2014-ETN No. 642893. The authors are supported by the "Centre National de la Recherche Scientifique" and the "Muséum National d'Histoire Naturelle.

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.

Publisher's note

All claims expressed in this article are solely those of the authors and do not necessarily represent those of their affiliated organizations, or those of the publisher, the editors and the reviewers. Any product that may be evaluated in this article, or claim that may be made by its manufacturer, is not guaranteed or endorsed by the publisher.

References

1. Hu G, Lin C, He M, Wong AOL. Neurokinin B and reproductive functions: "KNDy neuron" model in mammals and the emerging story in fish. *Gen Comp Endocrinol* (2014) 208:94–108. doi: 10.1016/j.ygcen.2014.08.009

2. Nässel DR, Zandawala M, Kawada T, Satake H. Tachykinins: Neuropeptides that are ancient , diverse , widespread and functionally pleiotropic. *Front Neurosci* (2019) 13:1262. doi: 10.3389/fnins.2019.01262

3. Vanden Broeck J, Torfs H, Poels J, Van Poyer W, Swinnen E, Ferket K, et al. Tachykinin-like peptides and their receptors: A review. *Ann N Y Acad Sci* (1999) 897:374–87. doi: 10.1111/j.1749-6632.1999.tb07907.x

4. Severini C, Improta G, Falconieri Erspamer G, Salvadori S, Erspamer V, Falconieri-Erspamer G, et al. The tachykinin peptide family. *Pharmacol Rev* (2002) 54:285–322. doi: 10.1016/0166-2236(81)90084-9

5. Pennefather JN, Lecci A, Candenas ML, Patak E, Pinto FM, Maggi CA. Tachykinins and tachykinin receptors: A growing family. *Life Sci* (2004) 74:1445–63. doi: 10.1016/j.lfs.2003.09.039

6. Liu L, Burcher E. Tachykinin peptides and receptors: Putting amphibians into perspective. *Peptides* (2005) 26:1369–82. doi: 10.1016/j.peptides.2005.03.027

7. Van Loy T, Vandersmissen HP, Poels J, Van Hiel MB, Verlinden H, Vanden Broeck J. Tachykinin-related peptides and their receptors in invertebrates: A current view. *Peptides* (2010) 31:520–4. doi: 10.1016/j.peptides.2009.09.023

8. Satake H, Aoyama M, Sekiguchi T, Kawada T. Insight into molecular and functional diversity of tachykinins and their receptors. *Protein Pept Lett* (2013) 20:615–27. doi: 10.2174/0929866511320060002

9. Dufour S, Quérat B, Tostivint H, Pasqualini C, Vaudry H, Rousseau K. Origin and evolution of the neuroendocrine control of reproduction in vertebrates, with special focus on genome and gene duplications. *Physiol Rev* (2020) 100:869–943. doi: 10.1152/physrev.00009.2019

10. Volkoff HV, Canosa LF, Unniappan S, Cerdá-reverter JM, Bernier NJ, Kelly SP, et al. Neuropeptides and the control of food intake in fish. *Gen Comp Endocrinol* (2005) 142:3–19. doi: 10.1016/j.ygcen.2004.11.001

11. Volkoff H. The neuroendocrine regulation of food intake in Fish: A review of current knowledge. *Front Neurosci* (2016) 10:540. doi: 10.3389/fnins.2016.00540

12. Ronnestad I, Gomes AS, Murashita K, Angotzi R, Jonsson E, Volkoff H. Appetite-controlling endocrine systems in teleosts. *Front Endocrinol (Lausanne)* (2017) 8:73. doi: 10.3389/fendo.2017.00073

13. van de Pol I, Flik G, Gorissen M. Comparative physiology of energy Metabolism: Fishing for endocrine signals in the early vertebrate pool. *Front Endo* (2017) 8:36. doi: 10.3389/fendo.2017.00036

14. Shahjahan M, Kitahashi T, Parhar IS. Central pathways integrating metabolism and reproduction in teleosts. *Front Endocrinol (Lausanne)* (2014) 5:36. doi: 10.3389/fendo.2014.00036

15. Volkoff H, London S. Nutrition and reproduction in fish. In: *Encyclopedia of reproduction* (Oxford: Academic Press), vol. 9 p. 743–8. doi: 10.1016/B978-0-12-809633-8.20624-9

16. Navarro VM, Tena-sempere M. Neuroendocrine control by kisspeptins: role in metabolic regulation of fertility. *Nat Publ Gr* (2012) 8:40–53. doi: 10.1038/nrendo.2011.147

17. Navarro VM. Metabolic regulation of kisspeptin — the link between energy balance and reproduction. *Nat Rev Endocrinol* (2020) 16:407–20. doi: 10.1038/s41574-020-0363-7

18. Castellano JM, Bentsen AH, Mikkelsen JD, Tena-sempere M. Kisspeptins: Bridging energy homeostasis and reproduction. *Brain Res* (2010) 1364:129–38. doi: 10.1016/j.brainres.2010.08.057

19. Blanco AM. Hypothalamic- and pituitary-derived growth and reproductive hormones and the control of energy balance in fish. *Gen Comp Endocrinol* (2020) 287:113322. doi: 10.1016/j.ygcen.2019.113322

20. Trudeau VL. Neuroendocrine control of reproduction in teleost Fish: Concepts and controversies. *Annu Rev Anim Biosci* (2022) 10:107–30. doi: 10.1146/annurev-animal-020420-042015

21. Zhang Y, Lu L, Furlonger C, Wu GE, Paige CJ. Hemokinin is a hematopoietic-specific tachykinin that regulates B lymphopoiesis. *Nat Immunol* (2000) 1:392–7. doi: 10.1038/80826

22. Maggi CA. Principles of tachykininergic co-transmission in the peripheral and enteric nervous system. *Regul Pept* (2000) 93:53–64. doi: 10.1016/S0167-0115(00)00177-4

23. Patacchini R, Lecci A, Holzer P, Maggi CA. Newly discovered tachykinins raise new questions about their peripheral roles and the tachykinin nomenclature. *Trends Pharmacol Sci* (2004) 25:1–3. doi: 10.1016/j.tips.2003.11.005

24. Maggi CA. The troubled story of tachykinins and neurokinins. *Trends Pharmacol Sci* (2000) 21:173–5. doi: 10.1016/S0165-6147(00)01463-2

25. Patacchini R, Maggi CA. The nomenclature of tachykinin receptors. In: Holzer P, editor. *Tachykinins*. (Berlin: Heidelberg: Springer Science & Business Media). (2004) p. 121–39.

26. Zhou W, Li S, Liu Y, Qi X, Chen H, Cheng CHK, et al. The evolution of tachykinin/tachykinin receptor (TAC/TACR) in vertebrates and molecular identification of the TAC3/TACR3 system in zebrafish (Danio rerio). *Mol Cell Endocrinol* (2012) 361:202–12. doi: 10.1016/j.mce.2012.04.007

27. Conlon JM, Larhammar D. The evolution of neuroendocrine peptides. *Gen Comp Endocrinol* (2005) 142:53–9. doi: 10.1016/j.ygcen.2004.11.016

28. Dehal P, Boore JL. Two rounds of whole genome duplication in the ancestral vertebrate. *PloS Biol* (2005) 3:1700–8. doi: 10.1371/journal.pbio.0030314

29. Donoghue PCJ, Purnell MA. Genome duplication, extinction and vertebrate evolution. *Trends Ecol Evol* (2005) 20:312–9. doi: 10.1016/j.tree.2005.04.008

30. Meyer A, Van De Peer Y. From 2R to 3R: Evidence for a fish-specific genome duplication (FSGD). *BioEssays* (2005) 27:937–45. doi: 10.1002/bies.20293

31. Braasch I, Postlethwait JH. Polyploidy in fish and the teleost genome duplication. In: Soltis PS, Soltis DE, editors. *Polyploidy and genome evolution*. Eugene: Springer-Verlag Berlin Heidelberg. (2012) p. 341–83. doi: 10.1007/978-3-642-31442-1

32. Almeida TA, Rojo J, Nieto PM, Pinto FM, Hernandez M, Martin JD, et al. Tachykinins and tachykinin receptors: structure and activity relationships. *Curr Med Chem* (2004) 11:2045–81. doi: 10.2174/0929867043364748

33. Pennefather JN, Patak E, Ziccone S, Lilley A, Pinto FM, Page NM, et al. Regulation of the stimulant actions of neurokinin a and human hemokinin-1 on the human uterus: A comparison with histamine. *Biol Reprod* (2006) 75:334–41. doi: 10.1095/biolreprod.106.051508

34. Steinhoff MS, von Mentzer B, Geppetti P, Pothoulakis C, Bunnett NW. Tachykinins and their receptors: contributions to physiological control and the mechanisms of disease. *Physiol Rev* (2014) 94:265–301. doi: 10.1152/physrev.00031.2013

35. Nawa H, Kotani H, Nakanishi S. Tissue-specific generation of two preprotachykinin mRNAs from one gene by alternative RNA splicing. *Nature* (1984) 312:550. doi: 10.1038/311525a0

36. Kawaguchi Y, Hoshimaru M, Nawa H, Nakanishi S. Sequence analysis of cloned cDNA for rat substance P precursor: Existence of a third substance P precursor. *Biochem Biophys Res Commun* (1986) 139:1040–6. doi: 10.1016/S0006-291X(86)80282-0

37. Lai J, Douglas SD, Rappaport E, Wu JM, Ho W. Identification of a δ isoform of preprotachykinin mRNA in human mononuclear phagocytes and lymphocytes. *J Neuroimmunol* (1998) 91:121–8. doi: 10.1016/S0165-5728(98)00170-2

38. Page NM, Morrish DW, Weston-Bell NJ. Differential mRNA splicing and precursor processing of neurokinin B in neuroendocrine tissues. *Peptides* (2009) 30:1508–13. doi: 10.1016/j.peptides.2009.04.023

39. Page NM, Bell NJ, Gardiner SM, Manyonda IT, Brayley KJ, Strange PG, et al. Characterization of the endokinins: Human tachykinins with cardiovascular activity. *Pharmacology* (2003) 100:6245–50. doi: 10.1073/pnas.0931458100

40. Page NM. New challenges in the study of the mammalian tachykinins. *Peptides* (2005) 26:1356–68. doi: 10.1016/j.peptides.2005.03.030

41. Page NM, Dakour J, Morrish DW. Gene regulation of neurokinin B and its receptor NK3 in late pregnancy and pre-eclampsia. *Mol Hum Reprod* (2006) 12:427–33. doi: 10.1093/molehr/gal025

42. Naono R, Nakayama T, Ikeda T, Matsushima O, Nishimori T. Leucine at the carboxyl-terminal of endokinins C and D contributes to elicitation of the antagonistic effect on substance p in rat pain processing. *Brain Res* (2007) 1165:71–80. doi: 10.1016/j.brainres.2007.05.062

43. Nishimori T, Naono-Nakayama R, Ikeda T. New tachykinin peptides and nociception. *Jpn Dent Sci Rev* (2013) 49:27–34. doi: 10.1016/j.jdsr.2012.11.002

44. Maggi CA. The mammalian tachykinin receptors. *Gen Pharmacol* (1995) 26:911–44. doi: 10.1016/0306-3623(94)00292-U

45. Patak E, Pinto FM, Story ME, Pintado CO, Fleming A, Page NM, et al. Functional and molecular characterization of tachykinins and tachykinin receptors in the mouse uterus. *Biol Reprod* (2005) 72:1125–33. doi: 10.1095/biolreprod.104.036814

46. von Euler U, Gaddum J. An unidentified depressor substance in certain tissue extracts. *J Physiol* (1931) 612:74–87.

47. Hokfelt T, Pernow B, Wahren J. Substance P: a pioneer amongst neuropeptides. *J Intern Med* (2001) 249:27–40.

48. Chang M, Leeman S, Niall H. Amino-acid sequence of substance P. *Nat New Biol* (1971) 232:86–7.

49. Tregear GW, Niall HD, Potts JT, Leeman SE, Chang MM. Synthesis of substance P. *Nat New Biol* (1971) 232:87–9. doi: 10.1038/newbio232087a0

50. Kimura S, Okada M, Sugita Y, Kanazawa I, Munekata E, Ebashi S. Novel neuropeptides, neurokinin α and β, isolated from porcine spinal cord. *Proc Japan Acad Ser B* (1983) 59:101–4. doi: 10.2183/pjab.59.101

51. Nawa H, Hirose T, Takashima H, Inayama S, Nakanishi S. Nucleotide sequences of cloned cDNAs for two types of bovine brain substance P precursor. *Nature* (1983) 306:32–6.

52. Minamino N, Kangawa K, Fukuda A, Matsuo H. Neuromedin L: A novel mammalian tachykinin identified in porcine spinal cord. *Neuropeptides* (1984) 4:157–66. doi: 10.1016/0143-4179(84)90126-4

53. Tatemoto K, Lundberg JM, Jörnvall H, Mutt V. Neuropeptide K: Isolation, structure and biological activities of a novel brain tachykinin. *Biochem Biophys Res Commun* (1985) 128:947–53. doi: 10.1016/0006-291X(85)90138-X

54. Kage R, McGregor GP, Thim L, Conlon JM. Neuropeptide-gamma: a peptide isolated from rabbit intestine that is derived from gamma-preprotachykinin. *J Neurochem* (1988) 50:1412–7. doi: 10.1111/j.1471-4159.1988.tb03024.x

55. Von Euler US, Ostlund E. Occurrence of a substance P-like polypeptide in fish intestine and brain. *Br J Pharmacol* (1956) 11:323–5.

56. Conlon JM, Harte FO, Peter RE, Kah Y. Carassin: A tachykinin that is structurally related to neuropeptide-y from the brain of the goldfish. *J Neurochem* (1990) 56:1432–6. doi: 10.1111/j.1471-4159.1991.tb11442.x

57. Jensen J, Conlon JM. Substance-P-related and neurokinin-a-related peptides from the brain of the cod and trout. *Eur J Biochem* (1992) 206:659–64. doi: 10.1111/j.1432-1033.1992.tb16971.x

58. Lin X, Peter RE. Goldfish γ-preprotachykinin mRNA encodes the neuropeptides substance P, carassin, and neurokinin A. *Peptides* (1997) 18:817–24. doi: 10.1016/S0196-9781(97)00013-2

59. López-Bellido R, Barreto-Valer K, Rodríguez RE. Substance P mRNA expression during zebrafish development: influence of mu opioid receptor and cocaine. *Neuroscience* (2013) 242:53–68. doi: 10.1016/j.neuroscience.2013.03.022

60. Ogawa S, Ramadasan PN, Goschorska M, Anantharajah A, We Ng K, Parhar IS. Cloning and expression of tachykinins and their association with kisspeptins in the brains of zebrafish. *J Comp Neurol* (2012) 520:2991–3012. doi: 10.1002/cne.23103

61. Hu G, He M, Ko WKW, Wong AOL. TAC1 gene products regulate pituitary hormone secretion and gene expression in prepubertal grass carp pituitary cells. *Endocrinology* (2017) 158:1776–97. doi: 10.1210/en.2016-1740

62. Shi X, Ye C, Qin X, Zhou L, Xia C, Cai T, et al. Novel pituitary actions of TAC4 gene products in teleost. *Int J Mol Sci* (2021) 22:1–20.

63. Duarte CR, Schütz B, Zimmer A. Incongruent pattern of neurokinin B expression in rat and mouse brains. *Cell Tissue Res* (2006) 323:43–51. doi: 10.1007/s00441-005-0027-x

64. Chawla MK, Gutierrez GM, Young WS, Mcmullen NT, Rance NE. Localization of neurons expressing substance P and neurokinin B gene transcripts in the human hypothalamus and basal forebrain. *J Comp Neurol* (1997) 384:429–42. doi: 10.1002/(SICI)1096-9861(19970804)384:3<429::AID-CNE8>3.0.CO;2-5

65. Pinto FM, Pintado CO, Pennefather JN, Patak E, Candenas L. Ovarian steroids regulate tachykinin and tachykinin receptor gene expression in the mouse uterus. *Reprod Biol Endocrinol* (2009) 7:77. doi: 10.1186/1477-7827-7-77

66. Page NM. Hemokinins and endokinins. *Cell Mol Life Sci* (2004) 61:1652–63. doi: 10.1007/s00018-004-4035-x

67. Kangawa K, Minamino N, Fukuda A, Matsuo H. Neuromedin K: A novel mammalian tachykinin identified in porcine spinal cord. *Biochem Biophys Res Commun* (1983) 114:533–40. doi: 10.1016/0006-291X(83)90813-6

68. Kotani H, Hoshimaru M, Nawa H, Nakanishi S. Structure and gene organization of bovine neuromedin K precursor. *Proc Natl Acad Sci U.S.A.* (1986) 83:7074–8. doi: 10.1073/pnas.83.18.7074

69. Bonner TI, Affolter H-U, Young AC, Young WS. A cDNA encoding the precursor of the rat neuropeptide, neurokinin B. *Mol Brain Res* (1987) 2:243–9. doi: 10.1016/0169-328X(87)90031-3

70. Page NM, Lowry PJ. Is "pre-eclampsia" simply a response to the side effects of a placental tachykinin? *J Endocrinol* (2000) 167:355–61. doi: 10.1677/joe.0.1670355

71. Biran J, Palevitch O, Ben-Dor S, Levavi-Sivan B. Neurokinin Bs and neurokinin B receptors in zebrafish-potential role in controlling fish reproduction. *Proc Natl Acad Sci* (2012) 109:10269–74. doi: 10.1073/pnas.1119165109

72. Biran J, Golan M, Mizrahi N, Ogawa S, Parhar IS, Levavi-Sivan B. Direct regulation of gonadotropin release by neurokinin B in tilapia (Oreochromis niloticus). *Endocrinology* (2014) 155:4831–42. doi: 10.1210/en.2013-2114

73. Qi X, Zhou W, Li S, Liu Y, Ye G, Liu X, et al. Goldfish neurokinin B: Cloning, tissue distribution, and potential role in regulating reproduction. *Gen Comp Endocrinol* (2015) 221:267–77. doi: 10.1016/j.ygcen.2014.10.017

74. Zmora N, Wong T-T, Stubblefield J, Levavi-Sivan B, Zohar Y. Neurokinin B regulates reproduction *via* inhibition of kisspeptin in a teleost, the striped bass. *J Endocrinol* (2017) 233:159–74. doi: 10.1530/JOE-16-0575

75. Hu G, He M, Ko WKW, Lin C, Wong AOL. Novel pituitary actions of TAC3 gene products in fish model: Receptor specificity and signal transduction for prolactin and somatolactin α regulation by neurokinin B (NKB) and NKB-related peptide in carp pituitary cells. *Endocrinology* (2014) 155:3582–96. doi: 10.1210/en.2014-1105

76. Campo A, Lafont AG, Lefranc B, Leprince J, Tostivint H, Kamech N, et al. Tachykinin-3 genes and peptides characterized in a basal teleost, the European eel: Evolutionary perspective and pituitary role. *Front Endocrinol (Lausanne)* (2018) 9:304. doi: 10.3389/fendo.2018.00304

77. Chen H, Xiao L, Liu Y, Li S, Li G, Zhang Y, et al. Neurokinin B signaling in hermaphroditic species, a study of the orange-spotted grouper (Epinephelus coioides). *Gen Comp Endocrinol* (2018) 260:125–35. doi: 10.1016/j.ygcen.2018.01.009

78. Zhang Z, Wen H, Li Y, Li Q, Li W, Zhou Y. TAC3 gene products regulate brain and digestive system gene expression in the spotted Sea bass (Lateolabrax maculatus). *Front Endocrinol (Lausanne)* (2019) 10:556. doi: 10.3389/fendo.2019.00556

79. Wang B, Cui A, Zhang Y, Xu Y, Wang W, Jiang Y, et al. Neurokinin B in a flatfish species, the half-smooth tongue sole (Cynoglossus semilaevis), and its potential role in reproductive functions. *Aquac Rep* (2021) 20:100651. doi: 10.1016/j.aqrep.2021.100651

80. Kurtz MM, Wang R, Clements MK, Cascieri M a., Austin CP, Cunningham BR, et al. Identification, localization and receptor characterization of novel mammalian substance-P peptides. *Gene* (2002) 296:205–12. doi: 10.1016/S0378-1119(02)00861-2

81. Harada Y, Takahashi T, Kuno M, Nakayama K, Masu Y, Nikanishi S. Expression of two different tachykinin receptors in xenopus oocytes by exogenous mRNAs. *J Neurosci* (1987) 7:3265–73. doi: 10.1523/JNEUROSCI.07-10-03265.1987

82. Masu Y, Nakayama K, Tamaki H, Harada Y, Kuno M, Nakanishi S. cDNA eloping of bovine substance-K receptor through oocyte expression system. *Nature* (1987) 329:836. doi: 10.1038/329836a0

83. Yokota Y, Sasai Y, Tanaka K, Fujiwara T, Tsuchida K, Shigemoto R, et al. Molecular characterization of a functional cDNA for rat substance P receptor. *J Biol Chem* (1989) 264:17649–52. doi: 10.1016/S0021-9258(19)84619-7

84. Shigemoto R, Yoshifumi Y, Tsuchida K, Nakanishi S. Cloning and expression of a rat neuromedin K receptor cDNA. *J Biol Chem* (1990) 265:623–8. doi: 10.1016/S0021-9258(19)40095-1

85. Gerard NP, Bao L, Ziao-Ping H, Gerard C. Molecular aspects of the tachykinin receptors. *Regul Pept* (1993) 43:21–35. doi: 10.1016/0167-0115(93)90404-V

86. Krause JE, Sachais BS, Blount P. Tachykinin receptors. In: Peroutka SJ, editor. *Handb recept channels*. Boca Raton, FL: CRC (1994). p. 277–98.

87. Nakanishi S. Mammalian tachykinin receptors. *Annu Rev Neurosci* (1991) 14:123–36. doi: 10.1146/annurev.ne.14.030191.001011

88. Liu Y, Wang Q, Wang X, Meng Z, Liu Y, Li S, et al. NKB / NK3 system negatively regulates the reproductive axis in sexually immature goldfish (Carassius auratus). *Gen Comp Endocrinol* (2019) 281:126–36. doi: 10.1016/j.ygcen.2019.05.020

89. Xu S, Zhou L, Guo S, Hu Q, Shi X, Xia C, et al. Different pituitary action of NK3Ra and NK3Rb in grass carp. *Gen Comp Endocrinol* (2021) 313:113829. doi: 10.1016/j.ygcen.2021.113829

90. de Roux N, Genin E, Carel J-C, Matsuda F, Chaussain J-L, Milgrom E. Hypogonadotropic hypogonadism due to loss of function of the KiSS1-derived peptide receptor GPR54. *Proc Natl Acad Sci U.S.A.* (2003) 100:10972–6. doi: 10.1073/pnas.1834399100

91. Funes S, Hedrick JA, Vassileva G, Markowitz L, Abbondanzo S, Golovko A, et al. The KiSS-1 receptor GPR54 is essential for the developement of the murine reproductive system. *Biochem Biophys Res Commun* (2003) 312:1357–63. doi: 10.1016/j.bbrc.2003.11.066

92. Seminara SB, Messager S, Chatzidaki EE, Thresher RR, Acierno JS, Shagoury JK, et al. The GPR54 gene as a regulator of puberty. *N Engl J Med* (2003) 349:1614–27. doi: 10.1056/NEJMoa035322

93. Oakley AE, Clifton DK, Steiner RA. Kisspeptin signaling in the brain. *Endocr Rev* (2009) 30:713–43. doi: 10.1210/er.2009-0005

94. Uenoyama Y, Inoue N, Nakamura S, Tsukamura H. Central mechanism controlling pubertal onset in Mammals: A triggering role of kisspeptin. *Front Endocrinol (Lausanne)* (2019) 10:312. doi: 10.3389/fendo.2019.00312

95. Uenoyama Y, Inoue N, Nakamura S, Tsukamura H. Kisspeptin neurons and estrogen – estrogen receptor α Signaling: Unraveling the mystery of steroid feedback system regulating mammalian reproduction. *Int J Mol Sci* (2021) 22:1–16. doi: 10.3390/ijms22179229

96. Goodman RL, Lehman MN, Smith JT, Coolen LM, De Oliveira CVR, Jafarzadehshirazi MR, et al. Kisspeptin neurons in the arcuate nucleus of the ewe express both dynorphin A and neurokinin B. *Endocrinology* (2007) 148:5752–60. doi: 10.1210/en.2007-0961

97. Fergani C, Navarro VM. Expanding the role of tachykinins in the neuroendocrine control of reproduction. *Reproduction* (2017) 153:R1–R14. doi: 10.1530/REP-16-0378

98. Lehman MN, Coolen LM, Goodman RL. Minireview: Kisspeptin/neurokinin B/dynorphin (KNDy) cells of the arcuate nucleus: A central node in the control of gonadotropin-releasing hormone secretion. *Endocrinology* (2010) 151:3479–89. doi: 10.1210/en.2010-0022

99. Rance NE, Krajewski SJ, Smith MA, Cholanian M, Dacks PA. Neurokinin B and the hypothalamic regulation of reproduction. *Brain Res* (2010) 1364:116–28. doi: 10.1016/j.brainres.2010.08.059

100. Grachev P, Millar RP, O'Byrne KT. The role of neurokinin B signalling in reproductive neuroendocrinology. *Neuroendocrinology* (2014) 99:7–17. doi: 10.1159/000357734

101. Moore AM, Coolen LM, Porter DT, Goodman RL, Lehman MN. KNDy cells revisited. *Endocrinology* (2018) 159:3219–34. doi: 10.1210/en.2018-00389

102. Li Q, Millar RP, Clarke IJ, Smith JT. Evidence that neurokinin B controls basal gonadotropin-releasing hormone secretion but is not critical for estrogen-positive feedback in sheep. *Neuroendocrinology* (2015) 101(2), 161–74.

103. Yamamura T, Wakabayashi Y, Ohkura S, Navarro V, Okamura H. Effects of intravenous administration of neurokinin receptor subtype-selective agonists on gonadotropin-releasing hormone pulse generator activity and luteinizing hormone secretion in goats. *J Reprod Dev* (2015) 61(1) 20–29. doi: 10.1262/jrd.2014-109

104. Noritake KI, Matsuoka T, Ohsawa T, Shimomura K, Sanbuissho A, Uenoyama Y, et al. Involvement of neurokinin receptors in the control of pulsatile luteinizing hormone secretion in rats. *J Reprod Dev* (2011) 11:1–21. doi: 10.1262/jrd.11-002S

105. de Croft S, Boehm U, Herbison AE. Through multiple tachykinin receptors in the Male mouse. *Endocrinology* (2013) 154:2750–60. doi: 10.1210/en.2013-1231

106. Leon S, Navarro VM. Novel biology of tachykinins in gonadotropin-releasing hormone secretion. *Semin Reprod Med* (2019) 37:109–18. doi: 10.1055/s-0039-3400252

107. Debeljuk L, Lasaga M. Tachykinins and the control of prolactin secretion. *Peptides* (2006) 27:3007–19. doi: 10.1016/j.peptides.2006.07.010

108. Billings HJ, Connors JM, Altman SN, Hileman SM, Holaskova I, Lehman MN, et al. Neurokinin B acts *via* the neurokinin-3 receptor in the retrochiasmatic area to stimulate luteinizing hormone secretion in sheep. *Endocrinology* (2010) 151:3836–46. doi: 10.1210/en.2010-0174

109. Simavli S, Thompson IR, Maguire CA, Gill JC, Carroll RS, Wolfe A, et al. Substance P regulates puberty onset and fertility in the female mouse. *Endocrinology* (2015) 156:2313–22. doi: 10.1210/en.2014-2012

110. Maguire CA, Song YB, Wu M, León S, Carroll RS, Alreja M, et al. Tac1 signaling is required for sexual maturation and responsiveness of gnrh neurons to kisspeptin in the male mouse. *Endocrinology* (2017) 158:2319–29. doi: 10.1210/en.2016-1807

111. De Felipe C, Herrero JF, O'brien JA, Palmer JA, Doyle CA, Smith AJH, et al. Altered nociception, analgesia and aggression in mice lacking the receptor for substance P. *Nature* (1998) 392:394. doi: 10.1038/32904

112. Torres E, Velasco I, Franssen D, Heras V, Gaytan F, Leon S, et al. Congenital ablation of Tacr2 reveals overlapping and redundant roles of NK2R

signaling in the control of reproductive axis. *Endocrinol Metab* (2021) 320:496–511. doi: 10.1152/ajpendo.00346.2020

113. Pintado CO, Pinto FM, Pennefather JN, Hidalgo A, Baamonde A, Sanchez T, et al. A role for tachykinins in female mouse and rat reproductive function. *Biol Reprod* (2003) 69:940–6. doi: 10.1095/biolreprod.103.017111

114. Ohtsuka S, Miyake A, Nishizaki T, Tasaka K, Aono T, Tanizawa O. Substance P stimulates gonadotropin-releasing hormone release from rat hypothalamus *in vitro* with involvement of oestrogen. *Acta Endocrinol (Copenh)* (1987) 115:247–52. doi: 10.1530/acta.0.1150247

115. Navarro VM, Bosch MA, León S, Simavli S, True C, Pinilla L, et al. The integrated hypothalamic tachykinin-kisspeptin system as a central coordinator for reproduction. *Endocrinology* (2015) 156:627–37. doi: 10.1210/en.2014-1651

116. Saffroy M, Torrens Y, Glowinski J, Beaujouan JC. Autoradiographic distribution of tachykinin NK2 binding sites in the rat brain: comparison with NK1 and NK3 binding sites. *Neuroscience* (2003) 116:761–73. doi: 10.1016/S0306-4522(02)00748-0

117. Tsuruo Y, Kawano H, Hisano S, Kagotani Y, Daikoku S, Zhang T, et al. Substance P-containing neurons innervating LHRH-containing neurons in the septo-preoptic area of rats. *Neuroendocrinology* (1991) 53:236–45. doi: 10.1159/000125724

118. Dudás B, Merchanthaler I. Close juxtapositions between LHRH immunoreactive neurons and substance P immunoreactive axons in the human diencephalon. *J Clin Endocrinol Metab* (2002) 87:2946–53. doi: 10.1210/jcem.87.6.8558

119. Borsay BÁ, Skrapits K, Herczeg L, Ciofi P, Bloom SR, Ghatei MA, et al. Hypophysiotropic gonadotropin-releasing hormone projections are exposed to dense plexuses of kisspeptin , neurokinin B and substance P immunoreactive fibers in the Human: A study on tissues from postmenopausal women. *Neuroendocrinology* (2014) 100:141–52. doi: 10.1159/000368362

120. Fergani C, Mazzella L, Coolen LM, McCosh RB, Hardy SL, Newcomb N, et al. Do substance P and neurokinin A play important roles in the control of lh secretion in ewes? *Endocrinology* (2016) 157:4829–41. doi: 10.1210/en.2016-1565

121. Sahu A, Kalra P. Effects of tachykinins on luteinizing hormone release in female Rats: Potent inhibitory action of neuropeptide K. *Endocrinology* (1992) 130:1571–7. doi: 10.1210/endo.130.3.1371455

122. Leon S, Fergani C, Talbi R, Simavli S, Maguire CA, Gerutshang A, et al. Characterization of the role of NKA in the control of puberty onset and gonadotropin release in the female mouse. *Endocrinology* (2019) 160:2453–63. doi: 10.1210/en.2019-00195

123. Rahdar P, Khazali H. Central injection of substance p antagonizes the RF amide-related peptide-3 impacts on hypothalamic KISS-1 and GnRH gene expressions in Male wistar rats. *Int J Basic Sicence Med* (2018) 3:127–32. doi: 10.15171/ijbms.2018.23

124. Hrabovszky E, Borsay BÁ, Rácz K, Herczeg L, Ciofi P, Bloom SR, et al. Substance P immunoreactivity exhibits frequent colocalization with kisspeptin and neurokinin B in the human infundibular region. *PloS One* (2013) 8:1–11. doi: 10.1371/journal.pone.0072369

125. Okamura H, Yamamura T., Wakabayashi Y. Mapping of KNDy neurons and immunohistochemical analysis of the interaction between KNDy and substance P neural systems in goat. *Journal of Reproduction and Development* (2017) 63:571–580. doi: 10.1262/jrd.2017-103

126. Kalil B, Ramaswamy S, Plant TM. The distribution of substance P and kisspeptin in the mediobasal hypothalamus of the Male rhesus monkey and a comparison of intravenous administration of these peptides to release GnRH as reflected by LH secretion. *Neuroendocrinology* (2016) 103:711–23. doi: 10.1159/000442420

127. Ogawa S, Ramadasan PN, Anthonysamy R, Parhar IS. Sexual dimorphic distribution of hypothalamic Tachykinin1 cells and their innervations to GnRH neurons in the zebrafish. *Front Endocrinol (Lausanne)* (2021) 11:534343. doi: 10.3389/fendo.2020.534343

128. Larsen PJ, Mikkelsen JD, Mau S, Særmark T. Binding and internalization of a iodinated substance P analog by cultured anterior pituitary cells. *Mol Cell Endocrinol* (1989) 65:91–101. doi: 10.1016/0303-7207(89)90169-X

129. Larsen PJ, Mikkelsen JD, Saermark T. Binding of a iodinated substance P analog to a NK-1 receptor on isolated cell membranes from rat anterior pituitary. *Endocrinology* (1989) 124:2548–57. doi: 10.1210/endo-124-5-2548

130. Kerdelhue B, Tartar A, Lenoir V, El Abed A, Hublau P, Millar RP. Binding studies of substance P anterior pituitary binding sites: changes in substance P binding sites during the rat estrous cycle. *Regul Pept* (1985) 10:133–43. doi: 10.1016/0167-0115(85)90008-4

131. Pisera D, Candolfi M, De Laurentiis A, Seilicovich A. Characterization of tachykinin NK2 receptor in the anterior pituitary gland. *Life Sci* (2003) 73:2421–32. doi: 10.1016/S0024-3205(03)00650-7

132. Debeljuk L, Lam EW, Bartke A. Effects of castration and sex steroids on neurokinin A concentrations in the anterior pituitary of male rats. *Neuroendocr Lett* (1991) 13:5–14.

133. Coslovsky R, Evans RW, Leeman SE, Braverman LE, Aronin N. The effects of gonadal steroids on the content of substance P in the rat anterior pituitary. *Endocrinology* (1984) 115:2285–9. doi: 10.1210/endo-115-6-2285

134. De Palatis LR, Khorram O, McCann SM. Age-, sex-, and gonadal steroid-related changes in immunoreactive substance P in the rat anterior pituitary gland. *Endocrinology* (1985) 117:1368–73. doi: 10.1210/endo-117-4-1368

135. Brown ER, Harlan RE, Krause JE. Gonadal steroid regulation of (SP) and SP-encoding messenger ribonucleic acids in the rat anterior pituitary and hypothalamus. *Endocrinology* (1990) 126:330–40. doi: 10.1210/endo-126-1-330

136. Makara GB, Kakucska I, Lenoir V, Kerdelhue B, Palkovits M. A substance P-containing hypothalamic neuronal system projects to the median eminence. *Brain Res* (1986) 374:399–401. doi: 10.1016/0006-8993(86)90438-5

137. Jessop DS, Chowdrey HS, Larsen PJ, Lightman SL. Substance P: multifunctional peptide in the hypothalamo-pituitary system? *J Endocrinol* (1991) 132:331–7. doi: 10.1677/joe.0.1320331

138. Rønnekleiv OK, Kelly MJ, Eskay RL. Distribution of immunoreactive substance P neurons in the hypothalamus and pituitary of the rhesus monkey. *J Comp Neurol* (1984) 224:51–9. doi: 10.1002/cne.902240105

139. Fisher GH, Humphries J, Folkers K, Pernow B, Bowers CY. Synthesis and some biological activities of substance P. *J Med Chem* (1974) 17:843–6. doi: 10.1021/jm00254a013

140. Vijayan E, McCann SM. *In vivo* and *in vitro* effects of substance P and neurotensin on gonadotropin and prolactin release. *Endocrinology* (1979) 105:64–8. doi: 10.1210/endo-105-1-64

141. Shamgochian MD, Leeman SE. Substance P stimulates luteinizing hormone secretion from anterior pituitary cells in culture. *Endocrinology* (1992) 131:871–5. doi: 10.1210/endo.131.2.1379165

142. Duval P, Lenoir V, Moussaoui S, Garret C, Kredelhue B. Substance P and neurokinin A variations throughout the rat estrous Cycle; comparison with ovariectomized and Male Rats: II. trigeminal nucleus and cervical spinal cord. *J Neurosci Res* (1996) 45:610–6. doi: 10.1002/(SICI)1097-4547(19960901)45:5<610::AID-JNR10>3.0.CO;2-2

143. Hidalgo-Díaz C, Castaño JP, López-Pedrera R, Malagón MM, García-Navarro S, Gracia-Navarro F. A modulatory role for substance P on the regulation of luteinizing hormone secretion by cultured porcine gonadotrophs. *Biol Reprod* (1998) 58:678–85. doi: 10.1095/biolreprod58.3.678

144. Hidalgo-Díaz C, Malagón MM, García-Navarro S, Luque RM, González de Aguilar JL, Gracia-Navarro F, et al. Role of Ca2+ in the secretory and biosynthetic response of porcine gonadotropes to substance P and gonadotropin-releasing hormone. *Regul Pept* (2003) 116:43–52. doi: 10.1016/S0167-0115(03)00176-9

145. Kalra PS, Sahu A, Bonavera JJ, Kalra SP. Diverse effects of tachykinins on luteinizing hormone release in male rats: mechanism of action. *Endocrinology* (1992) 131:1195–201. doi: 10.1210/en.131.3.1195

146. Arisawa M, De Palatis L, Ho R, Snyder GD, Wen HY, Pan G, et al. Stimulatory role of substance P on gonadotropin release in ovariectomized rats. *Neuroendocrinology* (1990) 51:523–9. doi: 10.1159/000125386

147. Ruiz-Pino F, Garcia-Galiano D, Manfredi-Lozano M, Leon S, Sánchez-Garrido MA, Roa J, et al. Effects and interactions of tachykinins and dynorphin on FSH and LH secretion in developing and adult rats. *Endocrinology* (2015) 156:576–88. doi: 10.1210/en.2014-1026

148. Coiro V, Volpi R, Caiazza A, Marcato A, Bocchi R, Colla R, et al. Luteinizing hormone response to an intravenous infusion of substance P in normal men. *Metabolism* (1992) 41:689–91. doi: 10.1016/0026-0495(92)90305-T

149. Traczyk WZ, Pau KYF, Kaynard AH, Spies HG. Modulatory role of substance P on gonadotropin and prolactin secretion in the rabbit. *J Physiol Pharmacol* (1992) 43:279–98.

150. Picanço-Diniz DLW, Valença MM, Franci CR, Antunes-Rodrigues J. Role of substance P in the medial preoptic area in the regulation of gonadotropin and prolactin secretion in normal or orchidectomized rats. *Neuroendocrinology* (1990) 51:675–82. doi: 10.1159/000125409

151. Kerdelhué B, Williams R, Lenoir V, Fardin V, Kolm P, Hodgen GD, et al. Variations in plasma levels of substance P and effects of a specific substance P antagonist of the NK1 receptor on preovulatory LH and FSH surges and progesterone secretion in the cycling cynomolgus monkey. *Reprod Neuroendocrinol* (2000) 71:228–36. doi: 10.1159/000054540

152. Moons L, Batten TFC, Vandesande F. Comparative distribution of substance P (SP) and cholecystokinin (CCK) binding sites and immunoreactivity in the brain of the sea bass (Dicentrarchus labrax). *Peptides* (1992) 13:37–46. doi: 10.1016/0196-9781(92)90137-R

153. Weld MM, Maler L. Substance P-like immunoreactivity in the brain of the gymnotiform fish Apteronotus leptorhynchus: Presence of sex differences. *J Chem Neuroanat* (1992) 5:107–29. doi: 10.1016/0891-0618(92)90038-R

154. Topaloglu AK, Reimann F, Guclu M, Yalin AS, Kotan LD, Porter KM, et al. TAC3 and TACR3 mutations in familial hypogonadotropic hypogonadism reveal a

key role for neurokinin B in the central control of reproduction. *Nat Genet* (2009) 41:354–8. doi: 10.1038/ng.306

155. Guran T, Tolhurst G, Bereket A, Rocha N, Porter K, Turan S, et al. Hypogonadotropic hypogonadism due to a novel missense mutation in the first extracellular loop of the neurokinin B receptor. *J Clin Endocrinol Metab* (2009) 94:3633–9. doi: 10.1210/jc.2009-0551

156. Young J, Bouligand J, Francou B, Raffin-Sanson ML, Gaillez S, Jeanpierre M, et al. TAC3 and TACR3 defects cause hypothalamic congenital hypogonadotropic hypogonadism in humans. *J Clin Endocrinol Metab* (2010) 95:2287–95. doi: 10.1210/jc.2009-2600

157. Gianetti E, Tusset C, Noel SD, Au MG, Dwyer AA, Hughes VA, et al. TAC3/TACR3 mutations reveal preferential activation of gonadotropin- releasing hormone release by neurokinin B in neonatal life followed by reversal in adulthood. *J Clin Endocrinol Metab* (2010) 95:2857–67. doi: 10.1210/jc.2009-2320

158. Fukami M, Maruyama T, Dateki S, Sato N, Yoshimura Y, Ogata T. Hypothalamic dysfunction in a female with isolated hypogonadotropic hypogonadism and compound heterozygous TACR3 mutations and clinical manifestation in her heterozygous mother. *Horm Res Paediatr* (2010) 73:477–81. doi: 10.1159/000313373

159. Francou B, Bouligand J, Voican A, Amazit L, Trabado S, Fagart J, et al. Normosmic congenital hypogonadotropic hypogonadism due to TAC3/TACR3 mutations: Characterization of neuroendocrine phenotypes and novel mutations. *PloS One* (2011) 6:e25614. doi: 10.1371/journal.pone.0025614

160. Silveira LFG, Teles G, Trarbach EB, Latronico AC. Role of kisspeptin / GPR54 system in human reproductive axis. *Front Horm Res* 39:13–24.

161. True C, Alam SN, Cox K, Chan YM, Seminara SB. Neurokinin B is critical for normal timing of sexual maturation but dispensable for adult reproductive function in female mice. *Endocrinology* (2015) 156:1386–97. doi: 10.1210/en.2014-1862

162. Yang JJ, Caligioni CS, Chan YM, Seminara SB. Uncovering novel reproductive defects in neurokinin B receptor null mice: Closing the gap between mice and men. *Endocrinology* (2012) 153:1498–508. doi: 10.1210/en.2011-1949

163. Li Y, Zhao T, Liu Y, Lin H, Li S, Zhang Y. Knockout of tac3 genes in zebrafish shows no impairment of reproduction. *Gen Comp Endocrinol* (2021) 311:1–11. doi: 10.1016/j.ygcen.2021.113839

164. Spicer OS, Wong T, Zmora N, Zohar Y. Targeted mutagenesis of the hypophysiotropic Gnrh3 in zebrafish (Danio rerio) reveals no effects on reproductive performance. *PloS One* (2016) 11:1–22. doi: 10.1371/journal.pone.0158141

165. Marvel M, Sivan BL, Wong TT, Zmora N, Zohar Y. Gnrh2 maintains reproduction in fasting zebrafish through dynamic neuronal projection changes and regulation of gonadotropin synthesis , oogenesis , and reproductive behaviors. *Sci Rep* (2021) 11:1–16. doi: 10.1038/s41598-021-86018-3

166. Tang H, Liu Y, Luo D, Ogawa S, Yin Y, Li S, et al. The kiss / kissr systems are dispensable for zebrafish Reproduction: Evidence from gene knockout studies. *Neuroendocrinology* (2015) 156:589–99. doi: 10.1210/en.2014-1204

167. Trudeau VL. Facing the challenges of neuropeptide gene Knockouts: Why do they not inhibit reproduction in adult teleost Fish? *Front Neurosci* (2018) 12:302. doi: 10.3389/fnins.2018.00302

168. Wakabayashi Y, Yamamura T, Sakamoto K, Mori Y, Okamura H. Electrophysiological and morphological evidence for synchronized GnRH pulse generator activity among kisspeptin / neurokinin B /Dynorphyn A (KNDy) neurons in goats. *J Reprod Dev* (2013) 59:40–8. doi: 10.1262/jrd.2012-136

169. Krajewski SJ, Anderson MJ, Iles-Shih L, Chen KJ, Urbanski HF, Rance NE. Morphologic evidence that neurokinin B modulates gonadotropin-releasing hormone secretion *via* neurokinin 3 receptors in the rat median eminence. *J Comp Neurol* (2005) 489:372–86. doi: 10.1002/cne.20626

170. Todman MG, Han S, Herbison AE. Profiling neurotransmitter receptor expression in mouse gonadotropin-release hormone neurons using green fluorescent protein-promoter transgenics and microarrays. *Neuroscience* (2005) 132:703–12. doi: 10.1016/j.neuroscience.2005.01.035

171. Corander MP, Challis BG, Thompson EL, Jovanovic Z, Loraine Tung YC, Rimmington D, et al. The effects of neurokinin B upon gonadotrophin release in male rodents. *J Neuroendocrinol* (2010) 22:181–7. doi: 10.1111/j.1365-2826.2009.01951.x

172. Navarro VM, Gottsch ML, Chavkin C, Okamura H, Clifton DK, Steiner RA. Regulation of gonadotropin-releasing hormone secretion by kisspeptin/dynorphin/neurokinin B neurons in the arcuate nucleus of the mouse. *J Neurosci* (2009) 29:11859–66. doi: 10.1523/JNEUROSCI.1569-09.2009

173. Amstalden M, Coolen LM, Hemmerle AM, Billings HJ, Connors JM, Goodman RL, et al. Neurokinin 3 receptor immunoreactivity in the septal region, preoptic area and hypothalamus of the female sheep: Colocalisation in neurokinin B cells of the arcuate nucleus but not in gonadotrophin-releasing hormone neurones. *J Neuroendocrinol* (2010) 22:1–12. doi: 10.1111/j.1365-2826.2009.01930.x

174. Glidewell-Kenney CA, Shao PP, Iyer AK, Grove AMH, Meadows JD, Mellon PL. Neurokinin B causes acute GnRH secretion and repression of GnRH transcription in GT1-7 GnRH neurons. *Mol Endocrinol* (2013) 27:437–54. doi: 10.1210/me.2012-1271

175. Navarro VM. New insights into the control of pulsatile GnRH release: The role of Kiss1/neurokinin B neurons. *Front Endocrinol (Lausanne)* (2012) 3:48. doi: 10.3389/fendo.2012.00048

176. Mittelman-Smith MA, Krajewski-Hall SJ, McMullen NT, Rance NE. Ablation of KNDy neurons results in hypogonadotropic hypogonadism and amplifies the steroid-induced lh surge in female rats. *Endocrinology* (2016) 157:2015–27. doi: 10.1210/en.2015-1740

177. Wakabayashi Y, Nakada T, Murata K, Ohkura S, Mogi K, Navarro VM, et al. Neurokinin B and dynorphin A in kisspeptin neurons of the arcuate nucleus participate in generation of periodic oscillation of neural activity driving pulsatile gonadotropin-releasing hormone secretion in the goat. *J Neurosci* (2010) 30:3124–32. doi: 10.1523/JNEUROSCI.5848-09.2010

178. Lehman MN, He W, Goodman RL, Coolen LM, Levine JE. Does the KNDy model for the control of gonadotropin-releasing hormone pulses apply to monkeys and humans? *Semin Reprod Med* (2019) 37:71–83. doi: 10.1055/s-0039-3400254

179. Young J, George JT, Tello A, Francou B, Bouligand J, Guiochon-Mantel A, et al. Kisspeptin restores pulsatile LH secretion in patients with neurokinin B signaling deficiencies: physiological, pathophysiological and therapeutic implications. *Neuroendocrinology* (2013) 97:193–202. doi: 10.1159/000336376

180. Garcia JP, Guerriero KA, Keen KL, Kenealy BP, Seminara SB, Terasawa E. Kisspeptin and neurokinin B signaling network underlies the pubertal increase in GnRH release in female rhesus monkeys. *Endocronology* (2017) 158:3269–80. doi: 10.1210/en.2017-00500

181. Garcia JP, Keen KL, Kenealy BP, Seminara SB, Terasawa E. Role of kisspeptin and neurokinin B signaling in Male rhesus monkey puberty. *Endocrinology* (2018) 159:3048–60. doi: 10.1210/en.2018-00443

182. Goodman RL, Coolen M, Lehman MN. A role for neurokinin B in pulsatile GnRH secretion in the ewe. *Neuroendocrinology* (2014) 99:18–32. doi: 10.1159/000355285

183. Kinsey-jones JS, Grachev P, Li XF, Lin YS, Milligan SR, Lightman SL, et al. The inhibitory effects of neurokinin B on GnRH pulse generator frequency in the female rat. *Neuroendocrinology* (2012) 153:307–15. doi: 10.1210/en.2011-1641

184. Gaskins GT, Glanowska KM, Moenter SM. GnRH release in a location-dependent but kisspeptin-independent manner in adult mice. *Endocrinology* (2013) 154:3984–9. doi: 10.1210/en.2013-1479

185. Mizrahi N, Gilon C, Atre I, Ogawa S, Parhar IS, Levavi-sivan B. Deciphering direct and indirect effects of neurokinin B and GnRH in the brain-pituitary axis of tilapia. *Front Endocrinol (Lausanne)* (2019) 10:469. doi: 10.3389/fendo.2019.00469

186. Servili A, Le Page Y, Leprince J, Caraty A, Escobar S, Parhar IS, et al. Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish. *Endocrinology* (2011) 152:1527–40. doi: 10.1210/en.2010-0948

187. Jin YH, Park JW, Kim J, Kwon JY. Neurokinin B-related peptide suppresses the expression of GnRH I , Kiss2 and tac3 in the brain of mature female Nile tilapia oreochromis niloticus. *Dev Rreproduction* (2016) 20:51–61. doi: 10.12717/DR.2016.20.1.051

188. Zuo C, Lyu L, Zou W, Wen H, Qi X. TAC3/TACR3 system function in the catadromous migration teleost, Anguilla japonica. *Front Endocrinol (Lausanne)* (2022) 13. doi: 10.3389/fendo.2022.848808

189. Marvel M, Spicer OS, Wong T, Zmora N, Zohar Y. Knockout of the gnrh genes in zebrafish: effects on reproduction and potential compensation by reproductive and feeding-related neuropeptides. *Biol Reprod* (2018) 0:1–13. doi: 10.1093/biolre/ioy078

190. Takahashi A, Kanda S, Abe T, Oka Y. Evolution of the hypothalamic-pituitary-gonadal axis regulation in vertebrates revealed by knockout medaka. *Endocrinology* (2016) 157:3994–4002. doi: 10.1210/en.2016-1356

191. Nakajo M, Kanda S, Karigo T, Takahashi A, Akazome Y, Uenoyama Y, et al. Evolutionally conserved function of kisspeptin neuronal system is nonreproductive regulation as revealed by nonmammalian study. *Endocrinology* (2018) 159:163–83. doi: 10.1210/en.2017-00808

192. Liu Y, Tang H, Xie R, Li S, Liu X, Lin H, et al. Genetic evidence for multifactorial control of the reproductive axis in zebrafish. *Endocrinology* (2017) 158:604–11. doi: 10.1210/en.2016-1540

193. Whitlock KE, Postlethwait J, Ewer J. Neuroendocrinology of reproduction: Is gonadotropin-releasing hormone (GnRH) dispensable? *Front Neuroendocrinol* (2019) 53:100738. doi: 10.1016/j.yfrne.2019.02.002

194. Muñoz-cueto JA, Zmora N, Paullada-salmerón JA, Marvel M. The gonadotropin-releasing hormones: Lessons from fish. *Gen Comp Endocrinol* (2020) 291:1–19. doi: 10.1016/j.ygcen.2020.113422

195. Somoza GM, Mechaly AS, Trudeau VL. Kisspeptin and GnRH interactions in the reproductive brain of teleosts. *Gen Comp Endocrinol* (2020) 298:113568. doi: 10.1016/j.ygcen.2020.113568

196. Ohga H, Selvaraj S, Matsuyama M. The roles of kisspeptin system in the reproductive physiology of fish with special reference to chub mackerel studies as main axis. *Front Endocrinol (Lausanne)* (2018) 9:147. doi: 10.3389/fendo.2018.00147

197. Dupré SM, Miedzinska K, Duval CV, Yu L, Goodman RL, Lincoln GA, et al. Report identification of Eya3 and TAC1 as long-day signals in the sheep pituitary. *Curr Biol* (2010) 20:829–35. doi: 10.1016/j.cub.2010.02.066

198. Jakimiuk A, Podlasz P, Wasowicz K. Characterisation, localisation and expression of porcine TACR1 , TACR2 and TACR3 genes. *Vet Med* (2017) 62:443–55. doi: 10.17221/23/2017-VETMED

199. Czelejewska W, Zmijewska A, Dziekonski M, Okrasa S. The potential implocation of neurokinin B in the modulation of prolactin secretion at the pituitary level in cyclic gilts. *J Physiol Pharmacol* (2020) 71:257–64. doi: 10.26402/jpp.2020.2.10

200. Ramaswamy S, Seminara SB, Ali B, Ciofi P, Amin NA, Plant TM. Neurokinin B stimulates GnRH release in the male monkey (Macaca mulatta) and is colocalized with kisspeptin in the arcuate nucleus. *Endocrinology* (2010) 151:4494–503. doi: 10.1210/en.2010-0223

201. Mijiddorj T, Kanasaki H, Purwana IN, Oride A, Sukhbaatar U, Miyazaki K. Role of neurokinin B and dynorphin A in pituitary gonadotroph and somatolactotroph cell lines. *Endocr J* (2012) 59:631–40. doi: 10.1507/endocrj.EJ11-0401

202. Grachev P, Li XF, Lin YS, Hu MH, Elsamani L, Paterson SJ, et al. GPR54-dependent stimulation of luteinizing hormone secretion by neurokinin B in prepubertal rats. *PloS One* (2012) 7:3–9. doi: 10.1371/journal.pone.0044344

203. Navarro VM, Castellano JM, McConkey SM, Pineda R, Ruiz-Pino F, Pinilla L, et al. Interactions between kisspeptin and neurokinin B in the control of GnRH secretion in the female rat. *Am J Physiol Endocrinol Metab* (2011) 300:E202–10. doi: 10.1152/ajpendo.00517.2010

204. Ruiz-Pino F, Navarro VM, Bentsen AH, Garcia-Galiano D, Sanchez-Garrido MA, Ciofi P, et al. Neurokinin B and the control of the gonadotropic axis in the Rat: Developmental changes , sexual dimorphism, and regulation by gonadal steroids. *Neuroendocrinology* (2012) 153:4818–29. doi: 10.1210/en.2012-1287

205. Nakamura S, Wakabayashi Y, Yamamura T, Ohkura S, Matsuyama S. A neurokinin 3 receptor-selective agonist accelerates pulsatile luteinizing hormone secretion in lactating cattle. *Biol Reprod* (2017) 97:81–90. doi: 10.1093/biolre/iox068

206. Ramaswamy S, Seminara SB, Plant TM. Evidence from the agonadal juvenile male rhesus monkey (Macaca mulatta) for the view that the action of neurokinin B to trigger gonadotropin-releasing hormone release is upstream from the kisspeptin receptor. *Neuroendocrinology* (2011) 94:237–45. doi: 10.1159/000329045

207. Fraser GL, Hoveyda HR, Clarke IJ, Ramaswamy S, Plant TM, Rose C, et al. The NK3 receptor antagonist ESN364 interrupts pulsatile LH secretion and moderates levels of ovarian hormones throughout the menstrual cycle. *Endocrinology* (2015) 156:4214–25. doi: 10.1210/en.2015-1409

208. Goodman RL, Hileman SM, Nestor CC, Porter KL, Connors JM, Hardy SL, et al. Kisspeptin, neurokinin B, and dynorphin act in the arcuate nucleus to control activity of the GnRH pulse generator in ewes. *Endocrinology* (2013) 154:4259–69. doi: 10.1210/en.2013-1331

209. Sakamoto K, Murata K, Wakabayashi Y, Yayou K, Ohkura S, Takeuchi Y, et al. Central administration of neurokinin B activates Kisspeptin/NKB neurons in the arcuate nucleus and stimulates luteinizing hormone secretion in ewes during the non-breeding season. *J Reprod Dev* (2012) 58:700–6. doi: 10.1262/jrd.2011-038

210. Nestor CC, Briscoe AMS, Davis SM, Valent M, Goodman RL, Hileman SM. Evidence of a role for kisspeptin and neurokinin B in puberty of female sheep. *Endocrinology* (2012) 153:2756–65. doi: 10.1210/en.2011-2009

211. Amodei R, Gribbin K, He W, Lindgren I, Corder KR, Jonker SS, et al. Role for kisspeptin and neurokinin B in regulation of luteinizing hormone and testosterone secretion in the fetal sheep. *Endocrinology* (2020) 161:1–14. doi: 10.1210/endocr/bqaa013

212. Jayasena CN, Comninos AN, De Silva A, Abbara A, Veldhuis JD, Nijher GMK, et al. Effects of neurokinin B administration on reproductive hormone secretion in healthy men and women. *J Clin Endocrinol Metab* (2014) 99:19–27. doi: 10.1210/jc.2012-2880

213. Narayanaswamy S, Prague JK, Jayasena CN, Papadopoulou DA, Mizamtsidi M, Shah AJ, et al. Investigating the KNDy hypothesis in humans by coadministration of kisspeptin, neurokinin B, and naltrexone in men. *J Clin Endocrinol Metab* (2016) 101:3429–36. doi: 10.1210/jc.2016-1911

214. Skorupskaite K, George JT, Veldhuis JD, Anderson RA. Neurokinin B regulates gonadotropin secretion, ovarian follicle growth and the timing of ovulation in healthy women. *J Clin Endocrinol Metab* (2017) 103:95–104. doi: 10.1210/jc.2017-01306

215. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Interactions between neurokinin B and kisspeptin in mediating estrogen feedback in healthy women. *J Clin Endocrinol Metab* (2016) 101:4628–36. doi: 10.1210/jc.2016-2132

216. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Neurokinin 3 receptor antagonism reveals roles for neurokinin B in the regulation of gonadotropin secretion and hot flashes in postmenopausal women. *Neuroendocrinology* (2018) 106:148–57. doi: 10.1159/000473893

217. Skorupskaite K, George JT, Veldhuis JD, Anderson RA. Neurokinin B regulates gonadotropin secretion, ovarian follicle growth, and the timing of ovulation in healthy women. *J Clin Endocrinol Metab* (2018) 103:95–104. doi: 10.1210/jc.2017-01306

218. Sandoval-Guzmán T,E, Rance N. Central injection of senktide, an NK3 receptor agonist, or neuropeptide y inhibits LH secretion and induces different patterns of fos expression in the rat hypothalamus. *Brain Res* (2004) 1026:307–12. doi: 10.1016/j.brainres.2004.08.026

219. Navarro VM, Gottsch ML, Wu M, García-Galiano D, Hobbs SJ, Bosch MA, et al. Regulation of NKB pathways and their roles in the control of Kiss1 neurons in the arcuate nucleus of the male mouse. *Endocrinology* (2011) 152:4265–75. doi: 10.1210/en.2011-1143

220. Skorupskaite K, George JT, Veldhuis JD, Millar RP, Anderson RA. Neurokinin 3 receptor antagonism decreases gonadotropin and testosterone secretion in healthy men. *Clin Endocrinol (Oxf)* (2017) 87:748–56. doi: 10.1111/cen.13445

221. Ye C, Xu S, Hu Q, Zhou L, Qin X, Jia J, et al. Global view of neuropeptides and their receptors in the brain and pituitary of grass carp (Ctenopharyngodon idellus). *Aquaculture* (2019) 512:734360. doi: 10.1016/j.aquaculture.2019.734360

222. Xu S, Zhou L, Chen X, Hu Q, Shi X, Xia C. Novel pituitary actions of NKB for anorectic peptides regulation in grass carp. *Aquaculture* (2021) 531:735857. doi: 10.1016/j.aquaculture.2020.735857

223. Mun SH, Oh HJ, Kwon JY. Response of pituitary cells and tissues to neurokinin B and F in the Nile tilapia. *Dev Reprod* (2022) 26:13–21. doi: 10.12717/DR.2022.26.1.13

224. Debeljuk L. Tachykinins and ovarian function in mammals. *Peptides* (2006) 27:736–42. doi: 10.1016/j.peptides.2005.08.002

225. Ojeda SR, Costa ME, Katz KH, Hersh LB. Evidence for the existence of substance P in the prepubertal rat ovary. I. biochemical and physiologic studies. *Biol Reprod* (1985) 33:286–95. doi: 10.1095/biolreprod33.2.286

226. Angelova P, Davidoff M, Kanchev L, Baleva-Ivanova K. Substance P: immunocytochemical localization and biological role in hamster gonads during ontogenensis. *Funct Dev morphol* (1991) 1:3–8.

227. Miyamoto A, Brückman A, von Lützow H, Schams D. Multiple effects of neuropeptide y, substance P and vasoactive intestinal polypeptide on progesterone and oxytocin release from bovine corpus luteum in vitro. *J Endocrinol* (1993) 3:451–8. doi: 10.1677/joe.0.1380451

228. Pitzel L, Jarry H, Wuttke W. Effects of substance-P and neuropeptide-y on *in vitro* steroid release by porcine granulosa and luteal cells. *Endocrinology* (1991) 129:1059–65. doi: 10.1210/endo-129-2-1059

229. Cintado CG, Pinto FM, Devillier P, Merida A, Candenas ML. Increase in neurokinin B expression and in tachykinin NK(3) receptor-mediated response and expression in the rat uterus with age. *J Pharmacol Exp Ther* (2001) 299:934–8.

230. Pinto FM, Cintado CG, Devillier P, Candenas ML. Expression of preprotachykinin-B, the gene that encodes neurokinin B, in the rat uterus. *Eur J Pharmacol* (2001) 425:R1–2. doi: 10.1016/S0014-2999(01)01186-4

231. Patak E, Candenas ML, Pennefather JN, Ziccone S, Lilley A, Martín JD, et al. Tachykinins and tachykinin receptors in human uterus. *Br J Pharmacol* (2003) 139:523–32. doi: 10.1038/sj.bjp.0705279

232. Pinto FM, Almeida TA, Hernandez M, Devillier P, Advenier C, Candenas ML. mRNA expression of tachykinins and tachykinin receptors in different human tissues. *Eur J Pharmacol* (2004) 494:233–9. doi: 10.1016/j.ejphar.2004.05.016

233. Hamlin GP, Williams MJ, Nimmo AJ, Crane LH. Hormonal variation of rat uterine contractile responsiveness to selective neurokinin receptor agonists. *Biology of reproduction* (2000) 62(6):1661–6.

234. Candenas ML, Magraner J, Armesto CP, Anselmi E, Nieto PM, Martín JD, et al. Changes in the expression of tachykinin receptors in the rat uterus during the course of pregnancy. *Biol Reprod* (2001) 65:538–43. doi: 10.1095/biolreprod65.2.538

235. González-Santana A, Marrero-Hernández S, Dorta I, Hernández M, Pinto FM, Báez D, et al. Altered expression of the tachykinins substance P/neurokinin A/hemokinin-1 and their preferred neurokinin 1/neurokinin 2 receptors in uterine leiomyomata. *Fertil Steril* (2016) 106:1521–9. doi: 10.1016/j.fertnstert.2016.07.007

236. Candenas L, Lecci A, Pinto FM, Patak E, Maggi CA, Pennefather JN. Tachykinins and tachykinin receptors: effects in the genitourinary tract. *Life Sci* (2005) 76:835–62. doi: 10.1016/j.lfs.2004.10.004

237. Fisher L, Pennefather JN. Potencies of agonists acting at tachykinin receptors in the oestrogen-primed rat uterus: effects of peptidase inhibitors. *Eur J Pharmacol* (1997) 335:221–6. doi: 10.1016/S0014-2999(97)01229-6

238. Patak EN. Activation of neurokinin NK2 receptors by tachykinin peptides causes contraction of uterus in pregnant women near term. *Mol Hum Reprod* (2000) 6:549–54. doi: 10.1093/molehr/6.6.549

239. Torricelli M, Giovannelli A, Leucci E, Florio P, De Falco G, Torres PB, et al. Placental neurokinin B mRNA expression increases at preterm labor. *Placenta* (2007) 28:1020–3. doi: 10.1016/j.placenta.2007.04.006

240. Page NM. Neurokinin B and pre-eclampsia: A decade of discovery. *Reprod Biol Endocrinol* (2010) 8:4–13. doi: 10.1186/1477-7827-8-4

241. Page NM, Woods RJ, Gardiner SM, Lomthaisong K, Gladwell RT, Butlin DJ, et al. Excessive placental secretion of neurokinin B during the third trimester causes pre-eclampsia. *Nature* (2000) 405:797–800. doi: 10.1001/archinte.160.6.777

242. Oride A, Kanasaki H, Mijiddorj T, Sukhbaatar U, Ishihara T, Kyo S. Regulation of kisspeptin and gonadotropin-releasing hormone expression in rat placenta: study using primary cultures of rat placental cells. *Reprod Biol Endocrinol* (2015) 13:1–19. doi: 10.1186/s12958-015-0083-3

243. Qi X, Salem M, Zhou W, Sato-Shimizu M, Ye G, Smitz J, et al. Neurokinin B exerts direct effects on the ovary to stimulate estradiol production. *Endocrinology* (2016) 157:3355–65. doi: 10.1210/en.2016-1354

244. Debeljuk L, Rao JN, Bartke A. Tachykinins and their possible modulatory role on testicular function: A review. *Int J Androl* (2003) 26:202–10. doi: 10.1046/j.1365-2605.2003.00401.x

245. Blasco V, Pinto FM, Gonzalez-Ravina C, Santamaria-Lopez E, Candenas L, Fernandez-Sanchez M. Tachykinins and kisspeptins in the regulation of human Male fertility. *Clin Med* (2020) 9:113–24.

246. Angelova PA, Davidoff S, Kanchev LN. Substance P-induced inhibition of leydig cell steroidogenesis in primary culture. *Andrologia* (1991) 23:325–7. doi: 10.1111/j.1439-0272.1991.tb02572.x

247. Angelova P, Davidoff MS, Bakalska M, Kanchev L. *In vitro* effects of substance P and arginine-vasopressin on testosterone production in leydig cells of short and long photoperiodic hamsters. *Andrologia* (1996) 28:321–6. doi: 10.1111/j.1439-0272.1996.tb02809.x

248. Pinto FM, Ravina CG, Subiran N, Cejudo-Román A, Fernández-Sánchez M, Irazusta J, et al. Autocrine regulation of human sperm motility by tachykinins. *Reprod Biol Endocrinol* (2010) 8:104. doi: 10.1186/1477-7827-8-104

249. Ravina CG, Seda MA, Pinto FM, Fernandez-Sanchez M, Pintado OC, Candenas ML. Characterization of tachykinin receptors in human sperm. *Fertil Steril* (2007) 88:S362. doi: 10.1016/j.fertnstert.2007.07.1205

250. Palea S, Corsi M, Artibani W, Ostardo E, Pietra C. Pharmacological characterization of tachykinin NK2 receptors on isolated human urinary bladder, prostatic urethra and prostate. *J Pharmacol Exp Ther* (1996) 277:700–5.

251. Atre I, Mizrahi N, Levavi-sivan B. Characteristics of neurokinin-3 receptor and its binding sites by mutational analysis. *Biol (Basel)* (2021) 10:968–93. doi: 10.3390/biology10100968

252. Delgado MJ, Cerdá-reverter JM, Soengas JL. Hypothalamic integration of metabolic, endocrine, and circadian signals in Fish: Involvement in the control of food intake. *Front Neurosci* (2017) 11:354. doi: 10.3389/fnins.2017.00354

253. Soengas JL, Cerdá-Reverter JM, Delgado MJ. Central regulation of food intake in fish: an evolutionary perspective. *J Mol Endocrinol* (2018) 60:171–99. doi: 10.1530/JME-17-0320

254. Volkoff H. Fish as models for understanding the vertebrate endocrine regulation of feeding and weight. *Mol Cell Endocrinol* (2019) 497:110437. doi: 10.1016/j.mce.2019.04.017

255. Heisler LK, Lam DD. An appetite for life: brain regulation of hunger and satiety. *Curr Opin Pharmacol* (2017) 37:100–6. doi: 10.1016/j.coph.2017.09.002

256. Crespo CS, Cachero AP, Jiménez LP, Barrios V, Arilla Ferreiro E. Peptides and food intake. *Front Endocrinol (Lausanne)* (2014) 5:58. doi: 10.3389/fendo.2014.00058

257. Cuomo R, Sarnelli G. Food intake and gastrointestinal motility: a complex interplay. *Nutr Metab Cardiovasc Dis* (2004) 14:173–9. doi: 10.1016/S0939-4753(04)80001-2

258. Mittelman-Smith MA, Williams H, Krajewski-Hall SJ, Lai J, Ciofi P, McMullen NT, et al. Arcuate kisspeptin/neurokinin B/dynorphin (KNDy) neurons mediate the estrogen suppression of gonadotropin secretion and body weight. *Endocrinology* (2012) 153:2800–12. doi: 10.1210/en.2012-1045

259. Navarro VM, Ruiz-pino F, Sa MA, García-galiano D, Hobbs SJ, Manfredi-lozano M, et al. Role of neurokinin B in the control of female puberty and its modulation by metabolic status. *J Neurosci* (2012) 32:2388–97. doi: 10.1523/JNEUROSCI.4288-11.2012

260. Yang JA, Yasrebi A, Snyder M, Roepke TA. The interaction of fasting, caloric restriction, and diet-induced obesity with 17β-estradiol on the expression of KNDy neuropeptides and their receptors in the female mouse. *Mol Cell Endocrinol* (2016) 437:35–50. doi: 10.1016/j.mce.2016.08.008

261. True C, Grove KL, Smith MS. Beyond leptin: emerging candidates for the integration of metabolic and reproductive function during negative energy balance. *Front Endocrinol (Lausanne)* (2011) 2:53. doi: 10.3389/fendo.2011.00053

262. Matsuzaki T, Iwasa T, Kinouchi R, Yoshida S, Murakami M, Gereltsetseg G, et al. Fasting reduces the KiSS-1 mRNA levels in the caudal hypothalamus of gonadally intact adult female rats. *Endocrine Journal* (2011) 58:1003–12. doi: 10.1507/endocrj

263. Merkley CM, Renwick AN, Shuping SL, Harlow K, Sommer JR, Nestor CC. Undernutrition reduces kisspeptin and neurokinin B expression in castrated male sheep. *Reprod Fertil* (2020) 1:21–33. doi: 10.1530/RAF-20-0025

264. Harlow K, Griesgraber M, Seman A, Shuping S, Sommer J, Hileman S, et al. Chronic undernutrition inhibits KNDy neurons in ovariectomized ewe lambs. *J Endocr Soc* (2021) 5:2021. doi: 10.1210/jendso/bvab048

265. Merkley CM, Shuping SL, Nestor CC. Domestic animal endocrinology neuronal networks that regulate gonadotropin-releasing hormone / luteinizing hormone secretion during undernutrition: evidence from sheep. *Domest Anim Endocrinol* (2020) 73:106469. doi: 10.1016/j.domaniend.2020.106469

266. Venancio JC, Margatho LO, Rorato R, Ribeiro R, Rosales C, Debarba LK, et al. Short-term high-fat diet increases leptin activation of CART neurons and advances puberty in female mice. *Endocrinology* (2017) 158:3929–42. doi: 10.1210/en.2017-00452

267. Li XF, Lin YS, Kinsey-jones JS, O'Byrne KT. High-fat diet increases LH pulse frequency and kisspeptin-neurokinin P expression in puberty-advanced female rats. *Neuroendocrinology* (2012) 153:4422–31. doi: 10.1210/en.2012-1223

268. Maggi CA. The mammalian tachykinin receptors. *General Pharmacology* (1995) 26:911–44.

269. Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part I . Expression, release and motor function. *Pharmacol Ther* (1997) 73:173–217. doi: 10.1016/S0163-7258(96)00195-7

270. Lecci A, Altamura M, Capriati A, Maggi CA. Tachykinin receptors and gastrointestinal motility: focus on humans. *Eur Rev Med Pharmacol Sci* (2008) 12:69–80.

271. Sahu A, Kalra PS, Dube MG, Kalra SP. Neuropeptide K suppresses feeding in the rat. *Regul Pept* (1988) 23:135–43. doi: 10.1016/0167-0115(88)90021-3

272. Kalra SP, Sahu A, Dube MG, Kalra PS. Effects of various tachykinins on pituitary LH secretion, feeding, and sexual behavior in the rat. *Ann N Y Acad Sci* (1991) 632:332–8. doi: 10.1111/j.1749-6632.1991.tb33120.x

273. Achapu M, Pompei P, Polidori C, De Caro G, Massi M. Central effects of neuropeptide K on water and food intake in the rat. *Brain Res Bull* (1992) 28:299–303. doi: 10.1016/0361-9230(92)90191-Y

274. Dib B. Food and water intake suppression by intracerebroventricular administration of substance P in food- and water-deprived rats. *Brain Res* (1999) 830:38–42. doi: 10.1016/s0006-8993(99)01379-7

275. Magoul R, Tramu G. Tachykinin-induced changes in β-endorphin gene expression in the rat arcuate nucleus. *Neurosci Lett* (1997) 223:93–6. doi: 10.1016/S0304-3940(97)13407-3

276. Karagiannides I, Torres D, Tseng YH, Bowe C, Carvalho E, Espinoza D, et al. Substance P as a novel anti-obesity target. *Gastroenterology* (2008) 134:747–55. doi: 10.1053/j.gastro.2007.12.032

277. Trivedi C, Shan X, Tung YCL, Kabra D, Holland J, Amburgy S, et al. Tachykinin-1 in the central nervous system regulates adiposity in rodents. *Endocrinology* (2015) 156:1714–23. doi: 10.1210/en.2014-1781

278. Maguire CA, León S, Carroll RS, Kaiser UB, Navarro VM. Altered circadian feeding behavior and improvement of metabolic syndrome in obese Tac1- deficient mice. *Nat Publ Gr* (2017) 41:1798–804. doi: 10.1038/ijo.2017.185

279. Holzer P, Holzer-Petsche U. Tachykinins in the gut. Part II. Roles in neural excitation, secretion and inflammation. *Pharmacol Ther* (1997) 73:219–63. doi: 10.1016/S0163-7258(96)00196-9

280. Holzer P, Holzer-petsche U. Tachykinin receptors in the gut: physiological and pathological implications. *Curr Opin Pharmacol* (2001) 1:583–90. doi: 10.1016/S1471-4892(01)00100-X

281. Peyon P, Saied H, Lin X, Peter RE. Preprotachykinin gene expression in goldfish brain: Sexual, seasonal, and postprandial variations. *Peptides* (2000) 21:225–31. doi: 10.1016/S0196-9781(99)00190-4

282. London S, Volkoff H. Effects of fasting on the central expression of appetite-regulating and reproductive hormones in wild-type and casper zebrafish (Danio rerio). *Gen Comp Endocrinol* (2019) 282:113207. doi: 10.1016/j.ygcen.2019.06.011

283. Sharma SC, Berthoud VM, Breckwoldt R. Distribution of substance P-like immunoreactivity in the goldfish brain. *J Comp Neurol* (1989) 279:104–16. doi: 10.1002/cne.902790109

284. Rao SD, Rao PDP, Nahorniak CS, Peter RE. Brain and pituitary immunocytochemistry of Carassin in the goldfish, Carassius auratus - A new neurohormone peptide. *Zoolog Sci* (1996) 13:415–20. doi: 10.2108/zsj.13.415

285. Weld MM, Kar S, Maler L, Quirion R. The distribution of tachykinin bingind sites in the brain of an electric fish (Apteronotus leptorhynchus). *J Chem Neuroanat* (1994) 7:123–39. doi: 10.1016/0891-0618(94)90024-8

286. He S, Liang X, Sun J, Li L, Yu Y, Huang W, et al. Insights into food preference in hybrid F1 of mandarin fish through transcriptome analysis. *BMC Genomics* (2013) 14:601. doi: 10.1186/1471-2164-14-601

287. Olsson C, Holmgren S. The control of gut motility. *Comp Biochem Physiol - A Mol Integr Physiol* (2001) 128:481–503. doi: 10.1016/S1095-6433(00)00330-5

288. Holmgren S. Neuropeptide functions in the fish gut. *Peptides* (1985) 6:363–8. doi: 10.1016/0196-9781(85)90398-5

289. Jensen J, Olson KR, Conlon JM. Primary structures and effects on gastrointestinal motility of tachykinins from the rainbow trout. *Am J Physiol Integr Comp Physiol* (1993) 265:R804–10. doi: 10.1152/ajpregu.1993.265.4.R804

290. Jensen J, Holmgren S, Jönsson A-C. Substance P-like immunoreactivity and the effects of tachykinins in the intestine of the Atlantic cod, Gadus morhua. *J Auton Nerv Syst* (1987) 20:25–33. doi: 10.1016/0165-1838(87)90078-6

291. Jensen J, Karila P, Jnsson A, Aldman G, Holmgren S. Effects of substance P and distribution of substance P-like immunoreactivity in nerves supplying the stomach of the cod. *Gadus morhua* (1993) 12:237–47. doi: 10.1007/BF00004371

292. Kitazawa T, Kimura A, Furuhashi H, Temma K, Kondo H. Contractile response to substance P in isolated smooth muscle strips from the intestinal bulb of the carp (Cyprinus carpio). *Comp Biochem Physiol C* (1988) 89:277–85. doi: 10.1016/0742-8413(88)90224-1

293. Jensen J, Holmgren S. Tachykinins and intestinal motility in different fish groups. *Gen Comp Endocrinol* (1991) 83:388–96. doi: 10.1016/0016-6480(91)90144-U

294. Kitazawa T, Kudo K, Ishigami M, Furuhashi H, Temma K, Kondo H. Evidence that a substance P-like peptide mediates the non-cholinergic excitatory response of the carp intestinal bulb (Cyprinus carpio). *Naunyn Schmiedebergs Arch Pharmacol* (1988) 338:68–73. doi: 10.1007/BF00168814

295. Holmberg A, Schwerte T, Pelster B, Holmgren S. Ontogeny of the gut motility control system in zebrafish Danio rerio embryos and larvae. *J Exp Biol* (2004) 207:4085–94. doi: 10.1242/jeb.01260

296. Anderson KC, Knuckey R, Cánepa M, Elizur A. A transcriptomic investigation of appetite-regulation and digestive processes in giant grouper Epinephelus lanceolatus during early larval development. *J Fish Biol* (2018) 93:694–710. doi: 10.1111/jfb.13798

297. Kitazawa T, Kaiya H. Regulation of gastrointestinal motility by motilin and ghrelin in vertebrates. *Front Endocrinol (Lausanne)* (2019) 10:278. doi: 10.3389/fendo.2019.00278

298. Azimi E, Reddy VB, Shade KC, Anthony RM, Talbot S, Juliana P, et al. Dual action of neurokinin-1 antagonists on mas-related GPCRs. *JCI Insight* (2016) 1:1–10. doi: 10.1172/jci.insight.89362

299. Chompunud C, Ayudhya N, Roy S, Alkanfari I, Ganguly A, Ali H. Identification of gain and loss of function missense variants in MRGPRX2's transmembrane and intracellular domains for mast cell activation by substance p. *Int J Mol Sci* (2019) 20:1–16. doi: 10.3390/ijms20215247

300. Green DP, Limjunyawong N, Gour N, Pundir P, Dong X. A mast-Cell-Specific receptor mediates neurogenic inflammation and pain. *Neuron* (2019) 101:412–420.e3. doi: 10.1016/j.neuron.2019.01.012

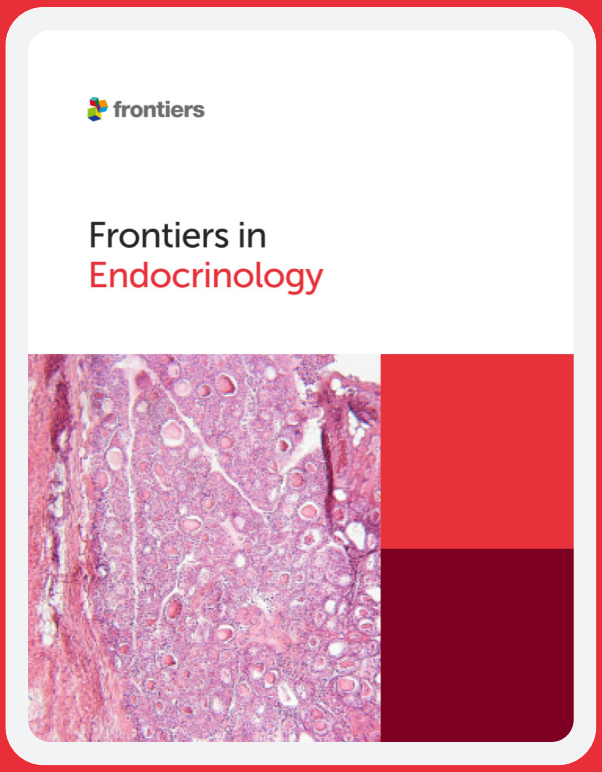
Frontiers in Endocrinology

Explores the endocrine system to find new therapies for key health issues

The second most-cited endocrinology and metabolism journal, which advances our understanding of the endocrine system. It uncovers new therapies for prevalent health issues such as obesity, diabetes, reproduction, and aging.

Discover the latest Research Topics

See more →



Frontiers

Avenue du Tribunal-Fédéral 34
1005 Lausanne, Switzerland
frontiersin.org

Contact us

+41 (0)21 510 17 00
frontiersin.org/about/contact