Characterization and Role of Secretoneurin in the Ovulatory Cycle of Zebrafish

Di Peng

Thesis submitted to the University of Ottawa
in partial Fulfillment of the requirements for the Ph. D. degree

Department of Biology
Faculty of Science
University of Ottawa

Acknowledgements

Finishing this thesis signifies the end of my 5-year life in Canada as a Ph.D. candidate. In these five years, there are many people whom I must express my sincere gratitude as they supported me gratuitously in my research and my life.

First and foremost, I would like to extend my sincerest gratitude to my supervisor Dr. Vance Trudeau. It was you that believed me in my academic ability, gave me the opportunity, and supported me to realize my scientific aspiration. You are not only the strict professor in the lab but also an energetic and hospitable friend in daily life. Thank you for inviting me to the Christmas parties with your family. Thank you for inviting me to your concerts. Thank you for your homemade wine and beer. Thank you for your bread. Thank you for your BBQ. Every piece of my memory about you will be the invaluable treasure in the rest of my life.

I would like to thank my committee members, Dr. Michael Jonz, Dr. Nafissa Ismail, and Dr. Jan Mennigen, for all their help on my project and their valuable suggestions. I would also like to thank Dr. Berta Levavi-Sivan from the Hebrew University of Jerusalem for the LHb-RFP and FSHb-eGFP transgenic zebrafish lines, which helped me to achieve significant results. I would also like to thank Dr. Wei Hu from the Institute of Hydrobiology, Chinese Academy of Science, Wu Han for the wonderful interactions and the constructive ideas from our international collaboration.

I would like to thank the past and present members of Teamendo.ca for helping my project by sharing experiences and assisting with my experiments. I would like to especially thank Kimberly Mitchell and Chunyu Lu. Kim helped me to familiarize the lab and the facility when I started my research and always answered my questions with patience. Chunyu Lu, as my collaborator and friend, taught me chemical knowledge and always supported me when facing challenges. I would like to acknowledge the University of Ottawa and the China Scholarship Council for their financial support in my life and research.

Finally, and most importantly, I would like to thank my parents in China for their love and encouragement. I would not be here without their support. I cannot wait to fly back home and tell them “I love you” face to face.

Abstract

Secretoneurin (SN) is a 31-42 amino acid neuropeptide, derived from the proteolytic processing of the precursor protein secretogranin-II (Scg2). In zebrafish, SNa and SNb are respectively 34 and 31 amino acids long, deriving from selective processing of the distinct Scg2a and Scg2b precursors. Our lab recently reported that frameshift mutations in Scg2 leads to reduces sexual behavior and disrupted spawning. This defect was partially rescued by injection of SNa. In my work, we determined the distribution of SNa in relation to other known reproductive hormones in zebrafish brain and pituitary by double immunofluorescent staining. SNa-immunoreactivity (ir) was observed in neuronal cell bodies in the ventral telencephalon, preoptic area (POA) and hypothalamus. Neuronal fibers staining for SNa projecting from the magnocellular POA passed through the pituitary stalk and terminated largely in the neurointermediate lobe (NIL). The SNa-ir fibers were less abundant but clearly present in the pars distalis. Moreover, SNa colocalized with isotocin in cell bodies in the POA and fibers in the NIL. Using the *lhb*-RFP x *fshb*-eGFP transgenic zebrafish line, we observed SNa-ir near gonadotroph cell bodies but not in them. Peptidomic analysis uncovered shorter processed fragments of both in SNa and SNb in whole brain and pituitary. We performed mass spectrometry to determine natural periovulatory variations and studied their potential bioactivities. Both SNa1-34 and SNa1-14 in the brain varied during the ovulatory cycle, while SNb-related peptides were relatively stable. The levels of SNa1-34 in brain peaked coincident with increased Gnrh3 at the time of the luteinizing hormone (Lh) surge. The levels of SNa1-14 in brain and ovaries peaked at the time of ovulation. To investigate the potential bioactivity of SNa1-34 and SNa1-14, we performed intraperitoneal injections and analyzed the expression numerous reproductive genes. The results suggested that SNa1-34 could induce ovulation by stimulating time-dependent expression of *gnrh3* in brain, *cga* and *lhb* in pituitary and *npr* in ovaries. In contrast, SNa1-14 exhibited far fewer effects, but stimulated the expression of *gnrh2* but suppressed *gnrhr2*, so its natural biological function remains unknown. After a single injection of SNa1-34 in females isolated from males, 61% (11/18) zebrafish ovulated. This compares favorably with the effects of the Lh analog human chorionic gonadotropin, inducing ovulation in 72% (13/18) of females. Natural variations in levels of SN in relation to other well-known neuropeptides and biological activity

data in the zebrafish model support the hypothesis that SNa is a new stimulatory reproductive hormone. The SN peptides are conserved in evolution so what we uncover in fish may help us speculate on its importance in other vertebrates.

Résumé

La sécrétoneurine (SN) est un neuropeptide de 31-42 acides aminés, dérivé de la digestion protéolytique de la protéine précurseur sécrétogranine-II (Scg2). Chez le poisson zèbre, la SNa et la SNb font respectivement 34 et 31 acides aminés de longueur, provenant du clivage sélectif des précurseurs Scg2a et Scg2b distincts. Notre laboratoire a récemment rapporté que des mutations avec décalage du cadre de lecture dans le gène Scg2 conduisent à une réduction du comportement sexuel et à une perturbation du frai. Ce défaut a été partiellement corrigé par l'injection du neuropeptide SNa. Dans mon travail, j'ai déterminé la répartition de la SNa en relation avec d'autres hormones reproductives connues dans le cerveau et l'hypophyse du poisson zèbre par double coloration immunofluorescente. L'immunoréactivité (ir) de la SNa a été observée dans les corps cellulaires neuronaux du télencéphale ventral, de l'aire préoptique (POA) et de l'hypothalamus. Les fibres neuronales colorées pour la SNa projetant à partir de la POA magnocellulaire passaient par la tige pituitaire et terminaient en grande partie dans le lobe neurointermédiaire (NIL). Les fibres SNa-ir étaient moins abondantes mais clairement présentes dans la *pars distalis*. De plus, la SNa a été colocalisée avec l'isotocine dans les corps cellulaires de la POA et dans les fibres de la NIL. En utilisant la lignée de poisson zèbre transgénique lhb-RFP x fshb-eGFP, nous avons observé un marquage SNa-ir près des corps cellulaires des gonadotrophes mais pas dans ceux-ci. L'analyse peptidomique a mis en évidence des fragments plus courts des deux SNa et SNb dans l'ensemble du cerveau et dans l'hypophyse. Nous avons réalisé une spectrometrie de masse pour déterminer les variations péri-ovulatoires naturelles de ces demi-peptides et étudier leur bioactivité potentielle. Les taux de SNa1-34 et SNa1-14 dans le cerveau varient au cours du cycle ovulatoire, tandis que les demi-peptides dérivés de SNb sont relativement stables. Les niveaux de SNa1-34 dans le cerveau ont atteint un pic coïncidant avec l'augmentation de Gnrh3 au moment du pulse pré-ovulatoire de l'hormone lutéinisante (LH). Les niveaux de SNa1-14 dans le cerveau et les ovaires ont atteint un pic au

moment de l'ovulation. Pour étudier la bioactivité potentielle de SNa1-34 et SNa1-14, nous avons effectué des injections intrapéritonéales et analysé l'expression de nombreux gènes impliqués dans la fonction de reproduction. Les résultats suggèrent que SNa1-34 peut induire l'ovulation en stimulant l'expression dans au cours du temps de *gnrh*3 dans le cerveau, *cga* et *lhb* dans l'hypophyse et *npr* dans les ovaires. En revanche, SNa1-14 présentait beaucoup moins d'effets, mais stimulait l'expression de *gnrh2* et réprimait l'expression de *gnrhr2*, de sorte que sa fonction biologique naturelle reste inconnue. Après une seule injection de SNa1-34 dans des femelles isolées des mâles, 61% (11/18) des poissons zèbres ont ovulé. Ceci se compare favorablement aux effets de la gonadotrophine chorionique humaine, analogue de LH, qui induit l'ovulation chez 72% (13/18) des femelles. Les variations naturelles des niveaux de SN par rapport à d'autres neuropeptides bien connus et les données sur l'activité biologique dans le modèle du poisson zèbre soutiennent l'hypothèse que la SNa est une nouvelle hormone stimulant la reproduction. Les peptides SN étant conservés au cours de l'évolution, ce que nous découvert chez le poisson zèbre peut nous aider à prévoir son importance chez d'autres vertébrés.

Table of Contents

List of figures

List of tables

Abbreviation

11-KT	11-ketotestosterone
5HT	Serotonin
ACN	Acetonitrile
ACTH	Adrenocorticotropic hormone
AH	Adenohypophysis
ANR	Anterior neural ridge
AR	Androgen receptor
avp	Vasotocin
BLAST	Basic Local Alignment Search Tool
Cg	Glycoprotein
CgA	Chromogranin A
CgB	Chromogranin B
cGnRH-II	Chicken GnRH II
DA	Dopamine
ddpcr	Digital droplet PCR
DHP	Dihydroxy-4-pregnen-3-one
E1	Estrone
E2	Estradiol
E3	Estriol
ECL	External cellular layer
ef1a	Elongation factor alpha
ERK	Extracellular signal-regulated kinases
ER	Estrogen receptor
EtOH	Ethanol
FA	Formic acid
FDR	False discovery rate
Fsh	Follicle stimulating hormone
FSHr	FSH receptor
GL	Glomerular layer
GnIH	Gonadotropin inhibitory hormone
Gnrh	Gonadotropin-releasing hormone
GnRHr	GnRH receptor
Gper	G protein-coupled estrogen receptor
GSI	Gonadosomatic index
Hc	Caudal zone of periventricular hypothalamus
hCG	Human chorionic gonadotropin
HPG	Hypothalamus-pituitary-gonads
HPLC	High-performance liquid chromatography
Hv	Ventral zone of periventricular hypothalamus

Hyp	hypothalamus
i.p.	Intraperitoneal
ir	Immunoreactivity
Kiss	Kisspeptin
Lh	Luteinizing hormone
lhcgr	Luteinizing hormone receptor
MIS	Maturation- inducing steroid
mpf	Months post-fertilization
MS	Mass spectrometry
NH	Neurohypophysis
NIL	Neurointermediate lobe
NPO	Nucleus preopticus
npr	Nuclear Progesterone Receptor
ONL	Olfactory nerve layer
OSN	Olfactory sensory neurons
OVP	Ovaprim
Oxt	Isotocin
PC	Prohormone convertase
PD	Pars distalis
PI	Pars intermedia
PKA	Protein kinase A
PM	Magnocellular preoptic nucleus
PN	Pars nervosa
POA	Preoptic area
POF	Peripheral olfactory fascicle
PPD	Proximal pars distalis
PR	Posterior recess
RPD	Rostral pars distalis
Scg2	Secretogranin 2
sGnrh	Salmon GnRH
SN	Secretoneurin
SPE	Solid phase extraction
star1	Steroidogenic acute regulatory protein 1
T	Testosterone
TALEN	Targeted transcription activator-like effector nuclease
Tel	telencephalon
Vd	Dorsal parts of the ventral telencephalon
Vl	Lateral parts of the ventral telencephalon
Vs	Supracommissural part of the ventral telencephalon
Vv	Ventral telencephalon
WT	Wildtype

Chapter 1 General Introduction

1.1 Thesis rationale and objectives

The hypothalamic-pituitary-gonadal (HPG) axis is central for vertebrate reproduction. This axis includes neuroendocrine networks that regulate pituitary function and in turn gonadal development, oogenesis, and ovulation in females. Gonadotropin-releasing hormone (Gnrh) is produced in the preoptic area and projected by hypothalamic neural fibers to the teleost pituitary and stimulate the expression and secretion of two types of gonadotropins, luteinizing hormone (Lh) and follicle stimulating hormone (Fsh) (Somoza, Mechaly et al. 2020). Lh is a major hormone of the pituitary to induce the oocyte maturation and ovulation (Chu, Li et al. 2015). In teleosts, increased Gnrh stimulation accompanied by a decrease in inhibitory dopaminergic tone drives the ovulatory Lh surge (Trudeau 1997). Research on zebrafish indicated that although gonads appear to develop normally, Lh-knockout female zebrafish are infertile since there is failure of oocyte maturation and ovulation while male is fertile (Zhang, Zhu et al. 2015). Fsh plays essential roles in regulating of teleost reproductive processes such as gametogenesis and follicular growth (Cahoreau, Klett et al. 2015). In contrast with Lh, both sexes in Fsh-knockout zebrafish are fertile, although gonadal development is delayed (Zhang, Zhu et al. 2015).

Secretoneurin (SN) is 31-42 amino acid neuropeptides derived from Scg2 precursor. In teleosts, including goldfish and zebrafish, two SN variants have been identified and named as SNa and SNb which are derived from the processing of large Scg2a and Scg2b precursor proteins, respectively. Note that the potential hormonal roles of SN have been reviewed by our group, a project in which I also had a co-author role (Mitchell, Mikwar et al. 2020). Previous research from our lab has indicated that SNa can increase expression of *cga*, *fshb*, and *lhb* in goldfish (Zhao, Grey et al. 2010). Moreover, this study also demonstrated that co-incubation with anti-SN antiserum reduces the stimulatory effect of Gnrh3 on Lh release while Gnrh3 can also increase the expression of pituitary *scg2* mRNA in goldfish (Zhao, Grey et al. 2010). These data suggested that SN may control reproduction by interaction with Gnrh3, Lh and Fsh in goldfish. Research on Scg2a/b TALEN frameshift double-mutants revealed that the breeding

rate of these zebrafish was less than 10%, while *in vitro* fertilization rate remains normal (Mitchell, Zhang et al. 2020). However, after injecting human chorionic gonadotropin (hCG), which is an Lh-like protein (Bulun 2008), breeding rate recovered to more than 30% (Mitchell, Zhang et al. 2020). These data suggested that the mechanism by which Scg2a/b mutations impacts zebrafish reproduction is associated with disrupted gonadotropin production.

Previous research has already revealed stimulatory effects of SN on reproductive hormones including Gnrh, Lh and Fsh in subfertile mutants. Yet, the roles and potential mechanism of action of SN in regulating reproduction in normal fish is still unknown. Therefore, in my project, we used the wildtype (WT) zebrafish as the experimental model to investigate the roles of SNa and SNb in reproduction.

We tested the hypothesis that the Scg2/SN system is controlling the ovulatory process in zebrafish. To specifically investigate this, we performed a series of experiments with the following objectives:

1. Characterization of the distribution of Scg2a/SNa-ir and its colocalization with reproductive hormones in brain and pituitary (Chapter 2)
2. Confirmation the presumed amino-acid sequences of SNa and SNb (Chapter 3)
3. Determination of possible differences in the production of SN peptides and classic reproductive hormones (Chapter 3)
4. Comparison of the levels of SN peptides with classic reproductive hormones in the periovulatory period (Chapter 4)
5. Perform intraperitoneal injection with SN peptides to investigate their biological actions in females (Chapter 5)

Below we review key aspects of the hypothalamic-pituitary-gonadal axis and the hormones regulating it. Thereafter, we present data chapters and present a summary and proposed new areas of investigation. As each chapter is presented as an independent manuscript, necessarily, there is some overlap in the introductory statements, although we have attempted to keep this to a minimum wherever possible.

1.2 The physiological anatomy of the hypothalamic-pituitary-gonadal axis

The hypothalamus is the major regulating center responsible for reproduction, growth, food intake and stress in the vertebrates (Gorissen and Flik 2016, Soengas, Cerda-Reverter et al. 2018, Li, Zhou et al. 2021). The structure and cell type in the hypothalamus are conserved among the species. It can be separated into four parts: preoptic, anterior, tuberal and mammillary regions (Xie and Dorsky 2017). The preoptic area (POA) is a functional area with various types of cell bodies to secret hormones regulating reproduction, behavior, and thermoregulation. There are two types of neurosecretory cells in POA defined as parvocellular neurosecretory cells and magnocellular neurosecretory cells (Wulliman, Rupp et al. 2012). The parvocellular cells secret the corticotropin-releasing hormone, which controls in the stress response by stimulating pituitary production of adrenocorticotropic hormone (Charmandari, Tsigos et al. 2005). As for magnocellular cells, oxytocin and vasopressin are produced and transported to the posterior pituitary through the median eminence in mammals (Moller 2021). While in teleost, isotocin and vasotocin, the non-mammalian homologues of oxytocin and vasopressin, are delivered through the prolonged magnocellular neuronal axons projecting into the posterior pituitary directly (Eaton, Holmqvist et al. 2008). In vertebrates, the pituitary is connected to the hypothalamus via a short stalk that enables the passage of hypothalamic signals. In mammals, trophic hormones are released by the hypothalamus and are transported through a network of capillaries, bypassing general circulation, to the pituitary gland. This structural network of capillaries, known as the median eminence, are not found in teleosts. Instead, teleost anterior pituitary endocrine cells are directly innervated by preoptic and hypothalamic neurons (Peter, Yu et al. 1990).

The pituitary gland is a critical component of vertebrate neuroendocrine systems. It is a functional link between the nervous system and the endocrine system; effectively acting as a relay between the hypothalamus and downstream effector organs and tissues. Hormones secreted by the pituitary regulate metabolism, reproduction, growth, lactation and maintains homeostasis (Scully and Rosenfeld 2002, Blanton and Specker 2007). Pituitary organogenesis is a complex and tightly regulated process controlled by many factors, both intrinsic and extrinsic. These include cell to cell interactions, signaling processes, transcription factors, co-activators

and repressors and all of which may be regulated temporally and spatially (Pogoda and Hammerschmidt 2009).The teleostean pituitary can be divided into two main parts based on its ontogeny and anatomy, the neurohypophysis (NH) and the adenohypophysis (AH). The NH is the result of a posteriorly directed downgrowth from the floor of the diencephalon that consists of neurosecretory nerve endings that project downward from the nucleus supraopticus and nucleus paraventricular of the hypothalamus (Zohar, Munoz-Cueto et al. 2010). Axonal termini of hypothalamic neuroendocrine cells project to the posterior pituitary and secrete the neuropeptides vasotocin and isotocin (Acher 1996). Unlike the NH, the AH is of non-neural origin derived from an epithelial upgrowth of Rathke's pouch. Following gastrulation, precursor AH cells are positioned near the anterior domain, the anterior neural ridge (ANR). As the ANR thickens, AH specific cells are observed followed by the AH becoming structurally distinct and positioned ventro-rostrally to the hypothalamus by 19 hpf in zebrafish. Subsequently, the AH can be subdivided into two regions, the pars distalis (PD) that can be further subdivided into the rostral pars distalis (RPD) and the proximal or caudal pars distalis (PPD); and the pars intermedia (PI) (Zohar, Munoz-Cueto et al. 2010).

Beginning at 26 hpf, the zebrafish AH placode becomes internalized into the head and reaches its final position between 48 and 72 hpf (Pogoda and Hammerschmidt 2009). The differentiations of endocrine AH cells begin at 24 hpf and over the next few hours they migrate to their final positions (Nica, Herzog et al. 2004, Pogoda and Hammerschmidt 2009). At least six different endocrine cell types are present in the AH. They are characterized by the different hormones released and are present in distinct domains. The anterior most domain of the AH contains the lactotropes that produce PRL, and corticotropes secreting adrenocorticotropic hormone (ACTH), with the lactotropes positioned ventral to corticotropes (Alsop and Vijayan 2009). The next domain contains thyrotropes producing thyroid stimulating hormone, somatotropes producing growth hormone and gonadotropes producing the gonadotropins Lh and Fsh and is located within the PPD (Trudeau and Somoza 2020) . The PI is the most posterior of the adenohypophysial regions and contains corticotropes and melanotropes producing α-melanocyte stimulating hormone (Liu, Huang et al. 2003). Somatolactin producing cells, somatolactotropes, are an additional endocrine cell type observed in the PD of embryonic and

PD as well as the PI of adult teleosts (Lopez, Nica et al. 2006, Zhu, Gleiberman et al. 2007).

Zebrafish ovaries are dynamic organs in which the follicles undergo asynchronous development. Different from seasonal spawning fish, zebrafish spawn every day after light on 0.5-1 hour and the ovulation time is approximately at about 1 hour before light on (Zhou, Tsang et al. 2011). The whole process of folliculogenesis from the primary growth to mature stage takes about 10 days in the zebrafish (Wang and Ge 2004). Zebrafish oocyte development can be divided into five stages, based on morphological criteria and on physiological and biochemical events (Selman, Wallace et al. 1993). In stage I (primary growth stage), oocytes reside in nests with other oocytes and then within a definitive follicle, where they greatly increase in size. In stage II (cortical alveolus stage), oocytes are distinguished by the appearance of variably sized cortical alveoli and the vitelline envelope becomes prominent. In stage III (vitellogenesis), yolk proteins appear in oocytes and yolk bodies with crystalline yolk accrue during this major growth stage. Oocytes develop the capacity to respond in vitro to the 17α,20β-dihydroprogesterone by undergoing oocyte maturation. In stage IV (oocyte maturation), oocytes increase slightly in size, become translucent, and their yolk becomes non-crystalline till final meiotic maturation. In stage V (mature egg), eggs are ovulated into the ovarian lumen, then oviposition (egg laying) happens.

1.3 Classic neurohormonal regulation of fish ovulation

In teleosts, there are various stimulatory and inhibitory regulators involved in the ovulatory process. A simplified overview of the neuroendocrine pathways regulating reproduction regulation are shown in Fig 1.1. More details have been covered recently in published reviews (Somoza, Mechaly et al. 2020, Trudeau 2022). The control centers around the gonadotrophs, so the roles of Lh and Fsh are first presented, followed by key neuroendocrine regulators.

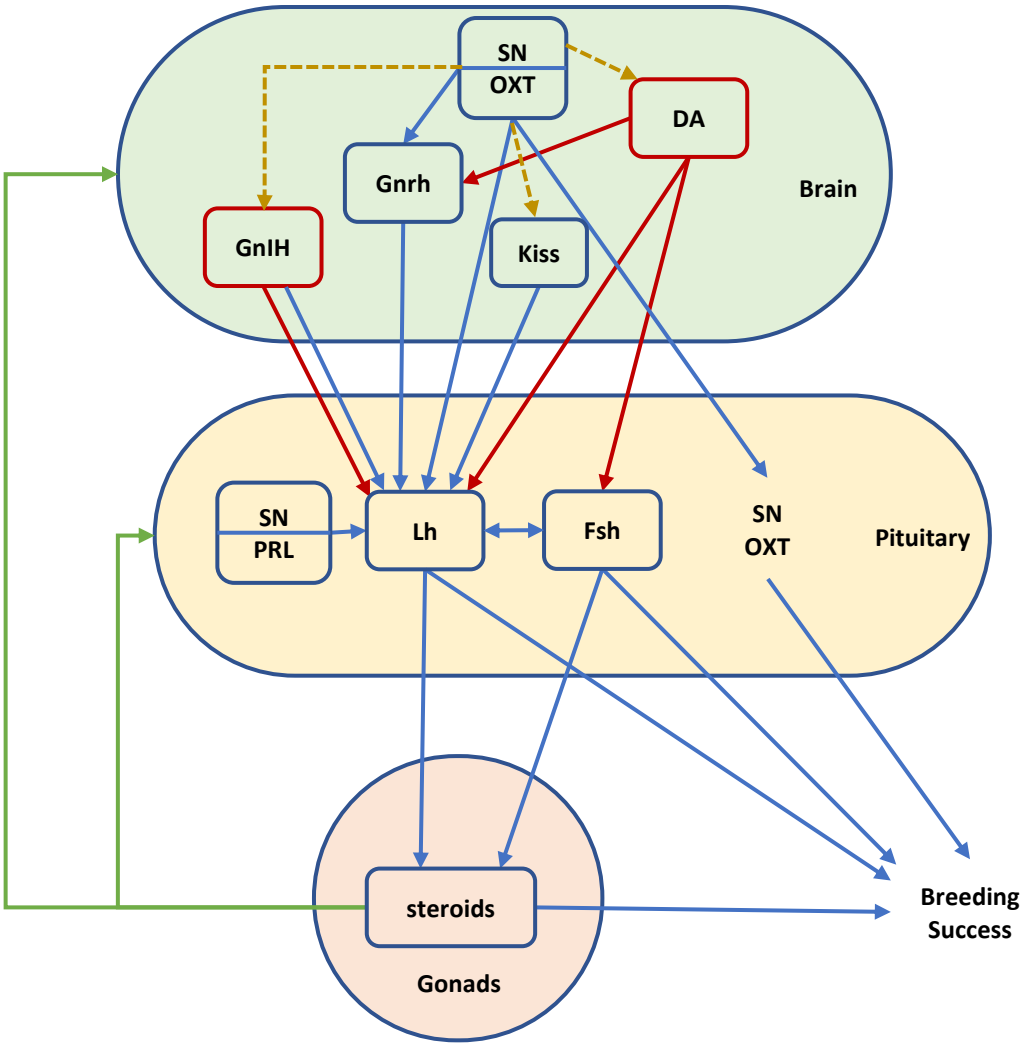

Fig 1.1 Proposed model for neuroendocrine pathway of teleost reproduction. Blue arrows show stimulatory regulation while red arrows show inhibitory regulation. Yellow dash arrows depict the unveiled regulatory pathway of SN. The green arrows depict the positive and negative feedback of sex steroids to the pituitary and brain. SN: secretoneurin, OXT: isotocin, DA: dopamine, Gnrh: gonadotropin-releasing hormone, GnIH: gonadotropin-inhibitory hormone, Kiss: kisspeptin, PRL: prolactin, Lh: luteinizing hormone, Fsh: follicle-stimulating hormone.

1.3.1 Pituitary gonadotropins

Luteinizing hormone (Lh) is composed of the glycoprotein α (Cgα) and Lhβ subunits and plays a critical role in vertebrate reproduction inducing oocyte maturation and ovulation (Lei, Mishra et al. 2001, Zhang, Poutanen et al. 2001). When Lh is secreted from PPD into the general circulation, it will bind to its receptors on the granulosa cells, the ovarian follicle starts the process of maturation, beginning with the production of the maturation- inducing steroid (MIS),

17α, 20β-progesterone. Binding of the MIS to its receptors on the oocyte plasma membrane is followed by activation of the maturation-promoting factor, a complex consisting of existing cdc2-kinase and newly synthesized cyclin B (Yaron and Levavi-Sivan 2011). The deletion of the *lhb* in mice causes infertility in both males and females. Lh-deficient females have defects in folliculogenesis including the degeneration of antral follicles and absence of corpora lutea in the ovaries. In males, Lh stimulates androgen production by the Leydig cells in the testes (Themmen and Huhtaniemi 2000). The lack of Lh leads to a decreased testes size, hypoplasia of Leydig cells, blockade of spermatogenesis at the round spermatid stage, and reduced expression of steroidogenic enzymes and secretion of testosterone (Ma, Dong et al. 2004). In zebrafish, the lack of Lh did not impact male reproduction while females were infertile because of failed oocyte maturation and ovulation (Zhang, Zhu et al. 2015). In normal female zebrafish, there is a significant increase of *fshb* mRNA and *lhb* mRNA from 1am to 4am as well as *cga* mRNA under controlled lighting when lights are on from 08:00- 22:00 (So, Kwok et al. 2005). The synthesis and release of Lh from the anterior pituitary is regulated by Gnrh that binds to membrane receptors on the gonadotrophs, triggering action potentials by rising the concentration of Ca^{2+} in cells, and releasing Lh into the circulation (Levavi-Sivan, Bogerd et al. 2010). In mammals and teleosts, the Lh surge is generated by the release of Gnrh (Trudeau 1997, Smith, Popa et al. 2006). The *lhb* gene promoters in teleost have been isolated and characterized in goldfish, tilapia, and zebrafish (Wang, Zhu et al. 2009). All *lhb* gene promoters in these species contain putative response elements, Sf-1 and Pitx1. The Sf-1 binding site on the Lhβ promoter is indispensable for promoter activity (Melamed, Zhu et al. 2006) with Pitx1 playing a role in the basal activation of the *lhb* gene (Melamed, Koh et al. 2002). Gnrh effects the Lhβ subunit via Gnrh-regulated c-jun, which plays a crucial role in mediating the Gnrh effect by interacting physically with Sf-1 and Pitx1 (Melamed, Zhu et al. 2006).

Follicle stimulating hormone (Fsh) is also a heterodimeric glycoprotein synthesized and secreted by the anterior pituitary. Like Lh, Fsh is composed of a glycoprotein α subunit (Cgα) and the Fshβ subunit that confers biological specificity (Cahoreau, Klett et al. 2015). In teleost reproduction Fsh regulates gametogenesis and follicular growth. The gonads respond to Fsh by secretion of estradiol-17β in females that promotes oogonial proliferation and vitellogenesis,

and progestogens such as dihydroxy-4-pregnen-3-one (DHP) that promotes initiation of germ cell meiosis and follicular maturation and ovulation (Szkudlinski 2015). In males, it is the androgens, mainly 11-ketotestosterone, that regulate spermatogenesis and spermiogenesis, as well as DHP that initiates the meiotic division of spermatogonia and controls the spermatozoa maturation and spermiation (Yaron and Levavi-Sivan 2011). In mice, Fsh-deficient males are fertile despite having smaller testes and oligospermia (Kumar, Wang et al. 1997). A similar result is also shown in zebrafish research. The absence of Fsh delays gonadal development but did not impact spawning (Zhang, Zhu et al. 2015). Previous research in teleost models suggests that Fsh plays a key role in the onset of puberty and early stages of reproductive development while Lh plays a role at the advanced stages of maturation (Yaron, Gur et al. 2003). However, studies in goldfish reported that Fsh activates the later stages of maturation (Hayakawa, Morita et al. 2008). Gene knockout studies of Fsh and Lh in zebrafish have confirmed that the Fsh-Fsh receptor (FSHr) signaling pathway is essential for puberty onset and gonadal growth in male and female fish (Zhang, Lau et al. 2015).

The gonadotropin receptors belong to the G protein-coupled receptor family (Boime, Garcia-Campayo et al. 2004). The first study about the distinct functions of fish Fsh and Lh came from salmonids. This earlier work demonstrated that Fsh is important in promoting oocyte growth while Lh critical in inducing final maturation (Swanson 1991). However, in the study of goldfish, the results suggest that Lh alone is probably enough to control the entire ovarian cycle and Fsh is likely functionally redundant (Yam, Yoshiura et al. 1999). As for zebrafish, it appears that Fsh is mainly responsible for promoting follicular growth, whereas Lh is mainly responsible for stimulating oocyte maturation and ovulation (Chu, Li et al. 2015). During ovarian and follicle development in zebrafish, So. et al. indicated that both FSHr and LHr have low expression in the follicles of primary growth stage. However, when the primary growth follicles grow to initiate vitellogenesis stage, there is a significant increase in FSHr but not LHr expression. The expression of LHr significantly increases in the mid-vitellogenic stage and peaks at the mature stage (So, Kwok et al. 2005). These results suggest that the two gonadotropin receptors have different functions in folliculogenesis, with FSHr being involved in follicle recruitment and

vitellogenic growth and LHr being important in the later stages of follicle development including final oocyte maturation.

1.3.2 Gonadotropin-releasing hormone

Gonadotropin-releasing hormone (Gnrh) is produced in the POA as well as hypothalamus and delivered to the PPD by neural fibers to stimulate the expression and secretion of Lh and Fsh in teleosts (Somoza, Mechaly et al. 2020). There are 15 different forms of Gnrh have been found in living vertebrates (Lethimonier, Madigou et al. 2004, Kavanaugh, Nozaki et al. 2008). A single teleost species usually has two or three forms with different primary amino acid sequences, referred to as GnRH1/2/3 (White, Eisen et al. 1998). It has been shown that the GnRH induces gonadotropin release from the pituitary and gonadal development, according to the relative abundance and seasonal fluctuation of mRNA and peptide levels in the brain (Kah, Lethimonier et al. 2007). GnRH1 is found primarily in POA, caudal hypothalamus and nerve fibers innervating the pituitary in mammals and frogs (Yoo, Kang et al. 2000, Somoza, Miranda et al. 2002). GnRH2 is represented by a single peptide, chicken Gnrh-II (cGnRH-II). It is found in the midbrain tegmentum in salmon as well as POA in goldfish (Kim, Oka et al. 1995) and plays a role in reproduction and metabolism (Kah, Lethimonier et al. 2007). In mammals, neurohormones such as Gnrh are released from nerve terminals into the median eminence and delivered to the pituitary by the hypothalamus-hypophyseal portal blood capillary system. In contrast, teleosts generally lack a well-developed median eminence, and thus a multitude of fibers directly innervate their target cells in the pars distalis of the pituitary. It has been shown that Gnrh2 fibers innervate the pituitary in several species of fish including the goldfish, tilapia (Peter, Chang et al. 1986). Moreover, it has been shown that Gnrh2 induces Lh release from the goldfish pituitary (Chang, Johnson et al. 2009). Gnrh3 is only found in fishes and encodes for a single peptide, salmon Gnrh (sGnrh). This peptide is located in the olfactory and POA in goldfish (Kim, Oka et al. 1995). The GnRH1 and GnRH3 neuronal elements have been shown to distribute in certain anterior forebrain regions in many species (Gonzalez-Martinez, Zmora et al. 2002). In zebrafish, Gnrh2 and Gnrh3 have been identified by HPLC (Powell, Krueckl et al. 1996), later confirmed isolation and sequencing of 2 separate genes (Torgersen, Nourizadeh-Lillabadi et al. 2002, Steven, Lehnen et al. 2003).

The Gnrh3 of adult zebrafish localizes to in the olfactory bulb- terminal nerve and POA-hypothalamus (Steven, Lehnen et al. 2003). Additionally, release of Gnrh3 in the zebrafish pituitary are shown to be 3- to 4-fold higher than those of Gnrh2. Therefore, Gnrh3 is considered to be the hypophysiotropic form (Powell, Krueckl et al. 1996, Steven, Lehnen et al. 2003). The function of Gnrh3 in the olfactory system is not clear, but it may play roles in reproductive behavior (Ogawa, Akiyama et al. 2006). Gnrh is indispensable in mammal reproduction (Bliss, Navratil et al. 2010). However, the studies on Gnrh2 and Gnrh3 mutant zebrafish indicates that the lack of Gnrh in zebrafish does not substantially impact reproduction (Spicer, Wong et al. 2016, Marvel, Spicer et al. 2019). This raising the distinct possibility that other known peptides may also be of fundamental importance (Munoz-Cueto, Zmora et al. 2020, Trudeau 2022).

There is considerable information on Gnrh receptors (Millar, Lu et al. 2004, Kah, Lethimonier et al. 2007, Tello, Wu et al. 2008). They belong to the G-protein coupled receptor superfamily and can be distinguished into four types (GnRHr1/2/3/4) based on their nucleotide sequences (Millar, Lu et al. 2004, Tello, Wu et al. 2008). GnRHr1 is found in mammals and fish, whereas GnRHr2 includes forms found primarily in amphibians and mammals, while GnRHr3 is mainly found in perciform species (Levavi-Sivan and Avitan 2005, Flanagan, Chen et al. 2007, Tello, Wu et al. 2008). One study of goldfish indicated that two forms of Gnrh receptor (GnRHr2 and GnRHr3) are expressed in the pituitary and GnRHr2 is more potent in releasing Lh (Illing, Troskie et al. 1999). Levavi-Sivan et al. identified two types of Gnrh receptor, GnRHr1 and GnRHr3 in Nile tilapia (Levavi-Sivan, Biran et al. 2006). In zebrafish, there are four Gnrh receptors, GnRHr3 was found mainly in brain areas related to reproductive function and was highly expressed in the posterior part of the pituitary that contains Lh- and Fsh-producing cells, suggesting that GnRHr3 may be important for regulating reproduction (Tello, Wu et al. 2008).

1.3.3 Gonadotropin-inhibitory hormone

Gonadotropin-inhibitory hormone (GnIH) was firstly found in quail (Tsutsui, Saigoh et al. 2000). After that, the inhibitory effect of GnIH on bird reproductive behavior was investigated (Bentley, Jensen et al. 2006, Ubuka, Ukena et al. 2006). Similarly with bird GnIH, the inhibitory effect of RFamide-related peptides, the mammalian GnIH homologue, in gonadotropin release

in pituitary was demonstrated in the studies of rat and sheep (Johnson, Tsutsui et al. 2007, Sari, Rao et al. 2009). However, studies in fish showed that the biological effects of GnIH varied in different species. In goldfish, there are three types of GnIH, named as gfGnIH-1, gfGnIH-2, gfGnIH-3, respectively. Some studies revealed that gfGnIH were able to decrease the Gnrh, Lh and Fsh serum levels and inhibit the expression of *gnrh2*, *gnrh3*, *lhb* and *fshb* (Qi, Zhou et al. 2013, Qi, Zhou et al. 2013, Choi, Kim et al. 2016). However, other studies demonstrated that gfGnIH stimulated the Gh, Lh and Fsh levels (Amano, Moriyama et al. 2006, Shahjahan, Ikegami et al. 2011, Shahjahan, Doi et al. 2016). Similar inducing effect of GnIH was also found in tilapia (Biran, Golan et al. 2014). In zebrafish, GnIH was found to decrease the Lh level in plasma and inhibit the expression of *gnrh3* and *lhb* (Zhang, Li et al. 2010, Spicer, Zmora et al. 2017). Moreover, the zebrafish study also indicated that GnIH inhibit the spermatogenesis in males as well as the oocytes maturation in females (Fallah, Tovo-Neto et al. 2019, Fallah and Habibi 2020, Fallah, Rodrigues et al. 2021).

1.3.4 Dopamine

Dopamine (DA) is the significant inhibitor of Gnrh as well as gonadotropins in teleosts. It is involved in sex maturation and ovulation (Peter, Chang et al. 1986, Trudeau 1997, Vidal, Pasqualini et al. 2004, Popesku, Martyniuk et al. 2008). Studies in goldfish indicated that DA had the inhibitory effect on gonadotropin releasing which is stimulated by Gnrh (Peter, Chang et al. 1986, Trudeau 1997). The destruction of the pituitary stalk to inhibit the release of DA leads to a significant increasing in Lh secretion (Chang and Peter 1983). It is now clear that DA inhibits basal and Gnrh-stimulated Lh secretion by several mechanisms. *In vitro* experiments with pituitaries from goldfish (Chang, Yu et al. 1990) demonstrated that it was D2-like, but not D1-like receptors were involved in the inhibition of Lh secretion while studies in teleost failed to detect a direct effect of DA on Lh synthesis (Melamed, Rosenfeld et al. 1998, Yaron, Gur et al. 2003), studies in European eel indicated that DA inhibited the Gnrh-induced Lh synthesis (Vidal, Pasqualini et al. 2004). Moreover, DA inhibits both Gnrh synthesis and release from the nerve terminals in goldfish (Yu and Peter 1990). In the preoptic area (POA), DA inhibits Gnrh by a D1-like receptor mechanism (Yu and Peter 1990, Yu and Peter 1992). DA also down-regulated the synthesis of Gnrh receptors to inhibit Gnrh activity (Kumakura, Okuzawa et al. 2003, Levavi-

Sivan, Safarian et al. 2004) and Gnrh binding capacity (De Leeuw, Habibi et al. 1989). These multiple actions account for the potent inhibitory effects of DA on Lh release in many teleost species. Some other studies are mainly focus on the regulation of DA on Fsh. Studies with rainbow trout indicated that DA may inhibit Fsh release, especially at the end of the reproductive cycle since gonadotropic cells have a high sensitivity to Gnrh at this time (Vacher, Mananos et al. 2000, Vacher, Ferriere et al. 2002). Bromocriptine, the dopamine D2 agonist, significantly inhibited basal Fsh release from cultured gonadotrophs of mature but not vitellogenic trout. Bromocriptine blocked Gnrh3-induced Fsh release from cultured gonadotrophs of both vitellogenic and mature trout (Vacher, Ferriere et al. 2002).

1.3.5 Kisspeptin

Kisspeptins (Kiss) are the neuropeptide family encoded by the *kiss1* gene. They are the endogenous ligands for the kisspeptin receptor, which is also known as the G protein-coupled receptor GPR54. In mammals, Kiss plays a key role in puberty as well as the maintenance of the reproductive axis (Seminara and Kaiser 2005, Kauffman, Clifton et al. 2007). In zebrafish, there are two types of *gpr54* genes and two types of kisspeptins, Kiss1 and Kiss2 (Biran, Ben-Dor et al. 2008). Several studies indicate that Kiss is involved in teleost reproduction. In the brains of zebrafish and medaka, *kiss1* is expressed in the ventromedial habenula and the periventricular hypothalamic nucleus while the expression of *kiss2* was observed in the posterior tuberal nucleus and the periventricular hypothalamic nucleus (Kitahashi, Ogawa et al. 2009). In the medaka, there were two clusters of hypothalamic neurons expressing the *kiss1* gene, they were at the nucleus posteriors periventricular and the nucleus ventral tuberis separately (Kitahashi, Ogawa et al. 2009). These two brain regions were important for the regulation of Lh and Fsh release. Moreover, this study also showed a significant increase of the expression of *kiss1, kiss2, gnrh2* and *gnrh3* during zebrafish development from the puberty till adulthood (Kitahashi, Ogawa et al. 2009). In adult female zebrafish, the study indicated that it was Kiss2 but not Kiss1 induced significantly *fshb* and *lhb* mRNAs in the pituitary (Kitahashi, Ogawa et al. 2009). However, the research on Kiss1/2 in zebrafish also indicates that Kiss/Kissr systems are dispensable because gene knockout does not have any effects on reproduction (Tang, Liu et al. 2015, Trudeau 2015).

1.3.6 Nonapeptides

Nonapeptides are an ancient neuropeptide family that are highly conserved across phyla and arose through the duplication of an ancestral vasotocin gene (Acher and Chauvet 1995). Since the first isolation and characterization of the mammalian nonapeptides oxytocin and vasopressin (Du Vigneaud, Ressler et al. 1953), 16 nonapeptides have been reported in vertebrates. All nonapeptides have an extremely conserved structure. They are cyclic peptides with two half cysteine residues at position 1 and 6, forming a disulphide bridge and a glycine amide at position 9. The various nonapeptides are classified by the amino acid at position 8 into two families: basic nonapeptide families and neutral nonapeptide families. All vertebrates have at least two nonapeptides one each from the two families (Urano and Ando 2011). In all vertebrates, from mammals to teleosts, nonapeptides are synthesized in the magnocellular neurons in preoptic area and project to the posterior pituitaries (Goodson 2008, Goodson, Kelly et al. 2012, Kelly 2014). In teleosts, the homologs of mammalian oxytocin and vasopressin are isotocin and vasotocin, respectively. It is now clear that Gnrh can directly modulate electrical activity of vasotocin neurons in the brain of rainbow trout (Saito, Hasegawa et al. 2003). Salmon Gnrh and chicken Gnrh are detected in rainbow trout brain, with fibers in close proximity of vasotocin neurons. Application of both Gnrh forms increased the frequency of cell-type-specific synchronous Ca^{2+} pulses in vasotocin neurons, suggesting that Gnrh may modulate these secretory neurons to regulate reproductive behavior (Saito, Hasegawa et al. 2003). In the bluehead wrasse, which exhibits a female-to- male sex change in response to social class, vasotocin neuronal phenotype is strongly influenced by social status which appears to be manifested independently of the gonads (Semsar and Godwin 2003). In rat, Gnrh acts as a neuromodulator of vasopressinergic neurons (Bojanowska, Guzek et al. 1999). A highly selective Gnrh agonist stimulates vasopressin release from the hypothalamo- neurohypophysial system *in vivo* and *in vitro* (Boczek-Leszczyk, Stempniak et al. 2010). Gnrh and its agonists also influence vasopressin secretion, and this effect is probably mediated by Gnrh receptors on the magnocellular neurons (Boczek-Leszczyk, Stempniak et al. 2010). Ovaprim (OVP) is a widely used spawning tool and contains a salmon Gnrh analog and domperidone, a dopamine receptor-2 (DA2) antagonist. In the catfish, an intraperitoneal injection of OVP induced

ovulation as well as increased brain and plasma vasotocin levels in a time-dependent manner (Chaube, Singh et al. 2014). Ovarian vasotocin level increased in a concentration- and time-dependent manner with the maximal response at 16h. Thus, OVP exerts effect more functions than the induction of the preovulatory Lh surge and acts at multiple sites other than the pituitary to stimulate vasotocin secretion. Concerning the mechanism of stimulation of brain vasotocin secretion, since OVP contains a salmon Gnrh analog and DA2 receptor blocker, the involvement of both the systems is likely. DA inhibits brain and plasma vasotocin level (Singh, Chaube et al. 2013). Thus, it is likely that domperidone in the OVP formulation may block the DA2 receptor, preventing the DA inhibition of vasotocin secretion. In the preoptic area and the nucleus preopticus (NPO), Gnrh may play a functional role in regulating interactions among vasotocin neurons. The same regulatory molecules are present in the fish ovaries and may be involved in the OVP-induced vasotocin secretion. The presence of Gnrh and Gnrh-binding sites has been described in catfish and the presence of DA in ovaries of catfish was reported (Singh and Joy 2010). Thus, salmon Gnrh and domperidone in the OVP preparation may act synergistically to stimulate ovarian vasotocin secretion. Moreover, the OVP-induced Lh surge may result in the increased levels of vasotocin. In summary, the Gnrh-gonadotropin axis is involved in regulating vasotocin secretion, which is further modulated by catecholamine and steroid hormones at the level of the brain and ovaries.

1.3.7 Secretogranin 2 and secretoneurin

The chromogranin family includes chromogranin A (CgA), which was first isolated from chromaffin cells of the adrenal medulla, chromogranin B (CgB), initially characterized in a rat pheochromocytoma cell line (Lee and Huttner 1983), and chromogranin C, also called secretogranin-II (Scg2) (Helle 2004). Chromogranins are in secretory cells of the nervous, endocrine, and immune system (Huttner, Gerdes et al. 1991, Winkler and Fischer-Colbrie 1992). They consist of single-polypeptide chains ranging from 180 to 700 amino acid and an amino-terminal signal peptide directs the movement of the Scg2 pre-proteins from ribosomes to the endoplasmic reticulum and the Golgi complex, where the pre-proteins can be further post-translationally modified. The modified proteins tend to bind Ca^{2+} with low affinity but high capacity and then aggregate in vitro at low pH in the presence of Ca^{2+} (Yoo and Albanesi 1991).

Secretogranin 2 (Scg2) was found to be widespread in peptide-secreting neuroendocrine cells in rats (Rosa, Hille et al. 1985) and it is also regionally conserved and expressed in brain, being highest in the hypothalamus and pituitary, less in the spinal cord and is virtually absent in the adrenal and other peripheral endocrine organs (Turquier, Vaudry et al. 1999). Later, *scg2a* mRNA was isolated and characterized in the pituitary of the goldfish (Blazquez, Bosma et al. 1998), therefore Scg2 becoming the first granin to be sequenced in fish. Different from CgA and CgB, the Scg2 precursor protein is poorly conserved among the vertebrates except for a short peptide known as secretoneurin (SN) that is located in the central core region of Scg2 (Blazquez, Bosma et al. 1998). By comparison with deduced amino acid sequences of mammalian and frog Scg2, goldfish Scg2 is 31% identical to rat Scg2. Studies in rats demonstrated that the prohormone convertases (PC)-1 and PC-2 start to process the pro-protein Scg2 starts as soon as the protein is first synthesized (Leitner, Kaufmann et al. 1997). The pro-protein Scg2 is fully processed to a free peptide SN, by PC1 and PC2. Both PC1 and PC2 are colocalized with SN in neurosecretory granules in the bovine posterior pituitary (Egger, Kirchmair et al. 1994). However, only PC1 can generate SN in neurons, suggesting that processing of Scg2 is tissue- and/or cell-type specific (Hoflehner, Eder et al. 1995). In another study, the PC producing cells transfected with either PC1 or PC2 revealed that both enzymes processed SgII into SN, although the ability to generate free SN peptide was more pronounced with PC2 than with PC1 (Laslop, Weiss et al. 1998). Moreover, in the absence of PC2, SN levels in mouse brain remain unchanged (Laslop, Becker et al. 2002). This result indicated the important role of PC1 in generating the SN peptide in a mammal *in vivo*. In comparison to other Scg2 derived peptides, the SN domain is highly conserved across evolution with a 90-100% sequence similarity between mammals and 84-87% sequence similarity between humans and cartilaginous fish and 45-67% identical between bony fish and mammals (Leitner, Schneitler et al. 1998, Fischer-Colbrie, Kirchmair et al. 2005, Zhao, Hu et al. 2010). In teleosts, including goldfish and zebrafish, two SN variants have been identified and are most likely the result of a gene duplication event of an ancestral Scg2. The two Scg2 paralogs are Scg2a and Scg2b that are then processed into SNa and SNb respectively through proteolytic processing by prohormone convertases. In teleosts, SNa shares some sequence identity with that of mammals

in the two conserved sequence regions that are “QYTP” and “LATLEQSVFQEEL”, respectively (Trudeau, Martyniuk et al. 2012, Mitchell, Mikwar et al. 2020). These regions of conservation are centered within the middle of the full amino acid sequence and are identical to their mammalian counterpart (Samia, Lariviere et al. 2004). This sequence conservation may be the determinants of the biological actions of SN (Zhao, Zhang et al. 2009). However, two stretches in the middle of the teleost SNb ‘‘EQYTPQSLA’’ and ‘‘FE(Q)ELG’’ are only moderately conserved (Zhao, Hu et al. 2010).

1.3.8 Anatomical distribution and biological effects of secretoneurin

Numerous studies have reported *Scg2* expression in the brain, pituitary, stomach, small and large intestine, thyroid cells, bronchial mucosa, ovaries, testes, synovial fluid, and the central nervous system of humans, rats, and bovine among others (Fischer-Colbrie, Laslop et al. 1995). Although, it is important to note that Scg2 distribution does not necessarily reflect SN localization since this is dependent on proteolytic processing of the precursor and the relative amounts and types of PCs present in each tissue. In rat adrenal medulla, Scg2 is found as intact protein whereas an intermediate processing occurs at the anterior pituitary and all other organs display a nearly complete processing of Scg2 into SN (Marksteiner, Kirchmair et al. 1993, Fischer-Colbrie, Laslop et al. 1995, Leitner, FischerColbrie et al. 1996). Similar results were observed in bovine in that a high degree of processing takes place in the brain, less in the adrenal medulla and least in the anterior pituitary (Kirchmair, Hogueangeletti et al. 1993). This is in contrast to *Xenopus laevis* intermediate pituitary cells where nearly all newly synthesized Scg2 is processed into its products and this occurs quickly (Van Horssen and Martens 1999). These results suggest that Scg2 processing to SN is tissue and species specific. SN-ir has been most extensively studied in the rat and is widespread throughout the hypothalamus and pituitary (Marksteiner, Kirchmair et al. 1993, Fischer-Colbrie, Laslop et al. 1995). Similar results were observed in cow (Kirchmair, Hogueangeletti et al. 1993). Goldfish brain and pituitary also have SNa distributed throughout; however, the highest SNa-ir was in the magnocellular and parvocellular cells of the preoptic nucleus that project to the pituitary (Canosa, Lopez et al. 2011). Various regions of the brain and discrete cells within each region differentially express SN. In rats, many SN immunoreactivity (SN-ir) cells was observed in several brain areas,

especially in the hypothalamus, olfactory bulb, and several areas of brainstem cell (Saria, Kaufmann et al. 1997). Rat serum also contains SN-ir as the free peptide, which is believed to be supplied by a large pool of SN from the intestines (Leitner, FischerColbrie et al. 1996). There is SNa-ir in magnocellular and parvocellular cells of the preoptic area in goldfish, several sites in the hypothalamus and lactotrophs of rostral pars distalis (RPD) of the anterior pituitary has also demonstrated that SN-ir (Zhao, Basak et al. 2009, Canosa, Lopez et al. 2011). More specifically, SNa-ir was colocalized in isotocin neurons in the goldfish (Canosa, Lopez et al. 2011).

Intraperitoneal injection of goldfish SNa in combination with a dopamine D2 receptor blocker indicated that SN could rapidly stimulate anterior pituitary function in vivo (Blazquez, Bosma et al. 1998). Saria et al. revealed that SN can evoke the release of dopamine from the rat caudate putamen (Saria, Troger et al. 1993). In rat hypothalamus, SN and oxytocin are secreted in the vesicles of hypothalamo-neurohyphysial neurons, and *scg2* and *oxt* mRNA are co-regulated as well (Mahata, Mahata et al. 1993). Furthermore, in goldfish, SN is colocalized with isotocin, the teleost homolog of oxytocin in mammals, and is potentially released from nerve terminals (Canosa, Lopez et al. 2011). These data support the hypothesis that SN of both neuroendocrine and paracrine origin regulates pituitary Lh release.

In the study of LβT2 mouse gonadotroph cell line, it was found that SN induced a significant increment of Lh release and production in vitro. Furthermore, SN can activate the protein kinase A (PKA) and cAMP-induced extracellular signal-regulated kinases (ERK) signaling pathways in the Lh-secreting mouse LβT2 pituitary cell line. Gnrh increased cellular *scg2a* mRNA level and total SN-immunoreactive protein release into the culture medium (Zhao, McNeilly et al. 2011). Moreover, Zhao et al. found that *fshb* mRNA in dispersed goldfish pituitary increases for 1.34-fold after the treatment of incubating SN (Zhao, Grey et al. 2010). Furthermore, evidence from our previous study indicates that SN may be functional in controlling reproductive hormone release. Previous studies in goldfish demonstrated that SN induced the expression of *cga*, *lhb* and *fshb* while anti-SN antiserum suppressed the effect of Gnrh3 on Lh stimulation (Zhao, Grey et al. 2010). Moreover, the mutant of Scg2a and Scg2b impact the zebrafish breeding successful rate significantly but the intraperitoneal injection with hCG rescued it partially (Mitchell, Zhang et al. 2020). All these data suggested that Scg2

modulates reproduction in teleosts by stimulating Gnrh3 and Lh. Many mechanisms of action and the physiology of Scg2 systems regulating teleosts reproduction are still unknown. To study this, we performed experiments to detect the natural variations of SN in WT zebrafish and investigate the effect of exogenous SN peptides on zebrafish ovulation.

1.4 Sex steroid regulation of ovulation in zebrafish

Sex steroids play a key role in regulating female reproduction in teleosts. For now, the positive and negative feedback of steroids on Gnrh and gonadotropins to regulate ovulatory processes in vertebrates have been revealed (Sugiura, Su et al. 2010, Fontaine, Royan et al. 2020). Immunostaining results revealed that the estrogen receptor 1 (ER 1) and androgen receptor (AR) were located in the Gnrh neurons in medaka and tilapia (Zempo, Karigo et al. 2018, Ogawa and Parhar 2020). In orange-spotted grouper the expression of ER can be found kisspeptin neurons (Guo, Wang et al. 2017). Similar result was also shown in goldfish (Wang, Sham et al. 2013). Further research in goldfish demonstrated the expression of ER in GnIH neurons and isotocin neurons (Mangiamele, Gomez et al. 2017, Qi, Zhou et al. 2017). In rainbow, ER is expressed in dopamine neurons (Linard, Anglade et al. 1996). Besides of brains, the expression of ER was detected in the gonadotrophs in teleost pituitary (Li, Tang et al. 2018, Fontaine, Ager-Wick et al. 2019, Hollander-Cohen, Golan et al. 2021). The studies in rats revealed that both E2 and T stimulate Gnrh (Tibolt and Childs 1985, Bauer-Dantoin, Weiss et al. 1995). As for teleosts, T was found to induced the development of Gnrh systems in immature African catfish (Dubois, Florijn et al. 1998) while E2 stimulated Gnrh levels in European sea bass (Gonzalez-Martinez, Madigou et al. 2004). Negative feedback effects of E2 also exist and involve stimulatory actions on DA-neurons that leads to inhibition of Lh release (Trudeau, Sloley et al. 1993). Moreover, the study in pituitary indicated that gonadotrophs are regulated by sex steroids. The study in juveniles goldfish revealed that the expression of *lhb* were stimulated by E2 and T injections (Kobayashi and Stacey 1993) . But in adult goldfish, Lh was induced by T in females and by E2 in males (Sohn, Suetake et al. 1998). These results not only revealed the distributions of ER in neurons related ovulation but also suggested that sex steroids were involved in the teleost ovulation by regulating the neuropeptides as well as gonadotropins.

1.5 Ethics statement

All experiments were approved by the University of Ottawa Protocol Review Committee and adhere to the guidelines established by the Canadian Council on Animal Care for the use of animals in research and teaching.

Chapter 2 Localization of SNa immunoreactivity in the brain and pituitary of zebrafish

2.1 Introduction

Secretoneurin immunoreactivity (SN-ir) has been most extensively studied in the rat and is widespread throughout the hypothalamus and pituitary (Marksteiner, Kirchmair et al. 1993, Fischer-Colbrie, Laslop et al. 1995). The hypothalamus is part of the diencephalon and it is the major regulating center responsible for reproductive, endocrine, food intake, stressed in the vertebrates (Sower, Freamat et al. 2009). The pituitary gland is a critical component of vertebrate neuroendocrine systems secreting the hormones involved in metabolism, reproduction, growth, lactation and maintains homeostasis (Scully and Rosenfeld 2002). Similar results were also observed in cow as well as other non-mammalian vertebrates (Kirchmair et al. 1993)(Canosa, Lopez et al. 2011). Goldfish brain and pituitary have Scg2, and SN distributed throughout; however, the highest SN-ir was in the magnocellular and parvocellular cells of the preoptic nucleus that project to the pituitary (Canosa, Lopez et al. 2011). The parvocellular cells secret the corticotropin releasing hormone, which involve in the stress response by stimulating pituitary producing adrenocorticotropic hormone (Charmandari, Tsigos et al. 2005). As for magnocellular cells, oxytocin and vasopressin are produced and transported to the pituitary through the median eminence in mammals(Landgraf and Neumann 2004). The structural network of capillaries known as the median eminence were not found in teleosts. Instead, teleost anterior pituitary endocrine cells are directly innervated by preoptic and hypothalamic neurons (Peter, Yu et al. 1990). Various regions of the brain and discrete cells within each region differentially express SN. In the electric fish *Brachyhypopomus gauderio*, SN-ir was found not only colocalized with vasotocin in the preoptic area and hindbrain, but also colocalized with prolactin in the rostral pars distalis in the pituitary. In goldfish, SN-ir is found in the lactotropes of the rostral pars distalis as well. Similarly, SN is co-localized with oxytocin and isotocin in the neurons of rat (Mahata, Mahata et al. 1993) and goldfish (Canosa, Lopez et al. 2011), respectively. Rat serum also contains SN-ir as the free peptide, which is believed to be supplied by a large pool of SN from the intestines (Leitner, FischerColbrie et al. 1996).

Here we investigated the distribution of SNa in the zebrafish forebrain and pituitary. This is the first attempt to explore the distribution of SNa peptide in the adult zebrafish. We also determined that SNa and isotocin are colocalized in zebrafish, thus giving support to the proposal that SNa is associated with another well-known reproductive peptide. Finally, we performed an immunofluorescent experiment in the *lhb*-RFP x *fshb*-eGFP transgenic zebrafish to determine the relationship between SNa producing neurons and cells and the gonadotrophs in the PPD. There are no antibodies that recognize SNb of teleosts, so this was not studied.

2.2 Materials and methods

2.2.1 Experimental animal husbandry

Experimental zebrafish (AB stain) were bred and kept in the University of Ottawa aquatics room at the temperature of 28°C. The lights on time were set for 14 hours from 0900h to 2300h. Wildtype zebrafish were provided from the University of Ottawa aquatics facility. The *lhb*-RFP transgenic zebrafish line and *fshb*-eGFP transgenic zebrafish line (Golan, Biran et al. 2014) were generously provided by Professor Berta Levavi-Sivan (The Hebrew University of Jerusalem) via the laboratory of Professor Yonathan Zohar (University of Maryland Baltimore County). The Nile tilapia Lh and Fsh promoter constructs driving *lhb*-RFP and *fshb*-eGFP transgene expression have been described in detail previously (Golan, Biran et al. 2014). We generated the double transgenic line by crossing the *lhb*-RFP transgenic line and *fshb*-eGFP transgenic line. The double transgenic line is housed in the University of Ottawa aquatics facility. All animal experiments followed the guidelines of the Canadian Council on Animal Care for the use of animals in research, approved by the University of Ottawa Animal Care Protocol Review Committee.

2.2.2 Paraffin sectioning

Sexually mature female zebrafish 6-9 mpf were anesthetized in an ice bath. After that, the heads were removed and incubated into 4% paraformaldehyde over night at room temperature. Then, the head samples were washed in PBS for 20 min for twice and decalcified in 0.5M EDTA at room temperature for 5-7 days. After washing in PBS 15 min for three times, the decalcified samples were dehydrated in ethanol (EtOH) in a series of 30-minute incubations

in increasing concentrations of EtOH (30, 50, 70, 80, 99%). Next, samples were incubated in the xylene for 2 X 30 min and transferred into melted paraffin at 60°C for 1 hour, then incubated in clean paraffin at 60°C overnight. The next morning, samples were embedded in metal molds and prepared for sectioning (10-20 μm) on the Microm HM350 micrometer (Heidelberg) and mounted on microscope glass slides (Fisherbrand, cat#12-550-15). One the day of immunofluorescence labelling, the sections were incubated in Coplin jars with xylene 2 x 20 min and rehydrated in EtOH with a series of 2 X 10-minute incubations in decreasing concentrations of EtOH (99, 95, 70%) and finally in PBS.

2.2.3 Cryosection

Female zebrafish heads were fixed and decalcified as described above. Then the samples were incubated for 6 hrs in increasing concentrations of sucrose (10, 20, 30%) and embedded in plastic molds with O.C.T compound (Fisher Science, cat# 23-730-571) in -80°C till cryosection. Cryosectioning procedure was performed on the Leica CM1850 Cryostat when operating temperature at -18°C and chamber temperature at -20°C. The cryosections (20μm) were mounted on microscope glass slides (Fisherbrand, cat#12-550-15) and stored in -80°C until use. One the day of immunofluorescence labelling, slides were thawed at room temperature and washed in 2 X 10 minutes PBS.

2.2.4 Immunofluorescence

Antigen retrieval is indispensable before the antibody binding step to expose the antigen binding sites of the primary antibody. In Coplin jars, slides were incubated in 0.01M sodium citrate solution at 80°C for 30 minutes, and then cooled to room temperature. Then the samples were blocked in blocking buffer with 1% skim milk and 0.3% Triton-100 for 30 minutes. For immunofluorescence, sections were incubated with our rabbit-anti-goldfish SN antiserum and guinea pig-anti-oxytocin antiserum (Abcam, cat#ab228508) 1:1000 as primary antibody overnight at 4°C. The specificity of the guinea pig-anti-oxytocin antiserum in goldfish (Canosa, Lopez et al. 2011) and zebrafish (Altmieme, Jubouri et al. 2019) has been previously validated. The rabbit-anti-goldfish SNa antiserum was generated and validated in our lab (Zhao, Basak et al. 2006, Zhao, Basak et al. 2009). It has been shown using western blotting that it will recognize

the precursor and any processed fragment containing the SNa epitope (Zhao, Basak et al. 2006). Therefore, we refer to this as Scg2a/SNa immunoreactivity (ir). The epitope (YTPQKLATLQSVFEE) recognized by the goldfish SNa antibody is identical to the central 15 amino acids in zebrafish SNa (zfSNa). Tao et al. also demonstrated that this antibody recognizes synthetic zebrafish SNa, with no cross-reaction with SNb on dot blots (Tao, Hu et al. 2018). On next day, after washed in PBS solution, the samples to perform colocalization of Scg2a/SNa-ir and oxytocin-ir were embedded in Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 (ThermoFisher, cat#A32731) diluted in 1:1500 for SN and Goat anti-Guinea Pig IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 594 (ThermoFisher, cat#A11076) diluted in 1:1500 for oxytocin at room temperature for 2 hours. As for the samples to investigate the distribution of Scg2a/SNa-ir in *lhb*-RFP X *fshb*-eGFP transgenic zebrafish line, they were embedded in Goat anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 488 (ThermoFisher, cat#A32731) diluted in 1:1500 at room temperature for 2 hours. Finally, mount coverslips on the slides with 20ul of antifade mount with DAPI (ThermoFisher, cat#P36971). Images were captured on Olympus Fluoview FV1000, and the brightness, contrast and color balance were adjusted by Olympus official software FV20-ASW Viewer.

2.3 Results

2.3.1 Verification of SNa antibody specificity

Co-incubations overnight (4°C) with zfSNa peptides (1nmol/μl) (Fig 2.1A) completely blocked the immunoreaction in zebrafish pituitary, confirming specificity. Moreover, the immunoreactivity of the anti-gfSN antibody was not decreased after incubation with zfSNb peptides (Fig 2.1B). These results indicated that the anti-gfSN antibody was specific to the Scg2a/SNa peptide in the zebrafish samples, but not specific to the Scg2b/SNb peptide.

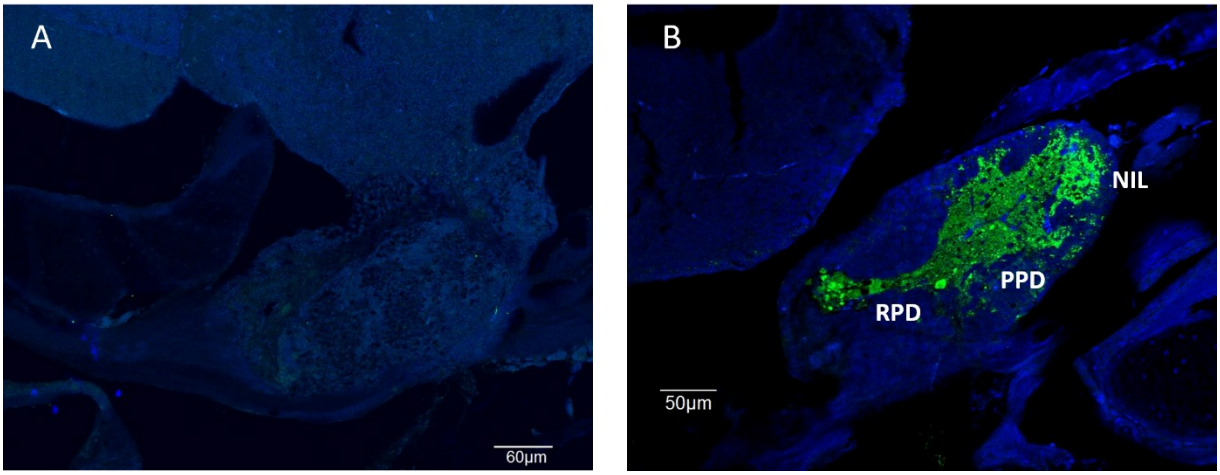

Fig 2.1 Specificity tests of anti-gfSNa antibody. (A) Immunofluorescent experiment performed by anti-gfSNa antibody incubated with zfSNa peptide. (B) Immunofluorescent experiment performed by anti-gfSNa antibody incubated with zfSNb peptide. Green fluorescence labels the SNa-ir nerve fibres and endocrine cells in female zebrafish pituitary. N=5

2.3.2 Distribution of Scg2a/SNa immunoreactivity (Scg2a/SNa-ir) in zebrafish brain

From the results, the immunoreactivity of Scg2a/SNa was in the olfactory bulb, telencephalon, preoptic area, and hypothalamus. There were no apparent differences we could identify between male and female brains.

In the olfactory bulb, strong Scg2a/SNa-ir were observed in the glomerular layer (GL), olfactory nerve layer (ONL) and peripheral olfactory fascicle (POF). Immunoreactivity most evident in neural fibers (Fig 2.2B). In the telencephalon, strong immunofluorescence was shown in the ventral part of the ventral telencephalon (Vv) while fluorescence was lower in dorsal (Vd) and lateral parts of the ventral telencephalon (Vl) (Fig 2.2C). In the preoptic area, the Scg2a/SNa-ir was located in cells bodies and fibres in the magnocellular preoptic nucleus (PM) (Fig 2.2D). The most conspicuous labelling was in cell bodies throughout the preoptic area (Fig 2.2E). In the hypothalamus, Scg2a/SNa-ir presented as punctate fibres following an inverted arc shape in the ventral zone of periventricular hypothalamus (Hv) (Fig 2.2F). A distinct pattern of Sgc2A/SNa-ir was evident in the caudal zone of periventricular hypothalamus (Hc) around the posterior recess (PR) of diencephalic ventricle (Fig 2.2G). Densely packed cells adjacent to the PR expressed the highest levels of Scg2a/SNa in the Hc, followed by a wider band of Scg2a/SNa-ir appearing like numerous boutons.

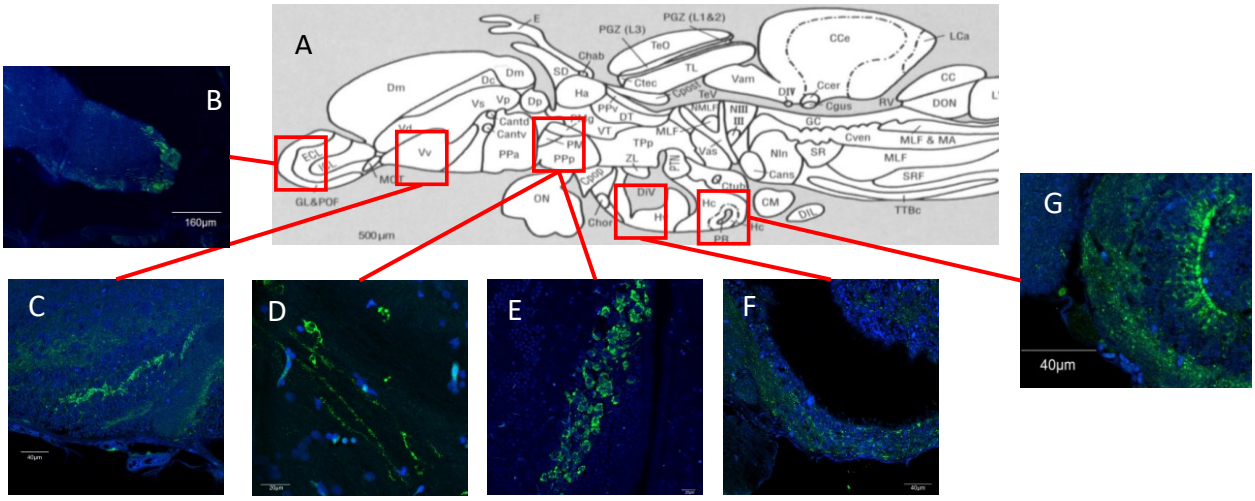

Fig 2.2 Distribution of Scg2a/SNa-ir in female zebrafish brain. (A) Schematic of the Scg2a/SNa-ir distribution of the zebrafish brain modified from the zebrafish atlas (Wullimann et al., 1996). (B) Scg2a/SNa-ir distributes in olfactory bulb. (C) Scg2a/SNa-ir distributes in telencephalon. (D) Neural fibers with Scg2a/SNa-ir in preoptic area. (E) Cell bodies with Scg2a/SNa-ir in preoptic area. (F) Scg2a/SNa-ir distributes in hypothalamus. (G) Scg2a/SNa-ir distributes in posterior recesses in hypothalamus. Green fluorescence labels the Scg2a/SNa-ir. N=10

2.3.3 Distribution of Scg2a/SNa-ir in zebrafish pituitary

Strong Scg2a/SNa-ir was found throughout the zebrafish pituitary. Heavily labelled fibres from the brain entered via the infundibular region (Fig 2.3A), passing centrally and terminating in the in the pars nervosa (PN). Lightly stained fibres can also be seen in the proximal pars distalis (PPD). A few Scg2a/SNa-ir pituitary cells were also evident in the rostral pars distalis (RPD), and pars intermedia (PI). Medially, the neurointermediate lobe (NIL) (Fig 2.3B) contained Scg2a/SNa-ir cells.

In the double *lhb*-RFP and *fshb*-eGFP transgenic zebrafish line (Fig 2.3C) it was clear that both Lh- and Fsh-expressing gonadotrophs were surrounded by Scg2/SNa-ir but were themselves devoid of it. Immunoreactivity for Scg2/SNa appeared as highly punctate fibres both near and surrounding Lh and Fsh cells (Fig 2.3D).

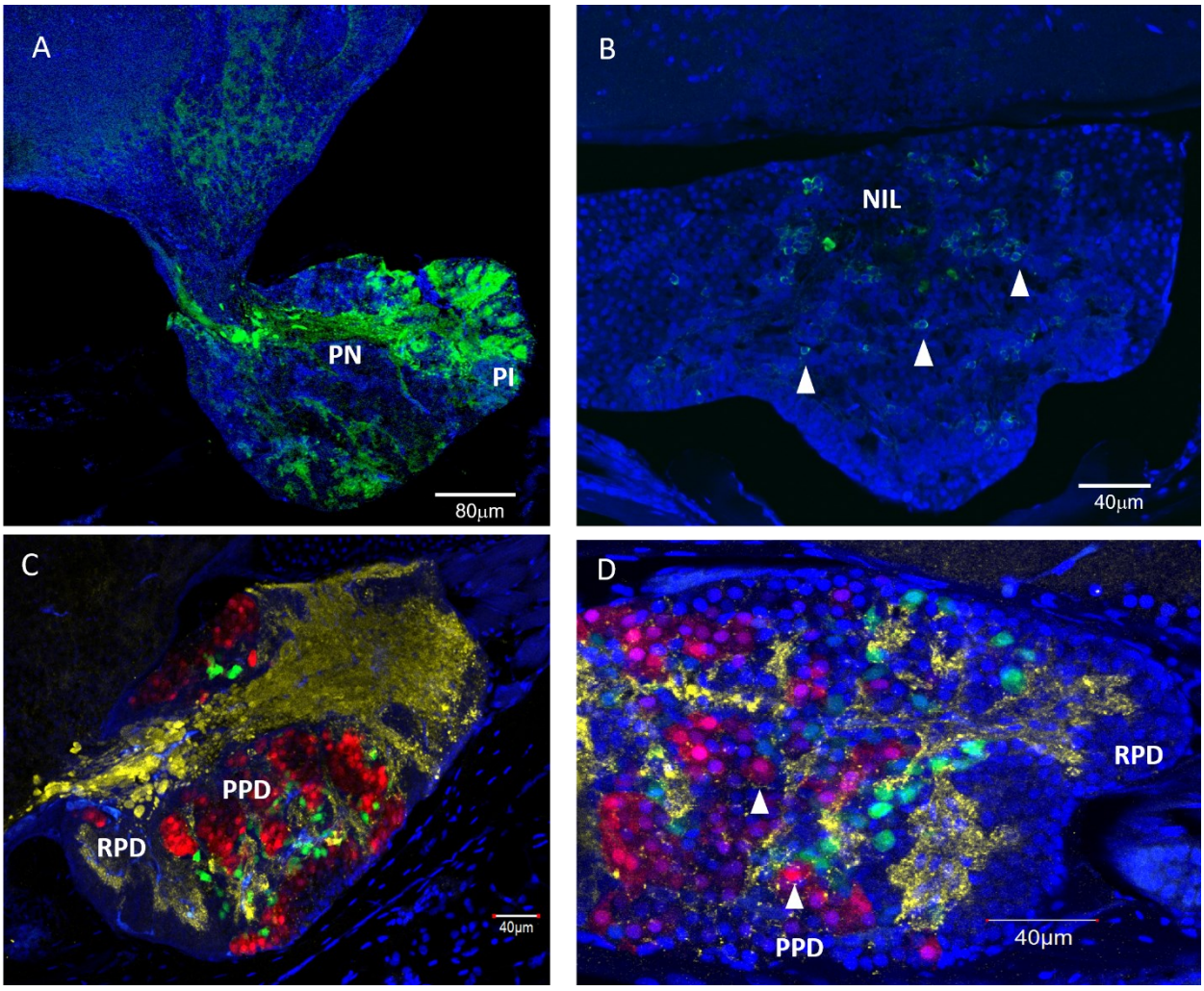

Fig 2.3 Distribution of Scg2a/SNa-ir in female pituitary. (A) Distribution of Scg2a/SNa-ir in PN and NIL in zebrafish pituitary. Green fluorescence labels the Scg2a/SNa-ir. (B) White arrow heads labelled cell bodies with Scg2a/SNa-ir in NIL in pituitary. Green fluorescence labels the Scg2a/SNa-ir. (C) Distribution of Scg2a/SNa-ir and Lh-producing gonadotrophs as well as Fsh-producing gonadotrophs. Yellow fluorescence labels the Scg2a/SNa-ir. Green fluorescence labels the Fsh-producing cells. Red fluorescence labels the Lh-producing cells. (D) White arrow heads labelled Scg2a/SNa-ir surrounding gonadotrophs. Yellow fluorescence labels the Scg2a/SNa-ir. Green fluorescence labels the Fsh-producing cells. Red fluorescence labels the Lh-producing cells. N=10

2.3.4 Colocalization of Scg2a/SNa and isotocin in zebrafish

Using an antibody against mammalian oxytocin, as expected, we labeled zebrafish preoptic magnocellular isotocin (Oxt) neurons and fibres. Clear co-colocalization of Scg2a/SNa-ir and Oxt-ir was observed in the zebrafish brain and pituitary (Fig 2.4&Fig 2.5). The double immunofluorescence was not only revealed in the cell bodies, but also visualized in numerous

fine projections. Fibres displaying both Scg2a/SNa- and Oxt-ir can be seem entering the pituitary via the infundibular stalk (Fig 2.5C). Note also that some Oxt-ir fibres do not express Scg2a/SNa-ir. Double fluorescence also labeled the neural fibers in Hv (Fig 2.5C). The intense co-labelling observed throughout the central portion of the pituitary terminating in the NIL (Fig 2.5D) originated on the preoptic region.

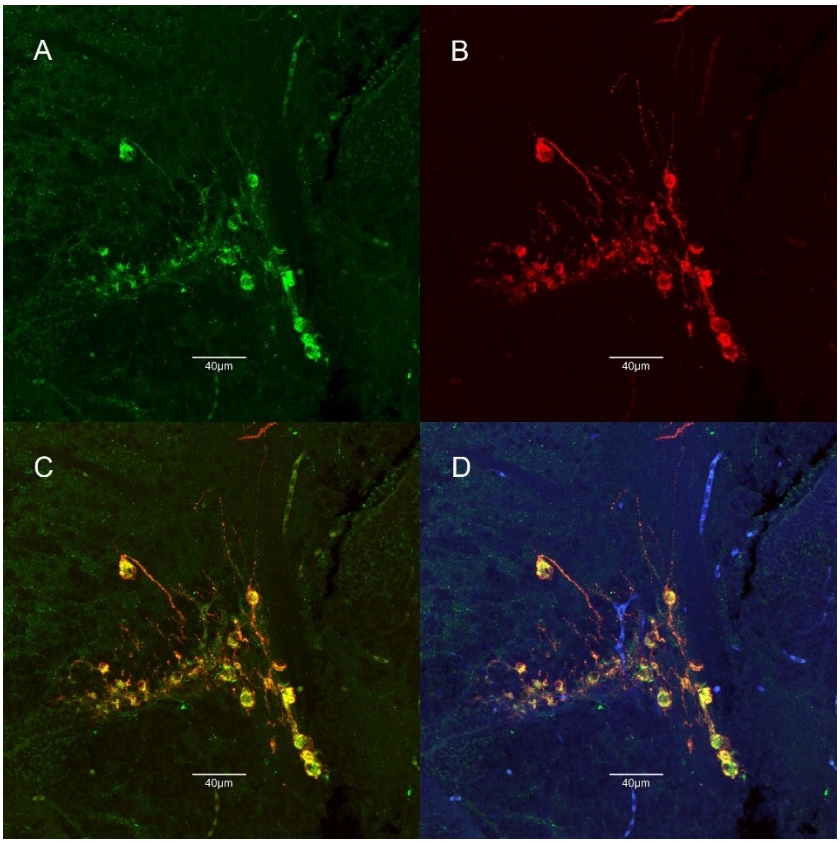

Fig 2.4 Colocalization of Scg2a/SNa-ir and Oxt-ir in preoptic area of female zebrafish. (A) Image of confocal microscope with green fluorescence labels the Scg2a/SNa-ir. (B) Image of confocal microscope with red fluorescence labels the Oxt-ir. (C) Image of confocal microscope shows the colocalization of Scg2a/SNa-ir and Oxt-ir. (D) Image of confocal microscope shows the colocalization of Scg2a/SNa-ir and Oxt-ir with DAPI. N=20

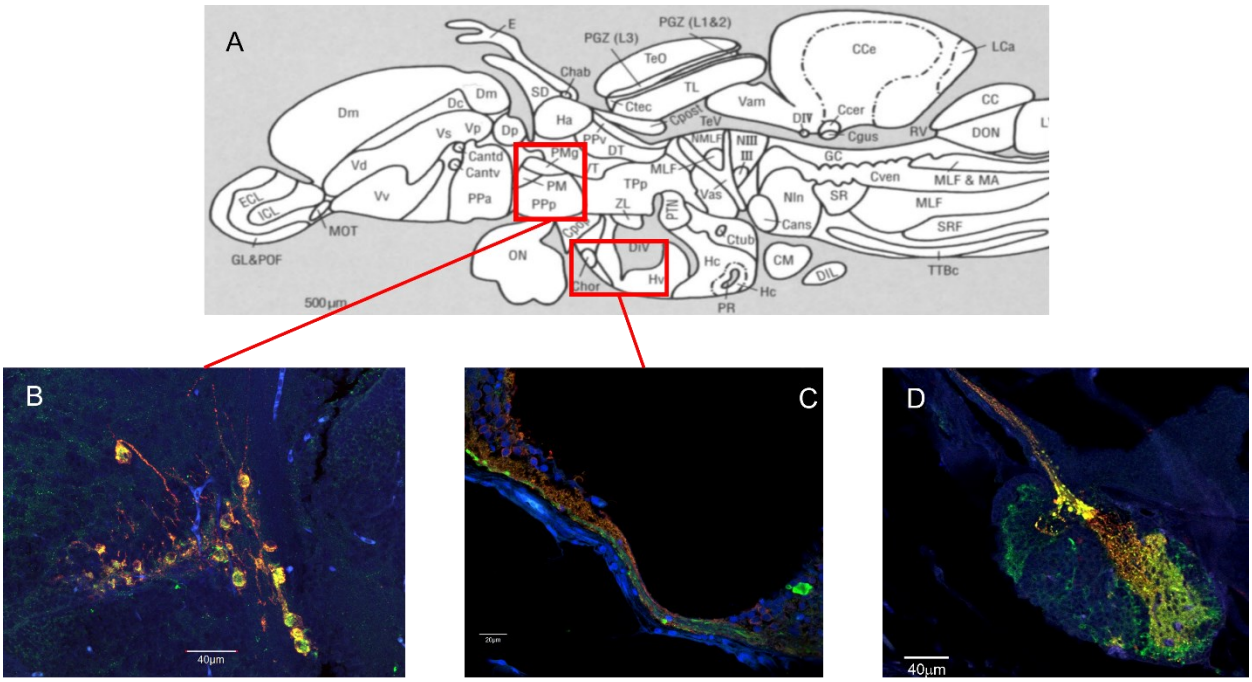

Fig 2.5 Colocalization of Scg2a/SNa-ir and Oxt-ir in female zebrafish. (A) Schematic of the colocalization of Scg2a/SNa-ir and Oxt-ir in the zebrafish brain. (B) Image of confocal microscope showed the colocalization of Scg2a/SNa-ir and Oxt-ir in preoptic area. Image is also presented in Fig 2.4 (C). Image of confocal microscope showed the distribution of Scg2a/SNa-ir and Oxt-ir in hypothalamus. (D) Image of confocal microscope showed the colocalization of Scg2a/SNa-ir and Oxt-ir in pituitary. Green fluorescence labels the Scg2a/SNa-ir. Red fluorescence labels the Oxt-ir. Yellow fluorescence shows the merge of green fluorescence and red fluorescence. N=20

2.4 Discussion

Specific Scg2a/SNa-ir was observed in the olfactory bulbs, ventral telencephalon, preoptic area, hypothalamus and pituitary of zebrafish. There were no obvious differences in the distribution of this immunoreactivity between males and females.

In the olfactory bulb, Scg2a/SNir located in the GL and POF. Similar results have been reported for goldfish using the same antibody (Canosa, Lopez et al. 2011). The rat olfactory bulb of rats was also observed containing the SN-ir cells(Leitner, Kaufmann et al. 1997). In the olfactory bulb of zebrafish, olfactory sensory neurons (OSN) in POF express a unique odorant receptor protein and project to the GL (Sato, Miyasaka et al. 2007, Braubach, Fine et al. 2012). In the zebrafish GL the lateral glomeruli are associated with feeding behavior (Wakisaka, Miyasaka et al. 2017), medial glomeruli are related to kin recognition (Gerlach, Tietje et al.

2019), while courtship behavior is controlled by ventromedial glomeruli (Yabuki, Koide et al. 2016). From previous *in situ* hybridization studies in our lab, *Scg2a* mRNA is expressed higher in external cellular layer (ECL) rather than in GL and POF (Mitchell 2018) while in the current study Scg2a/SNa-ir was not observed in the ECL. Given the distribution of SNa-ir throughout the GL and POF it is conceivable that Scg2a/SNa plays a role in feeding and reproductive behaviors by regulating the OSN in zebrafish.

In telencephalon, Scg2a/SNa-ir fibres were observed in the dorsal (Vd) and ventral (Vv) regions. Similarly, moderate to high density SNa-ir was observed in Vv and supracommissural part of the ventral telencephalon (Vs) of the goldfish, Electrolytic lesions in the Vs-Vv region impaired reproductive behavior in male goldfish by blocking the initiation of spawning (Kyle and Peter 1982, Kyle, Stacey et al. 1982), further implicating SNa in reproductive processes. High density Scg2a/SNa-ir was noted in Vv region (Fig 2.2C). Previous research from our lab had confirmed that telencephalon expresses reproductive genes in goldfish (Lado, Zhang et al. 2013). The existence of Scg2a/SNa-ir in Vv suggested that SNa may regulated reproduction by inducing the reproductive gene expression in Vv.

The most prominent Scg2a/SNa-ir was located in cells bodies in the preoptic area (POA). The POA includes magnocellular and parvocellular neurons that produce various reproductive neuropeptides and especially isotocin and vasotocin (Dulac, O'Connell et al. 2014). Immunoreactivity of Scg2a/SNa was shown in the cell bodies in PM. These Scg2a/SNa-ir positive fibers appear to project into and traverse the Hv before innervating the pituitary. The colocalization of SN peptides and the nonapeptides in neurons projecting to the NIL is a highly conserved feature in the vertebrate brain. This is supported by immunoelectron microscopy studies indicating that SN and oxytocin are co-stored in the large dense core vesicles of hypothalamo-neurohypophysial neurons and *Oxt* and *Scg2* mRNAs are coregulated in the lactating rat hypothalamus (Mahata, Mahata et al. 1993). It is therefore likely that SN is co-released with Oxt. Indeed, SN is found in human blood (Myhre, Ottesen et al. 2021). We previously described distribution of colocalized Scg2a/SNa and Oxt in the goldfish POA and pituitary (Canosa, Lopez et al. 2011) which is very similar to what we have observed in the zebrafish. Moreover, Scg2a/SNa colocalizes with vasotocin (fish equivalent to mammalian

vasopressin) and Oxt in cells and fibers of the preoptic area of the weakly electric fish *Brachyhypopomus gauderio* (Pouso, Quintana et al. 2015), further supporting data from the rat and goldfish.

The colocalization of Scg2a/SNa with the nonapeptide, and in particular Oxt strongly implicates SN in the hormonal control of reproduction (Trudeau, Martyniuk et al. 2012, Trudeau 2022) and in sociosexual behaviors (Saito, Hasegawa et al. 2003, Godwin and Thompson 2012, Pouso, Quintana et al. 2015). Both Oxt (Mennigen, Volkoff et al. 2017) and SNa (Mitchell, Mikwar et al. 2020) stimulate Lh release in goldfish. Male zebrafish treated with an oxytocin receptor antagonist exhibit reduced spawning success(Altmieme, Jubouri et al. 2019). Importantly, TALEN-mediated frameshift mutations of the zebrafish *Scg2a* precursor gene led to decreased expression of *oxt* in telencephalon and hypothalamus that was accompanied by significant decline in pituitary *lhb* subunit mRNA and lowered spawning success (Mitchell, Zhang et al. 2020).

High density Scg2a/SNa-ir was found in Hc around the posterior recess (PR), which is the caudal most extension of the diencephalic ventricle along the midline. These cells might produce dopamine (Pierre, Mahouche et al. 1997) or serotonin (5HT) (Kah and Chambolle 1983). The Scg2a/SNa-ir cells around the PR resemble tyrosine-positive catecholaminergic cells (Ma 2003), and their general morphology corresponds to 5HT neurons in goldfish (Kah and Chambolle 1983). Retrograde tracing studies indicate that cells around the PR in goldfish do not project to the pituitary and are thus not hypophysiotropic (Anglade, Zandbergen et al. 1993).

Fibres expressing Scg2a/SNa entered the pituitary via the infundibular stalk, projecting heavily to the NIL and PPD. In contrast to mammals, teleosts do not have a median eminence, and rather, a multitude of hypophysiotropic neurons directly innervate the anterior pituitary regions (Trudeau and Somoza 2020). In the rat, high levels of SN are found in the median eminence (Marksteiner, Kirchmair et al. 1993) and both anterior and posterior pituitary (Leitner, Kaufmann et al. 1997). In the zebrafish, the majority of fibres and nerve terminals in the pars nervosa were Scg2a/SNa positive Oxt neurons projecting from the POA. Very few fibres containing both Scg2a/SNa and Oxt were found in the RPD and PPD. However, Scg2a/SN-ir appeared as small intense boutons in close proximity to the gonadotrophs. It suggests that

some Lh and Fsh cells likely receive innervation by Scg2a/SNa producing neurons. Our result indicating that zebrafish gonadotrophs did not exhibit Scg2a/SNa-ir was also supported by the study in goldfish (Zhao, Basak et al. 2009). In contrast, several non-gonadotroph cells in the RPD, PPD, and PI displayed Scg2a/SNa-ir but their identities remain unknown.

Somatolactotropes and melanotropes can be found in the NIL (Sueiro, Carrera et al. 2007), and PRL as well as ACTH is found in the RPD (Laiz-Carrion, del Mar Segura-Noguera et al. 2003) so it will be important to determine which if any of these cells may produce Scg2a/SNa. Earlier *in situ* hybridization experiments revealed strong scg2a expression in the NIL (Mitchell 2018), supporting a local endocrine source of Scg2a/SNa in the pituitary. In goldfish, lactotrophs of the RPD are SNa-positive, and under the stimulation of Gnrh produce SNa that likely diffuses to the PPD to stimulate Lh cells by a paracrine mechanism (Zhao, Grey et al. 2010). It is known that SNa increases calcium entry and stimulates Lh release directly from dispersed goldfish pituitary cells (Zhao, Basak et al. 2009). Additionally, mouse SN activates the protein kinase A (PKA) and cAMP-induced ERK signaling pathways in the Lh-secreting LβT2 pituitary cell line (Zhao, McNeilly et al. 2011). Scg2a/SNa call be delivered to gonadotrophs both via neuroendocrine and intrapituitary paracrine pathways. Given the wide distribution of Scg2a/SN-ir in the pituitary other cell types are likely regulated by SNa. A 24-hr incubation with goldfish SNa significantly increased 565 transcripts and decreased 217 transcripts in dispersed pituitary cells *in vitro* (Trudeau, Martyniuk et al. 2012). At that time, a total of 308 of these differentially expressed genes were mapped to human homologs. The identified genes in the pituitary are likely involved in metabolic processes, transcription, signal transduction, membrane and ion transport, apoptosis, development, and phosphorylation and were affected by SNa.

In summary, we investigated the distribution of Scg2a/SNa in brain and pituitary in relation to other known reproductive hormones in normal and transgenic zebrafish by double immunofluorescent staining. Conspicuous Scg2a/SNa-ir was observed in neuronal cell bodies in the ventral telencephalon, POA and hypothalamus in female brain. Neuronal fibers staining for Scg2a/SNa projected from the magnocellular POA, passed through the pituitary stalk and terminated largely in the NIL. Some Scg2a/SNa-ir fibers were less abundant but clearly present

in the pars distalis. Moreover, Scg2a/SNa colocalized with Oxt in cell bodies in the POA and fibers in the NIL. Using the *lhb*-RFP x *fshb*-eGFP transgenic zebrafish line, we observed SNa-ir near gonadotrophs but not in them. Counterstaining nuclei with DAPI revealed that some endocrine cells within the NIL and RPD also expressed Scg2a/SNa-ir, but their identity is currently unknown. In situ hybridization confirmed that magnocellular neurons and cells in the NIL are sites for Scg2a/SNa production (Mitchell 2018). These data indicate that SNa or related peptides produced from the Scg2a precursor protein may affect gonadotrophs through neuroendocrine and paracrine pathways. These observations support the hypothesis that Scg2a/SNa is involved in reproductive processes.

Chapter 3 Sex differences in reproductive neuropeptides and steroid hormones in zebrafish

3.1 Introduction

The hypothalamus-pituitary-gonad (HPG) axis plays a key role in gonadal development as well as oogenesis and spermatogenesis. The reproductive hormones include not only the classical pituitary glycoprotein hormones and gonadal steroids but also an array neuropeptides and neurotransmitters implicated in the control of behaviours and pituitary hormone release (Trudeau 2022). Neuropeptides are produced in various isoforms by the multiple processing of protein precursors by a variety of prohormone convertases (Fricker 2012). Neurotransmitters are small molecular weight chemical messengers often derived from enzymatic conversion of amino acids (Fricker 2012). Both neuropeptides and neurotransmitters are stored in secretory vesicles and upon neuronal depolarization released into the synaptic cleft and exert their effects on post-synaptic receptors. In fish as in all vertebrates they have critical roles in the neuroendocrine reproduction. These include but are not limited to the neuropeptides Gnrh, kisspeptin, the nonapeptides, and SN, and the neurotransmitters dopamine, glutamate and gamma-aminobutyric acid (Trudeau 2021). Here we focus on neuropeptide and gonadal steroid production in the HPG axis of female and male zebrafish.

Different with sexual dimorphism which a characteristic has two differing forms in the sexes. In contrast, sex differences are endpoints that exist in both sexes on a continuum and the average is different between males and females (McCarthy, Arnold et al. 2012). There are major sex differences in both reproductive and non-reproductive physiology across the vertebrates. The impacts of sex are wide-ranging, yet the lack of studies on females relative to males was so pervasive that numerous research organizations instigated a requirement to examine sex differences (McCarthy, Arnold et al. 2012). For example, only 10 years ago did the U.S. National Institute of Mental Health convene the first workshop titled “Sex Differences in Brain, Behavior, Mental Health and Mental Disorders” on this issue. The workshop concluded: (1) that there is a paucity of research examining sex differences at a neurobiological and

mechanistic level; (2) there are pervasive sex differences in the brain, and (3) there is a need to incorporate sex as a variable in experimental designs (Health 2011).

While some typical sex hormones are well known, for example, the levels of circulating gonadal steroids, many others are not well-studied with the explicit goal of comparing females to males. In fish, there are few studies critically comparing the production of neuropeptides and gonadal hormones in brain and pituitary. Zebrafish has been a successful experimental model for human diseases including leukemia, obesity, and influenza (Bradford, Toro et al. 2017) as well as environmental toxicology (Fitzgerald, Konemann et al. 2021) and reproduction (Hoo, Kumari et al. 2016). In our study, the zebrafish were used for reproductive studies to investigate the new neuropeptide SN. Therefore, the sex differences of the reproductive hormones including SN in zebrafish were characterized in detail using mass spectrometry.

Gonadotropin-releasing hormone (Gnrh) is a 10-amino acid neuropeptide discovered 50 years ago (Zohar, Zmora et al. 2021). In the case of zebrafish and related cyprinids, they produce Gnrh2 and Gnrh3 (Munoz-Cueto, Zmora et al. 2020). The Gnrh peptide is produced in preoptic- hypothalamic neurons that sent their projections to the median eminence in tetrapods, but directly innervate the anterior pituitary in teleosts who have lost the median eminence over the course of evolution. The decapeptide activates well-characterized Gnrh receptors on the gonadotrophs in the pituitary to stimulate the expression and secretion of two types of gonadotropins Lh and Fsh (Somoza, Mechaly et al. 2020). Fsh stimulates the growth of granulosa cells in ovaries as well as that of Sertoli cells in testes to induce the development of oocytes and spermatogonia (Levavi-Sivan, Bogerd et al. 2010, Xie, Chu et al. 2017). As for Lh, it stimulates the maturation of follicles and the ovulatory process in females as well as the maturation of sperm in males (Levavi-Sivan, Bogerd et al. 2010, Xie, Chu et al. 2017).

The nonapeptides are members of an ancient neuropeptide family that is highly conserved across phyla and arose through the duplication of an ancestral vasotocin gene (Acher and Chauvet 1995). In teleosts, there are two nonapeptides, isotocin and vasotocin. Elegant studies in the midshipman fish revealed that vasotocin is involved in the vocalization patterns of females in courtship behavior while isotocin regulated the vocalization patterns of the males

(Goodson and Bass 2000). Moreover, isotocin injection stimulates Lh release and estradiol production in female goldfish (Popesku, Mennigen et al. 2011).

Kisspeptin is produced as a 145 amino acid pro-hormone which is cleaved into short mature peptides with 54, 14, 13, and 10 amino acids (Pinilla, Aguilar et al. 2012). In teleosts, there are two types of kisspeptin, named as Kiss1 and Kiss2 (Kitahashi, Ogawa et al. 2009, Kanda, Karigo et al. 2012). In zebrafish, the mutation of *kiss* gene did not impact the reproduction(Tang, Liu et al. 2015). But other studies indicates that Kiss is involved in the food intake and reproduction with Gnrh in teleosts (Somoza, Mechaly et al. 2020). In mammals, kisspeptin is indispensable to stimulate the release of Gnrh that in turn regulates the Lh secretion and gonadal function (Shahab, Mastronardi et al. 2005, Matsuda, Ohkura et al. 2019).

Secretoneurin (SN) is 31-42 amino-acid neuropeptide which is derived from the selective processing of a Scg2 precursor. Two isoforms of SN are predicted to be produced in teleost, named SNa and SNb, respectively (Mitchell, Mikwar et al. 2020). Results from our previous study indicates that SNa may be functional in controlling reproductive hormone release. Zhao et al. demonstrated that co-incubation with anti-SNa antiserum reduces the stimulatory effect of Gnrh3 on Lh release by 64% while Gnrh3 can also increase the expression of *scg2a* mRNA in goldfish (Zhao, Grey et al. 2010). This study also shows that SNa can upgrade the gene expression of Cgα-, Fshβ-, and Lhβ-subunits (Zhao, Grey et al. 2010). These data suggested that SNa may control reproduction by interaction with Gnrh, Lh and Fsh.

The gonadal sex steroids are the classical reproductive hormones in the vertebrates. Steroidogenesis in the gonads is regulated by the gonadotropins, thereafter exerting both positive and negative feedback on the hypothalamus and pituitary (Wierman 2007). Both of androgens and estrogens control reproductive behavior in vertebrates. In mice, for example, a rapid surge of T was detected at the copulatory behavior in males (James and Nyby 2002). In the Gulf toadfish study, the exogenous 11-KT induced the call rate to repel males and attract females (Remage-Healey and Bass 2005). As for E2, it stimulates singing behavior in birds (Remage-Healey, Coleman et al. 2010, Cornil, Seredynski et al. 2013). Moreover, estrogens are not only produced in ovaries and have effects in females involved in the vitellogenesis and oocyte maturation but are also indispensable in male reproduction (Nagahama, Yoshikuni et al.

1995, Okumura, Todo et al. 2002, Dammann, Shappell et al. 2011, Hess and Cooke 2018). Similarly, androgens are involved in female reproduction. Testosterone is not only produced and converted to E2 by aromatase in brain and ovaries, but exogenous T treatment also improves the sexual activity of female zebrafish (Diotel, Le Page et al. 2010, Liu, Yue et al. 2021).

In this chapter, we confirmed the amino acid sequences of SNa and SNb in zebrafish by targeted peptidomic analysis. This result offered the foundational evidence of endogenous SNa and SNb peptide synthesis and quantitative analysis of the SNa and SNb levels in zebrafish tissue samples by mass spectrometry. The results of untargeted peptidomics analysis revealed the existence of multiple shorter fragments of the SNa and SNb peptides. A hypothesis is proposed that some of the SNa1-34 and SNb1-31 fragments have biological activity. Moreover, other neuropeptides including Gnrh, nonapeptides and kisspeptin as well as sex steroids involved in zebrafish reproduction were detected by nano HPLC-MS and significant sexual differences was observed.

3.2 Materials and methods

3.2.1 Experimental animal husbandry

Zebrafish (AB strain) were bred and kept in the University of Ottawa Aquatics core facility at the temperature of 28°C. The lights-on time was set for 14 hours from 0900h to 2300h. All animal experiments followed the guidelines of the Canadian Council on Animal Care for the use of animals in research, approved by the University of Ottawa Animal Care Protocol Review Committee. Sexually mature zebrafish at the age of 6-9 months post-fertilization (mpf) were selected and separated by sex at least two weeks to synchronize cycles, and to eliminate possible acute influences of one sex on the other through pheromones or tactile interactions. All animals were dissections between 10:00-11:00. This is outside the normal nighttime periovulatory period to avoid possible variations due to ovarian cyclicity. Periovulatory changes in adult females in hormone levels are documented in Chapter 4.

3.2.2 Dissection and homogenization

The zebrafish were anaesthetized in 4.5% tricaine buffer before dissection. After dissection, brain, pituitary, and gonads were collected, placed in a homogenization buffer on ice and homogenized by ultrasonic homogenizer (Fisherbrand; Cat# FB120110) 3-5 sec. Before homogenization, the gonads were dissected and weigh for calculating the gonadosomatic index (GSI; gonads weight/total body weight*100) while brains were also dissected and weigh to calculate the brain-to-body mass ratio (brains weight/total body weight*100). The pituitaries were too tiny to weigh accurately. The homogenized samples were centrifuged at 4°C in 13,200 rpm for 10 min. After that, the supernatant was transferred into a new 1.5ml EP tube and dehydrated with the pellet in centrifugal vacuum concentrator (Labconco; Cat#7810014) at 45°C. Dehydrated samples were stored in -20°C.

3.2.3 Solid phase extraction (SPE)

The dehydrated supernatant samples were thawed from -20°C at room temperature and resuspended in loading buffer and subjected to SPE in a 96-well format. All details of the extraction are being considered for intellectual property protection and are in the thesis of my collaborator C. Lu. After collections the purified samples, the collection plate was transferred in the vacuum dried to be dehydrated and stored in -20°C till mass spectrometry detection.

3.2.4 Peptidomic analysis

In this section, we performed the targeted peptidomic analysis and untargeted peptidomic analysis. Targeted peptidomics was performed to confirm the predicted amino-acid sequence of SNa1-34 and SNb1-31 peptides when detected with standard compounds synthesized from our lab. To confirm the peptide sequences, we identified 5 of the most intense product ions (MS2) of standard peptides in the analyte samples for the same parent ion (MS1) at the same retention time with 30s detection window. Untargeted peptidomic analysis was used to identify the existence of SNa and SNb fragmental peptides in wildtype zebrafish samples.

Before loading in the HPLC-MS, the dehydrated samples were thawed at room temperature and resuspended in loading buffer. For targeted peptidomics, synthetic standard

peptides spiked into zebrafish sample were loaded together with experimental samples. The peptidomic analyses were done by HPLC-ESI-MS/MS. The system consisted of an Agilent 1100 micro HPLC system (Agilent Technologies, Santa Clara, CA, USA) coupled with an LTQ-Orbitrap mass spectrometer (ThermoFisher Scientific, San Jose, CA) equipped with a nano-electrospray interface operated in positive ion mode. The MS method consisted of one full MS scan from 300 to 1700 m/z followed by data-dependent MS/MS scan of the 5 most intense ions, with dynamic exclusion repeat count of 2, and repeat duration of 90s. As well, for the experiments on the Orbitrap MS the full MS was in performed in the Orbitrap analyzer with R=60000 defined at m/z 400, while the MS/MS analysis were performed in the LTQ. To improve the mass accuracy, all the measurements in Orbitrap mass analyzer were performed with internal recalibration (Lock Mass). On the Orbitrap, the charge state rejection function was enabled, with single and "unassigned" charge states rejected.

The raw files generated by the LTQ-Orbitrap were processed and analyzed using MaxQuant, Version 1.2.2.5 using the Uniprot protein FASTA database (2012, July version), including commonly observed contaminants. The following parameters were used: methionine oxidation. protein N-terminal acetylation, and no enzyme digestion. Precursor ion mass tolerances were 7 ppm, and fragment ion mass tolerance was 0.8 Da for MS/MS spectra. The false discovery rate (FDR) for peptide and protein was set at 1% and a minimum length of six amino acids was used for peptides identification. Quantification was performed using normalized LFQ intensity.

3.2.4 LC-MS analysis

The LC-MS system is Nano flow LC-MS/MS analysis system driven by an Agilent 1100 capillary liquid chromatography system (running on micro mode) and a Thermo Fisher LTQ Velos Pro Orbitrap Elite mass spectrometer (located in D. Figeys' lab, OHRI). This was operated by my collaborator C. Lu (Trudeau lab). The nano-HPLC was performed on narrow columns with 20-100 μm ID, and flow rates can range between 50-500 nL/min. During the analysis, the analytes in samples were loaded to a C18, 100μm×35mm, 5μm, 120Å, trapping column then separated by a C18, 50μm×150mm, 3μm, 100Å analytical column. LC elution is introduced into the mass analyzer by 2.5kV nano-electrospray ionization through a spray tip with a 1μm

opening. Through the entire analysis, the Fourier Transform mass analyzer is set to collect m/z information between 150 m/z and 1,500 m/z on scan mode under mass resolution 60,000. The LC-MS result files are processed by Xcalibur software (Thermo Xcalibur 2.2 SP1. Build 48). Target compounds are identified by retention time (RSD≤2.5%), MS1 (10 ppm mass accuracy), and MS2 (0.5 m/z mass accuracy). Chromatograms were plotted based on the MS1 mass intensity with 20 ppm mass accuracy. Peak area is used to calculate the concentration of analytes in the original sample with an external standard curve.

3.2.5 Statistical analysis

Statistical analysis was performed by GraphPad Prism 9 software (GraphPad Software Inc.). The normality and lognormality of the data was analyzed by Shapiro-Wilk test. The non-parametric two-tailed Mann-Whitney test was used to analyze the sex difference of peptides in samples. Information about the presentation of Mann-Whitney test and its associated statistics are described in each figure legend. Both GSI and the brain-to-body mass ratio of the zebrafish are presented as Mean ± SD.

3.3 Results

3.3.1 Sex differences in the levels of reproductive neuropeptides in zebrafish.

We quantified sex differences in the well-known neuropeptides involved in reproduction, including Gnrh2, Gnrh3, isotocin, vasotocin, Kiss1 and Kiss2. We also analyzed the GSI and the brain-to-body mass ratio in both males and females. The GSI in males was 1.09±0.02 (n=12), ranging from 0.84 to 1.27 while it in females was 14.46 ±11.82 (n=12), ranging from 10.01 to 22.21. As for the brain-to-body mass ratio, it was 1.00±0.08 in females (n=12), ranging from 0.71 to 1.44 and it was 1.63±0.03 in males (n=12), ranging from 1.32 to 1.89. However, zebrafish pituitary was too small to weigh accurately.

There was a significant sex difference in Gnrh2 in the pituitary but not in brain and ovaries (Fig 3.1A-C). Pituitary Gnrh2 levels were 2.2-fold higher in females than that in male ($p<0.05$) (Fig 3.1B). The mean values of the pituitary Gnrh2 in females was 16.23 fmol/tissue while that in males was 7.53 fmol/tissue. In the female brain, Gnrh3 were 3.1-fold higher than that in the male ($p<0.01$). The mean values of Gnrh3 were 683.9 fmol/tissue in female and 219

fmol/tissue in male (Fig 3.1D). There were no sex differences evident in pituitary Gnrh3 (Fig 3.1E). Ovarian Gnrh3 levels were 3.1-fold higher than the testes ($p<0.01$) (Fig 3.1F). The mean values of Gnrh3 in ovaries and testes were 143.8 fmol/tissue and 45.82 fmol/tissue, respectively.

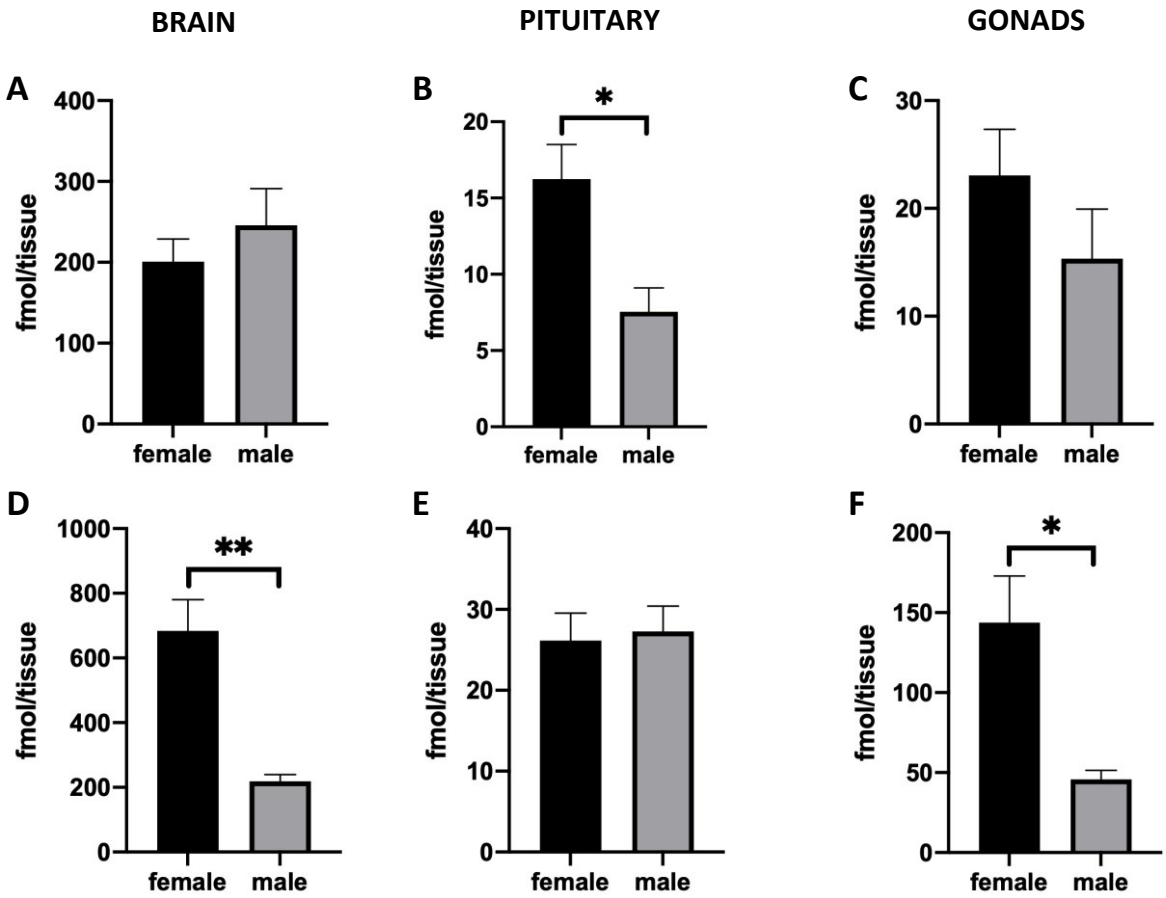

Fig 3.1 Differences in the levels of Gnrh2 and Gnrh3 in female and male zebrafish. Gnrh2 in brain (A), pituitary (B), gonads (C). Gnrh3 in brain (D), pituitary (E), gonads (F). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. *$p\leq0.05$ **$p\leq0.01$

Isotocin levels in the brain were 1.9-fold higher in female than that in male with the mean value of 1,728 fmol/tissue and 992 fmol/tissue, respectively ($p<0.05$) (Fig3.2A). On the other hand, male brain vasotocin had a mean value 109.7 fmol/tissue, which was 1.8-fold higher than that in female with the mean value of 195.4 fmol/tissue ($p<0.05$) (Fig3.2D). Isotocin

and vasotocin in the female in pituitary were respectively 2-fold ($p<0.05$) (Fig3.2B) and 2.2-fold higher ($p<0.01$) than in males (Fig3.2E). The mean value of isotocin and vasotocin were 40,961 fmol/tissue and 1,405 fmol/tissue in female while those in male were 20,335 fmol/tissue and 642.6 fmol/tissue separately. Isotocin levels in ovaries was 285.2 fmol/tissue which was 1.4-fold higher than that in testes ($p<0.01$) (Fig3.2C), while gonadal vasotocin levels were similar between the sexes.

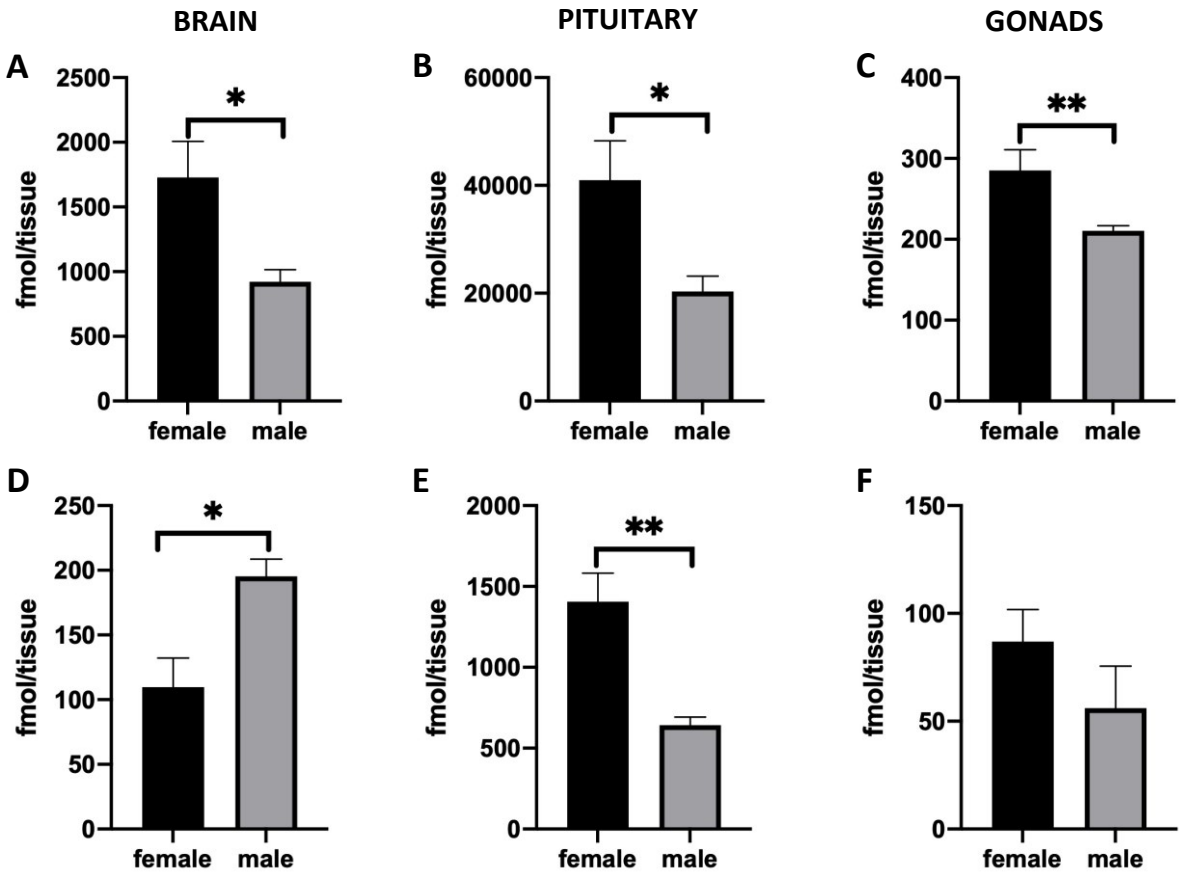

Fig 3.2 Differences in isotocin and vasotocin levels in female and male zebrafish. Isotocin in brain (A), pituitary (B), gonads (C). Vasotocin in brain (D), pituitary (E), gonads (F). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. *$p \leq 0.05$ **$p \leq 0.01$

Sex differences in the levels of Kiss1 but not Kiss2 were evident. In brain, the Kiss1 levels in males was 2.3-fold higher than that in females. Mean values of brain Kiss1 in females and males were respectively 32.63 fmol/tissue and 76.67 fmol/tissue ($p<0.05$) (Fig3.3A). There were no sex differences in Kiss1 in pituitary and gonads (Fig3.3B&C).

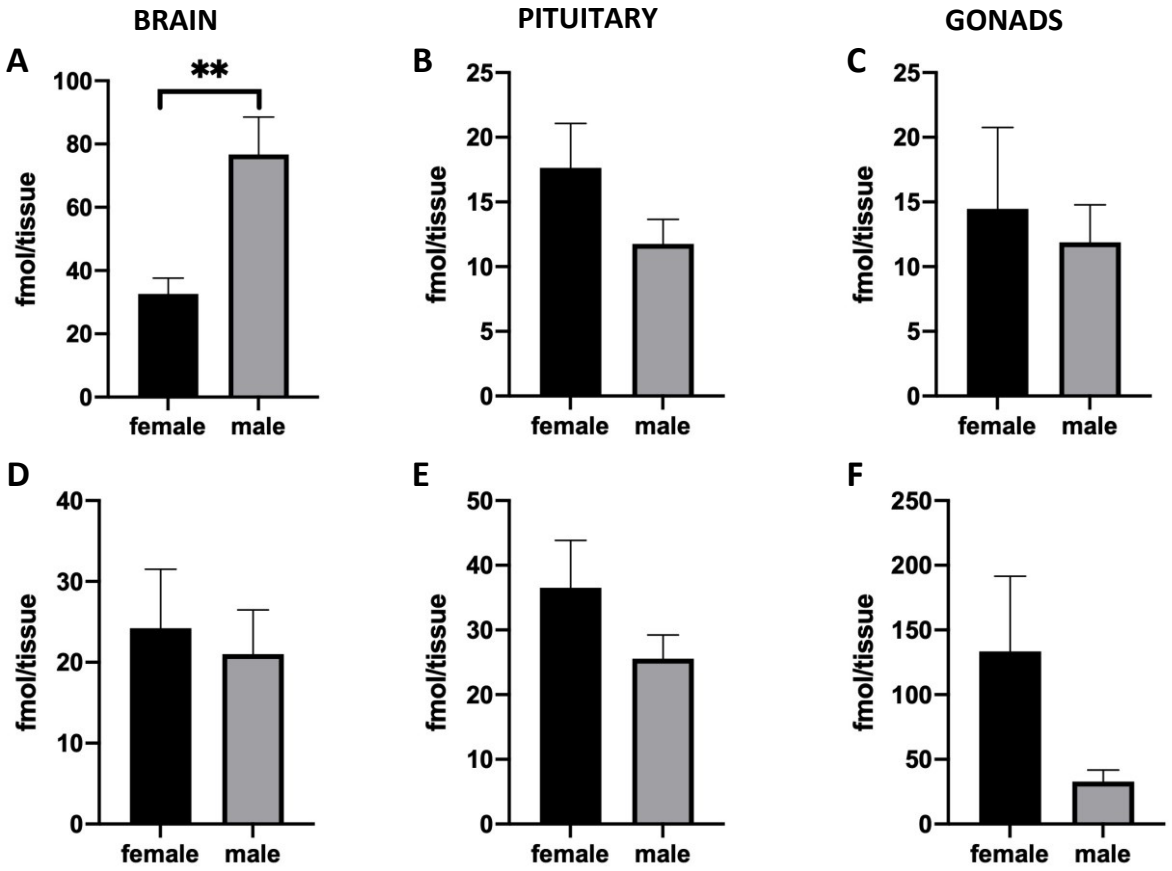

Fig 3.3 Differences in KISS1 and KISS2 levels in female and male zebrafish. KISS1 in brain (A), pituitary (B), gonads (C). KISS2 in brain (D), pituitary (E), gonads (F). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **$p \leq 0.01$

3.3.2 Amino acid sequences of SNa and SNb and related fragments

In this section, the predicted amino acid sequences of SNa1-34 and SNb1-31 derived from ZFIN database (https://zfin.org/) entries for Scg2a (ZDB-GENE-030131-8214; Chromosome 15) and Scg2b (ZDB-GENE-061103-160; Chromosome 2) were confirmed by targeted peptidomic analysis. They are highlighted in bold in Table 3.1. Moreover, we also identified by

untargeted peptidomics the existence of 6 fragmental peptides for SNa and 17 for SNb in the brain and pituitary samples in zebrafish. When comparing our peptidomic results with that in other research, we found that some fragments including SNb1-17, SNb19-31 were also detected by other groups study zebrafish (Van Camp, Baggerman et al. 2017). Moreover, similar fragmental peptides with similar cleavage sites were showed frequently in various research in mammals, including SNa1-14, SNa1-18, SNa19-34 (Che and Fricker 2005, Pan, Che et al. 2006, Boonen, Baggerman et al. 2007, Zhang, Che et al. 2008, Colgrave, Xi et al. 2011). Therefore, we selected these fragmental peptides as the candidates for quantification and assessment of biological activity (Chapter4, 5). The selected peptides were also shown in Table 3.1.

Table 3.1 Identified amino-acid sequence of zebrafish SNa and SNb peptides

Name	Sequence	
SNa1-34	**TNENAEEQYTPQKLATLQSVFEELSGIASSKTNT**	
SNa1-18	TNENAEEQYTPQKLATLQ	
SNa1-14	TNENAEEQYTPQKL	
SNa19-34	SVFEELSGIASSKTNT	
SNb1-31	**ATEDLDEQYTPQSLANMRSIFEELGKLSAAQ**	
SNb1-17	ATEDLDEQYTPQSLANM	Also detected by (Van Camp, Baggerman et al. 2017)
SNb19-31	SIFEELGKLSAAQ	Also detected by (Van Camp, Baggerman et al. 2017)

3.3.3 Sex differences in SNa-related peptides in zebrafish

SNa1-34 was detected in the zebrafish brain, pituitary and gonads in both male and female by HPLC-MS. In each tissue, the amount of SNa1-34 in female zebrafish was significantly higher than that in males. The SNa1-34 in female brain was 3.3-fold more than that in males ($p<0.01$). Mean values of brain SNa1-34 in female and male were 256,577 fmol/tissue and 78,718 fmol/tissue separately (Fig3.4A). While in the pituitary, the level of SNa1-34 in female was 1.4-fold higher than that in male with the mean value was 116,969 fmol/tissue in female and 82,421 fmol/tissue in male ($p<0.01$) (Fig3.4B). In gonads, SNa1-34 in female was 1.5-fold higher than that in male ($p<0.01$). The mean values of SNa1-34 in ovaries and testes were

100,543 fmol/tissue and 65,026 fmol/tissue, respectively (Fig3.4C).

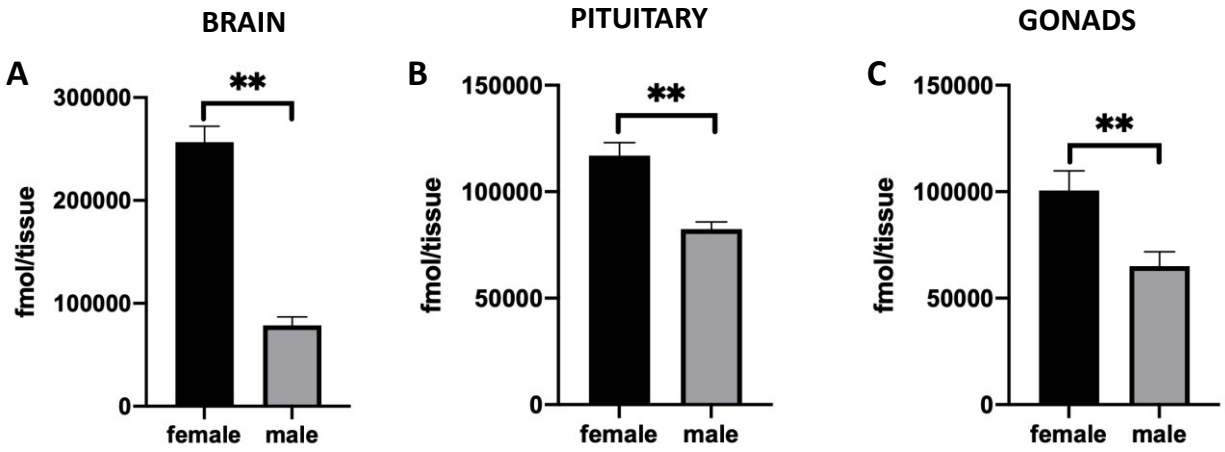

Fig 3.4 Differences in SNa1-34 levels in female and male zebrafish. SNa1-34 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **p≤0.01

Sex differences in SNa fragmental peptides were also evident. In contrast with the abundance of SNa1-34 in females, levels of SNa1-14 and SNa1-18 were significantly higher in males. Although there was no statistical difference of the SNa1-14 levels in brain between male and female, levels of SNa1-14 in pituitary and gonads were higher in males (Fig 3.5). In pituitary, the SNa1-14 level in male was 1.3-fold higher than that in female with the mean value was 1,431 fmol/tissue in female and 1, 920 fmol/tissue in male ($p<0.01$) (Fig 3.5B). Similar results were also noted for gonads. Levels of SNa1-14 in testes were 1.5-fold higher than that in ovaries($p<0.05$) (Fig 3.5C). There were significantly 2-fold, 1.3-fold and 1.6-fold higher levels of SNa1-18 in males than that in females in brain, pituitary and gonads respectively ($p<0.01$) (Fig 3.6). In brain, SNa19-34 levels in male were twice higher than that in female ($p<0.01$) (Fig 3.7A). In contrast, SNa19-34 levels in pituitary and gonads were similar in females and males (Fig 3.7B&C).

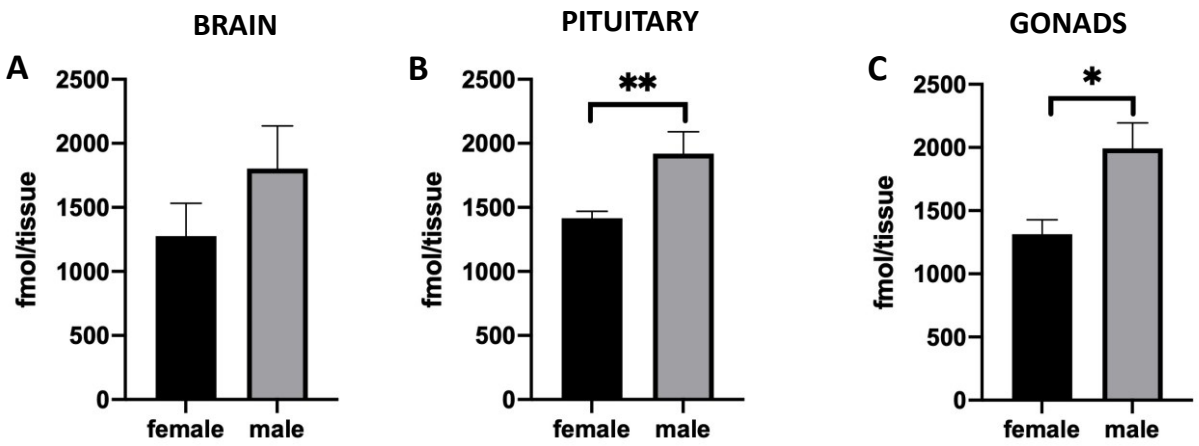

Fig 3.5 Differences in SNa1-14 levels in f emale and male zebrafish. SNa1-14 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. *p≤0.05 **p≤0.01

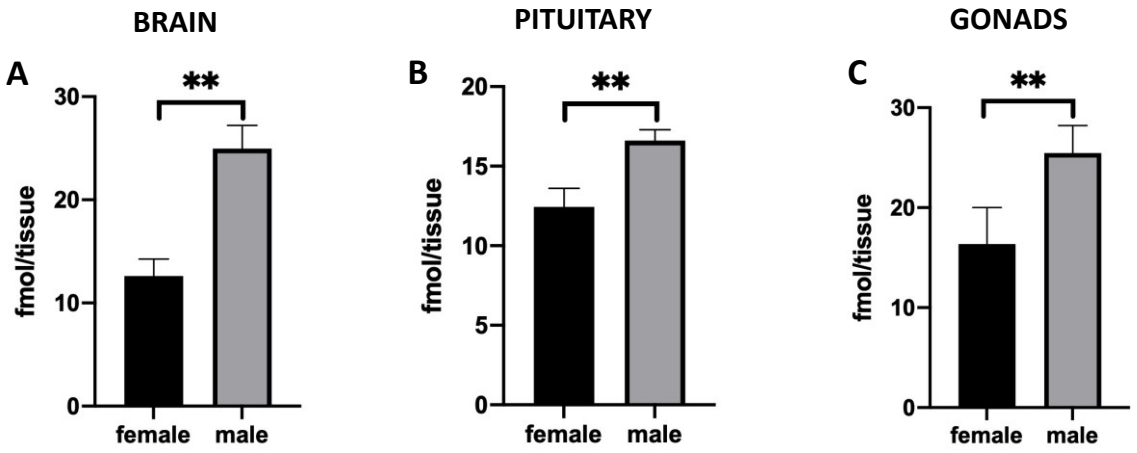

Fig 3.6 Differences in SNa1-18 levels in female and male zebrafish. SNa1-18 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **p≤0.01

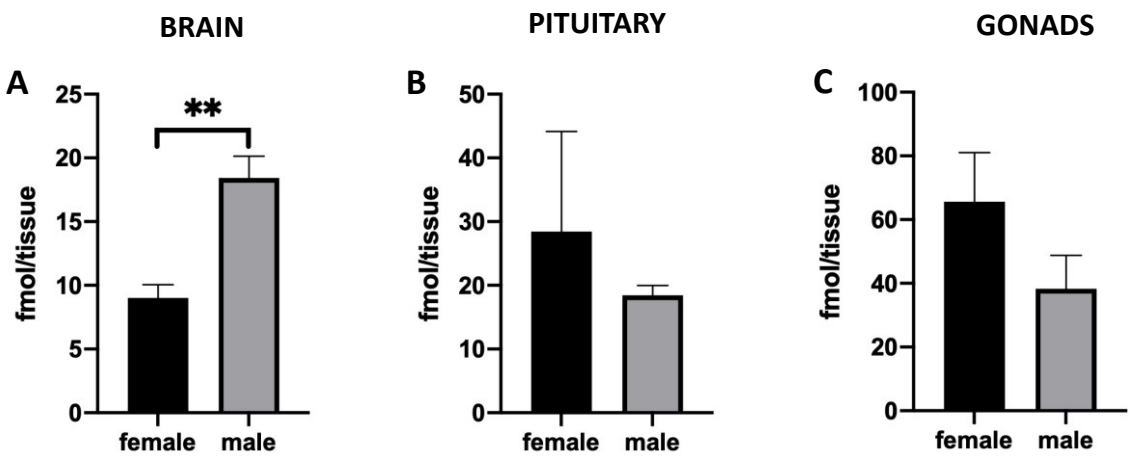

Fig 3.7 Differences in SNa19-34 levels in female and male zebrafish. SNa19-34 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the differe nt scales on the Y-axes. Mann-Whitney tests were performed. **$p \leq 0.01$

We also analyzed the ratio of SNa fragmental peptides to SNa1-34 to investigate potential sex differences in peptide processing. For all tissues both the SNa1-14 to SNa1-34 as well as the SNa1-18 to SNa1-34 ratios were higher in males than females (Fig3.8, Fig3.9). The largest differences were noted for brain and both ratios were 4.3-fold higher in males than females ($p<0.01$) (Fig3.8A, Fig3.9A). Although there were no significant sex differences for the ratio of SNa19-34 to SNa1-34 in the pituitary and gonads, it was 8-fold higher in males than that in females ($p<0.01$) (Fig 3.10).

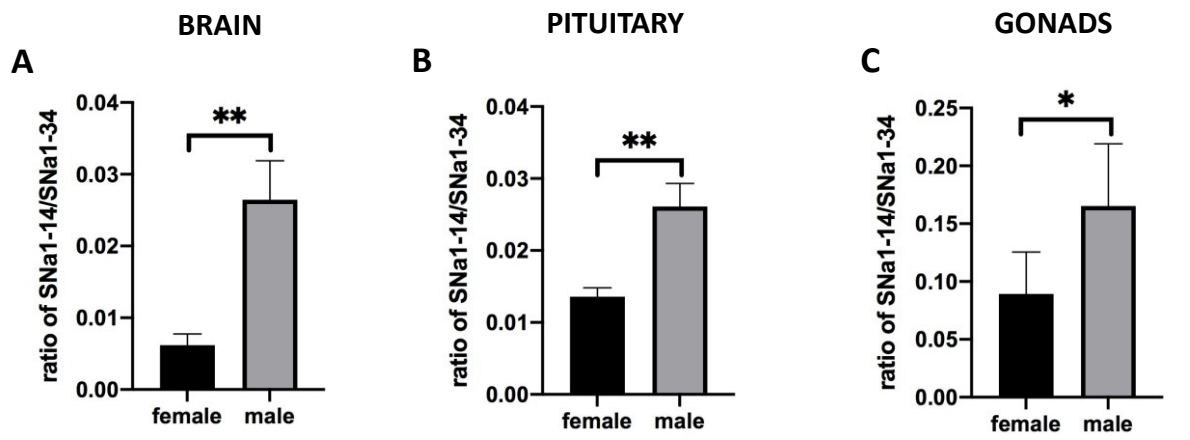

Fig 3.8 Differences in the ratio of SNa1-14 to SNa1-34 in female and male zebrafish. Ratio of SNa1-14 to SNa1-34 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. *p≤0.05 **p≤0.01

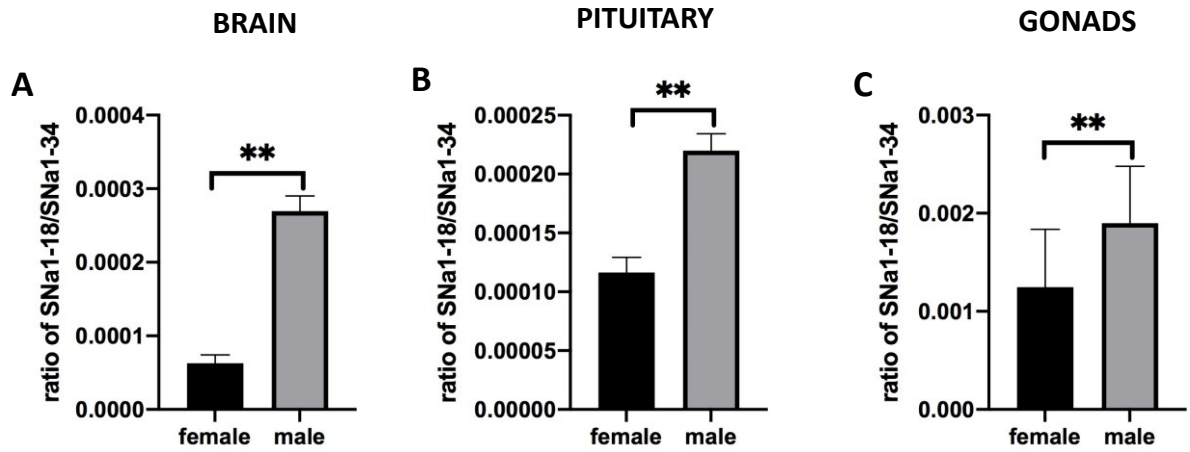

Fig 3.9 Differences in the ratio of SNa1-18 to SNa1-34 in female and male zebrafish. Ratio of SNa1-18 to SNa1-34 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **p≤0.01

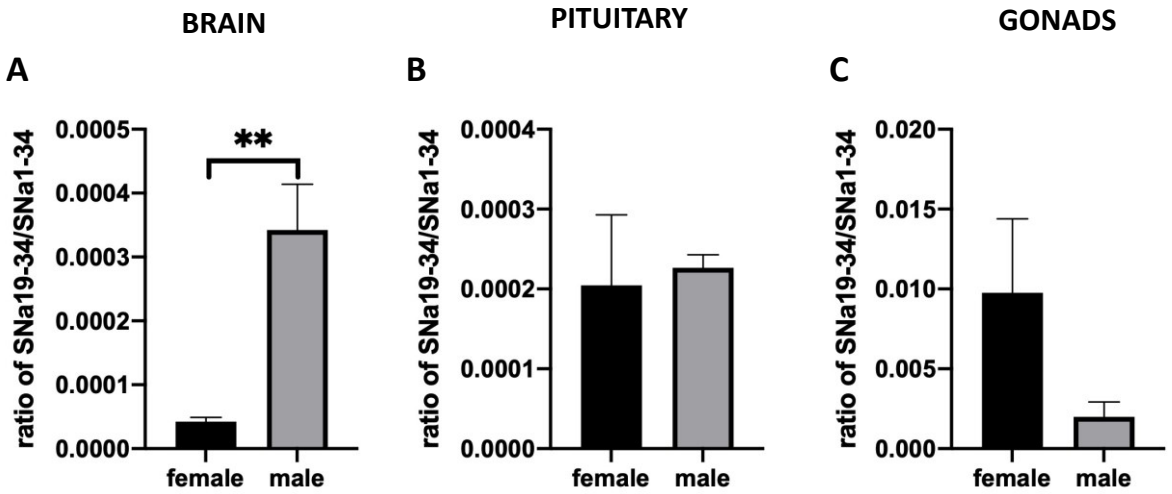

Fig 3.10 Differences in the ratio of SNa19-34 to SNa1-34 in female and male zebrafish. Ratio of SNa19-34 to SNa1-34 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **$p \leq 0.01$

3.3.4 Sex differences in SNb related peptides in zebrafish.

Levels of the SNb1-31 derived peptides levels were detected in the brain, pituitary and gonads of female and male zebrafish by HPLC-MS. There was a significant difference in the concentration of SNb1-31 in female and male. In the brain, the SNb1-31 levels in females were 3.2-fold higher than that in males. Mean values were 193,870 fmol/tissue in females and 60,502 fmol/tissue in males ($p<0.01$). In the pituitary, the SNb1-31 level in females was 4-fold higher than that in males. Mean values were 183,120 fmol/tissue in females and 44, 874 fmol/tissue in males ($p<0.01$). In gonads, the SNb1-31 level in female was 3.7-fold higher than that in males ($p<0.01$). The mean value of SNb1-31 in ovaries was 164,157 fmol/tissue and that in testes was 44,418 fmol/tissue (Fig 3.11).

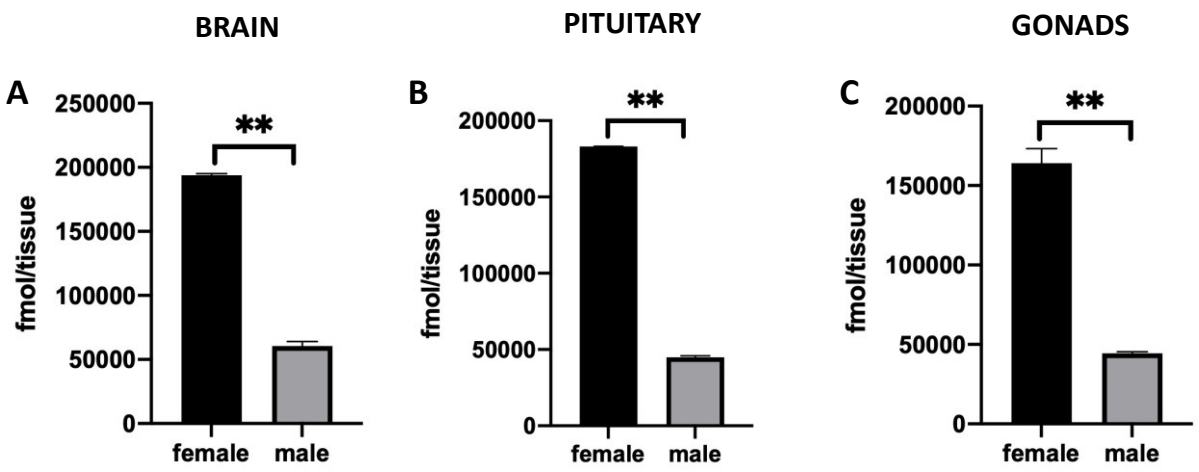

Fig 3.11 Differences in SNb1-31 levels in female and male zebrafish. SNb1-31 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **p≤0.01

The fragmental SNb1-17 and SNb19-31 peptides were detected in brain, pituitary and gonads. Different with SNa fragmental peptides, both SNb1 -17 and SNb19-31 were significant higher in female tissues rather than in males. SNb1-17 levels in female brain were twice higher than that in male brain. Means values of SNb1-17 were 262.9 and 129.4 for the brains of females and males, respectively ($p<0.01$) (Fig 3.12A). SNb1-17 also showed a significant difference in pituitary and gonads: peptides level in female were 1.2-fold higher than that in male (Fig 3.12B&C). As for SNb19-31, level was 2.6-fold higher in female than that in male brain. Means values of SNb19-31 were 129.7 and 50.64 for the brains of females and males, respectively ($p<0.01$) (Fig 3.13A). Moreover, a significant difference was also observed in pituitary ($p<0.01$) and gonads ($p<0.05$): SNb19-31 in females were 1.7-fold and 1.6-fold higher than that in males (Fig 3.13B&C).

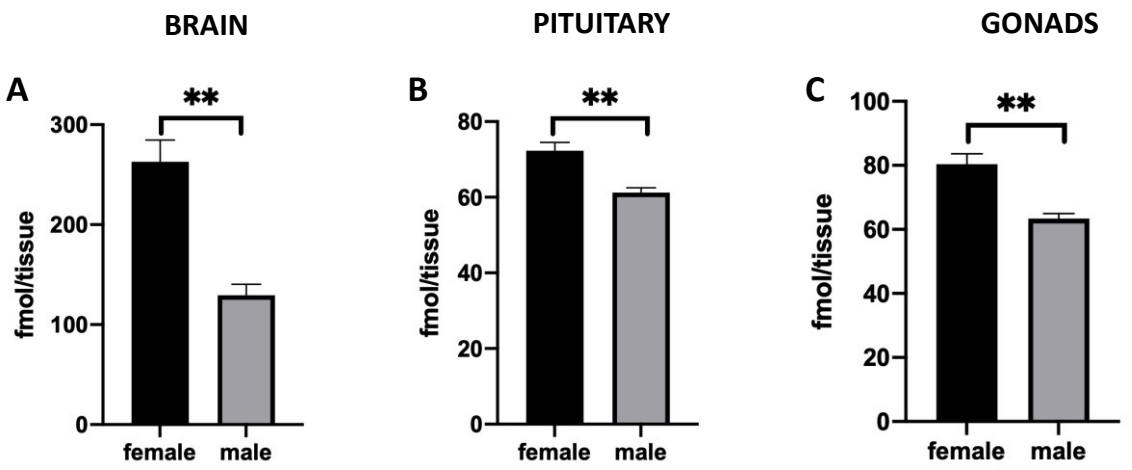

Fig 3.12 Differences in SNb1-17 levels in female and male zebrafish. SNb1-17 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **p≤0.01

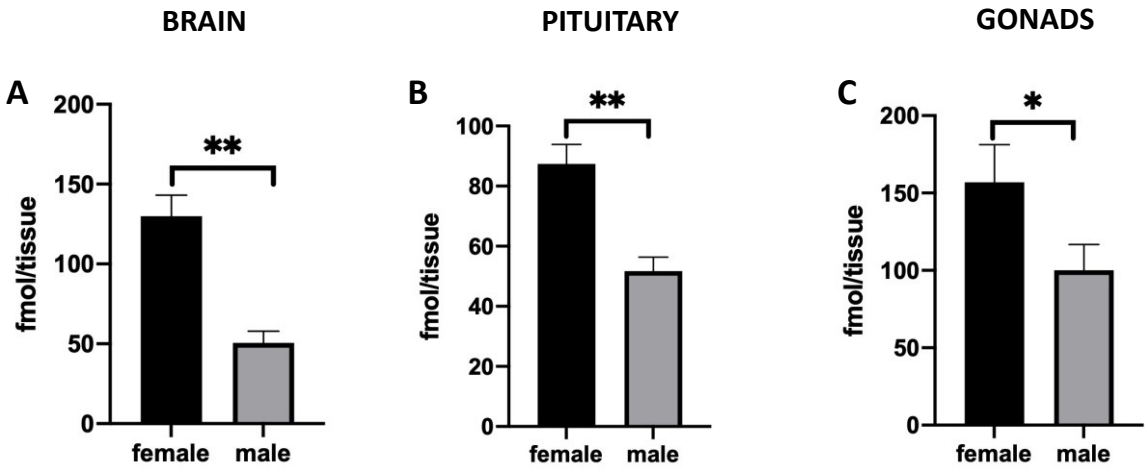

Fig 3.13 Differences in SNb19-31 levels in female and male zebrafish. SNb19-31 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. *p≤0.05 **p≤0.01

Sex differences the ratios of SNb1-17 to SNb1-31 and SNb19-31 to SNb1-31 were also evident in some tissues. In the brain, the SNb1-17 to SNb1-31 was found that the ratio in male was 1.7-fold than that in female ($p<0.01$) (Fig 3.14A). Similarly, the ratio of SNb1-17 to SNb1-31 in male was 3.5-fold higher than that in female in pituitary ($p<0.01$) and 2.3-fold higher than that in female in gonads ($p<0.01$) (Fig 3.14B&C). There was no sex difference in the ratio of

SNb19-31 to SNb1-31 in the brain (Fig 3.15A). In contrast, the SNb19-31 to SNb1-31 ratio was higher in male pituitary ($p<0.01$) and gonads ($p<0.01$) than in females. (Fig 3.15B&C).

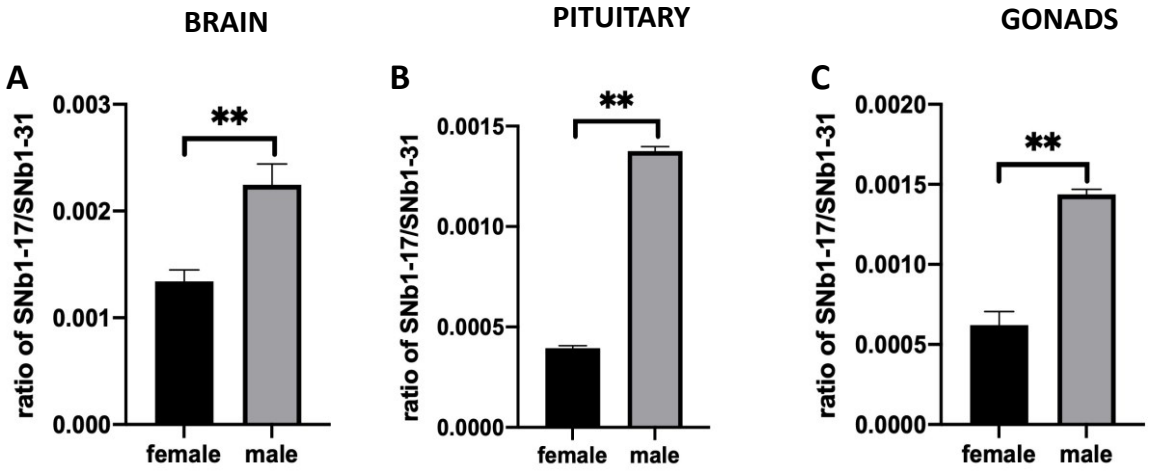

Fig 3.14 Differences in the ratio of SNb1-17 to SNb1-31 between female zebrafish and male zebrafish. Ratio of SNb1-17 to SNb1-31 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **$p \leq 0.01$

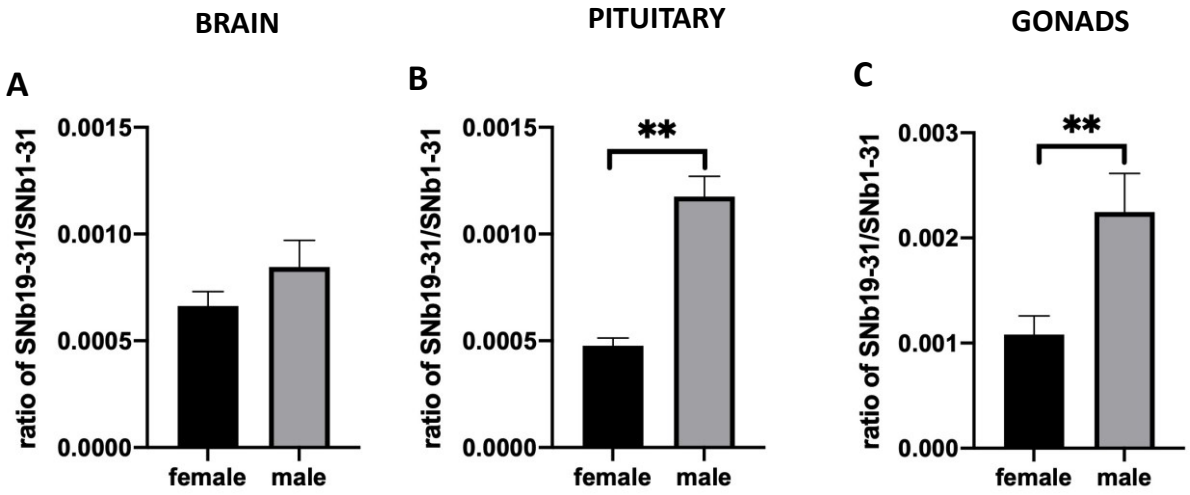

Fig 3.15 Differences in the ratio of SNb19-31 to SNb1-31 in female and male zebrafish. Ratio of SNb19-31 to SNb1-31 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **$p \leq 0.01$

3.3.5 Differences in sex steroid levels in female and male zebrafish.

Expectedly, there was a sex difference in testosterone (T) levels in the gonads ($p<0.01$, Fig 3.16C) but not in brain ($p>0.05$, Fig 3.16A) or pituitary ($p>0.05$, Fig 3.16B). The T levels in testes were 15-fold higher than that in ovaries. The mean level of T in testes was 175.8 fmol/tissue while that in ovaries was 11.67 fmol/tissue. In contrast of T, 11-ketotestosterone (11-KT) exhibited a male-based difference in brain ($p<0.01$, Fig 3.16D) and pituitary ($p<0.01$, Fig 3.16E) but not in gonads ($p>0.05$, Fig 3.16F). In brain, the mean level of 11-KT in males was 37.27 fmol/tissue which was about 7.5-fold higher than that of females with 5 fmol/tissue. The mean level of 11-KT in male pituitary was 10-fold higher than that in female, corresponding to 43.2 fmol/tissue and 4.36 fmol/tissue, respectively ($p<0.01$, Fig 3.16E).

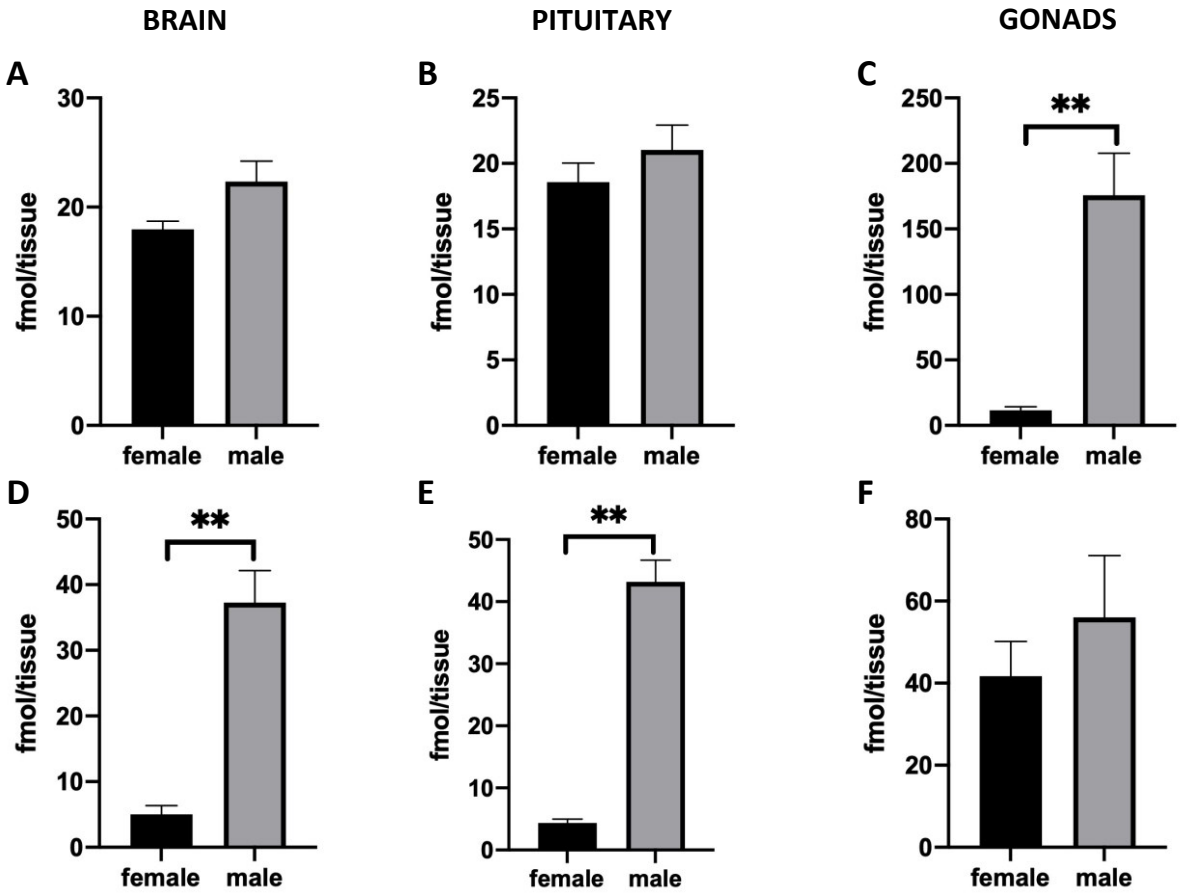

Fig 3.16 Differences in androgen levels between female and male zebrafish. T in brain (A), pituitary (B), gonads (C). 11-KT in brain (D), pituitary (E), gonads (F). Results were presented as

means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. **$p \leq 0.01$

There were female-biased differences in the levels of estrone (E1) in pituitary ($p<0.01$, Fig 3.17B) and gonads ($p<0.01$, Fig 3.17C) but not in brain ($p>0.05$, Fig 3.17A). In pituitary, the mean E1 level in female was 289.9 fmol/tissue which was 4.3-fold higher than that in male with 69.28 fmol/tissue. The E1 level in ovaries was 6.8-fold higher than that in testes. The mean level of E1 in the ovaries was 489.3 fmol/tissue while the testes the E1 level was 71.3 fmol/tissue. There were also female-biased differences in the levels of estradiol (E2) in brain ($p<0.05$, Fig 3.17D) and gonads ($p<0.01$, Fig 3.17F). There was no sex difference in the levels of pituitary E2 ($p>0.05$, Fig 3.17E). In brain, the mean level of E2 in female was 293.8 fmol/tissue and for male was 67.7 fmol/tissue and was 4.33-fold higher than that in male. The E2 levels in ovaries was 31-fold higher than that in testes. Mean E2 level in ovaries and testes were respectively 828.8 fmol/tissue and 26.31 fmol/tissue, respectively. A significant sex difference in estriol (E3) was only detected in gonads: E3 was 5.4-fold higher in female than male. Mean levels of E3 in ovaries and testes were 992.9 fmol/tissue and 184.4 fmol/tissue, respectively, $p<0.05$, Fig 3.17I). As for brain and pituitary, no significant sex differences in E3 were detected ($p>0.05$, Fig 3.17G&H).

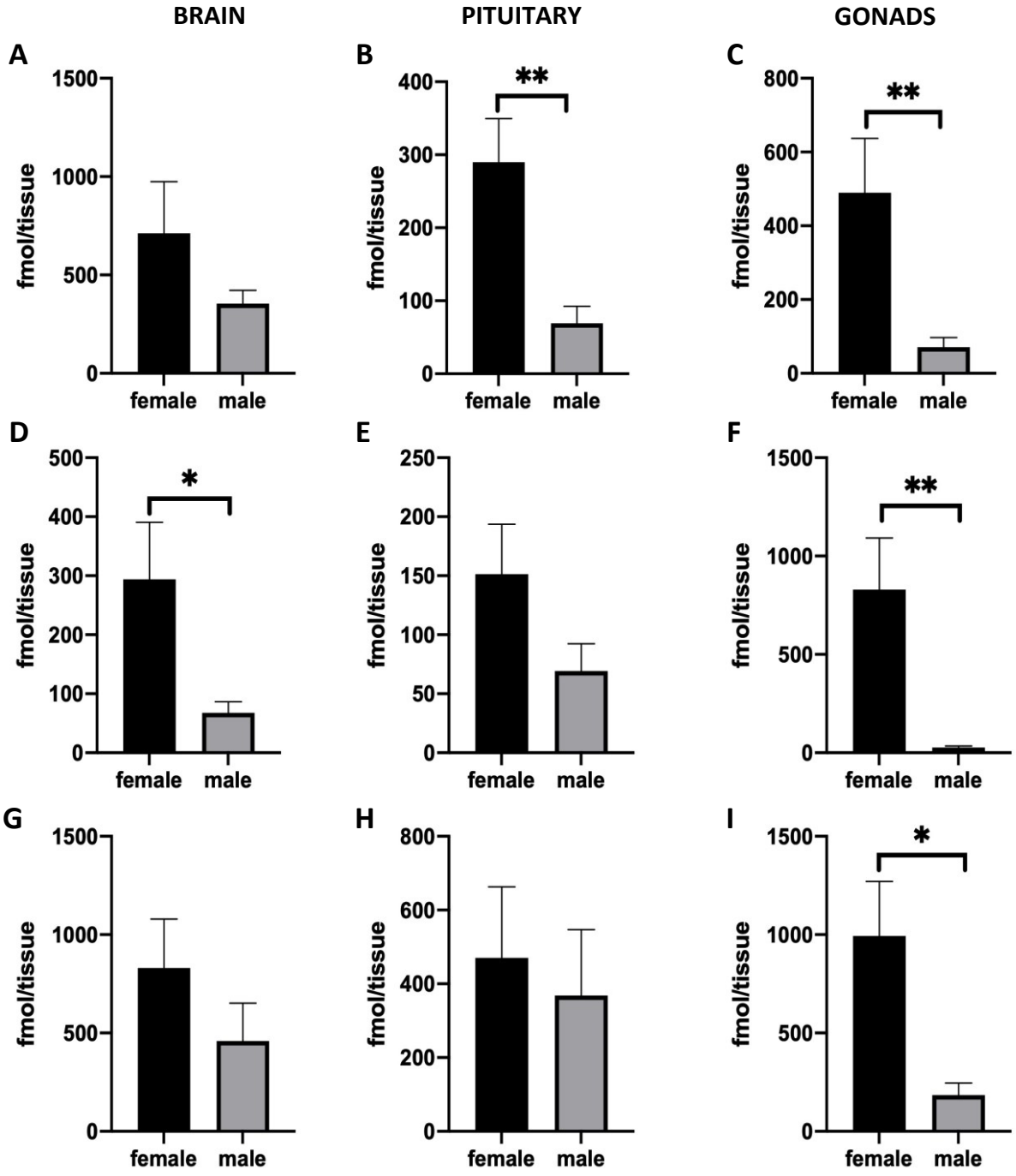

Fig 3.17 Differences in the levels of estrogens in **female and male zebrafish.** E1 in brain (A), pituitary (B), gonads (C). E2 in brain (D), pituitary (E), gonads (F). E3 in brain (G), pituitary (H), gonads (I). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. *p≤0.05 **p≤0.01

To investigate potential sex and tissue differences in metabolism of E2, the ratio of E2/T and the ratio of E3/E2 were analyzed. The results showed a significant female-biased difference in the ratio of E2/T (Fig 3.18). The most striking difference in ratio of E2/ T was in gonads ($p<0.01$, Fig 3.18C). The ratio in ovaries was about 200-fold higher than that in testes. In ovaries, the mean value of the E2 to T ratio was 199 while that in testes was only 1. In the brain, the E2/T ratio in female was 17.4 and that in male was 2.8 ($p<0.05$, Fig 3.18A). As for pituitary, the E2/T ratio in female was about 3-fold higher than that in male ($p<0.05$, Fig 3.18B). In female, the mean value of pituitary E2/T ratio was 9 while that in male pituitary was 2.9. There were no sex differences in E3/E2 ratios in any tissue ($p>0.05$, Fig 3.19).

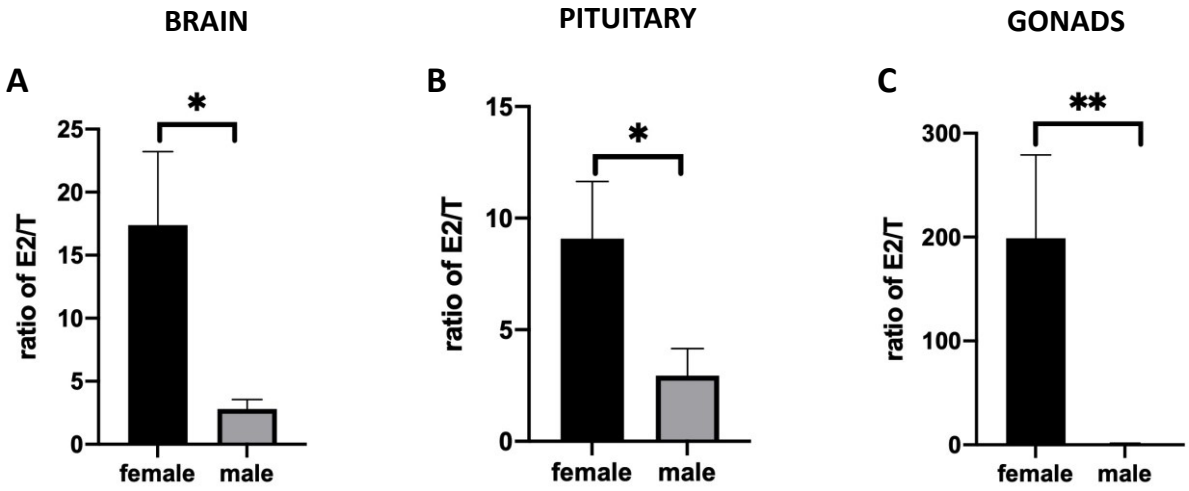

Fig 3.18 Differences in the ratio of E2 to T in female and male zebrafish. Ratio of E2 to T in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Mann-Whitney tests were performed. *$p \leq 0.05$ **$p \leq 0.01$

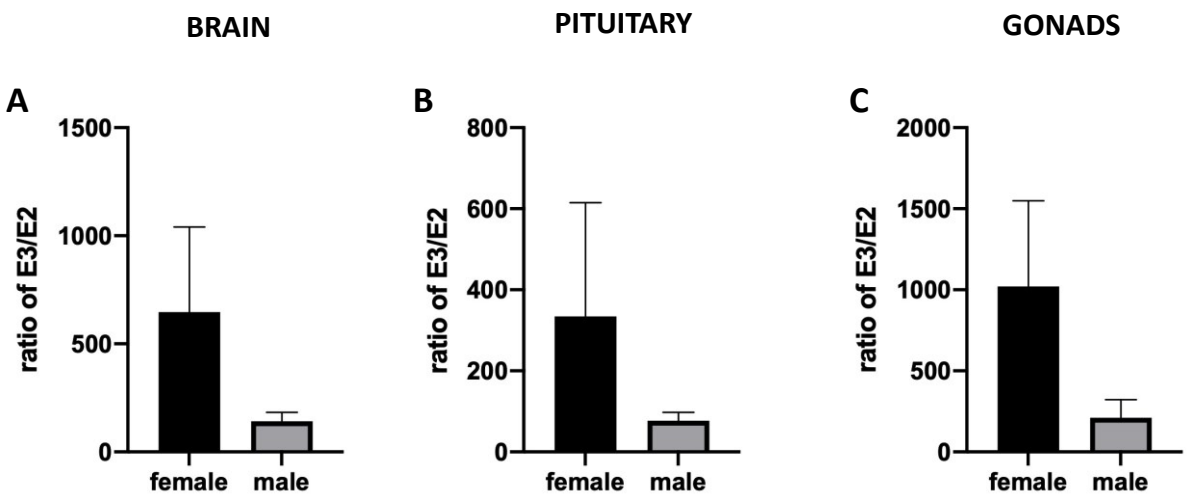

Fig 3.19 Differences in the ratio of E3 to E2 in female and male zebrafish. Ratio of E3 to E2 in brain (A), pituitary (B), gonads (C). Results were presented as means+SEM (n=12). Note the different scales on the Y-axes. Two-tailed unpaired t tests were performed.

3.4 Discussion

Hormonal sex differences were first reported for the rat in 1926 (Ball 1926). In 2005, the sex difference of gene expressions in humans were reported (Rinn and Snyder 2005). The indispensability of the sex differences scientific research has been discussed (McCarthy, Arnold et al. 2012). For now, hormonal and genetic sex differences have been well-studied in mammals (Marrocco and McEwen 2016, Gurvich, Thomas et al. 2020, Jennings and de Lecea 2020) and birds (Adkins-Regan 2007). As for teleosts, various researchers investigated the sex differences of reproductive hormones in goldfish (Bosma, Blazquez et al. 2001, Qi, Zhou et al. 2013) and medaka (Ohya and Hayashi 2006, Kawabata, Hiraki et al. 2012, Yamashita, Nishiike et al. 2021). Recent research revealed a sex difference in the distribution of the innervations of hypothalamic Tachykinin1 cells to Gnrh neurons in zebrafish (Ogawa, Ramadasan et al. 2020). But sex differences in reproductive hormones of zebrafish, a successful animal model for reproductive research (Hoo, Kumari et al. 2016) as well as human diseases (Dooley and Zon 2000), are still not well studied. In this section, we detected the reproductive neuropeptides including Gnrh, kisspeptin, nonapeptides and SN as well as sex steroids by nano LC/MS in males and females to reveal the major sex differences of these reproductive hormones in zebrafish.

The female dominant sex differences in Gnrh2 and Gnrh3 levels were detected. In the brains and gonads, the Gnrh3 levels in female was over 3-fold higher than that in males though no significant difference was found in the pituitary. Gene mutation studies indicate that Gnrh2 and Gnrh3 are dispensable for teleost reproduction (Marvel, Spicer et al. 2018). This is in marked contrast to mammals where normal expression and activation is indispensable for optimal reproduction. (Seeburg, Mason et al. 1987). Nevertheless, Gnrh3 is a major stimulator of Lh production and secretion in female teleosts (Zhao, Basak et al. 2009, Chen and Chiou 2010, Zohar, Zmora et al. 2021). Moreover, a role for Gnrh3 in zebrafish ovaries has been revealed. The research showed that *gnrh3* is expressed in the follicles and Gnrh3 stimulates GVBD as well as caspase-3 activity in the late-vitellogenic follicles to induce the oocytes maturation (Fallah and Habibi 2020). These results supported our data that Gnrh3 produced more in female brain and gonads rather than in males. However, there was no significant difference in pituitary Gnrh3 levels between male and female. This may be explained by the fact that the female samples were collected outside of the ovulatory period. In males, the role of Gnrh in regulating spermatogenesis is known in human (Behre, Kliesch et al. 2001) and teleost (Alvarado, Carrillo et al. 2015, Fallah, Rodrigues et al. 2020). *In vitro* studies in zebrafish showed that incubation with Gnrh3 stimulated the development of testes cell (Fallah, Rodrigues et al. 2020). In men, Gnrh antagonist treatments can suppress spermatogenesis (Behre, Kliesch et al. 2001). In male zebrafish GnRH3 is involved in mating behaviors (Li, Wojtowicz et al. 2017). In terms of Gnrh2, a sex difference was only detected in the pituitary. The Gnrh 2peptide levels in females were higher than that in males. This result suggests that Gnrh2 is involved in female reproduction in zebrafish. Female zebrafish with mutation of Gnrh2 exhibited smaller oocytes but can ovulate and produce embryos with higher mortality than WT (Marvel, Spicer et al. 2019), but suggested that Gnrh2 is involved in regulating the development of oocytes before ovulation. The length of Gnrh2 neural fibers to the pituitary increased 4-fold in fasting zebrafish while Gnrh3 neural fibers reduced 6-fold in length (Marvel, Levavi-Sivan et al. 2021). This research also indicated that the breeding success rate of wildtype zebrafish with 14-day fasting was significantly higher than that of Gnrh2 mutant zebrafish in both sexes (Marvel, Levavi-Sivan

et al. 2021). This supports our hypothesis that Gnrh2 is involved in the zebrafish reproduction by another pathway compared to Gnrh3.

From the results of LC-MS, levels of isotocin in female tissues were significantly higher than that in males. This implies that isotocin may have a major role in the female reproduction. As for vasotocin, levels were higher in female pituitary rather than in males, but in the brain samples, vasotocin levels in males were significantly higher than that in females. Perhaps vasotocin plays a more important role in male zebrafish reproduction rather than isotocin. These results are supported by studies in goldfish and medaka. In medaka brains, the transcript levels of isotocin and vasotocin were significantly higher in males (Ohya and Hayashi 2006, Kawabata, Hiraki et al. 2012). In goldfish, the isotocin injection showed a significant sexually difference as it increased the Lh and sex steroids levels in sexual regressed females (Popesku, Mennigen et al. 2011, Mennigen, Zamora et al. 2017). In the bluehead wrasse, which exhibits a female-to- male sex change in response to social class, vasotocin neuronal phenotype is strongly influenced by social status which appears to be manifested independently of the gonads (Semsar and Godwin 2003). In catfish, vasotocin levels in female were significantly higher than males during the recrudescent phase, especially the vasotocin levels in ovaries was 25-fold higher than males in the spawning phase (Singh and Joy 2008). The study of male zebrafish revealed the vasotocin was associated with the aggression. This study showed that vasotocin is produced in the large cells in the magnocellular preoptic area in dominant males while in the subordinate males, vasotocin was produced in the parvocellular preoptic area (Larson, O'Malley et al. 2006). Another study in male zebrafish indicated that the encircling behavior and breeding successful rate were suppressed significantly by injection of a vasotocin antagonist (Altmieme, Jubouri et al. 2019).

Except for Kiss1 levels in the brain, no other differences in Kiss1 and Kiss2 were evident. In the brain, Kiss1 in males was significantly higher than females which showed a contrast with the results in mammals (Kauffman, Gottsch et al. 2007, Hrabovszky, Ciofi et al. 2010). The contrasting results between teleost and mammals may relate to the different roles of kisspeptin in reproduction in the two animal groups. In mammals, kisspeptin regulates the reproductive process by stimulating the release of Gnrh (Irwig, Fraley et al. 2004). But in teleosts, gene

mutation studied indicate that kisspeptin is dispensable to the reproduction (Tang, Liu et al. 2015). There have been sex differences reported for the kisspeptin system in spotted snakehead and medaka (Kanda, Akazome et al. 2008, Bakshi and Rai 2019). In spotted snakehead, the expression of *kiss1* in males was significantly higher than females in the midbrain and gonads, but the male *kiss1* transcript levels in the anterior brain were significantly lower than that in females (Bakshi and Rai 2019). In medaka, the male dominant sexual differences of *kiss1* expression were found in nucleus ventral tuberis in the brain (Kanda, Akazome et al. 2008). However, contrasting results were reported in the study of chub mackerel. The expression level of *kiss1* in brain was significantly higher in females rather than in males (Selvaraj, Kitano et al. 2010). Our results showing nonsignificant sexual differences of Kiss2 are supported by the studies in goldfish and medaka (Mitani, Kanda et al. 2010, Kanda, Karigo et al. 2012). In European sea bass, the transcript levels of *kiss1* as well as *kiss2* increased significantly in the early spawning process (Migaud, Ismail et al. 2012). Moreover, Kiss1 was investigated to be involved in the zebrafish development. The expression of *Kiss1* was at peak in female zebrafish when ovaries contained mature oocytes while in male zebrafish, *kiss1* mRNA level was highest at the initial stage in spermatogenesis (Biran, Ben-Dor et al. 2008). Kiss2 is not as well-studied as Kiss1, but several research in teleost have indicated that the Kiss2 levels were regulated by E2 (Mitani, Kanda et al. 2010, Servili, Le Page et al. 2011).

In order to determine potential sex differences in SN peptides, we performed the targeted peptidomics to identify the amino acid sequences of SNa1-34 and SNb1-31 in zebrafish. Our peptidomic results showed the identical sequences of SNa and SNb with the predicted sequences derived from genomic data. This is the first research unequivocally identifying the existence of SNa and SNb peptides. It supplies the fundamental evidence to investigate the evolution Scg2 precursor among species and the biological function of SN peptides. Previous studies presumed two isoforms of SN peptides that were named SNa and SNb. The presumptive amino acid sequence of SNa was highly conserved with the mammalian homologs in the regions with "TNE", "QYTP" and "LATLEQSVFEEL" (Zhao, Hu et al. 2010). The conserved amino-acid sequence suggested that SNa peptides are biologically active. In zebrafish with Scg2a and Scg2b double mutations and impaired spawning, an i.p. injection with SNa

partially rescued the reproductive defects (Mitchell, Zhang et al. 2020). As for SNb, it conserved moderately with SNa and mammalian SN peptides in the regions with "QYTPQSLA" and "FEEL", but it was similar with the SN in Atlantic salmon in large regions with "ATEDLDEQY", "PQSLAN","RSIFEEL" and "KLS"(Zhao, Hu et al. 2010). Little is known about potential bioactivities, however, SNb injection increases the expression of *gnrh3*, *lhb*, *fshb* in grouper (Shu, Yang et al. 2018).

In addition to the identification of intact SNa1-34 and SNb1-31 peptides, 6 naturally occurring fragmental peptides for SNa and 17 for SNb were detected by untargeted peptidomics. This result led to the hypothesis that SNa1-34 and SNb1-31 are not the only Scg2a/2b peptides with biological functions. To select candidates, we reviewed peptidomic literature and found that SN1-14, SN1-18, SN19-33 peptides were detected in the bovine and human (Gupta, Bark et al. 2010, Colgrave, Xi et al. 2011). The amino acid sequences of mammalian SN1-14, SN1-18, SN19-33 were somewhat conserved with zebrafish SNa1-14, SNa1-18 and SNa19-34. Moreover, in the only other peptidomic analysis in zebrafish we could find, SNb1-17 peptides and SNb19-31 peptides were detected (Van Camp, Baggerman et al. 2017). These two fragmental peptides were also revealed in our peptidomic results. Peptides with similar sequences detected in multiple isolated studies indicated that these peptides are somewhat stable and potentially bioactive. Therefore, we selected SNa1-14, SNa1-18, SNa19-34, SNb1-17 and SNb19-31 peptides as candidates for subsequent experiments.

The results of LC-MS revealed sex differences of SNa1-34 and SNa fragmental peptides including SNa1-14, SNa1-18, SNa19-34. In female zebrafish, the levels of SNa1-34 were significantly higher than that in males in brain, pituitary and gonads. This implies that SNa1-34 plays a key role in female zebrafish rather than in males. Previous studies in goldfish indicated no sex difference of *scg2a* expression in pituitary (Zhao, Basak et al. 2006). In the study in goldfish, SNa stimulated the serum Lh level as well as the expression of *cga* and *lhb* genes (Zhao, Grey et al. 2010). Moreover, this study also revealed that the expression of scg2a gene was induced by Gnrh3 (Zhao, Grey et al. 2010). The reproductive defects in females caused by Scg2a/b mutation is partially rescued by exogenous SNa1-34 and hCG treatments while the Scg2a/b mutation did not have a major impact on reproduction in male zebrafish, pointing to

a sex difference in the importance of Scg2-derived peptides (Mitchell, Zhang et al. 2020). This is supported by our results that SNa1-34 is much higher in females than in males. In contrast to SNa1-34, SNa fragmental peptides were produced more in males rather than in females. Similarly, the ratio of fragmental SNa to intact SNa was statistically significant higher in males. It suggested that the metabolism of SNa peptides is more extensive in male zebrafish, as the result, less intact SNa1-34 was detected. Another hypothesis is that SNa1-34 is cleaved to produce smaller functional peptides regulating other functions. For example, intracerebroventricular injection of SNa rapidly induced food intake and locomotor behavior, but it is not known if this is due to SNa1-34 or because of processing to shorter peptides after injection (Mikwar, Navarro-Martin et al. 2016).

A somewhat different situation emerged for the SNb peptides, including SNb1-31 and fragmental peptides SNb1-17 and SNb19-31. All were higher in females rather than in males. Higher levels of SNb1-31 in female zebrafish suggests a role in females. However, a previous study in zebrafish revealed that exogenous SNb1-31 could not rescue impaired reproduction in Scg2a/2b double mutant fish (Mitchell, Zhang et al. 2020). On the other hand, in orange spotted grouper SNb1-31 had similar effects to SNa1-34, inducing the expression of *gnrh3*, *lhb*, *fshb* (Shu, Yang et al. 2018). Another study in larval zebrafish revealed a transient role of SNb1-31 in early neurovascular modeling via a MAPK and PI3K/AKT pathway (Tao, Hu et al. 2018). Interestingly, the ratio of SNb1-17 to SNb1-31 as well as ratio of SNb19-31 to SNb1-31 was significantly higher in male zebrafish. The high ratio of SNb fragmental peptides to SNb1-31 indicates more extensive processing in males. It is possible that there are different metabolic pathways and biological functions of SNb peptides in male zebrafish. Future research should focus on possible sex differences in the role of the SNb peptides.

Another major innovation in this study is the ability to detect and quantify neuropeptides and sex steroids in the same tissue samples simultaneously. Not only do the results confirm expectations of sex differences in gonadal steroid levels, but also now in other tissues. There was the clear male dominant sex difference of testicular T, versus the higher estrogens in ovaries. As a classic androgen, T plays a key role in testicular development as well as spermatogenesis while in females, it rises significantly in ovulation but the returns to basal

levels(Barannikova, Dyubin et al. 2002). A specific biological function for T in the teleost brain has yet to be established. However, T is converted to E2 by aromatase B, found exclusively in radial glial cells (Xing, Goswami et al. 2014, Diotel, Charlier et al. 2018). The main biologically active androgen in teleosts is 11-KT, being synthesized from T by 11β-hydroxylase and 11β-hydroxysteroid dehydrogenase (Rege, Turcu et al. 2018). Our results show that 11-KT levels were significantly higher in male brain and pituitary compared to females. In gonads, there was no statistical difference between ovarian 11-KT and testicular 11-KT, but when the difference of GSI is considered, the concentration of 11-KT in ovaries was approximately 13-fold lower than that in testis. The main biologically active androgen in teleosts is 11-KT, being synthesized from T by 11b-hydroxylase and 11 β -hydroxysteroid dehydrogenase (Rege, Turcu et al. 2018). Cyp11c1 was the key enzyme of the 11-KT synthesis from T (Bacila, Cunliffe et al. 2021). In female zebrafish, *cyp11c1* gene is expressed in ovaries by 30 dpf, then decreases after 40 dpf and is not detectable in adult ovaries (Oakes, Barnard et al. 2020, Zhang, Ye et al. 2020). In our result, the detection of 11-KT in zebrafish ovaries suggests that 11β-testosterone precursor is produced from other tissues and transfers to ovaries through blood. Several studies also detect 11-KT not only in zebrafish testis but also in ovaries (Zou, Liang et al. 2021, Mu, Qi et al. 2022). As for reproductive functions, 11-KT is involved in spermatogenesis in the Japanese eel (Miura, Yamauchi et al. 1991) and sexual development in zebrafish (Zhang, Ye et al. 2020). The biological function of 11-KT in ovary has also been studied in several fish species. The study in sterlet revealed that 11-KT induced the ovarian development and vitellogenin synthesis (Wang, Zhu et al. 2020). In coho salmon, 11-KT was also found to induce the primary follicle development in ovaries (Monson, Forsgren et al. 2017). But in zebrafish, 11-KT showed an inhibitory effect to the progression of follicles by inhibiting the cell cycle in granulosa-theca cell layer (Sousa, Silva et al. 2016). These studies not only support our results in the quantification of ovarian 11-KT, but also suggest the role of 11-KT in development of ovaries and follicular cells. This is suggestive of different metabolic routes for T in males versus females. In tilapia 11-KT induces that proliferation of GnRH3 neurons in brain (Narita, Tsutiya et al. 2018) , so perhaps there is a sex difference in this process driven by the relative levels of androgen to estrogens. A clear female dominant sex difference was observed in gonadal E1, E2 and E3. E2 is not only

produced in the follicle layers in oocytes, but also converted from T in brain (Diotel, Le Page et al. 2010). In zebrafish brain E2 inhibits neurogenesis (Diotel, Charlier et al. 2018). Previous studies in goldfish revealed i.p implantation pf silicon pellets releasing E2 enhances Gnrh-stimulated Lh release (Trudeau, Murthy et al. 1993). Moreover, T also induced the production of gonadotropins in goldfish (Habibi and Huggard 1998). These results revealed the stimulatory effects of sex steroids on the gonadotropins in the teleost pituitary.

In fathead minnows exogenous E1 induced hepatic vitellogenin production (Dammann, Shappell et al. 2011). Similarly, E2 plays a key role in the post-vitellogenesis stage to stimulate the oocyte maturation in the fish ovaries (Nagahama, Yoshikuni et al. 1995). In contrast, the biological role of E3 is not well-studied in fish. Moreover, the E2/T ratio was significantly higher in female brain, pituitary, and ovaries while no significant sex difference for E3/E2 ratios were evident. In the zebrafish steroidogenesis, E2 was conversed from T by aromatase and transformed into E3 (Piferrer and Blazquez 2005, Thomas and Potter 2013). Higher E2/T ratios and with no major differences in the E3/E2 ratio suggest that metabolism of E2 in males and females were similar. However, the E3/E2 ratio in ovaries appeared higher, but was more variable and not significantly different from males. More detailed studies are required to firmly establish metabolic activities in ovaries.

In conclusion, we confirmed the presumptive amino-acid sequence of SNa and SNb by targeted peptidomics and identified the fragmental peptides of SNa and SNb by untargeted peptidomics. Moreover, sex differences in the levels of many reproductive hormones were detected by LC-MS from brain, pituitary and gonads. These data suggest two main avenues for future research: (1) investigation of sex differences in the role and relative importance of importance of the SNs and other hormones, and (2) investigation of sex differences in the regulation of these neuropeptides and steroid hormones in the reproductive cycle. As a first step, we explored the periovulatory changes in these hormones, and the role of SNa on ovulation (Chapter 4).

Chapter 4 Periovulatory variations of reproductive hormone levels in the zebrafish

4.1 Introduction

In vertebrate reproduction, the hypothalamus-pituitary-gonads axis (HPG axis) plays a key role in gonadal development as well as oogenesis and spermatogenesis. Multiple neuropeptides and neurotransmitter produced in specific neuronal populations are axonally transported by neural fibers, then released at the nerve terminals to control pituitary functions in teleosts (Trudeau 2022).

Classically, it is thought that the principal regulator of vertebrate reproduction is the decapeptide Gnrh. In teleosts, neuroendocrine neurons directly innervate the anterior pituitary where the gonadotrophs are located (Somoza, Mechaly et al. 2020). Teleosts have lost the highly vascularized median eminence typical of mammals. Instead, Gnrh may be released from nerve terminal in close proximity to or by directly contacting the separate Lh and Fsh secreting cells (Somoza, Mechaly et al. 2020) The Gnrh decapeptide activates several forms of the G-protein-coupled Gnrh receptors on the gonadotrophs to stimulate the expression and secretion Lh and Fsh (Somoza, Mechaly et al. 2020).

In zebrafish, like other cyprinid fishes, there are two distinct isoforms of Gnrh, Gnrh2 and Gnrh3 arising from separate genes. Gnrh2, also known as chicken Gnrh-II (cGnRH-II), is found in the midbrain tegmentum in salmon as well as POA in teleost (Kim, Oka et al. 1995, Palevitch, Kight et al. 2007) and plays a role in reproduction and metabolism (Kah, Lethimonier et al. 2007). Gnrh3, also known as salmon Gnrh (sGnRH), is only found in fishes. This peptide is located in the olfactory and POA in teleosts (Kim, Oka et al. 1995, Palevitch, Kight et al. 2007). It has been shown that Gnrh fibers innervate the pituitary in several species of fish, including the goldfish and tilapia and induce Lh release from the pituitary (Peter, Chang et al. 1986, Chang, Johnson et al. 2009). Additionally, release of Gnrh3 in the zebrafish pituitary are shown to be 3- to 4-fold higher than those of Gnrh2 (Steven, Lehnen et al. 2003). Therefore, Gnrh3 is considered to be the hypophysiotropic form in several species (Powell, Krueckl et al. 1996,

Steven, Lehnen et al. 2003). The latest studies in zebrafish indicates that Gnrh2 is involved in reproductive regulation in fasting zebrafish (Marvel, Levavi-Sivan et al. 2021).

The gonadotropins, Lh and Fsh, are composed of the Cgα and Lhβ subunits and play a critical role in vertebrate reproduction (Lei, Mishra et al. 2001, Zhang, Poutanen et al. 2001, Cahoreau, Klett et al. 2015). The synthesis and release of gonadotropins from the anterior pituitary are regulated by Gnrh that binds to membrane receptors on the gonadotrophs, triggering action potentials by rising the concentration of Ca^{2+} in cells, and releasing hormones into the circulation (Levavi-Sivan, Bogerd et al. 2010, Chang and Pemberton 2018). In goldfish, increased Gnrh stimulation accompanied by a decrease in inhibitory dopaminergic tone drives the ovulatory Lh surge (Trudeau 1997). The Lh surge via activation of Lh receptors on granulosa cells, initiate ovarian follicle maturation (Yaron and Levavi-Sivan 2011). On the other hand, Fsh actives Fsh receptors on granulosa cells to enhance the secretion of estradiol-17β (E2) in females that promotes oogonial proliferation and vitellogenesis, and progestogens promoting initiation of germ cell meiosis and follicular maturation and ovulation (Szkudlinski 2015).

So far, $\sim$20 stimulatory and $\sim$10 inhibitory neuropeptides along with classical neurotransmitters are implicated in the neuroendocrine control of teleost reproduction (Trudeau 2022). Very few of these neurohormones have been measured in naturally ovulating fish. Moreover, gene mutation in zebrafish studies now bring into question the long thought indispensable role for Gnrh and kisspeptins in reproduction. Laser ablation of Gnrh3 neurons in the developing larvae abolishes ovulation in the adult (Abraham, Palevitch et al. 2010). Surprisingly, targeted transcription activator-like effector nuclease (TALEN) mediated frameshift mutations of both *gnrh2* and *gnrh3* have no effects on reproductive outcome (Spicer, Wong et al. 2016). Changes in the expression of *fshb*, *lhb*, and *cga* in young mutant fish mutants returned to normal in the adults. Tang et al. developed several zebrafish *kiss*/*kissr* mutants. All six mutation lines reproduced normally, challenging the importance of kisspeptinergic systems for teleost reproduction, in comparison to their essentiality in some mammals. Even in triple mutants with mutated *gnrh3* and two kiss genes, reproduction appears to be normal (Tang, Liu et al. 2015).

Such data point to the likelihood of other neurohormones being essential for reproduction. We and others are searching for novel candidates and alternative mechanism for the control of Lh release (Trudeau 2022). From previous research in our lab, it is clear that the secretogranin 2 (Scg2) proteins and the derived SN peptides are important. To date, the scg2 mutants are the only example in which neuropeptide precursor gene mutations impact reproduction in the zebrafish model (Mitchell, Zhang et al. 2020, Trudeau 2022). Female zebrafish with TALEN-mediated frameshift mutations of Scg2a and Scg2b had a significantly lower ovulation and oviposition rates but the mutant males were fertile (Mitchell 2018). Thus, Scg2a and Scg2b play key roles in reproduction. Some of these defects were partially rescued in double Scg2a/ Scg2b mutants by a single injection of SNa but not SNb. Moreover, our results from peptidomic (Chapter 3) analysis revealed the existence of the SNa fragment peptides (SNa1-14, SNa1-18 and SNa19-34) and SNb fragment peptides (SNb1-17 and SNb19-31). We hypothesized that SNa and SNb peptides or their smaller processed peptides may vary in relation to well-known neuropeptides and steroids controlling the ovulatory cycle in zebrafish.

We first had to develop a new method of single tissue sample purification and LC-MS analysis to detect a list of peptides and sex steroids hormones in the brain, pituitary and gonads obtained from a single zebrafish. Tissues were never pooled, so that important variations between individuals could be accurately measured for the first time. The detected hormones were SNa1-34, SNa1-14, SNa1-18, SNa19-34, SNb1-31, SNb1-17, SNb19-31, isotocin, vasotocin, Gnrh2, Gnrh3, Kiss1, Kiss2, 11-KT, testosterone (T), estrone (E1), estradiol (E2) and estriol (E3). Secondly, we used this method to quantify levels of these hormone in the female zebrafish at 3 different timepoints during the ovulatory cycle. Regression analysis over the periovulatory period revealed moderate to strong relationships between SNa1-34 and Gnrh3, and to a lesser extent Gnrh2. These data strongly implicate SN in the control of teleost reproduction.

4.2 Materials and methods

4.2.1 Experimental animal husbandry

Wildtype zebrafish (AB strain) were bred and kept in the University of Ottawa Aquatics core facility at the temperature of 28°C. The lights-on time was set for 14 hours from 0900h to 2300h. All animal experiments followed the guidelines of the Canadian Council on Animal Care for the use of animals in research, approved by the University of Ottawa Animal Care Protocol Review Committee. All the fish are fed twice a day. Sexually mature zebrafish at the age of 6-9 months post-fertilization (mpf) were selected and separated by sex for least two weeks to synchronize the reproductive cycles before the experiment.

4.2.2 Dissection and homogenization

On the afternoon before dissection, 30 males and 30 females were selected randomly and transferred into 30 breeding traps with one male and one female respectively separated by a transparent divider to avoid spawning. On the second day, these 30 couples were divided randomly in 3 groups of 10 couples each and anesthetized at 3 separate timepoints (Fig 4.1). The first timepoint was at 0200 since pituitary *lhb* and *cga* mRNAs level peaked at this time (So et al., 2005). The second timepoint was at 0500 because this is the time of the Lh surge (Tang et al., 2016). The last timepoint was at ovulation at 0830 (Zhou et al., 2011). During the anesthesia, brain, pituitary and gonads was collected separately, placed in a homogenization buffer on ice and homogenized by ultrasonic homogenizer (Fisherbrand; Cat# FB120110) 3-5 sec. The homogenized samples were centrifuged at 4°C in 13,200 rpm for 10 min. After that, the supernatant was transferred into a new 1.5ml EP tube and dehydrated with the pellet in centrifugal vacuum concentrator (Labconco; Cat#7810014) at 45°C. Dehydrated samples were stored in -20°C.

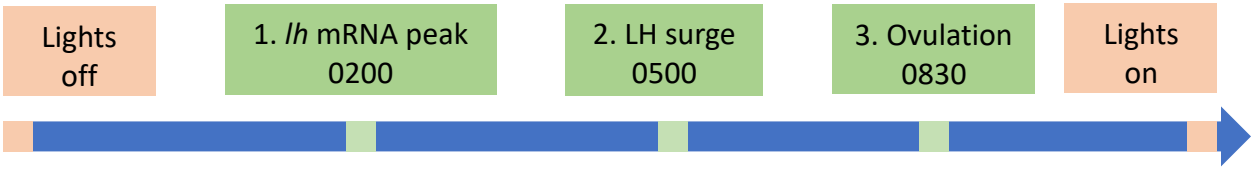

Fig 4.1 Timepoints of the female zebrafish dissection

4.2.3 Solid phase extraction (SPE) and mass spectrometry (MS) analysis

The SPE protocol and the recipe of the solutions were same as presented in Chapter 3. Before loading into the LC-MS system, the samples were thawed at the room temperature and resuspended in loading buffer. The LC-MS system and the process protocol is reported in Chapter 3.

All the peptides standard compounds were synthesized and purified by Chunyu Lu, a collaborating PhD student in our lab. All had purity >95%. The steroid hormones were purchased from Sigma-Aldrich Inc. All the information of these compounds is shown in Table 4.1.

4.2.4 Statistical analysis

The LC-MS data is presented as means+ SEM (n=10). All the results were statistically analyzed on GraphPad Prism 9.0 software. The Kruskal-Wallis one-way analysis of variance on ranks followed by a Tukey test was performed to analyze the difference of the data among different timepoints in each tissue. Non-linear regression analysis was performed to analyze the relation between reproductive hormones and SNa related peptides. The best models of non-linear regression analysis for each hormone were selected by GraphPad Prism 9.0 software. The software compares the third-order polynomial as the null hypothesis with the fourth-order polynomial as the alternative hypothesis. It accepts the alternative hypothesis when $p<0.05$ while it accepts the null hypothesis when $p>0.05$. In our project, the analysis of the relation between Gnrh2 and SNa1-34 and that between Gnrh3 and SNa1-14 were performed by the third-order polynomial while the other analysis was performed by the fourth-order polynomial. The results were present as R^2 value to reveal the goodness of fit. If R^2 value < 0.3, it is considered as not related; if $0.3 < R^2 < 0.5$, it is considered as weakly related; if $0.5 < R^2 < 0.7$, it is considered as moderately related; if R^2 value > 0.7, it is considered as strongly related (Moore, Notz et al. 2013).

Table 4.1 Standard compounds used for hormonal analysis by LC-MS

Compounds	**Amino acid sequence and information**
Isotocin	CYISNCPIG
Vasotocin	CYIQNCPRG
Kisspeptin1 (Kiss1)	YNLNSFGLRY
Kisspeptin2 (Kiss2)	FNYNPFGLRF
Gnrh2	QHWSHGWYPG
Gnrh3	QHWSYGWLPG
SNa1-34	TNENAEEQYTPQKLATLQSVFEELSGIASSKTNT
SNa1-14	TNENAEEQYTPQKL
SNa1-18	TNENAEEQYTPQKLATLQ
SNa19-34	SVFEELSGIASSKTNT
SNb1-31	ATEDLDEQYTPQSLANMRSIFEELGKLSAAQ
SNb1-17	ATEDLDEQYTPQSLANM
SNb19-31	SIFEELGKLSAAQ
Estrone (E1)	Sigma-Aldrich; Cat. # E9750
Estradiol (E2)	Sigma-Aldrich; Cat. # E1024
Estriol (E3)	Sigma-Aldrich; Cat. # E1253
Testosterone (T)	Sigma-Aldrich; Cat. # Y0002174
11-Ketotestosterone (11-KT)	Sigma-Aldrich; Cat. # K8250

4.3 Results

4.3.1 Brain Gnrh3 but not Gnrh2 level varied during the ovulatory cycle

Both of Gnrh2 and Gnrh3 were detected in the samples of whole brain, pituitary, and ovaries from a single fish (Fig 4.2). Pooled samples were never used. The results revealed that Gnrh3 level in the brain varied during the periovulatory period. In the brains of female zebrafish, Gnrh3 levels increased 1.6-fold from Timepoint 1 to Timepoint 2 and then decreased significantly at Timepoint 3 ($p<0.05$) (Fig 4.2A). Relative to brain, only low levels of Gnrh3 were detected in the pituitary, with a tendency for higher levels in some fish at Timepoint 3 (Fig 4.2B). Moreover, Gnrh3 was detected in ovaries, although with no significant variation during the cycle (Fig 4.2C). The levels of Gnrh3 in the zebrafish brain were significantly higher than that in the pituitary and ovaries (Mean of Gnrh3 in brain, pituitary and ovaries were respectively 1020.2, 51.35 and 519.7 fmol/tissue; $p<0.05$). In contrast with the variations observed in brain Gnrh3, there was no significant alteration of Gnrh2 levels during the zebrafish ovulatory process (Fig 4.2D-F). However, similar with Gnrh3, the levels of Gnrh2 in the zebrafish brain were significantly higher than that in the pituitary and ovaries. Mean levels of Gnrh2 in brain, pituitary and ovaries were respectively 211.8, 5.7 and 23.5 fmol/tissue ($p<0.05$).

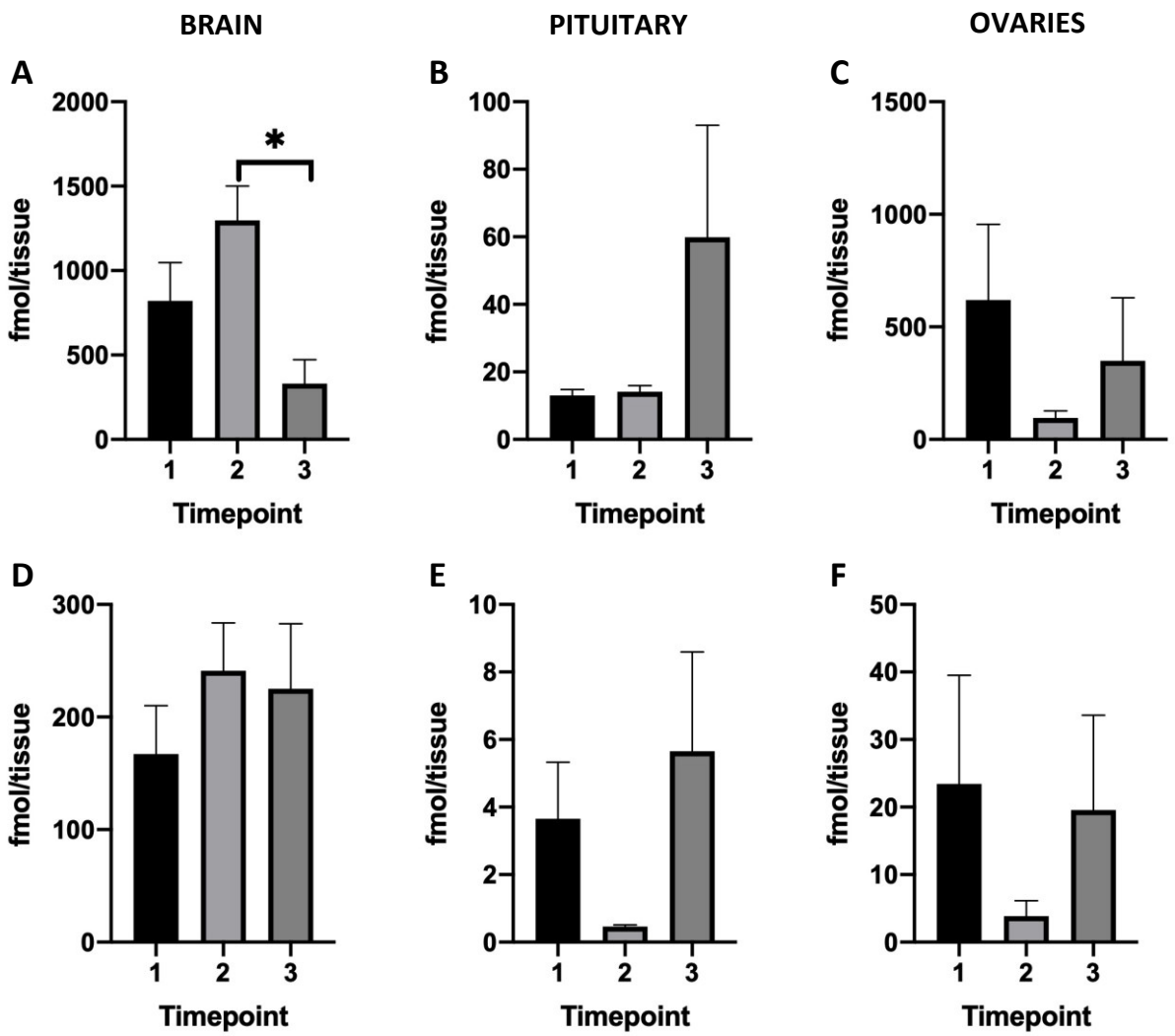

Fig 4.2 Gnrh3 and Gnrh2 levels during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. Gnrh3 in brain (A), pituitary (B), ovaries (C). Gnrh2 brain (D), pituitary (E), ovaries (F). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *p≤0.05.

4.3.2 Brain and pituitary isotocin but not vasotocin levels varied during the ovulatory cycle

We detected both isotocin and vasotocin in the zebrafish brain, pituitary, and ovaries. The results indicated that the level of isotocin was significantly higher than that of vasotocin in each tissue ($p<0.05$) and the variation of isotocin during the cycle was statistically significant. For isotocin, significant changes were observed in brain and pituitary. Moreover, isotocin variations in the brain (Fig 4.3A) and pituitary (Fig 4.3B) tended to be in opposite directions (Fig 4.3A). In brain, isotocin was low during Timepoint 1 and 2, but at Timepoint 3, it increased

significantly about 4.3 and 3.3-fold compared to the earliest sampling time. Mean isotocin levels at Timepoint 1,2 and 3 were respectively 1120, 1450 and 4760 fmol/tissue; ($p<0.01$). As for in the pituitary, isotocin decreased from Timepoint 1 to Timepoint 3 showing a significant variation ($p<0.01$) (Fig 4.2B). In ovaries, isotocin was below the detection limit (6.5fmol/tissue), except for 1 in 10 samples. For vasotocin, no significant changes were evident. Vasotocin levels in pituitary were ~ 17.5-fold and 3.2-fold higher than that in brain and ovaries, respectively. Mean levels of vasotocin in brain, pituitary and ovaries were respectively 75.4, 1323, 409.7 fmol/tissue.

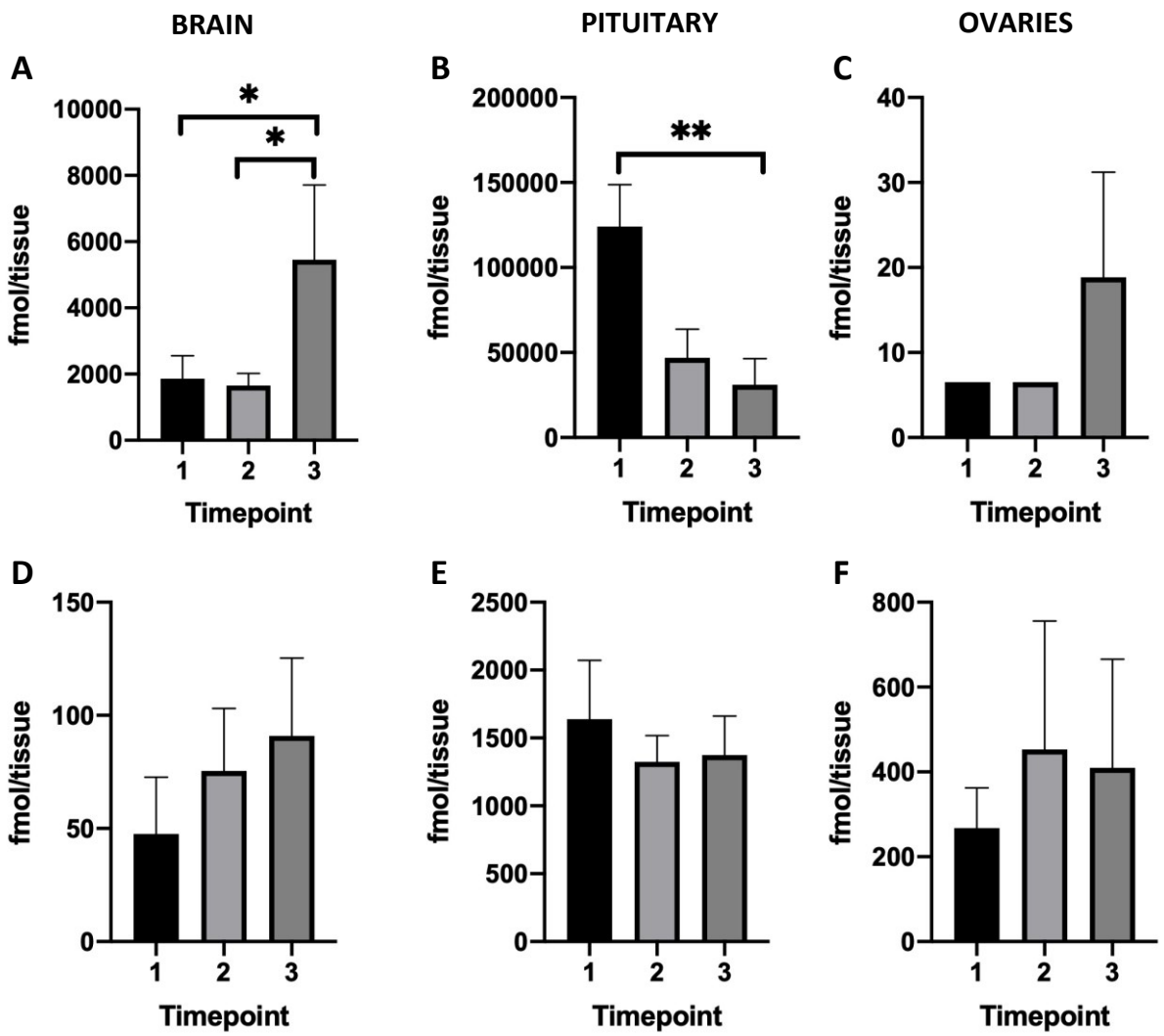

Fig 4.3 Isotocin and vasotocin levels during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. Isotocin in brain (A), pituitary (B), ovaries (C). Vasotocin in brain (D), pituitary (E), ovaries (F). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *p≤0.05 **p≤0.01

4.3.3 Kiss1 and Kiss2 levels did not change significantly during the ovulatory cycle

We detected Kiss1 and Kiss2 in the zebrafish brain, pituitary, and ovaries. However, significant periovulatory variations were not evident (Fig 4.4). The levels of Kiss1 in the brain were significantly higher than that in the pituitary (P<0.05) and ovaries (P<0.01). Mean Kiss1 levels in brain, pituitary and ovaries were respectively 57.5, 7.5 and 2.7 fmol/tissue. A different pattern of production was evident for Kiss2. The highest levels were found in the pituitary.

Mean levels of Kiss2 in brain, pituitary and ovaries were respectively 208.1, 727.7 and 580.7 fmol/tissue ($p<0.05$).

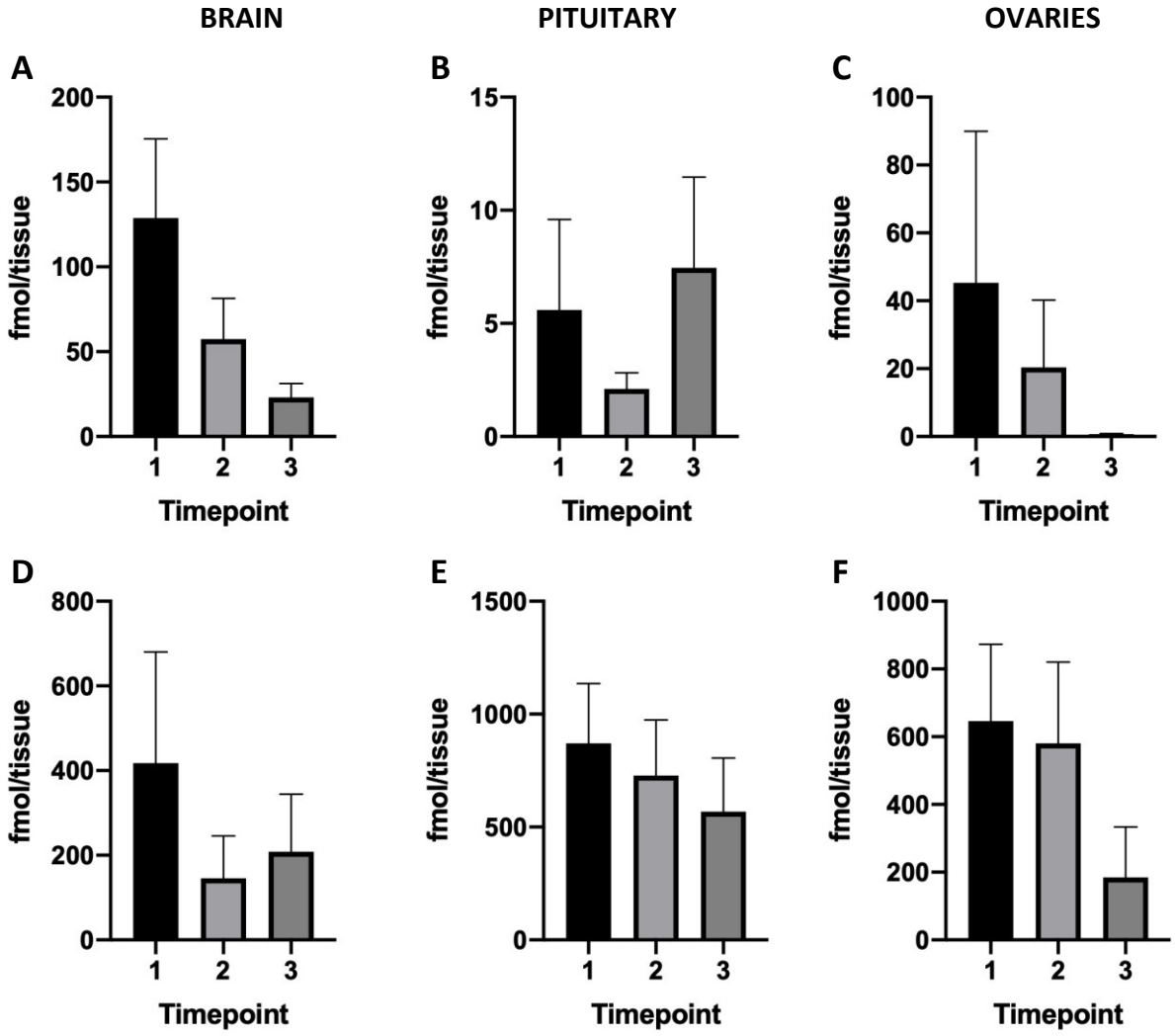

Fig 4.4 Kisspeptin levels during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. Kiss1 in brain (A), pituitary (B), ovaries (C). Kiss2 in brain (D), pituitary (E), ovaries (F). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. No significant differences were evident.

4.3.4 Sex steroid levels varied during the ovulatory cycle

We detected both estrogens and androgens in the zebrafish brain, pituitary and ovaries. We quantified E1, E2, E3 in estrogen as well as T and 11-KT in androgen. Moreover, we also analyzed the E2 to T ratio and the E3 to E2 ratio to gain an understanding of potential shifts in metabolic activity.

Both E1 and E2 varied significantly in the brain and pituitary, while the alteration in E3 levels was not statistically significant (Fig 4.5). E1 showed a significant variation in the brain and pituitary during the periovulatory period. In the brain, the E1 level in the brain decreased slightly without significant difference from Timepoint 1 to Timepoint 2 ($p>0.05$), but at Timepoint 3, the E1 level increased 3.7-fold ($p<0.01$). Mean E1 levels at Timepoint2 and Timepoint 3 were 168 fmol/tissue and 628.5 fmol/tissue separately (Fig 4.5A). In the pituitary, E1 level peaked at Timepoint 1 and decreased significantly from Timepoint 2 ($p<0.01$) (Fig 4.5B). Significant variation in E2 was only evident in brain. At Timepoint 1 and Timepoint 2, E2 was low (Mean of Timepoint 1= 137, Mean of Timepoint 2=105.5). But at Timepoint 3, the E2 level increased for 7.4-fold (Mean of Timepoint 3= 781) and showed a significant difference with that at Timepoint 1 ($p<0.05$) and Timepoint 2 ($p<0.01$) (Fig 4.5D). Levels of E3 decreased at Timepoint 2 and Timepoint 3 in all the tissues. Although there were no statistical differences over time (Fig 4.5G-I), the general pattern of E3 levels were very similar in the brain, pituitary and ovaries.

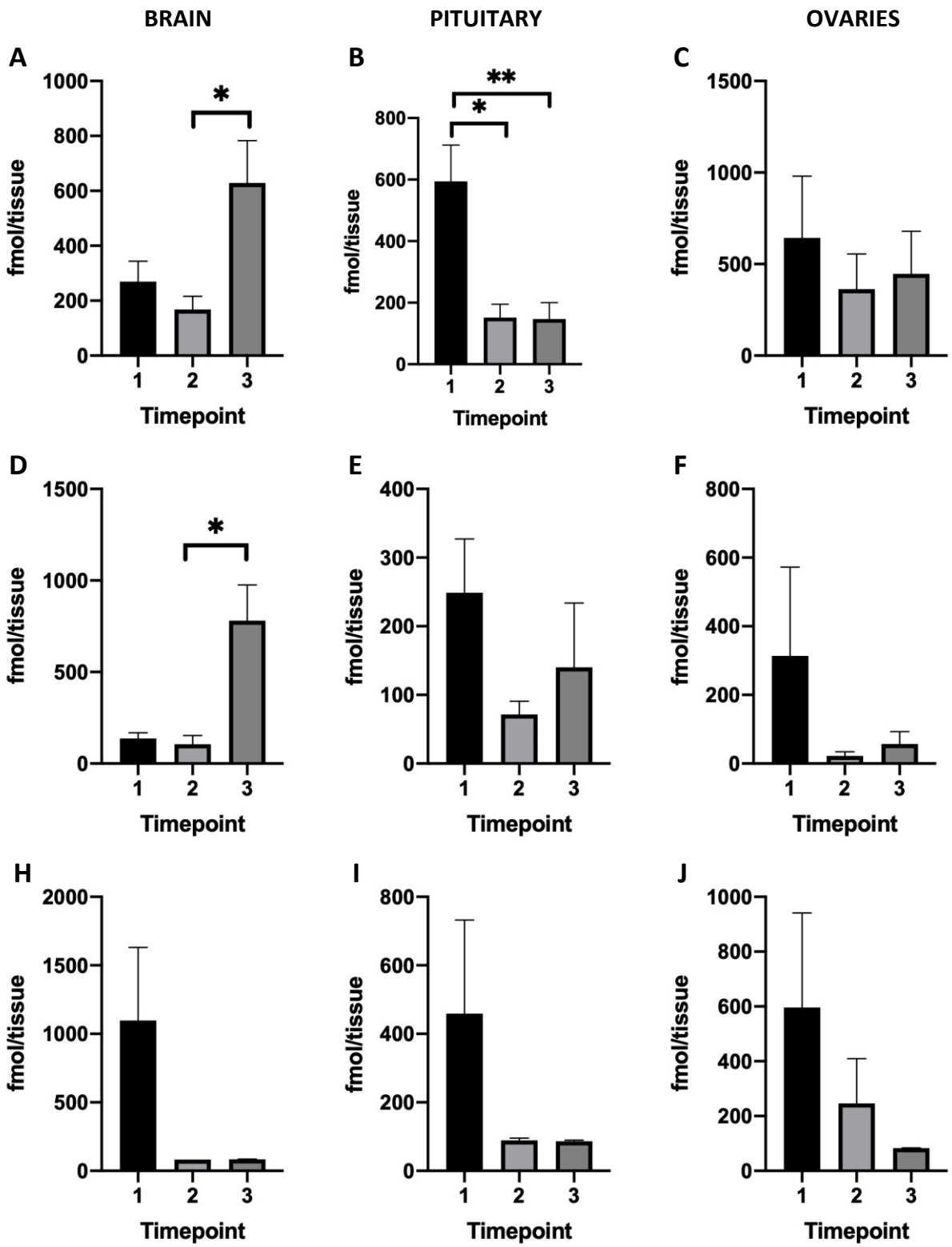

Fig 4.5 Estrogen levels during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. E1 in brain (A), pituitary (B), ovaries (C). E2 in brain (D), pituitary (E), ovaries (F). E3 in brain (G), pituitary

(H), ovaries (I). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *$p \leq 0.05$ **$p \leq 0.01$

Both T and 11-KT were detected in the brain, pituitary, and ovaries, but significant variations were only observed in the brain (Fig 4.6). T was at a relatively high level at Timepoint 1 as well as Timepoint 2 in brain but decreased significantly at Timepoint 3 at 10.2-fold ($p<0.05$) Mean of brain T levels at Timepoint 1, Timepoint 2 and Timepoint 3 was 11.96 fmol/tissue, 11.62 fmol/tissue and 1.14 fmol/tissue, respectively (Fig 4.6A). A similar trend was evident in brain 11-KT level: 11-KT level decreased slightly without a statistically significance from Timepoint 1 to Timepoint 2 (Mean of Timepoint 1: Mean of Timepoint 2= 50.38: 45.61; $p>0.05$) while it decreased sharply at Timepoint 3 with a statistically significance when compared with that at Timepoint 1 (Mean of Timepoint 1: Mean of Timepoint 3= 50.38: 21.56; $p<0.05$) (Fig 4.6D). Moreover, mean levels 11-KT was significantly higher than that of T in ovaries ($p<0.05$).

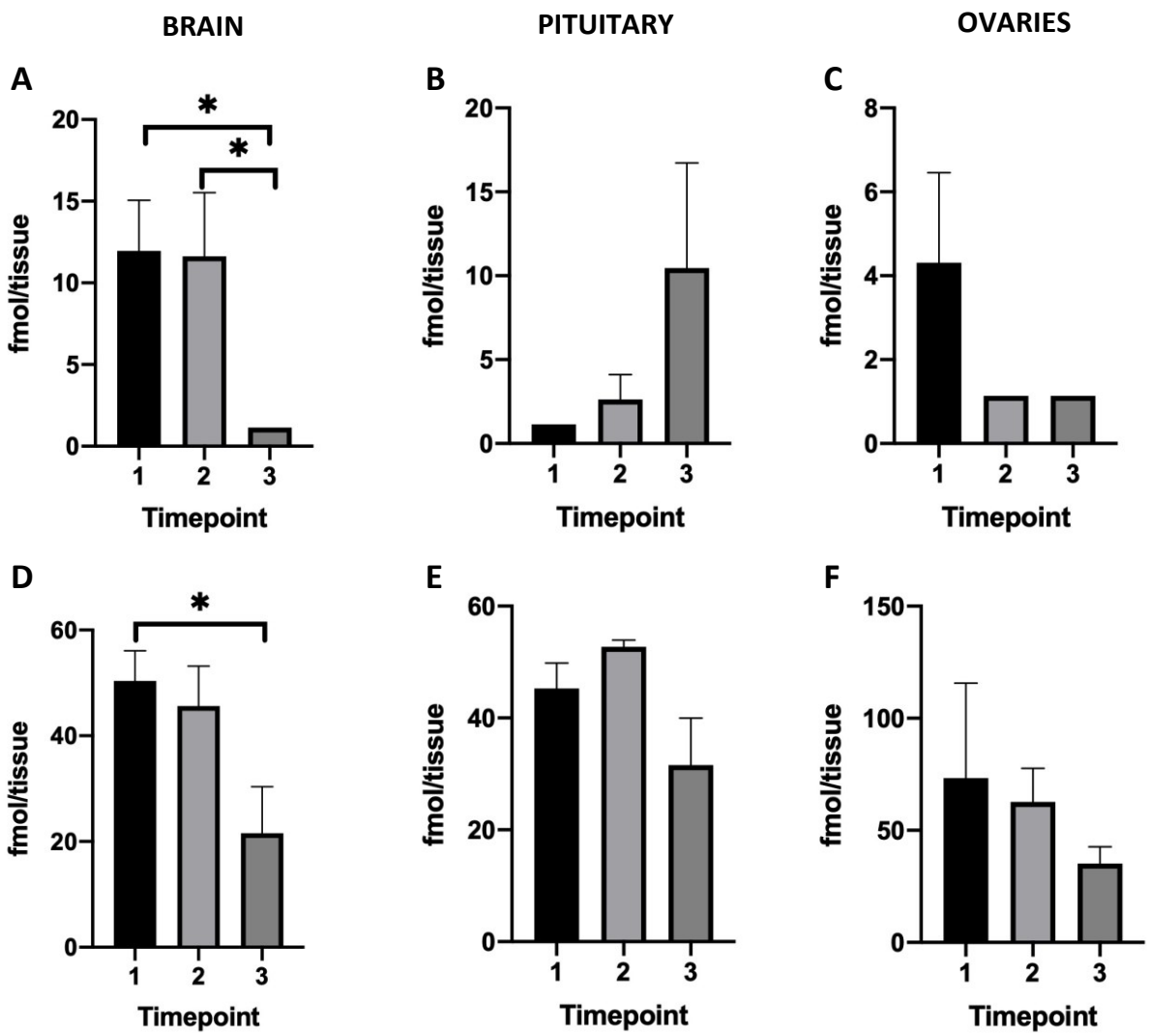

Fig 4.6 Androgen levels during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. T in brain (A), pituitary (B), ovaries (C). 11-KT in brain (D), pituitary (E), ovaries (F). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *$p \leq 0.05$

We also analyzed the E2/T and E3/E2 ratios in all tissues. From the results, we noted that the E2/T was increased significantly at Timepoint 3 in brain ($p<0.01$) (Fig 4.7A). Moreover, we observed a similar variation tendency of the E2/T ratio in the pituitary and ovaries although the variations were not statistically significant. In both pituitary and ovaries, the E2/T ratios were highest at Timepoint 1 and decreased at Timepoint 2 but increased slightly at Timepoint 3 (Fig 4.7B&C). In contrast with the E2/T ratio, the E3/E2 ratio decreased significantly from

Timepoint 1 to Timepoint 2 ($p<0.05$) and Timepoint 3 ($p<0.01$) (Fig 4.7D). Although there were no significant differences, the decreasing tendency of E3/E2 ratio from Timepoint 1 in pituitary and ovaries were evident ($p>0.05$) (Fig 4.7E&F).

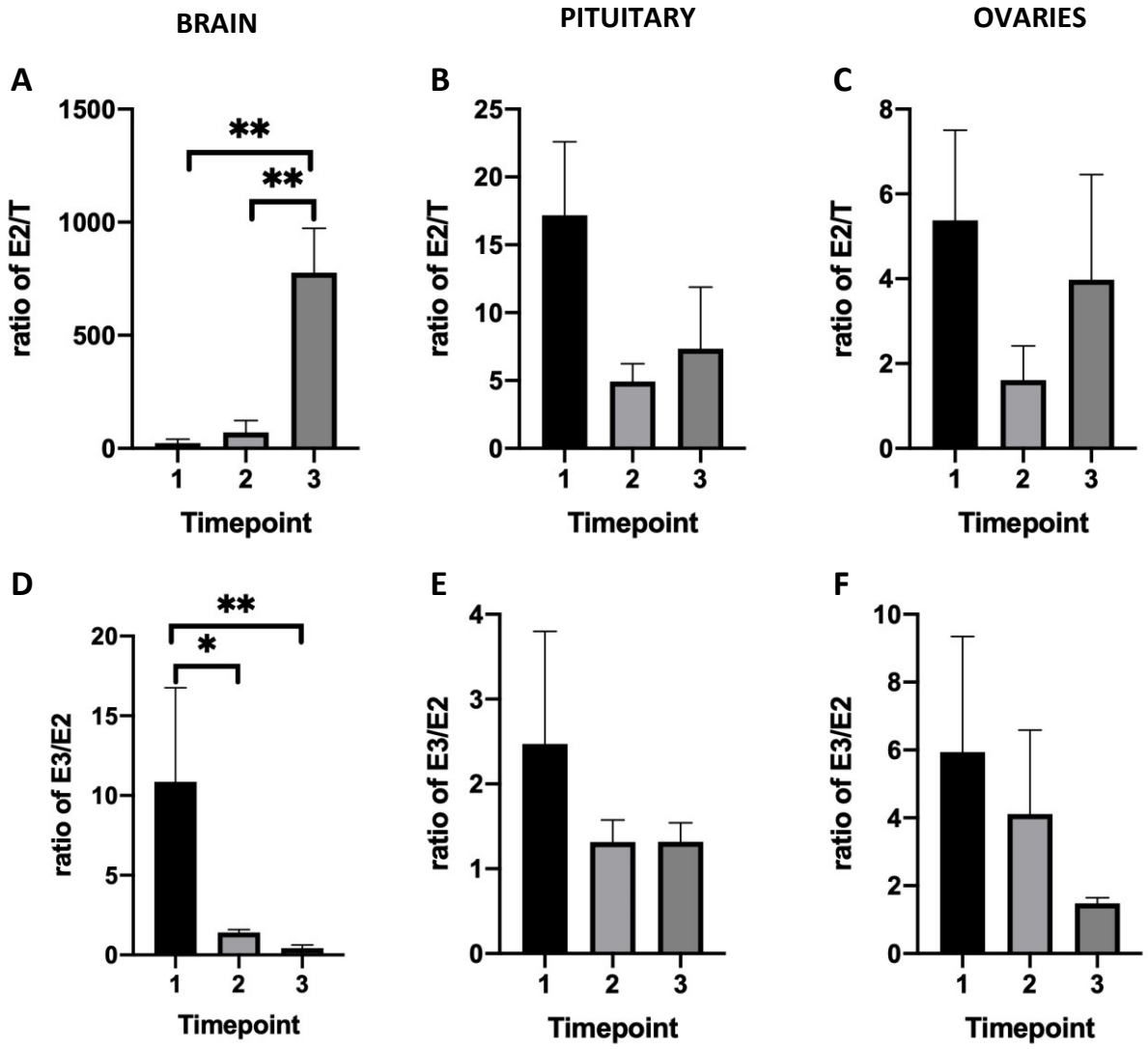

Fig 4.7 E2 to T ratios and E3 to E2 ratios during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. E2 to T ratios in brain (A), pituitary (B), ovaries (C). E3 to E2 ratios brain (D), pituitary (E), ovaries (F). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. **$p\leq0.01$ *$p\leq0.05$

4.3.5 SNa and related fragments fluctuated during the periovulatory period

Levels of the SNa-related peptides varied during the periovulatory period (Fig 4.8). In the whole brain (Fig 4.8A), SNa1-34 fluctuated significantly ($p<0.01$) and reached a peak at Timepoint 2 which was ~ 1.5-fold higher than at Timepoint 1 ($p<0.01$) and Timepoint 3 ($p<0.01$). Significant variations of SNa1-34 levels were also evident in the pituitary. SNa1-34 levels were similarly high at Timepoint 1 and Timepoint 2 ($p>0.05$) (Fig 4.8B), declining by ~50% at Timepoint 3 ($p<0.01$). In the ovaries (Fig 4.8C), the SNa1-34 levels declined gradually from Timepoint 1 to Timepoint 3 ($p<0.05$).

For the SNa1-14 fragment, significant variations were evident in the brain and ovaries, but not in pituitary (Fig 4.9). In contrast to SNa1-34, SNa1-14 levels increased gradually from Timepoint 1 to Timepoint 3 in both of brain and ovaries and peaked at Timepoint 3 (Fig 4.9A&C). In the brain, SNa1-14 at Timepoint 3 was ~5-fold higher than that at Timepoint 1 ($p<0.05$) while not different than that at Timepoint 2 (Fig 4.9A). In the ovaries, SNa1-14 levels were highest at Timepoint 3 and ~4-fold higher than at Timepoint 1 ($p<0.01$) and Timepoint 2 ($p<0.01$) (Fig 4.9C)

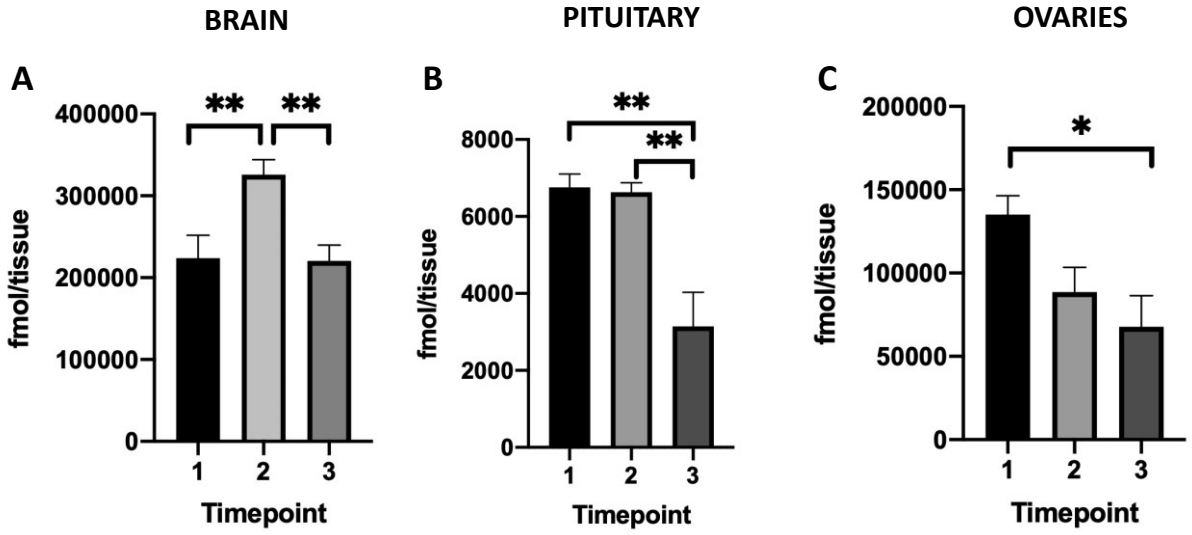

Fig 4.8 SNa1-34 levels vary during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. SNa1-34 in brain (A), pituitary (B), ovaries (C). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *$p\leq0.05$ **$p\leq0.01$

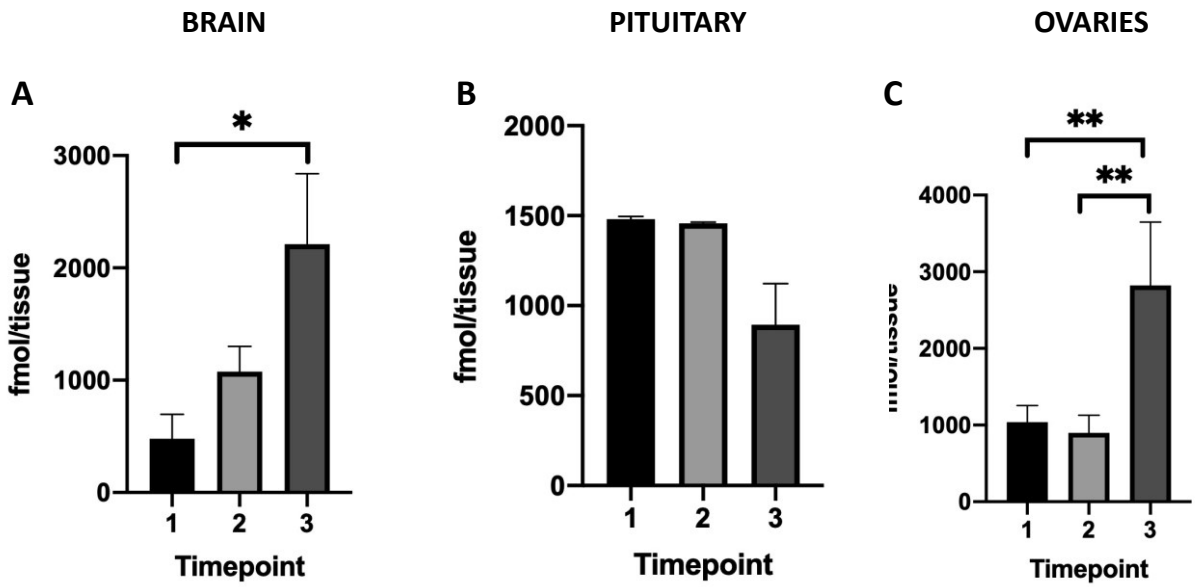

Fig 4.9 SNa1-14 levels vary during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. SNa1-14 in brain (A), pituitary (B), ovaries (C). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *$p \leq 0.05$ **$p \leq 0.01$

The SNa1-18 fragment was detected at a low level in the brain, pituitary and ovaries in the female zebrafish. Variation in SNa1-18 was only observed in the pituitary ($p<0.01$) (Fig 4.10B). The SNa1-18 peptide was not detectable in the pituitary at Timepoint 1 and Timepoint 2, but it was detectable at Timepoint 3 with the means value at 8.1 fmol/pituitary. For statistical purposes we used the minimum detection limit of SNa1-18 when it was not detectable.

The SNa19-34 fragment was detectable in all tissues (Fig 4.11). In brain, SNa19-34 level was lowest at Timepoint 2 and highest at Timepoint 3 ($p<0.05$) (Fig 4.11A). In pituitary, the pattern of SNa19-34 was overall similar to that in brain (Fig 4.11B) and varied significantly during the ovulatory cycle. Levels of SNa19-34 decreased significantly from Timepoint 1 to Timepoint 2 and was highest at timepoint 3 ($p<0.01$). In the ovaries (Fig 4.11C), SNa19-34 was highest at Timepoint 1, but this was not different from the other timepoints ($p>0.05$).

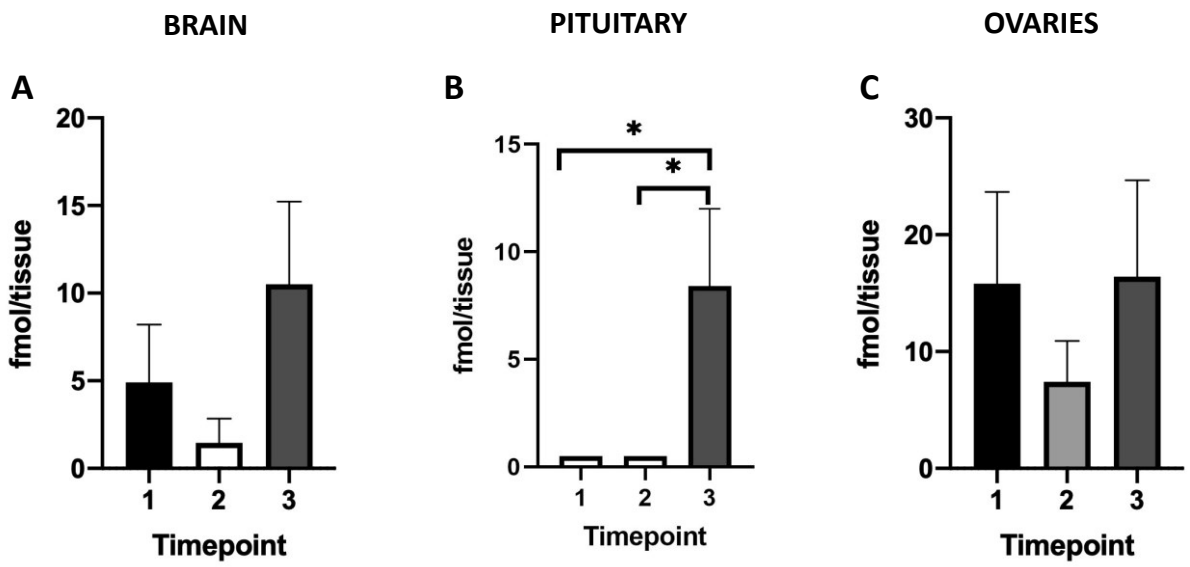

Fig 4.10 SNa1-18 levels vary during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. SNa1-18 in brain (A), pituitary (B), ovaries (C). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *p≤0.05

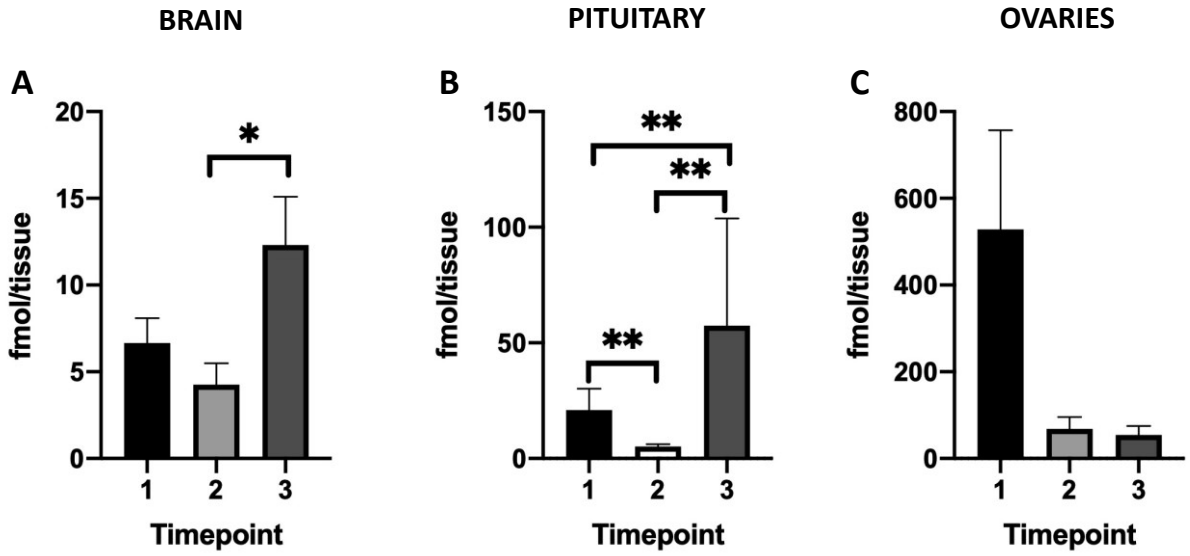

Fig 4.11 SNa19-34 levels vary during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. SNa19-34 in brain (A), pituitary (B), ovaries (C). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *p≤0.05

The data on SNa fragments suggests differential processing of SNa1-34 during the ovulatory cycle. Therefore, to investigate this, the ratios of the smaller SNa peptides to SNa1-34 were calculated. This was possible because we measured all of them in the tissues from individual animals. The SNa1-14/SNa1-34 ratios in the brain, pituitary and ovaries were all highest at Timepoint 3 (Fig 4.12). In brain, the SNa1-14/SNa1-34 was lowest at Timepoint 2, rising significantly thereafter at Timepoint 3 ($p<0.01$) (Fig 4.12A). The SNa1-14/SNa1-34 ratio in pituitary was similar at Timepoint 1 and Timepoint 2 and increased significantly at Timepoint 3 ($p<0.01$) (Fig 4.12B). In ovaries, the SNa1-14/SNa1-34 ratio increased gradually (Fig 4.12C), being different between Timepoint 1 to Timepoint 3 ($p<0.01$).

There was also significant variation of the SNa1-18/SNa1-34 ratio in the brain and pituitary but not in ovaries (Fig 4.13). In brain (Fig 4.13A), SNa1-18/SNa1-34 ratio decreased significantly from Timepoint 1 to Timepoint 2 ($p<0.05$) and was highest at Timepoint 3 ($p<0.05$). In pituitary, SNa1-18 was undetectable, so we calculated the ratio by the minimum detection limit of SNa1-18 levels. Nevertheless, SNa1-34 can be processed in the pituitary to SNa1-18 at Timepoint 3 (Fig 4.13B). Processing of SNa1-34 to SNa1-18 was very low in the ovaries at Timepoint 1 and Timepoint 2 (Fig 4.13C). In contrast the SNa1-18/SNa1-34 ratio variably increased at Timepoint 3, and thus was not statistically significant ($p>0.05$).

The SNa19-34/ SNa1-34 ratio varied significantly in brain and pituitary (Fig 4.14A&B). In both tissues, the SNa19-34/ SNa1-34 ratio was low in the first 2 time points and increased only at Timepoint 3 ($p<0.01$) (Fig 4.14A&B). In contrast, the SNa19-34/SNa1-34 ratio did not change ($P>0.05$) during the periovulatory period (Fig 4.14C).

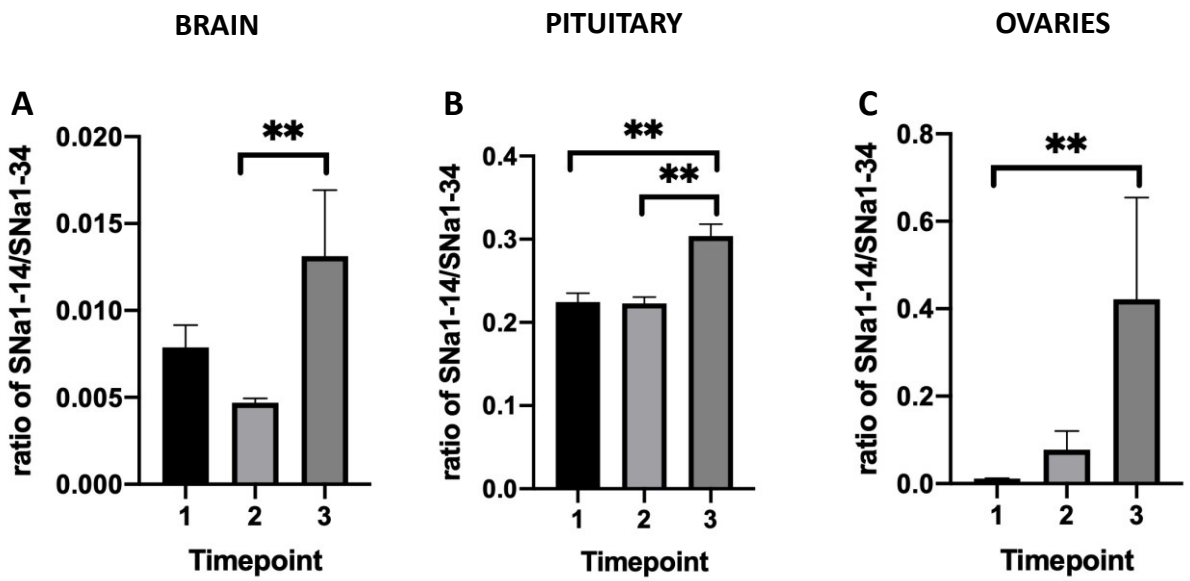

Fig 4.12 SNa1-14 to SNa1-34 ratio varied during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. SNa1-14 to SNa1-34 ratio in brain (A), pituitary (B), ovaries (C). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. **$p \leq 0.01$

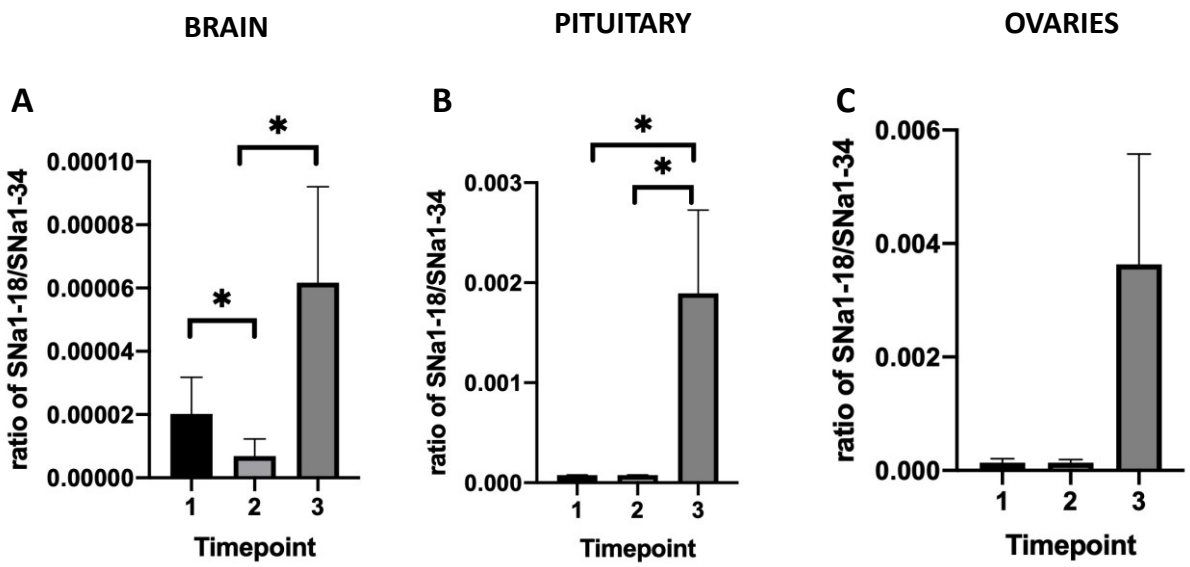

Fig 4.13 SNa1-18 to SNa1-34 ratio varied during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. SNa1-18 to SNa1-34 ratio in brain (A), pituitary (B), ovaries (C). Results are presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. *p≤0.05

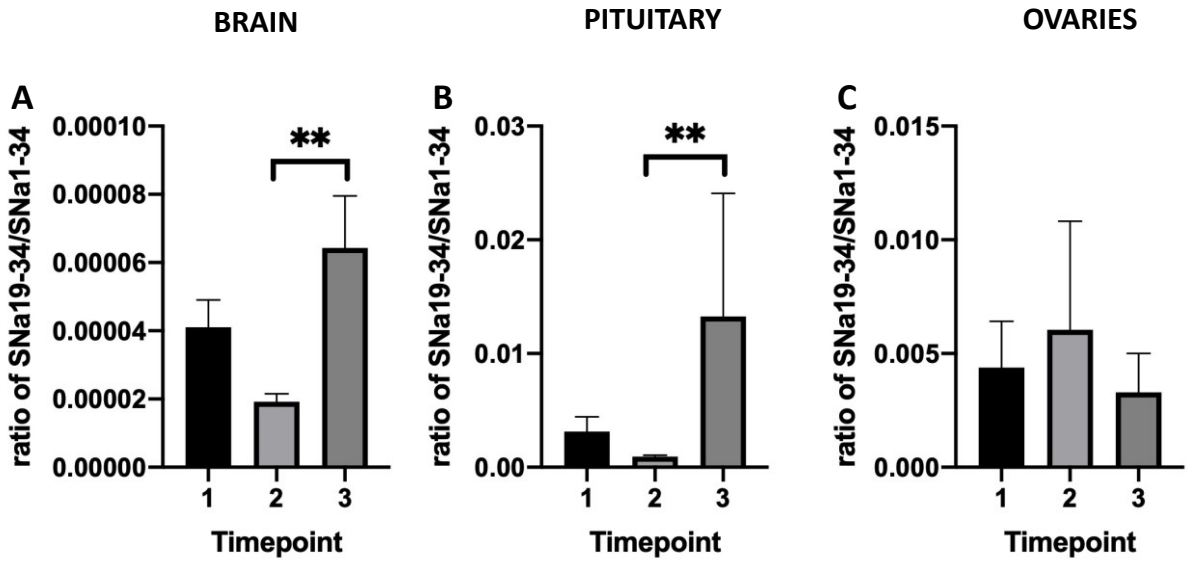

Fig 4.14 SNa19-34 to SNa1-34 ratio varied during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. SNa19-34 to SNa1-34 ratio in brain (A), pituitary (B), ovaries (C). Results were presented as means+SEM (n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test. **p≤0.01

4.3.6 SNa levels were strongly related to Gnrh in female zebrafish brain

Non-linear regression analysis was performed with fourth-order polynomial to investigate both moderate ($0.5 < R^2 < 0.7$) and strong ($R^2 > 0.7$) relations between SNa with the classical reproductive hormones including neuropeptides and sex steroids in the periovulation. The moderate and strong relations were only observed between SNa and Gnrh in brain (Table 4.2) while the R^2 values between SNa and other hormones in pituitary and ovaries were lower than 0.5. SNa1-34 levels were strongly related to levels of Gnrh3 in brain (R^2=0.7067). Meanwhile, the moderate relation was observed between SNa1-14 and Gnrh2 (R^2=0.5765). We performed regression analysis on all other hormones in relation to the SNa fragments, and none were significant (data not shown).

Table 4.2 Non-linear regression analysis of reproductive neuropeptides and SNa related peptides during the periovulatory period in brain of female zebrafish. Results are presented as R^2 value to show the goodness of fit (n=30). * $0.5 < R^2 < 0.7$, ** $R^2 > 0.7$

	SNa1-34	**SNa1-14**
Gnrh2	0.4710	0.5765*
Gnrh3	0.7067**	0.1124
Isotocin	0.3133	0.2629
Vasotocin	0.0626	0.1391
Kiss1	0.0866	0.1030
Kiss2	0.3098	0.0289

4.3.7 SNb and related fragments did not fluctuate during the periovulatory period.

The full SNb1-31 smaller and the smaller SNb1-17 and SNb1-19 fragments were detected in the brain, pituitary and ovaries. In contrast with the SNa-related peptides, there was no significant difference of SNb and related peptides among different timepoints in any tissues ($p>0.05$) (Fig 4.15).

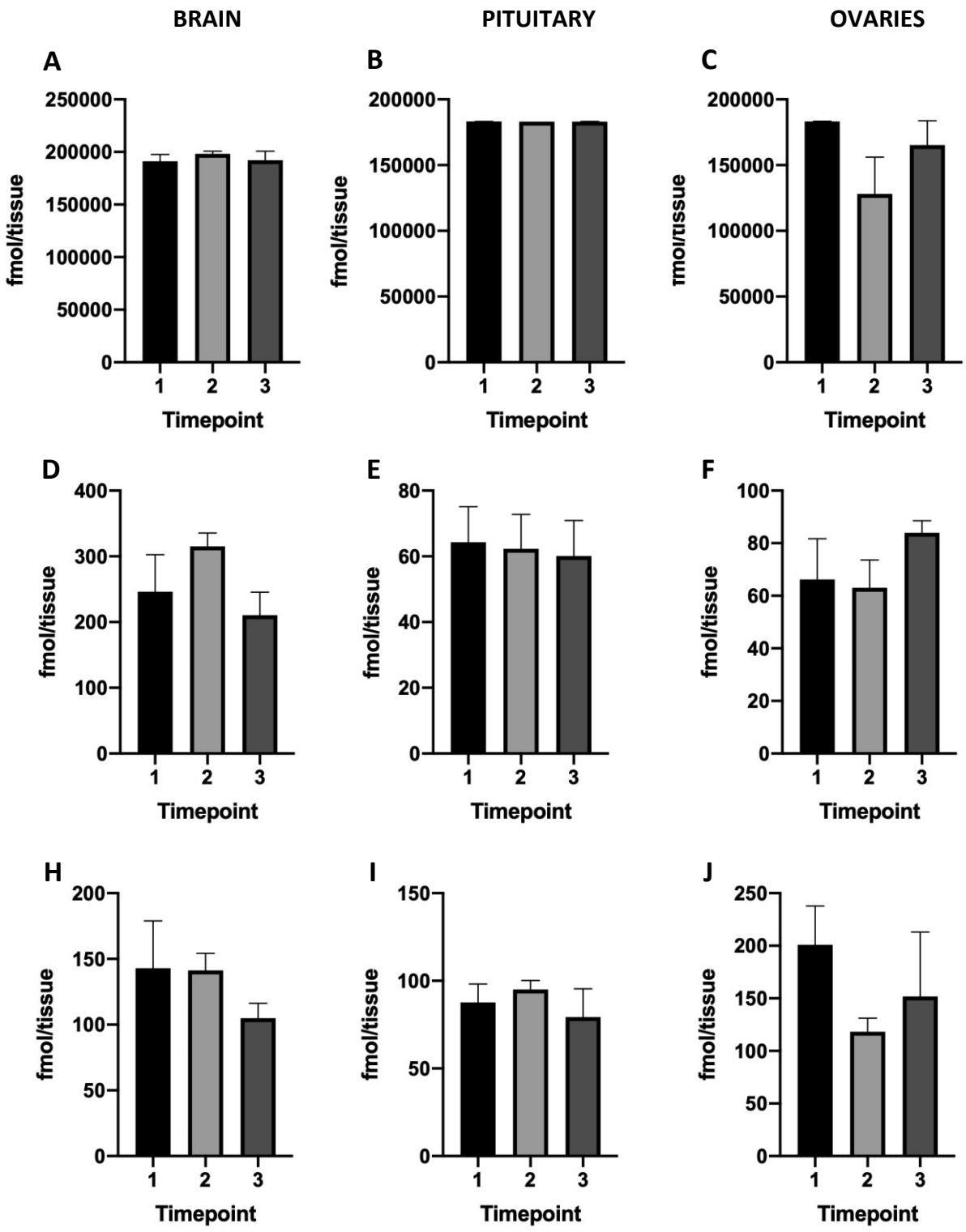

Fig 4.15 SNb related peptides levels during the periovulatory period in brain, pituitary and ovaries of female zebrafish. The timepoints represent (1) 02:00h, (2) 05:00h and (3) 08:30h. SNb1-31 in brain (A), pituitary (B) and ovaries (C). SNb1-17 in brain (D), pituitary (E) and ovaries (F). SNb19-31 in brain (H), pituitary (I) and ovaries (J). Results were presented as means+SEM

(n=10). Note the different scales on the Y-axes. The Kruskal-Wallis one-way analysis of variance on ranks was performed followed by a Tukey test.

4.4 Discussion

In this study, various reproductive hormones were detected in brain, pituitary, and ovaries during the female zebrafish ovulatory process by HPLC-MS. We investigated the hormonal fluctuation of the peptide hormones including Gnrh, nonapeptides, kisspeptin and SN as well as estrogens and androgens. The results revealed that Gnrh3, isotocin, SNa related peptides as well as the steroid hormones including E1, E2, T and 11-KT varied during the periovulatory period while other hormones like Gnrh2, vasotocin, kisspeptin, SNb related peptides and E3 were relatively stable.

Gonadotropin-releasing hormone (Gnrh) is one of the most important reproductive hormones in the vertebrates. It is involved in regulating the ovulation in mammals from human to mouse by stimulating the Lh surge (Ron-El, Raziel et al. 2000, Kanda, Nobukiyo et al. 2021). In mice, a Gnrh agonist can induce the oocyte maturation as well as increase ovulation rate (Kanter, Yildiz et al. 2004). Different with mammals, there are two types of Gnrh peptides in zebrafish, named Gnrh2 and Gnrh3 separately. Moreover, controversial data suggests that Gnrh in zebrafish may be less important than in some mammalian models. The research showed that both of the reduction of Gnrh levels and the loss of Gnrh neurons induce the infertility in mice (Papalexi, Persson et al. 2005, Tata, Huijbregts et al. 2014). But in zebrafish, the lack of both Gnrh2 and Gnrh3 did not impact reproduction significantly (Marvel, Spicer et al. 2018). Yet animals lacking GnRH3 neurons following larval neuronal ablation of exhibited arrested oocyte development as adults (Abraham, Palevitch et al. 2010). It may be that Gnrh neurons produce more than one stimulatory peptide (Trudeau 2018). Nevertheless, variation of brain Gnrh3 levels was observed in our study. This suggests that Gnrh3 plays a key role in the ovulatory process in zebrafish. From the results, higher Gnrh3 levels were detected in the brain and ovaries rather than that in pituitary while Gnrh2 were detected highest in the brain only. Moreover, in each tissue, the amount of Gnrh3 were more than Gnrh2. Gnrh3 is associated with the steroidogenesis and gonadal synchrony process in goldfish (Andreu-Vieyra and Habibi 2000). The existence of Gnrh3 in ovaries shown in our study is supportive of an intragonadal

role. Additionally, Gnrh3 regulates the primordial germ cells proliferation and sex differentiation in embryonic zebrafish (Feng, Cui et al. 2020). These results indicate that the role of Gnrh2 and Gnrh3 in zebrafish development and reproduction is important despite the conclusions from gene mutation studies. The Gnrh3 levels in zebrafish brain were significantly higher during the Lh surge. It suggested that Gnrh3 in zebrafish performs the function of GnRH1, the mammalian homologue, which can regulate the Lh production and secretion to stimulate the Lh surge. The focus of most studies has been on Gnrh3 and Gnrh1, so much remains to be discovered about Gnrh2. In goldfish, both Gnrh2 and Gnrh3 stimulated Lh release, invoking somewhat different intracellular signaling pathways (Chang and Pemberton 2018). A very recent in vivo study in zebrafish revealed that fasting can induce the development of Gnrh2 neural fibers indicating that Gnrh2 neurons may be activated during fasting to help maintain reproduction (Marvel, Levavi-Sivan et al. 2021). Moreover, both Gnrhs stimulate growth hormone release from the goldfish pituitary, which can have both reproductive and somatic roles (Chang and Pemberton 2018). Although the Gnrh2 peptide could be detected in the brain, pituitary and ovaries, the concentrations of it in the pituitary and ovaries were significantly lower than that in the brain. This result was consistent with another study in 2017 (Corchuelo, Martinez et al. 2017). As for the functions, we suggest Gnrh2 may not be the key player in the Lh surge since it did not increase at that time.

There was no significant variation of Kiss1 and Kiss2 levels during the Lh surge or at ovulation in our studies in zebrafish. This suggests that Kiss1 and Kiss2 are not involved in zebrafish ovulation, at least during the times we have studied. This proposal is supported by other observations. Mutation of kisspeptin and kisspeptin receptors did not impact zebrafish reproduction (Tang, Liu et al. 2015) , while in mammals the lack of kisspeptin leads to abnormalities of gonadal development and infertility (Hellier, Brock et al. 2018). Kisspeptin is a hypothalamic peptide which plays a key role in the reproduction in mammals (Asaba, Osakada et al. 2017). Kisspeptin is able to regulate reproductive behavior, such as olfaction (Kauffman, Park et al. 2007), audition (Comninos, Wall et al. 2017) and partner preference (Clarkson, d'Anglemont de Tassigny et al. 2008, Colledge and d'Anglemont de Tassigny 2010, Piet, Kalil et al. 2018) in mammals. Multiple studies in teleost have demonstrated that kisspeptin is

necessary to induce Lh surge and ovulation by stimulating Gnrh via GPR54, the kisspeptin receptor (Tang, Liu et al. 2015). Data on kisspeptins in fish is far less clear (Somoza, Mechaly et al. 2020). In some fish species, Kiss1 and Kiss2 may stimulate gonadotropin synthesis and secretion, yet their actions appear not to be mediated by Gnrh neurons as in mammalian models. In zebrafish and medaka, at least, hypophysiotropic Gnrh neurons do not express Kiss receptors. These contrasting results support the idea that teleosts and mammals have evolved different neuroendocrine control mechanisms.

Variations in isotocin and vasotocin levels during in the periovulatory period were also assessed. They are the respective homologs of mammalian oxytocin and vasopressin. Interestingly, patterns of variations in isotocin in brain was different from that in pituitary. In the zebrafish brain, isotocin was low during the Lh surge but increased significantly at the time of ovulation. However, in the pituitary, isotocin was highest at the earliest sampling, a time when we expected highest *lhb* mRNA levels, and then decreased significantly thereafter. The nonapeptides are highly conserved multifunctional hormones in vertebrates. Their roles in social behavior in various species has been revealed (Madden and Clutton-Brock 2011, Wircer, Blechman et al. 2017, Ataei Mehr, Garner et al. 2020). Considerable research has focused on the role of the nonapeptides in reproductive behavior (Kleszczynska, Sokolowska et al. 2012, Yokoi, Okuyama et al. 2015). Although much evidence is lacking, our results suggest a possible role for isotocin in the Lh surge. In rats, exogenous oxytocin not only stimulates the Lh surge but also induces lordosis behavior (Caldwell, Prange et al. 1986, Johnston, Lopez et al. 1990). However, isotocin has only a minor stimulatory effect on Lh release from dispersed goldfish pituitary cells *in vitro* (Mennigen, Volkoff et al. 2017) but more robustly enhanced Lh in the ricefield eel (Yang, Zhang et al. 2021). A recent study in *Gambusia affinis* revealed that isotocin could increase female avoidance of males in the coercive mating (Ramsey, Fry et al. 2019). These results indicated that isotocin may not only be involved in the social behavior but has a potential role for Lh release in fish. On the other hand, *oxt* gene function is required for mate choice based on familiarity recognition in medaka (Yokoi, Naruse et al. 2020). They found that oxt is important for female recognition of familiar males. Moreover, mutation of *oxt* or *oxtr1* did not affect spawning success in these medaka, supporting the concept that the nonapeptides

modulate sociosexual behaviors rather than ovulation itself. As for vasotocin levels, there was no significant fluctuation during the ovulation period in zebrafish. In female halfspotted goby, both the size and number of the vasotocin-producing cells in the preoptic area were increased in the spawning season (Maruska, Mizobe et al. 2007).

We were able to quantify steroids in the various tissues even though steroids are not stored but tend to be produced and quickly released when the steriodogenic pathway is activated. Estrogens are a group of sex steroid hormones including E1, E2 and E3. Previous studies in zebrafish revealed that E2 plays a key role in vitellogenesis (Nagahama, Yoshikuni et al. 1995). During ovarian follicle growth, androstenedione and T are respectively converted to E1 and E2 by aromatase A in the granulosa cells. In the brain and pituitary aromatase B predominates (Goto-Kazeto, Kight et al. 2004) and also converts the androgen precursors to E1 and E2. In the case of the teleost brain, the radial glial cells are the exclusive site of aromatase B expression and thus is the steroidogenic cell (Xing, Goswami et al. 2014, Diotel, Charlier et al. 2018).

Along the course of ovarian development, ovarian E1 and E2 are secreted into the blood and subsequently activate nuclear estrogen receptors in hepatocytes to stimulate vitellogenin synthesis (Okumura, Todo et al. 2002, Dammann, Shappell et al. 2011, Trudeau, Heyne et al. 2011). All 3 estrogens exhibit significant transactivational activities for all 3 zebrafish nuclear ERs(Menuet, Pellegrini et al. 2002, Asnake, Modig et al. 2019). Over the period of our assessment, ovarian E2 and E3 levels were highest at timepoint 1, but decreasing thereafter, being lowest at timepoint 3. At timepoint 1 there was high variation between females, so there were no statistically significant variations in ovarian E1, E2 and E3. Intraovarian E2 (and presumably also E1) plays a key inhibitory role via G protein-coupled estrogen receptor (Gper), controlling the onset of oocyte maturation and meiotic cell-cycle progression (Peyton and Thomas 2011). After the ovulatory Lh surge, steroidogenesis favors the increased synthesis of a maturation-inducing steroid (MIS, e.g., 17α,20β-dihydroxy-4-pregnen-3-one and related progestins) that induces final maturation of the oocyte via nongenomic actions involving a membrane progesterone receptor (Zhu, Rice et al. 2003). Ovarian T levels were generally low,

while 11-KT was higher, levels did not vary significantly over the study. This implies that most of the T is converted to E2 and 11-KT.

The surprising results of steroid analysis relate to brain and pituitary. The highest levels of pituitary E1 were found at timepoint 1, about 6 hrs before ovulation. The highest levels of brain E1 were noted at timepoint 3, at ovulation. Highest levels of E2 occurred at timepoint 3, coincident with the lowest brain T levels. Calculation of the E2/T ratios for brain indicate a major shift to E2 production at ovulation. That the brain E2/E3 ratios were their lowest at ovulation suggests a decrease in E2 metabolism (Thomas and Potter 2013), contributing to high E2 at timepoint 3. While whole brain tissue steroid levels may also reflect some uptake from the periphery, these results more likely reflect the unsurpassed capacity of the teleost brain, and specifically, radial glial cells to synthesize estrogens (Xing, Goswami et al. 2014, Diotel, Charlier et al. 2018). The functions of androgens and estrogens in the teleost brain are manyfold. On a timescale of a week or more, 11-KT and E2 can respectively increase or decrease neurogenesis in teleosts (Xing, Goswami et al. 2014, Diotel, Charlier et al. 2018). In particular, 11-KT modulates the expression of genes related to the cell cycle processes and can enhance Gnrh3 neuronal production in tilapia (Narita, Tsutiya et al. 2018). Long term reductions in total E2 following aromatase inhibition with fadrozole modulates expression of numerous estrogen-responsive genes in the goldfish telencephalon related to regulation of calcium signaling pathway and autoregulation of estrogen receptor action, among other processes (Zhang, Popesku et al. 2009). Estrogens can also regulate both the glutamic acid decarboxylase and ornithine decarboxylase/putrescine pathways for GABA synthesis in goldfish, indicating that estrogens also modulate the stimulatory GABAergic tone on Gnrh neurons and on Lh release in this model species.

The roles of brain estrogens on the short timescale of the periovulatory period studies here is a matter of speculation but may alter the synthesis of several neurotransmitters and neuropeptides, given the distribution of the nuclear ERs (Menuet, Pellegrini et al. 2002) and androgen receptors (Gorelick, Watson et al. 2008) in zebrafish brain. It is important to note that radial glial cells surround the isotocin neurons of the goldfish preoptic area express Gper, the membrane estrogen receptor (Mangiamele, Gomez et al. 2017). That isotocin and E2 levels

are both maximal at ovulation suggests a strong relationship that should be further investigated. Estrogens can increase neuronal firing rates in reproductively relevant brain circuits in midshipman fish within minutes (Remage-Healey and Bass 2007), or within 2 days in Gnrh1 neurons of medaka (Ikegami, Kajihara et al. 2022). Thus, the observed rapid shift in steroid dynamics in the female zebrafish brain over the 6.5 hr period is on a physiologically relevant timescale.

Similar result was also shown in our study, E1 and E2 were significantly increased in the brain during ovulation at timepoint 3. The association between E1 and ovulation is still not well studied. Results presented here suggest a reproductive role for E1 in fish. Moreover, the variations of E1 and E2 levels in the pituitary were on the contrary, they were at the peak during the expression of Lh and decreased significantly after the Lh surge occurred. The same tendency was observed in goldfish and mares (Kobayashi, Sorensen et al. 2002, Ginther, Beg et al. 2009). Both stimulatory (Herbison 1998) and inhibitory (Ng, Wolfe et al. 2009) effects of ovarian E2 feedback on Gnrh and Lh regulation are well-studied in mammals, so it is tempting to speculate that brain E2 has a role in fishes. However, in terms of the preovulatory Lh surge that is driven by E2 in mammalian models, the patterns observed in zebrafish, and especially the ovulatory increases in E1 and E2 indicate other roles. It is possible that increased brain estrogen levels could regulate post-ovulatory spawning behaviours, for example, egg laying. Data from our lab indeed suggest that brain estrogens control the timing of oviposition in female zebrafish (K. Shaw and V.L. Trudeau, unpublished). As for E3, it is a main estrogen in in pregnant mammals (Ding and Zhu 2016). There were no major changes in E3 during the zebrafish ovulatory cycle.

We detected T and 11-KT during the periovulatory period in female zebrafish. Studies in mice indicated that androgens affected granulosa at preantral and antral stages (Lenie and Smitz 2009). Moreover, androgens also play a key to enhance Fsh stimulation of follicular differentiation (Hossein Rashidi, Hormoz et al. 2009). Consistently, our results revealed that steroidal levels of these two androgens in brain were high before ovulation but decreased significantly at the timepoint of ovulation. It suggested that androgens are not specifically involved in the ovulatory process. A significant increase in the E2/T ratio was observed in the

brain at ovulation (timepoint 3). The significant increase of E2 and decrease of T is at the same time. E2 is produced from T by aromatase B in fish brain and aromatase A in gonads (Piferrer and Blazquez 2005). Meanwhile, the significantly decreasing E3/E2 ratio in brain in the ovulatory process suggests the rising of E2 levels and coincident falling of E3 levels. E3 is a product of E2 metabolism (Thomas and Potter 2013).

The main goal of this work was to compare periovulatory changes in the classical hormones with that of the emerging reproductive neuropeptides SNa and SNb that are derived from selective processing of the Scg2a and Scg2b precursor proteins. We detected SNa and SNb in the 3 tissues examined. While we have already detected SNa using immunocytochemistry, the presence of these peptides in ovaries was surprising. This raises the possibility that SN may regulate reproduction not only in the hypophysiotropic system, but also in gonads. To our knowledge only a single study has examined Scg2 in the mammalian ovaries (Hannon, Duffy et al. 2018). Luteinizing hormone increases Scg2 in human, primate and rodent granulosa cells during the periovulatory period. It is also likely that SN modulates ovulatory angiogenesis in the human ovaries in the periovulatory period. Moreover, female zebrafish produced more SNa and SNb than males (Chapter 3). It may be that SN peptides have a more important role in females than males. This hypothesis is supported by the previous research in our lab, which indicated that frameshift mutations in Scg2a and Scg2b impacted female zebrafish more than males (Mitchell, Zhang et al. 2020). This research also showed that there was no significant difference of the *scg2* transcript levels between male and females (Mitchell, Zhang et al. 2020).

Peptidomic analysis revealed the production of four SNa related peptides (SNa1-34, SNa1-14, SNa1-18, SNa19-34) while three SNb related peptides were detected (SNb1-31, SNb1-17, SNb19-31). While we are the first to attempt quantification of the specific fragments, an earlier report in the bovine hypothalamus did detect SN1-33, SN1-14, SN1-18 and SN19-33 (Colgrave, Xi et al. 2011). In comparing zebrafish SNa with the single bovine SN, there is a very similar cleavage pattern. We hypothesized that some SN peptides could be biologically active after cleavage. To confirm the candidate fragments we synthesized all the fragments from SNa and SNb and generated standard compounds for HPLC-MS.

Of the various SN forms, SNa1-34 was the highest. As for the SNa fragments, SNa1-14 levels were significantly more than SNa1-18 and SNa19-34. Moreover, significant periovulatory variations were observed only for SNa1-34 and SNa1-14, suggested that both of SNa1-34 and SNa1-14 may play different roles in regulating the ovulation. The SNa1-34 peptide was highest in the first 2 timepoints when the respective expected peaks in *lh* mRNA and the Lh surge occur. In goldfish, SNa injection *in vivo* can increase serum Lh in 30 minutes (Blazquez, Bosma et al. 1998). *In vitro*, a single treatment of SNa can sustain Lh release over a 6-12h period (Zhao, Basak et al. 2006, Zhao, Basak et al. 2009, Zhao, Grey et al. 2010). Mouse SN also rapidly stimulates *lhb* mRNA and Lh release via the protein kinase A (PKA) and cAMP-induced ERK signaling pathways within 3 hrs in LβT2 gonadotrophs (Zhao, McNeilly et al. 2011). It is also important to note that Gnrh3 stimulates Scg2a mRNA and Scg2a precursor protein cleavage in the goldfish pituitary (Zhao, McNeilly et al. 2011). Importantly, a single SNa injected in subfertile carrying frameshift double mutations in Scg2a and Scg2 enhances spawning by ~3 fold, similar to hCG in the same mutant line (Mitchell, Zhang et al. 2020). Thus these coorindated changes in brain and pituitary SNa levels support these other observations and strongly implicates SN in the ovulatory process.

Interestingly, SNa1-14 levels peaked at ovulation (Timepoint 3) in both brain and ovaries. Since there is no related research on SN fragments it is tempting to speculate on the importance of these observations. It is possible that SNa1-14 is involved in zebrafish or that SNa1-14 (and other fragments) are inactive degradation products of SNa1-34. The SNa1-14 to SNa1-34 ratio significantly increased in all the three tissues at ovulation. To investigate this further, we i.p. injected SNa1-14 into females and studied gene expression responses and ovulation (Chapter 5). Levels of SNa1-18 and SNa19-34 very low compared to all other fragments, although significant alterations of the SNa1-18 and SNa19-34 levels were still observed. Analysis of their ratios to SNa1-34 ratio suggests a higher production at ovulation in several tissues. These data suggest increased degradation of SNa1-34 following the Lh surge, but the biological significance remains to be investigated.

Noteworthy is the observation that whole brain SNa and Gnrh3 peaked at timepoint 2 at the time of the Lh surge, proceeding ovulation by ~3.5 hrs. Therefore, non-linear regression

analysis was performed to analyze the association of SNa1-34 and SNa1-14 peptides with other reproductive hormones. There was a strong relation of SNa1-34 with Gnrh3 (R^2=0.7067). This result suggests the role of SNa1-34 in inducing the Lh surge by stimulating the production of Gnrh3. This is supported by the observation that i.p. injection of SNa1-34 increased *gnrh3* within 3 hrs (Mitchell 2018). As for Gnrh2, it was not strongly related with SNa1-34 (R^2=0.471), but moderately associated with SNa1-14 (R^2=0.5765). The nonsignificant variation of Gnrh2 in the periovulatory period implies that Gnrh2 was not involved in the ovulation. However, *Gnrh2* mutant zebrafish spawn but oocyte quality is reduced (Marvel, Spicer et al. 2019). Therefore, we subsequently investigated the biological activities of SNa1-14 in Chapters 5.

In marked contrast with SNa, SNb related peptides levels did not vary in the periovulatory period in either brain, pituitary or ovaries. One the one hand this was surprising, since Scg2b mutant zebrafish have decreased spawning rates compared to wildtype. However, SNb injection does not rescue spawning in subfertile mutants (Mitchell, Zhang et al. 2020). Much remains to be discovered on the role of Scg2b/SNb in teleost.

In conclusion, this study details the rapid periovulatory changes in 18 reproductive hormones in the zebrafish brain, pituitary and ovaries. These observations strongly implicate SNa in the ovulatory process. In subsequent experiments, we have focused on the biological activities of SNa1-34 and SNa1-14 in female zebrafish (Chapter 5).

Chapter 5 Intraperitoneal injection of SNa activates the brain-pituitary-gonadal axis and stimulates ovulation in female zebrafish

5.1 Introduction

Ovulation is the process in female reproduction whereby mature oocytes are released from follicle. In order to start ovulation, the mature follicular cells around the oocytes must be stimulated by various associated hormones, inducing key morphological changes (Espey and Richards 2006). During this process, it is the Lh secretion surge that is indispensable to induce the follicular cells to produce the ovarian maturation-inducing hormone (Aizen, Pang et al. 2018). Studies in zebrafish and medaka revealed that the absence of Lh causes infertility in females by disrupting the ovulation (Zhang, Zhu et al. 2015, Takahashi, Kanda et al. 2016).

Previous results in presented above have revealed the natural variations of SNa1-34 and SNa1-14 peptides as well as other classic reproductive hormones including Gnrh, nonapeptides and sex steroids during the periovulatory period (Chapter 4). Moreover, polynomial regression revealed strong relationships between SNa and both Gnrh3 and Gnrh2 in whole brain samples (Chapter 4). Gnrh is the well-known stimulator of the Lh surge and ovulation in vertebrates (Atkins, Busch et al. 2008, Herbison, Porteous et al. 2008, Zohar, Zmora et al. 2021). Double immunofluorescence revealed the colocalization of SNa-ir and isotocin (Oxt)-ir in preoptic area and posterior pituitary which suggested that SNa may be co-released with isotocin (Chapter 2). Oxytocin release is associated with the Lh surge and ovulation in mammals (Johnston, Lopez et al. 1990, Melli, Tagavi et al. 2007, Roushangar, Soleymanirad et al. 2009). These results suggested that SNa peptides may be involved in the zebrafish ovulatory process by inducing other reproductive hormones leading to the Lh surge. This hypothesis is also supported by previous studies in our lab which indicated that SNa1-34 stimulates Lh secretion in goldfish (Zhao, Basak et al. 2009).

To reveal the regulatory effects of SNa peptides on reproductive processes, we performed the intraperitoneal (i.p.) injections with SNa1-34 and SNa1-14 to female zebrafish and detected the expression of the reproductive genes by ddPCR. Moreover, the ovulatory

status was analyzed after SNa1-34 and SNa1-14 i.p. injection to investigate the role of SNs peptide in ovulation.

5.2 Materials and methods

5.2.1 Experimental animal husbandry

Experimental zebrafish (AB stain) were bred and kept in the University of Ottawa aquatics room at the temperature of 28°C. The lights-on time was set for 14 hours from 0900h to 2300h. Wildtype zebrafish were provided from the University of Ottawa aquatics facility and fed twice a day. All animal experiments followed the guidelines of the Canadian Council on Animal Care for the use of animals in research, approved by the University of Ottawa Animal Care Protocol Review Committee.

5.2.2 Intraperitoneal injection and tissue dissection

The SNa1-14 and SNa1-34 peptides were synthesized and purified by our lab (see Chapter 3). The purity of the peptides was over 95% based on absorbance spectra. The amino acid sequence of the zebrafish SNa1-14 is TNENAEEQYTPQKL and for zebrafish SNa1-34 is TNENAEEQYTPQKLATLQSVFEELSGIASSKTNT.

Intraperitoneal (i.p.) injection was performed in female zebrafish to analyze the *in vivo* effect of SNa1-14 and SNa1-34 on reproductive gene expression. All fish were sexually mature and between 6-9 mpf. To synchronize their reproductive cycle females were separated from males and kept in groups of 12 in 3L tanks for two weeks before the injection. On the experimental day, zebrafish (n=16) were randomly assigned to three groups, anaesthetized in 4.3% tricaine solution and performed i.p. injection by a 32-gauge Hamilton syringe between 10:00-110:0 with saline (pH=7; 10 µL/g body weight), SNa1-14 (1 nmol/g body weight) and SNa 1-34 (1nmol/g body weight). At 3 and 6 hours after injection, 8 zebrafish in each group were sacrificed on ice. After that, telencephalon, hypothalamus, pituitary and ovaries were dissected from each individual and collected in 1.5 ml Eppendorff tubes. The samples were flash frozen in liquid nitrogen after collection and kept at -80°C until RNA extraction.

5.2.3 Total RNA isolation

Total RNA from the telencephalon, hypothalamus and ovaries were isolated by RNeasy Plus Mini Kit (Qiagen, Cat#74136) while the total RNA from pituitary were extracted by RNeasy Plus Micro Kit (Qiagen, Cat#74034). The RNA extraction protocol was followed the manufacturer's handbook. After isolation, the total RNA in each sample was quantified immediately.

5.2.4 RNA quantification

Because of the low amount of the total RNA in the zebrafish tissues, Quant-iTTM RiboGreen® RNA Reagent and Kit (Invitrogen, Cat#R11490) was selected to quantify total RNA. The protocol followed by the manufacturer's handbook and was optimized in our laboratory.

5.2.5 cDNA synthesis

After quantification, 50 ng total RNA per sample were reverse transcribed into cDNA immediately by using Sensiscript Reverse Transcription Kit (Qiagen, Cat# 205213). The reverse transcription reaction followed by the manufacturer's handbook. The reaction mixture was incubated at 37°C for 60 min and stored at -20°C until PCR quantification.

5.2.6 Primers design for gene expression analysis

Primer design followed Mitchell et al. (Mitchell, Zhang et al. 2020) and was performed online (http://bisearch.enzim.hu/). Primers were synthesized by Integrated DNA Technologies. The annealing temperature of all the primers were 58°C. The primers were validated by RT-PCR and the single product amplification was confirmed by the melting curve methods. The PCR products were sequenced, and the specificity was confirmed by Basic Local Alignment Search Tool (BLAST). The primer information is shown in Table 5.3.

Table 5.1 Information of primers for ddPCR

Gene (abbreviation)	Tissue analysed	Sequence (5`-3`)	Amplicon size(bp)
Elongation factor alpha (*ef1a*)	Tel, Hyp, Pituitary, Ovaries	Fw: CAAGGAAGTCAGCGCATACA Rv: ACCGCTAGCATTACCCTCCT	166
Gonadotropin-releasing hormone 2 (*gnrh2*)	Tel, Hyp	Fw: CAGAGGTTTCAGAGGAAGTGAAGC Rv: TGAGGGCATCCAGCAGTATTG	154
Gonadotropin-releasing hormone 3 (*gnrh3*)	Tel, Hyp	Fw: ATGGAGGCAACATTCAGGATGT Rv: CCTTTCAGAGGCAAACCTTCA	131
Isotocin (*oxt*)	Tel, Hyp	Fw: GATCTGCTGCTGAAGCTCCT Rv: TACAAAAGTGGGTGGCGAGT	134
Vasotocin (*avp*)	Tel, Hyp	Fw: AGGTCTGCATGGAAGAGGAG Rv: CTGCCTTCAGGACAGTCTGG	146
Follicle stimulating hormone beta (*fshb*)	Pituitary	Fw: TGTGGAGAGCGAAGAATGTG Rv: AGACCTTCTGGGTGTGCTGT	116
Gonadotropin- releasing hormone receptor 2 (*gnrhr2*)	Pituitary	Fw: TCCTCAACCCTCTGTCCATC Rv: TGCTTTGGGGAATCAATCTC	123
Glycoprotein alpha (*cga*)	Pituitary	Fw: CTGCTGCTTTTCGAGAGCTT Rv: AGTGGCAGTCTGTGTGGTTG	155
Luteinizing hormone beta (*lhb*)	Pituitary	Fw: AATGCCTGGTGTTTCAGACC Rv: AACAGTCGGGCAGGTTAATG	144
cyp19a1a	Ovaries	Fw: ACTGAAAGGGCTCAGGACAA Rv: TCCAACTGACCTGGAATGTG	174
Luteinizing hormone receptor (*lhcgr*)	Ovaries	Fw: TGAAAGAGCAGCCAGGTACT Rv: TGCTAAATTTCCTTTCGCCG	166
Nuclear Progesterone Receptor (*npr*)	Ovaries	Fw: GGGCCACTCATGTCTCGTCTA Rv: TCTCCACTCTGAAAATATGTGGACTTT	198
Steroidogenic acute regulatory protein 1 (s*tar1*)	Ovaries	Fw: GGTCTGAGGAAGAATGCAATGAT Rv: CCAGGTCCGGAGAGCTTGT	169

5.2.7 Digital droplet PCR (ddPCR)

All cDNA samples were diluted to a final concentration in the range of 10 to 100 copies/µl in the ddPCR reaction. The recipe of the ddPCR reaction followed the manufacturer's handbook. 20 µl of the ddPCR reaction mixture and 65 µl of droplet generation oil (Bio-Rad, Cat# 1864006) were added into the wells of the droplet generation cartridges (Bio-Rad, Cat# 1864007) and mixed in the QX200 droplet generator (Bio-Rad, Cat# 1864002) to generate

between 10,000 to 20,000 droplets. After that, the mixture was transferred into the ddPCR 96-well PCR plate (Bio-Rad, Cat# 12001925) and sealed with the PCR plate heat seal foil (Bio-Rad, Cat# 1814040) in the PCR plate sealer (Bio-Rad, Cat# 1814000). The sealed PCR plate was placed in the thermal cycler (Bio-Rad, Cat# 1851196) and performed the PCR reaction with the following cycling conditions: 1× (95 °C for 5 min); 50× (95 °C for 30 s, 58°C for 1 min, 4 °C for 5 min) with a ramp rate of −2 °C/s and 1× (90 °C for 5 min).After the PCR reaction, the plate was sent in the QX200 Droplet Reader (Bio-Rad, Cat# 17005228) to analyze the fluorescent signals.

5.2.8 Analysis of ovulation

The goal of this experiment was to determine if SN could induce ovulation in the absence of males, and outside the normal nighttime preovulatory period, given that a single injection of SNa increased the expression of key genes in the brain, pituitary, and ovaries. Seventy-two adult female zebrafish at the age of 7-8 mpf were selected and isolated from males for 2 weeks. On the experimental day, they were randomly separated into 4 groups (n=18) and anaesthetized in 4.3% tricaine solution and performed i.p. injection by a 32-gauge Hamilton syringe with saline (pH=7; 10 µL/g body weight), SNa1-14 (1nmol/g body weight) and SNa 1-34 (1nmol/g body weight). The Lh analog hCG (50 IU/g body weight) (Millipore, cat# 230734-1MG) served as a positive control as it is a well-characterized stimulator of ovulation in zebrafish (Mitchell, Zhang et al. 2020). At 6 hours after injection, the fish were anesthetized in 4.3% tricaine solution and dissected to collect ovaries. In order to weaken the attachment between oocytes and follicular layer, the dissected ovaries were incubated in modified Cortland's medium without calcium and magnesium which contained 0.125M NaCl, 0.012M $NaHCO_3$, 0.003M Na_2HPO_4, 0.005M KCl, 0.001M Na_2EDTA, 1g BSA, 5.2g HEPES, 0.03g penicillin, 0.05g streptomycin in 1L of ultrapure water (Tse and Ge 2010). After that, the numbers of ovulated and non-ovulated eggs for each fish were counted.

5.2.9 Data analysis

The ddPCR data were analyzed using the Bio-Rad QuantaSoft 1.7 software. The data for each tissue except the expression of *ef1a* were normalized using the Norma-Gene algorithm (Heckmann, Sorensen et al. 2011) and presented as means+SEM (n=8). These gene expression

data were statistically analyzed on GraphPad Prism 9.0 software. Normality and homoscedasticity were tested using the Shapiro-Wilk and Levene's tests, respectively. Two-way ANOVA followed by Tukey's multiple comparisons test. The number of females ovulating in each treatment group was analyzed using Fisher's Exact. It was found that only those injected with SNa1-34 or hCG ovulated, and we determined the percentage of ovulated eggs in each and compared this using the Mann-Whitney U test.

5.3 Results

5.3.1 Reference gene ef1a was not affected by SNa treatments in the female zebrafish telencephalon, hypothalamus, pituitary and ovaries

Although we did not take the reference gene approach to PCR data normalization, incorporation of a traditional "reference gene" in the NORMA-GENE normalization procedure is the approach taken in our laboratory for over a decade. Eukaryotic translation elongation factor 1 alpha (*ef1a)* is involved in elongation during protein synthesis and exhibits high expression, often very stable within treatment groups. Levels of *ef1a* in telencephalon, hypothalamus, pituitary and ovaries showed no significant change following injection of either SNa1-14 or SNa1-34 (Fig 5.1). In telencephalon, ef1a expression was not affected by time ($F=0.162$, $p=0.699$) or treatment effect ($F=1.051$, $p=0.373$) and the time X treatment interaction was also not significant ($F=0.480$, $p=0.600$). In hypothalamus, no significant effect was detected for time ($F=3.41$, $p=0.107$), treatment ($F=0.497$, $p=0.539$) and the time X treatment interaction ($F=0.514$, $p=0.569$) for expression of *ef1a.* Similarly, time ($F=0.656$, $p=0.445$), treatment ($F=3.43$, $p=0.069$), and time X treatment interaction ($F=1.134$, $p=0.347$) did influence *ef1a* s in pituitary. In ovaries, time ($F=0.0001$, $p=0.991$), treatment ($F=1.102$, $p=0.350$) or time X treatment interaction ($F=0.550$, $p=0.537$) had no impact on the expression of *ef1a*. Moreover, the consistent expression levels of *ef1a* in each sample indicates a stable experimental methodology.

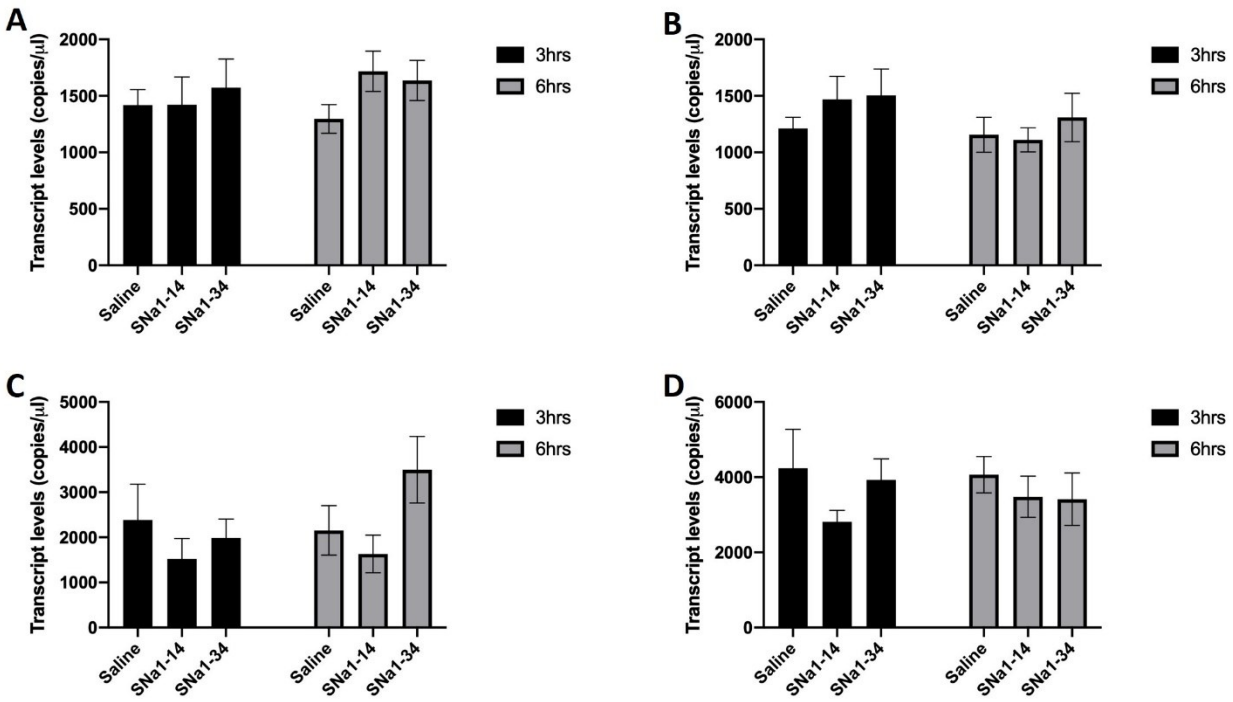

Fig 5.1 Levels of ef1a in female zebrafish tissues at 3hrs and 6hrs after IP injection with saline, SNa1-14 and SNa1-34. Expression of ef1a in the telencephalon (A), hypothalamus (B), pituitary (C), and ovaries (D) of female zebrafish is stable. Results are presented as means+SEM (n=8) of copies/µl.

5.3.2 SNa1-14 and SNa1-34 differentially increase expression of key neuroendocrine genes in the female zebrafish telencephalon

In telencephalon, all the target reproductive genes were detectable. Except for *avp*, all the other genes were influenced significantly by treatment and time (Fig 5.2). Levels of *gnrh2* were low but detectable by ddPCR. There was an overall effect of SNa1-14 and SNa1-34 to increase *gnrh2* ($F=7.735$, $p=0.012$), while time ($F=3.515$, $p=0.103$) and the treatment X time interaction were not significant ($F=4.325$, $p=0.070$). At 6 hr, this represented a 3 -fold with the mean value of *gnrh2* in saline treatment and SNa1-14 treatment was 1.319 and 3.975 respectively ($p<0.05$). As for SNa1-34 treatment, the expression level of *gnrh2* increased 2.2.-fold fold with the mean value of *gnrh2* with saline treatment and SNa1-34 treatment was 1.319 and 2.725 respectively ($p<0.05$) (Fig 5.2A).

For *gnrh3*, there were significant effects of treatment ($F=8.390$, $p=0.021$), but not time ($F=3.723$, $p=0.095$). Importantly, the treatment X time interaction ($F=6.270$, $p=0.029$), revealed that SNa1-34 had a time-dependent stimulatory effect on *gnrh3*. Levels of *gnrh3* rapidly

increased 8.9-fold at 3hrs ($p<0.05$) but returned to control levels by 6hrs. In contrast, SNa1-14 did not affect *gnrh3* in the telencephalon (Fig 5.2B).

The SNa peptides exerted differential effects on *oxt* and *avp* in the telencephalon. Levels of *oxt* were affected by treatment ($F=24.05$, $p<0.0001$), time ($F=6.340$, $p=0.039$), exhibiting a significant treatment X time interaction ($F=7.325$, $p=0.013$). Injection of SNa1-14 and SNa1-34 respectively stimulated a 7-fold ($p<0.05$) and 8.9-fold ($p<0.05$) increase in *oxt* at 3 hr. While the levels of *oxt* remained somewhat elevated at 6 hr in those fish injected with SNa1-34, overall levels were lower than at 3 hr and were not different from saline ($p>0.05$) (Fig 5.2C), while were elevated relative to the SNa1-14 group ($p<0.05$). In contrast to *oxt*, the levels of *avp* were not affected by SNa1-14 or SNa 1-34 treatment ($F=4.250$, $p=0.076$) at either timepoint ($F=2.601$, $p=0.151$) as well as the treatment X time interaction ($F=2.385$, $p=0.163$) (Fig 5.2D).

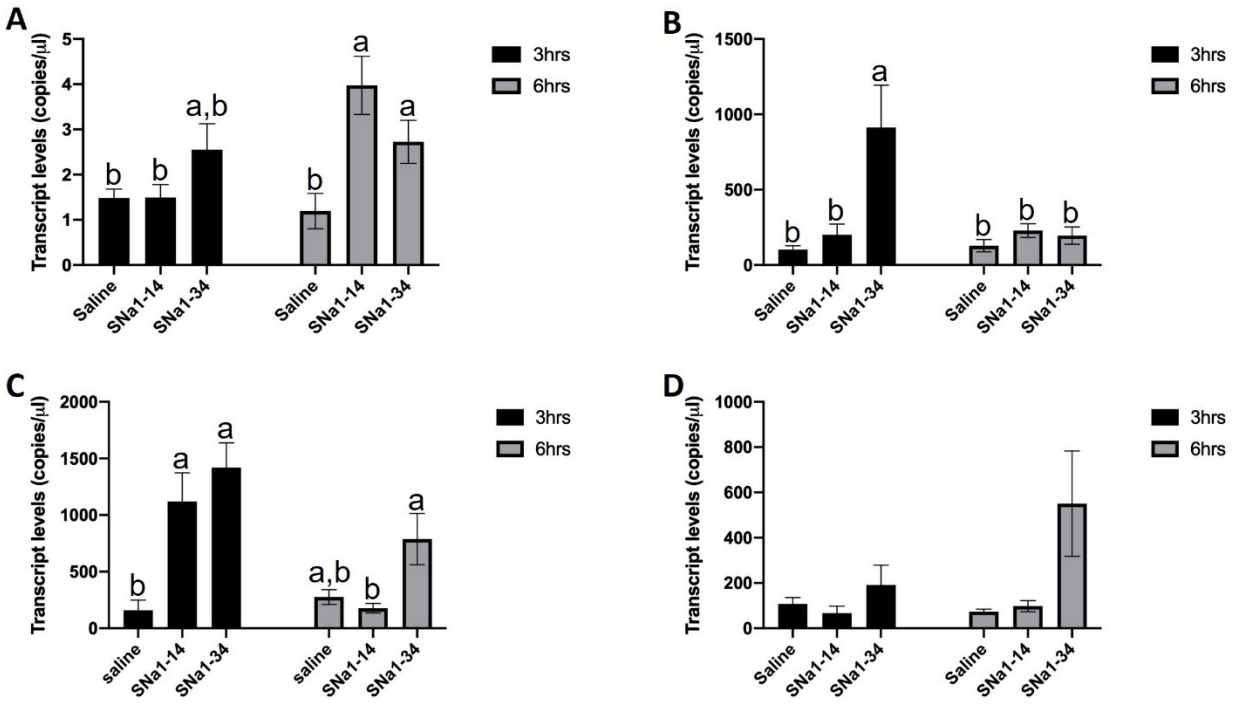

Fig 5.2 Effects of SNa1-14 and SNa1-34 on reproductive neuropeptide transcript levels in telencephalon of female zebrafish. Levels of (A) *gnrh2,* (B) *gnrh3*, (C) *oxt*, (D) *avp* are presented (means+SEM; n=8). Means with different letters (a.b) indicate a significant difference (Tukey's test; $p<0.05$).

5.3.3 SNa1-14 and SNa1-34 differentially increase expression of key neuroendocrine genes in the female zebrafish hypothalamus

In hypothalamus, the same reproductive genes quantified in the telencephalon were also detected. However, the patterns of expression of these genes were different than those observed for the telencephalon. Levels of *gnrh2* in hypothalamus were higher than that in telencephalon (Mean values of *gnrh2* in telencephalon and hypothalamus were respectively 1.49 and 106.9). Levels of *gnrh2* was not affected by treatment ($F=4.248$, $p=0.072$) and there was no treatment X time interaction ($F=3.381$, $p=0.073$). There was an overall trend for *gnrh2* to be higher at 6 hr than 3 hrs ($F=10.14$, $p=0.015$) (Fig 5.3A).

Injection of SNa1-34 but not SNa1-14 increased hypothalamic *gnrh3* ($F= 19.98$, $p=0.002$) in a time-dependent manner ($F= 12.39$, $p=0.009$), with a clear treatment X time interaction ($F= 13.40$, $p=0.007$) evident (Fig 5.3B). At 3hrs after injection of SNa1-34, *gnrh3* increased 7.6-fold (Mean value of *gnrh3* after saline injection and SNa1-34 injection were 23.83 and 180.0 respectively, $p<0.05$). By 6 hrs, *gnrh3* levels decreased significantly ($p<0.05$) although it remained somewhat higher ($p<0.05$) than the saline treatment (Fig 5.3B).

In a pattern similar to that observed for *gnrh3*, injection of SNa1-34 but not SNa1-14 increased hypothalamic *oxt* ($F= 28.39$, $p<0.0001$) in a time-dependent manner ($F= 8.489$, $p=0.022$), with a treatment X time interaction ($F= 5.800$, $p=0.033$) evident (Fig 5.3C).
At 3hrs after injection of SNa1-34, the expression of *oxt* increased 9.4-fold ($p<0.05$), then declined significantly ($p<0.05$) to basal levels at 6 hrs.

Injection of SNa1-14 but not SNa1-34 had an overall stimulatory effect on hypothalamic *avp* ($F= 6.116$, $p= 0.040$) to a similar level at both times ($F= 0.1825$, $p=0.682$) (Fig 5.3D). No treatment X time interaction ($F= 0.1795$, $p=0.703$) was evident. At 3 and 6 hrs following SNa1-14 injection *avp* was increased respectively 3.87-fold ($p>0.05$) and 5.35-fold ($p<0.05$) compared to the saline groups.

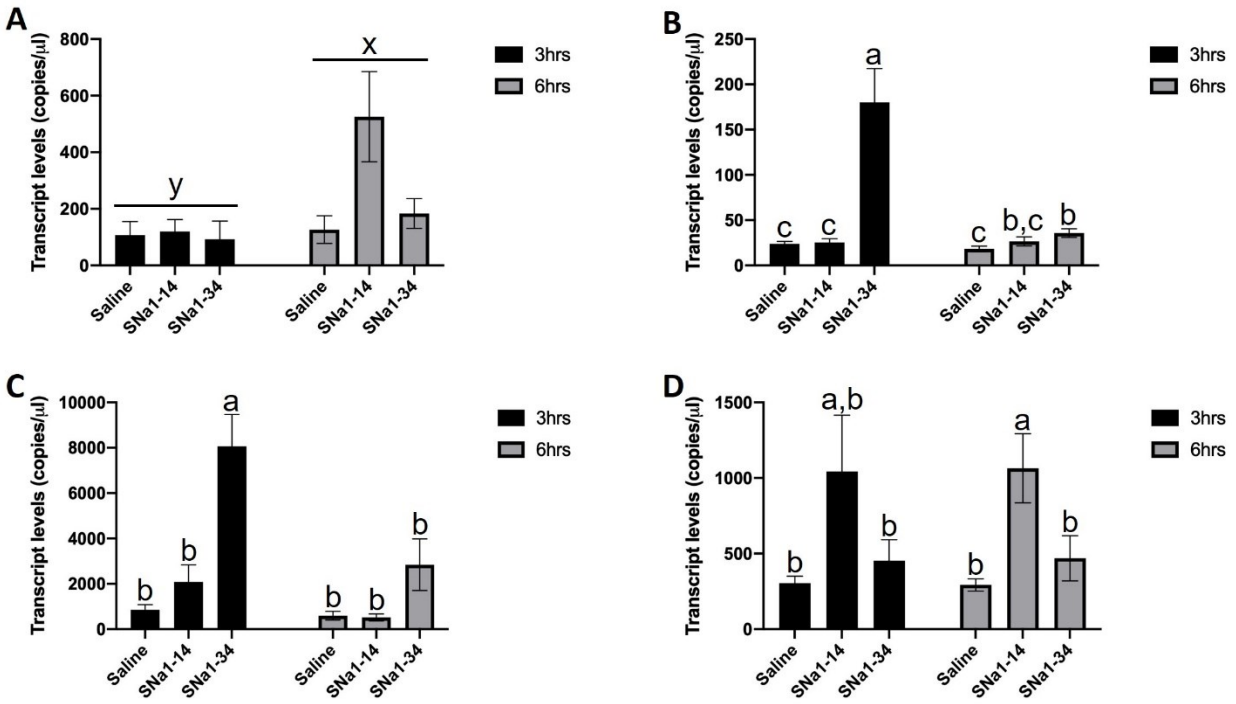

Fig 5.3 Effects of SNa1-14 and SNa1-34 on reproductive neuropeptide transcript levels in hypothalamus of female zebrafish. Expression of *gnrh2* (A), *gnrh3*(B), *oxt* (C), *avp* (D) are presented (means+SEM; n=8). In panel A, the different superscripts (x,y) indicate a significant main effect of time (F=10.14, p=0.015). In panels B, C and D, means with different letters (a,b) indicates a significant difference (Tukey's test; p<0.05).

5.3.4 SNa1-34 increases gonadotropin subunit gene expression in the female zebrafish pituitary

In the pituitary, *gnrhr2, cga, lhb, fshb* gene expressions were analyzed. Except for *fshb*, all others were affected by SNa1-34. It was found that treatment with the SNa peptides had an overall effect to decrease *gnrhr2* (F=11.81, p=0.001), but this was not affected by time (F=2.239, p=0.178) and there was no treatment X time interaction (F=1.158, p=0.331) (Fig 5.4A).

There was a significant main effect of treatment to increase the levels of *cga* (F=15.23, p=0.002) that was independent of time (F=0.1142, p=0.745) and there was no treatment x time interaction (F=1.530, p=0.256) (Fig 5.4B). Injection of SNa1-34 increased *cga* 10-fold and 3- fold at 3 and 6 hrs, respectively. In contrast SNa1-14 did not influence *cga*.

Similarly, there was a significant main effect of treatment to increase *lhb* (F=8.898, p=0.019) (Fig 5.4D). This was not affected by time (F=0.4146, p=0.540) and no treatment by time interaction was evident (F=0.6299, p=0.460). Injection of SNa1-34 increased *lhb* 7.7-fold

and 3.3- fold at 3 and 6 hrs, respectively, while SNa1-14 did not influence lhb. In contrast to the results on cga and lhb, *fshb* not affected by SNa1-14 or SNa1-34.

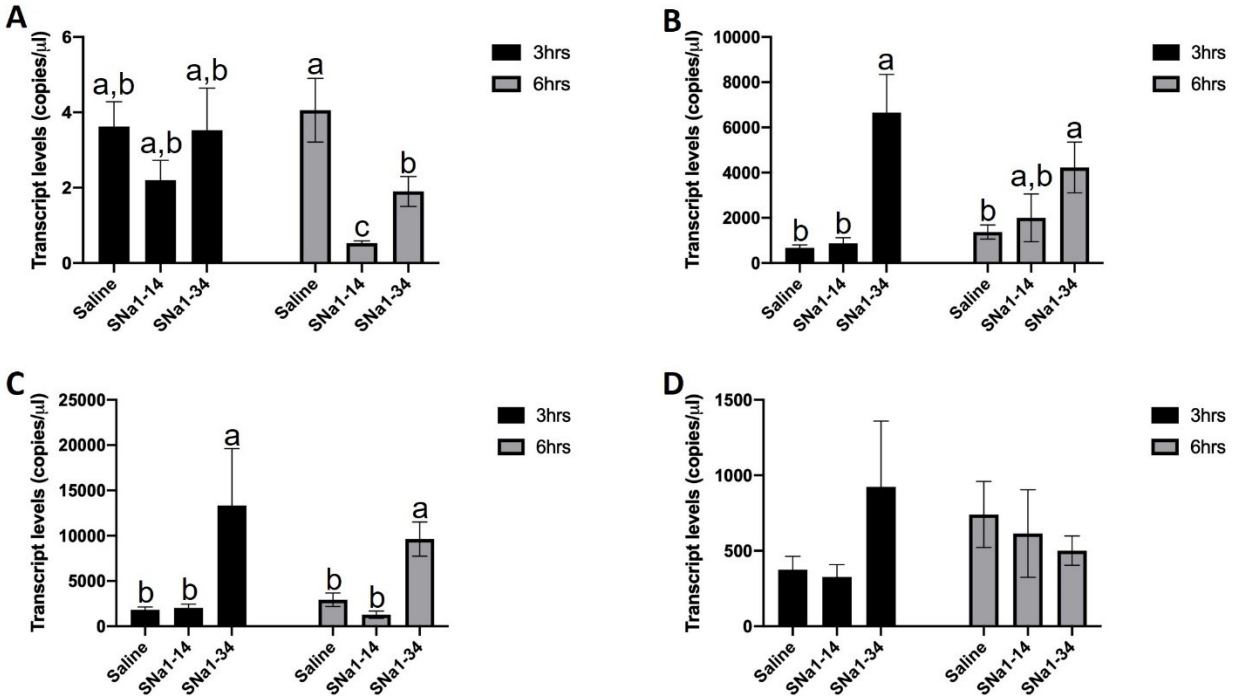

Fig 5.4 Effects of SNa1-14 and SNa1-34 on pituitary gene expression levels in female zebrafish. Expression of *gnrhr2* (A), *cga* (B), *lhb* (C), *fshb* (D) are presented as mean+SEM (n=8). Means with different letters (a,b,c) indicates a significant difference (Tukey's test; $p<0.05$).

5.3.5 SNa1-34 increases expression of key ovarian genes

Given the clear stimulatory effects on *cga* and *lhb* at the level of the pituitary, we sought evidence that ovarian function may also be altered by SNa1-34. Injection of SNa1-34 increased levels of *lhcgr* ($F=4.392$, $p=0.036$), a time-dependent- effect ($F=6.113$, $p=0.042$), yet no treatment X time interaction was evident ($F=2.551$, $p=0.139$). Levels of *lhcgr* were 2-fold higher in the SNa1-34 group at 3 hr compared to the other groups (Mean value of *lhcgr* after saline injection and SNa1-34 injection were 33.19 and 84.38 respectively, $p<0.05$), then returning to basal levels 6 hr. In contrast SNa1-14 did not affect *lhcgr* expression (Fig 5.4A).

The main effect of treatment with SNa1-34 was to increase *star1* ($F=1.401$, $p=0.048$). While the increase in *star1* was at 6 hr, the main effect of time ($F=1.624$, $p=0.243$) and the treatment X time interaction ($F=2.077$, $p=0.183$) were not statistically significant (Fig 5.4B).

Levels of *cyp19a1a* remained stable across treatments (F=0.603, p=0.504), time (F=3.448, p=0.105) and treatment X time interaction (F=2.892, p=0.098) (Fig 5.4C).

The main effect of treatment with SNa1-34 was to increase ovarian *npr* (F=1.733, p=0.036) (Fig 5.4D). While the 3.4-fold increase in *npr* was at 3 hr (p<0.05), the main effect of time (F=2.319, p=0.171) and the treatment x time interaction (F=1.279, p=0.304) were not statistically significant.

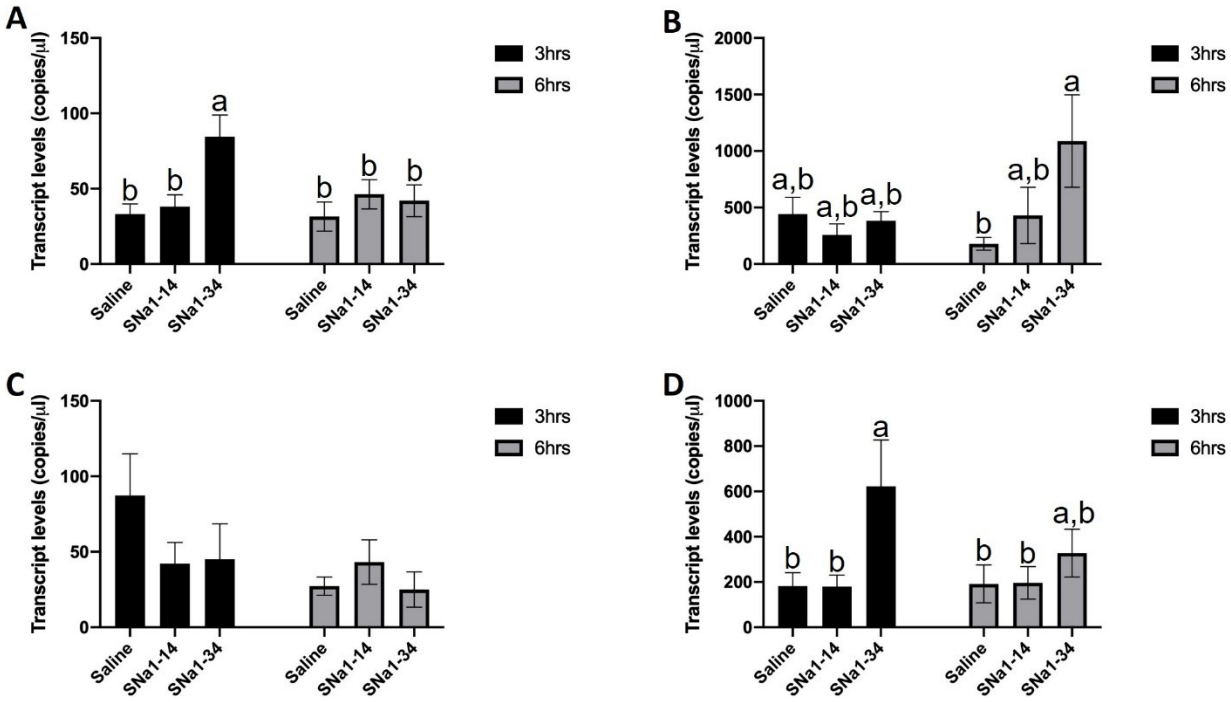

Fig 5.5 Effects of SNa1-14 and SNa1-34 on gene expression in the ovaries. Expression of *lhcgr* (A), *star1*(B), *cyp19a1a* (C), *npr* (D) are presented as means+SEM (n=8). Means with different letters (a,b) indicates a significant difference (Tukey's test; p<0.05).

5.3.6 A single i.p. injection of SNa1-34 stimulates ovulation in female zebrafish

The proceeding gene expression results strongly suggest that SNa1-34 can activate the brain-pituitary-ovarian axis. We wanted to determine if this could lead to a stimulation of ovulation. In this experiment, female zebrafish were dissected at 6 hours after injection. No fish ovulated following treatments with saline or SNa1-14. For the SNa1-34 injected group, 11/18 (61%) females ovulated. In the hCG-injected group 13 of 18 (72%) females ovulated (Fig 5.6A). Fisher's exact test revealed significant differences between the non-ovulating saline and both

the SNa1-34 ($p<0.01$) and hCG-injected groups ($p<0.01$). Ovulation rates were similar in the SNa1-34 and hCG groups ($p>0.05$). Median values of % ovulated eggs after injected SNa1-34 and hCG were 80% and 90% respectively ($p>0.05$, Fig 5.6B).

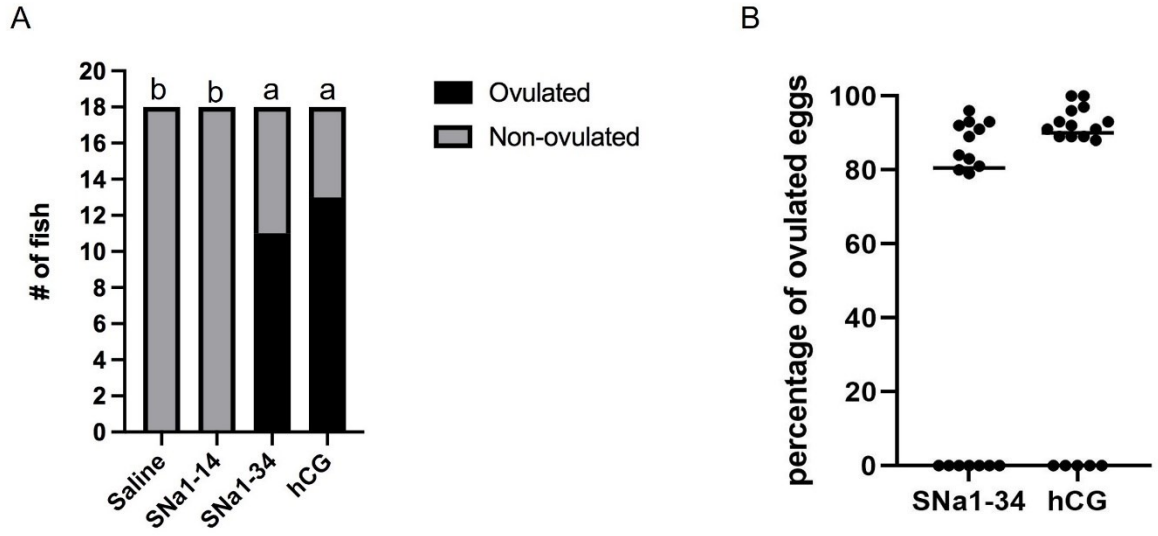

Fig 5.6 Ovulation of female zebrafish at 6hrs after IP injection with saline, SNa1-14, SNa1-34 and hCG. (A) The number of ovulating females was compared using Fisher's exact test. Means with different letters indicates the significant difference ($p<0.01$). (B) The percentage of ovulated eggs in female zebrafish injected with SNa1-34 or hCG. Results are presented as means+SEM (n=18); Mann-Whitney U-test.

5.4 Discussion

We report that injection of SNa1-34 increased the expression of key genes associated with the neuroendocrine control of reproduction at all levels of the brain-pituitary-ovarian axis. Particularly, as early as 3 hr post-injection SNa1-34 coordinately increased *gnrh3* and *oxt* in both telencephalon and hypothalamus, *cga* and *lhb* in pituitary, and both *lhcgr* and *npr* in ovaries. In contrast, the shorter SNa1-14 fragment had fewer effects, increasing *oxt* and *gnrh2* in the telencephalon and 3 and 6hr, respectively. The only change noted in the hypothalamus was an increase in *avp* at 6 hrs after SNa1-14 treatment. In marked contrast, SNa1-14 did not significantly alter gene expression in the pituitary or ovaries. These data are important because SNa1-34 and not SNa1-14 induced ovulation at 6 hr following i.p. injection to a level equivalent to that of the Lh agonist hCG.

These results are supported by previous studies in our lab (Mitchell, Zhang et al. 2020). We demonstrated that frameshift mutations in the *scg2a* gene led to reduced spawning success in zebrafish. Moreover, SNa1-34 was able to partially rescue failed reproduction in those fish carrying mutations in both *scg2a* and *scg2b* precursor genes. It was hypothesized that failed spawning was linked to reduced ovulation in these females (Mitchell, Zhang et al. 2020). Spawning is the coordinated release of sperm from males and eggs from females for optimum fertilization. Spawning success is thus a composite measure of reproduction but is especially indicative of successful ovulation followed by egg laying in females interacting with a male. The earlier work was also suggestive of possible mechanisms by which SNa1-34 may increase reproductive activity. It was shown that *scg2a* homozygous mutant females had reduced expression of *gnrh3* in hypothalamus and *cga, lhb* and *fshb* in pituitary. Here we have focused on the effects of two related SNa peptides on brain-pituitary-ovarian function in wild-type female zebrafish.

The positive associations between the brain levels of SNa1-14 and Gnrh2, and SNa1-34 and Gnrh3, over the periovulatory period (Data shown in Chapter 4), led to the hypothesis that one of the SNa forms may stimulate ovulation. Particularly, SNa1-34 and Gnrh3 peptide levels were both highest at the expected time of the Lh surge. In contrast, SNa1-14 increased later at the time of ovulation but Gnrh2 levels in brain were relatively stable over the periovulatory period. Both Gnrh2 and Gnrh3 are hypophysiotropic, stimulating Lh release in several fish species(Munoz-Cueto, Zmora et al. 2020, Trudeau 2022). It is also known that goldfish SNa1-34 stimulates Lh release *in vivo* (Blazquez, Bosma et al. 1998, Zhao, Basak et al. 2006). *In vitro* the absence of Gnrh, goldfish SNa1-34 and mouse SN1-33 stimulate Lh release from dispersed goldfish pituitary cells (Zhao, Basak et al. 2009) and the LβT2 gonadotroph cell line (Zhao, Basak et al. 2009, Zhao, McNeilly et al. 2011). Until now, the possible biological functions of any shorter SNa fragments have not been tested. We found that SNa1-14 injection increased *gnrh2* transcript levels in the telencephalon at 6 hrs, but SNa1-34 had no effect. On the other hand, SNa1-34 robustly increased *gnrh3* 8.9-fold in telencephalon and 7.6-fold in hypothalamus after 3 hrs, returning to baseline by 6 hrs. Injection of SNa1-14 had no effects on *gnrh3*. An additional effect of SNa1-34 was to increase *gnrh2* in the telencephalon at 6 hrs. In a study of the orange

spotted grouper, a commercially important fish, SNa increased the expression of *gnrh1* and *gnrh3* ~3-fold in hypothalamic fragments exposed *in vitro* (Shu, Yang et al. 2018).

It is also important to note that both SNa1-14 and SNa1-34 increased *oxt* in the telencephalon at 3 hrs. Levels of isotocin peptide were highest in pituitary before the time of the Lh surge, and highest in brain at the time of ovulation (Data shown in Chapter 4). Isotocin, the fish homolog of mammalian oxytocin, also stimulates Lh release in goldfish (Mennigen, Volkoff et al. 2017). Given the results on *gnrh* and *oxt* transcript levels and the demonstrated stimulatory role of Gnrh and Oxt in the ovulatory Lh surge in several vertebrates (Bakker and Baum 2000, Evans 2002, Munoz-Cueto, Zmora et al. 2020), we exanined whether SNa peptide injections *in vivo* may also affect gonadotropin subunit gene expression in the pituitary.

The most striking effect of SNa1-34 was the robust and concurrent increase in both the subunits composing Lh. The expression of *cga* and *lhb* increased 10- and 7 -fold at 3hrs, declining but remaining somewhat elevated at 6 hrs after injection. Yet, *fshb* was not affected, and SNa1-14 had no effects on any of the gonadotropin subunits. In teleosts such as the zebrafish, Lh and Fsh are produced in distinct cell types in the pars distalis, contrasting the situation in mammals where the two hormones are largely produced in the same cell (Trudeau and Somoza 2020). Our *in vivo* data are supported by previous data in goldfish and the mouse LβT2 cell line showing direction actions of SN on gonadotrophs, but these studies did not explore effects on Fsh (Zhao, Basak et al. 2006, Zhao, McNeilly et al. 2011). On the other hand, the only other species where effects of SNa on pituitary have been tested is the grouper. In that case SNa treatment of dispersed pituitary cells increased both *lhb* and *fshb* 2-3-fold, and thus to a similar degree to Gnrh1 and Gnrh3 (Shu, Yang et al. 2018). While we were also able to detect *gnrhr2 in* single pituitaries, levels were low, and at the limit of quantification. Thus, data must be interpreted with caution. Nevertheless, *gnrhr2* remained unchanged at 3 hrs, but tended to decrease at 6 hr. Considering the clear SNa-immunoreactive fibres heavily innervating the pituitary, presence of SNa immunoreactivity in several pituitary cell types (Data shown in Chapter 2), and that Lh and Fsh are SNa-negative, it can be proposed that SNa1-34 can stimulate Lh cells from the pituitary. The Lh surge drives ovulation and is indispensable in zebrafish reproduction. The absence of Lhb following TALEN-mediate mutations led to the

infertility in female zebrafish because of failed of ovulation (Zhang, Zhu et al. 2015). Female zebrafish carrying frameshift mutations in the *scg2a* precursor (Mitchell, Zhang et al. 2020) have a very similar phenotype to the Lhb mutants. Both have well-formed ovaries, but do not ovulate. Moreover, pituitary *cga* and *lhb* levels were decreased by >90%, while *fshb* decreased by 80% in *scg2a* mutant females (Mitchell, Zhang et al. 2020).

The observed changes in gene expression in the brain and pituitary largely followed expectations of hormonal changes required to initiate ovulation. Next, we measured the expression of ovarian *lhcgr, star1, cyp19a1a* and *npr* following treatments. The main effects of SNa1-34 were increased levels of *lhcgr* and *npr.* In contrast SNa1-34 did not affect *star1* or *cyp19a1a* and SNa1-14 fragment was without effects in the ovaries, suggesting no major effects on the steroidogenic pathway. It is important to note that SNa1-34 induced *lhcgr* at the same time we observed major increases in *cga* and *lhb.* A range of physiological, pharmacological, and gene mutational studies indicate that Fsh is important for oocyte development from primary ovarian growth to cortical alveolar stages, whereas Lh is necessary for the vitellogenesis stage, and then on to maturation and ovulation (Li and Ge 2020). Co-activation of Lh-producing gonadotrophs and ovarian Lh receptors is the hallmark of the ovulatory process (Andersson, Nijenhuis et al. 2009, Ogiwara, Fujimori et al. 2013). Critically, *npr* was also stimulated by exogenous SNa1-34 at 3 hours after injection. Nuclear progesterone receptor is important for ovulation (Akison and Robker 2012, Li and Ge 2020). Both Lhcgr and Npr are critical to the transformation of oocytes from the maturational to ovulation stages (Li and Ge 2020). Specifically in zebrafish, *npr* is stimulated by Lh and npr is highest full-grown stage of the follicles. Importantly, female zebrafish with mutated *npr* are infertile due to ovulation defects (Tang, Liu et al. 2016).

We then compared the ovulation-inducing potential of the SNa peptides to the Lh analog hCG. Firstly, it is known the hCG activates the key Lh-dependent pathways in the zebrafish ovaries (Li and Ge 2020), so can serve as an excellent positive control. Indeed, a single injection of hCG induced ovulation in female zebrafish. Similarly, SNa1-34 induced ovulation within 6 hrs. The average percentage of ovulated eggs in the SNa and hCG injected groups were also similar, 80% eggs ovulated after SNa injection, and 90% eggs ovulated after hCG treatment

separately. In contrast, SNa1-14, which did not affect pituitary or ovarian gene expression did not induce ovulation in any of the injected animals.

Our experimental design was such that females were isolated from males for 2 weeks, and injected in daytime, outside the normal ovulatory period at the end of a dark cycle. Thus, we show that activation of the brain-pituitary-ovarian axis by SNa1-34 can drive the ovulatory process without the presence of males. Several other observations require further investigation but nevertheless support a reproductive role for the SNa peptides. The surprising stimulatory effects of SNa1-34 on *oxt* and SNa1-14 on *avp* are potentially important. This was initially surprising because SN is produced in both Oxt and Avp neurons and are likely co-released with them from nerve terminals in the posterior pituitary (Mitchell, Mikwar et al. 2020). There are other sources of SN-ir in the brain as well. The robust stimulatory effects of the SN peptide on the nonapeptides could be part of a positive feedback loop or could be representative of a stimulatory pathway from other neurons. Increased activity of Oxt and Avp induced by SNa1-34 and/or SNa1-14 may serve to regulate socio-sexual behaviours after ovulation. In medaka, Oxt is required for mate choice based on familiarity recognition (Yokoi, Naruse et al. 2020). Moreover, mutation of Oxt or Oxt receptor1 did not affect spawning success in these medaka, supporting the concept that the nonapeptides modulate sociosexual behaviors rather than ovulation itself. However, there are data in some species demonstrating actions of Avp on teleost ovaries (Joy and Chaube 2015). The differential actions of SNa1-34 and SNa1-14 requires further study and raises the possibility for distinct roles of small SN-derived peptides we have uncovered (Data shown in Chapter 3). Together, these observations support our hypothesis that SN is a new reproductive hormone (Trudeau, Martyniuk et al. 2012, Trudeau 2022).

Chapter 6 General discussion and conclusion

6.1 Overview

Secretoneurin (SN) as a part of Scg2 precursor has been studied for more than 20 years (Blazquez, Bosma et al. 1998, Mitchell, Mikwar et al. 2020). Previous research in our lab has indicated that SN played a role in regulating gonadotropin release in goldfish *in vivo* and *in vitro* (Zhao, Basak et al. 2009, Zhao, Grey et al. 2010). Moreover, SN was demonstrated to stimulate the releasing of Lh in mouse pituitary gonadotropin cells LβT2 (Zhao, McNeilly et al. 2011). After that, studies with Scg2a/b TALEN mutant zebrafish revealed that the Scg2a/b double-mutant females has a significantly lower breeding success rate which could be partially rescued by human chorionic gonadotropin (hCG), an Lh-like protein (Mitchell, Zhang et al. 2020). These results suggested that SN is involved in the female zebrafish reproduction by modulating Gnrh and Lh. Based on these achievements, we investigated the hypothesis that SN regulates the female zebrafish reproduction by inducing ovulation.

6.2 Thesis summary

The first study in my project investigated the distribution of Scg2/SNa-immunoreactivity (ir) in zebrafish by immunofluorescence (Chapter 2). From the results, we figured out that Scg2a/SNa-ir located in the neurons in the ventral telencephalon, preoptic area (POA) and hypothalamus as well as fibres in pituitary stalk and neurointermediate lobe (NIL). This result indicates that Scg2a producing cells in the POA can potentially release SN peptides from the NIL. Other SNa-ir was found in nerve terminals in close proximity to both Lh and Fsh cells in the *lhb*-RFP x *fshb*-eGFP transgenic zebrafish line. The Scg2a/SNa-ir was located near gonadotrophs but never in them. There were other endocrine cells in the pituitary that we SNa-ir. Thus, SNa can reach gonadotrophs by both neuroendocrine and paracrine routes. The production of SNa in POA magnocellular cells, and in some pituitary cells types is supported by in situ hybridization localization studies in zebrafish (Mitchell 2018). Moreover, the colocalization of Scg2a/SNa-ir and Oxt-ir in the POA and NIL suggests SNa can work in concert with Oxt to

regulate reproduction. This is supported by similar neuroanatomical studies in goldfish (Canosa, Lopez et al. 2011).

For the next step, we performed targeted peptidomics to confirm the presumed amino-acid sequences of SNa and SNb peptides. We also detected the fragments of SNa and SNb and selected SNa1-14, SNa1-18, SNa19-34, SNb1-17, SNb19-31 as candidates to investigate the biological variation in the periovulatory period (Chapter 3). We also performed nano HPLC/MS to study the sex difference in the levels of reproductive hormones (Chapter 3). This is the first study in zebrafish to investigate the sex difference of reproductive hormones. The results showed that SNa1-34 levels were significantly higher in females but the ratios of SNa fragmental peptides to intact SNa peptides were higher in males. The results of SNb were similar with SNa. This indicated that the metabolism of SNa as well as SNb peptides was different in both sexes. Moreover, the sex differences in the E2/T and E3/ E2 ratios, and different production of 11-KT revealed different metabolic outcomes for steroids in the sexes.

After establishing sex differences in SNa and SNb peptide levels, we performed nano HPLC/MS to investigate the natural variation of the reproductive hormones in the periovulatory process of female zebrafish (Chapter 4). We detected the significant increases in while brain SNa1-34 at the time of the Lh surge while SNa1-14 in in brain and ovaries increased later at ovulation time. In contrast, SNb1-31 and smaller derived fragments did not vary during the ovulatory cycle. This is the first study on the natural variations of SN peptides in the periovulatory period in any vertebrate species. At this stage of the research, we hypothesized that SNa1-34 should play a key role in stimulating Lh surge in zebrafish while SNa1-14 should be more associated with the ovulatory process. Moreover, polynomial regression analysis revealed a significant relation of SNa1-34 and SNa1-14 with Gnrh2 and Gnrh3. This suggested that SNa-related peptides may induce zebrafish ovulation by regulating one or both Gnrh peptides.

To reveal the direct biological function of SNa on zebrafish ovulation, we performed the intraperitoneal (i.p.) injection with SNa1-34 peptides as well as SNa1-14 peptides to female zebrafish (Chapter 5). The results indicated a significant stimulation of SNa1-34 to the expression of *gnrh3* and *oxt* in brain, *cga* and *lhb* transcript levels in pituitary, and *lhcgr* and *npr* mRNA levels in ovaries. Various studies have demonstrated the association of these

reproductive genes with Lh surge and ovulation in teleosts and mammals (Bakker and Baum 2000, Evans 2002, Zhang, Zhu et al. 2015, Li and Ge 2020, Munoz-Cueto, Zmora et al. 2020). Our results support the regulatory effects of SNa1-34 on the reproductive genes expression. Moreover, SNa1-14 stimulated the expression of *gnrh2* and *avp* in brain. We hypothesized that SNa1-14 could modulate zebrafish ovulation by another pathway. The ovulation study clearly demonstrated that SNa1-34 has a similar stimulatory effect with hCG in zebrafish, while SNa1-14 did not induce ovulation. This result is supported by the observation that SNa1-34 injection partially rescued spawning in reproductively impaired zebrafish carrying TALEN-mediated double mutations in Scg2a and Scg2b (Mitchell, Zhang et al. 2020).

6.3 Future directions

The results in this thesis have revealed numerous novel aspects of SNa1-34 peptides and their involvement in zebrafish ovulation, but there are still many questions remaining. A few key areas for investigation are proposed below.

6.3.1 Roles of SNa in ovaries

The stimulatory effect of SNa in zebrafish ovulation has been revealed and the SNa peptides have been detected in ovaries by LC-MS, but the distribution of SNa-ir in ovaries needs to be investigated. According to the distribution, more novel aspects of SNa may become evident including possible roles in oogenesis, oocyte maturation as well as the ovulatory process.

6.3.2 Characterization of SNa1-14

SNa1-14 was investigated as the candidate of the SNa functional fragments. The results indicated that SNa1-14 varied naturally during the ovulation in brain and ovaries and its significant relation with Gnrh2 was detected. Moreover, study with SNa1-14 injection demonstrated the significantly stimulatory effect of SNa1-14 to the expression of *gnrh2* and *avp* in zebrafish brain. These results suggest that SNa1-14 regulates zebrafish reproduction by an unknown pathway.

6.3.3 Characterization of SNb

Although the distribution of SNa-ir has been studied in zebrafish brain and pituitary, the distribution of SNb-ir is still unknown. The lab previously tried to generate SNb antibodies, but this was not successful. The distribution of SNb-ir and the colocalization of SNb-ir with reproductive hormones in the brain, pituitary and gonads will be an important step to determine the biological functions of SNb Moreover, the female dominant sex difference of SNb peptides was evident in zebrafish gonads. This suggests that SNb may play a role in female reproduction although the natural variation of SNb peptides was not significant during the short periovulatory period examined here. Therefore, the investigation of SNb in female will be necessary to reveal possible SNb functions in gonadal development, oogenesis as well as oocyte maturation.

6.3.4 Investigation of SN receptors

The peptides levels of SNa and SNb have been detected in the zebrafish brain, pituitary, and gonads. The SNa-ir has been localized in the brain and pituitary. The SN receptors are still unidentified. The results from previous research suggested that the putative SN receptor should be a G-protein coupled membrane receptor. When the SN receptors are identified, the cytobiological mechanisms of SN regulation will be revealed.

6.4 Concluding remarks

This thesis research contributes basic knowledge about SN and demonstrates that SNa1-34 specifically can induce ovulation to a level comparable to the well-known pituitary hormone Lh. This strongly supports the proposal that SN is a key vertebrate reproductive hormone.

References

Abraham, E., O. Palevitch, Y. Gothilf and Y. Zohar (2010). "Targeted gonadotropin-releasing hormone-3 neuron ablation in zebrafish: effects on neurogenesis, neuronal migration, and reproduction." Endocrinology **151**(1): 332-340.

Acher, R. (1996). "Molecular evolution of fish neurohypophysial hormones: Neutral and selective evolutionary mechanisms." General and Comparative Endocrinology **102**(2): 157-172.

Acher, R. and J. Chauvet (1995). "The neurohypophysial endocrine regulatory cascade: precursors, mediators, receptors, and effectors." Front Neuroendocrinol **16**(3): 237-289.

Adkins-Regan, E. (2007). "Hormones and the development of sex differences in behavior." Journal of Ornithology **148**(1): 17-26.

Aizen, J., Y. Pang, C. Harris, A. Converse, Y. Zhu, M. A. Aguirre and P. Thomas (2018). "Roles of progesterone receptor membrane component 1 and membrane progestin receptor alpha in regulation of zebrafish oocyte maturation." Gen Comp Endocrinol **263**: 51-61.

Akison, L. K. and R. L. Robker (2012). "The critical roles of progesterone receptor (PGR) in ovulation, oocyte developmental competence and oviductal transport in mammalian reproduction." Reprod Domest Anim **47 Suppl 4**: 288-296.

Alsop, D. and M. Vijayan (2009). "The zebrafish stress axis: molecular fallout from the teleost-specific genome duplication event." Gen Comp Endocrinol **161**(1): 62-66.

Altmieme, Z., M. Jubouri, K. Touma, G. Cote, M. Fonseca, T. Julian and J. A. Mennigen (2019). "A reproductive role for the nonapeptides vasotocin and isotocin in male zebrafish (Danio rerio)." Comp Biochem Physiol B Biochem Mol Biol **238**: 110333.

Alvarado, M. V., M. Carrillo and A. Felip (2015). "Melatonin-induced changes in kiss/gnrh gene expression patterns in the brain of male sea bass during spermatogenesis." Comp Biochem Physiol A Mol Integr Physiol **185**: 69-79.

Amano, M., S. Moriyama, M. Iigo, S. Kitamura, N. Amiya, K. Yamamori, K. Ukena and K. Tsutsui (2006). "Novel fish hypothalamic neuropeptides stimulate the release of gonadotrophins and growth hormone from the pituitary of sockeye salmon." J Endocrinol **188**(3): 417-423.

Andersson, E., W. Nijenhuis, R. Male, P. Swanson, J. Bogerd, G. L. Taranger and R. W. Schulz (2009). "Pharmacological characterization, localization and quantification of expression of gonadotropin receptors in Atlantic salmon (Salmo salar L.) ovaries." Gen Comp Endocrinol **163**(3): 329-339.

Andreu-Vieyra, C. V. and H. R. Habibi (2000). "Factors controlling ovarian apoptosis." Can J Physiol Pharmacol **78**(12): 1003-1012.

Anglade, I., T. Zandbergen and O. Kah (1993). "Origin of the pituitary innervation in the goldfish." Cell Tissue Res **273**(2): 345-355.

Asaba, A., T. Osakada, K. Touhara, M. Kato, K. Mogi and T. Kikusui (2017). "Male mice ultrasonic vocalizations enhance female sexual approach and hypothalamic kisspeptin neuron activity." Horm Behav **94**: 53-60.

Asnake, S., C. Modig and P. E. Olsson (2019). "Species differences in ligand interaction and activation of estrogen receptors in fish and human." J Steroid Biochem Mol Biol **195**: 105450.

Ataei Mehr, B., S. R. Garner and B. D. Neff (2020). "Effect of isotocin on shoaling behaviour of the Guppy (Poecilia reticulata)." Anim Cogn **23**(4): 827-831.

Atkins, J. A., D. C. Busch, J. F. Bader, D. H. Keisler, D. J. Patterson, M. C. Lucy and M. F. Smith (2008). "Gonadotropin-releasing hormone-induced ovulation and luteinizing hormone release in beef heifers: effect of day of the cycle." J Anim Sci **86**(1): 83-93.
Bacila, I., V. T. Cunliffe and N. P. Krone (2021). "Interrenal development and function in zebrafish." Mol Cell Endocrinol **535**: 111372.
Bakker, J. and M. J. Baum (2000). "Neuroendocrine regulation of GnRH release in induced ovulators." Front Neuroendocrinol **21**(3): 220-262.
Bakshi, A. and U. Rai (2019). "Tissue-specific sexual dimorphism in the expression of kisspeptin and its receptors in spotted snakehead Channa punctatus." Current Science (00113891) **116**(5).
Ball, J. (1926). "The female sex cycle as a factor in learning in the rat." American Journal of Physiology-Legacy Content **78**(3): 533-536.
Barannikova, I. A., V. P. Dyubin, L. V. Bayunova and T. B. Semenkova (2002). "Steroids in the control of reproductive function in fish." Neurosci Behav Physiol **32**(2): 141-148.
Bauer-Dantoin, A. C., J. Weiss and J. L. Jameson (1995). "Roles of estrogen, progesterone, and gonadotropin-releasing hormone (GnRH) in the control of pituitary GnRH receptor gene expression at the time of the preovulatory gonadotropin surges." Endocrinology **136**(3): 1014-1019.
Behre, H. M., S. Kliesch, B. Lemcke, S. von Eckardstein and E. Nieschlag (2001). "Suppression of spermatogenesis to azoospermia by combined administration of GnRH antagonist and 19-nortestosterone cannot be maintained by this non-aromatizable androgen alone." Hum Reprod **16**(12): 2570-2577.
Bentley, G. E., J. P. Jensen, G. J. Kaur, D. W. Wacker, K. Tsutsui and J. C. Wingfield (2006). "Rapid inhibition of female sexual behavior by gonadotropin-inhibitory hormone (GnIH)." Horm Behav **49**(4): 550-555.
Biran, J., S. Ben-Dor and B. Levavi-Sivan (2008). "Molecular identification and functional characterization of the kisspeptin/kisspeptin receptor system in lower vertebrates." Biol Reprod **79**(4): 776-786.
Biran, J., M. Golan, N. Mizrahi, S. Ogawa, I. S. Parhar and B. Levavi-Sivan (2014). "LPXRFa, the piscine ortholog of GnIH, and LPXRF receptor positively regulate gonadotropin secretion in Tilapia (Oreochromis niloticus)." Endocrinology **155**(11): 4391-4401.
Blanton, M. L. and J. L. Specker (2007). "The hypothalamic-pituitary-thyroid (HPT) axis in fish and its role in fish development and reproduction." Crit Rev Toxicol **37**(1-2): 97-115.
Blazquez, M., P. T. Bosma, J. P. Chang, K. Docherty and V. L. Trudeau (1998). "Gamma-aminobutyric acid up-regulates the expression of a novel secretogranin-II messenger ribonucleic acid in the goldfish pituitary." Endocrinology **139**(12): 4870-4880.
Blazquez, M., P. T. Bosma, J. P. Chang, K. Docherty and V. L. Trudeau (1998). "Gamma-aminobutyric acid up-regulates the expression of a novel secretogranin-II messenger ribonucleic acid in the goldfish pituitary." Endocrinology **139**(12): 4870-4880.
Bliss, S. P., A. M. Navratil, J. Xie and M. S. Roberson (2010). "GnRH signaling, the gonadotrope and endocrine control of fertility." Front Neuroendocrinol **31**(3): 322-340.
Boczek-Leszczyk, E., B. Stempniak and M. Juszczak (2010). "Vasopressin release from the rat hypothalamo-neurohypophysial system: effects of gonadotrophin-releasing hormone (GnRH), its analogues and melatonin." J Physiol Pharmacol **61**(4): 459-466.

Boime, I., V. Garcia-Campayo and A. Hsueh (2004). "The glycoprotein hormones and their receptors." Reproductive Endrocrinology, 5th ed. Philadelphia: Elsevier Saunders: 75-92.
Bojanowska, E., J. W. Guzek and R. Dabrowski (1999). "Luteinizing hormone-releasing hormone and function of the magnocellular vasopressinergic system." Neuropeptides **33**(4): 301-305.
Boonen, K., G. Baggerman, W. D'Hertog, S. J. Husson, L. Overbergh, C. Mathieu and L. Schoofs (2007). "Neuropeptides of the islets of Langerhans: a peptidomics study." Gen Comp Endocrinol **152**(2-3): 231-241.
Bosma, P. T., M. Blazquez, E. J. Fraser, R. W. Schulz, K. Docherty and V. L. Trudeau (2001). "Sex steroid regulation of glutamate decarboxylase mRNA expression in goldfish brain is sexually dimorphic." J Neurochem **76**(4): 945-956.
Bradford, Y. M., S. Toro, S. Ramachandran, L. Ruzicka, D. G. Howe, A. Eagle, P. Kalita, R. Martin, S. A. Taylor Moxon, K. Schaper and M. Westerfield (2017). "Zebrafish Models of Human Disease: Gaining Insight into Human Disease at ZFIN." ILAR J **58**(1): 4-16.
Braubach, O. R., A. Fine and R. P. Croll (2012). "Distribution and functional organization of glomeruli in the olfactory bulbs of zebrafish (Danio rerio)." J Comp Neurol **520**(11): 2317-2339, Spc2311.
Bulun, S. E. (2008). "The physiology and pathology of the female reproductive axis." Williams textbook of endocrinology: 541-614.
Cahoreau, C., D. Klett and Y. Combarnous (2015). "Structure-function relationships of glycoprotein hormones and their subunits' ancestors." Front Endocrinol (Lausanne) **6**: 26.
Caldwell, J. D., A. J. Prange, Jr. and C. A. Pedersen (1986). "Oxytocin facilitates the sexual receptivity of estrogen-treated female rats." Neuropeptides **7**(2): 175-189.
Canosa, L. F., G. C. Lopez, E. Scharrig, K. Lesaux-Farmer, G. M. Somoza, O. Kah and V. L. Trudeau (2011). "Forebrain mapping of secretoneurin-like immunoreactivity and its colocalization with isotocin in the preoptic nucleus and pituitary gland of goldfish." J Comp Neurol **519**(18): 3748-3765.
Canosa, L. F., G. C. Lopez, E. Scharrig, K. Lesaux-Farmer, G. M. Somoza, O. Kah and V. L. Trudeau (2011). "Forebrain mapping of secretoneurin-like immunoreactivity and its colocalization with isotocin in the preoptic nucleus and pituitary gland of goldfish." Journal of Comparative Neurology **519**(18): 3748-3765.
Chang, J. P., J. D. Johnson, G. R. Sawisky, C. L. Grey, G. Mitchell, M. Booth, M. M. Volk, S. K. Parks, E. Thompson, G. G. Goss, C. Klausen and H. R. Habibi (2009). "Signal transduction in multifactorial neuroendocrine control of gonadotropin secretion and synthesis in teleosts-studies on the goldfish model." Gen Comp Endocrinol **161**(1): 42-52.
Chang, J. P. and J. G. Pemberton (2018). "Comparative aspects of GnRH-Stimulated signal transduction in the vertebrate pituitary - Contributions from teleost model systems." Mol Cell Endocrinol **463**: 142-167.
Chang, J. P. and R. E. Peter (1983). "Effects of dopamine on gonadotropin release in female goldfish, Carassius auratus." Neuroendocrinology **36**(5): 351-357.
Chang, J. P., K. L. Yu, A. O. Wong and R. E. Peter (1990). "Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release in vitro in goldfish." Neuroendocrinology **51**(6): 664-674.
Charmandari, E., C. Tsigos and G. Chrousos (2005). "Endocrinology of the stress response." Annu Rev Physiol **67**: 259-284.

Chaube, R., R. K. Singh and K. P. Joy (2014). "Effects of ovaprim, a commercial spawning inducer, on vasotocin and steroid hormone profiles in the catfish Heteropneustes fossilis: in vivo and in vitro studies." Gen Comp Endocrinol **195**: 190-200.
Che, F. Y. and L. D. Fricker (2005). "Quantitative peptidomics of mouse pituitary: comparison of different stable isotopic tags." J Mass Spectrom **40**(2): 238-249.
Chen, J. Y. and M. J. Chiou (2010). "Molecular cloning and functional analysis of the zebrafish luteinizing hormone beta subunit (LH<beta>) promoter." Fish Physiol Biochem **36**(4): 1253-1262.
Choi, Y. J., N. N. Kim, H. R. Habibi and C. Y. Choi (2016). "Effects of gonadotropin inhibitory hormone or gonadotropin-releasing hormone on reproduction-related genes in the protandrous cinnamon clownfish, Amphiprion melanopus." Gen Comp Endocrinol **235**: 89-99.
Chu, L., J. Li, Y. Liu and C. H. Cheng (2015). "Gonadotropin Signaling in Zebrafish Ovary and Testis Development: Insights From Gene Knockout Study." Mol Endocrinol **29**(12): 1743-1758.
Clarkson, J., X. d'Anglemont de Tassigny, A. S. Moreno, W. H. Colledge and A. E. Herbison (2008). "Kisspeptin-GPR54 signaling is essential for preovulatory gonadotropin-releasing hormone neuron activation and the luteinizing hormone surge." J Neurosci **28**(35): 8691-8697.
Colgrave, M. L., L. Xi, S. A. Lehnert, T. Flatscher-Bader, H. Wadensten, A. Nilsson, P. E. Andren and G. Wijffels (2011). "Neuropeptide profiling of the bovine hypothalamus: thermal stabilization is an effective tool in inhibiting post-mortem degradation." Proteomics **11**(7): 1264-1276.
Colledge, W. H. and X. d'Anglemont de Tassigny (2010). "The role of kisspeptin signalling in the regulation of the GnRH-gonadotrophin ovarian axis in mice." Ann Endocrinol (Paris) **71**(3): 198-200.
Comninos, A. N., M. B. Wall, L. Demetriou, A. J. Shah, S. A. Clarke, S. Narayanaswamy, A. Nesbitt, C. Izzi-Engbeaya, J. K. Prague, A. Abbara, R. Ratnasabapathy, V. Salem, G. M. Nijher, C. N. Jayasena, M. Tanner, P. Bassett, A. Mehta, E. A. Rabiner, C. Honigsperger, M. R. Silva, O. K. Brandtzaeg, E. Lundanes, S. R. Wilson, R. C. Brown, S. A. Thomas, S. R. Bloom and W. S. Dhillo (2017). "Kisspeptin modulates sexual and emotional brain processing in humans." J Clin Invest **127**(2): 709-719.
Corchuelo, S., E. R. M. Martinez, A. J. Butzge, L. B. Doretto, J. M. B. Ricci, F. N. Valentin, L. S. O. Nakaghi, G. M. Somoza and R. H. Nobrega (2017). "Characterization of Gnrh/Gnih elements in the olfacto-retinal system and ovary during zebrafish ovarian maturation." Mol Cell Endocrinol **450**: 1-13.
Cornil, C. A., A. L. Seredynski, C. de Bournonville, M. J. Dickens, T. D. Charlier, G. F. Ball and J. Balthazart (2013). "Rapid control of reproductive behaviour by locally synthesised oestrogens: focus on aromatase." J Neuroendocrinol **25**(11): 1070-1078.
Dammann, A. A., N. W. Shappell, S. E. Bartell and H. L. Schoenfuss (2011). "Comparing biological effects and potencies of estrone and 17beta-estradiol in mature fathead minnows, Pimephales promelas." Aquat Toxicol **105**(3-4): 559-568.
De Leeuw, R., H. R. Habibi, C. S. Nahorniak and R. E. Peter (1989). "Dopaminergic regulation of pituitary gonadotrophin-releasing hormone receptor activity in the goldfish (Carassius auratus)." J Endocrinol **121**(2): 239-247.
Ding, J. and B. T. Zhu (2016). "Unique dose-dependent effects of the human pregnancy hormone estriol on the ratio of blood IgM to IgG in female mice." Mol Med Rep **13**(1): 447-452.

Diotel, N., T. D. Charlier, C. Lefebvre d'Hellencourt, D. Couret, V. L. Trudeau, J. C. Nicolau, O. Meilhac, O. Kah and E. Pellegrini (2018). "Steroid Transport, Local Synthesis, and Signaling within the Brain: Roles in Neurogenesis, Neuroprotection, and Sexual Behaviors." Front Neurosci **12**: 84.
Diotel, N., Y. Le Page, K. Mouriec, S. K. Tong, E. Pellegrini, C. Vaillant, I. Anglade, F. Brion, F. Pakdel, B. C. Chung and O. Kah (2010). "Aromatase in the brain of teleost fish: expression, regulation and putative functions." Front Neuroendocrinol **31**(2): 172-192.
Dooley, K. and L. I. Zon (2000). "Zebrafish: a model system for the study of human disease." Curr Opin Genet Dev **10**(3): 252-256.
Du Vigneaud, V., C. Ressler and S. Trippett (1953). "The sequence of amino acids in oxytocin, with a proposal for the structure of oxytocin." J Biol Chem **205**(2): 949-957.
Dubois, E. A., M. A. Florijn, M. A. Zandbergen, J. Peute and H. J. Goos (1998). "Testosterone accelerates the development of the catfish GnRH system in the brain of immature African catfish (Clarias gariepinus)." Gen Comp Endocrinol **112**(3): 383-393.
Dulac, C., L. A. O'Connell and Z. Wu (2014). "Neural control of maternal and paternal behaviors." Science **345**(6198): 765-770.
Eaton, J. L., B. Holmqvist and E. Glasgow (2008). "Ontogeny of vasotocin-expressing cells in zebrafish: selective requirement for the transcriptional regulators orthopedia and single-minded 1 in the preoptic area." Dev Dyn **237**(4): 995-1005.
Egger, C., R. Kirchmair, S. Kapelari, R. Fischer-Colbrie, R. Hogue-Angeletti and H. Winkler (1994). "Bovine posterior pituitary: presence of p65 (synaptotagmin), PC1, PC2 and secretoneurin in large dense core vesicles." Neuroendocrinology **59**(2): 169-175.
Espey, E. and J. Richards (2006). Ovulation. Knobil and Neill's Physiology of Reproduction, edited by Jimmy D. Neill, Elsevier.
Evans, J. J. (2002). "Peptides interact in gonadotrophin regulation." Arch Physiol Biochem **110**(1-2): 154-161.
Fallah, H. P. and H. R. Habibi (2020). "Role of GnRH and GnIH in paracrine/autocrine control of final oocyte maturation." Gen Comp Endocrinol **299**: 113619.
Fallah, H. P., M. S. Rodrigues, S. Corchuelo, R. H. Nobrega and H. R. Habibi (2020). "Role of GnRH Isoforms in Paracrine/Autocrine Control of Zebrafish (Danio rerio) Spermatogenesis." Endocrinology **161**(2).
Fallah, H. P., M. S. Rodrigues, M. Zanardini, R. H. Nobrega and H. R. Habibi (2021). "Effects of gonadotropin-inhibitory hormone on early and late stages of spermatogenesis in ex-vivo culture of zebrafish testis." Mol Cell Endocrinol **520**: 111087.
Fallah, H. P., A. Tovo-Neto, E. C. Yeung, R. H. Nobrega and H. R. Habibi (2019). "Paracrine/autocrine control of spermatogenesis by gonadotropin-inhibitory hormone." Mol Cell Endocrinol **492**: 110440.
Feng, K., X. Cui, Y. Song, B. Tao, J. Chen, J. Wang, S. Liu, Y. Sun, Z. Zhu, V. L. Trudeau and W. Hu (2020). "Gnrh3 Regulates PGC Proliferation and Sex Differentiation in Developing Zebrafish." Endocrinology **161**(1).
Fischer-Colbrie, R., R. Kirchmair, C. M. Kahler, C. J. Wiedermann and A. Saria (2005). "Secretoneurin: A new player in angiogenesis and chemotaxis linking nerves, blood vessels and the immune system." Current Protein & Peptide Science **6**(4): 373-385.

Fischer-Colbrie, R., A. Laslop and R. Kirchmair (1995). "Secretogranin-II - molecular-properties, regulation of biosynthesis and processing to the neuropeptide secretoneurin." Progress in Neurobiology **46**(1): 49-70.
Fitzgerald, J. A., S. Konemann, L. Krumpelmann, A. Zupanic and C. Vom Berg (2021). "Approaches to Test the Neurotoxicity of Environmental Contaminants in the Zebrafish Model: From Behavior to Molecular Mechanisms." Environ Toxicol Chem **40**(4): 989-1006.
Flanagan, C. A., C. C. Chen, M. Coetsee, S. Mamputha, K. E. Whitlock, N. Bredenkamp, L. Grosenick, R. D. Fernald and N. Illing (2007). "Expression, structure, function, and evolution of gonadotropin-releasing hormone (GnRH) receptors GnRH-R1SHS and GnRH-R2PEY in the teleost, Astatotilapia burtoni." Endocrinology **148**(10): 5060-5071.
Fontaine, R., E. Ager-Wick, K. Hodne and F. A. Weltzien (2019). "Plasticity of Lh cells caused by cell proliferation and recruitment of existing cells." J Endocrinol **240**(2): 361-377.
Fontaine, R., M. R. Royan, K. von Krogh, F. A. Weltzien and D. M. Baker (2020). "Direct and Indirect Effects of Sex Steroids on Gonadotrope Cell Plasticity in the Teleost Fish Pituitary." Front Endocrinol (Lausanne) **11**: 605068.
Fricker, L. D. (2012). Neuropeptides and other bioactive peptides: from discovery to function. Colloquium series on neuropeptides, Morgan & Claypool Life Sciences.
Gerlach, G., K. Tietje, D. Biechl, I. Namekawa, G. Schalm and A. Sulmann (2019). "Behavioural and neuronal basis of olfactory imprinting and kin recognition in larval fish." J Exp Biol **222**(Pt Suppl 1).
Ginther, O. J., M. A. Beg, E. L. Gastal, M. O. Gastal and D. A. Cooper (2009). "Treatment with human chorionic gonadotropin (hCG) for ovulation induction is associated with an immediate 17beta-estradiol decrease and a more rapid LH increase in mares." Anim Reprod Sci **114**(1-3): 311-317.
Godwin, J. and R. Thompson (2012). "Nonapeptides and social behavior in fishes." Horm Behav **61**(3): 230-238.
Golan, M., J. Biran and B. Levavi-Sivan (2014). "A novel model for development, organization, and function of gonadotropes in fish pituitary." Front Endocrinol (Lausanne) **5**: 182.
Gonzalez-Martinez, D., T. Madigou, E. Mananos, J. M. Cerda-Reverter, S. Zanuy, O. Kah and J. A. Munoz-Cueto (2004). "Cloning and expression of gonadotropin-releasing hormone receptor in the brain and pituitary of the European sea bass: an in situ hybridization study." Biol Reprod **70**(5): 1380-1391.
Gonzalez-Martinez, D., N. Zmora, S. Zanuy, C. Sarasquete, A. Elizur, O. Kah and J. A. Munoz-Cueto (2002). "Developmental expression of three different prepro-GnRH (gonadotrophin-releasing hormone) messengers in the brain of the European sea bass (Dicentrarchus labrax)." J Chem Neuroanat **23**(4): 255-267.
Goodson, J. L. (2008). "Nonapeptides and the evolutionary patterning of sociality." Prog Brain Res **170**: 3-15.
Goodson, J. L. and A. H. Bass (2000). "Forebrain peptides modulate sexually polymorphic vocal circuitry." Nature **403**(6771): 769-772.
Goodson, J. L., A. M. Kelly and M. A. Kingsbury (2012). "Evolving nonapeptide mechanisms of gregariousness and social diversity in birds." Horm Behav **61**(3): 239-250.
Gorelick, D. A., W. Watson and M. E. Halpern (2008). "Androgen receptor gene expression in the developing and adult zebrafish brain." Dev Dyn **237**(10): 2987-2995.

Gorissen, M. and G. Flik (2016). The endocrinology of the stress response in fish: an adaptation-physiological view. Fish physiology, Elsevier. **35:** 75-111.
Goto-Kazeto, R., K. E. Kight, Y. Zohar, A. R. Place and J. M. Trant (2004). "Localization and expression of aromatase mRNA in adult zebrafish." Gen Comp Endocrinol **139**(1): 72-84.
Guo, Y., Q. Wang, G. Li, M. He, H. Tang, H. Zhang, X. Yang, X. Liu and H. Lin (2017). "Molecular mechanism of feedback regulation of 17beta-estradiol on two kiss genes in the protogynous orange-spotted grouper (Epinephelus coioides)." Mol Reprod Dev **84**(6): 495-507.
Gupta, N., S. J. Bark, W. D. Lu, L. Taupenot, D. T. O'Connor, P. Pevzner and V. Hook (2010). "Mass spectrometry-based neuropeptidomics of secretory vesicles from human adrenal medullary pheochromocytoma reveals novel peptide products of prohormone processing." J Proteome Res **9**(10): 5065-5075.
Gurvich, C., N. Thomas and J. Kulkarni (2020). "Sex differences in cognition and aging and the influence of sex hormones." Handb Clin Neurol **175**: 103-115.
Habibi, H. R. and D. L. Huggard (1998). "Testosterone regulation of gonadotropin production in goldfish." Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **119**(3): 339-344.
Hannon, P. R., D. M. Duffy, K. L. Rosewell, M. Brannstrom, J. W. Akin and T. E. Curry, Jr. (2018). "Ovulatory Induction of SCG2 in Human, Nonhuman Primate, and Rodent Granulosa Cells Stimulates Ovarian Angiogenesis." Endocrinology **159**(6): 2447-2458.
Hayakawa, Y., T. Morita, W. Kitamura, S. Kanda, A. Banba, H. Nagaya, K. Hotta, Y. C. Sohn, G. Yoshizaki and M. Kobayashi (2008). "Biological activities of single-chain goldfish follicle-stimulating hormone and luteinizing hormone." Aquaculture **274**(2-4): 408-415.
Health, N. I. o. M. (2011). Sex differences in brain, behavior, mental health and mental disorders. Workshop.
Heckmann, L. H., P. B. Sorensen, P. H. Krogh and J. G. Sorensen (2011). "NORMA-Gene: a simple and robust method for qPCR normalization based on target gene data." BMC Bioinformatics **12**: 250.
Helle, K. B. (2004). "The granin family of uniquely acidic proteins of the diffuse neuroendocrine system: comparative and functional aspects." Biol Rev Camb Philos Soc **79**(4): 769-794.
Hellier, V., O. Brock, M. Candlish, E. Desroziers, M. Aoki, C. Mayer, R. Piet, A. Herbison, W. H. Colledge, V. Prevot, U. Boehm and J. Bakker (2018). "Female sexual behavior in mice is controlled by kisspeptin neurons." Nat Commun **9**(1): 400.
Herbison, A. E. (1998). "Multimodal influence of estrogen upon gonadotropin-releasing hormone neurons." Endocr Rev **19**(3): 302-330.
Herbison, A. E., R. Porteous, J. R. Pape, J. M. Mora and P. R. Hurst (2008). "Gonadotropin-releasing hormone neuron requirements for puberty, ovulation, and fertility." Endocrinology **149**(2): 597-604.
Hess, R. A. and P. S. Cooke (2018). "Estrogen in the male: a historical perspective." Biol Reprod **99**(1): 27-44.
Hoflehner, J., U. Eder, A. Laslop, N. G. Seidah, R. Fischer-Colbrie and H. Winkler (1995). "Processing of secretogranin II by prohormone convertases: importance of PC1 in generation of secretoneurin." FEBS Lett **360**(3): 294-298.
Hollander-Cohen, L., M. Golan and B. Levavi-Sivan (2021). "Differential Regulation of Gonadotropins as Revealed by Transcriptomes of Distinct LH and FSH Cells of Fish Pituitary." Int J Mol Sci **22**(12).

Hoo, J. Y., Y. Kumari, M. F. Shaikh, S. M. Hue and B. H. Goh (2016). "Zebrafish: A Versatile Animal Model for Fertility Research." Biomed Res Int **2016**: 9732780.
Hossein Rashidi, B., B. Hormoz, E. Shahrokh Tehraninejad, M. Shariat and A. Mahdavi (2009). "Testosterone and dehydroepiandrosterone sulphate levels and IVF/ICSI results." Gynecol Endocrinol **25**(3): 194-198.
Hrabovszky, E., P. Ciofi, B. Vida, M. C. Horvath, E. Keller, A. Caraty, S. R. Bloom, M. A. Ghatei, W. S. Dhillo, Z. Liposits and I. Kallo (2010). "The kisspeptin system of the human hypothalamus: sexual dimorphism and relationship with gonadotropin-releasing hormone and neurokinin B neurons." Eur J Neurosci **31**(11): 1984-1998.
Huttner, W. B., H. H. Gerdes and P. Rosa (1991). "The granin (chromogranin/secretogranin) family." Trends Biochem Sci **16**(1): 27-30.
Ikegami, K., S. Kajihara, C. Umatani, M. Nakajo, S. Kanda and Y. Oka (2022). "Estrogen upregulates the firing activity of hypothalamic gonadotropin-releasing hormone (GnRH1) neurons in the evening in female medaka." J Neuroendocrinol: e13101.
Illing, N., B. E. Troskie, C. S. Nahorniak, J. P. Hapgood, R. E. Peter and R. P. Millar (1999). "Two gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (Carassius auratus)." Proc Natl Acad Sci U S A **96**(5): 2526-2531.
Irwig, M. S., G. S. Fraley, J. T. Smith, B. V. Acohido, S. M. Popa, M. J. Cunningham, M. L. Gottsch, D. K. Clifton and R. A. Steiner (2004). "Kisspeptin activation of gonadotropin releasing hormone neurons and regulation of KiSS-1 mRNA in the male rat." Neuroendocrinology **80**(4): 264-272.
James, P. J. and J. G. Nyby (2002). "Testosterone rapidly affects the expression of copulatory behavior in house mice (Mus musculus)." Physiol Behav **75**(3): 287-294.
Jennings, K. J. and L. de Lecea (2020). "Neural and Hormonal Control of Sexual Behavior." Endocrinology **161**(10).
Johnson, M. A., K. Tsutsui and G. S. Fraley (2007). "Rat RFamide-related peptide-3 stimulates GH secretion, inhibits LH secretion, and has variable effects on sex behavior in the adult male rat." Horm Behav **51**(1): 171-180.
Johnston, C. A., F. Lopez, W. K. Samson and A. Negro-Vilar (1990). "Physiologically important role for central oxytocin in the preovulatory release of luteinizing hormone." Neurosci Lett **120**(2): 256-258.
Joy, K. P. and R. Chaube (2015). "Vasotocin--A new player in the control of oocyte maturation and ovulation in fish." Gen Comp Endocrinol **221**: 54-63.
Kah, O. and P. Chambolle (1983). "Serotonin in the brain of the goldfish, Carassius auratus. An immunocytochemical study." Cell Tissue Res **234**(2): 319-333.
Kah, O., C. Lethimonier, G. Somoza, L. G. Guilgur, C. Vaillant and J. J. Lareyre (2007). "GnRH and GnRH receptors in metazoa: a historical, comparative, and evolutive perspective." Gen Comp Endocrinol **153**(1-3): 346-364.
Kanda, A., A. Nobukiyo and Y. Sotomaru (2021). "Effect of Cetrorelix administration on ovarian stimulation in aged mice." Exp Anim **70**(1): 31-36.
Kanda, S., Y. Akazome, T. Matsunaga, N. Yamamoto, S. Yamada, H. Tsukamura, K. Maeda and Y. Oka (2008). "Identification of KiSS-1 product kisspeptin and steroid-sensitive sexually dimorphic kisspeptin neurons in medaka (oryzias latipes)." Endocrinology **149**(5): 2467-2476.

Kanda, S., T. Karigo and Y. Oka (2012). "Steroid sensitive kiss2 neurones in the goldfish: evolutionary insights into the duplicate kisspeptin gene-expressing neurones." J Neuroendocrinol **24**(6): 897-906.
Kanter, M., C. Yildiz, I. Meral, A. Koc and I. Tasal (2004). "Effects of a GnRH agonist on oocyte number and maturation in mice superovulated with eCG and hCG." Theriogenology **61**(2-3): 393-398.
Kauffman, A. S., D. K. Clifton and R. A. Steiner (2007). "Emerging ideas about kisspeptin- GPR54 signaling in the neuroendocrine regulation of reproduction." Trends Neurosci **30**(10): 504-511.
Kauffman, A. S., M. L. Gottsch, J. Roa, A. C. Byquist, A. Crown, D. K. Clifton, G. E. Hoffman, R. A. Steiner and M. Tena-Sempere (2007). "Sexual differentiation of Kiss1 gene expression in the brain of the rat." Endocrinology **148**(4): 1774-1783.
Kauffman, A. S., J. H. Park, A. A. McPhie-Lalmansingh, M. L. Gottsch, C. Bodo, J. G. Hohmann, M. N. Pavlova, A. D. Rohde, D. K. Clifton, R. A. Steiner and E. F. Rissman (2007). "The kisspeptin receptor GPR54 is required for sexual differentiation of the brain and behavior." J Neurosci **27**(33): 8826-8835.
Kavanaugh, S. I., M. Nozaki and S. A. Sower (2008). "Origins of gonadotropin-releasing hormone (GnRH) in vertebrates: identification of a novel GnRH in a basal vertebrate, the sea lamprey." Endocrinology **149**(8): 3860-3869.
Kawabata, Y., T. Hiraki, A. Takeuchi and K. Okubo (2012). "Sex differences in the expression of vasotocin/isotocin, gonadotropin-releasing hormone, and tyrosine and tryptophan hydroxylase family genes in the medaka brain." Neuroscience **218**: 65-77.
Kelly, A. M. (2014). Vasopressin and oxytocin mechanisms of avian social phenotypes, Indiana University.
Kim, M. H., Y. Oka, M. Amano, M. Kobayashi, K. Okuzawa, Y. Hasegawa, S. Kawashima, Y. Suzuki and K. Aida (1995). "Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, Carassius auratus." J Comp Neurol **356**(1): 72-82.
Kirchmair, R., R. Hogueangeletti, J. Gutierrez, R. Fischercolbrie and H. Winkler (1993). "Secretoneurin - a neuropeptide generated in brain, adrenal medulla and other endocrine tissues by proteolytic processing of secretogranin-II (chromogranin C) " Neuroscience **53**(2): 359-365.
Kitahashi, T., S. Ogawa and I. S. Parhar (2009). "Cloning and expression of kiss2 in the zebrafish and medaka." Endocrinology **150**(2): 821-831.
Kleszczynska, A., E. Sokolowska and E. Kulczykowska (2012). "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 **175**(2): 290-296.
Kobayashi, M., P. W. Sorensen and N. E. Stacey (2002). "Hormonal and pheromonal control of spawning behavior in the goldfish." Fish Physiology and Biochemistry **26**(1): 71-84.
Kobayashi, M. and N. Stacey (1993). "Prostaglandin-induced female spawning behavior in goldfish (Carassius auratus) appears independent of ovarian influence." Horm Behav **27**(1): 38-55.
Kumakura, N., K. Okuzawa, K. Gen and H. Kagawa (2003). "Effects of gonadotropin-releasing hormone agonist and dopamine antagonist on hypothalamus-pituitary-gonadal axis of pre-pubertal female red seabream (Pagrus major)." Gen Comp Endocrinol **131**(3): 264-273.

Kumar, T. R., Y. Wang, N. Lu and M. M. Matzuk (1997). "Follicle stimulating hormone is required for ovarian follicle maturation but not male fertility." Nat Genet **15**(2): 201-204.
Kyle, A. L. and R. E. Peter (1982). "Effects of forebrain lesions on spawning behaviour in the male goldfish." Physiol Behav **28**(6): 1103-1109.
Kyle, A. L., N. E. Stacey and R. E. Peter (1982). "Ventral telencephalic lesions: effects on bisexual behavior, activity, and olfaction in the male goldfish." Behav Neural Biol **36**(3): 229-241.
Lado, W. E., D. Zhang, J. A. Mennigen, J. M. Zamora, J. T. Popesku and V. L. Trudeau (2013). "Rapid modulation of gene expression profiles in the telencephalon of male goldfish following exposure to waterborne sex pheromones." Gen Comp Endocrinol **192**: 204-213.
Laiz-Carrion, R., M. del Mar Segura-Noguera, M. del Pilar Martin del Rio and J. M. Mancera (2003). "Ontogeny of adenohypophyseal cells in the pituitary of the American shad (Alosa sapidissima)." Gen Comp Endocrinol **132**(3): 454-464.
Landgraf, R. and I. D. Neumann (2004). "Vasopressin and oxytocin release within the brain: a dynamic concept of multiple and variable modes of neuropeptide communication." Front Neuroendocrinol **25**(3-4): 150-176.
Larson, E. T., D. M. O'Malley and R. H. Melloni, Jr. (2006). "Aggression and vasotocin are associated with dominant-subordinate relationships in zebrafish." Behav Brain Res **167**(1): 94-102.
Laslop, A., A. Becker, I. Lindberg and R. Fischer-Colbrie (2002). "Proteolytic processing of chromogranins is modified in brains of transgenic mice." Ann N Y Acad Sci **971**: 49-52.
Laslop, A., C. Weiss, D. Savaria, C. Eiter, S. A. Tooze, N. G. Seidah and H. Winkler (1998). "Proteolytic processing of chromogranin B and secretogranin II by prohormone convertases." J Neurochem **70**(1): 374-383.
Lee, R. W. and W. B. Huttner (1983). "Tyrosine-O-sulfated proteins of PC12 pheochromocytoma cells and their sulfation by a tyrosylprotein sulfotransferase." J Biol Chem **258**(18): 11326-11334.
Lei, Z. M., S. Mishra, W. Zou, B. Xu, M. Foltz, X. Li and C. V. Rao (2001). "Targeted disruption of luteinizing hormone/human chorionic gonadotropin receptor gene." Mol Endocrinol **15**(1): 184-200.
Leitner, B., R. FischerColbrie, G. Scherzer and H. Winkler (1996). "Secretogranin II: Relative amounts and processing to secretoneurin in various rat tissues." Journal of Neurochemistry **66**(3): 1312-1317.
Leitner, B., W. A. Kaufmann, J. Marksteiner, J. Hoflehner, H. Traurig, A. Saria, R. Fischer-Colbrie and H. Winkler (1997). "Ontogenic development of secretogranin II and of its processing to secretoneurin in rat brain." Brain Res Dev Brain Res **100**(2): 161-168.
Leitner, B., C. Schneitler, H. Klocker, W. Volknandt, H. Zimmermann, H. Winkler and R. Fischer-Colbrie (1998). "Formation and sequence analysis of secretoneurin, a neuropeptide derived from secretogranin II, in mammalian, bird, reptile, amphibian and fish brains." Neuroscience Letters **248**(2): 105-108.
Lenie, S. and J. Smitz (2009). "Functional AR signaling is evident in an in vitro mouse follicle culture bioassay that encompasses most stages of folliculogenesis." Biol Reprod **80**(4): 685-695.
Lethimonier, C., T. Madigou, J. A. Munoz-Cueto, J. J. Lareyre and O. Kah (2004). "Evolutionary aspects of GnRHs, GnRH neuronal systems and GnRH receptors in teleost fish." Gen Comp Endocrinol **135**(1): 1-16.

Levavi-Sivan, B. and A. Avitan (2005). "Sequence analysis, endocrine regulation, and signal transduction of GnRH receptors in teleost fish." Gen Comp Endocrinol **142**(1-2): 67-73.
Levavi-Sivan, B., J. Biran and E. Fireman (2006). "Sex steroids are involved in the regulation of gonadotropin-releasing hormone and dopamine D2 receptors in female tilapia pituitary." Biol Reprod **75**(4): 642-650.
Levavi-Sivan, B., J. Bogerd, E. L. Mananos, A. Gomez and J. J. Lareyre (2010). "Perspectives on fish gonadotropins and their receptors." Gen Comp Endocrinol **165**(3): 412-437.
Levavi-Sivan, B., H. Safarian, H. Rosenfeld, A. Elizur and A. Avitan (2004). "Regulation of gonadotropin-releasing hormone (GnRH)-receptor gene expression in tilapia: effect of GnRH and dopamine." Biol Reprod **70**(6): 1545-1551.
Li, G., H. Tang, Y. Chen, Y. Yin, S. Ogawa, M. Liu, Y. Guo, X. Qi, Y. Liu, I. S. Parhar, X. Liu and H. Lin (2018). "Estrogen directly stimulates LHb expression at the pituitary level during puberty in female zebrafish." Mol Cell Endocrinol **461**: 1-11.
Li, J. and W. Ge (2020). "Zebrafish as a model for studying ovarian development: Recent advances from targeted gene knockout studies." Mol Cell Endocrinol **507**: 110778.
Li, L., J. L. Wojtowicz, J. H. Malin, T. Huang, E. B. Lee and Z. Chen (2017). "GnRH-mediated olfactory and visual inputs promote mating-like behaviors in male zebrafish." PLoS One **12**(3): e0174143.
Li, S., Y. Zhou, C. Yang, S. Fan, L. Huang, T. Zhou, Q. Wang, R. Zhao, C. Tang, M. Tao and S. Liu (2021). "Comparative analyses of hypothalamus transcriptomes reveal fertility-, growth-, and immune-related genes and signal pathways in different ploidy cyprinid fish." Genomics **113**(2): 595-605.
Linard, B., I. Anglade, M. Corio, J. M. Navas, F. Pakdel, C. Saligaut and O. Kah (1996). "Estrogen receptors are expressed in a subset of tyrosine hydroxylase-positive neurons of the anterior preoptic region in the rainbow trout." Neuroendocrinology **63**(2): 156-165.
Liu, C., S. Yue, J. Solarz, J. Lee and L. Li (2021). "Improving the sexual activity and reproduction of female zebrafish with high testosterone levels." Sci Rep **11**(1): 3822.
Liu, N. A., H. Huang, Z. G. Yang, W. Herzog, M. Hammerschmidt, S. Lin and S. Melmed (2003). "Pituitary corticotroph ontogeny and regulation in transgenic zebrafish." Molecular Endocrinology **17**(5): 959-966.
Lopez, M., G. Nica, P. Motte, J. A. Martial, M. Hammerschmidt and M. Muller (2006). "Expression of the somatolactin beta gene during zebrafish embryonic development." Gene Expression Patterns **6**(2): 156-161.
Ma, P. M. (2003). "Catecholaminergic systems in the zebrafish. IV. Organization and projection pattern of dopaminergic neurons in the diencephalon." J Comp Neurol **460**(1): 13-37.
Ma, X., Y. Dong, M. M. Matzuk and T. R. Kumar (2004). "Targeted disruption of luteinizing hormone beta-subunit leads to hypogonadism, defects in gonadal steroidogenesis, and infertility." Proc Natl Acad Sci U S A **101**(49): 17294-17299.
Madden, J. R. and T. H. Clutton-Brock (2011). "Experimental peripheral administration of oxytocin elevates a suite of cooperative behaviours in a wild social mammal." Proc Biol Sci **278**(1709): 1189-1194.
Mahata, S. K., M. Mahata, H. Hortnag, R. Fischer-Colbrie, H. J. Steiner, O. Dietze and H. Winkler (1993). "Concomitant changes of messenger ribonucleic acid levels of secretogranin II, VGF,

vasopressin and oxytocin in the paraventricular nucleus of rats after adrenalectomy and during lactation." J Neuroendocrinol **5**(3): 323-330.
Mahata, S. K., M. Mahata, H. Hortnagl, R. Fischercolbrie, H. J. Steiner, O. Dietze and H. Winkler (1993). "Concomitant changes of messenger ribonucleic acid levels of secretogranin II, VGF, vasopressin and oxytocin in the paraventricular nucleus of rats after adrenalectomy and during lactation." Journal of Neuroendocrinology **5**(3): 323-330.
Mangiamele, L. A., J. R. Gomez, N. J. Curtis and R. R. Thompson (2017). "GPER/GPR30, a membrane estrogen receptor, is expressed in the brain and retina of a social fish (Carassius auratus) and colocalizes with isotocin." J Comp Neurol **525**(2): 252-270.
Marksteiner, J., R. Kirchmair, S. K. Mahata, M. Mahata, R. Fischer-Colbrie, R. Hogue-Angeletti, A. Saria and H. Winkler (1993). "Distribution of secretoneurin, a peptide derived from secretogranin II, in rat brain: an immunocytochemical and radioimmunological study." Neuroscience **54**(4): 923-944.
Marksteiner, J., R. Kirchmair, S. K. Mahata, M. Mahata, R. Fischercolbrie, R. Hogueangeletti, A. Saria and H. Winkler (1993). "Distribution of secretoneurin, a peptide derived from secretogranin-II, in rat-brain - an immunocytochemical and radioimmunological study." Neuroscience **54**(4): 923-944.
Marrocco, J. and B. S. McEwen (2016). "Sex in the brain: hormones and sex differences." Dialogues Clin Neurosci **18**(4): 373-383.
Maruska, K. P., M. H. Mizobe and T. C. Tricas (2007). "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 A Mol Integr Physiol **147**(1): 129-144.
Marvel, M., B. Levavi-Sivan, T. T. Wong, N. Zmora and Y. Zohar (2021). "Gnrh2 maintains reproduction in fasting zebrafish through dynamic neuronal projection changes and regulation of gonadotropin synthesis, oogenesis, and reproductive behaviors." Sci Rep **11**(1): 6657.
Marvel, M., O. S. Spicer, T. T. Wong, N. Zmora and Y. Zohar (2018). "Knockout of the Gnrh genes in zebrafish: effects on reproduction and potential compensation by reproductive and feeding-related neuropeptides." Biol Reprod **99**(3): 565-577.
Marvel, M. M., O. S. Spicer, T. T. Wong, N. Zmora and Y. Zohar (2019). "Knockout of Gnrh2 in zebrafish (Danio rerio) reveals its roles in regulating feeding behavior and oocyte quality." Gen Comp Endocrinol **280**: 15-23.
Matsuda, F., S. Ohkura, F. Magata, A. Munetomo, J. Chen, M. Sato, N. Inoue, Y. Uenoyama and H. Tsukamura (2019). "Role of kisspeptin neurons as a GnRH surge generator: Comparative aspects in rodents and non-rodent mammals." J Obstet Gynaecol Res **45**(12): 2318-2329.
McCarthy, M. M., A. P. Arnold, G. F. Ball, J. D. Blaustein and G. J. De Vries (2012). "Sex differences in the brain: the not so inconvenient truth." J Neurosci **32**(7): 2241-2247.
Melamed, P., M. Koh, P. Preklathan, L. Bei and C. Hew (2002). "Multiple mechanisms for Pitx-1 transactivation of a luteinizing hormone beta subunit gene." J Biol Chem **277**(29): 26200-26207.
Melamed, P., H. Rosenfeld, A. Elizur and Z. Yaron (1998). "Endocrine regulation of gonadotropin and growth hormone gene transcription in fish." Comp Biochem Physiol C Pharmacol Toxicol Endocrinol **119**(3): 325-338.
Melamed, P., Y. Zhu, S. H. Tan, M. Xie and M. Koh (2006). "Gonadotropin-releasing hormone activation of c-jun, but not early growth response factor-1, stimulates transcription of a luteinizing hormone beta-subunit gene." Endocrinology **147**(7): 3598-3605.

Melli, M. S., S. Tagavi, M. Alizadeh, M. Ghojazadeh and M. Sheshvan (2007). "Comparison the effect of oxytocin and human chorionic gonadotropin on ovulation." Journal of Medical Sciences **7**(7): 1126-1134.
Mennigen, J. A., H. Volkoff, J. P. Chang and V. L. Trudeau (2017). "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 **254**: 38-49.
Mennigen, J. A., J. M. Zamora, J. P. Chang and V. L. Trudeau (2017). "Endocrine disrupting effects of waterborne fluoxetine exposure on the reproductive axis of female goldfish, Carassius auratus." Comp Biochem Physiol C Toxicol Pharmacol **202**: 70-78.
Menuet, A., E. Pellegrini, I. Anglade, O. Blaise, V. Laudet, O. Kah and F. Pakdel (2002). "Molecular characterization of three estrogen receptor forms in zebrafish: binding characteristics, transactivation properties, and tissue distributions." Biol Reprod **66**(6): 1881-1892.
Migaud, H., R. Ismail, M. Cowan and A. Davie (2012). "Kisspeptin and seasonal control of reproduction in male European sea bass (Dicentrarchus labrax)." Gen Comp Endocrinol **179**(3): 384-399.
Mikwar, M., L. Navarro-Martin, L. Xing, H. Volkoff, W. Hu and V. L. Trudeau (2016). "Stimulatory effect of the secretogranin-ll derived peptide secretoneurin on food intake and locomotion in female goldfish (Carassius auratus)." Peptides **78**: 42-50.
Millar, R. P., Z. L. Lu, A. J. Pawson, C. A. Flanagan, K. Morgan and S. R. Maudsley (2004). "Gonadotropin-releasing hormone receptors." Endocr Rev **25**(2): 235-275.
Mitani, Y., S. Kanda, Y. Akazome, B. Zempo and Y. Oka (2010). "Hypothalamic Kiss1 but not Kiss2 neurons are involved in estrogen feedback in medaka (Oryzias latipes)." Endocrinology **151**(4): 1751-1759.
Mitchell, K. (2018). Characterization and Role of Secretogranin-II/Secretoneurin in Zebrafish Reproduction, Université d'Ottawa/University of Ottawa.
Mitchell, K., M. Mikwar, D. Da Fonte, C. Lu, B. Tao, D. Peng, W. Erandani, W. Hu and V. L. Trudeau (2020). "Secretoneurin is a secretogranin-2 derived hormonal peptide in vertebrate neuroendocrine systems." Gen Comp Endocrinol **299**: 113588.
Mitchell, K., W. S. Zhang, C. Lu, B. Tao, L. Chen, W. Hu and V. L. Trudeau (2020). "Targeted mutation of secretogranin-2 disrupts sexual behavior and reproduction in zebrafish." Proc Natl Acad Sci U S A **117**(23): 12772-12783.
Miura, T., K. Yamauchi, H. Takahashi and Y. Nagahama (1991). "Hormonal induction of all stages of spermatogenesis in vitro in the male Japanese eel (Anguilla japonica)." Proc Natl Acad Sci U S A **88**(13): 5774-5778.
Moller, M. (2021). "Vasopressin and oxytocin beyond the pituitary in the human brain." Handb Clin Neurol **180**: 7-24.
Monson, C., K. Forsgren, G. Goetz, L. Harding, P. Swanson and G. Young (2017). "A teleost androgen promotes development of primary ovarian follicles in coho salmon and rapidly alters the ovarian transcriptome." Biol Reprod **97**(5): 731-745.
Moore, D., W. Notz and M. Flinger (2013). "Scatterplots and Correlation." The basic practice of statistics **6**.

Mu, X., S. Qi, J. Liu, H. Wang, L. Yuan, L. Qian, T. Li, Y. Huang, C. Wang, Y. Guo and Y. Li (2022). "Environmental level of bisphenol F induced reproductive toxicity toward zebrafish." Sci Total Environ **806**(Pt 1): 149992.
Munoz-Cueto, J. A., N. Zmora, J. A. Paullada-Salmeron, M. Marvel, E. Mananos and Y. Zohar (2020). "The gonadotropin-releasing hormones: Lessons from fish." Gen Comp Endocrinol **291**: 113422.
Myhre, P. L., A. H. Ottesen, A. L. Faaren, S. H. Tveit, J. Springett, J. Pyylampi, M. Stridsberg, G. Christensen, A. D. Hoiseth, T. Omland and H. Rosjo (2021). "Performance of a Novel Research-Use-Only Secretoneurin ELISA in Patients with Suspected Acute Coronary Syndrome: Comparison with an Established Secretoneurin Radioimmunoassay." Cardiology **146**(5): 566-574.
Nagahama, Y., M. Yoshikuni, M. Yamashita, T. Tokumoto and Y. Katsu (1995). "Regulation of oocyte growth and maturation in fish." Curr Top Dev Biol **30**: 103-145.
Narita, Y., A. Tsutiya, Y. Nakano, M. Ashitomi, K. Sato, K. Hosono, T. Kaneko, R. D. Chen, J. R. Lee, Y. C. Tseng, P. P. Hwang and R. Ohtani-Kaneko (2018). "Androgen induced cellular proliferation, neurogenesis, and generation of GnRH3 neurons in the brain of mature female Mozambique tilapia." Sci Rep **8**(1): 16855.
Ng, Y., A. Wolfe, H. J. Novaira and S. Radovick (2009). "Estrogen regulation of gene expression in GnRH neurons." Mol Cell Endocrinol **303**(1-2): 25-33.
Nica, G., W. Herzog, C. Sonntag and M. Hammerschmidt (2004). "Zebrafish pit1 mutants lack three pituitary cell types and develop severe dwarfism." Molecular Endocrinology **18**(5): 1196-1209.
Oakes, J. A., L. Barnard, K. H. Storbeck, V. T. Cunliffe and N. P. Krone (2020). "11beta-Hydroxylase loss disrupts steroidogenesis and reproductive function in zebrafish." J Endocrinol **247**(2): 197-212.
Ogawa, S., G. Akiyama, S. Kato, T. Soga, Y. Sakuma and I. S. Parhar (2006). "Immunoneutralization of gonadotropin-releasing hormone type-III suppresses male reproductive behavior of cichlids." Neurosci Lett **403**(3): 201-205.
Ogawa, S. and I. S. Parhar (2020). "Single-Cell Gene Profiling Reveals Social Status-Dependent Modulation of Nuclear Hormone Receptors in GnRH Neurons in a Male Cichlid Fish." Int J Mol Sci **21**(8).
Ogawa, S., P. N. Ramadasan, R. Anthonysamy and I. S. Parhar (2020). "Sexual Dimorphic Distribution of Hypothalamic Tachykinin1 Cells and Their Innervations to GnRH Neurons in the Zebrafish." Front Endocrinol (Lausanne) **11**: 534343.
Ogiwara, K., C. Fujimori, S. Rajapakse and T. Takahashi (2013). "Characterization of luteinizing hormone and luteinizing hormone receptor and their indispensable role in the ovulatory process of the medaka." PLoS One **8**(1): e54482.
Ohya, T. and S. Hayashi (2006). "Vasotocin/isotocin-immunoreactive neurons in the medaka fish brain are sexually dimorphic and their numbers decrease after spawning in the female." Zoolog Sci **23**(1): 23-29.
Okumura, H., T. Todo, S. Adachi and K. Yamauchi (2002). "Changes in hepatic vitellogenin mRNA levels during oocyte development in the Japanese eel, Anguilla japonica." Gen Comp Endocrinol **125**(1): 9-16.

Palevitch, O., K. Kight, E. Abraham, S. Wray, Y. Zohar and Y. Gothilf (2007). "Ontogeny of the GnRH systems in zebrafish brain: in situ hybridization and promoter-reporter expression analyses in intact animals." Cell Tissue Res **327**(2): 313-322.
Pan, H., F. Y. Che, B. Peng, D. F. Steiner, J. E. Pintar and L. D. Fricker (2006). "The role of prohormone convertase-2 in hypothalamic neuropeptide processing: a quantitative neuropeptidomic study." J Neurochem **98**(6): 1763-1777.
Papalexi, E., A. Persson, M. Bjorkqvist, A. Petersen, B. Woodman, G. P. Bates, F. Sundler, H. Mulder, P. Brundin and N. Popovic (2005). "Reduction of GnRH and infertility in the R6/2 mouse model of Huntington's disease." Eur J Neurosci **22**(6): 1541-1546.
Peter, R. E., J. P. Chang, C. S. Nahorniak, R. J. Omeljaniuk, M. Sokolowska, S. H. Shih and R. Billard (1986). "Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish." Recent Prog Horm Res **42**: 513-548.
Peter, R. E., K. L. Yu, T. A. Marchant and P. M. Rosenblum (1990). "Direct neural regulation of the teleost adenohypophysis." Journal of Experimental Zoology: 84-89.
Peyton, C. and P. Thomas (2011). "Involvement of epidermal growth factor receptor signaling in estrogen inhibition of oocyte maturation mediated through the G protein-coupled estrogen receptor (Gper) in zebrafish (Danio rerio)." Biol Reprod **85**(1): 42-50.
Pierre, J., M. Mahouche, E. I. Suderevskaya, J. Reperant and R. Ward (1997). "Immunocytochemical localization of dopamine and its synthetic enzymes in the central nervous system of the lamprey Lampetra fluviatilis." J Comp Neurol **380**(1): 119-135.
Piet, R., B. Kalil, T. McLennan, R. Porteous, K. Czieselsky and A. E. Herbison (2018). "Dominant Neuropeptide Cotransmission in Kisspeptin-GABA Regulation of GnRH Neuron Firing Driving Ovulation." J Neurosci **38**(28): 6310-6322.
Piferrer, F. and M. Blazquez (2005). "Aromatase distribution and regulation in fish." Fish Physiol Biochem **31**(2-3): 215-226.
Pinilla, L., E. Aguilar, C. Dieguez, R. P. Millar and M. Tena-Sempere (2012). "Kisspeptins and reproduction: physiological roles and regulatory mechanisms." Physiol Rev **92**(3): 1235-1316.
Pogoda, H. M. and M. Hammerschmidt (2009). "How to make a teleost adenohypophysis: Molecular pathways of pituitary development in zebrafish." Molecular and Cellular Endocrinology **312**(1-2): 2-13.
Popesku, J. T., C. J. Martyniuk, J. Mennigen, H. Xiong, D. Zhang, X. Xia, A. R. Cossins and V. L. Trudeau (2008). "The goldfish (Carassius auratus) as a model for neuroendocrine signaling." Mol Cell Endocrinol **293**(1-2): 43-56.
Popesku, J. T., J. A. Mennigen, J. P. Chang and V. L. Trudeau (2011). "Dopamine D1 receptor blockage potentiates AMPA-stimulated luteinising hormone release in the goldfish." J Neuroendocrinol **23**(4): 302-309.
Pouso, P., L. Quintana, G. C. Lopez, G. M. Somoza, A. C. Silva and V. L. Trudeau (2015). "The secretogranin-II derived peptide secretoneurin modulates electric behavior in the weakly pulse type electric fish, Brachyhypopomus gauderio." Gen Comp Endocrinol **222**: 158-166.
Powell, J. F., S. L. Krueckl, P. M. Collins and N. M. Sherwood (1996). "Molecular forms of GnRH in three model fishes: rockfish, medaka and zebrafish." J Endocrinol **150**(1): 17-23.
Qi, X., W. Zhou, S. Li, D. Lu, S. Yi, R. Xie, X. Liu, Y. Zhang and H. Lin (2013). "Evidences for the regulation of GnRH and GTH expression by GnIH in the goldfish, Carassius auratus." Mol Cell Endocrinol **366**(1): 9-20.

Qi, X., W. Zhou, D. Lu, Q. Wang, H. Zhang, S. Li, X. Liu, Y. Zhang and H. Lin (2013). "Sexual dimorphism of steroidogenesis regulated by GnIH in the goldfish, Carassius auratus." Biol Reprod **88**(4): 89.
Qi, X., W. Zhou, Q. Wang, L. Guo, D. Lu and H. Lin (2017). "Gonadotropin-Inhibitory Hormone, the Piscine Ortholog of LPXRFa, Participates in 17beta-Estradiol Feedback in Female Goldfish Reproduction." Endocrinology **158**(4): 860-873.
Ramsey, M. E., D. Fry and M. E. Cummings (2019). "Isotocin increases female avoidance of males in a coercive mating system: Assessing the social salience hypothesis of oxytocin in a fish species." Horm Behav **112**: 1-9.
Rege, J., A. F. Turcu, J. Z. Kasa-Vubu, A. M. Lerario, G. C. Auchus, R. J. Auchus, J. M. Smith, P. C. White and W. E. Rainey (2018). "11-Ketotestosterone Is the Dominant Circulating Bioactive Androgen During Normal and Premature Adrenarche." J Clin Endocrinol Metab **103**(12): 4589-4598.
Remage-Healey, L. and A. H. Bass (2005). "Rapid elevations in both steroid hormones and vocal signaling during playback challenge: a field experiment in Gulf toadfish." Horm Behav **47**(3): 297-305.
Remage-Healey, L. and A. H. Bass (2007). "Plasticity in brain sexuality is revealed by the rapid actions of steroid hormones." J Neurosci **27**(5): 1114-1122.
Remage-Healey, L., M. J. Coleman, R. K. Oyama and B. A. Schlinger (2010). "Brain estrogens rapidly strengthen auditory encoding and guide song preference in a songbird." Proc Natl Acad Sci U S A **107**(8): 3852-3857.
Rinn, J. L. and M. Snyder (2005). "Sexual dimorphism in mammalian gene expression." Trends Genet **21**(5): 298-305.
Ron-El, R., A. Raziel, M. Schachter, D. Strassburger, E. Kasterstein and S. Friedler (2000). "Induction of ovulation after gnRH antagonists." Hum Reprod Update **6**(4): 318-321.
Rosa, P., A. Hille, R. W. Lee, A. Zanini, P. De Camilli and W. B. Huttner (1985). "Secretogranins I and II: two tyrosine-sulfated secretory proteins common to a variety of cells secreting peptides by the regulated pathway." J Cell Biol **101**(5 Pt 1): 1999-2011.
Roushangar, L., J. Soleymanirad, P. NIKPOU and M. Sayahmeli (2009). "Effect of oxytocin injection on folliculogenesis, ovulation and endometrial growth in mice."
Saito, D., Y. Hasegawa and A. Urano (2003). "Gonadotropin-releasing hormones modulate electrical activity of vasotocin and isotocin neurons in the brain of rainbow trout." Neurosci Lett **351**(2): 107-110.
Samia, M., K. E. Lariviere, M. H. Rochon, B. M. Hibbert, A. Basak and V. L. Trudeau (2004). "Seasonal cyclicity of secretogranin-II expression and its modulation by sex steroids and GnRH in the female goldfish pituitary." General and Comparative Endocrinology **139**(3): 198-205.
Sari, I. P., A. Rao, J. T. Smith, A. J. Tilbrook and I. J. Clarke (2009). "Effect of RF-amide-related peptide-3 on luteinizing hormone and follicle-stimulating hormone synthesis and secretion in ovine pituitary gonadotropes." Endocrinology **150**(12): 5549-5556.
Saria, A., W. A. Kaufmann, J. Marksteiner, B. Leitner, H. Traurig, R. Fischer-Colbrie and H. Winkler (1997). "Distribution and processing of secretoneurin in the developing rat brain." Ann N Y Acad Sci **814**: 90-96.

Saria, A., J. Troger, R. Kirchmair, R. Fischer-Colbrie, R. Hogue-Angeletti and H. Winkler (1993). "Secretoneurin releases dopamine from rat striatal slices: a biological effect of a peptide derived from secretogranin II (chromogranin C)." Neuroscience **54**(1): 1-4.
Sato, Y., N. Miyasaka and Y. Yoshihara (2007). "Hierarchical regulation of odorant receptor gene choice and subsequent axonal projection of olfactory sensory neurons in zebrafish." J Neurosci **27**(7): 1606-1615.
Scully, K. M. and M. G. Rosenfeld (2002). "Development - Pituitary development: Regulatory codes in mammalian organogenesis." Science **295**(5563): 2231-2235.
Seeburg, P. H., A. J. Mason, T. A. Stewart and K. Nikolics (1987). "The mammalian GnRH gene and its pivotal role in reproduction." Recent Prog Horm Res **43**: 69-98.
Selman, K., R. A. Wallace, A. Sarka and X. Qi (1993). "Stages of oocyte development in the zebrafish, Brachydanio rerio." J Morphol **218**(2): 203-224.
Selvaraj, S., H. Kitano, Y. Fujinaga, H. Ohga, M. Yoneda, A. Yamaguchi, A. Shimizu and M. Matsuyama (2010). "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 **169**(1): 28-38.
Seminara, S. B. and U. B. Kaiser (2005). "New gatekeepers of reproduction: GPR54 and its cognate ligand, KiSS-1." Endocrinology **146**(4): 1686-1688.
Semsar, K. and J. Godwin (2003). "Social influences on the arginine vasotocin system are independent of gonads in a sex-changing fish." J Neurosci **23**(10): 4386-4393.
Servili, A., Y. Le Page, J. Leprince, A. Caraty, S. Escobar, I. S. Parhar, J. Y. Seong, H. Vaudry and O. Kah (2011). "Organization of two independent kisspeptin systems derived from evolutionary-ancient kiss genes in the brain of zebrafish." Endocrinology **152**(4): 1527-1540.
Shahab, M., C. Mastronardi, S. B. Seminara, W. F. Crowley, S. R. Ojeda and T. M. Plant (2005). "Increased hypothalamic GPR54 signaling: a potential mechanism for initiation of puberty in primates." Proc Natl Acad Sci U S A **102**(6): 2129-2134.
Shahjahan, M., H. Doi and H. Ando (2016). "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 **227**: 77-83.
Shahjahan, M., T. Ikegami, T. Osugi, K. Ukena, H. Doi, A. Hattori, K. Tsutsui and H. Ando (2011). "Synchronised expressions of LPXRFamide peptide and its receptor genes: seasonal, diurnal and circadian changes during spawning period in grass puffer." J Neuroendocrinol **23**(1): 39-51.
Shu, H., L. Yang, Y. Zhang, X. Liu, H. Lin and S. Li (2018). "Identification and functional characterization of two Secretogranin II genes in orange-spotted grouper (Epinephelus coioides)." Gen Comp Endocrinol **261**: 115-126.
Singh, R. K., R. Chaube and K. P. Joy (2013). "Differential and reproductive stage-dependent regulation of vasotocin secretion by catecholamines in the catfish Heteropneustes fossilis." Comp Biochem Physiol A Mol Integr Physiol **166**(4): 619-626.
Singh, V. and K. P. Joy (2008). "Immunocytochemical localization, HPLC characterization, and seasonal dynamics of vasotocin in the brain, blood plasma and gonads of the catfish Heteropneustes fossilis." Gen Comp Endocrinol **159**(2-3): 214-225.
Singh, V. and K. P. Joy (2010). "An involvement of vasotocin in oocyte hydration in the catfish Heteropneustes fossilis: A comparison with effects of isotocin and hCG." Gen Comp Endocrinol **166**(3): 504-512.

Smith, J. T., S. M. Popa, D. K. Clifton, G. E. Hoffman and R. A. Steiner (2006). "Kiss1 neurons in the forebrain as central processors for generating the preovulatory luteinizing hormone surge." J Neurosci **26**(25): 6687-6694.
So, W. K., H. F. Kwok and W. Ge (2005). "Zebrafish gonadotropins and their receptors: II. Cloning and characterization of zebrafish follicle-stimulating hormone and luteinizing hormone subunits--their spatial-temporal expression patterns and receptor specificity." Biol Reprod **72**(6): 1382-1396.
Soengas, J. L., J. M. Cerda-Reverter and M. J. Delgado (2018). "Central regulation of food intake in fish: an evolutionary perspective." J Mol Endocrinol **60**(4): R171-R199.
Sohn, Y. C., H. Suetake, Y. Yoshiura, M. Kobayashi and K. Aida (1998). "Structural and expression analyses of gonadotropin Ibeta subunit genes in goldfish (Carassius auratus)." Gene **222**(2): 257-267.
Somoza, G. M., A. S. Mechaly and V. L. Trudeau (2020). "Kisspeptin and GnRH interactions in the reproductive brain of teleosts." Gen Comp Endocrinol **298**: 113568.
Somoza, G. M., L. A. Miranda, P. Strobl-Mazzulla and L. G. Guilgur (2002). "Gonadotropin-releasing hormone (GnRH): from fish to mammalian brains." Cell Mol Neurobiol **22**(5-6): 589-609.
Sousa, M. L., A. Silva, F. Malhao, M. J. Rocha, E. Rocha and R. Urbatzka (2016). "Reproductive hormones affect follicular cells and ooplasm of Stage I and II oocytes in zebrafish." Reprod Fertil Dev **28**(12): 1945-1952.
Sower, S. A., M. Freamat and S. I. Kavanaugh (2009). "The origins of the vertebrate hypothalamic-pituitary-gonadal (HPG) and hypothalamic-pituitary-thyroid (HPT) endocrine systems: New insights from lampreys." General and Comparative Endocrinology **161**(1): 20-29.
Spicer, O. S., T. T. Wong, N. Zmora and Y. Zohar (2016). "Targeted Mutagenesis of the Hypophysiotropic Gnrh3 in Zebrafish (Danio rerio) Reveals No Effects on Reproductive Performance." PLoS One **11**(6): e0158141.
Spicer, O. S., N. Zmora, T. T. Wong, M. Golan, B. Levavi-Sivan, Y. Gothilf and Y. Zohar (2017). "The gonadotropin-inhibitory hormone (Lpxrfa) system's regulation of reproduction in the brain-pituitary axis of the zebrafish (Danio rerio)." Biol Reprod **96**(5): 1031-1042.
Steven, C., N. Lehnen, K. Kight, S. Ijiri, U. Klenke, W. A. Harris and Y. Zohar (2003). "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 **133**(1): 27-37.
Sueiro, C., I. Carrera, S. Ferreiro, P. Molist, F. Adrio, R. Anadon and I. Rodriguez-Moldes (2007). "New insights on Saccus vasculosus evolution: a developmental and immunohistochemical study in elasmobranchs." Brain Behav Evol **70**(3): 187-204.
Sugiura, K., Y. Q. Su, Q. Li, K. Wigglesworth, M. M. Matzuk and J. J. Eppig (2010). "Estrogen promotes the development of mouse cumulus cells in coordination with oocyte-derived GDF9 and BMP15." Mol Endocrinol **24**(12): 2303-2314.
Swanson, P. (1991). "Salmon gonadotropins: reconciling old and new ideas." Reproductive physiology of fish: 2-7.
Szkudlinski, M. W. (2015). "New Frontier in Glycoprotein Hormones and Their Receptors Structure-Function." Front Endocrinol (Lausanne) **6**: 155.

Takahashi, A., S. Kanda, T. Abe and Y. Oka (2016). "Evolution of the Hypothalamic-Pituitary-Gonadal Axis Regulation in Vertebrates Revealed by Knockout Medaka." Endocrinology **157**(10): 3994-4002.
Tang, H., Y. Liu, J. Li, Y. Yin, G. Li, Y. Chen, S. Li, Y. Zhang, H. Lin, X. Liu and C. H. Cheng (2016). "Gene knockout of nuclear progesterone receptor provides insights into the regulation of ovulation by LH signaling in zebrafish." Sci Rep **6**: 28545.
Tang, H., Y. Liu, D. Luo, S. Ogawa, Y. Yin, S. Li, Y. Zhang, W. Hu, I. S. Parhar, H. Lin, X. Liu and C. H. Cheng (2015). "The kiss/kissr systems are dispensable for zebrafish reproduction: evidence from gene knockout studies." Endocrinology **156**(2): 589-599.
Tao, B., H. Hu, K. Mitchell, J. Chen, H. Jia, Z. Zhu, V. L. Trudeau and W. Hu (2018). "Secretogranin-II plays a critical role in zebrafish neurovascular modeling." J Mol Cell Biol **10**(5): 388-401.
Tata, B., L. Huijbregts, S. Jacquier, Z. Csaba, E. Genin, V. Meyer, S. Leka, J. Dupont, P. Charles, D. Chevenne, J. C. Carel, J. Leger and N. de Roux (2014). "Haploinsufficiency of Dmxl2, encoding a synaptic protein, causes infertility associated with a loss of GnRH neurons in mouse." PLoS Biol **12**(9): e1001952.
Tello, J. A., S. Wu, J. E. Rivier and N. M. Sherwood (2008). "Four functional GnRH receptors in zebrafish: analysis of structure, signaling, synteny and phylogeny." Integr Comp Biol **48**(5): 570-587.
Themmen, A. P. N. and I. T. Huhtaniemi (2000). "Mutations of gonadotropins and gonadotropin receptors: elucidating the physiology and pathophysiology of pituitary-gonadal function." Endocr Rev **21**(5): 551-583.
Thomas, M. P. and B. V. Potter (2013). "The structural biology of oestrogen metabolism." J Steroid Biochem Mol Biol **137**: 27-49.
Tibolt, R. E. and G. V. Childs (1985). "Cytochemical and cytophysiological studies of gonadotropin-releasing hormone (GnRH) target cells in the male rat pituitary: differential effects of androgens and corticosterone on GnRH binding and gonadotropin release." Endocrinology **117**(1): 396-404.
Torgersen, J., R. Nourizadeh-Lillabadi, H. Husebye and P. Alestrom (2002). "In silico and in situ characterization of the zebrafish (Danio rerio) gnrh3 (sGnRH) gene." BMC Genomics **3**(1): 25.
Trudeau, V. L. (1997). "Neuroendocrine regulation of gonadotrophin II release and gonadal growth in the goldfish, Carassius auratus." Rev Reprod **2**(1): 55-68.
Trudeau, V. L. (2015). "Kiss and tell: Deletion of kisspeptins and receptors reveal surprising results see article in Endocrinology February 2015;156: 589-599." Endocrinology **156**(3): 769-771.
Trudeau, V. L. (2018). "Facing the Challenges of Neuropeptide Gene Knockouts: Why Do They Not Inhibit Reproduction in Adult Teleost Fish?" Front Neurosci **12**: 302.
Trudeau, V. L. (2021). "Neuroendocrine Control of Reproduction in Teleost Fish: Concepts and Controversies." Annu Rev Anim Biosci.
Trudeau, V. L. (2022). "Neuroendocrine Control of Reproduction in Teleost Fish: Concepts and Controversies." Annu Rev Anim Biosci **10**: 107-130.
Trudeau, V. L., B. Heyne, J. M. Blais, F. Temussi, S. K. Atkinson, F. Pakdel, J. T. Popesku, V. L. Marlatt, J. C. Scaiano, L. Previtera and D. R. Lean (2011). "Lumiestrone is Photochemically

Derived from Estrone and may be Released to the Environment without Detection." Front Endocrinol (Lausanne) **2**: 83.
Trudeau, V. L., C. J. Martyniuk, E. Zhao, H. Hu, H. Volkoff, W. A. Decatur and A. Basak (2012). "Is secretoneurin a new hormone?" Gen Comp Endocrinol **175**(1): 10-18.
Trudeau, V. L., C. K. Murthy, H. R. Habibi, B. D. Sloley and R. E. Peter (1993). "Effects of sex steroid treatments on gonadotropin-releasing hormone-stimulated gonadotropin secretion from the goldfish pituitary." Biol Reprod **48**(2): 300-307.
Trudeau, V. L., B. D. Sloley, A. O. Wong and R. E. Peter (1993). "Interactions of gonadal steroids with brain dopamine and gonadotropin-releasing hormone in the control of gonadotropin-II secretion in the goldfish." Gen Comp Endocrinol **89**(1): 39-50.
Trudeau, V. L. and G. M. Somoza (2020). "Multimodal hypothalamo-hypophysial communication in the vertebrates." Gen Comp Endocrinol **293**: 113475.
Tse, A. C. and W. Ge (2010). "Spatial localization of EGF family ligands and receptors in the zebrafish ovarian follicle and their expression profiles during folliculogenesis." Gen Comp Endocrinol **167**(3): 397-407.
Tsutsui, K., E. Saigoh, K. Ukena, H. Teranishi, Y. Fujisawa, M. Kikuchi, S. Ishii and P. J. Sharp (2000). "A novel avian hypothalamic peptide inhibiting gonadotropin release." Biochem Biophys Res Commun **275**(2): 661-667.
Turquier, V., H. Vaudry, S. Jegou and Y. Anouar (1999). "Frog chromogranin A messenger ribonucleic acid encodes three highly conserved peptides. Coordinate regulation of proopiomelanocortin and chromogranin A gene expression in the pars intermedia of the pituitary during background color adaptation." Endocrinology **140**(9): 4104-4112.
Ubuka, T., K. Ukena, P. J. Sharp, G. E. Bentley and K. Tsutsui (2006). "Gonadotropin-inhibitory hormone inhibits gonadal development and maintenance by decreasing gonadotropin synthesis and release in male quail." Endocrinology **147**(3): 1187-1194.
Urano, A. and H. Ando (2011). "Diversity of the hypothalamo-neurohypophysial system and its hormonal genes." Gen Comp Endocrinol **170**(1): 41-56.
Vacher, C., F. Ferriere, M. H. Marmignon, E. Pellegrini and C. Saligaut (2002). "Dopamine D2 receptors and secretion of FSH and LH: role of sexual steroids on the pituitary of the female rainbow trout." Gen Comp Endocrinol **127**(2): 198-206.
Vacher, C., E. L. Mananos, B. Breton, M. H. Marmignon and C. Saligaut (2000). "Modulation of pituitary dopamine D1 or D2 receptors and secretion of follicle stimulating hormone and luteinizing hormone during the annual reproductive cycle of female rainbow trout." J Neuroendocrinol **12**(12): 1219-1226.
Van Camp, K. A., G. Baggerman, R. Blust and S. J. Husson (2017). "Peptidomics of the zebrafish Danio rerio: In search for neuropeptides." J Proteomics **150**: 290-296.
Van Horssen, A. M. and G. J. M. Martens (1999). "Biosynthesis of secretogranin II in Xenopus intermediate pituitary." Molecular and Cellular Endocrinology **147**(1-2): 57-64.
Vidal, B., C. Pasqualini, N. Le Belle, M. C. Holland, M. Sbaihi, P. Vernier, Y. Zohar and S. Dufour (2004). "Dopamine inhibits luteinizing hormone synthesis and release in the juvenile European eel: a neuroendocrine lock for the onset of puberty." Biol Reprod **71**(5): 1491-1500.
Wakisaka, N., N. Miyasaka, T. Koide, M. Masuda, T. Hiraki-Kajiyama and Y. Yoshihara (2017). "An Adenosine Receptor for Olfaction in Fish." Curr Biol **27**(10): 1437-1447 e1434.

Wang, Q., K. W. Sham, S. Ogawa, S. Li, I. S. Parhar, C. H. Cheng, X. Liu and H. Lin (2013). "Regulation of the two kiss promoters in goldfish (Carassius auratus) by estrogen via different ERalpha pathways." Mol Cell Endocrinol **375**(1-2): 130-139.
Wang, S., Y. Zhu and P. Melamed (2009). "The molecular regulation of Chinook salmon gonadotropin beta-subunit gene transcription." Gen Comp Endocrinol **161**(1): 34-41.
Wang, W., H. Zhu, Z. Tian, A. Sun, Y. Dong, T. Dong and H. Hu (2020). "Effects of 11-Ketotestosterone on Development of the Previtellogenic Ovary in the Sterlet, Acipenser ruthenus." Front Endocrinol (Lausanne) **11**: 115.
Wang, Y. and W. Ge (2004). "Developmental profiles of activin betaA, betaB, and follistatin expression in the zebrafish ovary: evidence for their differential roles during sexual maturation and ovulatory cycle." Biol Reprod **71**(6): 2056-2064.
White, R. B., J. A. Eisen, T. L. Kasten and R. D. Fernald (1998). "Second gene for gonadotropin-releasing hormone in humans." Proc Natl Acad Sci U S A **95**(1): 305-309.
Wierman, M. E. (2007). "Sex steroid effects at target tissues: mechanisms of action." Adv Physiol Educ **31**(1): 26-33.
Winkler, H. and R. Fischer-Colbrie (1992). "The chromogranins A and B: the first 25 years and future perspectives." Neuroscience **49**(3): 497-528.
Wircer, E., J. Blechman, N. Borodovsky, M. Tsoory, A. R. Nunes, R. F. Oliveira and G. Levkowitz (2017). "Homeodomain protein Otp affects developmental neuropeptide switching in oxytocin neurons associated with a long-term effect on social behavior." Elife **6**.
Wulliman, M. F., B. Rupp and H. Reichert (2012). Neuroanatomy of the zebrafish brain: a topological atlas, Birkhäuser.
Xie, Y., L. Chu, Y. Liu, K. W. Y. Sham, J. Li and C. H. K. Cheng (2017). "The highly overlapping actions of Lh signaling and Fsh signaling on zebrafish spermatogenesis." J Endocrinol **234**(3): 233-246.
Xie, Y. and R. I. Dorsky (2017). "Development of the hypothalamus: conservation, modification and innovation." Development **144**(9): 1588-1599.
Xing, L., M. Goswami and V. L. Trudeau (2014). "Radial glial cell: critical functions and new perspective as a steroid synthetic cell." Gen Comp Endocrinol **203**: 181-185.
Yabuki, Y., T. Koide, N. Miyasaka, N. Wakisaka, M. Masuda, M. Ohkura, J. Nakai, K. Tsuge, S. Tsuchiya, Y. Sugimoto and Y. Yoshihara (2016). "Olfactory receptor for prostaglandin F2alpha mediates male fish courtship behavior." Nat Neurosci **19**(7): 897-904.
Yam, K. M., Y. Yoshiura, M. Kobayashi and W. Ge (1999). "Recombinant goldfish activin B stimulates gonadotropin-Iβ but inhibits gonadotropin-IIβ expression in the goldfish, Carassius auratus." General and comparative endocrinology **116**(1): 81-89.
Yamashita, J., Y. Nishiike, T. Fleming, D. Kayo and K. Okubo (2021). "Estrogen mediates sex differences in preoptic neuropeptide and pituitary hormone production in medaka." Commun Biol **4**(1): 948.
Yang, W., N. Zhang, Y. Wu, L. Zhang, L. Zhang and W. Zhang (2021). "Oxytocin-like signal regulates Lh cells directly but not Fsh cells in the ricefield eel Monopterus albusdagger." Biol Reprod **104**(2): 399-409.
Yaron, Z., G. Gur, P. Melamed, H. Rosenfeld, A. Elizur and B. Levavi-Sivan (2003). "Regulation of fish gonadotropins." Int Rev Cytol **225**: 131-185.

Yaron, Z. and B. Levavi-Sivan (2011). "Endocrine regulation of fish reproduction." Encyclopedia of fish physiology: from genome to environment **2**: 1500-1508.
Yokoi, S., K. Naruse, Y. Kamei, S. Ansai, M. Kinoshita, M. Mito, S. Iwasaki, S. Inoue, T. Okuyama, S. Nakagawa, L. J. Young and H. Takeuchi (2020). "Sexually dimorphic role of oxytocin in medaka mate choice." Proc Natl Acad Sci U S A **117**(9): 4802-4808.
Yokoi, S., T. Okuyama, Y. Kamei, K. Naruse, Y. Taniguchi, S. Ansai, M. Kinoshita, L. J. Young, N. Takemori, T. Kubo and H. Takeuchi (2015). "An essential role of the arginine vasotocin system in mate-guarding behaviors in triadic relationships of medaka fish (Oryzias latipes)." PLoS Genet **11**(2): e1005009.
Yoo, M. S., H. M. Kang, H. S. Choi, J. W. Kim, B. E. Troskie, R. P. Millar and H. B. Kwon (2000). "Molecular cloning, distribution and pharmacological characterization of a novel gonadotropin-releasing hormone ([Trp8] GnRH) in frog brain." Mol Cell Endocrinol **164**(1-2): 197-204.
Yoo, S. H. and J. P. Albanesi (1991). "High capacity, low affinity Ca2+ binding of chromogranin A. Relationship between the pH-induced conformational change and Ca2+ binding property." J Biol Chem **266**(12): 7740-7745.
Yu, K. L. and R. E. Peter (1990). "Dopaminergic regulation of brain gonadotropin-releasing hormone in male goldfish during spawning behavior." Neuroendocrinology **52**(3): 276-283.
Yu, K. L. and R. E. Peter (1992). "Adrenergic and dopaminergic regulation of gonadotropin-releasing hormone release from goldfish preoptic-anterior hypothalamus and pituitary in vitro." Gen Comp Endocrinol **85**(1): 138-146.
Zempo, B., T. Karigo, S. Kanda, Y. Akazome and Y. Oka (2018). "Morphological Analysis of the Axonal Projections of EGFP-Labeled Esr1-Expressing Neurons in Transgenic Female Medaka." Endocrinology **159**(2): 1228-1241.
Zhang, D., J. T. Popesku, C. J. Martyniuk, H. Xiong, P. Duarte-Guterman, L. Yao, X. Xia and V. L. Trudeau (2009). "Profiling neuroendocrine gene expression changes following fadrozole-induced estrogen decline in the female goldfish." Physiol Genomics **38**(3): 351-361.
Zhang, F. P., M. Poutanen, J. Wilbertz and I. Huhtaniemi (2001). "Normal prenatal but arrested postnatal sexual development of luteinizing hormone receptor knockout (LuRKO) mice." Mol Endocrinol **15**(1): 172-183.
Zhang, Q., D. Ye, H. Wang, Y. Wang, W. Hu and Y. Sun (2020). "Zebrafish cyp11c1 Knockout Reveals the Roles of 11-ketotestosterone and Cortisol in Sexual Development and Reproduction." Endocrinology **161**(6).
Zhang, X., F. Y. Che, I. Berezniuk, K. Sonmez, L. Toll and L. D. Fricker (2008). "Peptidomics of Cpe(fat/fat) mouse brain regions: implications for neuropeptide processing." J Neurochem **107**(6): 1596-1613.
Zhang, Y., S. Li, Y. Liu, D. Lu, H. Chen, X. Huang, X. Liu, Z. Meng, H. Lin and C. H. Cheng (2010). "Structural diversity of the GnIH/GnIH receptor system in teleost: its involvement in early development and the negative control of LH release." Peptides **31**(6): 1034-1043.
Zhang, Z., S. W. Lau, L. Zhang and W. Ge (2015). "Disruption of Zebrafish Follicle-Stimulating Hormone Receptor (fshr) But Not Luteinizing Hormone Receptor (lhcgr) Gene by TALEN Leads to Failed Follicle Activation in Females Followed by Sexual Reversal to Males." Endocrinology **156**(10): 3747-3762.
Zhang, Z., B. Zhu and W. Ge (2015). "Genetic analysis of zebrafish gonadotropin (FSH and LH) functions by TALEN-mediated gene disruption." Mol Endocrinol **29**(1): 76-98.

Zhao, E., A. Basak, K. Crump and V. L. Trudeau (2006). "Proteolytic processing and differential distribution of secretogranin-II in goldfish." Gen Comp Endocrinol **146**(2): 100-107.
Zhao, E., A. Basak and V. L. Trudeau (2006). "Secretoneurin stimulates goldfish pituitary luteinizing hormone production." Neuropeptides **40**(4): 275-282.
Zhao, E., A. Basak, A. O. Wong, W. Ko, A. Chen, G. C. Lopez, C. L. Grey, L. F. Canosa, G. M. Somoza, J. P. Chang and V. L. Trudeau (2009). "The secretogranin II-derived peptide secretoneurin stimulates luteinizing hormone secretion from gonadotrophs." Endocrinology **150**(5): 2273-2282.
Zhao, E., A. Basak, A. O. Wong, W. Ko, A. Chen, G. C. López, C. L. Grey, L. F. Canosa, G. M. Somoza, J. P. Chang and V. L. Trudeau (2009). "The secretogranin II-derived peptide secretoneurin stimulates luteinizing hormone secretion from gonadotrophs." Endocrinology **150**(5): 2273-2282.
Zhao, E., C. L. Grey, D. Zhang, J. A. Mennigen, A. Basak, J. P. Chang and V. L. Trudeau (2010). "Secretoneurin is a potential paracrine factor from lactotrophs stimulating gonadotropin release in the goldfish pituitary." Am J Physiol Regul Integr Comp Physiol **299**(5): R1290-1297.
Zhao, E., H. Hu and V. L. Trudeau (2010). "Secretoneurin as a hormone regulator in the pituitary." Regul Pept **165**(1): 117-122.
Zhao, E., H. X. Hu and V. L. Trudeau (2010). "Secretoneurin as a hormone regulator in the pituitary." Regulatory Peptides **165**(1): 117-122.
Zhao, E., J. R. McNeilly, A. S. McNeilly, R. Fischer-Colbrie, A. Basak, J. Y. Seong and V. L. Trudeau (2011). "Secretoneurin stimulates the production and release of luteinizing hormone in mouse L{beta}T2 gonadotropin cells." Am J Physiol Endocrinol Metab **301**(2): E288-297.
Zhao, E., D. Zhang, A. Basak and V. L. Trudeau (2009). "New insights into granin-derived peptides: evolution and endocrine roles." General and Comparative Endocrinology **164**(2-3): 161-174.
Zhou, R., A. H. Tsang, S. W. Lau and W. Ge (2011). "Pituitary adenylate cyclase-activating polypeptide (PACAP) and its receptors in the zebrafish ovary: evidence for potentially dual roles of PACAP in controlling final oocyte maturation." Biol Reprod **85**(3): 615-625.
Zhu, X. Y., A. S. Gleiberman and M. G. Rosenfeld (2007). "Molecular physiology of pituitary development: Signaling and transcriptional networks." Physiological Reviews **87**(3): 933-963.
Zhu, Y., C. D. Rice, Y. Pang, M. Pace and P. Thomas (2003). "Cloning, expression, and characterization of a membrane progestin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes." Proc Natl Acad Sci U S A **100**(5): 2231-2236.
Zohar, Y., J. A. Munoz-Cueto, A. Elizur and O. Kah (2010). "Neuroendocrinology of reproduction in teleost fish." Gen Comp Endocrinol **165**(3): 438-455.
Zohar, Y., N. Zmora, V. L. Trudeau, J. A. Munoz-Cueto and M. Golan (2021). "A half century of fish gonadotropin-releasing hormones: Breaking paradigms." J Neuroendocrinol: e13069.
Zou, T., Y. Q. Liang, X. Liao, X. F. Chen, T. Wang, Y. Song, Z. C. Lin, Z. Qi, Z. F. Chen and Z. Cai (2021). "Metabolomics reveals the reproductive abnormality in female zebrafish exposed to environmentally relevant levels of climbazole." Environ Pollut **275**: 116665.