Characterization and Role of Secretogranin-II/Secretoneurin in Zebrafish Reproduction

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ABSTRACT

At the hypothalamo-pituitary interface, multiple neurotransmitters and neuropeptides interact to control luteinizing hormone and follicle stimulating hormone release from gonadotrophs. The luteinizing hormone surge is essential for fertility as it triggers ovulation in females and sperm release in males. While it is well-established that gonadotropin-releasing hormone and/or kisspeptin are required for pulsatile and surge release of luteinizing hormone in mammalian species, their essentiality is challenged by studies showing knockouts in zebrafish and medaka do not block reproduction. In goldfish, secretoneurin-a, a neuropeptide derived from secretogranin-IIa processing, stimulates luteinizing hormone release *in vivo* and from dispersed pituitary cells *in vitro*. Secretoneurin does not bind to the human gonadotropin releasing hormone receptor and can enhance luteinizing hormone release when applied directly to mouse LbetaT2 cells. Our study indicates the presence of secretogranin-IIa and secretogranin-IIb mRNA in specific regions of the zebrafish brain and pituitary that have been implicated in the control of reproductive processes. I also observed that secretogranin-II knockout disrupts normal morphology of the pectoral fins by reducing the number of breeding tubercule rows, breeding tubercule width and breeding tubercule cluster length which could potentially lead to a reduced spawning success. Knocking out the secretogranin-IIa and secretogranin-IIb genes using TALENs in zebrafish indicates that these genes are required for optimal reproduction. Rates of oviposition for double secretogranin-II knockout females are 6% and 11% when crossed with double secretogranin-II knockout and wild-type males, respectively, compared to 62% in virgin wild-type pairings. Comprehensive video analysis demonstrates that secretogranin-II knockout reduces all stereotypical male courtship behaviours. Severe reductions in the expression of gonadotropin releasing hormone 3 in the hypothalamus and luteinizing hormone in the pituitary suggest that secretogranin-II-derived peptides drive the gonadotropin releasing hormone-luteinizing hormone control system. Spawning success is rescued in double secretogranin-II knockouts following one injection of synthetic secretoneurin-a in which it increases from 11% to 30% thereby supporting the hypothesis that secretoneurin-a is a key reproductive hormone. However, embryo survival rate of secretoneurin-a injected double secretogranin-II knockout was lower than saline-injected wild-type pairings. Injection of human chorionic gonadotropin, a hormone that mimics the action of luteinizing hormone on binding and activating the luteinizing hormone receptor in fish, increased double secretogranin-II knockout spawning success to 38%, thus comparing favourably to 47% in saline-injected wild-type controls. My data provides support that

secretogranin-II is required for optimal reproductive functions although the precise mechanisms behind this reduced capacity in zebrafish lacking the secretogranin-II genes remain to be investigated. Moreover, the high conservation of secretoneurin from lamprey to human suggests a broader importance of this emerging peptide family.

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ABBREVIATIONS

11-KT	11-ketotestotsterone
17,20βP	17,20-beta-dihydroxy-4-pregnen-3-one
17,20βP-S	17,20-dihydroxy-4-pregnen-3-one-20- sulfate
A	Anterior thalamic nucleus
AD	Androstenedione
ANOVA	Analysis of variance
AR	Androgen receptor
ATN	Anterior tuberal nucleus
BCIP	5-Bromo 4-chloro 3-indolyl phosphate
BLAST	Basic Local Alignment Search Tool
BT	Breeding tubercle
cAMP	Cyclic AMP
Cg	Chromogranin
CgA	Chromogranin A
CgB	Chromogranin B
CgC	Chromogranin C
CIL	Central nucleus of the inferior lobe
CM	Corpus mamillare
CP	Central posterior thalamic nucleus
CPN	Central pretectal nucleus
CRF	Corticotropin-releasing factor
D	Dorsal telencephalic area
DAO	Dorsal accessory optic nucleus
Dc	Central zone of D
Dd	Dorsal zone of D
ddPCR	Droplet digital PCR
DEPC	Diethyl pyrocarbonate
DHT	5α-dihydrotestosterone
DIG	Digoxigenin
DIL	Diffuse nucleus of the inferior lobe
DiV	Diencephalic ventricle
Dl	Lateral zone of D
Dm	Medial zone of D
DOT	Dorsomedial optic tract
DP	Dorsal posterior thalamic nucleus
Dp	Posterior zone of D
DTN	Dorsal tagmental nucleus
E2	17β-estradiol
ECL	External cellular layer of olfactory bulb including mitral cells
EDTA	Ethylenediaminetetraacetic acid
ENd	Entopeduncular nucleus, dorsal part

ENv	Entopeduncular nucleus, ventral part
ERK	Extracellular signal-regulated kinase
EtOH	Ethanol
EV	Early-vitellogenic follicle
EW	Edinger-Westphal nucleus
FG	Full-grown follicle
FSH	Follicle stimulating hormone
GABA	Gamma-aminobutyric acid
GFP	Green fluorescent protein
GH	Growth hormone
GL	Glomerular layer of olfactory bulb
GnRH	Gonadotropin releasing hormone (mammals)
Gnrh	Gonadotropin releasing hormone (in fish)
GnRHR	GnRH receptor
GSI	Gonadosomatic index
Gt	Gonadotrope
Had	Dorsal habenular nucleus
Hav	Ventral habenular nucleus
HBTU	N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate
Hc	Caudal zone of periventricular hypothalamus
Hd	Dorsal zone of periventricular hypothalamus
HEK	Human embryonic kidney
hpf	Hours post fertilization
HPG	Hypothalamic-pituitary-gonadal
HPLC	High performance liquid chromatography
HUVEC	Human umbilical vein endothelial cells
Hv	Ventral zone of periventricular hypothalamus
HYP	Hypothalamus
i.p.	Intraperitoneal
ICL	Internal cellular layer of olfactory bulb
ir	Immunoreactive
IST	Isotocin
Jak	Janus kinase
KO	Knockout
LH	Lateral hypothalamic nucleus
Lh	Luteinizing hormone
LLF	Lateral longitudinal fascicle
LOT	Lateral olfactory tract
MAPK	Mitogen-activated protein kinase
MCH	Melanin-concentrating hormone
MLF	Medial longitudinal fascicle
MOT	Medial olfactory tract
MSH	Melanocyte-stimulating hormone

MV	Mid-vitellogenic follicle
NBT	Nitro Blue Tetrazolium
NIII	Oculomotor nucleus
NIL	Neurointermediate lobe
NIV	Trochlear nucleus
NKB	Neurokinin B
NKF	Neurokinin F
NMLF	Nucleus of MLF
NMM	N-Methylmorpholine
NPY	Neuropeptide Y
NT	Nucleus taeniae
NTMT	Alkaline phosphatase buffer
OB	Olfactory bulb
OX	Orexin
OXT	Oxytocin
PBS	Phosphate buffered-saline
PC	Prohormone convertases
PCR	Polymerase chain reaction
PD	Pars distalis
PFA	Paraformaldehyde
PG	Primary growth follicle
$PGF_{2\alpha}$	Prostaglandin $F_{2\alpha}$
PGI	Lateral preglomerular nucleus
PGm	Medial preglomerular nucleus
PGZ	Periventricular gray zone of optic tectum
PI	Pars intermedia
Pit	Pituitary
PKA	Protein kinase A
PKC	Protein kinase C
PM	Magnocellular preoptic nucleus
POF	Primary olfactory fiber layer
POMC	Pro-opiomelanocortin
Ppa	Parvocellular preoptic nucleus, anterior part
PPd	Periventricular pretectal nucleus, dorsal part
PPD	Proximal pars distalis
PPp	Parvocellular preoptic nucleus, posterior part
PPv	Periventricular pretectal nucleus, ventral part
PRL	Prolactin
PSm	Magnocellular superficial pretectal nucleus
PSp	Parvocellular superficial pretectal nucleus
PTN	Posterior tuberal nucleus
PV	Pre-vitellogenic follicle

REST	RE-1 silencing transcription factor
RNA	Ribonucleic acid
RPD	Rostral pars distalis
RT-PCR	Real-time PCR
SC	Spermatocytes
SC	Suprachiasmatic nucleus
scg2	Secretogranin II gene
SD	Dorsal sac
Sg	Secretogranin
SG	Spermatogonia
SG	Subglomerular nucleus
SgII	Secretogranin II
SgIII	Secretogranin III
SgIV	Secretogranin IV
SgV	Secretogranin V
SgVI	Secretogranin VI
SgVII	Secretogranin VII
SN	Secretoneurin
SNR	SN receptor
ST	Spermatids
Stat	Signal Transducer and Activator of Transcription
SZ	Spermatozoa
T	Testosterone
TALEN	Transcription activator effector nucleases
TBST	Tris-Buffered Saline and Tween-20
TEL	Telencephalon
TelV	Telencephalic ventricle
TeO	Tectum opticum
TGN	Tertiary gustatory nucleus
TL	Torus longitudinalis
Tla	Torus lateralis
TPM	Tractus pretectomamillaris
TPp	Periventricular nucleus of posterior tuberculum
TS	Torus semicircularis
TSc	Central nucleus of torus semicircularis
TSH	Thyroid stimulating hormone
TSvl	Ventrolateral nucleus of torus semicircularis
TTB	Tractus tectobulbaris
V	Ventral telencephalic area
Val	Lateral division of valvula cerebelli
Vam	Medial division of valvula cerebelli
Vas	Vascular lacuna of area postrema
Vc	Central nucleus of V

Vd	Dorsal nucleus of V
VEGF	Vascular endothelial growth factor
Vl	Lateral nucleus of V
VL	Ventrolateral thalamic nucleus
VlM	Ventromedial thalamic nucleus
VM	Ventromedial thalamic nucleus
VOT	Ventrolateral optic tract
Vp	Postcommissural nucleus of V
VP	Vasopressin
Vs	Supracommissural nucleus of V
VSCC	Voltage-sensitive calcium channels
VT	Vasotocin
Vv	Ventral nucleus of V
wpf	Weeks post fertilization
WT	Wild-type

Chapter 1: General Introduction

This chapter was adapted from:

Mitchell KM[1]., Da Fonte D, Mikwar M and Trudeau VL. The role of the secretogranin-II-derived peptide secretoneurin in central and peripheral neuroendocrine systems. Invited review chapter for *Granin-derived Peptides*. Ed. Josef Troger (Austria). Research Signpost. Accepted January 2016.

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1.1 Granin protein family

The granins are a group of proteins that have characteristics of acidity and heat-stability and are distributed in a wide range of secretory granules in cells of the endocrine, neuroendocrine, and neuronal systems (Huttner et al., 1991). The granin proteins chromogranin A (CgA), chromogranin B (CgB) and secretogranin II (SgII) play essential roles in regulated secretory pathways that are responsible for secretory granule formation and subsequent secretion of peptides, hormones and neurotransmitters. The mechanism by which granins are likely controlling the regulated secretory pathway to both sort and package proteins in secretory granules occurs at the trans-Golgi network (TGN) through multiple steps. This process begins with the biosynthesis of granin proteins in the rough endoplasmic reticulum then, through vesicular transport, they are targeted to the TGN. Conditions such as low pH and high calcium ion concentrations in the TGN cause granins to aggregate while interacting with sorting receptors to form secretory granules. Finally, secretory granules fuse with the cell membrane and upon specific stimulation, the secretory granule contents (i.e., hormones) are released from the cell (Bartolomucci et al., 2011; Courel et al., 2010; Fischer-Colbrie et al., 1995; Ozawa and Takata, 1995).

Granin proteins share many common characteristics such as hydrophobicity, high proportion of acidic amino acids, calcium binding and the ability to form aggregates at low pH levels (Ozawa and Takata, 1995). Granins are also precursor proteins for smaller biologically active peptides through the proteolytic processing by prohormone convertases (PCs) at multiple dibasic amino acid cleavage sites (Helle, 2004; Huttner et al., 1991). The granin family is composed of two groups, the chromogranins (Cg) distinguished by the presence of a disulfide-bonded loop at the N-terminus and the secretogranins (Sg), where the disulfide loop is absent (Zhao et al., 2009b). The Cg family is divided into two subgroups including chromogranin A (CgA) and chromogranin B (CgB) while the Sg family consists of seven members: secretogranin II (SgII), secretogranin III (SgIII), secretogranin IV (SgIV), secretogranin V (SgV), secretogranin VI (SgVI), secretogranin VII (Sg VII), and proSAAS (Zhao et al., 2009b). The Cg proteins have a high degree of DNA and amino acid similarity and are conserved throughout vertebrate evolution. In contrast, the Sgs are not as well conserved compared to the Cgs (Zhao et al., 2009b). The first identified peptides generated from the granin family were pancreastatin from CgA, $CgB_{1\text{-}14}$ from CgB and secretoneurin (SN) from SgII (Helle, 2004). Secretogranin-II is unique among the known granins, as the large precursor is not well-conserved, while comparisons of the short core SN domain show clear sequence similarities across the vertebrate taxa. Here I review the evidence for the neuroendocrine functions of SgII and SN.

1.2 Secretogranin-II and Secretoneurin

Secretogranin-II is an approximately 600 amino acid long, acidic, tyrosine-sulfated preproprotein processed into small peptides by PCs at several dibasic cleavage sites in secretory granules of vertebrate endocrine and neuronal cells (Fischer-Colbrie et al., 1995). Also known as chromogranin C (CgC), SgII was originally isolated and characterized in the bovine anterior pituitary (Rosa and Zanini, 1981) and the rat PC12 cell line (Helle, 2010). Like other granins, SgII is involved in the biogenesis of secretory granules. Depletion of SgII by siRNA decreases both the number and size of dense-core secretory granules in PC12 cells (Courel et al., 2010). Because of its granulogenic properties, SgII is thought to participate in the sorting and packaging of hormones and neuropeptides in secretory granules (Miyazaki et al., 2011).

The primary sequence of mammalian SgII is well-conserved, being 79-87% identical between mammalian species. In marked contrast, the SgII sequences of some non-mammalian vertebrates has a low degree of conservation compared to mammalian SgII sequences (Blazquez et al., 1998; Montero-Hadjadje et al., 2008). Only a discrete domain in the middle of the SgII precursor, representing the SN peptide, is relatively well-conserved across all vertebrate taxa (Trudeau et al., 2012). Tetrapods such as mammals and amphibians have only one SgII gene and produce one SN peptide. However, teleosts (i.e., the bony fishes) have two SgII genes named SgIIa and SgIIb, that likely arose due to the whole genome duplication event that occurred in teleost fishes (Zhao et al., 2010b). The two teleost SgII subtype precursors generate their respective SNa and SNb peptides (Trudeau et al., 2012; Zhao et al., 2009b). Searches of the sea lamprey *(Petromyzon marinus)* genome revealed a single copy of a SgII-like gene with a potential SN-related segment (Trudeau et al., 2012). While the central core sequence is conserved and clearly identified as an SgII/SN family member, this predicted lamprey SgII precursor was unusually short compared to gnathostome SgII sequences. Secretogranin-II gene locus is found upstream of genomic regions coding for the serine protease inhibitor serpin in human (Chromosome 2), zebrafish (Chromosome 15), and lamprey (Sf_5643.1-182288), exhibiting conservation of synteny, and implying a shared ancestry (Trudeau et al., 2012). Currently, it is not possible to determine which of the SgII forms is evolutionarily more ancient, as SgII sequence information is lacking in many non-teleost fishes and diverse agnathans. The SgII or SN-like sequences in protochordates have yet to be identified.

Secretoneurin is found across vertebrate taxa and is moderately conserved. For example, the predicted goldfish SN ($SgII_{214–248}$) amino acid sequence shares 59% identity to human SN and more than 75% identity with some other vertebrate SN sequences (Blazquez et al., 1998). The SgII precursor

is processed at dibasic cleavage sites to yield free SN peptide by PCs. Some, but not all these dibasic cleavage sites are conserved between mammals and fish, allowing for significant species variation in SgII processing (Blazquez and Shennan, 2000). From the PC family members (PC1/PC3, PC2, PACE 4, PC4, PC5/PC6 and furin/PACE), only PC1 and PC2 have been shown to cleave SgII to generate both SN-containing intermediately-sized fragments and the free SN peptide. Both PC1 and PC2 are colocalized with SN in neurosecretory granules in the bovine posterior pituitary (Egger et al., 1994). However, only PC1 can generate SN in neurons, suggesting that processing of SgII is tissue- and/or cell-type specific (Hoflehner et al., 1995). In another study, PC12 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 et al., 1998). Moreover, in the absence of PC2, SN levels in mouse brain remain unchanged, indicating the important role of PC1 in generating the SN peptide in a mammal *in vivo* (Laslop et al., 2002). Evidence supports the role of both PC1 and PC2 in generating SN from SgII, although, the relative involvement of either PC depends on the tissue studied and the experimental system employed.

Originally, we proposed that the core YTPQ-X-LA-X_7-EL sequence defined the SN peptide family (Trudeau et al., 2012), however, position 15 (i.e., the 'A') is variable. For example, the predicted amino acid sequences of SN (Fig. 1.1), all contain an 'A' at position 15 except for trout SNb. It is interesting to note that the predicted SNa sequences for lamprey and shark are 43 and 42 amino acids long respectively, whereas in the elephant shark it is more like other SN forms at 31 amino acids (Fig. 1.1). The C-terminal dibasic processing site for lamprey is a prediction and could conceivably be at or near an earlier single K position thereby resulting in a mature SN peptide of 32 amino acids long. The native SN peptides in agnathan and chondrychthian species must be identified, as has been done in frog (Chartrel et al., 1991), rat (Kirchmair et al., 1993) and goldfish (Zhao et al., 2009a) in order to resolve such data gaps.

```
Lamprey          VQENIEDEYTPQNLARLQVILQELGFFDRAGGKTPARPESRGV  43
Shark            TNEIVEEQYTPQSLATLESAFRELGKYAGPYKEQGRLEEEHF-  42
Elephant shark   TNEIVEEQYTPQSLATLESAFQELGKYTGAY------------  31
Eel              ANERAEEQYTPQGLATLQSVFQELGKLSAAKN-----------  32
Trout SNa        TNENMEGKYTPQNLANLQSVFEELGRIANAK------------  31
Trout SNb        ATEDLKEKYTPQSLNNLRSIFKELGKPSTSNNQ----------  33
Zebrafish SNa    TNENAEEQYTPQKLATLQSVFEELSGIASSKTNT---------  34
Zebrafish SNb    ATEDLDEQYTPQSLANMRSIFEELGKLSAAQ------------  31
Frog             TSEIVEGQYTPQNLATLQSVFQELGKLKGQANP----------  33
Chicken          TNEIVEEQYTPQSLATLESVFQELGKMAGPSNH----------  33
Human            TNEIVEEQYTPQSLATLESVFQELGKLTGPNNQ----------  33
                 . *  . :**** *  :   : **.
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Figure 1.1 Predicted amino acid sequences of SN. The predicted amino acid sequences for lamprey (*Petromyzon marinus*), shark (*Squalus acanthius*), elephant shark (*Callorhinchus milii*), eel (*Anguilla anguilla*), zebrafish (*Danio rerio*) SNa, zebrafish SNb, frog (*Xenopus tropicalis*), chicken (*Gallus gallus*) and human (*Homo sapiens*) SN are shown. An asterisk (*) indicates a fully conserved residue, a colon (:) indicates conservation between groups of strongly similar properties, a period (.) indicates conservation between groups of weekly similar properties. The number to the right indicates the length of the peptide. For reference, the YTPQ-X-L-X8-EL signature is underlined in the human SN sequence. The Multiple Sequence Comparison by Log-Expectation (MUSCLE) program (http://www.ebi.ac.uk/Tools/msa/muscle/) was used to align the sequences.

Besides SN, only two other peptides have been identified in mammalian SgII, which are EM66 and manserin (Fischer-Colbrie et al., 1995; Yajima et al., 2004). Flanking SN at the C-terminus is the 66-amino acid peptide EM66 which is well conserved in human and other tetrapods but is not at all conserved in fish (Anouar et al., 1998; Zhao et al., 2009c). Manserin, the most recently discovered peptide processed from SgII, was first investigated in the rat anterior lobe of the pituitary and hypothalamus, suggesting that manserin might have a role in the neuroendocrine system (Yajima et al., 2004). No specific biological activity has yet been reported for either EM66 or manserin.

1.3 Secretoneurin Activates Signal Transduction Pathways that Indicate the Existence of a Specific G-Protein Coupled Receptor

High conservation of the SN peptide within the SgII precursor indicates important physiological functions that may be similar in the different vertebrate classes. Despite considerable efforts to map signaling pathways regulated by SN, a bona fide SN receptor has yet to be

identified. Regardless, there is evidence obtained using several cell lines that support the hypothesis that the SN receptor is a G-protein coupled membrane receptor. Specific binding sites assessed by classical radio-receptor ligand assays demonstrate high affinity binding in the human Mono Mac 6 monocyte cell line (Schneitler et al., 1998) and isolated human monocytes (Kong et al., 1998). Several studies indicate SN effects are sensitive to pertussis toxin, providing evidence that the SN receptor is a pertussis toxin-sensitive G-protein. Migration of human monocytes *in vitro* is inhibited by pertussis toxin and an inhibitor of protein kinase C, indicating signaling is coupled to Giα/o subunits of G proteins (Schratzberger et al., 1996). Secretoneurin binds to a cell surface receptor expressed on monocytes and activates signaling pathways, which are sensitive to both cholera and pertussis toxins (Kong et al., 1998). Additionally, SN stimulates granule cell differentiation through a signaling mechanism that is also coupled to pertussis toxin-sensitive G-proteins (Gasser et al., 2003). In both human monocytes (Schratzberger et al., 1996) and goldfish gonadotrophs (Zhao et al., 2011) SN activates protein kinase C (PKC) and induces a rise in intracellular calcium levels. Several other cell lines activate PKC-dependent pathways in response to SN (Dunzendorfer et al., 1998; Gruber et al., 1997; Kong et al., 1998; Kähler et al., 2002). In mouse LβT2 gonadotropin cells, SN increases cyclic AMP (cAMP) and activates both protein kinase A (PKA) and PKC causing the subsequent activation of extracellular signal-regulated kinase (ERK) signaling pathways (Zhao et al., 2011). Secretoneurin also mediates its effects in mouse endothelial progenitor cells (Kirchmair et al., 2004a) and epithelial cell lines (Xu et al., 2015) through ERK-dependent pathways. Together, these data lend support to the hypothesis that the putative SN receptor is G-protein-coupled (Fig. 1.2A).

1.4 Secretogranin-II and Secretoneurin in the Hypothalamus

Secretoneurin-immunoreactivity (SN-ir) has been most extensively studied in the rat and is widespread throughout the hypothalamus and pituitary (Fischer-Colbrie et al., 1995; Marksteiner et al., 1993; Zhao et al., 2009a). However, SN localization is dependent on proteolytic processing of the SgII precursor and the relative amounts and types of PCs present. In the rat brain, SgII processing to SN reaches 89–97%, 49% in the adrenal medulla, and only 26% in the anterior pituitary (Kirchmair et al., 1993). 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 et al., 1993). Secretoneurin is also overexpressed in certain neuroendocrine tumors (Fischer-Colbrie et al., 1995; Montero-Hadjadje et al., 2008).

In goldfish, SN-like ir fibers and presumptive nerve terminals are located in neuroendocrine territories of the brain such as the periventricular preoptic nucleus, ventrocaudal aspect of the nucleus of the lateral recess, and the pituitary (Canosa et al., 2011). Consistent with observations in the laboratory rat (Mahata et al., 1993a), the most conspicuous SN-ir is in the goldfish magnocellular and parvocellular cells of the preoptic nucleus that express isotocin (fish equivalent to oxytocin) and project heavily to the neural lobe of the pituitary (Canosa et al., 2011). Given that SN and oxytocin co-localize in dense core vesicles in the rat, they are likely to be co-released into the blood (Mahata et al., 1993b) from the posterior pituitary. More recently, we examined SN-ir distributions in the weakly pulse-type electric fish, *Brachyhypopomus gauderio*. Secretoneurin also colocalized with isotocin and vasotocin (fish equivalent to vasopressin) in cells and fibers of the preoptic area of this species (Pouso et al., 2015), further supporting data from the rat and goldfish.

Surprisingly, the possible function of SN co-released from neurons secreting either oxytocin (OXT) or vasopressin (VP) has not been explored. Colocalization with the OXT/VP family of nonapeptides supports a reproductive role for SN given the importance of neurons expressing these nonapeptides for the control of reproductive and social behaviors in numerous vertebrates (Goodson and Bass, 2001). Additionally, VP is an antidiuretic hormone controlling blood pressure, osmoregulation, hypertension, stress and many other functions (Mavani et al., 2015). Therefore, VP and SN co-release implies a role for SN in at least some of these diverse processes, however, these hypotheses await rigorous testing.

Using the electric fish model, it was shown that SN modulates a key social behaviour that is dependent on vasotocin. The South American electric fish (*Brachyhypopomus gauderio*) display electric organ discharges that signal arousal, dominance and subordinate status and constitute easily measurable, distinctive electric behaviors that depend on neural circuits (Caputi, 1999). Some SN-positive fibers were colocalized with vasotocin in the hindbrain, at the level of the command nucleus of the electric behaviour (pacemaker nucleus). Moreover, the rate of electric organ discharges that signals arousal and dominance and subordinate status was modulated by intraperitoneal injection of goldfish SN in a context-dependent manner (Pouso et al., 2015). Secretoneurin also increased pacemaker nucleus firing rate in brainstem slices *in vitro*, partially mimicking effects *in vivo* (Pouso et al., 2015). Therefore, SN acts through either neuroendocrine and/or endocrine mechanisms at the level of the electric fish pacemaker nucleus.

1.5 Secretogranin-II and Secretoneurin in the Pituitary Gland

An elevated level of SN is present in the median eminence of the rat and infundibular area of the goldfish suggesting a conserved hypophysiotropic role. Additionally, SN has been found in some endocrine cells of the pituitary gland. For example, SN-ir has been colocalized to varying degrees with LH, follicle stimulating hormone (FSH), thyroid-stimulating hormone (TSH), growth hormone (GH), prolactin (PRL) and adrenocorticotropic hormone in various mammalian species (Crawford and McNeilly, 2002; Fischer-Colbrie et al., 1995; Trudeau et al., 2012; Zhao et al., 2010b).

More precisely, SgII has been localized in normal rat mammotrophs and GH3B6 cells (Tougard et al., 1989b). Secretogranin-II is preferentially colocalized in secretory granules with TSH and Lh, rather than GH and PRL in the bovine pituitary gland (Bassetti et al., 1990a; Hashimoto et al., 1990). Rundle et al. (Rundle et al., 1986) also determined that ovine mammotrophs did not appear to contain SgII. On the other hand, at the electron microscopy level, heterogeneous distributions of PRL and SgII within secretory granules have been observed. Most cells exhibit colocalization of PRL and SgII in granules, however, there are some granules in rat pituitary cells that exhibit labeling for only PRL or only SgII (Ozawa et al., 1994; Tougard et al., 1989a). There may be distinct subpopulations of secretory granules containing granins or PRL in the bovine pituitary (Bassetti et al., 1990b). Some GH4C1 cells also store PRL in secretory granules in the absence of detectable levels of SgII (Thompson et al., 1992). An early report also indicated that SgII was mainly localized with the glycoprotein hormone (LH, FSH and TSH) producing cells of the rat anterior pituitary, with no apparent immunoreactivity detected in PRL cells (Conn et al., 1992). Vasauskas et al. (2011) showed that primate chorionic gonadotropin (Lh-like) and SgII are found in gonadotrophs in the squirrel monkey pituitary gland, yet PRL and SgII were found in distinct and separate cell populations. In the goldfish anterior pituitary, SN-ir is nearly exclusively found in the highly regionalized lactotrophs in the rostral region of the gland (Zhao et al., 2010b). Similarly, many cells in the electric fish pituitary, were both SN- and PRL-positive; however, there were also SN-ir cells in the proximal region of the pituitary, where both Lh and GH cells are regionally located (Pouso et al., 2015). There is nevertheless a strong association between SgII, SN-ir and PRL protein in the rat, bovine, goldfish and several PRL-secreting tumor cell lines. The lactotroph is therefore an evolutionarily conserved but not exclusive source of SN-ir products in the vertebrate anterior pituitary (Trudeau et al., 2012).

It is clear there are also species differences in the localization of SN-ir with the classical anterior pituitary hormones (Trudeau et al., 2012). There is yet no obvious pattern regarding the conservation of the association between SgII/SN colocalization with anterior pituitary cell types other

than the situation described for lactotrophs. This variation across the vertebrate taxa should be examined more extensively if we are to gain a comprehensive understanding of the evolution and function of SN in the pituitary. Another essential question relates to the differential release and function of SgII and derived peptides compared to the classical main hormones produced in pituitary endocrine cells (Bassetti et al., 1990b; Thompson et al., 1992). It is therefore possible, depending on physiological condition or species, that SN could be either co-released with pituitary hormones or independently released from cells secreting these classical hormones. The release could be local for autocrine or paracrine effects, or into the general circulation to affect peripheral functions.

1.6 Functions of Secretoneurin in the Anterior Pituitary

To date, the best described action of SN in a neuroendocrine system is the positive regulation of Lh in the anterior pituitary (Trudeau et al., 2012). Luteinizing hormone is the main hormone released to the circulation to control reproductive function. This hormone is essential in stimulating gonadal maturation, steroid synthesis and gamete release in vertebrates (Blazquez et al., 1998). Initially, an mRNA coding for the SgII protein was identified by a PCR differential display strategy, isolated and sequenced from the goldfish pituitary because its expression was increased in association with Lh release *in vivo* after robust stimulation with a γ-aminobutyric acid metabolism inhibitor (Blazquez et al., 1998). Only one of the 2 fish SN paralogs was known then and this known SN peptide was later named type A SN (SNa) (Zhao et al., 2010b). Intraperitoneal injection of SNa in combination with a dopamine D2 receptor blocker indicated, for the first time, that SN could rapidly stimulate anterior pituitary function *in vivo* (Blazquez et al., 1998). *In vitro* studies have since established that SN stimulates Lh production and release (Zhao et al., 2006a; Zhao et al., 2006b; Zhao et al., 2009a; Zhao et al., 2010a) in the goldfish model and from the mouse LβT2 gonadotroph cell line (Zhao et al., 2011). Moreover, SN stimulates calcium entry into isolated cultured goldfish gonadotrophs (Zhao et al., 2009a), and activates cAMP production and ERK through PKC-MAPK mediated pathways in LβT2 cells (Fig. 1.2A) leading to an increase in Lh production and release (Zhao et al., 2011). Although these signaling pathways are gonadotropin releasing-hormone (Gnrh)-like, SN does not modify Gnrh action *in vitro* nor does it activate the Gnrh receptor (Zhao et al., 2011). This evidence indicates that SN is an independent stimulator of Lh release. Secretoneurin can regulate Lh cells potentially by neuroendocrine, endocrine, paracrine and autocrine pathways.

In the goldfish model, direct innervation of the anterior pituitary by SN-producing isotocin neurons and intense SN-ir in lactotrophs (Canosa et al., 2011) supports the hypothesis that SN of both

neuroendocrine and paracrine origin may regulate pituitary Lh release (Fig. 1.2B). *In vitro* studies of dispersed goldfish pituitary cells showed that Gnrh stimulates the release of SN-ir proteins concomitantly with Lh and PRL (Conn et al., 1992; Nicol et al., 2002; Zhao et al., 2011). Furthermore, pre-incubation with a specific SN antibody partially blocked the stimulatory effect of Gnrh on Lh release, demonstrating that goldfish PRL cells release SN to affect nearby Lh cells by a paracrine mechanism (Zhao et al., 2011). Similarly, mouse LβT2 cells concomitantly release SN and Lh in response to GnRH, and SN alone can stimulate Lh production and release from these cells (Zhao et al., 2011). Moreover, SN can be released from native rat gonadotrophs in culture following GnRH stimulation (Tilemans et al., 1994; Wei et al., 1995). These data support the existence of an active, SN-dependent, autocrine positive feedback loop controlling Lh release (Trudeau et al., 2012). Even though SN has been shown to stimulate Lh release *in vivo* and *in vitro*, there is a critical absence of data regarding the involvement of SN in major reproductive processes such as ovulation, sperm release or an essential reproductive behaviour.

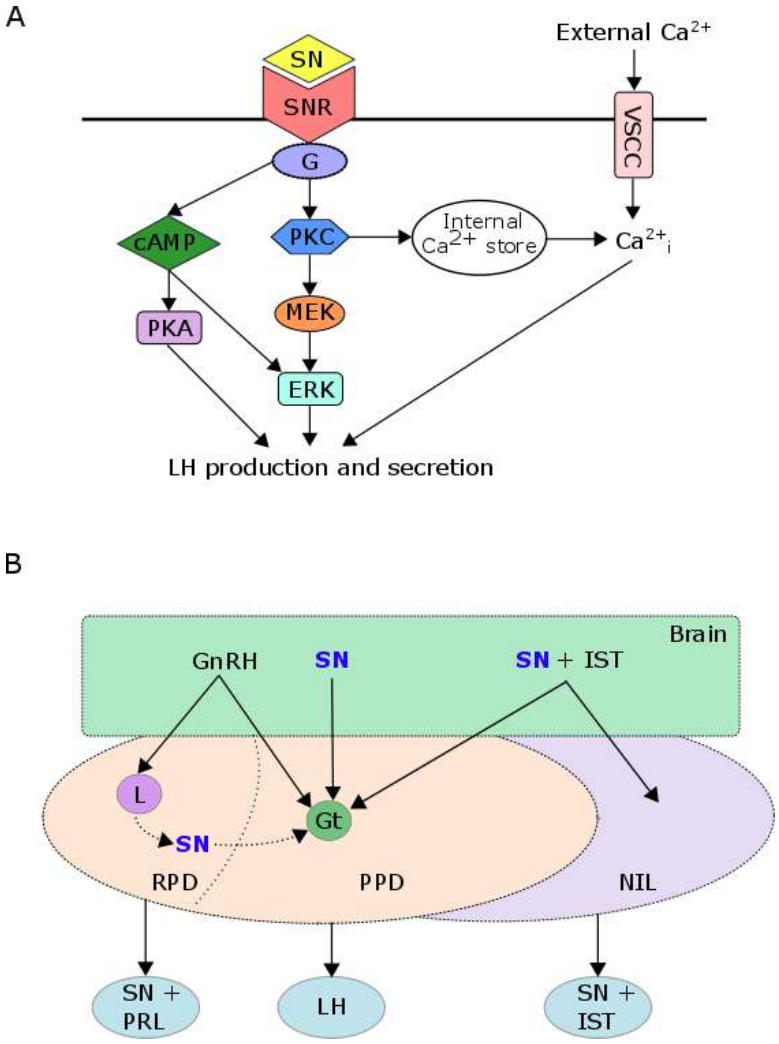

Figure 1.2 Proposed mechanism of action of secretoneurin (SN) in the gonadotrope (A) and role of SN in the regulation of Lh release in the goldfish model (B). (A) Exogenous SN activates both protein kinase A (PKA) and protein kinase C-dependent signaling cascades in the mouse LβT2 cell line. Secretoneurin increases cyclic AMP (cAMP) levels that in turn leads to activation of PKA-dependent luteinizing hormone (Lh) production and secretion. Activation of PKC leads to activation of the multiple kinase pathway involving mitogen-activated protein kinase MEK (MAPK/ERK kinase), the extracellular signal-regulated kinase (ERK), thus enhancing Lh. In several cell types, including the goldfish gonadotroph, SN-induced calcium ion entry via voltage-sensitive calcium channels (VSCC), may also contribute to Lh production and release. The membrane SN receptor (SNR) is proposed to be a G-protein (G)-coupled, but direct evidence is still lacking. (B) Gonadotropin-releasing hormone- (GnRH) producing neurons in the basal preoptic region directly project to the pituitary to regulate Lh release by two pathways. GnRH directly stimulates Lh release from the gonadotropes (Gt). Secretoneurin is localized to lactotrophs (L) in the rostral pars distalis (RPD) of the pituitary. Under the stimulation of GnRH, SN is released from the lactotrophs and acts on the Gt to stimulate Lh release in a paracrine manner (dotted lines). It is possible but not demonstrated that SN and PRL could be co-released to the circulation. Magoncellular neurons in the preoptic area are immunoreactive for SN and isotocin (IST) and project to the neurointermediate lobe (NIL), where it is possible that the 2 neuropeptides are co-released into the circulation. Additionally, SN-immunoreactive neurons containing IST also project to the proximal pars distalis (PPD) near the Gts. Some SN-immunopositive/IST-negative neurons of unknown origin also innervate the PPD and could stimulate Lh release by a neuroendocrine mechanism.

A microarray screen of dispersed goldfish pituitary cells incubated for 24 hours with SNa suggests that SN may regulate other functions of the anterior pituitary (Trudeau et al., 2012). Genes expressed in the pituitary involved in transport, metabolic processes, transcription, signal transduction, membrane and ion transport, apoptosis, development and phosphorylation were altered by SN treatment. This likely reflects both the diversity of endocrine cell types in the primary cultures of pituitary cells and the length of exposure to SNa *in vitro* (Trudeau et al., 2012).

It is suggested that SN is an intra-pituitary factor regulating cellular metabolism and hormone synthesis and release. It should be noted that while free SN peptide is produced by some pituitary cells, much of the SgII precursor is not fully processed, and thus there are larger SN-ir fragments (Zhao et al., 2006a) produced in the pituitary that could also be hormone-like factors. Biological activity has been tested solely for the SN peptide, thus the possibility that other SgII-derived peptides also hold functional significance is an open question worthy of investigation.

1.7 Thesis hypotheses and objectives

The aim of this thesis was to characterize the distribution and function of secretogranin-II/secretoneurin in zebrafish (*Danio rerio*). In this thesis, I tested the hypothesis that SgII regulates reproductive processes in zebrafish. To address this hypothesis, I had the following objectives:

1) Examine the levels of *scg2a* and *scg2b* in WT zebrafish brain during key developmental stages;
2) Observe the distribution of *scg2a* and *scg2b* in the brain and pituitary of adult wild-type (WT) zebrafish;
3) Measure the levels of *lhb, fshb*, *cga*, *oxt*, *avp, kiss2, tac3a* and g*nrh3* in zebrafish brain or pituitary by means of real-time PCR and ddPCR to determine if SgII-KO impacts key reproductive regulators;
4) Compare secondary sex characteristics, like breeding tubercles, between WT and SgII-KO zebrafish;
5) Determine if gene knockout of *scg2a* and/or *scg2b* alters spawning success, fecundity, fertility and courtship behaviour;
6) Perform morphological and histological analyses on SgII-KO zebrafish gonads and compare to WTs to determine if SgII-KO alters normal gonadal development;
7) Measure whole body zebrafish levels of testosterone, 11-ketotestosterone and estradiol to assess hormone levels in SgII-KO zebrafish;

8) Determine if SN injection can rescue the reduced reproductive capabilities of SgII-KO zebrafish;
9) Determine the mechanism behind the reduced reproductive capabilities of SgII-KO zebrafish.

1.8 Generation of SgII knockout zebrafish

In this study I comprehensively characterized the reproductive functions of SgII in zebrafish. To do so, Tao et al. (2018) previously generated three knockout models in zebrafish, by disrupting the SgIIa and SgIIb genes *(scg2a* and *scg2b*) using transcription activator-like effector nuclease (TALEN) technology. The three mutant lines generated contain mutations in *scg2a* [*scg2a*$^{-/-}$ or SgIIa-knockout (KO)], *scg2b* [*scg2b*$^{-/-}$ or SgIIb-KO] or both *scg2a* and *scg2b* [*scg2a*$^{-/-}$*;scg2b*$^{-/-}$ or SgII(a+b)-KO], resulting in a complete loss of all SgII peptides (Fig. 1.3). The SgIIa-KO mutants used in this research contained a seven base pair deletion and two base pair insertion (M1 line, Fig. 1.3A). The SgIIb-KO mutants used in this research contained a five base pair deletion and seven base pair insertion (M1 line, Fig. 1.3C). All lines assessed were homozygous mutants. Throughout the thesis these lines are referred to as SgIIa-KO, SgIIb-KO and SgII(a+b)-KO.

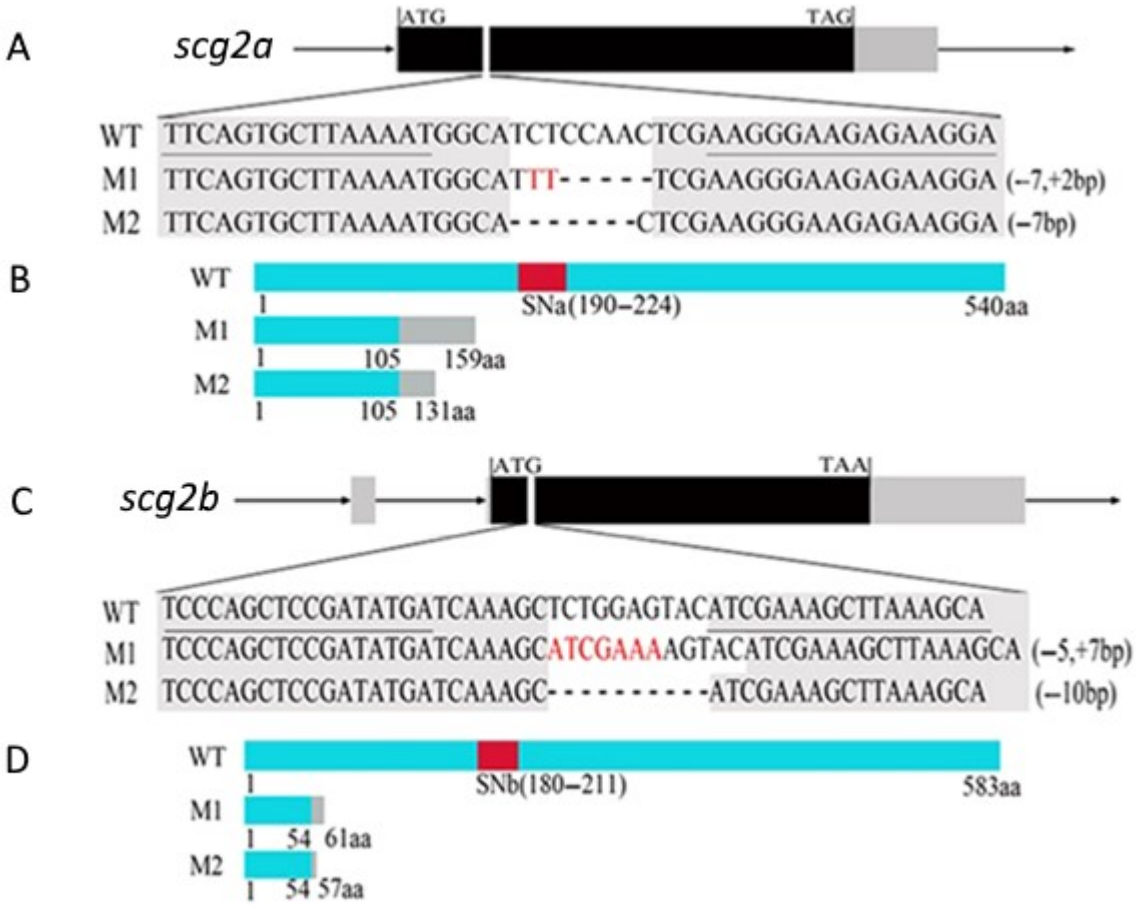

Figure 1.3 Generating secretogranin-II knockout zebrafish. Location of the TALEN-binding sites (underlined) on zebrafish *scg2a* or *scg2b* genes and two mutant lines of TALEN-targeted *scg2a* alleles (A) or *scgIIb* alleles (C). Deletions and insertions are indicated by dashes and red letters, respectively. (B and D) Schematic representation of the putative WT, SgIIa or SgIIb protein and two mutated SgIIa (B) or SgIIb (D) proteins. Figure adapted from Tao et al. (2018).

1.9 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.

1.10 Thesis presentation

This doctoral thesis is organized in 4 data chapters (Chapters 2-5). Chapter 2 addresses objectives 1-3; chapter 3 focuses on objective 4; chapter 4 examines objectives 4-7; chapter 5 focuses on objective 8; appendix 1 touches on objective 9.

1.11 Contribution of collaborations

This doctoral thesis also served as a source of projects for undergraduate students and volunteers; therefore, it involved the collaboration of many students. The students who were involved in honours projects, co-op student programs or who held NSERC summer scholarships are listed as co-authors of the chapters they participated in. The contributions of all co-authors are stated on the first page of each chapter.

Chapter 2: Maturational profile and consequences of *scg2a* and *scg2b* deletion for gene expression in the brain and pituitary of zebrafish

Kimberly Mitchell[1], Marissa Northorp[2], Wei Hu[3] and Vance L. Trudeau[4]

[1]Contributed to original ideas, conducted all experiments, performed data analysis and manuscript preparation.

[2]Helped prepare *in situ* probes

[3]Developed and provided SgII mutant zebrafish and collaborated on this study

[4]Contributed to original ideas and revised the manuscript

2.1 Introduction

The hypothalamic-pituitary-gonadal axis (HPG) plays a critical role in the development and regulation of reproductive systems. In all vertebrates, gonadotropin-releasing hormone (GnRH) is a neuropeptide with a key role in the control of reproduction. Phylogenetic analyses support the existence of three paralogous genes for GnRH (GnRH1, GnRH2 and GnRH3) in the vertebrate lineage (Okubo and Nagahama, 2008). In general, all species investigated to date, posses two or three distinct forms of GnRH. The most conserved form, GnRH2 is present in all jawed vertebrates, together with a species-specific form and a possible third form (Dubois et al., 2002). The species-specific forms vary but if a third form is present, like in modern teleost's, then it is always the GnRH3 form. A loss of other paralogous genes has also occurred. The GnRH3 and GnRH2 genes have been deleted or silenced in certain mammalian species whereas the GnRH1 or GnRH3 gene is lost in some teleosts. For example, only GnRH2 and GnRH3 genes are present in zebrafish (Powell et al., 1996). The two or three forms of GnRH are localized in different brain regions and have different embryonic origins (Dubois et al., 2002). GnRH2 cells are localized to the anterior midbrain and the posterior diencephalon whereas GnRH3, is present in the terminal nerve and ventral telencephalon (Dubois et al., 2002). The species-specific GnRH is present in the pre-optic area and regions of the hypothalamus (Dubois et al., 2002). GnRH is essential for the synthesis and secretion of the pituitary gonadotropins luteinizing hormone (Lh) and follicle stimulating hormone (Fsh). The gonadotropins bind to their receptors in the gonads, stimulating steroidogenesis and gametogenesis. Sex steroids produced by the gonads in turn feedback on the brain to either increase or decrease gonadotropin release.

Mammals possess a well-developed portal vascular system known as the median eminence, therefore, GnRH is secreted and travels to the pituitary via this portal system. However, the portal system in not well developed in teleost fish, therefore pituitary endocrine cells are directly innervated by a multitude of neurons (Peter et al., 1990) that secrete neuropeptides like Gnrh and secretoneurin (SN), and also classical neurotransmitters such as dopamine (Trudeau, 1997).

In teleosts, there is a complex interaction between a multitude of stimulatory and inhibitory factors in the brain that regulate gonadotropin release (Trudeau, 1997; Zohar et al., 2010). Notable neuropeptides and neurotransmitters include Gnrh, dopamine, gamma-aminobutyric acid (GABA), and neuropeptide-Y (NPY); Although recent research has also focused on kisspeptin (Kiss), gonadotropin inhibiting hormone (GnIH), tachykinin3a and secretoneurin (Trudeau, 2018). In fish, dopamine is one of a few inhibitory neurohormones and acts through multiple pathways and

mechanisms to inhibit the control of Lh release. More specifically, dopamine (1) is released from nerve terminals then activates D_2 receptors on the gonadotrophs to inhibit Lh secretion (Peter et al., 1986); (2) may decrease GnRH receptor binding to the pituitary; (3) interferes with intracellular GnRH signal transduction pathways inhibiting Lh release (Chang et al., 1993); and (4) has inhibitory effects on GnRH release at the pituitary nerve terminal (Peter et al., 1990). In contrast is the amino acid neurotransmitter GABA, that stimulates the release of Lh in goldfish by increasing Gnrh release and decreasing dopaminergic activity (Trudeau et al., 1993). Similarly, NPY stimulates Lh release at the levels of the hypothalamus and pituitary gonadotrophs. In the goldfish, NPY stimulates Gnrh release *in vitro* (Peng et al., 1993a). *In vitro* treatment with NPY also stimulates Lh release from the pituitary cells of the goldfish (Peng et al., 1993b). In mammals, the neuropeptide kisspeptin stimulates GnRH neurons by activating its kisspeptin receptor, GPR54. Kisspeptin has been shown to be essential for normal fertility in mammals, however, this essentiality in teleosts has been recently challenged. Kisspeptin mutant mice are infertile, fail to go through puberty, have reduced gonad sizes and decreased levels of FSH (Kirilov et al., 2013). Intraperitoneal administration of goldfish Kiss1 to mature females results in increased serum Lh levels; although, this peptide does not influence Lh release from goldfish pituitary cells in primary culture (Li et al., 2009). In zebrafish, kisspeptin mutant lines are not reproductively impaired thereby demonstrating that the kisspeptin systems play nonessential roles in reproduction in certain non-mammalian vertebrates (Tang et al., 2015). Neurokinin B (NKB), encoded by *tac3*, also plays a role in controlling fish reproduction. In zebrafish, Synthetic zebrafish NKBa and NKBb activates Tac3 receptors via PKC/Ca^{2+} and PKA/cAMP signal transduction pathways *in vitro* (Biran et al., 2012). The levels of Lh were also elevated in mature female zebrafish following a single intraperitoneal injection of NKBa and neurokinin F (NKF) (Biran et al., 2012). For my research, I have focused on the neuropeptide secretoneurin.

Secretoneurin is a peptide produced from the proteolytic processing of the precursor protein secretogranin II (SgII) by prohormone convertases (PCs) in secretory granules of vertebrate neuroendocrine cells (Fischer-Colbrie et al., 1995). The primary amino acid sequence of mammalian SgII is only moderately conserved through evolution, being 79-87% identical between mammalian species, but much less so in non-mammalian vertebrates (Blazquez et al., 1998; Montero-Hadjadje et al., 2008). Only a discrete domain in the middle of the SgII precursor, representing the SN peptide, is relatively well-conserved across all vertebrate taxa (Trudeau et al., 2012). Tetrapods, such as mammals and amphibians, has only one SgII gene and produce one SN peptide. However, teleosts have two SgII paralogs, SgIIa and SgIIb, likely a consequence of the early whole genome duplication

event that occurred in teleost fishes (Zhao et al., 2010b). The two teleost SgII subtype precursors generate SNa and SNb peptides, respectively (Trudeau et al., 2012; Zhao et al., 2009b), ranging in size from 31 to 43 amino acids.

The first SgII cDNA was cloned and sequenced from the common goldfish (Blazquez et al., 1998), which we know now is *scg2a*. Following this study, Samia et al. (2001) detected the presence of *scg2a* in the pituitary, hypothalamus, and ovary of goldfish which agreed with previous reports in mammals (Fischer-Colbrie et al., 1995). It was also determined that *scg2* levels varied seasonally in the female goldfish pituitary (Samia et al., 2004). The highest levels occurred in winter, when goldfish undergo sexual redevelopment and Lh expression and release are elevated whereas the lowest levels were in spring when goldfish are sexually mature, indicating a possible negative feedback mechanism (Samia et al., 2004). In zebrafish embryos and larvae, s*cg2a* expression is detectable by 10 hours post fertilization (hpf) and gradually increases until it stabilizes at 24 hpf, whereas *scg2b* expression is only weakly detected at 10 hpf and increases until it stabilizes at 36 hpf (Tao et al., 2018). Moreover, *scg2a* is highly expressed in the developing forebrain, midbrain and ventral part of the neural tube while *scg2b* is mainly expressed in the central nervous system (Tao et al., 2018). In adult zebrafish, *scg2a* is detected in the ventral telencephalon, preoptic area, midbrain, and dorsal hypothalamus (Marvel et al., 2018). Similarly, both *scg2a* and *scg2b* are highly expressed in select brain regions, including the telencephalon and hypothalamus, and the pituitary gland of the orange-spotted grouper (Shu et al., 2018).

Secretogranin-II and SN are also found in a variety of other tissues; however, SN localization is dependent on proteolytic processing of SgII and the relative amounts and types of prohormone convertases present. Therefore, SN displays differential processing in each tissue. The SgII protein is found in brain, pituitary, stomach, small and large intestine, thyroid cells, bronchial mucosa, ovary, testis, synovial fluid, aqueous humour, and the central nervous system of humans, rats, and bovine among others (see review by Fischer-Colbrie et al., 1995). Anatomical studies have shown that the most conspicuous secretoneurin-immunoreactivity (SN-ir) is in the preoptic nucleus and anterior pituitary of rat (Fischer-Colbrie et al., 1995; Marksteiner et al., 1993), goldfish (Canosa et al., 2011; Zhao et al., 2009b) and the weekly pulse-type electric fish (Pouso, et al. 2015) suggesting a role in the regulation of pituitary hormones. Furthermore, SN is also colocalized with isotocin (IST) and vasotocin (VT), the teleost homologs of oxytocin (OXT) and vasopressin (VP), respectively, in cells and fibers of the preoptic area in the weakly pulse-type electric fish (Pouso et al., 2015). A reproductive role for

SN is supported by its colocalization with the OXT/VP family of nonapepties which are important for reproductive and social behaviours in several vertebrates (Goodson and Bass, 2001).

SN stimulates Lh release *in vivo* and *in vitro* from pituitary fragments and dispersed pituitary cells of goldfish (Blazquez et al., 1998; Zhao et al., 2006b; Zhao et al., 2009a; Zhao et al., 2010b; Zhao et al., 2009b) and the LβT2 mouse gonadotrope cell line (Zhao et al., 2011), suggesting an evolutionarily conserved effect of SN on Lh release. In goldfish, SN also moderately up-regulates *lh*- and *fsh*-β subunit mRNAs (Zhao et al., 2010a). This body of evidence suggests that SgII and/or SN are involved in reproductive processes.

In this study, I first investigated the developmental expression of *scg2a* and *scg2b* in the brain of zebrafish from 2 weeks post fertilization (wpf) to 12 wpf since previous studies have either focused on embryos or adults. To understand their potential roles, I examined the gene expression of *scg2a* and *scg2b* in the male and female adult zebrafish brain and pituitary by *in situ* hybridization. To evaluate the physiological effect of *scg2* gene knockout on the reproductive axis, I measured gene expression of Gnrh 3 (*gnrh3*), Gnrh receptor 2 (*gnrhr2*), Fsh-β subunit (*fshb*), Lh-β subunit (*lhb*), glycoprotein-α subunit (*cga*), isotocin (*oxt*), vasotocin (*avp*), kisspeptin (*kiss2*), and tachykinin 3a (*tac3a*). Finally, to determine if genetic compensatory mechanisms (El-Brolosy and Stainier, 2017) are activated by the paralogous SgII gene, I measured SgIIa (*scg2a*) and SgIIb (*scg2b*).

2.2 Materials and Methods

2.2.1 Experimental animals

Wild-type (WT; AB strain) and SgII-KO zebrafish (Tao et al., 2018) were bred in house and raised according to standard husbandry procedures. Fish were maintained in 10-L tanks of dechloraminated City of Ottawa tap water at 28^{o}C on a 14:10 light-dark cycle in the University of Ottawa aquatics facility.

2.2.2 Tissue collection for gene expression

Wild-type zebrafish were collected at 2, 4, 6, 8, and 12 weeks post fertilization (wpf). At 2 wpf, 30 larvae were anesthetised in an ice bath then placed in a 2.0 ml safe-lock tube (Eppendorf). For all other time points, zebrafish were anesthetised in an ice bath to dissect and collect whole brains in 2.0 ml safe-lock tubes (8 fish brains pooled per tube). Fish were not distinguished by sex. A separate experiment was carried out with adult WT and SgII-KO zebrafish. Sexually mature adults (8 mpf) were

anesthetised in an ice bath, then their telencephalon, hypothalamus and pituitary were dissected and collected in 2.0 ml safe-lock tubes (4 fish pooled per tube). All samples were flash frozen in liquid nitrogen and stored at -80°C until RNA isolation took place.

2.2.3 Total RNA isolation and cDNA synthesis

Total ribonucleic acid (RNA) was extracted from heads, brains, telencephalon, hypothalamus and pituitary according to the manufacturer's protocols using Trizol™ (Thermo Scientific, cat# 15596018) and Phasemaker™ tubes (Invitrogen, cat# A33248) with a few modifications. Briefly, Trizol™ was added to each tube (300 µl for 2 wpf WT heads; 400 µl for 4, 6 and 8 wpf and 500 µl for 12 wpf WT brains; 200 µl for pituitaries; 400 µl for telencephalon and hypothalamus samples). The tissues were homogenized with stainless steel grinding balls in a mixer mill (Retsch mm301). The samples were centrifuged for 5 minutes at 12000 xg at 4°C then the supernatant was transferred to Phasemaker™ tubes and incubated for 5 minutes at room temperature. Chloroform was added to each tube (0.2 ml chloroform per 1 ml of Trizol™ used) which was then shaken vigorously for 15 seconds. The samples were left at room temperature for 5 min then centrifuged at 12000 xg, 4°C for 15 minutes. The supernatant was transferred to a new tube then isopropranol (0.5 ml of isopropanol per 1 ml of Trizol™) and 1 µl of GlucoBlue™ (Invitrogen, cat# AM9515) were added. The tubes were inverted 5-times, placed at -20°C for 30 min, then centrifuged at 12000 xg, 4°C for 10 min. The isopropanol was removed from each tube and the samples were rinsed by the addition of 0.5 ml 75% ethanol followed by centrifugation at 7500 xg, 4°C for 5 min. The ethanol was removed, and the samples were washed again with 0.5 ml 75% ethanol and centrifuged at 7500 xg, 4°C for 5 min. As much of the ethanol was removed as possible then the samples were left open to dry for 5 min in a fumehood. Samples were suspended in 15-25 µl ddH_2O depending on the amount of RNA present then heated at 60°C for 10 min. Total RNA concentrations and the 260/280 and 260/230 absorbance ratios were quantified using a spectrophotometer (NanoDrop 2000, Thermo Scientific). The RNA integrity was verified through gel electrophoresis (1% (w/v) agarose). RNA was frozen at –80°C until used. Genomic DNA was removed from 2, 4, 6, 8 and 12 wpf samples using AccuRT genomic DNA removal kit (abm, cat# G488) according to the manufacturer's protocol. Total complementary DNA (cDNA) was synthesized using the Maxima First Strand cDNA Synthesis Kit for RT-qPCR (Thermo Scientific, cat# K1642) according to the manufacturer's protocol. 1 µg of total RNA was used for cDNA synthesis (20 µl reactions) in all samples except the pituitary in which 250 ng was used. cDNA for each experiment and/or tissue type was synthesized at the same time then stored at -20°C.

2.2.4 Primer design for gene expression analysis

Gene specific primers were designed using the program Primer3 (version 0.4.0). Primers were supplied by Integrated DNA technologies. PCR products were verified for specificity by assessing sequenced products with the Basic Local Alignment Search Tool (BLAST). Annealing temperatures and cDNA dilutions were determined by performing thermal gradients (55-65°C) with cDNA collected from whole brain (1:10 dilution), telencephalon (1:5 – 1:200 dilution), hypothalamus (1:5 – 1:200 dilution) and pituitary (1:4 – 1:50 dilution). Primer sequences, annealing temperature and amplicon sizes are presented in Table 2.1.

Table 2.1 Droplet digital PCR primer sets and reaction conditions

Gene name	Gene abbreviation	Direction	Primer Sequence (5'-3')	Amplicon size	Annealing temperature (°C)
Luteinizing hormone beta	*lhb*	Forward Reverse	AATGCCTGGTGTTTCAGACC AACAGTCGGGCAGGTTAATG	144	59
Follicle stimulating hormone beta	*fshb*	Forward Reverse	TGTGGAGAGCGAAGAATGTG AGACCTTCTGGGTGTGCTGT	116	57
Glycoprotein alpha	*cga*	Forward Reverse	CTGCTGCTTTTCGAGAGCTT AGTGGCAGTCTGTGTGGTTG	155	59
Isotocin	*oxt*	Forward Reverse	GATCTGCTGCTGAAGCTCCT TACAAAAGTGGGTGGCGAGT	134	59
Vasotocin	*avp*	Forward Reverse	AGGTCTGCATGGAAGAGGAG CTGCCTTCAGGACAGTCTGG	146	58
Gonadotropin-releasing hormone 3	*gnrh3*	Forward Reverse	ATGGAGGCAACATTCAGGATGT CCTTTCAGAGGCAAACCTTCA	131	56
Gonadotropin-releasing hormone receptor 2	*ghrnr2*	Forward Reverse	TCCTCAACCCTCTGTCCATC TGCTTTGGGGAATCAATCTC	123	56
Neurokinin B	*tac3a*	Forward Reverse	GACTCATAACTTGCTGAAGAG ACTCTTTCCTCCATTGACGTC	188	58
Kisspeptin 2	*kiss2*	Forward Reverse	GCCTATGCCAGACCCCAAA TTTACTGCGTGCTAGTCGATGTTT	154	58

Gene name	Gene abbreviation	Direction	Primer Sequence (5’-3’)	Amplicon size	Annealing temperature (°C)
Secretogranin-IIa	*scg2a*	Forward Reverse	CAGGACGTACGGGTTATGCT GCGTTGGTCTTTGGTTTTGT	138	57
Secretogranin-IIb	*scg2b*	Forward Reverse	AAACAAAGCTCCGAGCAAAA AACTGGTGTCGGGATACTCG	116	57
Elongation factor alpha	*ef1α*	Forward Reverse	CAAGGAAGTCAGCGCATACA ACCGCTAGCATTACCCTCCT	150	59
Beta actin	*actβ1*	Forward Reverse	CTCTTCCAGCCTTCCTTCCT CTTCTGCATACGGTCAGCAA	166	61

2.2.5 Droplet digital PCR

Droplet digital PCR (ddPCR) set-up, droplet generation and transfer of emulsified samples to PCR plates were performed according to the manufacturer's protocols. Before beginning, cDNA samples were diluted based on previous ddPCR standard curve analyses. 23 µl ddPCR sample reactions [1 x EvaGreen ddPCR Supermix (Bio-Rad, cat# 1864034); 100 nM of gene specific forward and reverse primers; 5 µl of template cDNA and ddH2O] were prepared. 20 µl of the sample reactions and 70 µl of Droplet Generation oil for EvaGreen (Bio-Rad, cat# 186-4006) were loaded into the sample and oil wells of the droplet generation cartridges, respectively (Bio-Rad, cat# 1864007). 14000-20000 droplets were generated using the QX200 Droplet Generator (Bio-Rad), transferred to 96 well plates (Eppendorf) and sealed. The PCRs were performed on a Bio-Rad c1000 Touch thermal cycler according to the following cycling conditions: 1 x (95°C for 5 min); 40 x (95°C for 30 sec, annealing temp for 1 min, 4°C for 5 min) with a ramp rate of -2°C/sec; 1 x (90°C for 5 min). The fluorescent intensity of the droplets was measured with the QX200 Droplet Reader (Bio-Rad). Data analysis was performed with QuantaSoft software (Bio-Rad). For WT samples collected at 2, 4, 6, 8 and 12 wpf, the absolute transcript levels (copies/µl) were divided by the absolute transcript levels of a reference gene *ef1*α and presented as means + SEM (n = 7). The absolute transcript levels of telencephalon, hypothalamus and pituitary samples were normalized using the algorithm NORMA-Gene (Heckmann et al., 2011) and presented as means + SEM (n=5-7).

2.2.6 In situ hybridization

2.2.6.1 Tissue preparation

Sexually mature WT zebrafish were anesthetised in an ice bath. Tissues were prepared according to procedures outlined by Paul et al. (2016) with a few modifications. Briefly, fish heads were removed and placed immediately in 4% paraformaldehyde (PFA) in RNase-free phosphate buffered-saline (PBS; pH 7.4) and stored at 4°C for one week. Samples were then washed with PBS (2 x 30 min) and incubated in 0.5M Ethylenediaminetetraacetic acid (EDTA) for 7-10 days at room temperature for decalcification. The heads were washed in diethyl pyrocarbonate (DEPC)-H_2O (2 x 5 min) followed by a series of ethanol washes to dehydrate the tissues [30% EtOH (1 x 30 min); 50% EtOH (1 x 30 min); 70% EtOH (1 x 30 min); 99% EtOH (2 x 30 min). For the paraffin to infiltrate the tissue, samples were incubated in a 50% Citrisolv/50% EtOH mixture (1 x 30 min) followed by a 75% Citrisolv/25% EtOH mixture (1 x 30 min) and finally 100% Citrisolv (1 x 30 min) at room temperature.

Samples were subsequently incubated in a 50% Citrisolv/50% paraffin mixture at 60°C for one hour then 100% paraffin overnight. The following morning, samples were embedded in clean paraffin. Serial sections of 10 µm thickness were prepared using a motorized microtome (HM 350 Microm, Heidelberg) and spread on a water bath at 42°C. Once the sections expanded to normal size, they were mounted on Superfrost Plus slides (Fisher Scientific) and stored at 4°C until needed for *in situ* hybridization. Sections were used for *in situ* hybridization within two weeks of sectioning.

2.2.6.2 Antisense RNA probes

A 935 bp fragment of the zebrafish *scg2a* cDNA was cloned by RT-PCR from a cDNA library of adult zebrafish brain with forward primer 5'-TGAACCCGTTTCTTATGG-3' and reverse primer 5'-ACTGCATGGGTTTGACTTCC-3'. A 965 bp fragment of zebrafish *scg2b* was cloned the same way with forward primer 5'-CAACCCTCCTCCATACACTAA-3' and reverse primer 5'-GGCTGACAGCAGTTGTTTGA-3'. Digoxigenin (DIG)-labelled antisense RNA probes were generated as previously described (Thisse and Thisse, 2008).

2.2.6.3 *In situ* hybridization protocol

Prior to the hybridization, paraffin sections were warmed for 1 hour at 60°C then deparaffinized by washes in xylene (1 x 5 min, 1 x 10 min) and a series of ethanol washes [99% EtOH (1 x 5 min); 80% EtOH (1 x 10 min)]. Tissues were permeabilized by washing the slides with 0.3% Triton-X-100/DEPC-PBS (pH=7.4) (1 x 15 min) followed by washes DEPC-PBS (pH 7.4) (2 x 5 min). The slides were treated with proteinase K [5 µg/ml proteinase K, 0.1 M tris-HCl pH 8.0, 0.05 M EDTA and topped up with DEPC-H_2O); (1 x 5 min)] to improve permeability. The slides were washed with DEPC-PBS (2 x 5 min), fixed for 20 minutes in 4% PFA and washed with DEPC-PBS (2 x 5 min) again. To neutralize the changes produced during proteinase K treatment, slides were washed in acetylation mix [500 µl triethanolamine, 108 µl acetic anhydride and 40 ml DEPC-H_2O; (1 x 5 min)] made immediately prior to use. Slides were subsequently washed with DEPC-H_2O (2 x 5 min) and allowed to dry. All subsequent, steps were performed as previously described (Smith et al., 2008). Briefly, anti-sense DIG labelled RNA probes were diluted in hybridization buffer at a dilution of 1:300 and 1:500 for *scg2a* and *scg2b,* respectively. The probe mix was denatured at 70°C for 10 minutes, then transferred on ice. 450 µl of the probe mix was added to each slide that was then covered with the coverslip. Slides were hybridized at 70°C overnight (~17 hours). Slides were then washed in solution A [1x saline-sodium citrate (SSC), 50% deionized formamide, and 0.1% Tween-20; (2 x 30 min)] at

70°C, and in Tris-Buffered Saline and Tween-20 (TBST) at room temperature (2 x 30 min). Afterwards, slides were blocked in 10% calf serum in 1x TBST (blocking solution) for 1 hour at room temperature. 300 µl of 1:1000 diluted anti-DIG antibody conjugated with alkaline phosphatase (Roche) in blocking solution was applied to each slide and then they were covered with a coverslip and incubated at 4°C overnight. The following day the slides were washed in 1x TBST (4 x TBST) and equilibrated in alkaline phosphatase buffer (NTMT) staining buffer [100mM NaCl, 100mM Tris HCl pH 9.5, 50 mM $MgCl_2$, and 0.1% Tween-20; (1 x 10 min)]. The slides were stained overnight in opaque Coplin jars containing 40 ml of NTMT, 180 µl of Nitro Blue Tetrazolium (NBT) and 140 µl of 5-Bromo 4-chloro 3-indolyl phosphate (BCIP) at room temperature. The staining reaction was stopped by washes in distilled water (2 x 5 min) then slides were left to dry before mounting with coverslips using Aquamount (Polysciences). Slides were kept in the dark until visualization.

2.2.6.4 Microscopy

Microscopic images were taken using an Olympus DP70 digital microscope camera on a Zeiss Axiophot microscope. Images were processed with Zeiss (AxioVision4) software. Images were acquired in TIFF format and adjusted for light and contrast before being assembled in panels using ImageJ (FIJI) software. The nomenclature for the zebrafish brain followed Wullimann et al. (1996).

2.2.7 Data analysis

Statistical analyses on gene expression data were performed using SigmaPlot 11.0 software (Systat Software, Inc., Chicago, IL). Normality and homoscedasticity were tested using a Shapiro-Wilk test and Levene's test, respectively. One-way analyses of variance (ANOVA) followed by a Holm-Sidak post-hoc test was used to analyze the mRNA levels of *scg2a* and *scg2b* in WT fish throughout development. Two-way analyses of variance (ANOVA) followed by a Holm-Sidak post-hoc test was used to analyze the tissue specific mRNA levels. Information about the presentation of each two-way ANOVA and its associated statistics are described in each figure caption.

2.3 Results

2.3.1 Expression of scg2a and scg2b in the zebrafish brain during development

I aimed to determine the *scg2a* and *scg2b* expression profiles in the zebrafish brain during development and maturation. The level of *scgIIa* was low at 2-4 wpf but gradually increased except at 8-wpf when there was a slight but significant decrease in *scg2a* levels ($P < 0.001$) (Fig. 2.1A). Similarly, *scg2b* levels were low at 2-wpf and gradually increased except between 6 and 8-wpf when there was no significant difference ($P < 0.001$) (Fig. 2.1B). mRNA levels did not peak in the given time points I assessed but were considerable for both *scg2a* and *scg2b* in the brain by 12 wpf. Generally, there was a higher amount of *scg2b* than *scg2a* mRNA in the whole brain.

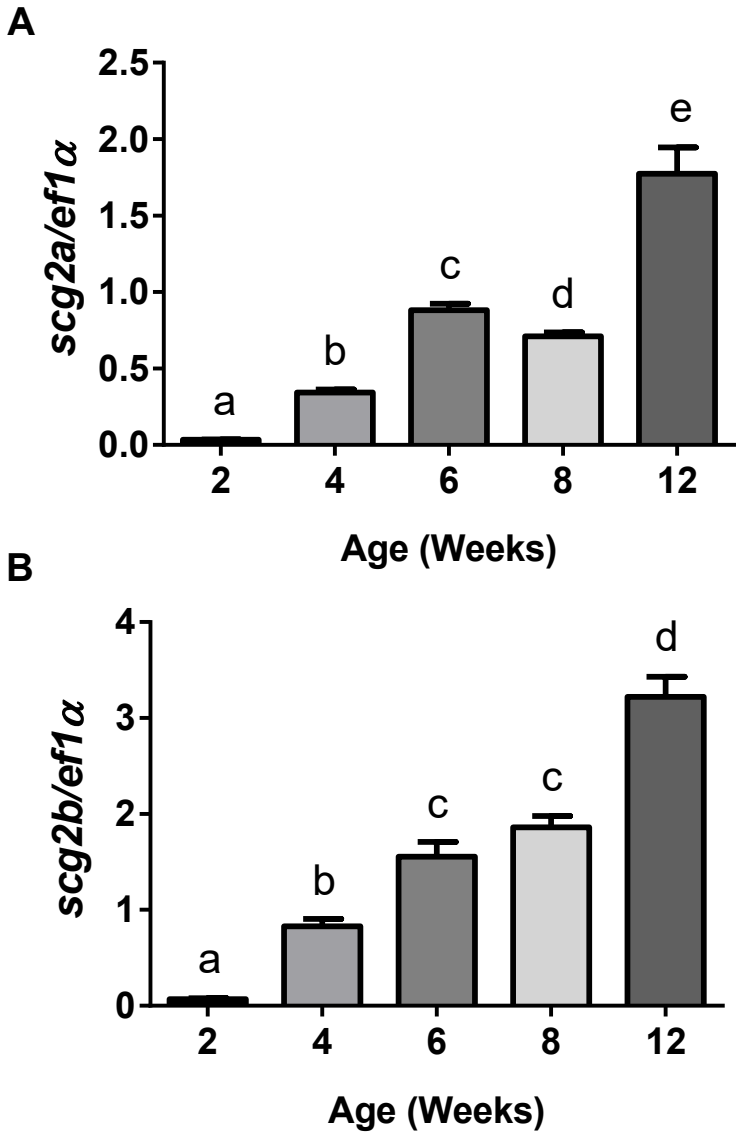

Figure 2.1 Relative expression of *scg2a* (A) and *scg2b* (B) in zebrafish head and brain at various ages. The relative abundance of the mRNAs (copies/µl) was normalized to the amount of elongation factor 1 α (*ef1α*). Results are presented as means + SEM (n = 7). Means with different letters denote significant differences ($P < 0.05$).

2.3.2 Localization of scg2a and scg2b in the brain and pituitary of adult zebrafish

In situ hybridization techniques were used to determine the localization of *scg2a* and *scg2b* transcripts in the brain and pituitary of adult zebrafish. Coronal sections from the olfactory bulbs to the medulla oblongata and midline sagittal sections of sexually mature males and females were examined. The general distribution patterns of expression of *scg2a* and *scg2b*, revealed by the antisense riboprobes are shown in figures 2.2, 2.3, 2.4 and table 2.2.

The overall patterns of expression of *scg2a* or *scg2b* were highly similar in all fish examined (4 males, 4 females). Furthermore, both *scg2a* or *scg2b* were expressed in the same areas and/or regions in both males and females. For this reason, micrographs presented in Figs 2.2 and 2.3 include both male and female brains although most are from females. Sections containing the pituitary of both males and females are presented for comparison in Fig. 2.4.

Strong staining of both *scg2a* and *scg2b* mRNA were detected in the central, dorsal, ventral and lateral nucleus of the ventral telencephalic area (Vc, Vd, Vv and Vl, respectively) (Fig. 2.2C2 and C4) as well as the supra- and post-commissural nuclei of the ventral telencephalic area (Vs and Vp, respectively) (Fig. 2.2D1 and E1; Fig. 2.2 D3 and E3) and the posterior zone of the dorsal telencephalic area (Dp) (Fig. 2.2F1 and F3). A high level of staining was also observed in the periventricular nucleus of the posterior tuberculum (TPp) (Fig. 2.3C1 and C4), posterior tuberal nucleus (PTN) (Fig. 2.3C3 and C6) and lateral and medial preglomerular nuclei (PGl and PGm, respectively) (Fig. 2.3C2 and C5). In the hypothalamus, staining was observed in the caudal, dorsal and ventral zones of the periventricular hypothalamus (Hc, Hd and Hv, respectively) (Fig 2.3B3, C3, D3, E3, B6, C6, D6 and E6) and the lateral hypothalamic nucleus (LH) (Fig. 2.3C3 and C6). The nucleus of the medial longitudinal fascicle (NMLF) showed staining for both *scg2a* and *scg2b* although *scg2b* appeared stronger (Fig. 2.3D2 and D5). Prominent staining was also observed in the central and ventrolateral nucleus of the semicircular torus (TSc and TSvl, respectively) (Fig. 2.3E1 and E4). See Table 2.2 for the complete distribution of *scg2a* and *scg2b* mRNA.

Serial pituitary sections were compared between *scg2a* and *scg2b* transcripts and they show clearly that *scg2a* mRNA is more prominent in the pituitary (Fig. 2.4A1, A2, B1 and B2) than *scg2b* (Fig. 2.4A3, A4, B3 and B4). Moreover, the highest expression was observed in the pars intermedia (PI) of the neurointermediate lobe (NIL) and low staining was observed in the rostral pars distalis (RPD) of females.

The distribution of *scg2a* and *scg2b* mRNA were similar, although *scg2b* was present in several regions where *scg2a* was not detected. These regions exhibiting only *scg2b* transcripts include

the medial zone of dorsal telencephalic area (Dm) (Fig 2.2D3), central nucleus of ventral telencephalic area (Vc) (Fig 2.2C4), dorsal habenuar nucleus (Had) (Fig 2.3B4), ventral habenular nucleus (Hav) (Fig 2.3B4), anterior thalamic nucleus (A) (data not shown), subglomerular nucleus (SG) (data not shown), lateral torus (TLa) (data not shown), oculomotor nucleus (NIII) (data not shown) and Edinger-Westphal nucleus (EW) (data not shown). Both *scg2a* and *scg2b* transcripts were present in the lateral zone of the dorsal telencephalon (Dl); however, there were few *scg2a* positive cells (Fig 2.2D3). The lateral division of valvula cerebelli (Val) and medial division of valvula cerebelli (Vam) were the only regions where *scg2a* expression was observed whereas *scg2b* was not detectable (Fig 2.3E1).

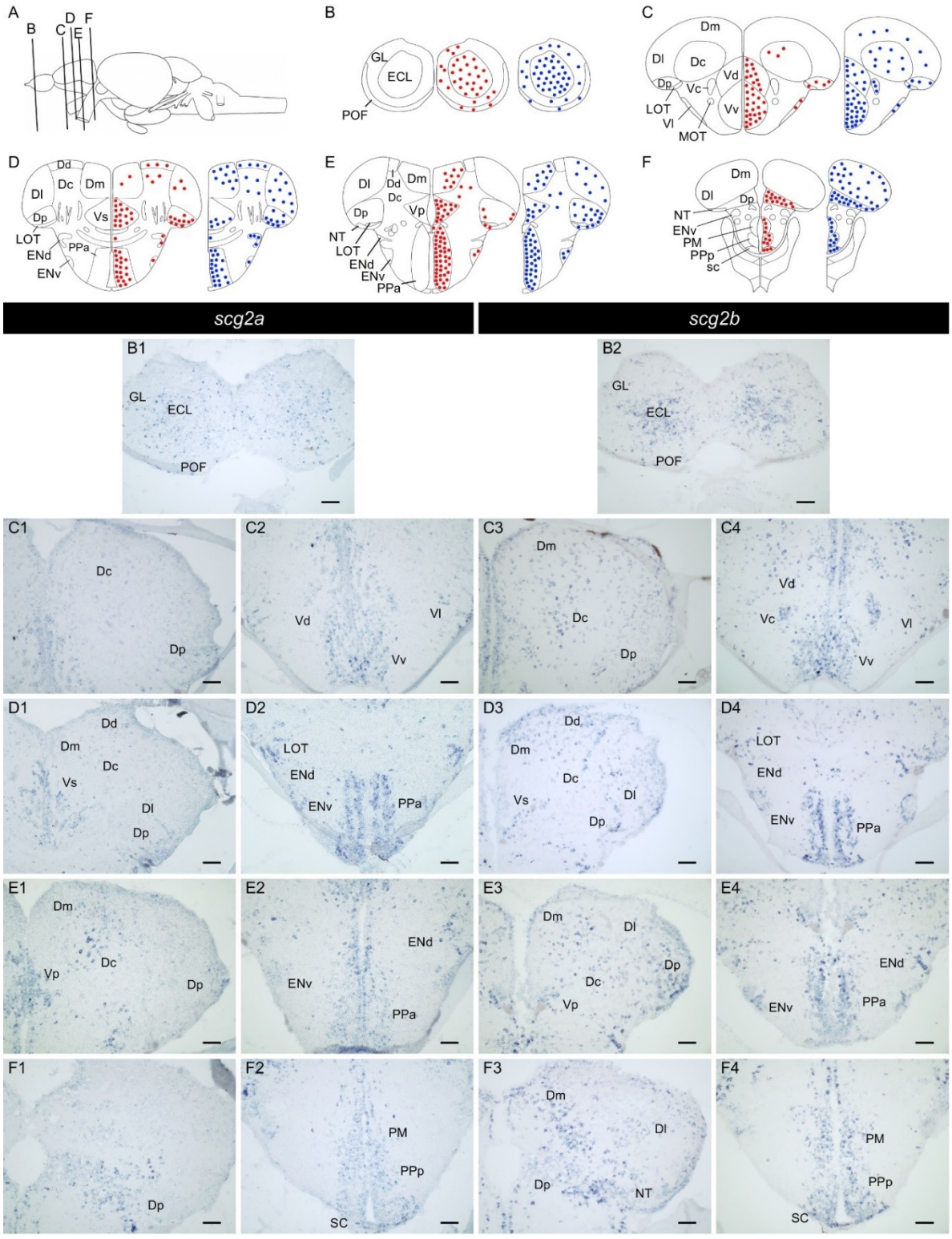
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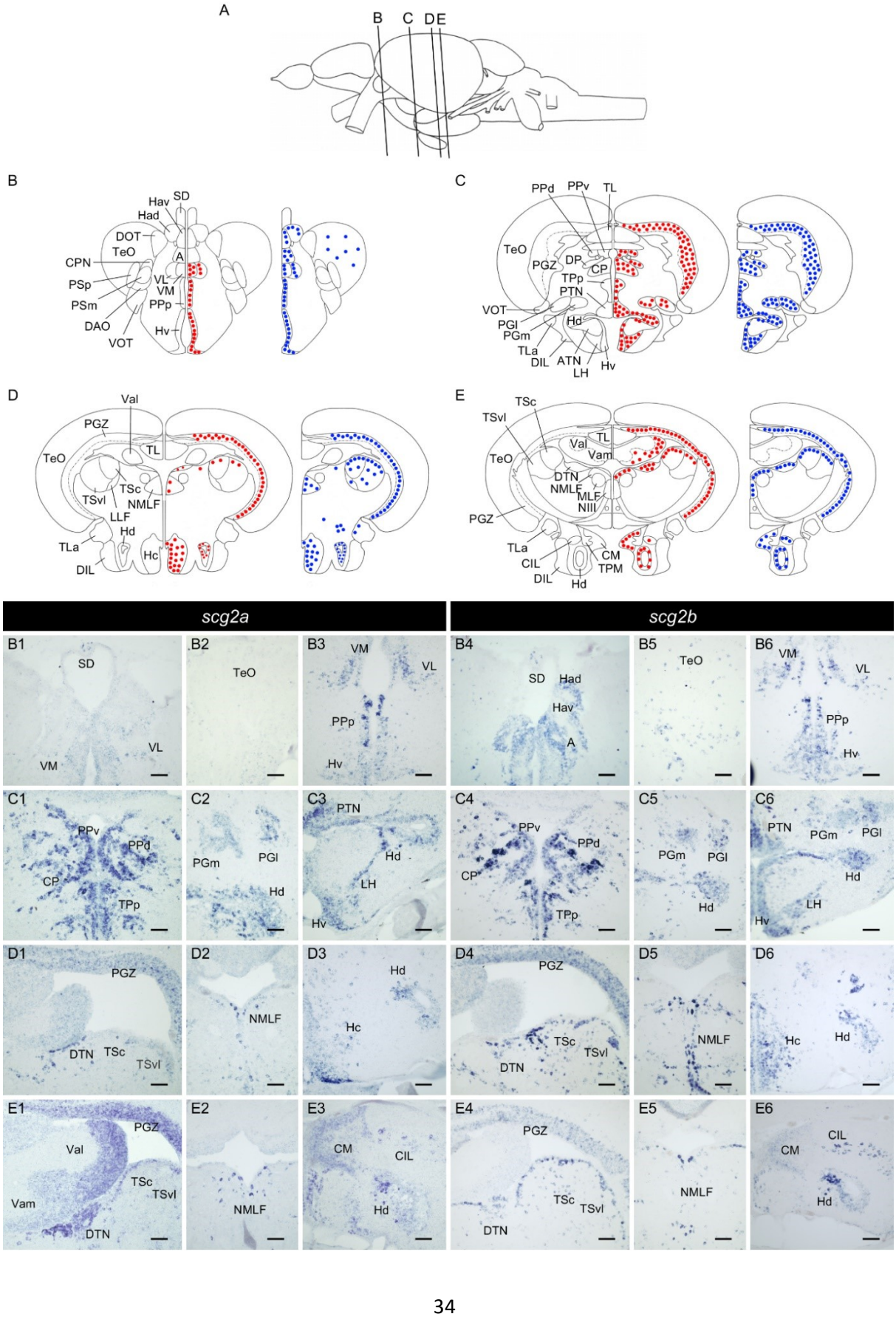
Figure 2.2 *scg2a* and *scg2b* distribution in cross sections through the adult zebrafish forebrain. (A) Schematic of the sagital view of the zebrafish brain. (B-F) Lines in A indicate the positions of coronal sections. A-E are schematic coronal brain drawings modified from the zebrafish atlas (Wullimann et al., 1996). *Scg2a* expressing cells are represented by red dots and and *scg2b* expressing cells are representated by blue dots. (B1, C1-2, D1-2, E1-2, F1-2) Micrographs of *scg2a* expressing cells in the 8 mpf female zebrafish olfactory bulb and telencephalon. (B2, C3-4, D3-4, E3-4, F3-4) Micrographs of *scg2b* expressing cells in 8 mpf female zebrafish olfactory bulb and telencephalon. Four females and three males were analyzed for both *scgIIa* and *scgIIb* transctiprs. Two-four sections were assessed for each position in every fish. For abbreviations, see abbreviation list. Scale bar = 50 µm.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E4
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DTN
E5
NMLF
E6
CIL
CM
Hd

Figure 2.3 *scg2a* and *scg2b* distribution in cross sections through the adult zebrafish midbrain. (A) Schematic of the sagittal view of the zebrafish brain. (B-E) Lines in A indicate the positions of coronal sections. A-E are schematic coronal brain drawings modified from the zebrafish atlas (Wullimann et al., 1996). *Scg2a* expressing cells are represented by red dots and *scg2b* expressing cells are represented by blue dots. (B1-3, C1-3, D1-3, E1-3) Micrographs of *scg2a* expressing cells in the 8 mpf female zebrafish midbrain. (B4-6, C4-6, D4-6, E4-6) Micrographs of *scg2b* expressing cells in 8 mpf female zebrafish midbrain. Four females and three males were analyzed for both *scgIIa* and *scgIIb* transctiprs. Two-four sections were assessed for each position in every fish. For abbreviations, see abbreviation list. Scale bar = 50 µm.

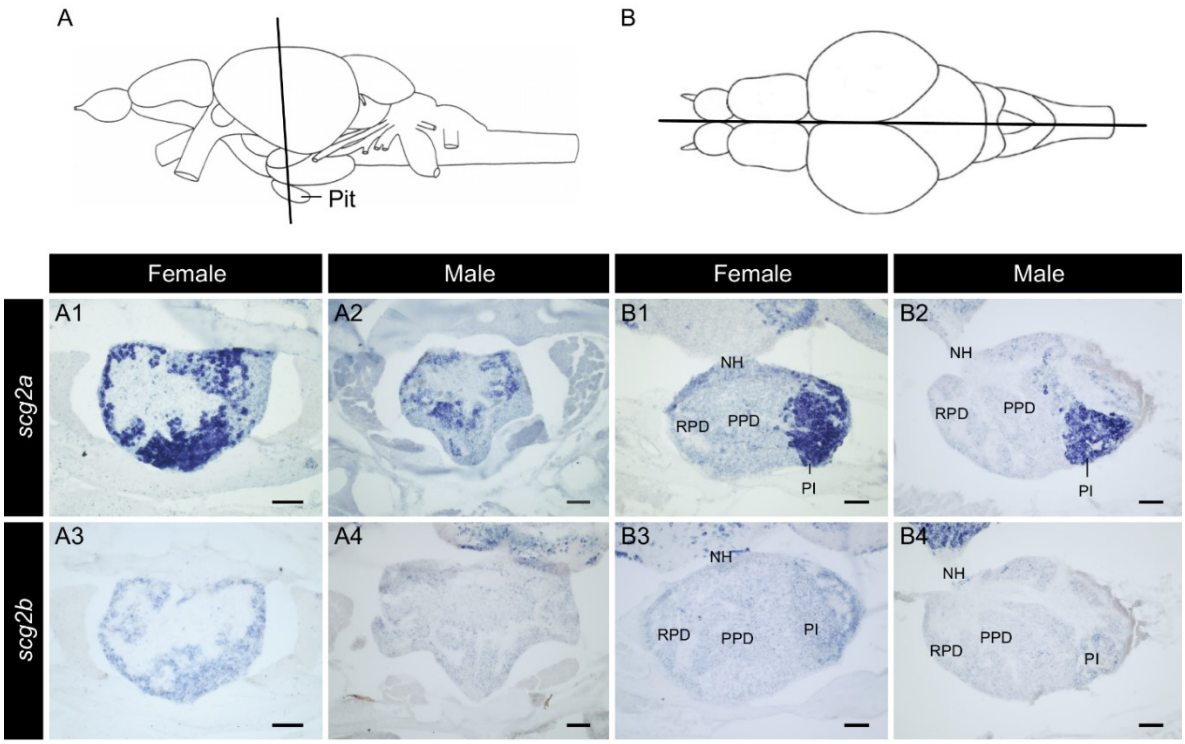

Figure 2.4 *scg2a* and *scg2b* distribution in the adult zebrafish pituitary. (A) Schematic of the coronal view of the zebrafish brain. (B) Schematic of the sagital view of the adult zebrafish brain. Lines in A and B indicate the positions of coronal and sagital sections, respectively. Micrographs of *scg2a* (A1-A2) and *scg2b* (A3-4) expressing cells in 8 mpf adult female and male coronal sections. Micrographs of *scg2a* (B1-A2) and *scg2b* (B3-4) expressing cells in 8 mpf adult female and male sagital sections. Pit, pituitary; RPD, rostral pars distalis; PPD, proximal pars distalis; PI, pars intermedia; NH, neurohypophysis. Four females and three males were analyzed for both *scgIIa* and *scgIIb* transcripts. Two-four sections were assessed for each position in every fish. Scale bar = 50 µm.

Table 2.2. Structures in the adult zebrafish brain showing *scg2a* and *scg2b* (+ = present; - = not observed).

Structure	*scg2a*	*scg2b*
Olfactory bulbs (OB)		
lateral olfactory tract (LOT)	-	-
medial olfactory tract (MOT)	-	-
primary olfactory fiber layer (POF)	+	+
glomerular layer of olfactory bulb (GL)	+	+
external cellular layer of olfactory bulb (ECL)	+	+
internal cellular layer of olfactory bulb (ICL)	+	+
Dorsal telencephalic area (D)		
central zone of dorsal telencephalic area (Dc)	+	+
lateral zone of dorsal telencephalon (Dl)	+	+
medial zone of dorsal telencephalic area (Dm)	+	+
posterior zone of dorsal telencephalic area (Dp)	+	+
Ventral telencephalic area (V)		
central nucleus of ventral telencephalic area (Vc)	-	+
dorsal nucleus of ventral telencephalic area (Vd)	+	+
postcommissural nucleus of ventral telencephalic area (Vp)	+	+
supracommissural nucleus of ventral telencephalic area (Vs)	+	+
ventral nucleus of ventral telencephalic area (Vv)	+	+
lateral nucleus of ventral telencephalic area (Vl)	+	+
entopeduncular nucleus, dorsal part (ENd)	+	+
entopeduncular nucleus, ventral part (ENv)	+	+
Nulceus preopticus (NPO)		
parvocellular preoptic nucleus, anterior part (PPa)	+	+
parvocellular preoptic nucleus, posterior part (PPp)	+	+
suprachiasmatic nucleus (SC)	+	+
magnocellular preoptic nucleus (PM)	+	+
Epithalamus		
dorsal habenular nucleus (Had)	-	+
ventral habenular nucleus (Hav)	-	+
dorsal sac (SD)	+	+
dorsal thalamus (DT)		
anterior thalamic nucleus (A)	-	+
dorsal posterior thalamic nucleus (DP)	+	+
central posterior thalamic nucleus (CP)	+	+
ventral thalamus (VT)		
ventrolateral thalamic nucleus (VL)	+	+

Structure	*scg2a*	*scg2b*
ventromedial thalamic nucleus (VM)	+	+
Posterior tuberculum		
periventricular nucleus of posterior tuberculum (TPp)	+	+
posterior tuberal nucleus (PTN)	+	+
lateral preglomerular nucleus (PGl)	+	+
medial preglomerular nucleus (PGm)	+	+
tertiary gustatory nucleus (TGN)	+	+
subglomerular nucleus (SG)	-	+
lateral torus (TLa)	-	+
Hypothalamus		
caudal zone of periventricular hypothalamus (Hc)	+	+
dorsal zone of periventricular hypothalamus (Hd)	+	+
ventral zone of periventricular hypothalamus (Hv)	+	+
anterior tuberal nucleus (ATN)	+	+
lateral hypothalamic nucleus (LH)	+	+
central nucleus of the inferior lobe (CIL)	+	+
hypophysis=pituitary (Pit)	+	+
Synencephalon		
nucleus of the medial longitudinal fascicle (NMLF)	+	+
parvocellular pretectal nucleus, dorsal part (PPd)	+	+
parvocellular pretectal nucleus, ventral part (PPv)	+	+
Tectum opticum (TeO)		
periventricular gray zone of optic tectum (PGZ)	+	+
semicircular torus (TS)		
central nucleus of semicircular torus (TSc)	+	+
ventrolateral nucleus of semicircular torus (TSvl)	+	+
Tegmentum		
oculomotor nucleus (NIII)	-	+
trochlear nucleus (NIV)	+	+
Edinger-Westphal nucleus (EW)	-	+
dorsal tegmental nucleus (DTN)	+	+
Cerebellum (Ce)		
cerebellar corpus (CCe)	+	+
lateral division of valvula cerebelli (Val)	+	-
medial division of valvula cerebelli (Vam)	+	-

2.3.3 Genes involved in reproduction are altered following SgII-KO in adult male and female zebrafish telencephalon

All genes assessed in the telencephalon except *gnrh3* showed no statistical main effects from sex or interactions between genotype and sex. In those cases, results were pooled across sex (Fig. 2.5).

There were significant inhibitory main effects from genotype [$F(3) = 4.638$, $P = 0.007$], no significant effects of sex [$F(1) = 2.663$, $P < 0.110$] but a significant interaction between genotype and sex [$F(3) = 3.318$, $P = 0.028$] on the levels of *gnrh3*. Post-hoc analyses indicated that SgIIb-KO and SgII(a+b)-KO females had significantly lower transcript levels compared to WT females ($P < 0.05$); whereas SgIIa-KO females were not statistically different from WT females. The levels of *gnrh3* were reduced by 61% and 63% in SgIIb-KO and SgII(a+b)-KO females compared to WT females. The levels of *gnrh3* were also reduced in all SgII-KO males however, none were statistically significant compared to the WT male controls ($P < 0.05$). Furthermore, only WT females had significantly higher *gnrh3* levels compared to males ($P < 0.05$) while this increase was not observed in the SgII-KO animals (Fig. 2.5A).

For *tac3a*, there was a significant main effect of genotype [$F(3) = 19.906$, $P < 0.001$]. However, there were no significant effects of sex [$F(1) = 0.309$, $P = 0.581$] or interactions between genotype and sex [$F(3) = 1.761$, $P = 0.168$]. Holm-Sidak post-hoc analyses revealed that the levels of *tac3a* were decreased significantly in SgIIa-KO and SgIIb-KO fish compared to WTs, but no significant differences were observed between SgII(a+b)-KO fish and WTs ($P < 0.05$). In SgIIa-KO and SgIIb-KO fish, *gnrh3* levels were decreased by 31% and 58%, respectively. The levels of *gnrh3* were also significantly higher in SgII(a+b)-KO fish compared to SgIIb-KO fish but not significantly different from SgIIa-KO or WT fish ($P < 0.05$) (Fig. 2.5B).

The levels of *oxt* showed a significant main effect from genotype [$F (3) = 4.241$, $P = 0.010$]. However, there were no significant effects of sex [$F(1) = 0.0185$, $P = 0.892$] or any interactions between genotype and sex [$F(3) = 0.837$, $P = 0.481$]. Post-hoc analyses showed that levels of *oxt* were decreased similarly in SgIIa-KO and SgIIb-KO fish compared to WTs and no significant differences were observed between SgII(a+b)-KO and WT fish ($P < 0.05$). The levels of *oxt* were decreased by 59% and 50% in SgIIa-KO and SgIIb-KO fish, respectively, compared to WTs. The levels of *oxt* appeared higher in SgII(a+b)-KO compared to single SgII-KO fish, although, this difference was not statistically significant ($P > 0.05$) (Fig. 2.5C).

A significant main effect of genotype was also observed for the levels of *avp* [f(3) = 7.306, P < 0.001]. No significant differences were observed for sex [F(1) = 2.353, P = 0.132] or interactions between genotype and sex [F(3) = 1.980, P = 0.130]. Post-hoc analysis revealed a significant decrease in *avp* levels in SgIIb-KO fish but no significant differences between SgIIa-KO and SgII(a+b)-KO fish compared to WTs (P < 0.05). The level of *avp* was decreased by 66% in SgIIb-KO fish compared to WTs. SgIIa-KO and SgII(a+b)-KO levels of *avp* were also significantly higher than those in SgIIb-KO fish (Fig. 2.5 D).

There was a significant main effect of genotype on the levels of *scg2a* [F(3) = 19.087, P <0.001]. However, there was no significant effect of sex [F(1) = 2.730, P = 0.106] or interactions between genotype and sex [F(3) = 0.885, P = 0.456]. There was a significant decrease in the *scg2a* levels in SgIIa-KO and SgII(a+b)-KO fish (P < 0.05) and no difference in SgIIb-KO compared to WTs. The levels of *scg2a* were decreased by 55% and 73% in SgIIa-KO and SgII(a+b)-KO fish, respectively, compared to WTs. The levels of *scg2a* were also higher in SgIIb-KO fish compared to SgIIa-KO and SgII(a+b)-KO fish (P < 0.05) (Fig. 2.5E).

The levels of *scg2b* had a main effect from genotype [F(3) = 40.231, P < 0.001]. No significant differences were observed from sex [F(1) = 0.0068, P = 0.934] or interactions between genotype and sex [F(3) = 1.488, P = 0.231]. There was a significant decrease in *scg2b* in all SgII-KO lines compared to WTs and each SgII-KO line was different from the other (P < 0.05). The levels of *scg2b* were decreased by 32%, 56% and 88% in SgIIa-KO, SgIIb-KO and SgII(a+b)-KO fish, respectively (Fig. 2.5F).

Telencephalon

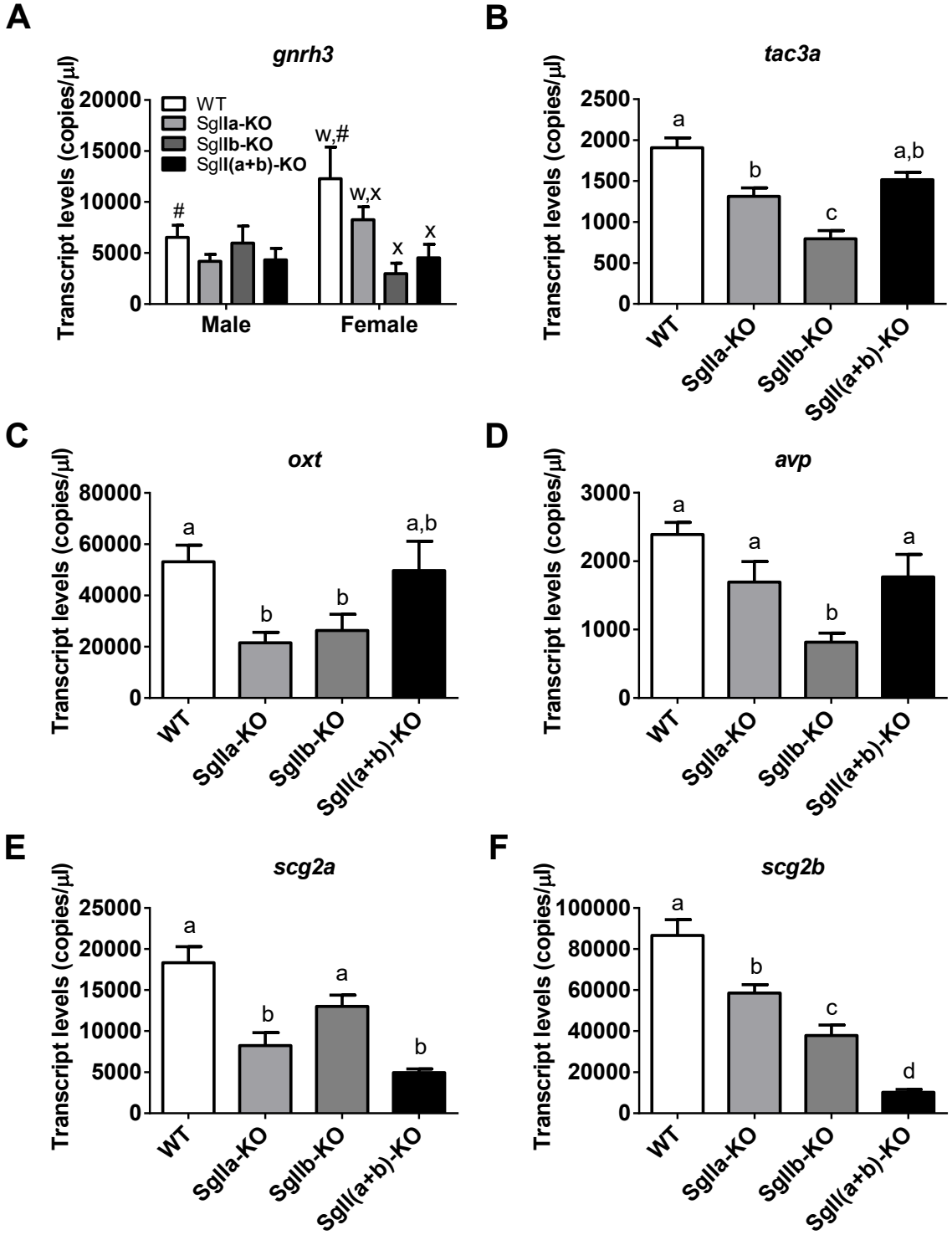

Figure 2.5 Gene expression in the adult zebrafish telencephalon. The relative abundance of the mRNAs (copies/µl) was normalized according to NORMA-gene. Results are presented as means + SEM (n = 5-14). Two-way ANOVA followed by Holm-Sidak post-hoc tests were performed. When interactions are present (A), means with different letters, w-z, represent statistical differences between genotypes in females and the pound sign (#) represents significant difference in sex within a genotype. Results were pooled across sex when there were no statistically significant differences in females versus males (B, C, D, E, F). In this case, means with different letters (a, b, c, d) denote significant differences between genotypes.

2.3.4 Genes involved in reproduction are altered following SgII-KO in adult male and female zebrafish hypothalamus

There were clear and significant changes in the levels of *gnrh3* in the hypothalamus of SgII-KO fish. There was a main inhibitory effect from genotype [F(3) = 36.421, P = <0.001] on the levels of *gnrh3*. However, there were no significant effects from sex [F(1) = 0.0960, P = 0.758] or interaction between genotype and sex [F(3) = 2.273, P = 0.093]. Given the absence of significant differences in sex or interactions, results were pooled across sex. The levels of *gnrh3* were significantly decreased in all SgII-KO fish (P < 0.05). In SgIIa-KO, SgIIb-KO and SgII(a+b)-KO lines, *gnrh3* was reduced by 71%, 62% and 73% compared to WTs. SgIIa-KO and SgII(a+b)-KO lines also had lower levels of *gnrh3* compared to SgIIb-KO (P < 0.05) (Fig. 2.6A).

There was also a main effect from genotype [F(3) = 8.536, P < 0.001] on the levels of *kiss2* while main effects of sex [F(1) = 1.367, P = 0.249] and interactions between genotype and sex [F(3) = 2.518, P = 0.070] were not evident. Given the absence of significant differences in sex or interactions, results were pooled across sex. Interestingly, the levels of *kiss2* were significantly elevated in SgII(a+b)-KOs compared to WTs and single KO fish (P < 0.05). No differences were observed between single KO fish compared to WTs (P < 0.05). The levels of *kiss2* were increased by 51% in SgII(a+b)-KO fish (Fig. 2.6B).

For *oxt,* there was no main effect of genotype [F(3) = 1.318, P = 0.281] but there was a main effect of sex [F(1) = 15.669, P < 0.001] and no statistically significant interaction between genotype and sex [F(3) = 1.087, P = 0.364]. The levels of *oxt* were highly variable but generally females had lower levels of *oxt* compared to males (P < 0.05). No significant differences (P > 0.05) were observed between genotypes but the levels of *oxt* did vary somewhat (Fig. 2.6C).

For the levels of *avp*, there was a statistically significant main effect from genotype [F(3) = 6.079, P < 0.001] but no main effect from sex [F(1) = 3.331, P = 0.074]. There was however an interaction between genotype and sex [F(3) = 6.693, P < 0.001]. Post-hoc analyses revealed that SgIIa-KO and SgII(a+b)-KO males had higher levels of *avp* compared to WTs and SgIIb-KO males (P < 0.05). The levels of *avp* were increased by 57% and 50% in SgIIa-KO and SgII(a+b)-KO males, respectively, compared to WT males. Furthermore, there was a significant decrease in the levels of *avp* in SgIIa-KO females compared to SgIIa-KO males (P < 0.05) but no other sex differences were observed in the other genotypes (Fig. 2.6D).

Comparable effects of gene knockout were also observed for the levels of *tac3a*. There was a statistically significant main effect of genotype [$F(3) = 10.052$, $P < 0.001$], no main effect of sex [$F(1) = 1.855$, $P = 0.180$] but an interaction between genotype and sex [$F(3) = 9.246$, $P < 0.001$]. Post-hoc tests showed that SgIIa-KO and SgII(a+b)-KO males had significantly higher levels of *tac3a* compared to WT and SgIIb-KO males ($p < 0.05$). The levels of *tac3a* were increased by 79% and 54% in SgIIa-KO and SgII(a+b)-KO males, respectively, compared to WT males. Single KO females had significantly lower levels of *tac3a* compared to WTs and SgII(a+b)-KO fish ($P < 0.05$). In females, the levels of *tac3a* were reduced by 44% and 41% in SgIIa-KO and SgIIb-KO fish compared to WTs. Moreover, there was a significant decrease in the levels of *tac3a* in SgIIa-KO females compared to SgIIa-KO males ($P < 0.05$) while no other sex differences were observed in the other genotypes (Fig. 2.6E).

There was a significant main effect of genotype [$F(3) = 9.612$, $P < 0.001$], no main effect of sex [$F(1) = 0.0197$, $P = 0.889$] but an interaction between genotype and sex [$F(3) = 3.330$, $P = 0.028$] on the levels of *scg2a*. Holm-Sidak post-hoc tests revealed that SgIIa-KO and SgII(a+b)-KO males had significantly decreased levels of *scg2a* compared to WTs and SgIIb-KOs ($P < 0.05$). In males, *scg2a* levels were reduced by 64% and 76 % in SgIIa-KOs and SgII(a+b)-KOs, respectively. In females, no significant differences were observed in the levels of *scg2a* between genotypes. Furthermore, SgII(a+b)-KO females had significantly higher levels of *scg2a* compared to males ($P < 0.05$) (Fig. 2.6F).

For *scg2b,* there was a significant main effect of genotype [$F(3) = 29.200$, $P < 0.001$] but no main effect of sex [$F(1) = 0.0088$, $P = 0.926$]. However, there was an interaction between genotype and sex [$F(3) = 3.212$, $P = 0.032$]. Post-hoc analyses showed a significant decrease in the levels of *scg2b* in SgIIb-KO and SgII(a+b)-KO males ($P < 0.05$). SgII(a+b)-KO males also had significantly lower levels of *scg2b* compared to SgIIb-KO males ($P < 0.05$). The levels of *scg2b* were decreased by 74% and 83% in SgIIb-KO and SgII(a+b)-KO males, respectively. All SgII-KO females had significantly lower levels of *scg2b* compared to WT females ($P < 0.05$). SgII(a+b)-KO females also had significantly lower levels of *scg2b* compared to the single KO lines ($P < 0.05$). The levels of *scg2b* were reduced by 72%, 70% and 88% in SgIIa-KO, SgIIb-KO and SgII(a+b)-KO females, respectively. Also, SgIIa-KO females had significantly lower levels of *scg2b* compared to SgIIa-KO males ($P < 0.05$) (Fig. 2.6G)

Hypothalamus

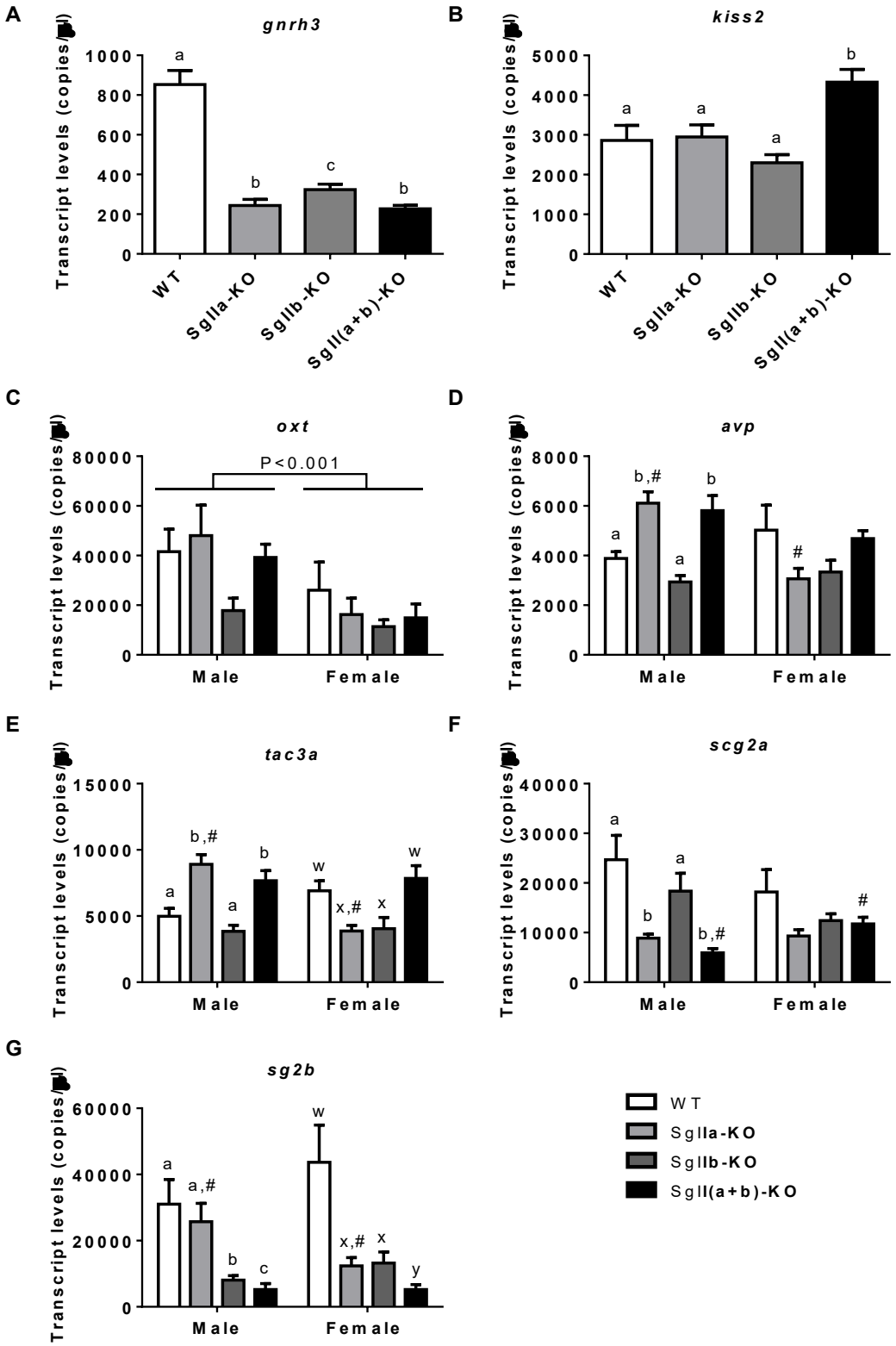

Figure 2.6 Gene expression in the adult zebrafish hypothalamus. The relative abundance of the mRNAs (copies/µl) was normalized according to NORMA-gene. Results are presented as means + SEM (n = 5-14). Two-way ANOVA followed by Holm-Sidak post-hoc tests were performed. Results were pooled across sex when there were no statistically significant differences in females versus males (A, B). In this case, means with different letters denote significant differences between genotypes. When no interactions are present but there is a main effect of sex (C) then P-values shown above the graph indicate significant differences compared between sexes. When interactions are present (D, E, F, G), means with different letters a-d and w-z represent statistical differences between genotypes in males and females, respectively, and the pound sign (#) represents significant differences in sex within a genotype.

2.3.5 Genes involved in reproduction are altered following SgII-KO in adult male and female zebrafish pituitary

Clear changes were observed in the levels of the gonadotropin subunits in the pituitary in response to *scg2* knockout in both male and female adult zebrafish. There were main effects from both genotype [$F(3) = 90.462$, $P < 0.001$] and sex [$F(1) = 34.312$, $P < 0.001$] on the levels of *lhb*. A significant interaction between genotype and sex [$F(3) = 3.341$, $P = 0.028$] was also evident for *lhb*. The post-hoc tests revealed that the levels of *lhb* were significantly lower in both male and female SgII-KO fish relative to WT controls ($P < 0.05$). In males, there was a 93%, 97% and 66% decrease in *lhb* in SgIIa-KO, SgIIb-KO and SgII(a+b)-KO fish, respectively compared to WT males. Similarly, SgIIa-KO, SgIIb-KO and SgII(a+b)-KO female levels of *lhb* were reduced by 91%, 92% and 60%, respectively compared to WT females. Furthermore, levels of *lhb* were significantly higher in SgIIa-KO and SgIIb-KO females compared to males ($P < 0.05$) (Fig. 2.7A) but no sex differences were observed in WTs or SgII(a+b)-KO fish.

The expression pattern observed for *fshb* was slightly different. Main effects of both genotype [$F(3) = 123.053$, $P < 0.001$] and sex [$F(1)$, 85.607, $P < 0.001$] were evident and there was a significant interaction between genotype and sex [$F(3) = 5.756$, $P = 0.002$]. Holm-Sidak post-hoc analysis indicated for both males and females that levels of *fshb* were lower in single SgII-KO lines compared to WTs ($P < 0.05$). Specifically, there was a 76% and 87% decrease in *fshb* in SgIIa-KO and SgIIb-KO males, respectively, compared to WT males. SgIIa-KO and SgIIb-KO females also had a reduction of 80% and 73% in *fshb* levels, respectively, compared to WT females. Interestingly, no difference was observed in SgII(a+b)-KOs males or females when compared to their same sex WT controls. All females had significantly higher transcript levels of *fshb* compared to males ($P < 0.05$) (Fig. 2.7B).

The levels of *cga* showed the same general trend as that of *lhb*. There were main effects from genotype [$F(3) = 26.472$, $P < 0.001$] and sex [$F(1) = 9.554$, $P = 0.004$]. Furthermore, there was a statistically significant interaction between genotype and sex [$F(3) = 3.181$, $P = 0.034$]. Post-hoc analysis revealed that levels of *cga* were significantly lower in all SgII-KO male and females compared to WTs ($P < 0.05$). Male levels of *cga* were decreased by 90%, 96% and 78% in SgIIa-KO, SgIIb-KO and SgII(a+b)-KO, respectively, compared to WT males. Female levels of *cga* were decreased by 94%, 95% and 61% in SgIIa-KO, SgIIb-KO and SgII(a+b)-KO, respectively, compared to WT females. Moreover, only WT females had significantly higher levels of *cga* compared to WT males whereas no sex differences were observed in any SgII-KO fish (Fig. 2.7C).

There were significant main effects from genotype [$F(3) = 36.466$, $P < 0.001$] and sex [$F(1) = 77.391$, $P < 0.001$] on the levels of *gnrhr2*. However, there were no significant interactions [$F(3) = 0.758$, $P = 0.524$]. Post-hoc analysis revealed that the levels of *gnrhr2* were lower in single SgII-KO males and females compared to their same sex WT controls ($P < 0.05$). Male *gnrhr2* levels were reduced by 86% and 82% in SgIIa-KO and SgIIb-KO. Furthermore, SgIIa-KO and SgIIb-KO females had higher transcript levels than their male counterparts ($P < 0.05$) (Fig. 2.7D).

Significant main effects of genotype [$F(3) = 19.238$, $P < 0.001$] and sex [$F(1) = 4.193$, $P = 0.047$] were observed for *scg2a* but no significant interactions were present [$F(3) = 1.477$, $P = 0.235$]. Post-hoc analyses revealed that the levels of *scg2a* were significantly lower in SgIIa-KO and SgIIb-KO males and SgIIa-KO, SgIIb-KO and SgII(a+b)-KO females compared to their same sex WT controls ($P < 0.05$). The levels of *scg2a* decreased by 51% and 61% in SgIIa-KO and SgIIb-KO males, respectively. The levels of *scg2a* decreased by 55%, 54% and 29% in SgIIa-KO, SgIIb-KO and SgII(a+b)-KO females, respectively. Single SgII-KO females also had higher *scg2a* levels than males ($P < 0.05$) and no sex differences were observed in WTs or SgII(a+b)-KO fish (Fig. 2.7E).

Significant main effects of genotype [$F(3) = 11.441$, $P < 0.001$) were also observed for the levels of *scg2b*. However, no significant effects of sex [$F(1) = 0.0268$, $P = 0.871$] nor interactions [$F(3) = 0.0894$, $P = 0.965$] were observed. Since there were no statistically significant differences in females versus males in the levels of *scg2b,* the results were pooled across sex. Post-hoc analyses showed that transcript levels of *scg2b* were decreased similarly in all SgII-KO lines compared to WTs ($P < 0.05$) (Fig 2.5F). The levels of *scg2b* were decreased by 54%, 74% and 68% in SgIIa-KO, SgIIb-KO and SgII(a+b)-KO fish, respectively (Fig. 2.7F).

Pituitary

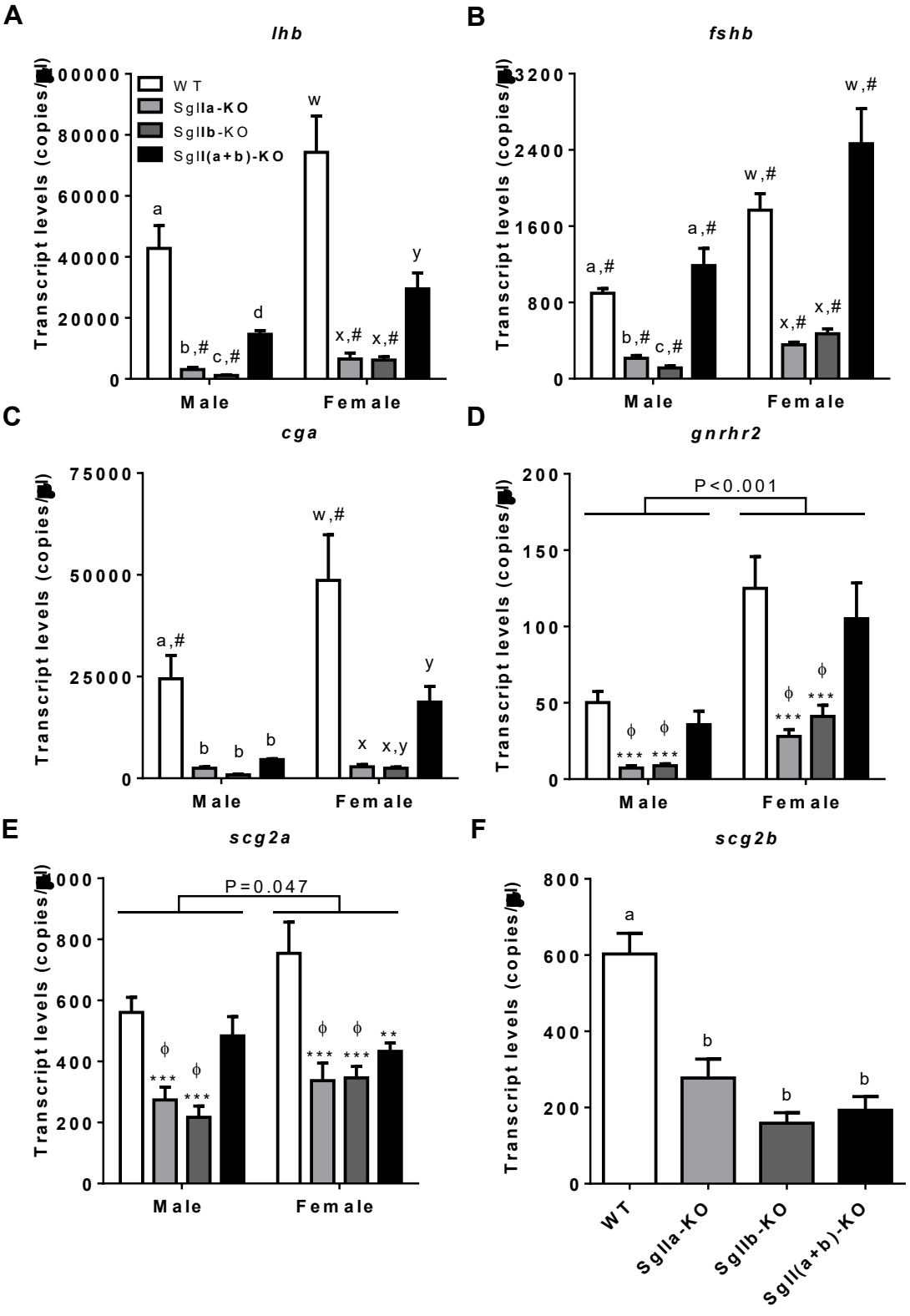
A
lhb
Transcript levels (copies/µl)
WT
Sgl**a**-KO
Sgl**b**-KO
Sgl**(a+b)**-KO
Male
Female
B
fshb
C
cga
D
gnrhr2
P<0.001
E
scg2a
P=0.047
F
scg2b
WT
Sgl**a**-KO
Sgl**b**-KO
Sgl**(a+b)**-KO

Figure 2.7 Gene expression in the adult zebrafish pituitary. The relative abundance of the mRNAs (copies/µl) was normalized according to NORMA-gene. Results are presented as means + SEM (n = 5-14). Two-way ANOVA followed by Holm-Sidak post-hoc tests were performed. When interactions are present (A, B, C), means with different letters a-d and w-z represent statistical differences between genotypes in males and females, respectively, and the pound sign (#) represents significant difference in sex within a genotype. When no interactions are present but there is a main effect of sex and genotype (D, E) then P-values shown above the graph indicate significant differences compared between sexes. Also, the asterisks (*) and phi (ϕ) represent significant differences between genotypes. *** $P \leq 0.001$ and ** $P \leq 0.01$ compared to WT; ϕ $P \leq 0.05$ compared to SgII(a+b)-KO. Since there were no statistically significant differences in females versus males in the levels of *scg2b,* the results were pooled across sex (D). In this case, means with different letters (a, b, c, d) denote significant differences between genotypes.

2.4 Discussion

In this study, I characterized the levels *scg2a* and *scg2b* during the first 3 months of life using ddPCR and described the *in-situ* localization of these transcripts in the adult (6mpf) brain and pituitary. Furthermore, I provide the first evidence for the impact of knockout of SgIIa/b on the expression of other key reproductive neuroendocrine regulators.

2.4.1 Levels of scg2a and scg2b increase with age in zebrafish

Using droplet digital PCR, I quantified *scg2a* and *scg2b* mRNA in the WT zebrafish. At 2 wpf I assessed whole heads but in each subsequent time point I measured *scg2a* and *scg2b* transcripts in whole brains. As *scg2a* and *scg2b* transcripts had been previously detected in zebrafish embryos as early as 10 and 14 hpf, respectively (Tao et al., 2018), it was not surprising that I detected *scg2a* and *scg2b* mRNA at 2 wpf, albeit at low levels. Their expression gradually increased until 6 wpf. At 8 wpf, the mRNA levels of *scg2a* decreased slightly whereas there was no difference in the levels of *scg2b* between 6 and 8 wpf. The highest mRNA levels of *scg2a* and *scg2b* were detected at 12 wpf. Also, neither *scg2a* nor *scg2b* mRNA levels peaked during the 12-week timeframe of the analysis.

At approximately 8 wpf zebrafish go through puberty and by 12 wpf spermatozoa and oocytes should be well developed (Maack and Segner, 2003). The relationship between gonadal histology and *scg2* mRNA levels should be described to examine if SgII is associated in specific reproductive developmental stages such as gonad differentiation, puberty onset, and gonad maturation.

2.4.2 Secretogranin-II is distributed throughout the adult zebrafish brain and pituitary

I performed *in situ* hybridizations on adult male and female zebrafish brains and pituitaries to determine the localization relationship between *scg2a* and *scg2b*. To our knowledge, this is the first study to compare the distribution of two SgII transcripts in adults. I found that both *scg2a* and *scg2b* are widely expressed in the brain and pituitary of adult male and female zebrafish. The highest transcript levels of *scg2a* were found in the pituitary followed by specific brain regions of the hypothalamus, telencephalon, midbrain and cerebellum. In contrast, *scg2b* staining was low in the pituitary and localized primarily in the hypothalamus, telencephalon and midbrain. I identified several principal areas in the brain where clearly labelled cell bodies containing both *scg2a* and *scg2b* were found. Specifically, cells were localized to the OB, Vc, Vd, Vv, Vl, Vs, Vp, Dp, TPp, PTN, PGl, PGm, Hc, Hd, Hv, LH, NMLF, TSc and TSvl in the brain. The spatial expression patterns observed in my study suggest that SgII may act within the central nervous system to regulate physiological processes such

as food intake and control pituitary functions. These distributions were qualitatively similar between both sexes, although this comparison was not quantified. Previous studies have focused on measuring *scg2* transcripts by *in situ* hybridization and RT-PCR in rat (Mahata et al., 1993a; Saria et al., 1997). In fish, *scg2a* has been examined using RT-PCR in goldfish (Zhao et al., 2006a), *in situ* hybridization and RT-PCR in zebrafish (Marvel et al., 2018; Tao et al., 2018) and RT-PCR in the orange-spotted grouper (*Epinephelus coioides*) (Shu et al., 2018). However, the only studies that have examined *scg2b* transcripts were conducted using *in situ* hybridization and RT-PCR in zebrafish embryos (Tao et al., 2018) and RT-PCR in the orange-spotted grouper (*Epinephelus coioides*).

Both *scg2a* and *scg2b* patterns of expression were evident in the OB. In particular, *scg2a* and *scg2b* transcripts were prominent in the ICL and ECL but were also present to a lesser degree in GL, and POF regions. Furthermore, *scg2b* labelled cells seemed to be more densely located in the ICL whereas *scg2a* was more dispersed throughout the OB. Immunoreactivity for SNa was also found throughout the OB in goldfish (Canosa et al., 2011). The OB is the primary processing center of odor information. In zebrafish, the OB is organized into four concentric layers: (1) POF, that is formed by olfactory sensory neuron axons (Sato et al., 2007); (2) GL, containing glomeruli (Braubach et al., 2012); (3) ECL, consisting of mitral and ruffed cell somas (Fuller and Byrd, 2005; Fuller et al., 2006); and (4) ICL, containing cell bodies of different interneurons such as juxtaglomerular, periglomerular and granular cells (Bundschuh et al., 2012; Edwards and Michel, 2002). In zebrafish, the mitral cells receive synaptic inputs from olfactory sensory neurons and extend their axons through the medial (MOT) and lateral olfactory tracts (LOT) to different brain centers. The MOTs and LOTs are separate axon bundles. In cod, electrical stimulation of the MOT induces an alarm reaction or reproductive behaviour while stimulation of the LOT induces feeding behaviours (Doving and Selset, 1980). In the goldfish and Crucian carp, the LOT transmits food-related odors (Dulka, 1993; Hamdani et al., 2001a; Hamdani et al., 2001b) while the MOT is involved exclusively with pheromonal and social signals (Demski and Dulka, 1984; Hamdani et al., 2000; Sorensen et al., 1991). In goldfish, the LOT specifically innervates the habenula while the MOTs project to the Vv (Vonbartheld et al., 1984). Electrical stimulation of the goldfish OB and administration of receptor antagonists indicates that the MOT sends glutamatergic signals to the ventral POA (Lado et al., 2014). In zebrafish, the mitral cells project to the Vv, Dp, the right habenula and the hypothalamus in the diencephalon (Gayoso et al., 2011; Miyasaka et al., 2009; Rink and Wullimann, 2004). Given the localization of *scg2* transcripts throughout the OB it is conceivable that SgII and/or SN may regulate similar behaviours such as fear conditioning, feeding and reproduction.

In my study, light to moderate staining of *scg2a* and *scg2b* was observed in the dorsal telencephalic areas, which are targets of LOT and MOT projections. Both *scg2a* and *scg2b* transcripts were present in the Dc, Dl, Dp and Dm although, only faint staining of *scg2a* was observed in the Dm. The Dl, Dm and Dp regions of the zebrafish brain have been shown to be homologous to the hippocampus, amygdala and piriform cortex in mammals (Mueller et al., 2011; von Trotha et al., 2014). Furthermore, studies in goldfish suggest that the Dl is involved in the spatial, relational, or temporal memory systems (Broglio et al., 2010; Portavella et al., 2004). In zebrafish, a subpopulation of neurons in the Dm have been shown to be essential for fear conditioning (Lal et al., 2018). The distribution of *scg2* in these areas in my study suggests that perhaps SgII is involved in the regulation of behaviours related to conditioning, learning and memory.

Considerable staining of *scg2a* and *scg2b* transcripts was also observed throughout the ventral telencephalic areas. This is consistent with the findings of Marvel et al. (2018). Similarly, SNa-ir distribution was moderate to high in the Vv/Vl and Vs of the goldfish (Canosa et al., 2011). In goldfish, male courtship and spawning behaviour were reduced by lesions to the Vs/Vv region (Kyle and Peter, 1982). The teleost Vv is the homolog of the septal area, a part of the limbic system, in mammals (Kermen et al., 2013). Very high SN-ir was observed in the medial and central amygdaloid nuclei of the rat (Marksteiner et al., 1993). In rodents, the amygdala has been implicated in regulating reproductive physiology since lesions in this area lead to disruption of ovarian cycles (Tyler and Gorski, 1980). The Vc is one area where *scg2a* mRNA was absent and *scg2b* was present but the functional consequence of this distribution remains to be examined. The Vc is known to contain GABAergic neurons (Adolf et al., 2006; Kim et al., 2004) but the functions of this brain region are not well understood.

In my study, the NPO and HYP in WT zebrafish were densely populated with *scg2a* and *scg2b* containing cells. This is in accordance with Marvel et al. (2018). More specifically, I observed *scg2a* and *scg2b* transcripts in the PPa, PPp, SC and PM of the NPO. Similarly, in goldfish, the most prominent staining of SNa-ir was observed in the magnocellular and parvocellular cells in the NPO, which were also colocalized with IST (Canosa et al., 2011). These cells are equivalent to the mammalian OXT expressing neurons located in the supraoptic and paraventricular nuclei. Previous studies have also shown that SN and OXT are co-stored in dense core vesicles and OXT and *scg2* are coregulated in the lactating rat hypothalamus (Mahata et al., 1993b). In the rat, the HYP was also the most intensively stained SN-ir area (Marksteiner et al., 1993). The colocalization of SN and OXT in both fish and mammals provides support of a conserved reproductive role for SN. However, it is

important to note that it is still unknown which form of the teleost SN (SNa or SNb) is equivalent to the mammalian SN. Moreover, colocalization studies with *scg2b* or SNb have yet to be undertaken.

Cells expressing *scg2a* and *scg2b* were detected in known hypophysiotropic brain regions involved in stress, feeding and reproduction. I observed *scg2b* transcripts in the Had/Hav where kisspeptin 1 (*kiss1)* (Ogawa et al., 2012; Servili et al., 2011) and tachykinin 3a (*tac3a*) (Biran et al., 2012) were previously shown to be expressed in zebrafish as well. Kisspeptin and tachykinin have also been implicated in the control of reproductive functions. Both *scg2a* and *scg2b* expressing cells were detected throughout the periventricular hypothalamus (Hc/Hd/Hv). In goldfish, neuropeptide Y (NPY) is expressed in the periventricular regions of the HYP and is involved in appetite regulation (Yokobori et al., 2012). Similarly, Melanin-concentrating hormone 2 (MCH2), which is expressed in the telencephalic area, NPO, thalamus, PTN, Hv, locus coeruleus and vagal motor nerves, has been shown to regulate food intake in zebrafish (Berman et al., 2009). Corticotropin-releasing factor (CRF), urotensin I and CRF-binding protein are expressed in the preoptic area, periventricular hypothalamus and tectal regions in zebrafish supporting stress and behavioural functions (Alderman and Bernier, 2007). The expression data from my study, showing *scg2a* and *scg2b* transcripts in hypophysiotropic brain regions suggests that these genes are likely involved in the control of feeding, stress and/or reproductive functions. This is also the first study to show the prominent expression of *scg2b* in regions of the zebrafish brain implicated in the control of reproduction.

In vertebrates, the pituitary is connected to the hypothalamus via a short stalk that enables the passage of hypothalamic signals. Many teleost species lack a median eminence that is typical in tetrapods; therefore, the teleost anterior pituitary is highly innervated by neurons that secrete neuropeptides and neurotransmitters (Peter et al., 1990). However, there is more recent evidence that zebrafish have a dual mode of gonadotrope regulation by Gnrh3 that combines neuroglandular and neurovascular components (Golan et al., 2015). In the pituitary, Fsh cells are closely associated with the vasculature whereas Lh cells reside in tight clumps and are located further away from blood vessels (Golan et al., 2015). Gnrh3 fibers form boutons, which are considered the release site of the peptide, upon reaching the pituitary (Golan et al., 2015). These Gnrh3 boutons are in the neurohypophysis rather than near the gonadotropes (Golan et al., 2015). Furthermore, Fsh cells and Gnrh3 boutons share a close association but Lh cells do not, suggesting that Fsh cells are more directly regulated than Lh cells. Gnrh3 axons closely follow the pituitary vasculature and the abundance of boutons suggests a neurovascular control of gonadotropes.

In my study I found that most *scg2a* positive cells were observed in the PI of the NIL. Our results are in contrast to previous RT-PCR studies indicating that *scg2a* mRNA levels are consistently higher in the pars distalis (PD) than in the NIL of goldfish pituitaries (Samia et al., 2004), albeit, those dissections were rather crude. Similarly, western blot analyses found that the levels of a ~57 kDa SgIIa-derived protein containing SNa were higher in the PD than in the NIL of male and female goldfish (Zhao et al., 2006a). The PI is the most posterior of the adenohypophysial regions and contains corticotropes and melanotropes producing α-melanocyte stimulating hormone (α-MSH) (Liu et al., 2003). I also detected *scg2a* transcripts in the rostral PD (RPD), a region of the pituitary where prolactin producing lactotrophs are situated, although staining was far less pronounced than in the PI. In goldfish, SNa-ir is present in the lactotrophs of the RPD (Zhao et al., 2009a). However, in mammals, SN localization is highly variable and found in all major anterior pituitary cell types (Zhao et al., 2010b), emphasizing the species-specific processing of SgII to SN. The neural lobe of the fish pituitary is innervated by the axonal termini of magnocellular neuroendocrine neurons that secrete the nonapeptides, IST and VT (Acher, 1996). Previous studies in goldfish report that SN and IST are colocalized in the NIL and in some fibers entering the PD and terminating near gonadotropes (Canosa et al., 2011). Magnocellular neuronal fibers that terminate in the NIL are also strongly positive for both SN and IST (Canosa et al., 2011). The observation that *scg2a* and *scg2b* transcripts are found in multiple hypophysiotropic brain regions and the pituitary supports the hypothesis of SgII being involved in reproductive control.

2.4.3 Mutation of SgII alters expression of genes involved in reproductive processes

The preoptic area of the telencephalon, hypothalamus and pituitary are the main neuroendocrine territories regulating reproductive processes in teleosts. Since *in situ* hybridization techniques showed that *scg2a* and *scg2b* transcripts were distributed throughout these regions, I quantified the mRNA levels to identify the tissues where these transcripts were most abundant. Furthermore, this is the first study to genetically mutate *scg2* in any organism and study the effects on genes regulating reproduction in adults. My results clearly show that genetic modification of *scg2* leads to altered expression patterns of key genes in the telencephalon, hypothalamus and pituitary.

Gonadotropin-releasing hormone is considered the main stimulator of the pituitary gonadotropins, Lh and Fsh (Schally et al., 1971; Zohar et al., 2010). In this study, I found that *gnrh3* was significantly downregulated in SgII-KO fish in both the telencephalon and hypothalamus, although this effect was most evident in the hypothalamus. In the hypothalamus of SgIIa-KO, SgIIb-

KO and SgII(a+b)-KO fish, *gnrh3* transcripts were reduced by 71%, 62% and 73% compared to WTs. These results are consistent with the homozygous, double Kiss-mutant zebrafish that also had significantly decreased levels of *gnrh3* in whole brains (Tang et al., 2015). However, Kiss-mutation did not affect reproductive phenotypes while our SgII-KO fish have impaired reproductive functions (see next chapters). One possibility for this difference is that other related genes are upregulated to compensate for the loss of protein function. Therefore, the expression pattern of other reproductive neuropeptides were also examined in SgII-KO and WT fish.

Neurokinin B, encoded by *tac3*, has been shown to play a role in controlling fish reproduction by increasing luteinizing hormone levels in mature female zebrafish (Biran et al., 2012). I measured *tac3a* in this study and found that it was significantly decreased in the telencephalon of SgII-KO female and male zebrafish. On the other hand, in the hypothalamus, *tac3a* mRNA was increased in SgIIa and SgII(a+b)-KO males and decreased in SgIIa-KO and SgIIb-KO females. My results suggest a region- and sex-related effect on *tac3a* mRNA due to SgII disruption. While *tac3a* levels have been measured in other KO lines they are difficult to compare to as other studies performed whole brain analyses rather than separating regions of the brain as I have done. In the *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish, males had an upregulation of *tac3a* and no differences were observed in females (Marvel et al., 2018). The triple knockout *kiss1*$^{-/-}$*;kiss2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish also had an increase in *tac3a* transcripts in both sexes (Liu et al., 2017). The OXT/VP (isotocin/vasotocin in teleosts) family of nonapeptides are important for reproductive and social behaviours in vertebrates (Goodson and Bass, 2001). The mRNA levels of *oxt* were not significantly affected by SgII-KO in the hypothalamus; however, SgIIa-KO and SgIIb-KO fish had decreased levels in the telencephalon and no differences in sex were observed. The mRNA levels of *avp* were decreased in the telencephalon of SgIIb-KO fish (no sex differences), whereas it was increased in the SgIIa-KO and SgII(a+b)-KO male hypothalamus. Previous studies in the *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish, show that *avp* is unaltered (Marvel et al., 2018). Interestingly, *kiss2* was increased in double SgII(a+b)-KO females and males. This is in contrast to the *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish in which no difference was observed for *kiss2* (Marvel et al., 2018).

In my study, I only measured the expression of one of the four Gnrh receptors (Gnrhr), *gnrhr2,* in the pituitary. Previous studies report that all four receptors are expressed in the pituitary and that each receptor was functional (Tello et al., 2008). The Gnrhr2 is equally sensitive to Gnrh2 and Gnrh3, and more sensitive to native Gnrh3 than all other Gnrhrs (Tello et al., 2008); hence why I chose to measure Gnrhr2 instead of the other Gnrhrs. I found that the single SgII-KO fish had lower mRNA levels of *gnrhr2* than WT or SgII(a+b)-KO males and females; SgIIa-KO and SgIIb-KO females also had

higher mRNA levels of *gnrhr2* than their male counterparts. This decrease in *gnrhr2* could lead to decreased synthesis and release of Gnrh and ultimately lead to lower gonadotropin levels. The expression patterns of the gonadotropin subunits (*lhb, fshb* and *cga*) were significantly lowered in the pituitaries of KO animals with the exception of *fshb* in double-KO fish. Interestingly, the SgII(a+b)-KO fish had slightly higher transcript levels than single knockout lines, like the situation with *gnrhr2*, suggesting that the organism is attempting to compensate for the deleterious effects of SgII(a+b)-KO. The expression patterns of *lhb* and *fshb* observed in our study follow a somewhat similar pattern for those reported in the Kiss mutant (*kiss*$^{-/-}$) zebrafish. In double kiss mutant lines (*kiss1*$^{-/-}$*:kiss2*$^{-/-}$), *fshb* was significantly down-regulated in both sexes and *lhb* was down regulated in only males (Tang et al., 2015). However, the reduction in *lhb* transcripts observed by Tang et al. (2015) was far less than the dramatic 60-97% reductions of *lhb* in my study. Our results contrast several other knockout studies. In the adult pituitaries of *gnrh3*$^{-/-}$ zebrafish (Spicer et al., 2016), *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish (Marvel et al., 2018), *kiss1*$^{-/-}$*;kiss2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish (Liu et al., 2017), and *gnrh1*$^{-/-}$ medaka (Takahashi et al., 2016), the gonadotropin transcript levels were similar between KO animals and WTs. These previous studies provide support that a compensatory mechanism exists in these knockout lines, likely to maintain normal gonadotropin levels. These studies also emphasize the probable importance of SgII in regulating genes involved in reproductive processes. Previous knockout studies have shown that single, double and triple gene knockouts of traditional reproductive regulators (e.g., Kiss and Gnrh) resulted in minor alterations in reproductive genes. The current study is thus in marked contrast, given that SgII mutants have major decreases in many of the neuroendocrine genes critically important for gonadotropin synthesis and secretion. Moreover, SgII-KO has major impacts on reproductive success in zebrafish (see chapter 4). The absence of mRNA changes in previous studies could be due to dilution of region-specific effects, since these other authors analysed whole brains.

I also measured *scg2a* and *scg2b* to determine if the paralogous gene would be upregulated in order to compensate for its loss. I found that *scg2a* or *scg2b* were significantly decreased in the SgIIa-KO, SgIIb-KO or SgII(a+b)-KO correspondingly, indicating a mechanism of nonsense mediated mRNA decay (Chang et al., 2007). Furthermore, our results show that the *scg2* paralog was not upregulated, so true genetic compensation is unlikely. Functional knockout of genes may activate a compensatory network to buffer against deleterious mutations such as the upregulation of related genes as a consequence of protein loss. This type of genetic compensation in response to gene knockout is a widespread phenomenon (El-Brolosy and Stainier, 2017). In my study I found that genetic compensation did not take place. This is similar to the Gnrh knockouts that demonstrated

that Gnrh2 is not compensating for the genetic loss of the Gnrh3 isoform. Furthermore, the genetic loss of both Gnrh2 and Gnrh3 resulted in no changes in reproductive functions. However, like in my study, there was a shift in the expression in other functional networks to compensate. In the *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ zebrafish there were increased levels of *gnih*, *pacap1, scg2a* and *tac3a* (Marvel et al., 2018). In the triple mutant zebrafish (*kiss1*$^{-/-}$;*kiss2*$^{-/-}$;*gnrh3*$^{-/-}$) there was increased expression of *npy*, *tac3a*, and *scg2a*, although the expression of the gonadotropins was not significantly altered (Liu et al., 2017). These previous studies suggest the existence of some sort of compensation mechanism to maintain reproduction following multiple gene mutations.

2.4.4 Conclusion

In this study, I identified that *scg2a* and *scg2b* transcripts are present in the brain and increasingly expressed until adulthood. These genes were also both expressed in the telencephalon, hypothalamus and pituitary. In the male and female adult pituitary, *scg2a* was highly expressed whereas *scg2b* staining was faint. Furthermore, SgII-KO altered numerous neuroendocrine genes involved in the regulation of reproduction. These data suggest that SgII plays a major modulatory role in fish reproduction.

Chapter 3: Secretogranin-II gene disruption reduces breeding tubercle development in male zebrafish pectoral fins

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[2]Conducted the experiments presented in this chapter

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[4]Contributed to original ideas

[5]Helped with experiments

[6]Provided SgII mutant zebrafish and collaborated on this study

[7]Contributed to original ideas and revised the manuscript

3.1 Introduction

Breeding tubercles (BT) are multicellular epidermal structures supporting a keratinized cap that are found in at least 15 families of fishes with most sharing a similar structure, although, differences exist in the size, shape, number and distribution between species (Wiley and Collette, 1970). In those species having them, BTs are present only on males or are more developed in males than females, and they can also appear seasonally during the spawning period (Wiley and Collette, 1970). In the Eurasian minnow (*Cyprinus phoxinus*), BTs have been observed on the dorsal side of the head, regions anterior to the pectoral fin base, posteroventral to the gill cleft and dorsal and ventral sides of the pectoral fin (Chen and Arratia, 1996). The ventral side of the pectoral fin has smaller and fewer BTs than those on the dorsal side. On the dorsal side, the BTs are arranged into rows (Chen and Arratia, 1996). Similarly, in male zebrafish, the most prominent presence of BTs is on the dorsal sides of anteromedial rays of pectoral fins (Kang et al., 2013). In most fishes, BTs begin to develop prior to the spawning period then following spawning, BTs may break off, be sloughed off, or gradually regress (Wiley and Collette, 1970). In zebrafish, BTs appear around sexually maturation (~2.5 months post-fertilization) and are maintained throughout adulthood (McMillan et al., 2013).

Several possible functions of pectoral fin BTs have been proposed. They include maintaining body contact during spawning, defending nests and or territories, stimulating females in spawning, and sex recognition (Wiley and Collette, 1970). Through the use of high-speed cameras to film zebrafish courtship behaviours and spawning, Kang et al. (2013) determined that male zebrafish use BTs on the pectoral fins to grasp females, promoting egg release and fertilization. Males maintain contact with the females by using BTs then place their posterior trunk over the female and contort against the female's body as eggs are released (Kang et al., 2013). Moreover, male fish that had both pectoral fins amputated failed to spawn, whereas partial amputation resulted in a graded effect on spawning and was less severe than double amputation (Kang et al., 2013). These results suggest that BTs on the pectoral fins play a key role in grasping females to stimulate egg lay (Kang et al., 2013).

In some species, aggressive behaviour has been correlated to an increased number and size of BTs. Kortet et al. (2004) speculated that individuals that had larger and more numerous BTs were also more dominant. High circulating levels of testosterone (T) and 11-ketotestosterone (11-KT) were associated with elaborate BT ornamentation in the male roach (*Rutilus rutilus*) (Kortet et al., 2003). Both dominant behaviours and the presence of BTs are regulated by androgens. Moreover, the formation of BTs is stimulated by androgens (Kang et al., 2013; Wiley and Collette, 1970). Androgens can induce the formation of BTs in female zebrafish and increase the BT cluster size in males

(McMillan et al., 2013). In contrast, male zebrafish treated with flutamide, an androgen receptor antagonist, had a significantly decreased number of BTs (Kang et al., 2013). Similarly, administration of 17β-estradiol (E2) and activation of estrogen receptors results in the inhibition of BT formation (McMillan et al., 2013). Furthermore, reduction in T following castration in medaka resulted in the regression of BTs (Yamamoto and Egami, 1974). These results indicate that the androgens to estrogens ratio regulates the development of BTs (Kang et al., 2013; McMillan et al., 2013).

Vascularization of BTs has been observed in some fish species (Wiley and Collette, 1970). Two processes regulate the development of the vascular system: vasculogenesis and angiogenesis. Vasculogenesis is the *de novo* assembly of the first blood vessels whereas angiogenesis is the coordinated growth of endothelial cells from pre-existing vasculature (Risau, 1997). Blood vessels are suggested to play a role in the maintenance of BTs during long periods of spawning (Wiley and Collette, 1970). In zebrafish, the growth of large BT clusters depends on the presence of androgens and the formation of a unique patterning of highly vascularized secondary blood vessel networks (McMillan et al., 2013). Inhibiting blood vessel formation by administration of an inhibitor of vascular endothelial growth factor (VEGF) receptor tyrosine kinases reduces the ability of androgens to induce BT formation in female zebrafish (McMillan et al., 2013). While it is now clear that androgen-driven angiogenesis is necessary for the formation and maintenance of BTs (McMillan et al., 2013), little is known about other hormones. In this regard, the pro-angiogenic SgII-derived peptide secretoneurin (SN) is a potential candidate.

Secretoneurin can induce neovascularization in mouse cornea *in vivo* and promote capillary tube formation in human umbilical vein endothelial cells (HUVECs) *in vitro* (Kirchmair et al., 2004b). *In vitro* studies in human coronary artery endothelial cells, show that the activation of mitogen-activated protein kinases by SN is dependent on vascular endothelial growth factor (VEGF) (Albrecht-Schgoer et al., 2012). In HUVECs, however, the angiogenic effects of SN are VEGF independent (Kirchmair et al., 2004b). Intravenous administration of SN is neuroprotective and promotes neurogenesis and angiogenesis in murine models of stroke (Shyu et al., 2008). Direct injection of a plasmid vector expressing mammalian SN induced therapeutic angiogenesis in the mouse hindlimb ischemia model (Schgoer et al., 2009a). In zebrafish, *in vivo* studies provide evidence that *scg2b* is critical in the neurovascular modeling in the hindbrain and is mediated by activation of the MAPK and PI3K/AKT pathways that are independent of VEGF (Tao et al., 2018). Neurons expressing *scg2b* are also aligned with central arteries in the hindbrain demonstrating a close neurovasculature

relationship (Tao et al., 2018). However, nothing is known about the role of endogenous SgII protein or SN neuropeptide in BT vascularization and development.

In this study, I analyzed the morphological differences of BTs on the pectoral fins in WT and SgII-KO fish. We hypothesized that disruption of the zebrafish SgII genes (*scg2a*$^{-/-}$, *scg2b*$^{-/-}$, and *scg2a*$^{-/-}$;*scg2b*$^{-/-}$) would result in changes in the morphology of BTs. We predicted that the size and distribution of BTs would be reduced in the SgII-KOs.

3.2 Materials and methods

3.2.1 Experimental animals

Wild-type (WT) and SgII-KO zebrafish were bred in house and raised according to standard husbandry procedures. Fish were maintained in 10-L tanks of dechloraminated City of Ottawa tap water at 28^{o}C on a 14:10 light-dark cycle.

3.2.2 Imaging and morphometric assessments

Fish were anaesthetized with tricaine (3-amino benzoic acid ethyl ester also called ethyl 3-aminobenzoate) according to standard zebrafish husbandry procedures (Westerfield, 2000) and placed on a 1% agarose plate containing a wedge that allowed for proper body positioning. The right pectoral fin was spread out until it was taught on the agar surface. All images were of the dorsal surface of the right pectoral fin. Images were captured using a Zeiss AxioCam HRm digital camera connected to a Leica MZ FLIII dissection microscope and Axiovision AC software (Carl Zeiss). All images were processed using ImageJ (NIH). The morphometric assessments that were made include the length of the fin rays and BT clusters on fin rays #1-8; the width and length of an individual BT on rays 3-5 as well as the width of the fin ray at that location; and the number of BT rows present. Lengths were quantified using the measurement tool in ImageJ (NIH). See figure 3.1 for a representation of the measurements taken.

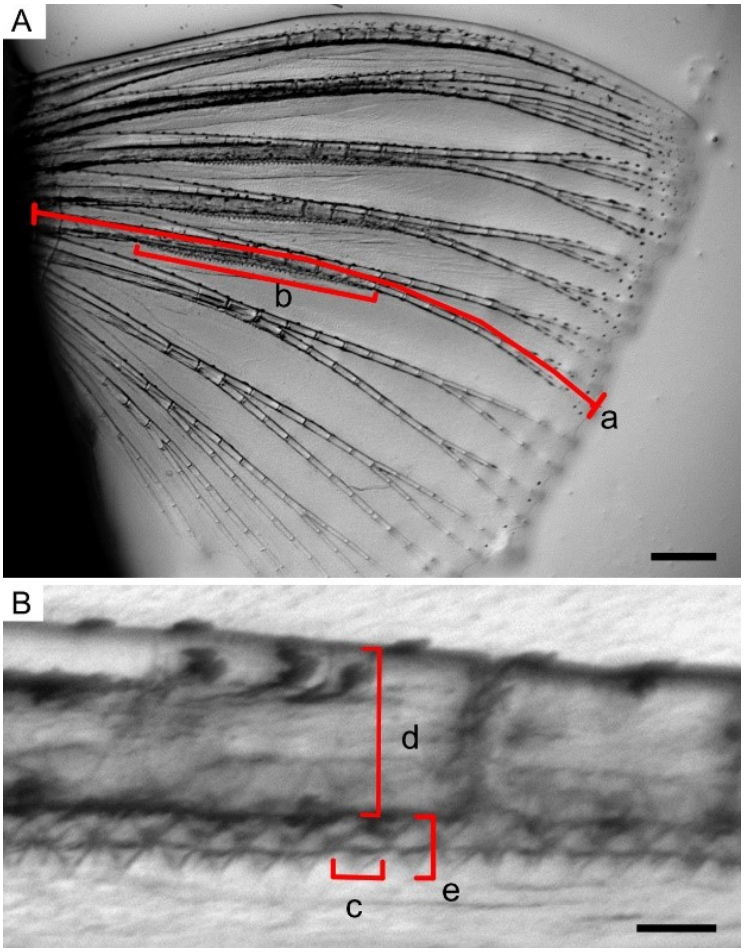

Figure 3.1 Description and visualization of measurements taken from the zebrafish pectoral fin. (A) The adult male zebrafish pectoral fin where the red line (a) represents the length of the fin ray and (b) is the length of the BT cluster. (B) Magnification of the fin ray showing the measurements for the individual BT length (c), fin ray width (d), and BT width (e). A: scale represents 500 µm; B: scale represents 100 µm.

3.2.3 Data analysis

Statistical analyses were performed using SigmaPlot 11.0 software (Systat Software, Inc., Chicago, IL). Normality and homoscedasticity were tested using a Shapiro-Wilk test and Levene's test respectively. One-way analyses of variance (ANOVA) followed by the Holm-Sidak post-hoc test was used to analyze the ratio between BT width/Fin ray width. The Kruskal-Wallis one-way analysis of variance on ranks followed by a Tukey test was used to analyze the number of BT rows, the ratio of the BT length/Fin ray length and the ratio between the BT length and BT cluster length.

3.3 Results

Representative images of pectoral fins from each experimental group are shown in Fig. 3.2A-D and magnified areas focusing on the BT clusters along each fin ray shown in Fig. 3.2E-H. Most BT clusters were observed on fin rays 3-5, therefore all subsequent morphometric analyses were conducted only on these rays. Clusters of BTs were observed on fin rays 2 and 6 in some cases, although this occurred less frequently than on rays 3-5. Moreover, there were few instances where BTs were found on fin ray 7 in SgII-KO fish.

There was a statistically significant difference in the number of BT rows between males of different genotypes [$H(3) = 48.406$, $P < 0.001$]. Post-hoc tests revealed that SgIIa-KO and SgII(a+b)-KO males had significantly fewer BT rows compared to WTs ($P < 0.05$). The number of BT rows in SgIIb-KO fish was not statistically different from WTs or SgIIa-KO fish. However, SgIIb-KOs did have more BT rows than SgII(a+b)-KO fish ($P < 0.05$) (Fig. 3.3A).

There was a significant difference due to genotype on the ratio of BT width to fin ray width [$F(3, 140) = 30.236$, $P < 0.001$). The SgIIa-KO and SgII(a+b)-KO males had a significantly decreased ratio compared to WTs ($P < 0.05$). The SgIIb-KO males had an intermediate BT to fin ray width and was significantly higher ($P < 0.05$) than in SgIIa and SgII(a+b)-KO animals ($P < 0.05$) (Fig. 3.3B).

The ratio between BT length and fin ray length was also significantly different between genotypes [$H(3) = 35.365$, $P < 0.001$]. Post-hoc analyses showed that all SgII-KO fish had a reduction in BT length/fin ray length compared to WTs ($P < 0.05$). All SgII-KO fish had similar BT lengths (Fig. 3.3C).

In contrast, there was no significant effect of genotype on the ratio between BT length and BT cluster length [$H(3) = 1.884$, $P = 0.597$]. All SgII-KO and WT males had similar ratios (Fig. 3.3D).

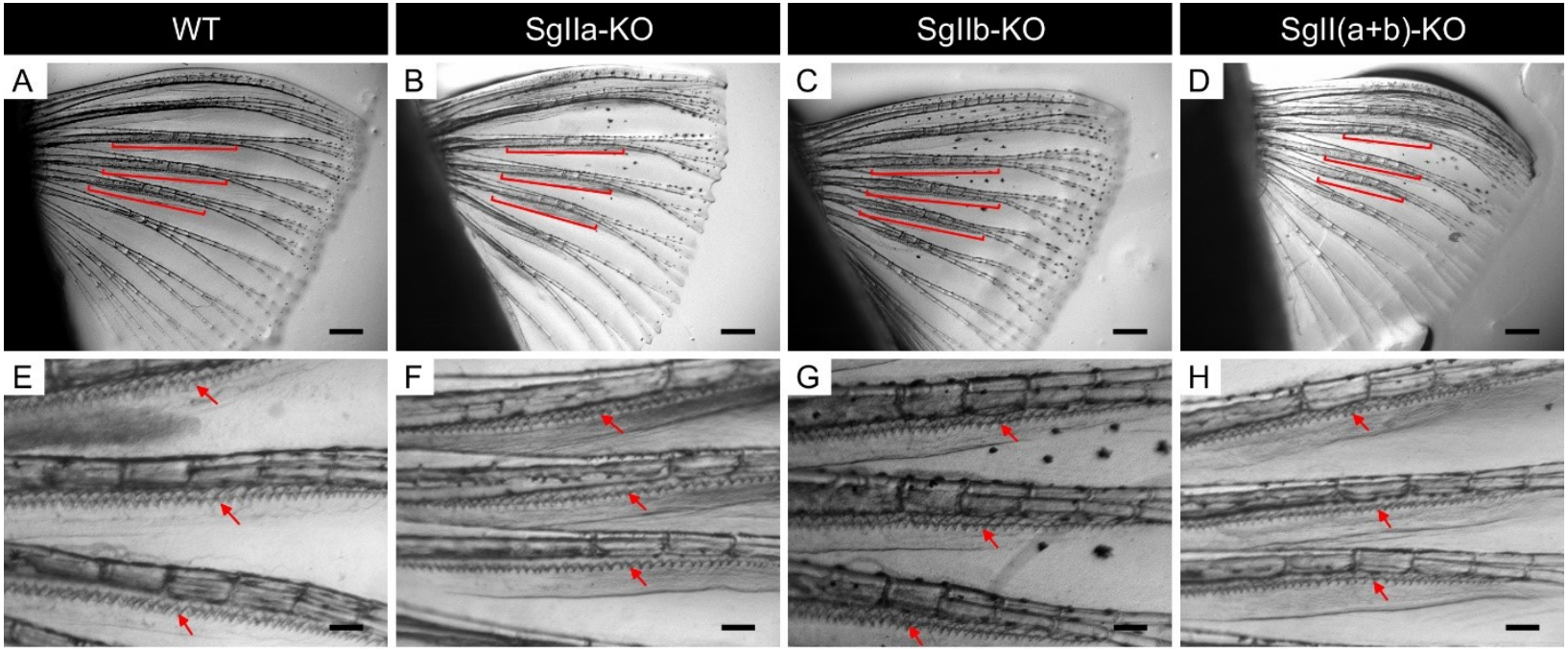

Figure 3.2 Characterization of male zebrafish pectoral fin BTs. (A-D) Brightfield images of pectoral fin BT clusters are indicated by red bars. (E-H) Higher magnifications of A-D. Red arrows indicate the BTs. Scale bars: 500 µm in A-D; 200 µm in E-H.

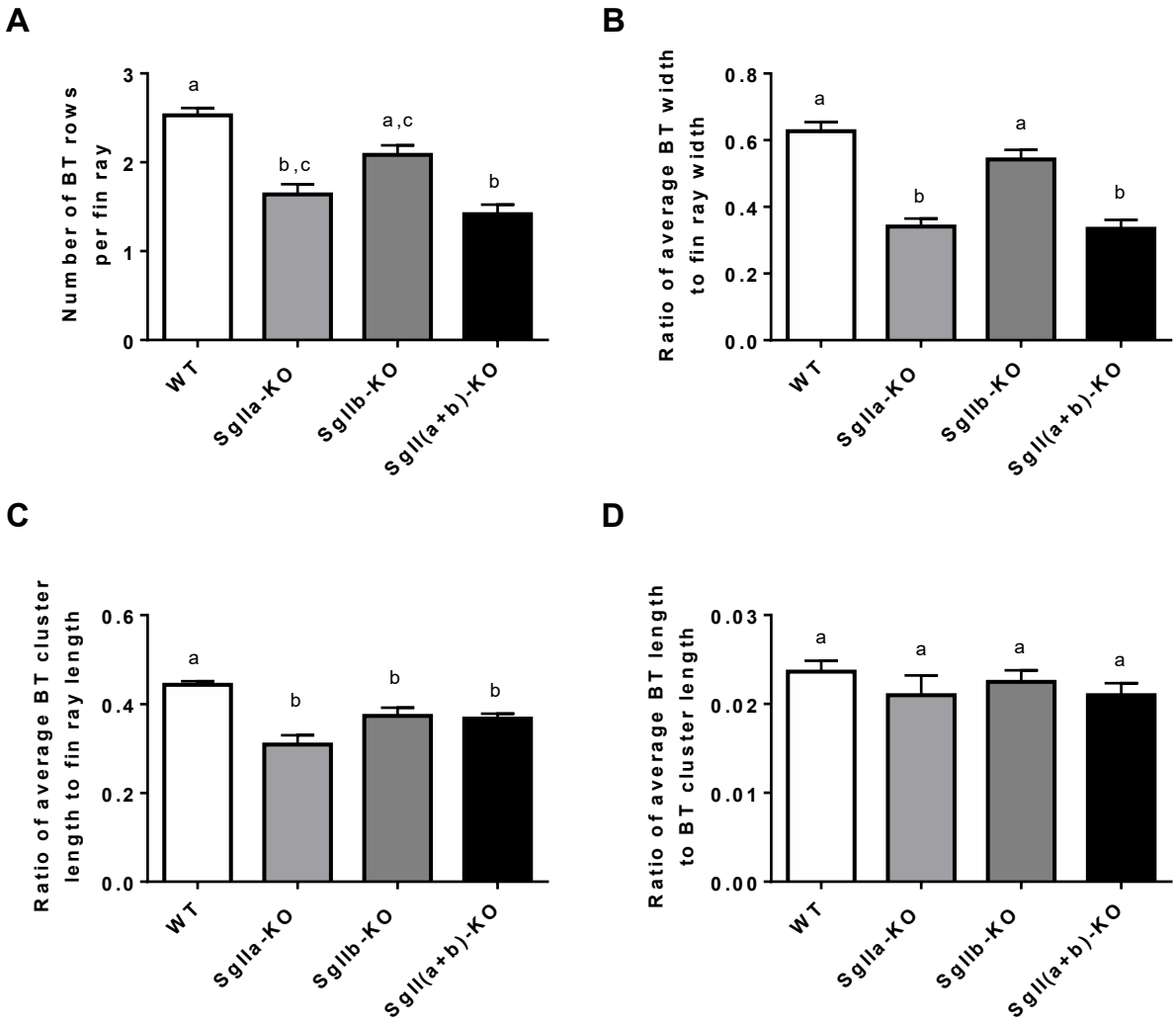

Figure 3.3 Analysis of male zebrafish pectoral fin BTs. (A) Number of BT rows per fin ray. (B) Ratio of the average BT width to fin ray width. (C) Ratio of the average BT cluster length to the fin ray length. (D) Ratio of the average BT length to the BT cluster length. Results are presented as means + SEM (n = 12 per group). Means with different letters denote significant differences between genotypes ($P<0.05$; Holm-Sidak post-hoc test).

3.4 Discussion

This is the first study to provide evidence that the morphology of BTs on male zebrafish pectoral fins are altered by SgII-KO. General observations of the SgII-KO fish while breeding and maintaining mutant lines led us to this initial BT assessment. We noticed that it was sometimes difficult to visually recognize and separate male and female SgII-KO fish from one another. Breeding tubercles are a secondary sex characteristic used to identify mature males. In WT males, BTs are clearly visible along the central rays of the pectoral fin whereas females lack BTs and therefore their pectoral fins appear translucent (McMillan et al., 2015). We determined through microscopic examination that the cluster distribution was similar between male WT and SgII-KO lines in that most BT clusters were found on rays 2 to 6, although the most prominent clusters were observed on rays 3 to 5. These results are consistent with the detailed and elegant observations of McMillan et al. (2013) in WT male zebrafish. However, BT clusters were also observed on ray 7 of some SgII-KO males, while this was not apparent in WTs suggesting that SgII may play a role in directing the development of BT clusters on fin rays. Kang et al. (2013) previously demonstrated that pectoral fins are necessary for successful spawning by allowing males to maintain contact with females and stimulate egg release. However, whether these extra BT clusters on the lower fin rays (rays 7 and onwards) play a significant reproductive role remains to be assessed. One can speculate that the lower fin rays, that are usually shorter than the other rays, would have less contact with the female's body and therefore be functionally redundant to contain BT clusters. Until further analyses are conducted, we are not able to determine the relevance of these extra BT clusters.

We measured several morphological parameters including the number of BT rows, the ratio between BT width and fin ray width, the ratio between BT cluster length and fin ray length and the ratio between BT cluster length and fin ray lengths. There were significantly fewer rows of BTs in male SgIIa-KOs and SgII(a+b)-KO fish compared to WTs. Similarly, the ratio between the BT width and fin ray width were reduced in SgIIa-KO and SgII(a+b)-KO male zebrafish. Moreover, all SgII-KO males had a reduced BT cluster length to fin ray length ratio compared to WTs. The only parameter where no significant differences were observed between WT and SgII-KO males was the ratio between BT cluster length and fin ray lengths. Interestingly though, the number of BT rows and average BT width in SgIIb-KO males was not significantly different from WTs. These results provide support that SgIIa and SgIIb may have different physiological roles given that SgIIa and SgII(a+b)-KO had a significant impact on BT morphology whereas the effect of SgIIb-KO was not as evident.

An explanation for the reduction in BTs is that SgII may be regulating angiogenesis in the zebrafish fin. In my study, BTs were still present in SgII-KO males although, their distribution and size were significantly reduced. McMillan et al. (2013) treated female zebrafish with the angiogenesis inhibitor PTK787 (Wood et al., 2000) and demonstrated that androgen-induced neo-angiogenesis is necessary for BT formation. There is considerable evidence for the pro-angiogenic effects of SN in mammalian models (Fischer-Colbrie et al., 2005). In mice, synthetic SN can induce neovascularization *in vivo* and promote capillary tube formation *in vitro* (Kirchmair et al., 2004b). Gene therapy with SN induces therapeutic angiogenesis, arteriogenesis, and vasculogenesis in a mouse hindlimb ischemia model by a nitric oxide-dependent mechanism (Schgoer et al., 2009b). Secretoneurin also promotes coronary angiogenesis in the rat (Albrecht-Schgoer et al., 2012). Recently, it was shown that SN-based gene therapy in mice ameliorates diabetic neuropathy, probably by induction of growth of vasa nervorum, the small capillaries nourishing peripheral nerves (Theurl et al., 2018). It is therefore plausible that SgII-KO leads to reduced angiogenic activity and thus reduced BT formation in males. Moreover, in zebrafish, *scg2b* is critical in the neurovascular modeling in the developing hindbrain (Tao et al., 2018). Using the same mutant lines presented here, we demonstrated previously that both SgIIb-KO and SgII(a+b)-KO mutant embryos were defective in hindbrain central artery development due to impairment of migration and proliferation of central artery cells (Tao et al., 2018). In the same study, no impairments were evident in SgIIa-KO zebrafish embryos. Neurons expressing *scg2b* are also aligned with central arteries in the hindbrain demonstrating a close neurovasculature relationship (Tao et al., 2018). This *scg2b*-angiogenesis relationship in embryos is in marked contrast to the results presented here for adult males. Most reductions in BT morphology were observed in SgIIa-KO or SgII(a+b)-KO males. Future studies should establish the link between *scg2* and the angiogenic control of BT formation. It will be important in the future to determine the relative contributions of *scg2a* versus *scg2b* to angiogenesis. Studies currently underway in the laboratory were designed to examine the vasculature of the zebrafish pectoral fins in SgII-KO zebrafish crossed with Tg(fli1a:EGFP). In this transgenic line, the promoter for endothelial marker fli1a drives the expression of EGFP in blood vessels thereby allowing their visualization and quantitative assessment.

The neuropeptide SN has previously been shown to stimulate the synthesis and release of luteinizing hormone (Lh) *in vivo* in goldfish and *in vitro* using the LβT2 mouse cell line (Zhao et al., 2006b; Zhao et al., 2009a; Zhao et al., 2011). Luteinizing hormone stimulates testicular androgen production, and androgens are clearly important for BT cluster formation (McMillan et al., 2013). I

initially hypothesized that SgIIa-KO may alter the androgens to estrogens ratio which has been proposed to regulate BT cluster formation in zebrafish (McMillan et al., 2013), thus leading to reduced BT numbers and size. However, whole body steroid levels provide little support for this hypothesis. The ratio of E2/T was significantly higher in SgIIa-KO males compared to WTs but no other significant differences in E2/T or E2/11-KT were observed (chapter 4). Perhaps circulating steroid levels would be more indicative of androgen and estrogen status. Previous studies have shown that androgens [T and 5α-dihydrotestosterone (DHT)] induce the formation of BTs through androgen receptors (ARs) (McMillan et al., 2013). McMillan et al. (2013) also found that treating zebrafish with the AR antagonist flutamide inhibited BT formation. It is therefore possible that it is not circulating androgens but rather the androgen receptor expression that is altered in SgII-KO male zebrafish, thereby leading to a reduction in BTs. Future studies could focus on measuring the androgen receptors and the effects of androgen administration in the SgII-KO fish to determine if it is possible to rescue the smaller BTs seen in SgII-KO fish. This may also allow for the determination of the mechanism by which *scg2* maintains BT morphology.

In this study we showed that SgII-KO disrupts normal morphology of the pectoral fins by reducing the number of BT rows, BT width and BT cluster length which could potentially lead to a reduced spawning success. We therefore assessed the spawning success of the SgII-KO lines in the following chapter (chapter 4).

Chapter 4: Secretogranin-II plays a critical role in courtship behaviour and reproductive success in zebrafish

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[1]Contributed to original ideas, conducted breeding experiments, performed data analysis and manuscript preparation.

[2]Helped with spawning experiments and embryo collections

[3]Analyzed courtship behaviours of zebrafish (video analysis)

[4]Performed *in vitro* fertilization experiment

[5]Provided SgII mutant zebrafish and collaborated on this study

[6]Contributed to original ideas and revised the manuscript

4.1 Introduction

Secretogranin-II is an ~600 amino acid long, acidic, tyrosine-sulfated preproprotein processed into small peptides by prohormone convertases (PCs) at several dibasic cleavage sites in secretory granules of vertebrate neuroendocrine cells (Fischer-Colbrie et al., 1995). Secretogranin-II is involved in the biogenesis of secretory granules and possibly participates in the sorting and packaging of hormones and neuropeptides in secretory granules (Miyazaki et al., 2011). The primary amino acid sequence of mammalian SgII is only moderately conserved through evolution, being 79-87% identical between mammalian species, but much less so in non-mammalian vertebrates (Blazquez et al., 1998; Montero-Hadjadje et al., 2008). Only a discrete domain in the middle of the SgII precursor, representing the SN peptide, is relatively well-conserved across all vertebrate taxa (Trudeau et al., 2012). Tetrapods, such as mammals and amphibians, posess only one SgII gene and produce one SN paralog. However, teleosts have two SgII genes, SgIIa and SgIIb, likely a consequence of the early whole genome duplication event that occurred in teleost fishes (Zhao et al., 2010b). The two teleost SgII subtype precursors generate SNa and SNb peptides, respectively (Trudeau et al., 2012; Zhao et al., 2009b), ranging in size from 31 to 43 amino acids. A large body of evidence suggests that SgII and/or SN are involved in reproduction (Zhao et al., 2006a; Zhao et al., 2006b; Zhao et al., 2009a; Zhao et al., 2010a; Zhao et al., 2011).

Secretoneurin stimulates Lh production and release in the goldfish model (Zhao et al., 2006a; Zhao et al., 2006b; Zhao et al., 2009a; Zhao et al., 2010a) and from the mouse LβT2 gonadotroph cell line (Zhao et al., 2011). Zhao et al. (2009a) showed in goldfish that magnocellular cells sending projections to the anterior and posterior pituitary were SN immunopositive, suggesting that SN may influence Lh release via stimulatory or inhibitory neurohormones. Furthermore, in goldfish, SN is co-released with isotocin (IST), the teleost homolog of the reproductive peptide oxytocin (OXT), from nerve terminals (Canosa et al., 2011). Since Gnrh innervates IST neurons that project into the pituitary, Gnrh may also innervate SN neurons. In rat hypothalamus, SN and OXT are co-stored in dense core vesicles of hypothalamo-neurophysial neurons, and SgII and OXT mRNA are co-regulated (Mahata et al., 1993). In rat pituitary and mouse LβT2 cells, GnRH also stimulates the release of SN immunoreactive proteins concomitantly with Lh (Conn et al., 1992; Nicol et al., 2002; Zhao et al., 2011). This previous data also suggests an evolutionarily conserved effect of SN on Lh release (Zhao et al., 2011).

To date, most of the studies on the SN-regulation of reproduction have been carried out using exogenously applied SN, usually on *in vitro* model systems. However, little is known about the effect

of the endogenous SgII precursor protein or the SN neuropeptide on reproductive processes. Until recently, there were no SgII mutant animal models, and human mutations have not yet been identified. Employing our recently developed zebrafish SgIIa and SgIIb single and double mutant lines (Tao et al., 2018) I present the first *in vivo* evidence that SgII plays a critical stimulatory role in vertebrate reproduction. This is in marked contrast to previous mutational studies in zebrafish that have shown that single, double and triple gene knockouts of traditional reproductive regulators (e.g., Gnrh and Kisspeptin) resulted in minor alterations in reproductive processes. For example, Gnrh is considered the main stimulator of Lh and Fsh (Schally et al., 1971; Zohar et al., 2010), however, the *gnrh3*$^{-/-}$ mutant zebrafish are fertile, display normal gametogenesis and reproductive performance in both males and females (Spicer et al., 2016). Similarly, *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ mutant zebrafish were fertile (Marvel et al., 2018). In zebrafish kisspeptin and the kisspeptin receptor mutant lines (*kiss1*$^{-/-}$, *kiss2*$^{-/-}$, *kiss1*$^{-/-}$;*kiss2*$^{-/-}$, *kissr1*$^{-/-}$, *kissr2*$^{-/-}$, *kissr1*$^{-/-}$;*kissr2*$^{-/-}$), spermatogenesis and folliculogenesis, as well as reproductive capabilities were not impaired in any of the six mutant lines. Moreover, the triple knockout mutants lacking *gnrh3* and the two *kiss* genes (*kiss1*$^{-/-}$;*kiss2*$^{-/-}$;*gnrh3*$^{-/-}$) undergo normal puberty and gonad maturation (Liu et al., 2017). These previous knockout studies challenge conventional ideas that Gnrh and kisspeptin are essential for reproduction across species. Instead, they provide evidence that different mechanisms have evolved for the neuroendocrine control of reproduction between mammals and fish.

Given the profound effects of SgII-KO on *gnrh3*, *lhb* and *fshb* expression (chapter 2) and on BT development (chapter 3), I investigated in detail the reproductive capabilities of SgII mutant zebrafish. I hypothesized that SgII is a reproductive regulator and predicted that gene mutation of *scg2a* and *scg2b* would reduce reproductive output and courtship behaviours. Gonadal development and whole-body sex steroid levels were also investigated.

4.2 Materials and Methods

4.2.1 Spawning trials

4.2.1.1 Animals

Secretogranin mutant lines (SgII-KO) (Tao et al., 2018) and wild-type (WT) zebrafish were reared according to standard husbandry procedures. Fish were maintained in 10-L tanks of dechloraminated City of Ottawa tap water at 28^{o}C on a 14:10 light-dark cycle.

4.2.1.2 Spawning

The reproductive capacity of SgII-KO zebrafish was assessed via mating crosses between pairs of fish that were within the same mutant lines and from reciprocal crosses with WT zebrafish. Two weeks prior to the start of the spawning experiment, fish were separated by sex into 3L tanks at a density of 4 fish/litre. The night before each experiment, fish were transferred to 1-L breeding tanks. Each tank contained one male and one female, separated overnight with an insert containing a divider. Breeding tanks were covered overnight with cardboard boxes to prevent visual disturbance. The morning of the experiment, covers were removed, water was changed, and tanks were placed in the recording arena. The dividers separating the male and female were removed, and the fish were filmed for 10 mins with either a Panasonic 16GB HC-V700M Full HD Camcorder or a Canon VIXIA HF R400 Digital Camcorder. Once 10 mins elapsed the tanks were set aside, and the next pair was filmed. All filming took place within one hour of first light (9 AM). The fish were left undisturbed for 1.5 hrs at which point oviposition was noted. Clutches were placed in E3 medium and fertilization rate was assessed. Embryos were kept in an incubator (28°C) until 24 hrs post fertilization (hpf) to assess survival rate.

4.2.1.3 Courtship behaviours

Zebrafish exhibit an elaborate pre-mating ritual consisting of a series of behaviours to attract mates (Darrow and Harris, 2004). The following courtship behaviours were assessed in male zebrafish: chase (following or swimming alongside female), tail-nose (touching the female's side or tail with nose or head), zig-zag (tail sweep and circle along female's body), encircle (circling around or in front of the female) and quiver (rapid tail oscillation against the female's side) (Darrow and Harris, 2004). The only female courtship behaviour that was assessed was egg lay (oviposition). Courtship behaviours were assessed by several blind observers. Videos were viewed using VideoPad Video Editor (http://www.nchsoftware.com/videopad/) at 40-50% speed and the start and stop times of a specific behaviour were manually recorded in an Excel spreadsheet. Due to time and manpower constraints, only 25% of the videos (n=20-22 per group) were analyzed. The number of non-spawning and spawning pairwise crosses analyzed in each group are proportionate to the total data set.

4.2.2 Gonads

4.2.2.1 Tissue collection and gonadosomatic index

Secretogranin-II knockout fish and WTs were bred within their respective lines and their offspring were reared according to standard husbandry procedures. Fish were maintained in 10-L tanks of water at 28°C on a 14:10 light-dark cycle until 3 months of age. Fish were randomly selected from each tank, anaesthetized in ice water, weighed, and decapitated. Gonads of mutant (n=20-50) and wild-type (n=90) fish were dissected and weighed to calculate the gonadosomatic index (GSI; gonad weight/body weight x 100). Of these, the gonads of 5 female and 5 male fish from each line were fixed in Bouin's solution overnight at 4°C. The fixed samples were washed twice in PBS, embedded in 1-1.5% agarose, trimmed and prepared for histological processing.

4.2.2.2 Histology

Samples were dehydrated in increasing ethanol (EtOH) concentrations, cleared with xylene, infiltrated with paraffin, and embedded in individual paraffin blocks. Serial sections of 5-8 µm thickness were prepared on a microtome (Kedee rotary microtome) and suspended on a water bath at 45°C. Once the sections expanded to normal size, they were mounted on glass slides (Citoglas, Citotest, China). Sections were deparaffinized in xylene, rehydrated in EtOH, stained with hematoxylin and eosin-Y, and covered with a cover slip (Citoglas, Citotest, China). A CRI Nuance multispectral imaging camera in conjunction with an Olympus BX53 microscope was used to capture digital images of the sections (Perkin-Elmer, Hopkinton, MA). Pictures were assigned a random number to perform unbiased, blind analyses.

4.2.2.3 Oogenesis and spermatogenesis

Zebrafish ovaries develop asynchronously and have follicles of all stages at any given time (Clelland and Peng, 2009). The follicles were divided into 5 groups according to size and general appearance: primary growth, pre-vitellogenic, early-vitellogenic, mid-vitellogenic and full-grown (Chen and Ge, 2013). Follicle stages were presented as a percentage of the total follicles examined from each female. In males, 4 major stages were examined: spermatogonia, spermatocytes, spermatids and spermatozoa (Maack and Segner, 2003). All other space or interstitial tissue is referred to as "other". Stages are presented as a percentage of the total area of each picture.

4.2.3 Sex steroids

Sex steroids were extracted using the Folch method (Folch et al., 1957). Whole zebrafish were individually frozen with liquid nitrogen, ground in a mortar and pestle, and poured into a 50 mL plastic centrifuge tube. Chloroform:methanol (2:1) (15 mL) was added to each sample. Samples were homogenized for 30 sec with a Polytron (Luzern, Switzerland) homogenizer, then incubated for 15 min at room temperature. Five mL 2 M KCl containing 5 mM ethylenediaminetetraacetic acid (EDTA) were added to each sample and the tubes were vortexed for 1 min. The samples were left at room temperature for 20 min to allow layer separation and then the lower layer containing steroids, was removed and placed into a 10 mL glass test tube. The samples were evaporated under a gentle stream of nitrogen in a fumehood. The lipid extract was reconstituted in 0.3 mL ethylene glycol monomethyl ether (EGME), transferred to a 1.5 mL conical centrifuge tube, and stored in a -20°C freezer until analyzed. Whole body extraction efficiencies were determined by spiking homogenates with known amounts of their respective radioactive isotope (^{14}C or ^{3}H). The efficiencies were 85% for 17β-estradiol (E2) and 89% for testosterone (Vera Chang, 2018). Extraction efficiencies were relatively high therefore results were not corrected for these differences. Testosterone (T) (TECO Diagnosis, Anaheim, CA, TEST-96), estradiol (E2) (TECO Diagnosis, Anaheim, CA, ESTRA-96) and 11-keto-testosterone (11-KT) (Cayman Chemical, Ann Arbor, MI, 582751) levels were assessed using enzyme immunoassay test kits according to the manufacturer's protocols.

4.2.4 Data analysis

Online freeware was used for statistical analysis of the spawning success data. The percent spawning success of mating pairs were compared to the WT within line cross with Fisher's exact test (http://graphpad.com/quickcalcs/contingency1/). All other statistical analyses were performed using SigmaPlot 11.0 software (Systat Software, Inc., Chicago, IL). Normality and homoscedasticity were tested using a Shapiro-Wilk test and Levene's test respectively. A Kruskal-Wallis test on ranks followed by a Dunn's multiple comparison test was used to analyze the percent fertilization, percent survival and all behavioural data since the data was not normally distributed. Two-way analysis of variance (ANOVA), with sex and genotype as main factors, was used to analyze GSI, oogenesis, spermatogenesis and sex steroid concentrations.

4.3 Results:

4.3.1 Secretogranin-II knockout reduces spawning success and courtship behaviours

I performed large-scale pair-wise mating and behavioural tests to gain insight into the potential reproductive roles of SgII. Sexually naïve WT males paired with WT females (within line cross), successfully spawned in 62% of the 101 spawning trials. Within line spawning success was significantly reduced to 37%, 44% and 6%, which corresponds to a 40%, 29% and 90% decrease in SgIIa-, SgIIb- and SgII(a+b)-KO fish, respectively (Fig. 4.1A). The spawning success of SgIIa-KO, SgIIb-KO and SgII(a+b)-KO males paired with WT females increased to 52%, 59% and 49% (Fig. 4.1A). Similarly, both SgIIa-KO and SgIIb-KO females paired with WT males increased their spawning success to 50% (Fig. 4.1A). However, the SgII(a+b)-KO females paired with WT males were unable to significantly elevate their spawning success and spawned in 11% of all trials (Fig. 4.1A). The fecundity (# embryos/clutch) was significantly higher in the SgII(a+b)-KO within line crosses as well as when SgII(a+b)-KO males were paired with WT females (Fig. 4.1B), although there was increased mortality (Fig. 4.1D). Furthermore, the percent fertilization was reduced significantly in SgIIa-KO and SgII(a+b)-KO within line crosses and increased in SgIIa-KO and SgIIb-KO males crossed with WT females compared to the WT within line cross (Fig. 4.1C). *In vitro* fertilization procedures were performed according to Westerfield et al. (2000). It was found that *in vitro* fertilization rates of SgIIa-KO, SgIIb-KO and SgII(a+b)-KOs were 90%, 89% and 89%, respectively, and not significantly different compared to WTs that were at 88% (data not shown). Similarly, no differences in fecundity were observed between WT and SgII-KO *in vitro* fertilizations.

Male zebrafish exhibit typical courtship behaviours towards females, including chasing, tail-nose, encircling, zig-zag and quiver (Darrow and Harris, 2004). The five-male courtship behaviours were observed in WT and SgII-KO spawning trials and followed a similar sequence of behaviours. As compared to WT within line crosses, SgII(a+b)-KO male within line crosses showed a significant reduction in the frequency and duration of chase, tail-nose, encircle and quiver behaviours (Fig. 4.2 and Fig. 4.3). The number of encircles and duration were also reduced in SgII(a+b)-KO females paired with WT males (Fig. 4.2 and Fig. 4.3). Zig-zags were observed sporadically throughout the mating trials and no significant differences were observed (data not shown). The total duration of male courtship behaviours was significantly reduced in the SgII(a+b)-KO within line crosses as well as when SgII(a+b)-KO females were paired with WT males (Fig. 4.4).

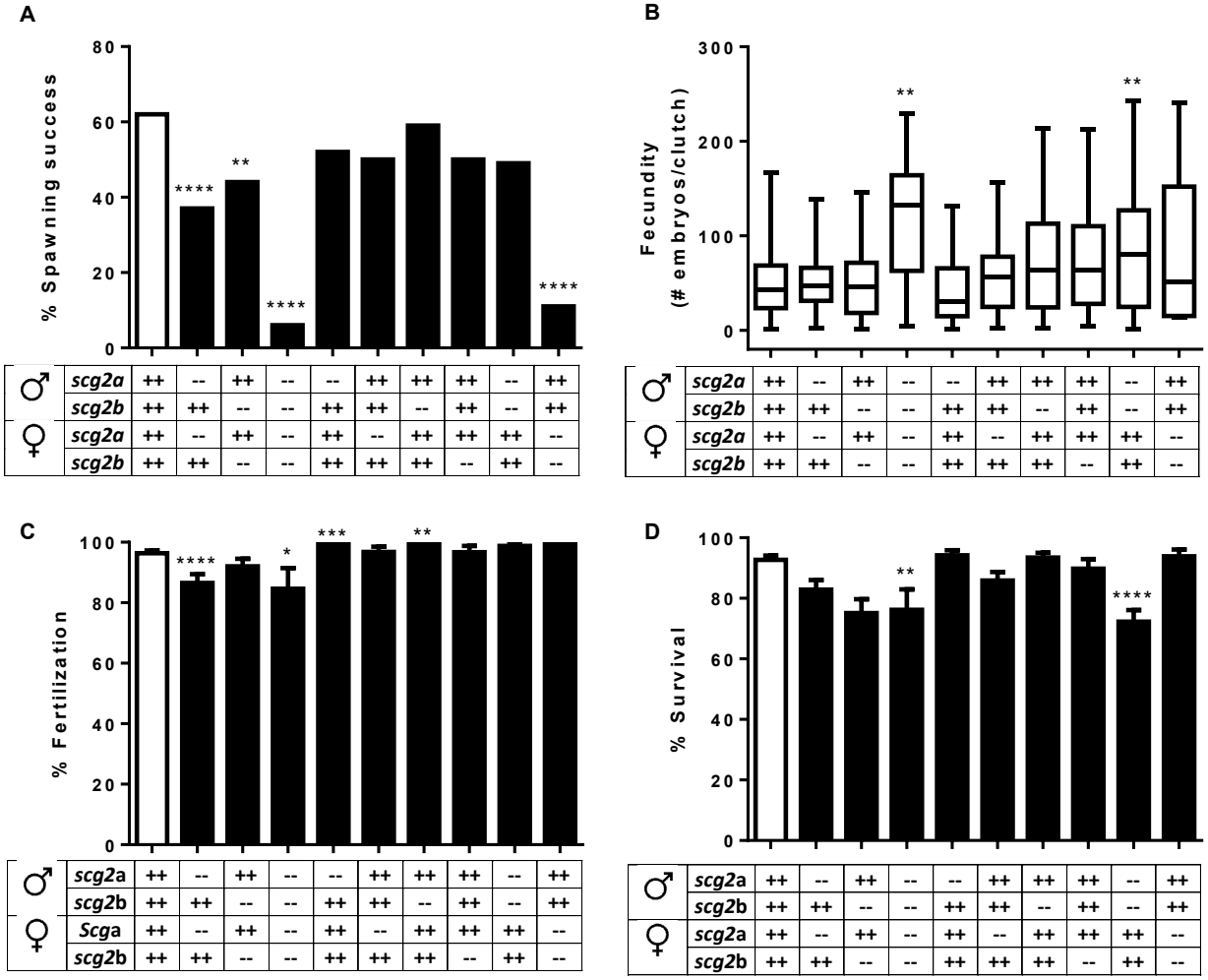
A
% Spawning success
80
60
40
20
0
****
**
****
****
scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
scg2a ++ -- ++ -- ++ -- ++ ++ ++ --
scg2b ++ ++ -- -- ++ ++ ++ -- ++ --
B
Fecundity
(# embryos/clutch)
300
200
100
0
**
**
scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
scg2a ++ -- ++ -- ++ -- ++ ++ ++ --
scg2b ++ ++ -- -- ++ ++ ++ -- ++ --
C
% Fertilization
100
80
60
40
20
0
****
*
***
**
scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
Scga ++ -- ++ -- ++ -- ++ ++ ++ --
scg2b ++ ++ -- -- ++ ++ ++ -- ++ --
D
% Survival
100
80
60
40
20
0
**
****
scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
scg2a ++ -- ++ -- ++ -- ++ ++ ++ --
scg2b ++ ++ -- -- ++ ++ ++ -- ++ --

Figure 4.1 Spawning success, fecundity, fertilization and survival of embryos produced during zebrafish pairwise matings. (A) % spawning success of within line (n=82-101 matings) and reciprocal crosses (n=80-90 matings). Significant differences were determined using Fisher's exact test. (B) Box plots of the number of embryos produced per couple. Horizontal lines represent median values; boxes represent interquartile ranges and whiskers represent minimum-maximum values (n=8-90). P values present the comparison of that group against the wild-type pair using Kruskal-Wallis and Dunn test for post hoc comparisons. (C) Mean (+SEM) % fertilization and (D) % embryo survival of mating crosses (n=8-90). Since the data was not normally distributed, significant differences were determined using a Kruskal-Wallis test on ranks followed by a Dunn's multiple comparison test. Asterisks denote a significant difference from the wild-type within line cross (white bar); ****$P \leq 0.0001$; *** $P \leq 0.001$; **$P \leq 0.01$; * $P \leq 0.05$.

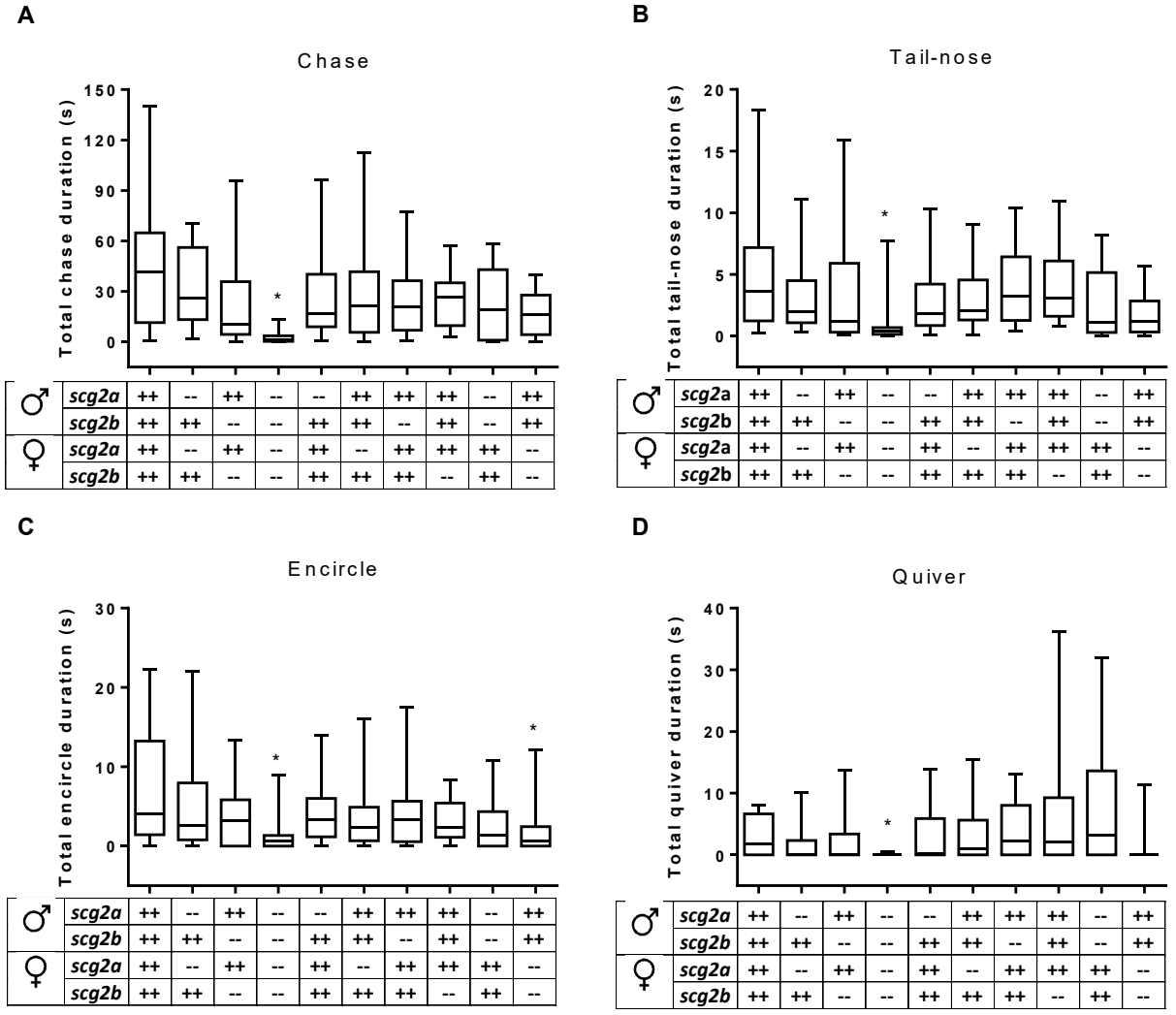
A
Chase
Total chase duration (s)
150
120
90
60
30
0
*
♂ scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
♂ scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
♀ scg2a ++ -- ++ -- ++ -- ++ ++ ++ --
♀ scg2b ++ ++ -- -- ++ ++ ++ -- ++ --
B
Tail-nose
Total tail-nose duration (s)
20
15
10
5
0
*
♂ scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
♂ scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
♀ scg2a ++ -- ++ -- ++ -- ++ ++ ++ --
♀ scg2b ++ ++ -- -- ++ ++ ++ -- ++ --
C
Encircle
Total encircle duration (s)
30
20
10
0
*
*
♂ scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
♂ scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
♀ scg2a ++ -- ++ -- ++ -- ++ ++ ++ --
♀ scg2b ++ ++ -- -- ++ ++ ++ -- ++ --
D
Quiver
Total quiver duration (s)
40
30
20
10
0
*
♂ scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
♂ scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
♀ scg2a ++ -- ++ -- ++ -- ++ ++ ++ --
♀ scg2b ++ ++ -- -- ++ ++ ++ -- ++ --

Figure 4.2 Box plots of total chase (A), tail-nose (B), encircle (C) and quiver (D) behaviour durations (s) assessed for 10 minutes in pairwise within-line and reciprocal mating crosses. Horizontal lines represent median values; boxes represent interquartile ranges and whiskers represent minimum-maximum values. Since the data was not normally distributed, significant differences were determined using a Kruskal-Wallis test on ranks followed by a Dunn's multiple comparison test (n=20-22). Asterisks denote a significant difference from the wild-type pair; ****P ≤ 0.0001; ***P≤0.001; **P ≤ 0.01; *P≤0.05.

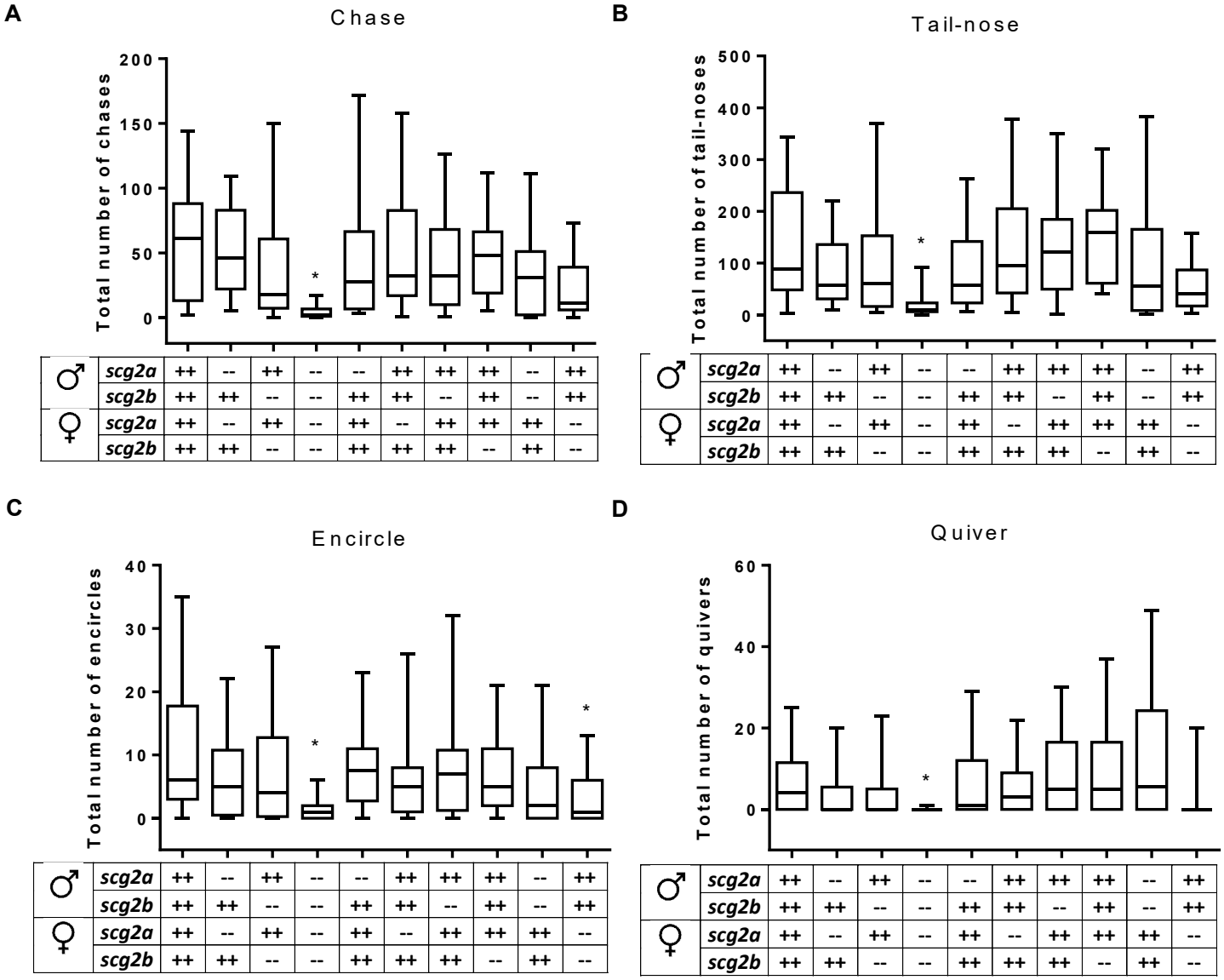
A
Chase
Total number of chases
200
150
100
50
0
*
B
Tail-nose
Total number of tail-noses
500
400
300
200
100
0
*
C
Encircle
Total number of encircles
40
30
20
10
0
*
*
D
Quiver
Total number of quivers
60
40
20
0
*
♂ scg2a ++ -- ++ -- -- ++ ++ ++ -- ++
scg2b ++ ++ -- -- ++ ++ -- ++ -- ++
♀ scg2a ++ -- ++ -- ++ -- ++ ++ ++ --
scg2b ++ ++ -- -- ++ ++ ++ -- ++ --

Figure 4.3 Box plots of number of chase (A), tail-nose (B), encircle (C) and quiver (D) behaviours assessed for 10 minutes in pairwise within-line and reciprocal mating crosses. Horizontal lines represent median values; boxes represent interquartile ranges and whiskers represent minimum-maximum values. Since the data was not normally distributed, significant differences were determined using a Kruskal-Wallis test on ranks followed by a Dunn's multiple comparison test (n=20-22). Asterisks denote a significant difference from the wild-type pair; *P≤0.05.

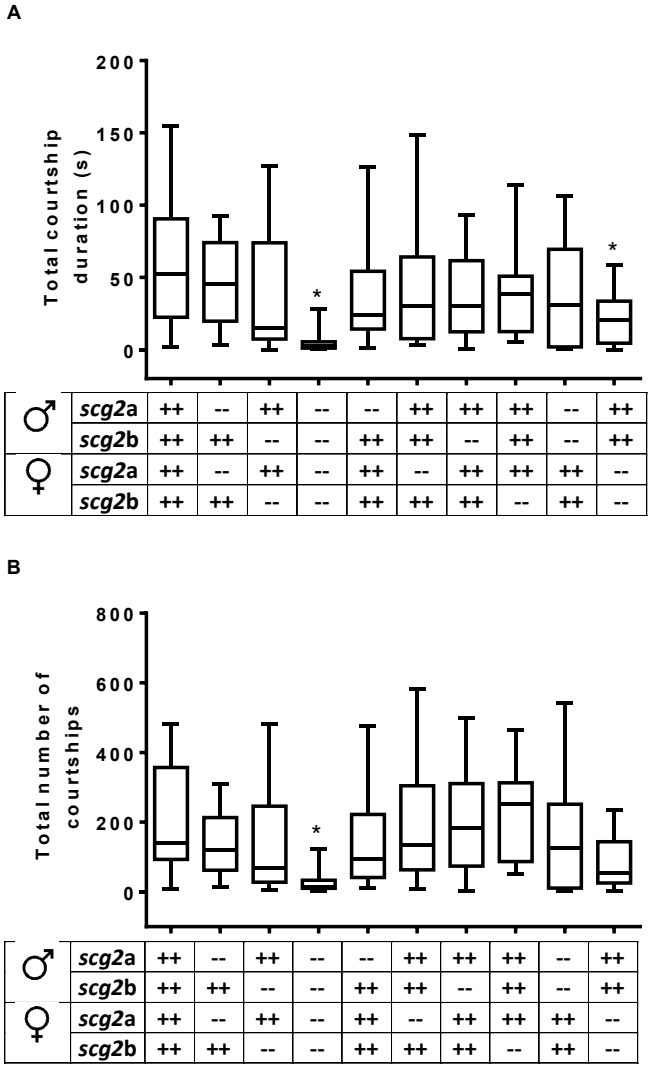

Figure 4.4 Median (min-max) of total courtship behaviour duration (s) (A) and total number of courtship behaviours (B) assessed for 10 minutes in pairwise within-line and reciprocal mating crosses. Horizontal lines represent median values; boxes represent interquartile ranges and whiskers represent minimum-maximum values. Since the data was not normally distributed, significant differences were determined using a Kruskal-Wallis test on ranks followed by a Dunn's multiple comparison test (n=20-22). Asterisks denote a significant difference from the wild-type pair; ****P ≤ 0.0001; *P≤0.05.

4.3.2 Gonadosomatic index is unaltered in SgII knockout zebrafish

The effects of SgII-KO on gonadosomatic index (GSI) of adult zebrafish are shown in Figure 4.5. There were no significant main effects of genotype [F(3) = 0.989, P = 0.398] but there was a main effect of sex [F(1) = 1001.425, P < 0.001]. No interactions between genotype and sex were observed [F(3) = 0.773, P = 0.510]. Post-hoc analyses showed that the GSI of females was higher than that of males (P < 0.05) (Fig. 3.5)

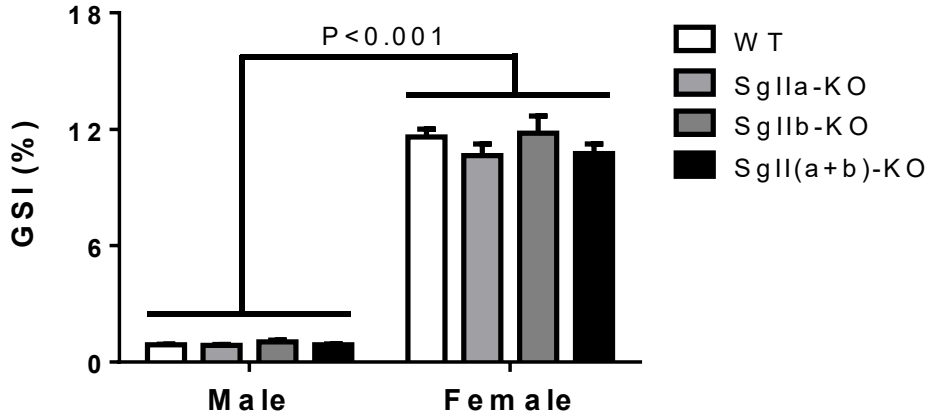

Figure 4.5 Gonadosomatic index (GSI) for SgII-KO and wild-type males and females. Means + SEM are presented (n = 20-90 fish per group). Lines connecting different bars represent the comparison made between the two.

4.3.3 Secretogranin-II knockout does not affect gonad morphology

The ovaries and testes of all SgII-KO fish appeared normal by histology (Fig. 4.6). In the testes, spermatogonia, spermatocytes, spermatids and spermatozoa were observable in all genotypes (Fig. 3.6) indicating that spermatogenesis could be completed in all the genotypes. In the ovaries, follicles of various stages including primary growth, pre-vitellogenic, early-vitellogenic, mid-vitellogenic and full-grown oocytes could be observed in all the genotypes (Fig. 4.6) indicating that folliculogenesis was not affected.

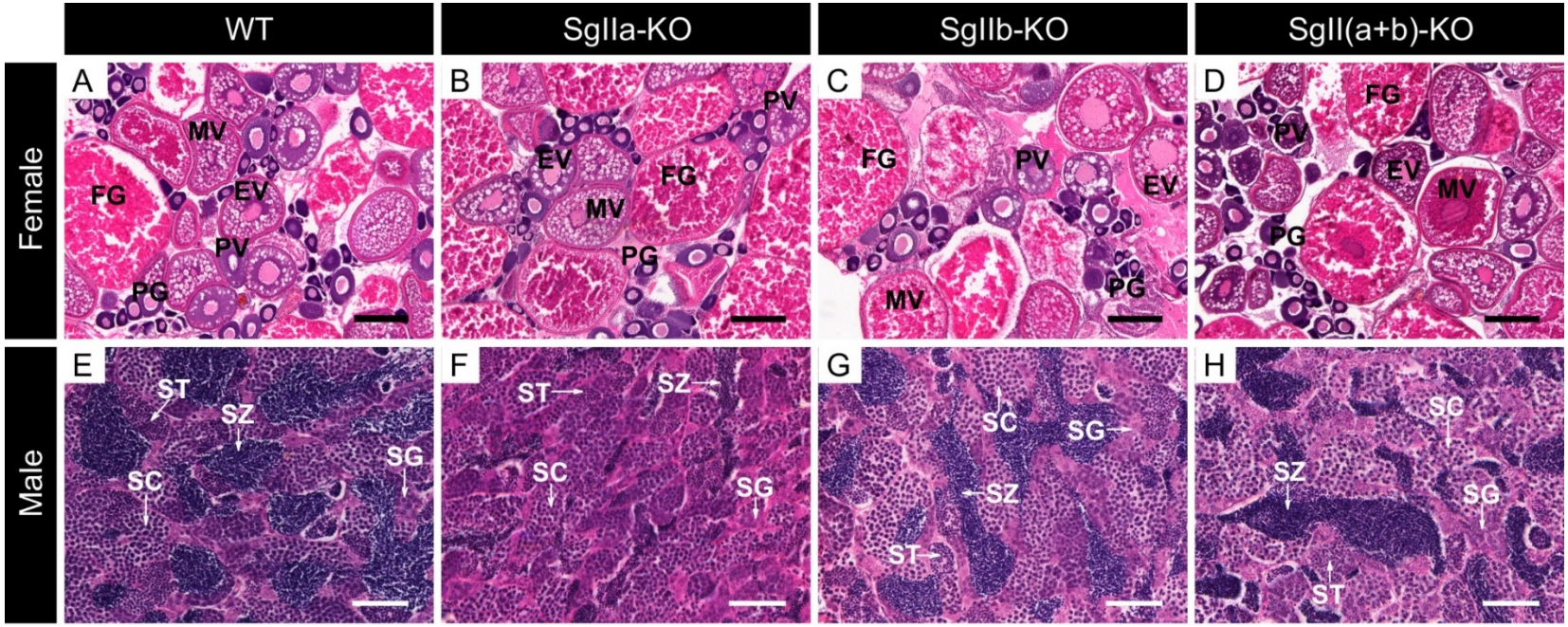

Figure 4.6 Representative histological sections of ovaries and testes of WT, SgIIa-KO, SgIIb-KO and SgII(a+b)-KO zebrafish at 4 months of age. Ovary follicle stages are labeled on top of their respective structure and abbreviated as follows: PG, primary growth follicle; PV, pre-vitellogenic follicle; EV, early-vitellogenic follicle; MV mid-vitellogenic follicle; FG, full-grown follicle. Sperm stages are marked with arrows; SG, spermatogonia; SC, spermatocytes; ST, spermatids; SZ, spermatozoa. Scale bars correspond to 200 µm for females and 50 µm for males. Sections are 5-8 µm thick and stained with hematoxylin-eosin stain.

4.3.4 Secretogranin-II knockout affects spermatogenesis but not oogenesis

Histological assessments of ovaries and testes were conducted to potentially delineate the cause of fertility defects observed in SgII-KO fish. In females, there was no effect of genotype [$F(3) = 0.0229$, $P = 0.995$] but there was a main effect of follicle stage [$F(4) = 20.266$, $P < 0.001$]. However, there were no interactions between follicle stage and genotype [$F(12) = 0.953$, $P < 0.500$], indicating that SgII-KO did not affect ovarian development. Holm-Sidak post-hoc analyses indicated that the levels of primary growth and pre-vitellogenic follicles were statistically different from early-vitellogenic, mid-vitellogenic and full-grown follicles ($P < 0.05$). However, there were no statistical differences between primary growth and pre-vitellogenic follicles as well as early-vitellogenic, mid-vitellogenic and full-grown follicles (Fig. 4.7A).

In males, there was no main effect of genotype [$F(3) = 0.000$, $P = 1.000$] but there was a statistically significant effect of sperm stage [$F(4) = 114.931$, $P < 0.001$] and a significant interaction between genotype and stage [$F(12) = 5.182$, $P < 0.001$]. Post-hoc analyses revealed that there was a significantly lower percentage of spermatogonia and spermatids compared to spermatocytes and spermatozoa ($P < 0.05$) although, spermatogonia and spermatid ratios were not significantly different from each other. There was also a higher percentage of spermatocytes compared to spermatozoa ($P < 0.05$). Within the spermatocyte stage, SgIIa-KO males had a higher percentage of spermatocytes compared to SgIIb-KO ($P < 0.05$) males, but neither were significantly different compared to WTs. The percentage of spermatocytes in WT, SgIIa-, SgIIb- and SgII(a+b)-KO males were 36%, 40%, 30% and 34%, respectively. Within the spermatozoa stage, SgIIa-KO and SgII(a+b)-KO males had significantly lower percentages compared to WTs ($P < 0.05$). The percentage of spermatozoa were decreased by 46% and 37% in SgIIa-KO and SgII(a+b)-KO males, respectively, indicating that SgII-KO did affect spermatic development (Fig. 4.7B).

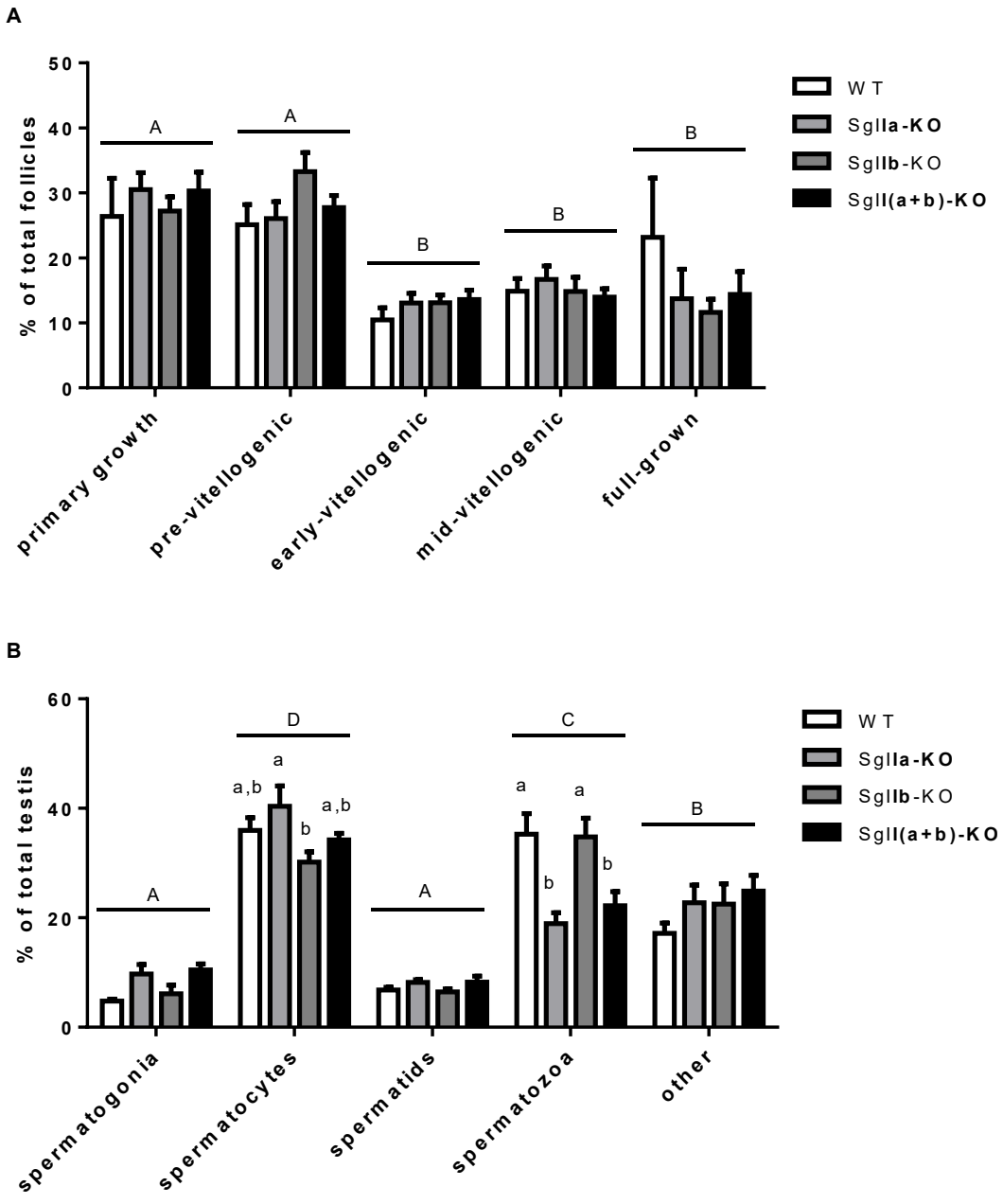

Figure 4.7 Effects of SgII knockout on oogenesis and spermatogenesis in zebrafish. (A) percentages of primary growth, pre-vitellogenic, early-vitellogenic, mid-vitellogenic and full-grown oocytes in ovaries from females (mean +SEM, n = 5). (B) percentages of spermatogonia, spermatocytes, spermatids, spermatozoa and other tissues in testes from males (mean +SEM, n = 5). Upper case letters (A, B, C, D) denote differences between gonad stages ($P < 0.05$) whereas lower case letters (a, b, c, d) signify differences between genotypes within a stage ($P < 0.05$).

4.3.5 Secretogranin-II knockout does not alter whole-body sex steroids

There was no main effect of genotype [$F(3) = 1.716$, $P = 0.172$] on the levels of T; however, there was a significant main effect of sex [$F(1) = 15.729$, $P < 0.001$] and no interaction between genotype and sex [$F(3) = 0.773$, $P = 0.513$]. Post-hoc tests indicated that males had significantly higher levels of T than females ($P < 0.05$) (Fig. 4.8A).

Similarly, for 11-KT, there was no main effect of genotype [$F(3) = 1.238$, $P = 0.302$] but there was a significant main effect of sex [$F(1) = 2065.543$, $P < 0.001$]. No interactions between sex and genotype were observed [$F(3) = 1.337$, $P = 0.270$]. Post-hoc tests indicated that males had significantly higher levels of 11-KT than females ($P < 0.05$) (Fig. 4.8B).

For the levels of E2, there was no main effect of genotype [$F(3) = 0.163$, $P = 0.921$], a significant main effect of sex [$F(1) = 15.425$, $P < 0.001$] and no interactions between sex and genotype [$F(3) = 2.579$, $P = 0.060$]. Post-hoc tests revealed that E2 levels were higher in females than males ($P < 0.05$) (Fig. 4.8C).

The ratio between E2 and T was analyzed and there was a significant main effect of genotype [$F(3) = 5.982$, $P < 0.001$] and sex [$F(1) = 77.221$, $P < 0.001$]. There were no interactions between genotype and sex [$F(3) = 2.699$, $P = 0.052$]. Post-hoc analyses revealed that females had higher E2/T ratios ($P < 0.05$). Moreover, SgIIa-KO fish had higher E2/T ratios than WTs and SgII(a+b)-KO fish ($P < 0.05$) (Fig. 4.8D).

Lastly, the ratio of E2 to 11-KT showed no significant main effect of genotype [$F(3) = 2.658$, $P = 0.055$]. There was a significant main effect of sex [$F(1) = 2153.817$, $P < 0.001$] but no interactions between genotype and sex [$F(3) = 1.188$, $P = 0.321$]. Post-hoc analyses reveal a significantly higher ratio of E2/11-KT in females compared to males ($P < 0.05$) (Fig. 4.8E).

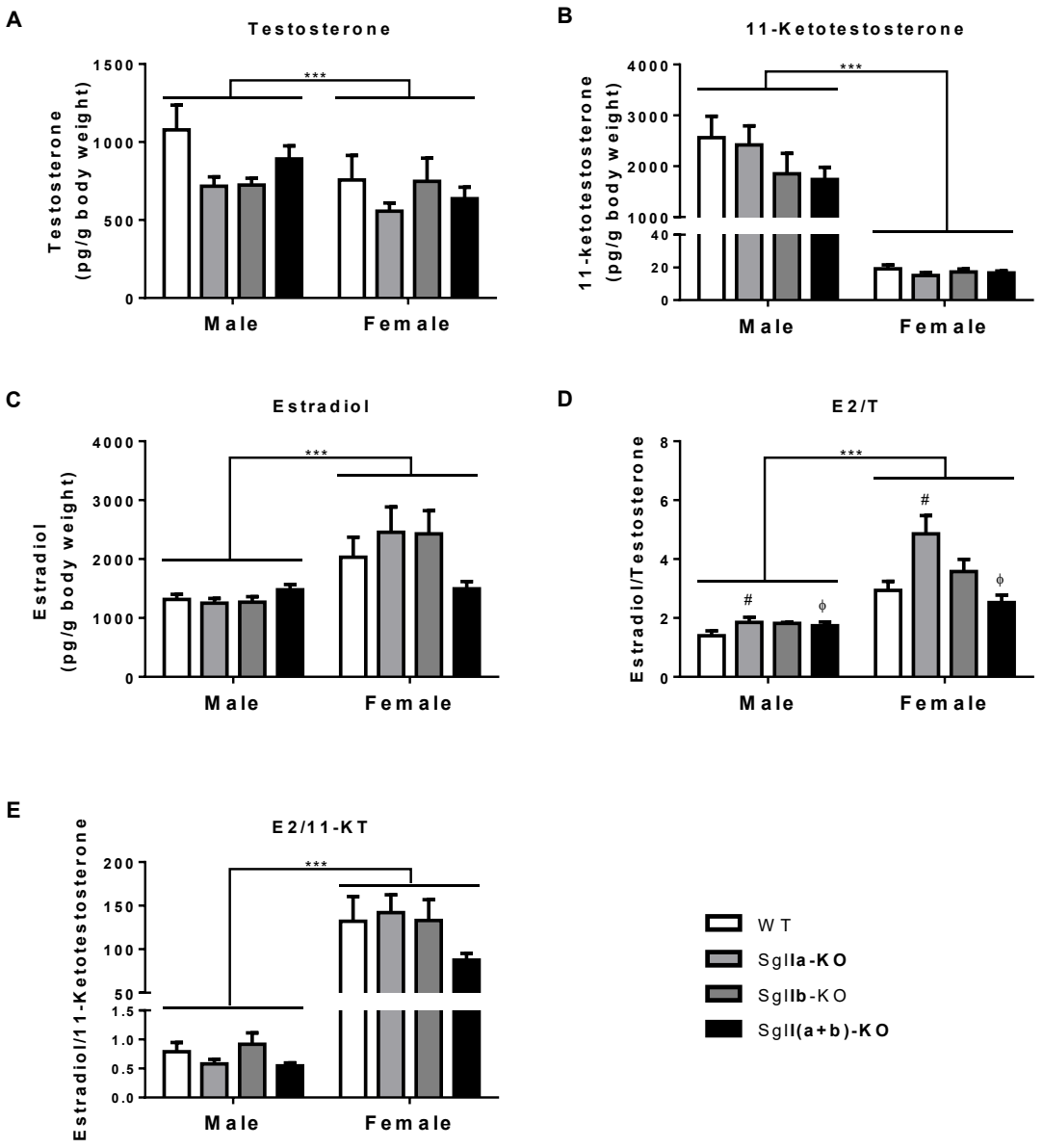

Figure 4.8 Effects of SgII knockout on whole-body steroid levels. (A) testosterone, (B) 11-ketotestosterone (C) estradiol (D) estradiol/testosterone (E2/T) ratio, (E) estradiol/11-ketotestosterone (E2/11-KT) ratio. Means + SEM are presented (n = 8-10). When no interactions are present but there is a main effect of sex (A, B, C, E) then lines above the graph indicate significant differences compared between sexes (*** $P \leq 0.001$). When no interactions are present but there is a main effect of sex and genotype (D) then pound (#) and phi (ϕ) represent significant differences between genotypes. #$P \leq 0.01$ compared to WT; ϕ $P \leq 0.01$ compared to SgII(a)-KO.

4.4 Discussion

4.4.1 Knockout of SgII disrupts reproductive output

Knockout of either *scgIIa* or *scgIIb* caused a significant reduction in the spawning success of within line breeding pairs of zebrafish. This negative effect was further amplified in double knockout animals. Interestingly, spawning success increased back to WT control levels when SgII-KOs were paired with WTs except when SgII(a+b)-KO females were paired with WT males. This is indicative of a female-specific effect. These results clearly indicate the importance of *scgII* in zebrafish reproduction. It has been demonstrated in fish that gene modification of pituitary hormones also leads to impaired reproduction. Zebrafish and medaka *lhb*$^{-/-}$ females paired with WT males are unable to spawn indicating that they are infertile (Chu et al., 2014; Takahashi et al., 2016; Zhang et al., 2015b), although, homozygous or heterozygous *lhb* receptor (*lhr*) KO zebrafish can spawn with WT males but the number of embryos produced is decreased slightly (Chu et al., 2014). On the other hand, male *lhb*$^{-/-}$ or *lhr*$^{-/-}$ zebrafish and *lhb*$^{-/-}$ medaka are fertile (Chu et al., 2014; Takahashi et al., 2016; Zhang et al., 2015a; Zhang et al., 2015b). Similarly, f*shb*$^{-/-}$ male and female zebrafish were fertile (Zhang et al., 2015b) whereas *fshb*$^{-/-}$ male medaka were fertile and females were infertile (Takahashi et al., 2016). Genetic modifications of genes further upstream in the hypothalamic-pituitary gonadal (HPG) axis reveal no major changes in reproductive capacity or phenotypes. For example, no changes were observed in the fecundity, fertilization rate or survival of progeny of *gnrh3*$^{-/-}$ zebrafish (Spicer et al., 2016). This result was surprising since cell ablation of Gnrh3, the hypophysiotropic Gnrh form in zebrafish, lead to infertility in *gnrh3*$^{-/-}$ females (paired with WT males) and a reduced reproductive capacity and fecundity in *gnrh3*$^{-/+}$ fish (Abraham et al., 2010). Spicer et al. (2016) suggested that these differences could be explained by compensatory mechanisms that exist to mitigate the effects of the lack of *gnrh3* and that these mechanisms are activated early in development. This compensation hypothesis is further supported by the knockout of the zebrafish kiss system (*kiss1*$^{-/-}$*, kiss2*$^{-/-}$*, kiss1*$^{-/-}$*;kiss2*$^{-/-}$ *and kissr1*$^{-/-}$*;kissr2*$^{-/-}$) which are all fertile (Tang et al., 2015). These previous knockout studies challenge conventional ideas that GnRH and kisspeptin are essential for reproduction across species. Instead, they provide evidence that different mechanisms have evolved for the neuroendocrine control of reproduction between mammals and fish. Also supported is that zebrafish appear to be able to compensate for gene silencing at the level of the brain as opposed to the pituitary or gonads (Spicer et al., 2016). For the SgII-KOs it is possible that the reduced levels of *gnrh3* and gonadotropins leads to a reduction in courtship behaviour and ultimately spawning success.

4.4.2 Mutation of SgII reduces courtship behaviour

Extensive video analysis of SgII-KO spawning trials revealed a decrease in frequency and duration of chase, tail-nose, encircle and quiver behaviours in SgII(a+b)-KO within line crosses, as well as the encircle behaviour in SgII(a+b)-KO females paired with WT males. Only two other gene silencing studies in zebrafish have examined courtship behaviour similarly to this study, likely due to the major challenges that exist to obtain and analyze such data. The first study knocked out the androgen receptor (AR) gene (*ar*) and assessed male courtship-like behaviours in fourteen mating pairs (Yong et al., 2017). Androgen receptors are widely expressed in the brain regions responsible for mating behaviours (Gorelick et al., 2008). As predicted, AR-KO resulted in impaired male courtship behaviours during mating trials (Yong et al., 2017). These *ar*$^{-/-}$ males were slower to initiate courtship and exhibited fewer chases, tail-noses, zig-zags and quivers; however, encircling was not significantly different between genotypes suggesting that it may not be under androgenic control (Yong et al., 2017). The second study consisted of knocking out the prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) receptor (*or114-1*$^{-/-}$) in zebrafish. The female reproductive hormone, $PGF_{2\alpha}$, facilitates ovulation and spawning (Kobayashi et al., 2002; Stacey and Goetz, 1982) but it also a sex pheromone that induces male reproductive behaviours in some fish (Yabuki et al., 2016a). The *or114-1*$^{-/-}$ males encircled and touched females less frequently and had shorter chase durations although the number of courtship bouts were not significantly different between *or114-1*$^{-/-}$ and WT males. Like previous studies have shown, not all behavioural components related to courtship are completely disrupted in all knockout individuals and in fact most behaviours are still exhibited but durations and frequencies are reduced. Furthermore, even though these behaviours were severely disrupted in the double mutant SgII lines, a very small percentage were still able to spawn. Taken together, these data provide support that multiple mechanisms and genes are involved in regulating courtship behaviour. My study as well as Yabuki (2016a) and Yong (2017), show that behaviour can be modulated through genetic changes. More importantly, my data suggests that SgII-KO also negatively impacts male fish.

4.4.3 Knockout of SgII does not affect oogenesis

Morphological and histological analysis showed that ovaries from all adult SgII mutant lines as well as WTs contained a full range of developing follicles from the primary growth (PG) stage (stage I) to full grown (FG) stage (stage III) indicating that SgII-KO did not affect ovarian development. This is consistent with the fact that these fish were able to spawn, although, at a reduced capacity. Similar effects were observed in *lhb*$^{-/-}$ KO zebrafish (Takahashi et al., 2016; Zhang et al., 2015b) and medaka

(Takahashi et al. 2016) lines in which there were no observable effects on ovarian development, but it did cause infertility due to failed oocyte maturation and ovulation. Furthermore, Chu et al. (2014) found that ovary size and GSI were increased in *lhr*$^{-/-}$ KO adult females and the ratio of PG stage follicles decreased whereas FG follicles increased, indicating that only ovulation was affected in the *lhr*$^{-/-}$ KO fish. Folliculogenesis was unaltered in *gnrh3*$^{-/-}$ zebrafish (Spicer et al., 2016) which was surprising given that Gnrh3 cell ablation led to incomplete oogenesis in which fish lacked oocytes past the vitellogenic (stage III) stage (Abraham et al., 2010). In medaka, *gnrh1*$^{-/-}$ (the hypophysiotropic form of Gnrh) and *lhb*$^{-/-}$ KO females had fully developed ovaries but none of them ovulated (Takahashi et al., 2016). In contrast, *fsh6*$^{-/-}$ in medaka caused severe disruption of folliculogenesis with no FG or late vitellogenic ovum observable (Takahashi et al., 2016). Similarly, the ovaries from *fshb*$^{-/-}$ zebrafish were blocked at the PG- pre-vitellogenic (PV) transition and delayed development into the vitellogenic growth; however, follicles could still develop into FG stage eventually after a delay (Zhang et al., 2015b). Interestingly, in fshb receptor (*fshr*$^{-/-}$) KO females, ovarian growth and follicle activation were completely halted, and all females eventually turned into males probably by sex reversal (Zhang et al., 2015a). These studies show that in females, Fsh is responsible for follicle growth whereas Lh is responsible for oocyte maturation and final ovulation (Chu et al., 2014; Zhang et al., 2015a; Zhang et al., 2015b). The SgII-KO female ovaries developed in a similar fashion to the *lhb*$^{-/-}$ zebrafish. The ratio of follicle stages and adult ovaries contained all stages therefore folliculogenesis was not affected. However, both *lhb*$^{-/-}$ and SgII-KO females were infertile or subfertile, respectively. Based on previous studies I hypothesize that SgII-KO leads to failed ovulation and thus impaired spawning. Future work will assess ovulation in the SgII-KO mutant fish to directly test this hypothesis.

4.4.4 Knockout of SgII affects sperm development

Morphological and histological analysis of SgII-KO testes showed that all stages of sperm cells including spermatogonia, spermatocytes, spermatids and spermatozoa were present; however, SgIIa-KO and SgII(a+b)-KO fish had fewer spermatozoa compared to WT suggesting that spermatic development was affected. Initially I hypothesized that the reduction in sperm production may have contributed towards the decreased fertilization success of within line breeding pairs; although it is unlikely given that *in vitro* fertilization rates were unaltered in any SgII-KOs. These results are comparable to other gene knockout studies. All stages of sperm cells, as described previously, were present in *fshb*$^{-/-}$, *lhb*$^{-/-}$ and *lhr*$^{-/-}$ zebrafish testes indicating normal development (Chu et al., 2014; Zhang et al., 2015b). However, Chu et al. (2014) observed a significant increase in the number of

spermatocytes in *lhb*$^{-/-}$ zebrafish suggesting that Lh signaling may regulate spermatogenesis, especially the process from meiosis stage to spermiogenesis (Chu et al., 2014). There was also a significant decrease in the GSI of *lh*b$^{-/-}$ but not in *lhr*$^{-/-}$ mutants (Chu et al., 2014). Double knockout of *fshb* and *lhb* (*fshb*$^{-/-}$;*lh*b$^{-/-}$) led to all male offspring (Zhang et al., 2015b). Knockout of *fshr*$^{-/-}$ resulted in a delayed initiation of spermatogenesis although the testis of adults showed normal development (Zhang et al., 2015a). Furthermore, *fshr*$^{-/-}$ zebrafish that spawned had normal spermatogenesis in the testis whereas those that didn't contained fewer spermatocytes and more spermatogonia (Zhang et al., 2015a). The *gnrh3*$^{-/-}$ zebrafish did not have altered testis development and all stages of sperm cells were present (Spicer et al., 2016), although, this contrasts to cell ablated *gnrh3*$^{-/-}$ zebrafish that resulted in an all female population suggesting that *gnrh3* is involved in sex determination (Abraham et al., 2010). In medaka, no differences were observed in testis development in any knockout lines tested (*gnrh1*$^{-/-}$, *lhb*$^{-/-}$ and *fshb*$^{-/-}$) (Takahashi et al., 2016). These studies show that in males Fsh and Lh signalling overlap but Fsh mainly plays a regulatory role in the initial stages of spermatogenesis, while Lh is involved in the final stages of maturation (Chu et al., 2015; Schulz et al., 2010). The mechanisms by which SgII affects spermatogenesis are still unknown, however, the effects of SgII-KO somewhat resemble those of the *lhb*$^{-/-}$ zebrafish. Future SgII-KO studies could examine sperm function by measuring sperm volume, density, motility and velocity.

4.4.5 Knockout of SgII does not alter whole body sex steroid hormones

Sex steroids are essential for spermatogenesis, spermiation, sexual differentiation and sexual maturation (Schulz et al., 2010). Sex steroids were analyzed in this study to further determine the possible mechanisms behind the fertility defects seen in SgII-KO fish. Whole body T, 11-KT and E2 levels were unaltered in any SgII-KO lines, however there was a significant increase in the E2/T ratio in SgIIa-KO males and females. The balance between sex steroid hormones plays a crucial role in the masculinization or feminization of fish (Pradhan and Olsson, 2015). At this point the mechanisms behind the increase in E2/T are unknown. In previous studies, serum T levels were increased in *lhb*$^{-/-}$ male zebrafish whereas 11-KT and T were decreased in *fsh*b$^{-/-}$;*lh*b$^{-/-}$ and *fsh*$^{-/-}$*r*;*lhr*$^{-/-}$ lines (Chu et al., 2015). Given that sex steroid hormones were for the most part unaltered in SgII-KOs, it is unlikely that they are contributing to the observed fertility defects. It is also possible that whole body estimates may not detect subtle changes in sex steroids therefore plasma samples should be used to confirm these findings.

4.4.6 Conclusion

In this study, I have demonstrated for the first time in any animal that SgII plays a critical role in courtship behaviour and reproductive success in zebrafish. Moreover, the reproductive phenotypes observed in the SgII-KO lines are similar to those of the *lhb*$^{-/-}$ zebrafish suggesting that SgII may negatively impact Lh. These data also support the hypothesis that SgII is a reproductive regulator.

Chapter 5: Injection of SN and hCG rescues spawning in SgII-mutant zebrafish

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[1]Contributed to original ideas, conducted breeding experiments, performed data analysis and manuscript preparation

[2]Synthesized and purified zebrafish SNa and SNb peptides

[3]Helped with injection experiments

[4]Analyzed courtship behaviours of zebrafish (video analysis)

[5]Developed and provided SgII mutant zebrafish and collaborated on this study

[6]Contributed to original ideas and revised the manuscript

5.1 Introduction

The secretoneurins (SN) are conserved 31-43 amino acid vertebrate neuropeptides derived from enzymatic processing of the secretogranin-II (SgII) precursor (Trudeau et al., 2012). Tetrapods such as mammals and amphibians have one SgII gene and produce one SN peptide. Teleosts such as goldfish, zebrafish, and medaka have two genes, *scg2a* and *scg2b*. These SgII proteins are processed by prohormone convertases to produce several peptides including SNa and SNb, in teleosts, respectively (Mahata et al., 1993b; Trudeau et al., 2012; Zhao et al., 2009c). In zebrafish, the SNa and SNb peptides are 34 and 31 amino acids long, respectively.

To date, the best described action of SN in a neuroendocrine system is the positive regulation of luteinizing hormone (Lh) in the anterior pituitary (Trudeau et al., 2012). Intraperitoneal (i.p.) injection of SN in goldfish in combination with a dopamine D2 receptor blocker indicated that SN could stimulate Lh release *in vivo* (Blazquez et al., 1998; Zhao et al., 2006b). Dopamine is an inhibitor of Gnrh- and GABA-induced Lh release in goldfish and thus it is necessary to pre-treat with dopamine antagonists to observe Gnrh-induced Lh secretion (Peter et al., 1986; Trudeau, 1997; Trudeau et al., 2000). Similarly, *in vitro* studies have established that SN stimulates Lh production and release (Zhao et al., 2006a; Zhao et al., 2006b; Zhao et al., 2009a; Zhao et al., 2010a) in dispersed pituitary cells of the goldfish model and from the mouse LβT2 gonadotrope cell line (Zhao et al., 2011) providing support that SN is an important regulator of Lh release in vertebrates. Recently, *in vitro* studies in the orange spotted grouper have revealed that SNa and SNb significantly elevate gonadotropin-releasing hormone 1 (*gnrh1*) and 3 (*gnrh3*) transcript levels in hypothalamic fragments, and follicle-stimulating hormone beta (*fshb*) and *lhb* levels in pituitary cells (Shu et al., 2018). In addition, peripheral injection of SNa or SNb stimulates *gnrh1*, *gnrh3*, *fshb* and *lhb* levels *in vivo* (Shu et al., 2018). In the electric fish, i.p. injection of goldfish SNa modulated the rate of electric organ discharge that signals arousal and dominant/subordinate status in a context-dependent manner (Pouso et al., 2015). Secretoneurin also increased pacemaker nucleus firing rate in brainstem slices *in vitro*, partially mimicking effects *in vivo* (Pouso et al., 2015). Therefore, SN is neuromodulatory through either neuroendocrine and/or endocrine mechanisms at the level of the electric fish pacemaker nucleus. Moreover, injection of SN into the third brain ventricle of female goldfish increased feeding and locomotory behaviours as well as increased the expression of hypothalamic neuropeptide Y and decreased hypothalamic cocaine- and amphetamine-regulated transcript at 2h and 5h post-injection, respectively (Mikwar et al., 2016). This study has raised important questions about major functions regulated directly by SN or indirectly by SN-responsive neurons.

Secretoneurin is one of a few identified peptides produced from the SgII precursor; however, it is the only fragment that has been studied for bioactivity. My previous results (chapter 4) demonstrate that SgII-KO fish have reduced spawning success and courtship behaviour indicating that SgII is required for optimal reproduction. In this chapter, I investigated the involvement of the SN peptides in regulating reproductive processes. I hypothesized that SN is the bioactive peptide and is acting as a reproductive regulator. I performed i.p. injections, using zebrafish SNa and SNb peptides, in the SgII(a+b)-KO fish and predicted that injection of SN would rescue spawning and courtship behaviour in the SgII(a+b)-KO fish.

5.2 Materials and Methods

5.2.1 Peptide synthesis

The zebrafish SNa amino acid sequence is TNENAEEQYTPQKLATLQSVFEELSGIASSKTNT while the zebrafish SNb amino acid sequence is ATEDLDEQYTPQSLANMRSIFEELGKLSAAQ. Peptide samples were prepared using an Intavis Multipep Peptide Synthesizer (Intavis Bioanalytical Instruments AG), using an Fmoc-tBu strategy. N,N,N',N'-Tetramethyl-O-(1H-benzotriazol-1-yl)uronium hexafluorophosphate (HBTU) and N-Methylmorpholine (NMM) were used as the coupling reagent system. All Fmoc protected amino acids were purchased from Chempep Inc. All other chemicals were purchased from Sigma Aldrich. Peptides were purified by semi-preparation HPLC (Varian ProStar) using Phenomenex Luna C-18 column (3 um 10X250 mm). The purity of each peptide was analyzed using high performance liquid chromatography (HPLC) with Jupitor analytical C-18 column and diode array detector. The purity of each peptide used in the experiment is over 95% based on absorbance spectrums. Peptide sequences were confirmed using LC-Orbitrap tandem mass spectrometry (Agilent Technologies, Santa Clara, CA, USA). All peptides were lyophilized and stored as trifluoroacetic salt at -80°C.

5.2.2 Pairwise spawning trials and SN injections

The reproductive capabilities of SgII-KO zebrafish following intraperitoneal (i.p.) injection of SNa and SNb were assessed via within line mating crosses. Two weeks prior to the spawning experiment, fish were separated by sex into 3L tanks at a density of 4 fish/litre. The night before each experiment, fish were transferred to 1L breeding tanks. Each tank contained one male and one female, separated overnight with an insert containing a divider. The morning of the experiment, both

male and female fish were anaesthetised with Tricaine (3-amino benzoic acid ethyl ester) according to standard zebrafish husbandry procedures (Westerfield, 2000) then weighed using an analytical balance. Intraperitoneal injection was performed into the abdominal cavity, posterior to the pelvic girdle. Fish were injected using a 32-gauge Hamilton syringe with either Ringer's solution (pH 7), human chorionic gonadotropin (hCG) (50 IU/g body weight) (Millipore, cat# 230734-1MG) zebrafish SNa peptide (4 µg/g body weight) or zebrafish SNb peptide (4 µg/g body weight). Fish were immediately returned to their breeding tanks to recover for 20-30 minutes, and then moved into the recording arena. Behaviour was monitored using a Zebracube system (ViewPoint Behavior Technology) consisting of a sound proof chamber with an infra-red camera [ViewPoint high sensitivity camera (Dragonfly2, DR2-HIBW, IEEE-1394; 60 frames/Sec)]. Tanks were filmed from above. Filming was started then dividers were removed. All filming began within one hour of first light (9 AM) and within 45 minutes of injection. Courtship behaviour was filmed for 6 hours at which point the absence or presence of a clutch was noted. Clutches were placed in E3 medium and fertilization rate was assessed. Embryos were kept in an incubator (28°C) until 24 hours post fertilization (hpf) at which point survival rate was assessed.

5.2.3 Courtship behaviour

Zebrafish exhibit an elaborate pre-mating ritual consisting of a series of behaviours to attract mates (Darrow and Harris, 2004). Several zebrafish courtship behaviours exist (see chapter 4) although only quiver, a rapid tail oscillation against the female's side, and egg lay (oviposition) were assessed in this study. Our previous results (chapter 4) showed that frequency and duration of zebrafish courtship behaviours are correlated ($r=0.9266$; $P<0.0001$) therefore only frequency was assessed here. Behavioural analyses were conducted using BORIS (Behavioral Observation Research Interactive Software, version 6.3), an opensource software for video coding, by several blind observers. Only the first 2 hours of each 6-hour experiment were analyzed.

5.2.4 Data analysis

Online freeware from GraphPad QuickCalcs was used for statistical analysis of the spawning success data. Spawning success of mating pairs were compared to the WT controls injected with Ringer's (hereafter referred to as WT controls) using Fisher's exact test (one-tailed) (http://graphpad.com/quickcalcs/contingency1/). Data are reported as percentages. All other statistical analyses were performed using SigmaPlot 11.0 software (Systat Software, Inc., Chicago, IL).

Normality and homoscedasticity were tested using a Shapiro-Wilk test and Levene's test, respectively. Two-way analysis of variance (ANOVA) followed by a Holm-Sidak post-hoc test was used to analyze all other data.

5.3 Results

5.3.1 Injection of hCG and SN rescues spawning success but not courtship behaviours of SgII(a+b)-KO zebrafish

Double KO zebrafish were i.p. injected with either SNa or SNb to determine if SN can rescue spawning success and courtship behaviour. Similarly, WT animals were injected with SNa and SNb to determine if SN could enhance spawning. Human chorionic gonadotropin (hCG), a hormone that mimics the action of Lh on binding and activating the Lh receptor in fish (Chu et al., 2014), was used as a positive control to induce spawning in our WTs but also to determine if the gonads of SgII-KO fish are sensitive to Lh. Under the conditions of handling, anaesthesia and i.p. injection, sexually naïve WT males paired with WT females successfully spawned in 47% of trials following injection of Ringer's solution (Fig. 4.1A). Spawning success was significantly increased to 83% in hCG-injected WTs ($P = 0.0013$) (Fig. 4.1A). Zebrafish SNa and SNb increased spawning success equally in WTs to 61%, although this was not statistically different from WT controls ($P = 0.2062$) (Fig. 5.1A). Spawning success in SgII(a+b)-KO pairs injected with Ringer's was 11% which is comparable to non-injected SgII(a+b)-KO at 6% and significantly lower than WT controls ($P = 0.0006$) (Fig. 5.1A). Injection of hCG in SgII(a+b)-KO fish significantly increased spawning success to 38% compared to Ringer's injected SgII(a+b)-KO fish ($P = 0.0063$), making this similar and not significantly different from WT controls ($P = 0.2662$) (Fig. 5.1A). Secretoneurin a increased spawning success in SgII(a+b)-KO fish almost 3-fold to 30%. Injection of SNa in SgII(a+b)-KO fish was statistically significant compared to SgII(a+b)-KO ($P = 0.0403$) and not statiscitally different compared to WT fish injected with saline control ($P = 0.0973$) (Fig. 5.1A). The spawning success of SNb injected SgII(a+b)-KO fish was 8% which was not different from Ringer's injected SgII(a+b)-KO fish ($P = 0.5$) but this was significantly lower compared to WT controls ($P = 0.0002$) (Fig.5.1A).

In the video analyses of courtship behaviour, there was a clear significant inhibitory effect of genotype [$F(1) = 10.845$, $P = 0.001$] on the number of quivers fish were performing. The SgII(a+b)-KO fish performed fewer quivers than WTs ($P < 0.05$). However, there were no significant main effects from injection [$F(3) = 2.269$, $P = 0.082$] or interactions between genotype and injection [$F(3) = 1.322$,

P = 0.269] (Fig. 5.2A). Similar results were also obtained in the latency to quiver. There was a significant inhibitory main effect from genotype [F(1) = 17.652, P < 0.001]. The SgII(a+b)-KO fish took longer to perform the first quiver than WTs (P < 0.05). However, there were no significant main effects from injection [F(3) = 0.299, P = 0.826] or interactions between genotype and injection [F(3) = 1.703, P = 0.170] (Fig. 5.2B).

There were no significant main effects from genotype [F(1) = 3.275, P = 0.073] or injection [F(3) = 1.338, P = 0.266] on the fecundity (# embryos/clutch). Furthermore, no interactions between genotype and injection were observed [F(3) = 0.964, P = 0.413] (Fig. 5.1B). For the fertilization rate, there were no significant main effects from genotype [F(1) =1.162, P = 0.284], injection [F(3) = 0.646, P = 0.587] or interactions between genotype and injection [F(3) = 0.201, P = 0.896] (Fig. 5.1C). There were significant negative effects from genotype [F(1) =6.244, P = 0.014] on embryo survival. Generally, SgII(a+b)-KO fish had a lower survival than WTs (P < 0.05). However, there were no significant effects of injection [F(3) = 2.650, P = 0.053] or interactions between genotype and injection [F(3) = 2.656, P = 0.0.52] (Fig. 5.1D).

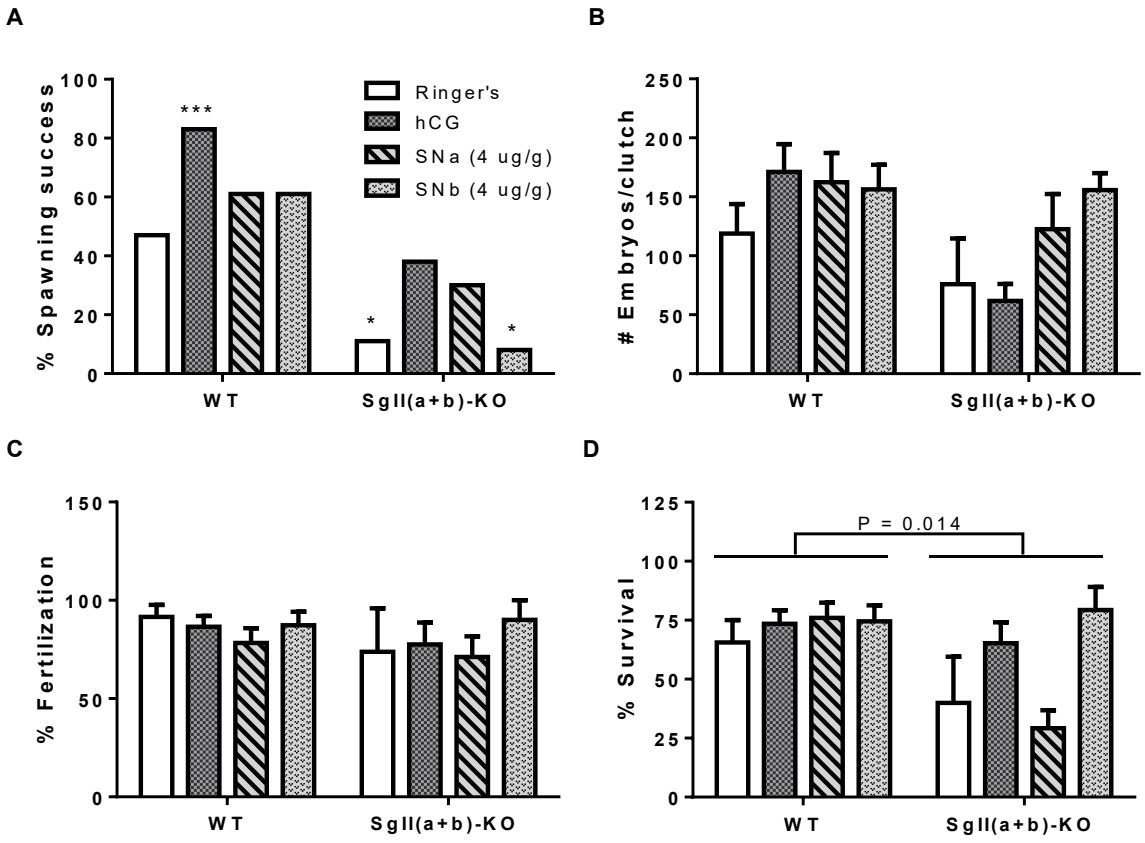

Figure 5.1 Spawning success, fecundity (# embryos/clutch), fertilization and survival of embryos produced during zebrafish pairwise spawning following i.p. injections. (A) % spawning success of WT and SgII(a+b)-KO mattings. Mean of 28-40 spawning trials are presented. Significant differences were determined using Fisher's exact test. Asterisks denote a significant difference from the wild-type controls; *** $P \leq 0.001$; * $P \leq 0.05$. (B) fecundity or the number of embryos produced per spawning couple. Mean +SEM of 3-30 successful spawnings are presented. (C) Mean + SEM % fertilization and (D) % embryo survival of mating crosses (n=3-30). Significant differences were determined using a two-way ANOVA followed by a Holm-Sidak post-hoc test. Lines connecting different bars represent the comparison between WT and SgII(a+b)-KO fish.

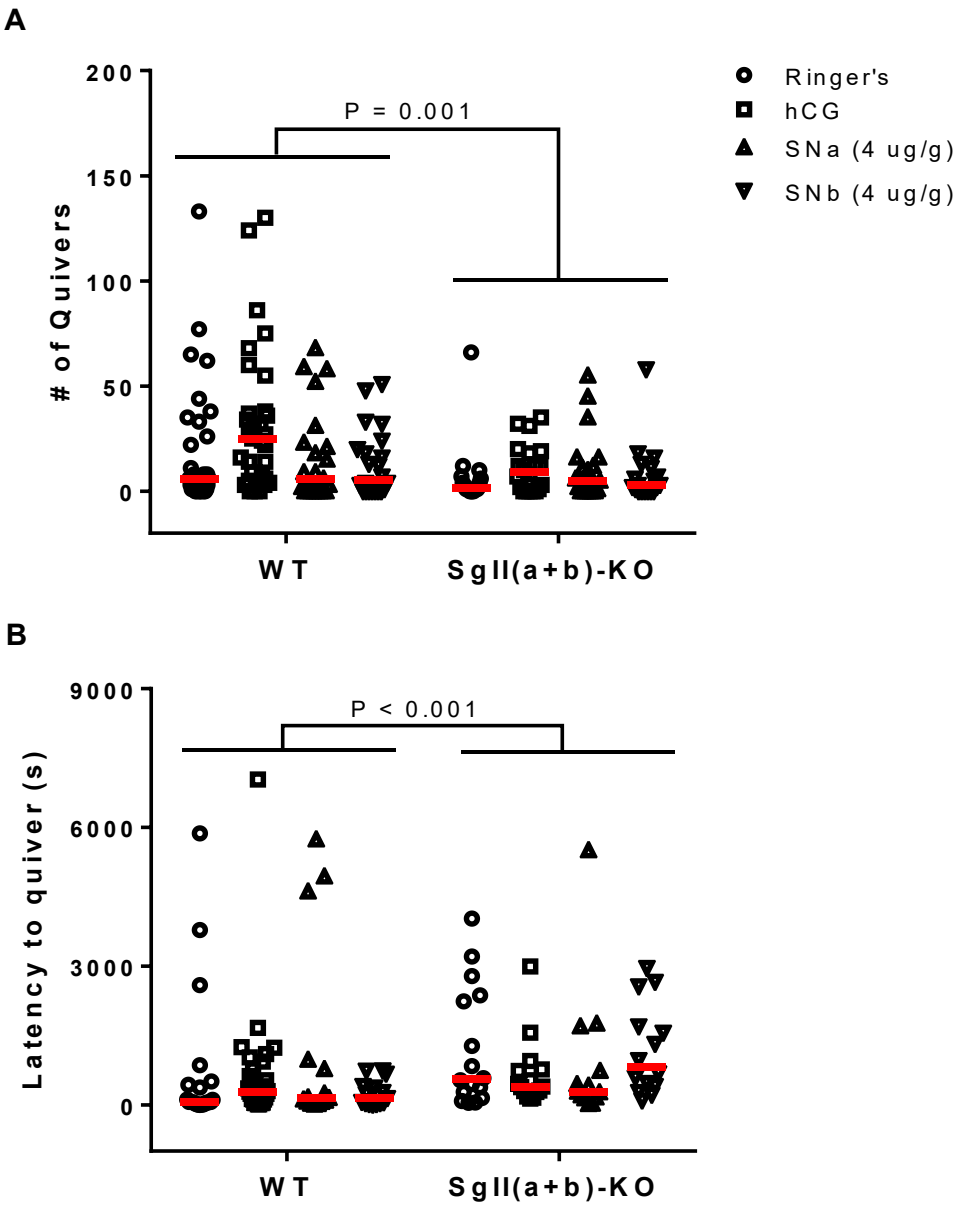

Figure 5.2 Number of quivers and latency to quiver of zebrafish during pairwise spawning following i.p. injections. (A) number of quivers of WT and SgII(a+b)-KO matings (n=20-33). (B) latency to quiver (s) (n=13-29). Red horizontal lines represent median values. Significant differences were determined using a two-way ANOVA followed by a Holm-Sidak post-hoc test. Lines connecting different bars represent the comparison between WT and SgII(a+b)-KO fish.

5.4 Discussion

My previous research shows that SgII-KO significantly disrupts spawning and courtship behaviours in zebrafish (chapter 4) providing evidence that SgII is required for reproduction. In this study, the spawning success of WT controls was about 15% lower compared to the WT within line crosses (chapter 4). It is likely that anesthesia, handling and i.p. injection stressed the animals, thus reducing spawning success. Surprisingly, i.p. injection did not significantly impact the spawning success of SgII(a+b)-KO fish since non-injected fish spawned 6% whereas in this study the Ringer's injected fish spawned 8% of the time. Intraperitoneal injection of hCG significantly elevated spawning success from 11% to 38% in SgII(a+b)-KO zebrafish confirming that SgII(a+b)-KO mutant gonads are sensitive to Lh. Since hCG induces oocyte maturation and ovulation leading to spawning in several fish species (Fernandes et al., 2013; Mollah and Tan, 1983), our results indicate that hormonal induction of ovulation and spawning is possible in SgII(a+b) mutant fish. Previous studies also show that hCG stimulates 11-ketotestosterone production in zebrafish testis (Fraz et al., 2018) and the plasma levels of free 17α, 20β-dihydroxy-4-pregnen-3-one and testosterone in female goldfish (Moriwaki et al., 1991). Injection of SNa increased spawning success in SgII(a+b) mutants by almost three-fold and this was statistically different from Ringer's injected mutants. No effect of SNb injection was observed. It is possible that SNb may also regulate these processes but perhaps the concentration used in this study was not optimal. Several lower concentrations of SN were injected in preliminary experiments (data not presented), however no SNb injections elevated spawning success in SgII(a+b)-KO mating trials. Higher concentrations of both SNa and SNb should be tested to determine if they are able to fully or partially rescue spawning success, respectively. The spawning success of SgII(a+b)-KO fish injected with hCG and SNa were not statistically different compared to WT controls indicating that spawning was rescued. These results also demonstrate the severe reproductive impairment in the double mutants given the difficulties to increase spawning to control levels even with hCG.

This is the only study to examine the sexual behaviour following SN injection. Surprisingly, neither SN nor hCG injection rescued quiver behaviour or altered the latency to quiver in SgII(a+b)-KO fish. Hormonal induction of reproduction may require more time, and we may have missed the rescued behaviours by only analyzing the first two out of six hours. Similarly, only quiver behaviour was assessed but it is plausible that other behaviours were altered. Furthermore, the many observers led to large variances in the data which may have masked subtle differences. I am hoping that new

behavioural software we are attempting to develop can be employed in the future to aid in analyzing our full data sets in a consistent manner.

No differences were observed in the fecundity (# embryos/clutch) or fertilization rate of any spawnings. However, this data should be interpreted cautiously given the low sample sizes of some groups. For example, Ringer's and SNb injected SgII(a+b)-KO fish only spawned 4 and 3 times, respectively. Survival was decreased in SgII(a+b)-KO fish compared to WT's suggesting that although SNa and hCG injection rescues spawning, the resulting embryos are similar to SgII(a+b)-KO fish injected with Ringer's and are clearly of suboptimal quality.

These data provide the first functional evidence in zebrafish that SNa has a stimulatory effect on reproduction. Blazquez et al. (1998) performed i.p injections in sexually regressed female goldfish and observed an increase in serum Lh levels in fish injected with the dopamine receptor antagonist domperidone in combination with SNa. Comparable results were observed in the orange spotted grouper. Both SNa and SNb peripheral injection stimulated *gnrh1*, and *gnrh3* in the hypothalamus, and *fshb* and *lhb* in the pituitary, *in vivo* (Shu et al., 2018). Results from *in vitro* studies in the orange spotted grouper were similar to *in vivo* results (Shu et al., 2018). Shu et al. (2018) suggest that SN can act directly at the pituitary level or indirectly through the action on Gnrhs in the brain. This is also supported by our findings here and in chapter 2. Alternatively, these results could suggest that SN may not be the only bioactive SgII-derived peptide capable of regulating reproductive processes. Besides SN, only two other peptides have been identified in mammalian SgII, which are EM66 and manserin (Fischer-Colbrie et al., 1995; Yajima et al., 2004). Flanking SN at the C-terminus is EM66, which is well conserved in human and other tetrapods but is not at all conserved in fish (Anouar et al., 1998; Zhao et al., 2009c). Manserin, the most recently discovered peptide processed from SgII, was first investigated in the rat anterior lobe of the pituitary and hypothalamus, suggesting that manserin might have a neuroendocrine role (Yajima et al., 2004). Manserin is also not conserved in fishes. No specific biological activity has yet been reported for either EM66 or manserin. Regardless, our data provides us with further evidence that SgII/SN regulates reproduction in vertebrates.

Chapter 6: General discussion and conclusions

Kimberly Mitchell[1]

[1]Contributed to original ideas and wrote the manuscript

6.1 Overview of the thesis

The research presented in this thesis aimed to elucidate the role of secretogranin-II (SgII) in the reproductive functions of zebrafish (*Danio rerio*). A review of the literature on the functions of SgII/secretoneurin (SN) provided support that they are involved in this process as they have been shown to stimulate the synthesis and release of luteinizing hormone (Lh) (Chapter 1). However, there was a clear absence of data regarding the involvement of the SgII/SN system in major reproductive processes such as ovulation, sperm release or essential reproductive behaviours. The hypothesis tested here was that SgII regulates reproductive processes in zebrafish. Therefore, my study focused on the reproductive physiology and behaviours that were altered following SgII-KO. Based on the data presented in this thesis, I provide evidence that SgII is acting in a yet unknown mechanism to regulate spawning, courtship behaviour and genes that positively regulate reproductive processes.

6.2 Secretogranin-II is distributed throughout the brain and pituitary

The first objective of my study was to characterize the spatial and temporal distribution of *scg2a* and *scg2b* transcripts in larvae and adult zebrafish brain and pituitary (chapter 2). I found that both *scg2a* and *scg2b* were expressed early in development, as has been shown by Tao et al. (2018) and continued to increase until adulthood.

Using *in situ* hybridization techniques on sections of adult zebrafish heads, I compared the spatial distribution patterns of expression of *scg2a* and s*cg2b*; this is the first study to do so in adults. Both *scg2a* and *scg2b* were located throughout the olfactory bulb. The olfactory bulb is the primary processing center of odor information and sends neuronal projections to the telencephalon (TEL) and hypothalamus (HYP). Given the localization of *scg2* throughout the OB it is conceivable that *scg2* may regulate behaviours such as fear conditioning, feeding and, most importantly, reproduction. Light to moderate staining was also observed in the dorsal and ventral telencephalic areas. The dorsal areas in which *scg2* was observed have been implicated in spatial, relational, temporal memory systems as well as conditioning, learning and memory (Broglio et al., 2010; Portavella et al., 2004) whereas the ventral telencephalic areas are involved in regulating reproductive physiology (Tyler and Gorski, 1980). Regions such as the nucleus preopticus (NPO) and the HYP of WT zebrafish were densely populated with *scg2a* and *scg2b* containing cells. Similarly, the most prominent staining of SNa-immunoreactivity (ir) was in the NPO of goldfish (Canosa et al., 2011).

Cells expressing *scg2a* and *scg2b* were detected in known hypophysiotropic brain regions. I observed *scg2b* in the dorsal and ventral habenular nucleus regions of the brain where kisspeptin 1 (*kiss1)* (Ogawa et al., 2012; Servili et al., 2011) and tachykinin 3a (*tac3a*) (Biran et al., 2012) were previously shown to be expressed in zebrafish. Both *scg2a* and *scg2b* expressing cells were detected throughout the periventricular HYP. In goldfish, neuropeptide Y (NPY) is expressed in the periventricular regions of the HYP and is involved in appetite regulation (Yokobori et al., 2012) and stimulates Lh release from pituitary fragments in the goldfish (Peng et al., 1993a). As previously mentioned, I detected *scg2a* and *scg2b* in the TEL, NPO and HYP. Similarly, Melanin-concentrating hormone 2 (MCH2) is expressed in the telencephalic area, NPO, HYP, locus coeruleus and vagal motor nerves, and has been shown to regulate food intake in zebrafish (Berman et al., 2009). Corticotropin-releasing factor, urotensin I and CRF-binding protein are also expressed in the NPO, periventricular HYP and tectal regions in zebrafish supporting stress and behavioural functions (Alderman and Bernier, 2007). The overlapping patterns of expression of *scg2* with previously identified hypophysiotropic brain areas suggests that *scg2* could be involved in similar functions such as reproduction, appetite regulation and stress.

In my study, I observed that *scg2a* expression was abundant in the pituitary (PIT) whereas *scg2b* staining was low. Most *scg2a* positive cells were observed in the pars intermedia (PI) which is in contrast to previous studies that detected higher levels of SNa in the pars distalis (PD) than in the neurointermediate lobe (NIL) of male and female goldfish (Zhao et al., 2006a); however, light staining of *scg2a* was also observed in the rostral PD (RPD) in the present study. Prominent staining of *scg2a* could possible indicate its involvement in the regulation of pituitary cell function.

Besides the pituitary, other key differences in the distribution between *scg2a* and *scg2b* transcripts were observed. The regions exhibiting only *scg2b* included the medial zone of dorsal telencephalic area (Dm), central nucleus of ventral telencephalic area (Vc), dorsal habenuar nucleus (Had), ventral habenular nucleus (Hav), anterior thalamic nucleus (A), subglomerular nucleus (SG), lateral torus (TLa), oculomotor nucleus (NIII) and Edinger-Westphal nucleus (EW). The lateral division of valvula cerebelli (Val) and medial division of valvula cerebelli (Vam) were the only regions where *scg2a* was expressed whereas *scg2b* was not detectable. The functions of each of these brain regions is still not fully understood; however, the differences in distributions could indicate that each *scg2* have distinct functions. Future studies could focus on these areas to delineate the roles *scg2a* or *scg2b* may play.

6.3 Knockout of SgII alters expression of genes involved in reproductive processes

The second objective was to determine how SgII-KO affects key reproductive genes. My results clearly show that genetic modification of *scg2* leads to altered expression patterns of key genes in the TEL, HYP and PIT. Gonadotropin-releasing hormone (Gnrh) is considered the main stimulator of the pituitary gonadotropins, Lh and Fsh (Schally et al., 1971; Zohar et al., 2010). Gonadotropin-releasing hormone 3 (*gnrh3*) was significantly downregulated in SgII-KO fish in the TEL and HYP. These results were consistent with the double Kiss-knockout (*kiss1*$^{-/-}$*;kiss2*$^{-/-}$*)* zebrafish that also had significantly decreased levels of *gnrh3* in whole brains (Tang et al., 2015). Other peptides such as neurokinin B (*tac3a*), that has been shown to play a role in controlling fish reproduction by increasing luteinizing hormone levels in mature female zebrafish (Biran et al., 2012) were assessed. Levels of *tac3a* in the TEL of single SgII-KO female and male zebrafish were decreased. In contrast, *tac3a* was increased in SgIIa-KO and SgII(a+b)-KO males and decreased in SgIIa-KO and SgIIb-KO females, in the HYP, suggesting tissue specific responses. An upregulation of *tac3a* was also observed in the male *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish (Marvel et al., 2018) and the triple knockout *kiss1*$^{-/-}$*;kiss2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish in both sexes (Liu et al., 2017). Furthermore, the isotocin/vasotocin family of nonapeptides which are important for vertebrate reproductive and social behaviours (Goodson and Bass, 2001) were also measured in SgII-KO fish. The mRNA levels of *oxt* were decreased in the TEL of SgIIa-KO and SgIIb-KO fish. The mRNA levels of *avp* were only decreased in the TEL of SgIIb-KO fish whereas it was increased in the HYP of SgIIa-KO and SgII(a+b)-KO males. Previous studies in the *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish, show that *avp* is unaltered (Marvel et al., 2018). Interestingly, k*iss2* was increased in double SgII(a+b)-KO females and males. This is in contrast to the *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish in which no difference was observed for *kiss2* (Marvel et al., 2018). In the pituitary, the Gnrh receptor 2 (*gnrhr2*) was significantly downregulated in single SgII-KO fish. The gonadotropins (*lhb, fshb* and *cga*), which stimulate steroidogenesis and gametogenesis were significantly lowered in the pituitaries of KO animals with the exception of *fshb* in double-KO fish. Interestingly, the SgII(a+b)-KO fish had slightly higher transcript levels of *fshb* and *gnrhr2* compared to single knockouts, although they were not significantly different from WT levels. The expression patterns of *lhb* and *fshb* observed in our study follow a somewhat similar pattern for those reported in the Kiss mutant (*kiss1*$^{-/-}$*;kiss2*$^{-/-}$) zebrafish. However, the gene expression patterns observed in the pituitary contrast with other knockout studies in that I documented major downregulation of gonadotropins here whereas this was clearly not the case for the *gnrh3*$^{-/-}$ zebrafish (Spicer et al., 2016), *gnrh2*$^{-/-}$*;gnrh3*$^{-/-}$ zebrafish

(Marvel et al., 2018), *kiss1*$^{-/-}$;*kiss2*$^{-/-}$;*gnrh3*$^{-/-}$ zebrafish (Liu et al., 2017), and gnrh1$^{-/-}$ medaka (Takahashi et al., 2016).

Previous knockout studies have shown that single, double and triple gene knockouts of traditional reproductive regulators (e.g., Kiss and Gnrh) resulted in minor alterations in reproductive genes. The current study is thus in marked contrast, given that SgII mutants have major decreases in many of the neuroendocrine genes critically important for gonadotropin synthesis and secretion. Like the *gnrh2*$^{-/-}$;*gnrh3*$^{-/-}$ zebrafish (Marvel et al., 2018), SgII-KO fish do not exhibit genetic compensation in response to gene knockout. Instead, there are shifts in the expression of other functional networks to compensate and maintain reproduction. Moreover, all previous gene knockouts of reproductive neuropeptides in zebrafish had little to no effect on spawning success. This is in clear contrast to my results, showing unequivocal depression of reproductive processes following SgII mutations.

6.4 Knockout of SgII disrupts reproductive processes

In the present study, I analyzed the functions of *scg2a* and *scg2b* in zebrafish reproduction. I first examined the characteristics of breeding tubercles (BT) (chapter 3), which are secondary sex characteristics used to identify mature males and used by males to maintain contact with females and stimulate egg release (Kang et al., 2013). The normal morphology of the pectoral fins was disrupted in SgII-KO males. The number of BT rows, BT width and BT cluster length were all reduced in SgII-KO males which could potentially lead to a reduced spawning success. Spawning trials with SgII-KO animals showed that *scg2a* and *scg2b* are required for optimal reproduction (chapter 4). Rates of oviposition for double SgII-KO females were 6% and 11% when crossed with double SgII-KO and wild-type (WT) males, respectively, compared to 62% in naïve WT pairings. Extensive video analysis of SgII-KO spawning trials revealed a decrease in frequency and duration of chase, tail-nose, encircle and quiver behaviours in SgII(a+b)-KO fish within line crosses as well as the encircle behaviour in SgII(a+b)-KO females paired with WT males. Morphological and histological analysis showed that ovaries from all adult SgII-KO mutant lines as well as WTs contained a full range of developing follicles, indicating that SgII-KO did not affect ovarian development. Analysis of SgII-KO male testes showed that all stages of sperm cells including spermatogonia, spermatocytes, spermatids and spermatozoa were present; however, SgIIa-KO and SgII(a+b)-KO fish had fewer spermatozoa compared to WT suggesting that spermatic development was affected. This reduced sperm production may have also contributed to the decreased fertilization success of within line breeding pairs. Whole body sex steroids were assessed since they are essential for spermatogenesis, spermiation, sexual

differentiation and sexual maturation (Schulz et al., 2010). However, testosterone, 11-keto testosterone and estradiol levels were unaltered in any SgII-KO lines. The results from chapter 4 clearly indicate the importance of *scg2a* and *scg2b* in the reproductive capacity of zebrafish.

I next wanted to determine if spawning success and courtship behaviours could be rescued in the SgII(a+b)-KO fish (Chapter 5). Intraperitoneal injection (i.p.) of human chorionic gonadotropin (hCG) significantly elevated spawning success in SgII(a+b)-KO zebrafish, indicating that the gonads of SgII fish are sensitive to Lh. However, hCG injected SgII(a+b)-KO fish were unable to increase spawning to WT ringer's injected levels, indicating that SgII(a+b)-KO fish are severely reproductively impaired. Similarly, i.p. injection of SNa rescued spawning success in the SgII(a+b)-KO fish whereas no effect was observed in SNb-injected fish. Furthermore, the resulting embryos from hCG and SNa injected SgII(a+b)-KO fish appeared to be of suboptimal quality. In addition to oviposition, quiver behaviour was also assessed in WT and SgII(a+b)-KO males following injection and surprisingly neither SN nor hCG injection altered the latency to quiver or rescue the quiver behaviour itself. From this data I speculate that SN injection is either stimulating the synthesis and/or release of Lh. The increased Lh levels alter steroidogenic pathways which induces oocyte maturation and ovulation.

My data also suggests that SN may not be the only bioactive SgII peptide capable of regulating reproductive processes, since SNa injection did not rescue the SgII mutants back to WT control spawning levels. There are nine conserved dibasic cleavage sites, based on the predicted goldfish SgII amino acid sequence, where prohormone convertases can produce bioactive small peptides (Blazquez et al., 1998). In a peptidomic analysis of whole zebrafish brain, Van Camp et al. (2017), identified 11 and 22 peptides from the SgIIa and SgIIb precursors, respectively. However, the production of numerous other peptides is theoretically possible given the number of cleavage sites. Other new peptides will need to be identified, localized and tested for biological activities and relevance to the control of reproduction. It is also possible that larger fragments of the SgII precursors that contain the SN peptide sequence may be necessary to exert full effects.

6.5 Possible mechanisms by which SgII-KO reduces spawning

I attempted to determine one of several potential mechanisms underlying the reduced reproductive capabilities of SgII-KO zebrafish. Circulating Lh levels could be influenced by SgII-KO thereby decreasing the production of sex pheromones. Therefore, I postulated that pheromonal status may be disrupted in SgII-KO zebrafish, and this leads to reduced courtship behaviours in males and ultimately reduced spawning success. First, I attempted to determine if SgII mutant males could

perceive the post-ovulatory pheromone $PGF_{2\alpha}$ with waterborne administrations; however, neither WT nor SgII(a+b)-KO males were attracted to it. This may be an artifact of the tank design (appendix 1). I then assessed if natural sex pheromones released into the water by WT fish would rescue SgII(a+b)-KO fish spawning success and courtship behaviour. I observed that WT males crossed with SgII(a+b)-KO females that were exposed to spawning water had a significantly higher spawning success and spent more time interacting with one another. These data suggest that the pheromonal cues present in the spawning water stimulated males to increase their courtship behaviours leading to a greater spawning success. I was unable to rescue the spawning success of SgII(a+b)-KO within line crosses back to WT control levels suggesting that these fish are dramatically impaired such that pheromonal signaling alone is insufficient. However, these data should be interpreted cautiously since these were preliminary studies, and many problems in the experimental design and execution were encountered.

Another equally plausible mechanism of SgII-KO spawning reduction is that SgII-KO alters ovulation. Ovulation in fish is the release of mature fertilizable oocytes from the surrounding follicle wall into the ovarian cavity (Lubzens et al., 2010). Ovulation is triggered by the Lh surge. Gene expression data in the present study shows dramatic reductions in *gnrh3*, *lhb,* and *fshb* in SgII-KO fish which could potentially lead to reduced circulating Lh levels, failed ovulation and failed spawning. The phenotype observed in my study is similar to *lhb*$^{-/-}$ zebrafish (Zhang et al., 2015b). Both male and female *lhb*$^{-/-}$ zebrafish showed normal gonadal growth but females failed to spawn. Ovaries were examined at first light and showed that control ovaries contained mature and ovulated eggs with the chorion membrane separated from the egg and expanded; the *lhb*$^{-/-}$ females had full grown but immature follicles that lacked germinal vesicle breakdown indicating a failure to undergo final oocyte maturation (Zhang et al., 2015b). Zhang et al. (2015b) did observe a few follicles that had undergone germinal vesicle breakdown in the ovary of the *lhb*$^{-/-}$ mutant fish however, they were not ovulated. Spawning was assessed in twenty-two pairs of *lhb*$^{-/-}$ zebrafish and all failed to spawn, but perhaps results would be different if more pairs were assessed since a few mature follicles were observed. In my study, I assessed eighty-two pairs of SgII(a+b)-KO fish given the failure to observe spawning with smaller sample sizes. It is possible that like the *lhb*$^{-/-}$ fish, the majority of SgII-KO females are unable to undergo natural final oocyte maturation (e.g., not injected with exogenous hormones), leading to failed ovulation and spawning.

6.6 Future directions

The data presented in this thesis shows that SgII/SN are involved in the regulation of reproductive processes, however there are many areas where more research is needed. Some future directions could include:

6.6.1 Mechanisms of SgII regulation of spawning

The mechanisms of SgII action to regulate reproduction are still unknown. Future studies should focus on how SgII regulates Lh release, whether that be directly acting on the pituitary, indirectly through Gnrh or by other means. Ovulation should be examined to determine if the failed spawnings were due to failed oocyte maturation and ovulation. Producing SgIIa and SgIIb transgenic reporter lines or crossing SgII-KO fish with and Gnrh, Lh and Fsh transgenic reporter zebrafish lines will allow us to elucidate the anatomical relationships between these regulators. The transcript (*gnrh, lhb* and *fshb*) and protein levels of (Gnrh, Lh, and Fsh) of SgII-KO fish should also be assessed.

6.6.2 Characterize endogenously present neuropeptides in the zebrafish brain and pituitary

The endogenous peptide content within a cell, tissue or organism is spatially and temporally dynamic. After processing by PC's, bioactive peptides can be stored in dense core vesicles prior to their release within the nervous system or peripheral organs. Upon binding to their receptor, they can modify physiological processes in response to internal or external stimuli. It is important to know which neuropeptides are present in each tissue as the identification of these peptides is likely to further elucidate neuropeptidergic signaling systems. Furthermore, performing peptidomic profiling studies in our SgII-KO models and WT zebrafish will allow us to determine other SgII-derived peptides.

6.6.3 Isolation and localization of the SN receptor(s)

The SN receptor remains unidentified although this project is currently underway. Evidence obtained from several cell lines support the hypothesis that the SN receptor is a G-protein coupled membrane receptor. Identifying the SN receptor will aid in identifying all SN target tissues and its respective functions. Once the receptor(s) is identified then it should be characterized.

6.6.4 Detection of SN in the zebrafish brain and pituitary

All the work presented in this thesis was at the transcript level. Zebrafish specific antibodies for SNa and SNb should be established to compare the protein distributions and levels in the zebrafish

brain and pituitary. Identifying the regional distributions of SNa and SNb may provide some insight into their functions and relationships to other hypophysiotropic factors.

6.7 Concluding remarks

The research presented in this thesis provides evidence that supports the hypothesis that SgII regulates reproductive functions in zebrafish. The data presented here show that SgII mRNAs are distributed in critical hypophysiotropic regions of the brain and pituitary of adult male and female zebrafish known to be involved in reproductive control. Genes known to be involved in reproductive processes are altered following SgII-KO. I have also demonstrated for the first time in any organism that SgII is critical for optimal sexual behaviour and reproduction. A single injection of SNa, one of the many possible bioactive peptides generated from the large SgIIa precursor, rescues spawning in SgII(a+b)-KO fish, placing this peptide family as one of the newest reproductive regulators. The proposed model of SgII action on the regulation of reproductive processes in adult zebrafish brain and pituitary as assessed in this thesis is shown in Fig. 6.1.

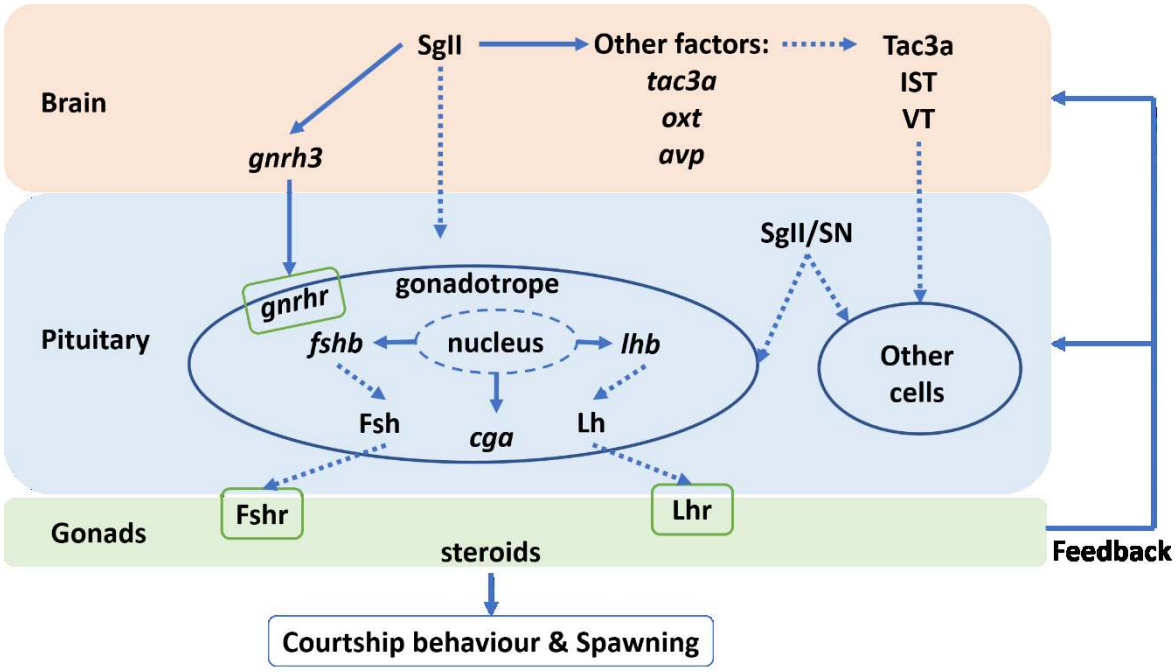

Figure 6.1 Proposed model of SgII action on the regulation of reproductive processes in the adult zebrafish brain and pituitary. Solid lines represent tested results while dashed lines are speculations. Note that the gonadotrope is shown as a single cell for convenience; Lh and Fsh would be secreted from separate specific cell types. Please see the discussion for details.

Appendix A: Effect of secretogranin-II knock-out on sex pheromone status in zebrafish (Danio rerio)

Kimberly Mitchell[1], Navpreet Langa[2], Paige Benson[2], and Vance L. Trudeau[3]

[1]Contributed to original ideas, conducted experiments, performed data analysis and manuscript preparation.

[2]Helped with experiments

[3]Contributed to original ideas and revised the manuscript

A.1 Introduction

Spawning, the controlled release of gametes by fish, is a complex reproductive event involving both internal hormonal signals and external cues including pheromones, temperature and spawning substrate (Kobayashi et al., 2002). Pheromonal signals have been best described in the goldfish model. Female goldfish release both preovulatory and postovulatory sex pheromones. The preovulatory signals are comprised of several dozen steroids; however, only three are primary components because they are detected with high specificity and sensitivity: 17,20-beta-dihydroxy-4-pregnen-3-one (17,20βP); 17,20-dihydroxy-4-pregnen-3-one-20- sulfate (17,20βP-S); and androstenedione (AD). The postovulatory pheromones are composed of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its metabolites (Sorensen et al., 1988). First, female gonadal growth and vitellogenesis is stimulated by 17β-estradiol (E2) which also causes the release of a recrudescent pheromone that attracts males. After vitellogenesis is complete, E2 production drops and plasma testosterone levels increase (Kobayashi et al., 1988). This sensitizes the females to environmental cues, eventually leading to a luteinizing hormone (Lh) surge that alters steroidogenic pathways favouring the production of progestins (Kobayashi et al., 2002). The progestin 17,20βP in plasma induces oocyte final maturation and ovulation but is also released to the water along with 17,20βP-S and AD to serve as preovulatory pheromones (Poling et al., 2001). In male goldfish, addition of 17,20-β and 17,20βP-S to fish water, increases Lh release and courtship behaviours in the form of nudging and chasing (Poling et al., 2001). Finally, ovulated female goldfish release $PGF_{2\alpha}$ and its metabolites as postovulatory pheromones thereby inducing male spawning behaviour which further increases male Lh and sperm production (Kobayashi et al., 2002).

Previous studies have established that the novel peptide secretoneurin (SN), that is produced from a precursor protein secretogranin-II (SgII), regulates Lh release from the pituitary in goldfish and mice (Zhao et al., 2009a; Zhao et al., 2010a; Zhao et al., 2011). Furthermore, my studies (chapter 4) have shown decreased spawning success and courtship behaviours in SgII-KO zebrafish even though they have what appear microscopically to be fully functional gonads. These results are sex-specific given that spawning success was rescued in all reciprocal breeding crosses of SgII-KOs with WT partners except SgII(a+b)-KO females paired with wildtype males. It is possible that SgII-KO could lower circulating Lh levels thereby decreasing the production of sex steroids and pheromones. In this study we test the hypothesis that pheromonal status is disrupted in SgII-KO zebrafish leading to reduced courtship behaviours in males and ultimately reduced spawning success. Two objectives were assessed. I attempted to determine if SgII mutant males could perceive the post-ovulatory

pheromone $PGF_{2\alpha}$; and if spawning success in SgII(a+b)-KO females could be rescued in the presence of zebrafish sex pheromones.

A.2 Materials and Methods

A2.1 Experimental animals

Wild type and SgII-KO zebrafish were maintained in 10-L tanks (Aquatic Habitats; Apopka, FL) in a recirculating system with a 14h light/10h dark cycle at 28°C.

A2.2 Preparation of odorants

Prostaglandin $F_{2\alpha}$ was purchased from Cayman chemicals (CAS #551-11-1) then a stock solution of 10^{-3} M $PGF_{2\alpha}$ was prepared in 99% ethanol. The final concentration of $PGF_{2\alpha}$ in the experimental tanks was 10^{-9} M. Ethanol was used as a vehicle control where its final concentration in the tank was 0.00099%.

A2.3 Attraction of male zebrafish to PGF2α

Prior to experiments, fish were separated by sex at the age of 6 months post-fertilization (mpf) then twenty-four fish (24 WT and 24 SgII(a+b)-KO) were randomly selected for the experiment. The WT and SgII(a+b)-KO fish were further separated and housed in 6 groups of 4 fish per tank and maintained in 3-L tanks. Behavioural analyses were performed with adult wild-type (WT) and SgII(a+b)-KO male zebrafish. Each experimental group of four fish (WT or SgII-KO) were transferred to a test tank (30 x 18 x 12 cm) filled with 6 L of fresh system water and allowed to acclimate for 60 min. After the acclimation, 600 µl of 10^{-5} M $PGF_{2\alpha}$ or EtOH was gently applied to one side of the tank through a silicon tube attached to a 3 ml syringe. Fish movements were recorded 10 minutes pre- and post pheromone administration, from the side of the tank using a ViewPoint high sensitivity camera (Dragonfly2, DR2-HIBW, IEEE-1394; 60 frames/Sec) and an infrared lighting system board as a backdrop. The experimental arena was covered so that the experimenter was not visible to the fish. Fish movements were tracked using ViewPoint software (Zebralab AVI, version 3.52). From the coordinate data, the mean position of each fish was plotted. In a preliminary experiment where the dissipation of a blue dye was monitored, it was determined that added solutions dissipate in about 3 minutes. Therefore, only data from the 3 minutes pre- and post-treatment were analyzed. A preference index was calculated for each group during each period (30 s) from the following equation as described by Yabuki et al. (2016b): preference index = $(T_{odor} - T_{control})/(T_{odor} + T_{center} + T_{control})$, where

T_{odor}, T_{center} and $T_{control}$ denote the time fish spend in the odor-applied side, control side and central area, respectively.

A2.4 Collection of wild-type spawning water

A separate batch of WT fish, used to generate spawning water containing a natural pheromone mixture, were set-up in 3-L breeding traps. Each trap contained two females and one male separated overnight with an insert containing a divider. For each experiment, sixteen breeding tanks were prepared. On the morning of the experiment the tank water for these WT pairs was changed, dividers were removed, and the WT fish were left to spawn for 30 minutes. Water was only collected from the tanks where a clutch of eggs was present then mixed all together. This spawning water was transferred to clean breeding traps and used for the spawning water experimental group.

A2.5 Pheromone rescue of spawning success

Spawning success and interaction time were compared by breeding fish in water where WT zebrafish had previously spawned (spawning water). The spawning success of sexually naïve fish (WT and SgII(a+b)-KOs, 8 months) was assessed via mating crosses: WT male (WTM) x WT female (WTF); WTM x SgII(a+b)-KO female (AB-KOF); AB-KOM x AB-KOF. Two weeks prior to the spawning experiment, fish were separated by sex into 3L tanks at a density of 4 fish/litre. The night before each experiment, fish were transferred to 1L breeding tanks. Each tank contained one male and one female, separated overnight with an insert containing a divider. Each experimental day contained several tanks of each experimental cross. On the morning of the experiment the fish in the control group were transferred to breeding tanks containing system water whereas the spawning water group were transferred into breeding tanks containing the collected WT spawning water.

Behaviour was monitored using a Zebracube system (ViewPoint Behavior Technology) consisting of a sound proof chamber with an infra-red camera. Dividers separating male and female were removed, and the fish were filmed for 20 minutes. Using Zebralab locomotion tracking software (ViewPoint Behaior Technology; Zebralab AVI, version 3.52) we calculated the ‘interaction time’; the amount of time the center of mass of the male and female fish were within 15 mm of one another. The fish were then left undisturbed for 1.5 hrs at which point the absence or presence of a clutch was noted. Clutches were placed in E3 medium and fertilization rate was assessed. Embryos were kept in an incubator (28°C) until 24 hours post fertilization (hpf) at which point survival rate was assessed.

A2.6 Data analysis

Statistical analyses of $PGF_{2\alpha}$ attraction data was performed using R Statistical Software (Foundation for Statistical Computing, Vienna, Austria), a free software environment for statistical computing. The mean positions of fish following odorant treatment were analyzed using t-Tests comparing positions before and after adminstation of either $PGF_{2\alpha}$ or EtOH. The preference index of each period was analyzed for a significant deviation from the centre (0) of the tank using a Wilcoxon signed-rank test. Online freeware from GraphPad QuickCalcs was used for statistical analysis of the spawning success data. Spawning success of each mating pairs were compared to the control/system water exposure with Fisher's exact test (http://graphpad.com/quickcalcs/contingency1/). Data are reported as percentages. All other statistical analyses were performed using SigmaPlot 11.0 software (Systat Software, Inc., Chicago, IL). Normality and homoscedasticity were tested using a Shapiro-Wilk test and Levene's test respectively. The Kruskal-Wallis test on ranks was used to analyze the percent fertilization and percent survival.

A.3 Results

Sexually naïve males were put into the test tank and, following acclimation, vehicle or $PGF_{2\alpha}$ was applied to one side of the tank. WT males were not attracted to $PGF_{2\alpha}$ based on their mean tank positions ($P > 0.05$) (Fig. A.1). SgII(a+b)-KO males were also not attracted to $PGF_{2\alpha}$ in our experimental system ($P > 0.05$) (Fig. A.1). To quantify the degree of attraction, we calculated the preference index of the group of fish for every 30 s. Similar results were obtained in that neither WT ($P = 0.083$) nor SgII(a+b)-KO mutants ($P = 0.321$) preferred the side in which $PGF_{2\alpha}$ was administered (Fig. A.2). However, WTs did prefer the EtOH side ($P = 0.018$) whereas SgII(a+b)-KOs did not ($P = 0.5565$) (Fig. A.2).

The spawning success of WT males crossed with WT females was 64% and 52% in control and spawning water, respectively. Fisher's exact test revealed no significant differences between these two exposures ($P = 0.4242$) (Fig. A.3A). Similarly, SgII(a+b)-KO within line crosses successfully spawned in 3% and 7% of control and spawning water exposures, but this was not significantly different ($P = 0.6115$) (Fig. A3A). In contrast, WT males crossed with SgII(a+b)-KO females (WTM X AB-KOF) did not spawn in any of the control water treatments but spawned successfully in 21% of the spawning water exposures. This was a significant increase compared to the respective control water exposure ($P = 0.0232$) (Fig. A.3A), suggesting that SgII(a+b)-KO females do not release adequate pheromone to induce male breeding behaviours.

The social interaction time fish spent during their courtship was assessed in control and spawning water exposed breeding crosses. The WT within line crosses spent significantly more time interacting with each other when exposed to spawning water compared to control water [$F(1, 59) = 14.946$, $P < 0.001$] (Fig. A.3B). A significant difference between spawning water and control water was also observed when WT males were paired with SgII(a+b)-KO females [$F(1, 59) = 36.484$, $P < 0.001$] (Fig. A.3B). However, no significant differences in interaction time were observed between control water and spawning water for SgII(a+b)-KO within line crosses [$F(1, 58) = 1.968$, $P < 0.166$] (Fig. 3B). These data should be interpreted with caution given that these observations are only preliminary.

The fertilization rate and percent survival were only assessed in WT within line crosses given the insufficient sample sizes in the other breeding crosses (data not shown). No significant differences were observed for the median percent fertilization since it was 100% in both control and spawning water [$H(1) = 2.325$, $P = 0.127$]. The median percent survival was 91% and 97% in control and spawning water exposed WT within line crosses, respectively; no significant differences were observed between these two groups [$H(1) = 1.292$, $P = 0.256$].

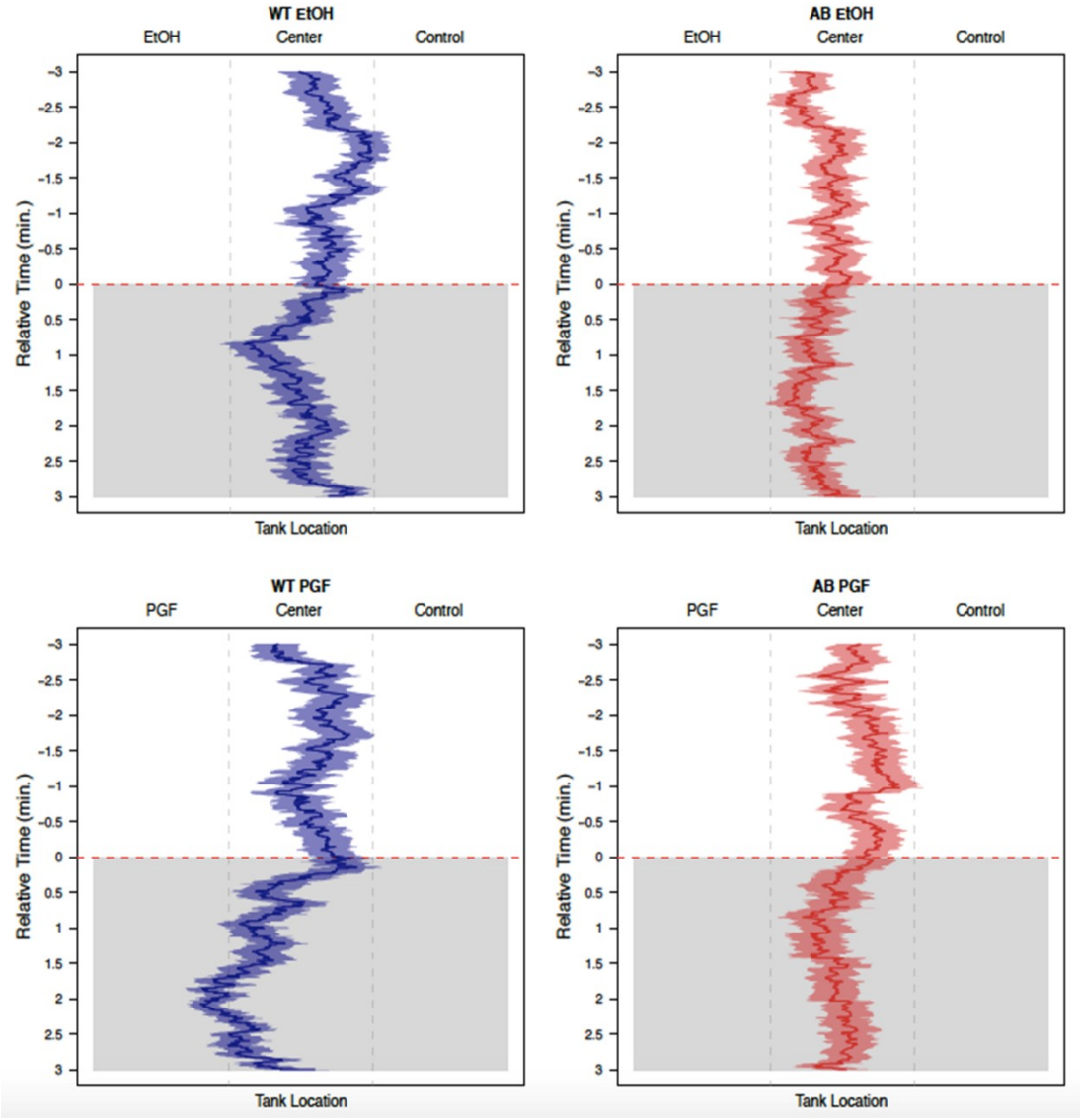

Figure A.1 Mean positions (solid line) and SEM (shading) of the 24 male WT or SgII(a+b)-KO fish in the tank 3 minutes pre- and post (grey shaded area) - $PGF_{2\alpha}$ or EtOH (control) exposure. Each tank contained four males (n=6). Significant differences were determined using a t-Test; $P \leq 0.05$. AB: SgII(a+b)-KO; WT: wild-type; PGF: prostaglandin $F_{2\alpha}$; EtOH: Ethanol.

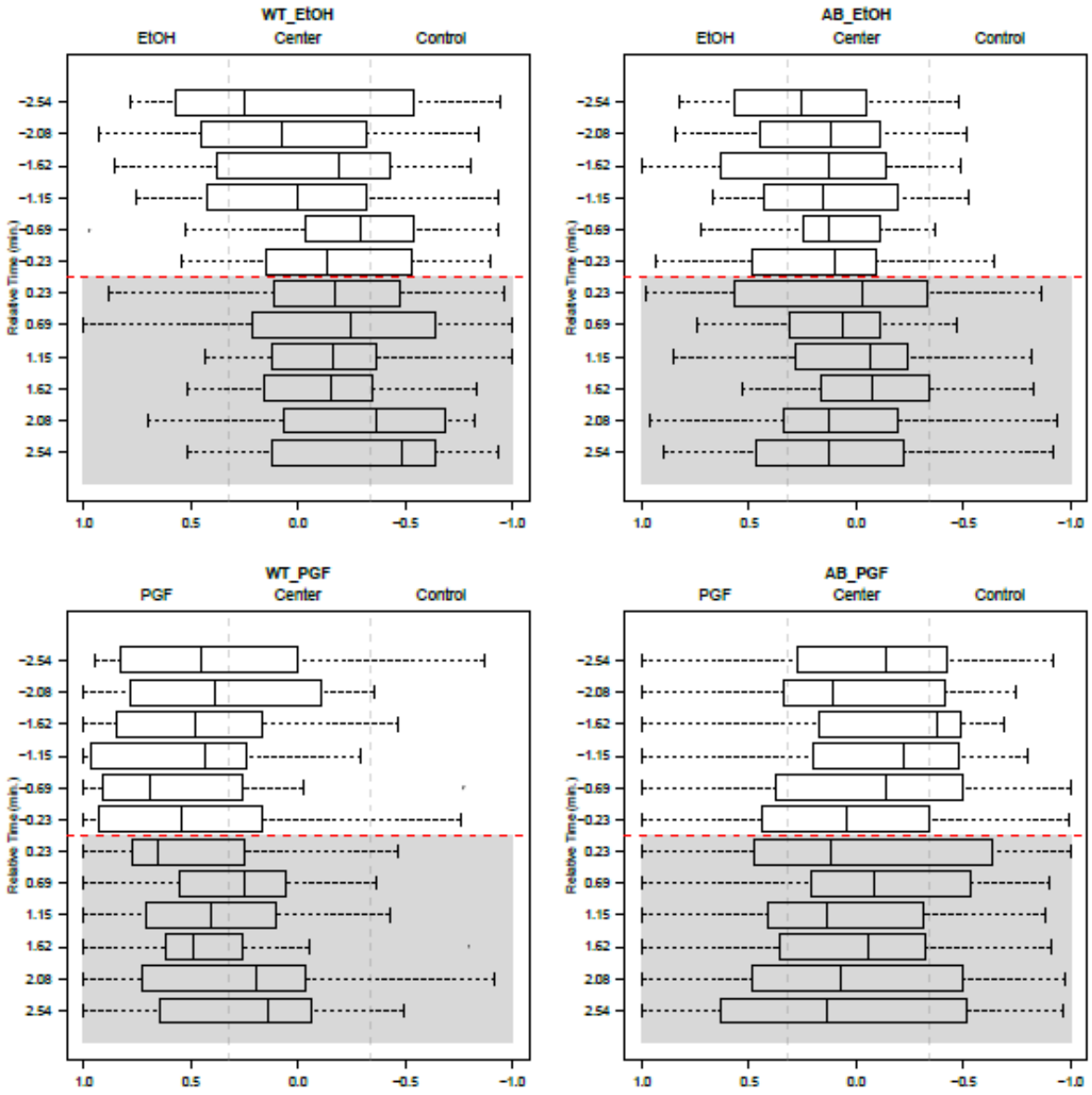

Figure A.2 Preference index in each time bin (30 s) of male WT or AB-KO fish in the tank 3 minutes pre- and post (grey shaded area) - PGF2α or EtOH (control) exposure. Box plots show median, quartiles (boxes) and range (whiskers). Each tank contained four males (n=6). The red horizontal dotted line indicates when the odorant was added. Significant differences were determined using a wilcoxen signed-rank test for significant deviation from 0. AB: SgII(a+b)-KO; WT: wild-type; PGF: prostaglandin $F_{2\alpha}$; EtOH: ethanol.

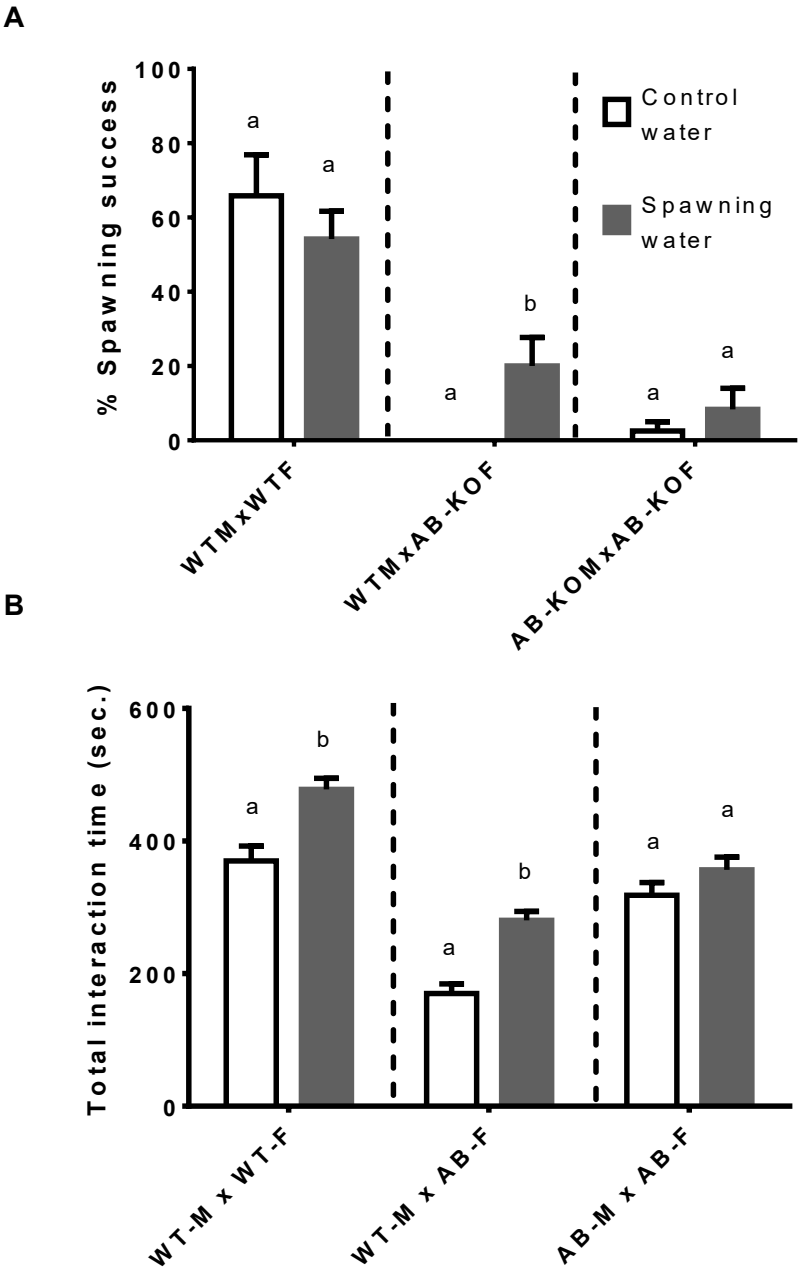

Figure A.3 Spawning success (A) and total interaction time (B) of pairwise crosses in spawning water vs control water. Successful spawning is indicated by the presence of a clutch after 1.5 hours of breeding. Statistical significance is determined using Fisher's exact test (A) and (B). Different letters denote statistical significance in between system and spawning water in each respective within line and reciprocal cross (n=32). WT: wild-type; AB: SgII(a+b)-KO; F: female; M: male.

A.4 Discussion

The hypothesis of this study was that SgII-KO alters the pheromonal status of zebrafish. We first set out to determine if male WT and SgII(a+b)-KOs could perceive the sex pheromone $PGF_{2\alpha}$ with waterborne administrations. We found that neither WT nor SgII(a+b)-KO males were attracted to $PGF_{2\alpha}$. These results were unexpected since previous studies report that WT male zebrafish exhibit a robust attraction to $PGF_{2\alpha}$ (Yabuki et al., 2016b). We, as well as Yabuki et al. (2016b), administered 10^{-9} M $PGF_{2\alpha}$ but perhaps higher concentrations should be tested with our fish. We believe the lack of response to $PGF_{2\alpha}$ was due to flaws in our experimental design. Yakubi et al. (2016b) used a larger experimental tank that was 45 x 30 x 23 cm filled with 25 L of water whereas in this experiment we used a tank that was 30 x 18 x 12 cm and filled with 6 L of water. A larger tank would have separated the odorant and control side of the tank more clearly and allow the fish to swim more freely from side to side. Our preliminary experiments led us to determine that methylene blue was no longer contained on the side in which it was administered after 3 minutes. A larger tank may have allowed us to analyze a longer period after administration of odorant. However, the attractive response of WT males to $PGF_{2\alpha}$ happens within the first 2 minutes (Yabuki et al., 2016b). We tried using tanks of different sizes however, none would allow us to track the fish using our ViewPoint behavior software. The WT-$PGF_{2\alpha}$ exposed fish also seemed to prefer the $PGF_{2\alpha}$ side of the tank as shown by the preference index plots, however, this preference was also observed before administration. Therefore, we are unable to determine for certain if WT male zebrafish were attracted to $PGF_{2\alpha}$. Odorant administration was randomly assigned to either the left or right side of the tank to prevent a possible bias caused by a fish's preference for left or right. It is possible that other environmental disturbances such as noise from the experimenter or vibrations from construction in a nearby building may have influenced our results. In the future these studies should be conducted in an isolated environment with an appropriate test tank/apparatus. Abreu et al. (2016) used a tank with two chambers leading to two lanes of water with laminar flow running in parallel to assess the attraction and aversion paradigm of various pharmaceuticals. There is a manifold for both chambers that allows the introduction of the test substance. Meanwhile, the fish is contained by mesh in the centre of the two lanes and can choose which chamber it prefers. A similar type of apparatus would be ideal to use for our experiments.

We also attempted to determine if natural sex pheromones released into the water by WT fish would rescue SgII(a+b)-KO spawning success and courtship behaviour. Courtship behaviour of animals relies on various sensory cues such as sex pheromones, visual appearance of female fish and

their motion and physical contact to accomplish efficient reproduction (Yabuki et al., 2016b). We predicted that introducing pheromones into tank water would increase courtship behaviour and thereby lead to increased spawning success. Spawning water did not have any impact on the percent spawning success of WT within line crosses, however there was a significant increase in the interaction time of these pairs. It is possible that WT fish had already reached their spawning capacity and could not spawn further but could continue to court each other. Interestingly, the WT males crossed with SgII(a+b)-KO females that were exposed to spawning water had a significantly higher spawning success and spent more time interacting with one another. Our data suggests that the pheromonal cues present in the spawning water stimulated males to increase their courtship behaviour leading to a greater spawning success. This data also provides support that a female specific defect is present. Comparable results were observed in male goldfish in that waterborne exposures to 10^{-10} M 17,20βP increased nudging, chasing, spawning with females and the milt volume of males (Defraipont and Sorensen, 1993). Moreover, waterborne exposure to 10^{-8} M $PGF_{2\alpha}$ dramatically increased swimming and social activity, chasing and nudging, of male goldfish (Sorensen et al., 1988). These data suggest that both pre- and post-ovulatory pheromones enhance male spawning success by physiological and behavioural mechanisms. In our study, we were unable to rescue spawning success and courtship in the SgII(a+b)-KO within line crosses back to WT control levels. It is possible that these fish are so impaired that pheromonal signaling alone is insufficient. It may also suggest that SgII-KO affects male olfactory sensitivity to reproductive pheromones. It is interesting to note that the time SgII(a+b)-KO fish spent interacting was not that low compared to the WT within line crosses although this data may be misleading. In this study we measured the interaction time instead of male courtship behaviours although we would have preferred the latter as we have done in the previous chapters (4 and 5). Due to time constraints, we used the social interaction module in ViewPoint Behavior Technology software (Version 3.52) which assesses the length of time the fish spend within a certain distance of one another. Therefore, our results may be misleading if fish were not in fact interacting and courting one another but simply spending their time next to each other. We attempted to calculate several parameters (distance, velocity, etc.) but the ViewPoint software mixed up the male and female fish and presented incorrect data. The struggles we encountered while working with this software also led us to develop our own courtship software (not completed yet). We attempted to repeat this experiment to include an SgII(a+b)-KO male and WT female cross however, we did not have enough SgII(a+b)-KO males to do so. Finally, the fertilization and survival of WT within line crosses was compared between system and spawning

water and there were no significant differences between them which was expected. Clutch size and viability have been shown to increase following exposure to male pheromones (Gerlach, 2006), although this is improbable for our study given an exposure length of 2 hours.

This study was unable to determine the sex pheromonal status of SgII(a+b)-KO zebrafish or elucidate the sex specific effects of the knockouts. However, it does suggest that pheromonal signaling may be upset. This study should be redesigned with an appropriate tank apparatus and assess all four fish crosses. Furthermore, we would like to measure the concentration of pheromones ($PGF_{2\alpha}$ and 17,20β-P) produced by the SgII(a+b)-KO females in courtship situations to determine if the reduced spawning success is due to lowered pheromone production. Alternatively, we could investigate the olfactory sensitivity of mature SgII-KO males to waterborne prostaglandins using electro-olfactogram recordings (EOG). These recordings are taken from the surface of the olfactory epithelium and reflects olfactory receptor potentials (Scott and Scott-Johnson, 2002).

Appendix B: Generating an lhb:mCherry transgenic zebrafish line

Kimberly Mitchell[1], Crystal Esau[2], Cynthia Solek[3], and Vance L. Trudeau[3]

[1]Contributed to original ideas, conducted experiments, performed data analysis and manuscript preparation.

[2]Helped with all experiments

[3]Designed the construct

[4]Contributed to original ideas and revised the manuscript

B.1 Introduction

Vertebrate reproduction is tightly regulated by a variety of feedback systems between the hypothalamus, pituitary, and gonads, known as the hypothalamus-pituitary-gonad (HPG) axis. Gonadotropin-releasing hormone (GnRH) is the main regulator of the HPG axis and stimulates the synthesis and secretion of pituitary gonadotropins, luteinizing hormone (Lh) and follicle stimulating hormone (Fsh). The gonadotropins are dimers comprised of a common α subunit and a hormone specific β subunit that confers biological specificity. These gonadotropins bind to their receptors in the gonads to stimulate steroidogenesis and gametogenesis (Li and Ford, 1998). Sex steroids that are produced in turn feedback onto the pituitary or brain to either increase or decrease gonadotropin release. In females, Fsh is primarily responsible for follicular development, whereas Lh is necessary for follicular maturation and ovulation (Li and Ford, 1998). In males, Lh binds to its receptors on Leydig cells, increasing steroidogenesis and Fsh binds to its receptors on the Sertoli cells to regulate spermatogenesis (Li and Ford, 1998). The regulation of Lh is of major interest because failure to activate Lh release leads to reduced fertility and often failed reproduction. Secretoneurin (SN), a peptide produced from the proteolytic cleavage of the precursor protein secretogranin-II (SgII), has been shown to promote the synthesis and release of Lh in the goldfish model (Zhao et al., 2006a; Zhao et al., 2006b; Zhao et al., 2009a; Zhao et al., 2010a) and from the mouse LβT2 gonadotroph cell line (Zhao et al., 2011). Our studies have also shown that the secretogranin-II genes are necessary for optimal reproductive function (chapters 4 and 5).

Several transgenic fish models have been produced to better understand the complex interactions involved in the HPG axis. Gonadotropin releasing neurons have been labeled in zebrafish (Abraham et al., 2008) and medaka (Karigo et al., 2012), Fsh in tilapia (Golan and Levavi-Sivan, 2013) and gonadotropins in medaka (Karigo et al., 2014).

The purpose of this project was to create an all-zebrafish transgenic line for identifying Lh producing cells. Once this transgenic line was established and characterized, I would have crossed them with the secretogranin-II mutant lines (SgII-KO). When we first started this study, no lines had been created to identify Lh and Fsh producing cells. However, in the process of our experiments, another group used regulatory elements from tilapia to drive fluorescent protein expression in zebrafish gonadotropes (Golan et al., 2014). Therefore, we halted further development of our transgenic zebrafish line to focus on other experiments. Moreover, we have obtained the lines of Golan et al. (2014) courtesy of B. Levavi-Sivan and Y. Zohar and will incorporate them into future studies. Here we present the work that was undertaken and suggest next steps.

B.2 Materials and Methods

B2.1 The *lhb* construct for transgenesis

Chen and Chiou (2010) previously cloned and characterized the zebrafish *lhb* gene structure and promoter region and found that proximal promoter regions between 3.4 and 3.7 kb in size displayed maximal promoter activity. Therefore, a 3.7 kb fragment from a zebrafish genomic fosmid library (BACPAC Resources Center) that was identified to contain the whole genomic sequence of *lhb* was used in our study, to amplify the promoter region of the *lhb*. The fluorescent protein (*mCherry*) was inserted in frame with *lhb* by homologous recombination into bacteria according to procedures described by Liu et al. (2003). Additionally, the *cmlc2* heart marker promoter in frame with the enhanced green fluorescent protein (EGFP) was included in the construct to drive eGFP expression in the heart. This was incorporated into the construct to facilitate screening because it is expressed at 1-day post fertilization (dpf), and earlier than *lhb.* Each piece of the construct was PCR amplified with the PCR primers in table B.1. Pieces of the construct were ligated through typical cloning techniques. A 6.9 kb fragment was then cloned into a Psp72-Tol2 vector in another round of homologous recombination. This *lhb*-mCherry construct was utilized for producing transgenic zebrafish lines. A diagram of the construct is shown in Figure. B.1.

B2.2 Transposase mRNA synthesis

The PCS2 plasmid in TOP10 cells that contained the transposase cDNA was linearized using NotI restriction enzyme. The RNA was synthesized using a mMessage mMachine® SP6 Transcription kit (Ambion) according to the manufacturer's protocols. The reaction was stopped, and the RNA was precipitated by adding 30 µl of Nuclease-free water and 30 µl of LiCl precipitation solution. The solution was chilled for 30 minutes at -20°C followed by centrifugation at 4°C for 15 min at 16000 revolutions per minute (RPM). The supernatant was removed, and the pellet was washed with 1 mL of 70% ethanol then re-centrifuged. The ethanol was removed, and the RNA was re-suspended in ddH_2O. Aliquots of RNA were stored at -80°C.

B2.3 Microinjection in zebrafish embryos

The injection mix was freshly prepared prior to injection with various DNA and mRNA concentrations along with 2% phenol red. DNA and mRNA concentrations ranged from 25-50 ng/µl and 35-70 ng/µl respectively. After several trial injections, 25 ng/µl of plasmid DNA and 70 ng/µl of

transposase mRNA produced the highest number of GFP-expressing cells in the injected embryos; therefore, these DNA and mRNA concentrations were used for all subsequent injections. The plasmid and transposase mRNA were co-injected into 1 cell zebrafish embryos according to established procedures (Xi et al., 2010). Microinjection was performed using a Narishige IM-300 microinjector.

B2.4 Screening of zebrafish embryos

Injected embryos were screened for eGFP in the heart at 1 dpf. GFP-positive individuals were raised to sexual maturity and are considered founder fish (F0). F0 individuals were then outcrossed with wild-type (WT) adult fish to identify transgenic carriers. If the offspring of F0 fish showed GFP fluorescence in the heart, then the F0 fish were regarded as a transgenic fish because the transgene was transmitted to its offspring. If no fluorescence was detected in 200 embryos, the F0 fish were not considered transgenic and not used. Fluorescent embryos from F0 transgenic fish were raised to make a stable transgenic line.

Table B.1 Primers used for each part of the *lhb* construct.

Construct piece	Forward (5'-3')	Reverse (5'-3')
lhβ	GTAAAGCTTGCCTAATTAACCATTAACCTGCC	GATTCTAGATCCTGGAGAATGAAGAAACAACAAT
mCherry	GTATCTAGAATGGTGAGCAAGGGCGA	AGTCCCGGGCTTGTACAGCTCGTCCATGCCGCC
polyA	GTAGGTACCACCTCGGACAACTCACATCAA	AGTGAATTCGGCCAAACTGTGCCATTAAA
cmlc2	GTAGATATCATAGCTTAAATCAGTTGTGTTAA	AGTGAATTCCTTGTTTATTGCAGCTTATAAT

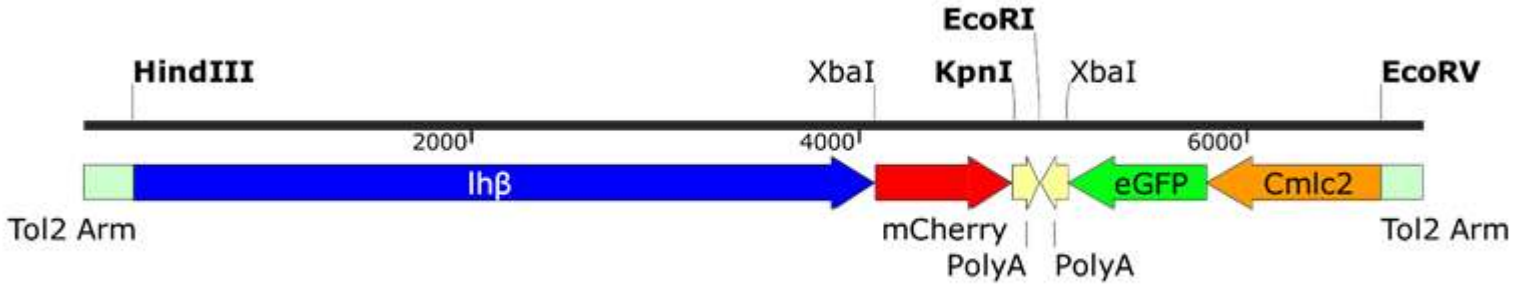

Figure B.1 Schematic of the lhb construct. The inserted DNA is a total of 6.9kb, containing the promoter region of *lhb* with mCherry inserted at the beginning of exon 1 and maintaining the same reading frame.

B.3 Results

More than 50% of embryos injected with the *lhb* construct in the presence of *tol2* tranaposase mRNA showed GFP expression in the heart at 1 dpf. These founder fish were raised according to standard husbandry procedures (Westerfield, 2000) and then crossed with WTs and screened for germ-line transmission of the transgene. Among the 115-founder fish, several were identified to have germ-line transmission although not all founder fish were assessed. Three fish, numbers 11, 44 and 53, showed prominent GFP expression in the heart and were selected to proceed with further experimentation. These F0 fish were crossed with WTs, their progeny was screened for GFP expression, and raised until adulthood, these were the F1 fish. The F1 fish were crossed with one another (i.e, male and female line 11 fish were crossed) to produce the F2 generation of fish. This progeny was again screened for GFP expression and raised until adulthood.

B.4 Future steps

MCherry expression in *Tg(lhb:mCherry)* fish will be determined by crossing suspected transgenic F2 fish with WTs and examining their progeny under a fluorescent microscope. Golan et al. (2014) detected *lhb* expression as early as 13 dpf therefore we examined the anaesthetised F1 progeny (F2 fish) at 13 dpf but were unable to detect mCherry expression. Therefore, it may be too early in our fish to detect Lh producing cells. In the future, a waterborne expose of the F2 or F3 fish to Gnrh may be conducted which would increase Lh thereby allowing us to detect the Lh producing cells more easily. Any fluorescent signals we detect could also be compared to *in situ* hybridization staining patterns to determine if the expression of the transgene is correct. These promising lines will be carried forward by another PhD student focussing on the role of SgII/SN to control Lh.

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