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Secretoneurin is a Neuropeptide Regulating Luteinizing Hormone Release from Pituitary Gonadotrophs

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Secretoneurin is a Neuropeptide Regulating Luteinizing Hormone Release from Pituitary Gonadotrophs

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Thesis submitted to the Faculty of Graduate and Postdoctoral Studies, University of Ottawa, in partial fulfillment of requirements of the PhD degree in the Ottawa-Carleton Institute of Biology.

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Abstract

Secretoneurin (SN) is a major functional peptide derived from the secretogranin II (SgII) precursor, one of granin family members. Our previous studies revealed that SN had a stimulatory action on luteinizing hormone (LH) secretion from goldfish pituitary *in vivo* and *in vitro*. In my PhD thesis, I isolated and identified free SN peptide in goldfish brain and pituitary by using reversed phase HPLC and western blots. Immunocytochemical localization showed that SN immunoreactivity (IR) was found within magnocellular neurons of the hypothalamic nucleus preopticus, prolactin producing lactotrophs in rostral pars distalis (RPD) of the pituitary and nerve fibres in pituitary neurointermediate lobe (NIL). These studies indicated the production of SN in goldfish brain and pituitary. I furthermore explored SN production under the regulation of hypothalamic gonadotropin releasing hormone (GnRH). Treatment of dispersed goldfish pituitary cells *in vitro* with GnRH increased both gene and protein expression of SgII and SgII-derived peptides which were significantly associated with that of prolactin. *In vitro* treatment with SN stimulated LH release from dispersed goldfish pituitary cells. Immunoneutralization experiments indicated that pituitary-sourced endogenous SN not only plays a paracrine role but also acts as a potential transduction factor to mediate GnRH-induced LH release in goldfish pituitary. In mouse LβT2 gonadotrophs, SN is able to up-regulate both LH secretion and the mRNA levels of multiple gonadotropin subunits. In LβT2 cells, SN also activates a mitogen-activated protein kinase (MAPK) cascade leading to phosphorylation of extracellular signal-regulated kinase (ERK). Further studies should focus on the isolation and localization of SN receptors in pituitary to expand the hypothesis that SN is a newly-identified hormone in the neuroendocrine system.

Résumé

La sécrétoneurine (SN) est un important peptide fonctionnel issu de la sécrétogranine II (SgII), un précurseur membre de la famille des granines. Des études antérieures ont révélé que la SN possède une action stimulatrice sur la sécrétion de la lutéostimuline (LH) dans l'hypophyse de poisson rouge (*Carassius auratus*), et ce, autant *in vivo* que *in vitro*. Dans le cadre de ma thèse doctorale, j'ai identifié et isolé des peptides correspondants à la SN en exécutant des HPLC en phase inverse et des buvardages de western. La localisation par marquage immunocytochimique a démontré que la SN se retrouve dans les neurones magnocellulaires des noyaux pré-optiques hypothalamiques, dans les cellules lactotropes de l'adénohypophyse et dans les fibres nerveuses du lobe intermédiaire de la neurohypophyse. Ces observations suggèrent que le cerveau et l'hypophyse de *C. auratus* produisent de la SN.

J'ai également étudié la régulation par la gonadolibérine (GnRH) de la production de la SN. Le traitement par la GnRH de cellules hypophysaires de *C. auratus* dispersées (*in vitro*) a élevé l'expression génique de la SgII et des peptides dérivés de la SgII. Ces augmentations étaient significativement corrélées avec l'accroissement de l'expression de la prolactine. Le traitement *in vitro* avec de la SN a stimulé la libération de LH. Des techniques de neutralisation immunologique ont indiqué que la SN produite dans l'hypophyse ne joue pas seulement un rôle de signalisation paracrine, mais qu'elle est potentiellement un facteur de transduction dans la libération de la GnRH induite par la LH dans l'hypophyse de *C. auratus*.

Dans la lignée cellulaire LβT2 (cellules gonadotropes de souris), la SN est également en mesure de stimuler la sécrétion de LH ainsi que la transcription de multiples sous-unités

constituant les gonadostimulines. Dans cette même lignée, la SN peut également enclencher une cascade de protéines kinases activées par des agents mitogènes (MAPK), causant la phosphorylation des kinases régulées par des signaux extracellulaires (ERK). Des études ultérieures devraient être axées sur l'isolation et la localisation des récepteurs de la SN dans l'hypophyse afin d'appuyer l'hypothèse que la SN est une nouvelle hormone du système neuroendocrine.

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"No pain, no palm; no thorns, no throne; no gall, no glory; no cross, no crown."
——*William Penn, British admiral*

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List of Abbreviations

AA	arachidonic acid
Ach	acetylcholine
ACN	acetonitrile
ANOVA	analysis of variance
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CaM	calmodulin
CaMK	CaM kinase
cAMP	cyclic adenosine monophosphate
CCB	C-terminal chromogranin B
cDNA	complementary deoxyribonucleic acid
Cg	chromogranin
CgA	chromogranin A
CgB	chromogranin B
cGnRH-II	chicken gonadotropin releasing hormone II
CPY	carboxypeptidase Y
CST	catestatin
DA	dopamine
DAG	diacylglycerol
DMEM	dulbecco's modified eagle's medium
DMSO	dimethyl sulfoxide
DNA	deoxyribonucleic acid
E	epinephrine
EGF	epidermal growth factor
ERK	extracellular signal-regulated kinase
Erg-1	early growth response-1
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
gf	goldfish
gfSN	goldfish secretoneurin
GH	growth hormone
GnRH	gonadotropin releasing hormone
GFR	growth factor receptor
GPCR	G protein-coupled receptor
Grb	growth factor-bound protein;
GTH	gonadotropin

HPLC	high performance liquid chromatography
IP3	(1,4,5) inositol trisphosphate
IR	immunoreactivity
JNK	c-Jun N-terminal kinase
LH	luteinizing hormone
MAP	multiple antigenic peptides
MAPK	MAP kinase, mitogen-activated protein kinase
Mek	mitogen/extracellular signal protein kinase
mGnRH	mammalian gonadotropin releasing hormone
mRNA	messenger ribonucleic acid
mSN	mouse secretoneurin
MW	molecular weight
M/Z	mass-to-charge ratio
NE	norepinephrine
NIL	neurointermediate lobe
NPO	nucleus preopticus
NRS	natural rabbit serum
PACAP	pituitary adenylate cyclase-activating polypeptide
PBS	phosphate buffered saline
PC	proprotein convertase
PCSK	proprotein convertase subtilisin kexin
PCR	polymerase chain reaction
PD	pars distalis
phospho-ERK	phosphorylated extracellular signal-regulated kinase
PI3-kinase	phosphatidylinositol 3-kinase
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PPD	proximal pars distalis
PRL	prolactin
PSI-Blast	position specific iterative BLAST
PST	pacreastatin
PTH	parathyroid hormone
PVDF	polyvinylidene difluoride
PWS	Prader-Willi syndrome
RAF	v-raf-1 murine leukemian viral oncogene
Ras	related RAS viral oncogene homolog
RIA	radioimmunoassay

RNA	ribonucleic acid
RPD	rostral pars distalis
Rt	retention time
RT-PCR	reverse transcriptase polymerase chain reaction
SELDI-TOF	surface enhanced laser desorption ionization time of flight
S.E.M.	standard error of mean
SF-1	steroidogenic factor-1
sGnRH	salmon gonadotropin releasing hormone
Sg	secretogranin
SgII	secretogranin II
SOS	son of sevenless
SN	secretoneurin
Tag	T-antigen oncogene
TBS	tris-HCl buffer
TFA	trifluoroacetic acid
VEGF	vascular endothelial growth factor
VS	vasostatin
VSCC	voltage-sensitive Ca^{2+} channels

CHAPTER 1: General Introduction

** Major contributors for this chapter*

E Zhao: Original ideas, major writing and organization.

Dapeng Zhang: Making the figures of phylogenetic trees and multiple alignments for granins.

1.1. Rationale and hypotheses of the thesis project

In my MSc research prior to this PhD work, I mainly explored the effect of secretoneurin (SN) on luteinizing hormone (LH) release *in vitro* and also its production in goldfish pituitary. First of all, I observed that SN not only had a direct stimulatory effect on LH release in static incubation of goldfish pituitary fragments but also induced a significant increase in LHβ subunit messenger RNA (mRNA) levels in pituitary fragments (Zhao et al., 2006a). These data provided the first *in vitro* evidence for a direct stimulatory action of SN on LH release and generation in the vertebrate pituitary, suggesting a potential reproductive role for SN. In order to conduct further in depth functional study of SN *in vitro*, we generated a new polyclonal antiserum against goldfish SN, and demonstrated for the first time the existence of two forms of secretogranin II (SgII)-derived protein (~57 and ~30 kDa) that contain the same SN sequence in goldfish pituitary. I also detected the presence of SN-immunoreactive product (~20 kDa) in hypothalamus. Moreover, my study revealed that levels of a major SgII-derived protein containing SN (~57 kDa) were higher in the pars distalis (PD, the anterior pituitary) than in the neurointermediate lobe (NIL, the posterior pituitary) of the goldfish pituitary (Zhao et al., 2006b). This was in consistent with the results obtained from a previous study indicating that SgII mRNA levels are consistently higher in the PD than in the NIL of goldfish (Samia et al., 2004). High production of SgII-derived proteins in the PD

supported the fact that goldfish SN plays an important role for PD function, namely LH release. Although my previous studies provide some primary evidence about the roles of SN in the physiological modulation of LH release and SgII processing, many questions remain unanswered and require proper interpretation. **In this thesis, I hypothesize that SN is a newly-identified hormone that regulates reproductive process in vertebrates.** To directly address this hypothesis, I attempted to answer the following major questions in my PhD research:

1. Is free SN peptide truly produced and secreted in the pituitary and brain?

2. Does the pituitary generated endogenous SN control gonadotrophs to secrete LH? Which cells produce and secrete SN in the goldfish pituitary? Does endogenous SN stimulate LH in a paracrine or autocrine manner?

3. Are SN-associated endocrine effects conserved during evolution? Does the stimulatory activity of SN on LH release and synthesis exist in mammalian pituitaries?

1.2. Overview of the thesis

My PhD studies were directed mainly to explore the above underlying themes by investigating SN-levels in the neuroendocrine system using dispersed goldfish pituitary cells and mouse LβT2 cell line. To answer the first question, I made efforts to detect free SN peptide in goldfish brain and pituitary. At first, I utilized reversed phase-HPLC method to isolate and purify free SN peptide from the extract of goldfish whole brain. The identity of the isolated peptide was fully confirmed by mass spectrum, endoproteinase Lys-C digestion and C-terminal sequence studies. In addition, I also found that this neuropeptide is present in the goldfish pituitary by using western blot analysis and high-density SDS-PAGE gel. These data

are the first isolation of free SN peptide in goldfish brain and pituitary and are presented in one part of Chapter 2 of the thesis. Another part of this chapter describes the detection of immunoreactive SN protein located in goldfish pituitary and brain. This study was carried out in collaboration with Dr. A.O.L. Wong and Dr. G.M. Somoza. The immunocytochemistry experiment demonstrated the presence of SN-immunoreactivity (IR) in prolactin (PRL) cells of the rostral pars distalis (RPD) of the anterior pituitary as well as in nerve fibers of the NIL part, namely the posterior pituitary. In addition to pituitary tissue, high SN-immunoreactive signals were also detected within magnocellular cells of nucleus preopticus (NPO) region in the goldfish brain. Based on these findings, I speculated that: 1) brain-associated SN peptide might play a neuroendocrine role on pituitary gonadotrophs via nerve fibers through the pituitary stalk and on the innervation of the neurointermediate and anterior lobes; 2) SN from lactotrophs (PRL cells) might regulate LH release within the pituitary, which is further investigated in Chapter 3. In Chapter 2, I also tested the SN stimulatory effects on LH using radioimmunoassay in another *in vitro* model, static culture of primary dispersed goldfish pituitary cells. This model system avoids interference from the residues of neuropeptides and neurotransmitters in comparison with static incubation of pituitary fragments in my previous study. The pituitary cells were quantified and cultured for static treatments with various levels of synthetic goldfish SN in order to determine whether SN has a direct stimulatory effect on LH release without any effect of other hypothalamic factors. Two individual experiments with different densities of pituitary cell showed that 1-10 nM SN is able to stimulate significantly LH release from gonadotrophs. Furthermore, an immunoneutralization experiment demonstrated the potential involvement of endogenous SN on LH regulation. The results concerning the generation, tissue-distribution and LH-regulatory activities of the SN peptide

are shown in Chapter 2. This provides the necessary theoretical foundation for further studies of this hormonal peptide in the brain-pituitary-gonad axis.

To further investigate LH secretory responses to SN, I performed a time-course study and found that SN induced significant increments of LH release after 6-hour and 12-hour static incubations of dispersed goldfish pituitary cells. Using real time RT-PCR, I demonstrated that SN slightly but significantly up-regulated the gene expression of common gonadotropinα, LHβ and FSHβ subunits. To prove the possibility of SN yielding in PRL cells under the control of hypothalamic factors, I performed another immunoneutralization experiment to investigate gonadotropin-releasing hormone (GnRH)-induced endogenous SN generation for LH regulation in the pituitary cells. The results showed that co-incubation with anti-SN antiserum partially blocked the stimulatory effect of salmon GnRH on LH increment value, suggesting the potential involvement of SN in GnRH-induced LH release. Furthermore, utilizing western blots and real time RT-PCR, I found that 12-hour incubation with GnRH stimulated the gene and protein expression of SgII (and/or its derived peptides) and PRL in goldfish pituitary cells during the sexual redevelopment season, and also there exists a significant correlation between the levels of the two proteins. The above results presented in Chapter 3 mainly addressed the second question based on my original hypothesis and affirmed that endogenous lactotroph-generated SN might exert a paracrine action on neighboring gonadotrophs to regulate LH release.

SN-IR was also detected in the gonadotrophs of mammalian pituitary (Vallet et al., 1997; Bassetti et al., 1990). Appendix I describes the possible SgII processing to produce SN in human and rat pituitary tissues. In order to answer the third question whether SN still conserved the regulatory effect on gonadotropin secretion in mammalians during evolution, I

adopted the mouse LβT2 gonadotropin cell line for the further study of mouse SN (mSN)-stimulated activity on LH in Chapter 4. The LβT2 cell line, generated by tumorigenesis in transgenic mice, exhibits functional characteristics consistent with those of normal pituitary gonadotropes such as LH secretion via a regulated pathway and changes in GnRH-receptors and LHβ gene expression in response to signaling by GnRH and steroid hormones (Turgeon et al., 1996). Moreover, there is a close correlation between LH and SgII storage and secretion under the regulation of GnRH in LβT2 cells (Nicol et al., 2002 and 2004). The static incubation conditions and SN concentrations for LβT2 cells were similar to those of goldfish pituitary cells. Mouse LH was measured by radioimmunoassay, in collaboration with Dr. A.S. McNeilly at the Queen's Medical Research Institute (UK). The expression changes of gonadotropin-associated genes were detected by real-time RT-PCR. I found that SN is able to up-regulate the release and production of LH in mouse pituitary cells. For clarifying the possible signaling transduction pathways to mediate LH secretion, I explored modifications of the SN-induced mitogen-activated protein kinases (MAPK) cascade which can be stimulated by GnRH in LβT2 cells (Liu et al., 2002). Western blot was utilized to analyze the protein expression of the one major MAPK subfamily, extracellular signal-regulated kinase (ERK). Similar to GnRH, mSN is capable of activating ERK for increasing LH secretion from mouse pituitary cells after acute stimulation. In addition, the possible step of SgII processing was revealed for SN production in mouse LβT2 gonadotrophs, using anti-goldfish SN antiserum (Zhao et al., 2006b). Moreover, GnRH was demonstrated as a regulator to induce SN production in this cell line.

Here, Chapter 1 describes a general introduction of background knowledge for my thesis, mainly focusing on reviewing the evolution and endocrine functions of granin-derived

peptides. In collaboration with Dr. D. Zhang, a previous PhD student in our lab, we used gene cloning and genome searches to reveal for the first time the existence of two paralogs of teleost SgII precursors that we name SgIIa and SgIIb (Fig. 1.1). Furthermore, we identified two teleost SN type A and B in some species, briefly SNa and SNb (Fig. 1.3). We also discussed the immunological distribution and biological activities of major peptides derived from the conserved parts of granin sequences, especially those linked to growth, glucose metabolism and reproduction of various vertebrates. The major part of this introduction has been published in a review paper (Zhao et al., 2009b).

In the last chapter of this thesis (Chapter 5), I summarize the major studies conducted during my PhD period concerning SN production and function in goldfish and mouse pituitary cell models. Until now, neither the SN receptor nor the mechanism of action of SN has been identified in the neuroendocrine system. For preparing the further studies, I not only reviewed the reported mechanisms concerning SN functions in the immune and inflammatory system, but also speculated the possible signal transduction pathway to mediate LH generation and secretion activated by the potential SN receptor in this chapter. This work has been published as part of an invited review article in Regulatory Peptides (Zhao et al., 2009c).

1.3. Granins and their products

The granins are a group of acidic, heat-stable proteins widely distributed in secretory granules of endocrine cells and neurons (Huttner et al., 1991). They are involved in regulating the secretory pathways of their co-localizing target neuropeptides and hormones (Blázquez and Shennan, 2000). They were first discovered in the chromaffin cells of the adrenal medulla by Blaschko et al. in 1967. At present, the granin family can be loosely divided into two

groups, the chromogranins (Cg) and the secretogranins (Sg), distinguishable by the presence of a disulfide-bonded loop at the N-terminus of Cg but not in Sg proteins (Taupenot et al., 2003; Helle, 2004). The chromogranin family is currently composed of chromogranin A (CgA) and chromogranin B (CgB) while the secretogranin group includes seven members namely secretogranin II (SgII), secretogranin III (SgIII or 1B1075), secretogranin IV (SgIV or HISL-19), secretogranin V (SgV or 7B2), secretogranin VI (SgVI or NESP55) (Montero-Hadjadje et al., 2008), SgVII (VGF) (Helle, 2004) and proSAAS (Fricker et al., 2000). Chromogranins possess relatively high amino acid and cDNA sequence similarities among different vertebrate species, indicating that these granins share well-conserved characteristics (Sato et al., 2000; Ait-Ali et al., 2002). The partial chromogranin A sequence encoding peptide functions as an "on/off" switch that controls dense-core secretory granule biogenesis and hormone sequestration in endocrine cells. In the absence of CgA-driven granule biogenesis, expression or storage of other granule components is diminished in PC12 endocrine cell line (Kim et al., 2001). However, SgII is poorly conserved in comparison with CgA and CgB. Only a short peptide known as SN in the central core region of SgII shows a high sequence similarity among the vertebrates (Blázquez et al., 1998a).

Granins serve as a precursor protein that can be proteolytically processed by prohormone or proprotein convertases (presently called Proprotein Convertase Subtilisin Kexins or PCSKs) (Chrétien et al., 2008; Seidah et al., 2008) at multiple cleavage sites to produce several bioactive small peptides (Natori and Huttner, 1994). The first reported peptides generated from granins were detected in the pancreatic β cell for CgA-derived peptide pancreastatin, in the bovine parathyroid cells for CgB_{1-41} and in the rat striatum for SgII-derived peptide SN (Helle, 2004). With further investigations, numerous granin-derived

peptides were identified among various species, suggesting that they are the most conserved parts in the granin squences and exert multiple biological activities. The most important functional peptides produced from granins are vasostatin I (bovine CgA_{1-76}), vasostatin II (bovine CgA_{1-113}), pacreastatin ($CgA_{240-288}$), bovine CgB_{1-41}, and secretoneurin (rat $SgII_{154-186}$ and goldfish $SgII_{154-187}$), among others (Taupenot et al., 2003; Blázquez et al., 1998a)

In this chapter, I will explore the evolution of the main members of the granin family, primarily focusing on comparison between mammals and teleosts. Based on their well-conserved characteristics in the precursor sequence, several granin-derived peptides will be considered across various vertebrate species. We focus on immunological and biological investigations of the endocrine system, especially those linked to growth, glucose metabolism and reproduction of various vertebrates. Previously, a number of excellent review articles on granins have been published but they are now considered as old (Huttner et al., 1991; Hendy et al., 1995), focused on specific members or functions such as sorting/biogenesis (Takeuchi and Hosaka, 2008), secretion (Tota et al., 2007) and their clinical significance as biomarkers (Feldman and Eiden, 2003). Therefore there is a great need for a more updated review covering recent advancements in the field focusing especially on their evolution, proteolysis and biological endocrine functions.

1.4. Revisiting the evolution of granin proteins

1.4.1. Lack of evidence for evolutionary relationships among the granins except for the chromogranin group

Although the granin family is generally considered as consisting of nine members, so far no extensive study has been conducted to clarify their evolutionary relationships and

conserved characteristics. Here we utilized comprehensive sequence analysis, phylogenetic tree construction and genomic information to elucidate these questions. Unexpectedly, our results show that except for the chromogranin group (CgA and CgB), there is no clear evolutionary relationship between the other granin groups. This is first revealed by position specific iterative BLAST (PSI-Blast), a sensitive sequence retrieval method (Altschul et al., 1997). Searching with either CgA or CgB retrieves the homologues of both CgA and CgB. However searching with other granins only retrieves similar within-group members. Secondly, we failed to detect any global similarity in the granins outside the CgA-CgB grouping using a profile-profile alignment strategy implemented in LogoMat-P program (Schuster-Bockler and Bateman, 2005). Furthermore, when the MEME motif discovery tool (Bailey and Elkan, 1994) was employed, no putative random conserved elements could be defined in granins outside the CgA-CgB grouping. Differences in species distribution and coding exon composition between these groups also indicate their different evolutionary origins (Table 1.1). In comparison to other granins that have multiple exons, both SgII and NESP55 genes have only one protein-coding exon. In contrast to NESP55 and VGF (a neurosecretory protein to regulate secretion of neurotransmitters; Possenti et al., 1989) which are restricted to mammals, CgA, CgB, SgII and SgIII all can be identified from fish to mammals, whereas SgV shows a phylogentically wider distribution being also found in invertebrates (Table 1.1). Taken together, these approaches suggest that there is no similarity in amino acid sequences between the granins except for the CgA/CgB group. The previously reported relationship between CgA/CgB and SgII, as well as between SgIII and SgV may be artifactual (Montero-Hadjadje et al., 2008; Helle, 2004). Notably, the CgA/CgB and SgII have been long thought to constitute a domain family by Pfam database; however, our analysis indicates that a

revision of this annotation is required. The incorrect grouping of seven members into one single family probably resulted from the high percentage of acidic residues in many of the granins. Another common character of these granins is their rigid structure, which might account for the relatively easy access for cleavage by proprotein convertase (PC) (Seidah and Chretien, 1999). These latter two characteristics, however, are not reflected in major sequence conservation, and thus the five so-called secretogranins (SgII-SgVI) do not share a common ancestor. As one example, the SgV gene codes for a polypeptide of ~185-220 amino acids in worm, fly, sea urchin and vertebrates. While it is certainly involved in endocrine function because it behaves as a chaperone and regulates prohormone convertase 2 activity (Mbikay et al., 2001), SgV (more commonly known as 7B2 protein) is clearly evolutionarily unrelated to the other secretogranins.

1.4.2. Two duplication events in CgA and SgII groups

In collaboration with Dr. D. Zhang, a previous PhD student in our lab, I updated the species distributions for each granin using PSI-Blast searching in the NCBI non-redundant database. Interestingly, we retrieved two CgA homologues in African clawed frog and two SgII homologs in zebrafish. Further phylogenetic analysis revealed two gene duplication events with high confidence (Fig. 1.1). The SgII duplication could be traced to the ancient teleost lineage that has long been recognized to have experienced a global genome duplication event (Amores et al., 1998). Goldfish SgII belongs to SgII subtype A (SgIIa) whereas pufferfish and grass carp SgIIs belong to SgII subtype B (SgIIb). Whether there exists another SgII subtype or if there is differential deletion in these fish species requires further investigations.

1.4.3. The conserved peptides derived from CgA, CgB and SgII

Multiple sequence alignment offers information on conservation of homologous sequences, which may reflect conservation of function. We utilized this strategy to define the conserved element for three granin proteins, CgA, CgB and SgII. Figure 1.2 shows the conserved elements that have been mostly studied. Some conserved elements are defined by adjacent conserved dibasic cleavage sites (**KK, RR, KR,** or **RK**), such as CgA-derived WE14 and catestatin, and SgII-derived SN. However, most of the putative peptides are generated by cleavage at relatively variable cutting sites or multiple cutting sites. Relatively variable cutting sites or cutting site shifts may indicate that the length of a given peptide is not a major determinant of function; whereas multiple cutting sites may guarantee the effective cutting by prohormone or proprotein convertases (PCs). As a result, diverse functional peptides are derived but harboring common conserved sequences, as exemplified by VS-I, VS-II and beta-granin in the CgA group.

Owing to the role of selected prohormone convertases in the processing granin proteins, it may be worthwhile to provide a brief introduction about these enzymes. The PCs are a group of endoproteolytic enzymes that belong to the mammalian subtilisin-kexin family. So far 9 members of this family have been discovered of which 7 are basic amino acid cleaving yeast kexin types. These PCs are capable of cleaving the granin precursor to generate small to intermediate-sized peptide fragments with potential biological activities. The nine PCs are PC1/PC3/PCSK1, PC2/PCSK2, furin/PACE/PCSK3 PC4/PCSK4, PC5/PC6 (PCSK5), PACE4 (PCSK6), PC7/PC8 (PCSK8) and NARC-1/PCSK9. Kexin-like PCs cleave peptide bonds at the carboxy terminal of Arg residue characterized by the sequence motif

R/K/H-X-X/K/R-R↓ (Seidah et al., 1994; Seidah and Chretien, 1999). The endocrine convertase PC1 has been shown to be the enzyme that is involved in tissue-specific processing of SgII to generate the neuropeptide SN in neurons (Hoflehner et al., 1995). It was also reported that the closely related PC2 enzyme also generates free peptides from granins, for example cleaving CgB to generate PE-11 in rat pituitary GH_3 cells; but PC2 failed to generate SN from SgII precursor in mouse pituitary GH_4C_1 cells (Hoflehner et al., 1995; Laslop et al., 1998). However, the immunoreactive signals of PC1 and PC2 within the large CgA-containing lucent granules of rat pituitary gonadotrophs indicated that these two enzymes participate in the the proteolytic processing of CgA (Uehara et al., 2001). Furthermore, PC1 was demonstrated to be an important enzyme for generating pancreastatin derived from CgA precursor in the human pancreatic carcinoid cell line (Udupi et al., 1999).

In the chromogranin group, the most highly conserved amino acid sequences are identified in the N- and C-terminal regions of the granin proteins (Fig. 1.2A-D). With respect to the CgA protein sequences in vertebrates, the C-terminal 314-430 amino acid region and N-terminal 177-amino acid region are over 80% similar among the mammals. Moreover, the N-terminal and C-terminal of CgA in frogs show 66.2% and 39.0% identity to those of horse CgA, respectively (Sato et al., 2000). Short peptides derived from N- and C- terminal domains of CgA, such as vasostatin, WE-14 and catestatin, might have conserved bioactivities, which we discuss in more detail in Section 1.5.1. Chromograin B was detected in several species of arthropods, fish, amphibians, birds and mammals. It has been found to possess a well-conserved molecular size, isoelectric point and common immunological epitopes (Rieker et al., 1988). While bovine CgB has 67%, 58% and 58% sequence identity with that of human, rat and mouse, respectively (Grandy et al., 1992), the entire CgB amino

acid sequence from frogs has only a 35%-40% overall identity as compared to those of other mammals. The N- and C-terminal domains of CgB were more similar (57%-65%) between frogs and mammals (Ait-Ali et al., 2002). Several peptides generated from the two termini of CgB proteolytic processing were identified in different vertebrate nervous and endocrine tissues; mainly including CCB [$CgB_{597-653}$, including secretolytin ($CgB_{614-626}$)], BAM-1745 (bovine $CgB_{547-560}$) and CgB_{1-41}, derived from the well-conserved C- and N- terminal ends (Helle, 2004; Montero-Hadjadje et al., 2008).

Secretogranin-II is a well-investigated granin protein in the secretogranin group. The primary sequence of mammalian SgII reveals a 79-87% identity between species (Fischer-Colbrie et al., 1995); however, the SgII sequences of some non-mammalian vertebrates appear to be very different from the mammalian SgII suggesting a low degree of conservation (Blázquez et al., 1998a). The middle domain of SgII shows a comparatively high sequence similarity relative to the entire SgII precursor among various vertebrate species (Fig. 1.2E, and Montero-Hadjadje et al., 2008), from which several functional peptides such as SN and EM66 have been generated. These two SgII-derived peptides have a wide distribution in the rat nervous and endocrine systems, including central nervous system, anterior pituitary, pancreas, and adrenal gland, suggesting their potential biological activities in these organs (Leitner et al., 1996; Anouar et al., 1998).

Free SN peptide is generated from the well-conserved sequence of the SgII precursors by cleavages at the carboxy (C-) terminal of a pair of basic amino acids (KR or RK). The entire SN sequence exhibits a high sequence similarity among the mammalian species, such as mouse, pig, hamster, rat and human (Kähler et al., 2000). SN is somewhat conserved in some non-mammalian species; for example, amphibian SN has two regions that are well

conserved and identical to those of mammalian SN. However, the 34-amino acid goldfish SN in general shares a relatively poor identity with the 33-amino acid SN in mammals. Only several stretches in the middle region of SN are well conserved, whereas its C-terminal end varies considerably between teleosts and tetrapods (Fig. 1.3). Moreover, gene cloning and genome searches reveal the existence of two paralogs of teleost SgII precursors, SgIIa and SgIIb. We place those sequences related to goldfish SgII in family "a" because the first SgII identified in teleosts was cloned from goldfish pituitary (Blázquez et al., 1998a). On the other hand, grass carp and pufferfish SgIIs belong to the SgIIb family. For example, both SNa and SNb have been found in some species (Fig. 1.1). Multiple sequence alignment offers information on conservation of sequences between different teleost SN subtypes and tetropod SN (Fig. 1.3). Also shown in the figure is the only shark SN peptide so far identified. Regarding teleost SNa, one domain in the N-terminus and two domains in the middle region including the sequences "TNE", "QYTP" and "LATLEQSVFE(Q)EL" are identical to the mammalian SN. However, only two stretches in the middle of teleost SNb [EQYTPQSLA and FE(Q)ELG] are conserved in comparison to the mammalian SN. The origins of 2 SgII precursors and thus the 2 SNs in teleosts likely arose because of gene duplication events in that lineage. In zebrafish, the SgIIa gene is located on chromosome 15 (43024170-43025789) and SgIIb gene on chromosome 2 (54110611-54112398). Comparison of zebrafish SNa and SNb indicates that only 13/33 (40%) amino acids located in the middle core are conserved. The biological activity of SNb has yet to be determined but is predicted to be different from that of SNa.

1.5. Functional aspects concerning the peptides generated from the chromogranin group

1.5.1. CgA-derived peptides

The CgA group is a family of highly acidic proteins that are co-stored and co-released with catecholamine in secretory granules of the adrenal gland (Hendy et al., 1995). Human CgA is a 439-amino acid long protein preceded by an 18-residue signal peptide. The CgA gene comprises 8 exons and 7 introns spanning approximately 11 kB. Three of its introns show some evolutionary conservation because their length and sequence are similar to a comparable region of another chromogranin member, CgB (Wu et al., 1991). CgA has been detected in tissues of several vertebrate species. For example, CgA-like proteins have been detected in the ultimobranchial glands and corpuscles of stannius in rainbow trout using immunohistochemistry (Deftos et al., 1987), the endocrine pancreas and intestine in amphibians and reptiles (Trandaburu and Ali, 1998; Trandaburu et al., 1999a and 1999b), the gizzard of chicken (Salvi et al., 1995) and the frog pituitary (Peinado et al., 2006). Regarding its distribution in mammalian endocrine cells, CgA mRNA has been detected in horse adrenal medulla, pituitary, spinal cord, liver, thyroid gland, striated muscle, lung, spleen, kidney, parotid gland and sublingual gland (Sato et al., 2000). Furthermore, there is a significant amount of CgA in the brain with the highest concentrations of CgA being found in the hypothalamus, amygdala/piriform cortex and hippocampus of rats (Weiler et al., 1990). CgA-related proteins/polypeptides have been identified in teleost endocrine glands, including pituitary, corpuscles and gastroendocrine cells (Deftos et al., 1987; Barrenechea et al., 1994). CgA is highly expressed in human neuroendocrine tumors, such as nonfunctioning tumors of the endocrine pancreas and medullary thyroid carcinomas (Nobels et al., 1997). In the adult male frog *Rana ridibunda*, CgA gene expression was detected in the pituitary, the whole brain,

the hypothalamus, the spinal cord, and the adrenal gland, suggesting its potential regulatory activities in the neuroendorine system (Turquier et al., 1999). In functional studies, CgA displays an autocrine inhibition of the secretion of proopiomelanocortin-derived peptides in mouse corticotropic cells in a concentration-dependent fashion (Wand et al., 1991). This chromogranin also suppresses cholecystokinin-induced amylase secretion from the human exocrine pancreas (Funakoshi et al., 1988) and inhibits acid secretion from parietal cells in the rabbit stomach (Lewis et al., 1988). These findings suggest that CgA may be important in controlling the endocrine and neuroendocrine systems in vertebrates.

1.5.1.1. Vasostatin and β-granin

Vasostatins (VS) are a group of 7-22 kDa polypepeptides derived from highly conserved N-terminal fragments of CgA and originally named for their vascular inhibitory bioactivities in human blood vessels (Fig. 1.1A and 1.2A, and Aardal et al., 1993). Two main types of vasostatins are defined as VS-I (CgA_{1-76}) and VS-II (CgA_{1-113}), which have been isolated and identified from the chromaffin granules of bovine adrenal medulla (Bauer et al., 1997). In addition, several short VS peptides, such as CgA_{1-40}, CgA_{7-57}, CgA_{47-66} and CgA_{67-76} were found to be present in the bovine chromaffin granules and co-secreted with catecholamines (Tota et al., 2007). These N-terminal fragments of CgA exhibit multiple biological activities that are summarized as: 1) inhibiting microbial growth (Lugardon et al., 2000), 2) relaxing blood vessels (Aardal et al., 1993) and hearts (Mazza et al., 2007), and 3) influencing hormone release and tumor growth in the endocrine system.

Parathyroid hormone (PTH) is secreted from the parathyroid gland when circulating calcium levels are low. The CgA precursor is located in parathyroid secretory granules and an

N-terminal VS fragment (26 kDa) is released in response to a low level of calcium ion (Ca^{2+}), indicating the co-storage and co-secretion of VS and PTH in the bovine (Drees and Hamilton, 1994). Moreover, the VS peptide has a paracrine and/or autocrine inhibitory effect on the low Ca^{2+}-responsive secretion of both PTH and CgA from dispersed bovine parathyroid cells (Drees et al., 1991). Russell et al. (1994) also reported that several N-terminal fragments of CgA markedly suppressed bovine PTH secretion in response to low extracellular Ca^{2+}. In comparison with CgA_{17-38}, CgA_{1-40} and CgA_{1-76} (VS-I) exhibited a potent inhibitory effect on PTH release; this biological activity is significantly reduced by disruption of the single disulfide bond between Cys_{17} and Cys_{38}. The CgA_{16-40} sequence is the most active part of VS in inhibiting the secretion of the bovine parathyroid cells *in vitro* (Angeletti et al., 1996).

Strong immunoreactivities of VS-I and VS-II were detected in rat adrenal PC12 phechromocytoma cells, suggesting the presence of the N-terminal CgA proteolytic products (VSs) in the neuroendocrine tumor (Cunningham et al., 1996). Moreover, VS-I and VS-II were expressed in various human pituitary adenoma and carcinoma, but with notably lower levels in the functional tumors of lactotrophs, adrenocorticotrophs, and somatotrophs than the nonfunctional gonadotroph tumor (Jin et al., 2003). In a study of neuroendocrine tumors in lungs, VS and other CgA-derived peptides were found to be general markers because of their different expression among various human lung neuroendocrine tumors. Specifically, CgA_{17-38} (a VS fragment) immunoreactivity was highly expressed in two kinds of neuroendocrine tumors, typical carcinoid and atypical carcinoid (Portela-Gomes et al., 2005). Recently, Belloni et al. (2007) demonstrated that VS-I suppressed the proangiogenic activity of vascular endothelial growth factor (VEGF) in human umbilical endothelial cells, indicating the potential inhibitory effect of VS on tumor growth, via depressing endothelial proliferation and

migration, or reducing the supply of nutrients and growth factors in endothelial cells. There are no reports concerning the endocrine effects of any VS peptides in teleosts. However, VS peptides exerted negative ionotropic action in fish heart (Imbrogno et al., 2004) which is similar to the human VS-I inhibitory effect of cardiac contraction and relaxation (Tota et al., 2008).

Derived from the CgA N-terminal fragment, β-granin (CgA_{1-128}) is highly similar between mammalian species. This CgA-derived peptide was demonstrated to be the 21-kDa granin protein distributed in adrenal medulla and secreted from the insulin granules, which suggested its potential bioactivities in the endocrine system (Hutton et al., 1985). β-granin is also highly expressed in rat pancreas, anterior and posterior pituitary, gastric antrum and small intestine, suggesting potential biological activities in the endocrine system (Hutton et al., 1988). However, there are few reports concerning the bioactivities of β-granin. Co-localized with insulin in the mouse pancreatic islet, β-granin showed an inhibitory effect on the glucose and potassium evoked insulin secretion via decreasing the Ca^{2+} influx *in vitro* (Schmid et al. 2007). In addition, β-granin was expressed in both luteinizing hormone (LH) and follicle stimulating hormone (FSH) producing pituitary cells in the male and dioestrous female rat, indicating that this CgA-derived peptide might potentially modulate the function of the brain-pituitary-gonad axis in the reproductive system (McVicar et al., 2003).

1.5.1.2. Catestatin

Catestatin (CST, bovine $CgA_{344-364}$ and human $CgA_{352-372}$) is generated from the C-terminus of CgA, and named as an endogenous inhibitor controlling nicotine-induced catecholamine secretion from chromaffin cells and neurons (Fig. 1.1A and 1.2B) (Montero-

Hadjadje et al., 2008; Preece et al., 2004). This peptide plays an inhibitory role in neuroendocrine secretion as a non-competitive antagonist at the nicotinic receptor on the rat chromaffin cell surface (Taupenot et al., 2000). Consistent with findings in rat, CST was capable of reducing the cellular Ca^{2+} signal and total secretion of catecholamine stimulated by acetylcholine (Ach) in the mouse chromaffin cell, indicating its interaction with different native nicotinic Ach receptor subtypes (Herrero et al., 2002). Three variant human CST peptides identified as Gly364Ser, Pro370Leu and Arg374Gln exhibited different inhibitory abilities on catecholamine secretion from rat PC12 pheochromocytoma cells. The rank order of their inhibitory potencies was Pro370Leu > wild type > Gly364Ser > Arg374Gln (Mahata et al., 2004). Furthermore, the CST Gly364Ser variant inhibits catecholamine secretion from human cultured neurons (Rao et al., 2007). Kennedy et al. (1998) first demonstrated that CST showed both vasodepressor and sympathoadrenal effects in rat via increasing endogenous histamine release and activating H1 receptors. Such CST stimulatory potency on histamine secretion was also shown uniquely in rat peritoneal and pleural mast cells, in comparison with other CgA-derived peptides (Krüger et al., 2003).

1.5.1.3. EL35

EL35 (human $CgA_{402-436}$, rat $CgA_{411-445}$ and frog $CgA_{344-378}$) is another well-conserved peptide derived from the C-terminal domain of CgA (Fig. 1.1A and 1.2B),,which shows 70-97% amino acid sequence identity between various vertebrate species; this peptide has 35 amino acids with glutamic aid (E) and leucine (L) at the N- and C-teminus (Montero-Hadjadje et al., 2002). There are few reports concerning the bioactivities of EL35, whereas the presence of EL35 in rat pituitary (gonadotropes and melanotropes), human adrenal gland

(adrenomedullary cells) and human pheochromocytoma suggests that this peptide plays an endocrine role (Montero-Hadjadje et al., 2002).

1.5.1.4. WE-14

WE-14 is a 14 amino-acid long peptide derived from the proteolytic processing within the C-terminal fragment of CgA (Fig. 1.1A and 1.2B). It has tryptophan (W) and glutamic acid (E) at the N- and C-terminus respectively, and was first isolated from a human ileal carcinoid tumor (Fig. 1.1A and 1.2B, and Curry et al., 1992). The WE-14 peptide shows 93% identity among human, bovine, rat, mouse and pig (Curry et al., 1992) and an antibody against bovine $CgA_{316-329}$ cross-reacted with pig, rat and chicken (Rieker et al., 1988), suggesting the high degree of conservation. WE-14 is widely generated and distributed in multiple endocrine systems. Immunohistochemical studies in the human and bovine using the antiserum against human $CgA_{315-321}$ (a C-terminal region of WE-14) showed that WE-14 was derived from CgA precursor in either normal pancreas gland and gastrointestinal system, or neuroendocrine tumors, such as ileal carcinoid and insulinomas (Gleeson et al., 1996). During early porcine fetal development, WE-14 immunoreactivity was detected in sympathetic ganglia and sympathoadrenal cells (Barkatullah et al., 2001). Furthermore, WE-14 is present not merely in distinct subpopulations of CgA-containing normal cells, especially in the pituitary, adrenal and parathyroid glands (Curry et al., 2002), but also in neuroendocrine tumorous tissues, such as human pheochromocytoma (Montero-Hadjadje et al., 2002). The physiological roles of WE-14 remain unclear. Forsythe et al. (1997) demonstrated that WE-14 increases histamine release induced by IgE antiserum and compound 48/80 in rat peritoneal mast cells but does not affect basal histamine secretion. Different from that of β-granin, a

weak immunoreactivity of WE-14 was discovered in only FSH producing gonadotrophs of the rat anterior pituitary during proestrous and oestrous, corresponding with the lowest cellular FSH level (McVicar et al., 2003), suggesting that CgA-derived peptide may play an important role in regulating vertebrate reproduction.

1.5.1.5. Pancreastatin

Pacreastatin (PST, $CgA_{240-288}$) is a 49 amino acid peptide located in the middle of CgA, showing a low sequence similarity in vertebrate species (Montero-Hadjadje et al., 2008). However, this CgA-derived peptide exerts several hormone-like regulatory effects in the endocrine system. It was first isolated and identified in the porcine pancreas, showing a potent inhibitory effect on glucose-induced insulin secretion (Tatemoto et al., 1986). PST might have a role against insulin release by hindering G-protein regulated and Ca^{2+}-mediated exocytosis in the rat pancreas RINm5F cell line (Hertelendy et al., 1996). Furthermore, PST was shown to have an enhancing effect on insulin-induced lipid generation and an inhibitory action on insulin-evoked glucose transport in rat epididymal adipocytes (Sánchez-Margalet and González-Yanes, 1998). It displays an inhibitory effect on insulin-evoked glycogen synthesis in adipocytes that is possibly mediated through a Ser/Thr phosphorylation of the insulin receptor by a staurosporine-sensitive mechanism (Sánchez-Margalet, 1999) associated with a stimulation of GSK-3 activity via activation of protein kinase C (PKC) (González-Yanes and Sánchez-Margalet, 2001). Pancreastatin also plays a regulatory role on lipid storage and metabolism in isolated rat adipocytes by both stimulating UCP-2 expression and inhibiting leptin secretion (González-Yanes and Sánchez-Margalet, 2003). In addition, pancreastatin has an inhibitory effect on growth and proliferation of hepatoma cells through activating NO

synthase to enhance NO production, which leads to activation of phospholipase C-β pathway followed by elevating intracellular Ca^{2+} level and activation of mitogen-activated protein kinase (MAPK) and PKC (Díaz-Troya et al., 2005). In humans, PST and its variants are specifically and potently active in type II diabetes, showing an ability to block glucose uptake (O'Connor et al., 2005).

In addition to its role in insulin release, pancreastatin has additional effects on hormone secretion from other CgA-positive endocrine tissues that express this peptide. Firstly, PST inhibits PTH release and production in an autocrine and/or paracrine fashion. In porcine and bovine parathyroid cells, PST down-regulates the secretion of both CgA and PTH under low Ca^{2+} conditions, indicating its physiological roles in regulation of parathyroid gland in mammals (Fasciotto et al., 1989; Drees and Hamilton, 1992). Furthermore, Zhang et al. (1994) demonstrated that PST dose-dependently inhibited the release and expression of PTH and CgA. Secondly, PST regulated catecholamine (epinephrine/E and norepinephrine/NE) secretion in some situations. In rat, gastroenteropancreatic PST reversibly inhibited epinephrine release from the adrenal medulla, in a dose-dependent manner, without modifying secretion of NE and dopamine (DA), suggesting a neuroendocrine role for PST in stress (Sánchez-Margalet and Goberna, 1993). Moreover, PST might influence catecholamine release from sympathetic nerves in humans with essential hypertension because there was a significant high correlation of PST-like immunoreactivity with NE, but not with E (Sánchez-Margalet et al., 1995). The correlated elevation of plasma levels of PST and NE/E was also determined in gestational diabetes, confirming the potential modulation of PST on catecholamines in humans (Sánchez-Margalet et al., 1998). Thirdly, PST may stimulate glucagon release from the pancreas. In 1987, Efendić et al. demonstrated that PST increased

arginine-induced glucagon release and inhibited glucose-stimulated insulin release from isolated rat islets. This PST action to modify the glucagon/insulin ratio was also discovered *in vivo* in mice (Ahrén et al., 1988). However, two individual labs in Japan and Spain in the same year reported that PST did not alter glucagon secretion from rat pancreas *in vivo* and *in vitro*, indicating more complex regulatory mechanism of PST on glucose level in mammals (Funakoshi et al., 1989; Peiró et al., 1989). Taken together, PST plays a role in the endocrine regulation of hormone (including insulin, glucagon, PTH and catecholamine) secretion.

Immunohistochemcial studies of PST indicate its presence in the gastro-entero-pancreatic neuroendorine system and adrenal glands in various vertebrate species, including teleosts (Reinecke et al., 1991; Reinecke and Maake, 1993). Pancreastatin-immunoreactivity was detected in endocrine cells and nerve fibers in the stomach and intestine of a flatfish, the turbot (*Scophthalmus maximus*) and in some cases was colocalized with other neuroendocrine factors (neurotensin, serotonin, gastrin/cholecystokinin) indicating a potential modulatory role in fish (Reinecke et al., 1997). More studies should be carried out to explore and clarify the physiological roles of PST in fish and other non-mammalian vertebrates.

1.5.2. CgB-derived peptides

CgB is a tyrosine-sulfated chromogranin protein found in a wide variety of peptidergic endocrine tissues. Human CgB is a 76-kDa polypeptide that contains 657 amino acids and is preceded by a cleaved N-terminal signal peptide of 20 residues (Benedum et al., 1987). Similar to CgA, CgB is found in chromaffin granules of bovine adrenal medulla (Grandy et al., 1992). Furthermore, CgB is localized in the terminals of mossy nerve fibers in the human hippocampus (Kandlhofer et al., 2000) and is significantly down-regulated in the dentate

gyrus and entorhinal cortex of the postmortem brains of Alzheimer's patients (Marksteiner et al., 2000). Clinically, in patients with endocrine pancreatic tumors and the multiple endocrine neoplasia 1 syndrome, elevated level of CgB is detected frequently in serum (88% increase) and to a much lesser degree in urine (15% increase) (Stridsberg et al., 1995). Similar to the biological action of CgA, CgB is involved in regulating the release of some peptide hormones. It was reported that a CgB antibody increased the insulin synthesis in the endocrine pancreas. This suggests that this granin, released from islet cells, might have an autocrine inhibitory effect on insulin secretion (Karlsson et al., 2000).

CCB (C-terminal CgB, $CgB_{597-653}$) is a peptide generated from the well-conserved CgB C-terminus, and was first isolated from human pituitary gland extracts (Fig. 1.1A and 1.2D, and Benjannet et al., 1987). This peptide is highly expressed in human glucagonoma tissue and small cell lung carcinoma (Nielsen et al., 1991; Iguchi et al., 1992). Although few CCB functions has been reported, the unique expression of CCB in human neuroendocrine bronchial tumors suggests a neuroendocrine role (Vieau et al., 1991). Wang et al. (2001) demonstrated that CCB was processed to produce another small peptide fragment, secretolytin. Secretolytin ($CgB_{614-626}$) was isolated and identified in the chromaffin granules of bovine adrenal medulla, which was generated via proteolysis of the C-terminus of CgB (Strub et al., 1995). Secretolytin has antibacterial activity due to its α-helical amphipathic structure, which might be of biological importance in the neuroendocrine system (Strub et al., 1995 and 1996). Although there is no report concerning secretolytin distribution in any other vertebrates except mammals, immunohistochemical detection in leech brain (*Theryomyzon tessulatum*) using antiserum against the bovine $CgB_{614-626}$ sequence, suggests its existence in annelids (Salzet and Verger-Bocquet, 2002). BAM-1745 (bovine $CgB_{547-560}$), another 1745-

Da peptide derived from the C-terminus of CgB is the most conserved domain in mammalian CgB (Fig. 1.1A and 1.2D; Flanagan et al., 1990; Grandy et al., 1992). However, distinct biological activities of BAM-1745 as well as CCB remain to be identified in the future. CgB_{1-41} is another conserved peptide derived from the N-terminal of CgB (VS-like peptide, Fig. 1A and 2C). It has inhibitory effects on PTH secretion from bovine parathyroid cells similar to two CgA-derived peptides, VS-I and VS-II (Russell et al., 1994).

1.6. Exploring the main member of the secretogranin group: SgII and its derived peptides

In contrast to CgA and CgB, much less is known about SgII, also known as CgC. Secretogranin II was first characterized in the bovine anterior pituitary (Rosa and Zanini, 1981). It is a ~600-amino acid, very acidic, tyrosine-sulfated protein expressed and typically processed to small peptides in soluble secretory granules of vertebrate endocrine cells (Mahata et al., 1991; Natori and Huttner, 1994; Blázquez et al., 1998a; Fischer-Colbrie et al., 1995). In 1991, the first peptide derived from the middle conserved domain of SgII was isolated and identified in the frog brain (Vaudry and Conlon, 1991). Afterward, another fragment in the SgII sequence was detected in the frog intestine (Trandaburu and Ali, 1998) and the snake enteric serotonin cells using the anti-SgII C26-3 antiserum (Trandaburu et al., 1999b). In addition, another SgII fragment, namely SgII C23-3, was discovered not only within enteric serotonin cells but also co-stored with neurotensin in the endocrine cells in lizards and snakes (Trandaburu et al., 1999b). SgII immunoreactivity is found in gonadotrophs, thyrotrophs and corticotrophs in human pituitaries (Vallet et al., 1997). SgII mRNA is expressed in some parts of rat brain, such as posterior intralaminar thalamic and

medial geniculate nuclei, the nucleus of the solitary tract, and also adrenal medulla (Mahata et al., 1991; Tsunashima et al., 1997). SgII was also observed in a subpopulation of vasopressinergic magnocellular neurons in rat supraoptic and paraventricular nucleus under osmotic stimulation (Ang et al., 1997). Amphibian SgII protein shows 48% sequence identity to that of mammals and was detected in the in *Xenopus laevis* intermediate pituitary (Holthuis and Martens, 1996). Moreover, van Horssen and Martens (1999) demonstrated that 84-kDa SgII precursor was generated in the intermediate lobe of the amphibian pituitary gland and derived to several small peptides, including a major product containing secretoneurin. In goldfish, SgII mRNA has been detected in the pituitary using semi-quantitative PCR and Northern blots (Blázquez et al., 1998a). Although the widespread distribution of SgII suggests many potential biological activities in the vertebrate neuroendocrine system, its putative roles are still unclear.

SgII synthesis and release are regulated by various factors in the neuroendocrine system. The chronic application of vasopressin induces an increase of SgII synthesis in rat brain (Mahata et al., 1992). In bovine chromaffin cells, histamine can significantly increase the SgII mRNA level without any significant effect on CgA and CgB (Bauer et al., 1993). The neuropeptide pituitary adenylate cyclase-activating polypeptide (PACAP) causes a great increment of SgII mRNA level by a rapid and long-lasting stimulation of bovine adrenochromaffin cells (Turquier et al., 2001). SgII is co-localized with LH in the rat anterior pituitary (Watanabe et al., 1991). Estradiol has a direct effect to decrease SgII mRNA steady-state levels in rat pituitary cells (Anouar and Duval, 1992). GnRH up-regulates SgII secretion from gonadotrophs in rat pituitary (Conn et al., 1992), and it was recently reported that there was a close correlation between the GnRH-induced release of SgII and LH in the mouse $L\beta T_2$

gonadotroph cell line (Nicol et al., 2002). The first teleost species from which SgII was cloned and sequenced was the common goldfish (Blázquez et al., 1998a). The γ-aminobutyric acid (GABA) metabolism inhibitor γ-vingl-GABA can specifically stimulate SgII gene expression in goldfish pituitary, concomitant with a decrease of LH cellular content and an increase of serum LH level (Blázquez et al., 1998a). Likewise, treatments *in vivo* with a GnRH agonist increase SgII mRNA expression in the goldfish anterior pituitary (Samia et al., 2004).

1.6.1. Secretoneurin (SN)

SN is the best characterized, among all the possible bioactive products generated from the middle domain of SgII (Fig. 1.1B and 1.2E). In mammals, birds and frogs, SN is 33 amino acid long (Anouar et al., 1996; Leitner et al., 1998) whereas in goldfish, it is a 34 amino acid long polypeptide (Blázquez et al., 1998a). The entire SN molecule is highly conserved among mammalian species, such as human, pig, hamster and rat (Kähler and Fischer-Colbrie, 2000). However, goldfish SN shares a relatively poor identity to mammalian SN; yet two sequences located in the middle part of goldfish SN peptide sequence are identical to those of mammals (Samia et al., 2001). These two domains might be the principal determinants of the biological activity of SN.

Secretoneurin plays an important role in the immune system and is involved in inflammatory responses. SN attracts human monocytes to increase their migration and locomotion in response to injury (Reinisch et al., 1993). This neuropeptide is present in human osteoarthritic joint and its levels decrease in the rheumatoid joint, indicating a function in inflammation (Eder et al., 1997). Furthermore, SN consistently triggers the migration of

endothelial cells via the combination of chemotactic and antiproliferative effects, suggesting that SN acts as a regulatory peptide of vascular cell functions (Kähler et al., 1997).

1.6.1.1. SN is a neuropeptide in mammals

1.6.1.1.1. Expression and localization:

SN is widely distributed in mammalian nervous and endocrine tissues. It is found in human central nervous system, adrenal medulla, thyroid C cells, thyroid stimulating hormone- and FSH/LH-producing cells of the anterior pituitary, A and B cells of pancreatic islets, endocrine cells of the gastrointestinal tract and the bronchial mucosa, and prostate. Particularly, there is a high concentration of SN in human anterior and posterior pituitary (Schmid et al., 1995). The highest levels of this peptide were also detected in rat hypothalamus, especially median eminence (Marksteiner et al., 1993a). Furthermore, SN is found in rodent central and sympathetic nervous systems (Marksteiner et al., 1994) and in parenchyma of the rat pineal gland and along the pineal stalk (Simonneaux et al., 1997). SN-like IR is also detected neurons of the rat hippocampal formation with a preferential localization in excitatory pathways (Schwarzer et al., 1997). The wide distribution indicated the important biological activities of SN in the central nervous system and various endocrine glands.

1.6.1.1.2. Hormonal activity

As a neuropeptide, SN exhibits a complex relationship with other neuropeptides and hormones. Local infusions of SN into the rat substantia nigra and neostriatum both caused an increment of extracellular glutamate and GABA levels in a concentration-dependent manner

(You et al., 1996). Moreover, SN has a stimulatory effect on DA release *in vivo* (Agneter et al., 1995; You et al., 1996). Rat SN and its 15-amino acid C-terminal sequence were determined to stimulate DA release in the striatum of awake rat, indicating that SN has a strong effect on DA secretion *in vivo* (Agneter et al., 1995). *In vitro,* SN stimulates DA release from rat striatal slices in a Ca^{2+} and dose-dependent fashion (Saria et al., 1993). Recently, reduced SgII expression was observed in fibroblasts derived from a female patient with Prader-Willi syndrome (PWS) resulting from maternal uniparental disomy. This observation has implicated SN in the etiology of some defects associated with PWS, which include neonatal hypotonia, hypogonadism, hyperphagia leading to obesity, and mental retardation (Horsthemke et al., 2003). In the pituitary, SN is co-secreted with LH from rat gonadotrophs *in vitro* in response to gonadotropin-releasing hormone (GnRH) (Watanabe et al., 1991; Wei et al., 1995). In the nervous system, preganglionic sympathetic neurons projecting to the superior cervical ganglion contain both substance P and SN, indicating a role for SN in sympathetic nerve activity (Klimaschewski et al., 1995).

1.6.1.2. SN production and function in teleosts

To explore proteolytic processing of SgII in goldfish, a polyclonal antiserum against goldfish SN (gfSN) was generated against a 15-amino acid conserved sequence (YTPQKLATLQSVFEE) in the middle region of gfSN. Our previous study demonstrated the existence of two SgII-derived proteins (~57 and ~30 kDa) containing the SN sequence in goldfish pituitary and potential distribution of a SN-immunoreactive product (~20 kDa) in hypothalamus. The levels of a major SgII-derived protein containing SN (~57 kDa) are higher in the PD (the anterior pituitary) than in the NIL (the posterior pituitary), suggesting that gfSN

was important for PD function, namely LH release to regulate fish reproduction (Zhao et al., 2006a). The other main source of gfSN is the brain. The expression of SgII gene and ~20-kDa SN-IR protein in hypothalamus indicated a potential processing from SgII to generate free SN peptide in goldfish brain (Zhao et al., 2006b). Teleosts do not possess a mammalian-like functional hypothalamo-hypophyseal portal blood system, thus neurons synthesizing neuropeptides and neurotransmitters can directly innervate the anterior pituitary to regulate hormone release (Blázquez et al., 1998b). Thus, we speculate that SN peptide might also be generated in the magnocellular neurons of nucleus preopticus (NPO) and released from nerve terminals in the NIL and PD to regulate pituitary function. Although SN-immunoreactive signals were previously detected in the pituitary and brain, the question whether free SN peptide is actually produced in these tissues remains unanswered. Therefore, the major study of Chapter 2 in the thesis describes the isolation and identification of free SN peptide in goldfish brain and pituitary. In addition, regarding to SgII processing to generate SN, we also wondered whether SgII is cleaved to produce SN in the pituitaries of other teleost and/or mammal species. To answer the question, I performed western blotting analysis of SN-IR proteins in mouse LβT2 pituitary cell line and pituitary tissues of human, rat and trout in Chapter 4 and Appendix I, respectively.

Brain regulation of vertebrate reproduction involves a complex interaction between neuroendocrine systems controlling pituitary hormone release. In particular, the neurotransmitter DA and the neuropeptide GnRH are the best studied in fish. Numerous *in vivo* and *in vitro* studies have shown that GnRH and DA are respectively the main stimulator and inhibitor of LH release from teleost gonadotrophs. LH stimulates sex steroid production, and controls ovulation in females or sperm production in males (Peter et al., 1986; Chang et

al., 1993; Trudeau, 1997). A decade ago, our research team for the first time found that intraperitoneal injection of synthetic SN *in vivo* could rapidly stimulate and increase serum LH concentration when the DA type-2 receptor was blocked by domperidone (Blázquez et al., 1998a). We also observed that gfSN had a direct stimulatory effect on LH release in static incubation of goldfish pituitary fragments *in vitro*. Moreover, gfSN significantly induced an increment in LHβ subunit messenger RNA (mRNA) levels in pituitary fragments *in vitro*. These data provide evidence for a direct stimulatory action of SN on LH release and synthesis in vertebrate pituitary, suggesting a potential reproductive role for SN (Zhao et al., 2006a). These facts indicate the possible existence of a sensitive SN receptor prone to down-regulation in the pituitary. However, several neuropeptide and neurotransmitter residues in teleost pituitary fragments might affect the direct action of SN to stimulate LH release. To avoid this interference, we adopted the static culture of primary dispersed goldfish pituitary cells for the studies of SN-induced LH release and production. The major results are presented and discussed in Chapter 2 and 3. Although SN-relative endocrine effect is well investigated in goldfish brain-pituitary-gonad axis, it is still unclear if this neuropeptide shows a similar biological action on LH in other vertebrates, especially in mammalian species. To address this query, I explored SN effect and its associated signal transductions within a mouse pituitary cell line, LβT2. The major data are described in Chapter 4.

1.6.2. EM66

EM66 ($SgII_{187-252}$) is a 66-amino acid peptide generated from SgII precursor by cleavage at conserved pairs of basic residues (Anouar et al., 1998). The EM66 peptide was first identified in adult and fetal human adrenal gland, and is so named because there are

glutamic acid (E) and methionine (M) residues at the N- and C-terminus of the sequence, respectively (Anouar et al., 1998). This peptide is moderately conserved in tetrapods, showing >70% amino acid identity (Fig. 1.1B and 1.2E), which is in contrast to low conservation in fish. The putative dibasic cleavage sites flanking this region, the E and M at the terminals and the length are variable between species. For example, the putative peptide from goldfish is predicted to be 67 amino acids, having E and V at the N- and C- terminals, and shows only ~30% identity with human EM66. Therefore, the characteristics of this peptide remain to be established in most vertebrate groups. Nevertheless, its immunoreactive distribution has been studied in some mammals. Double-labeling immunohistochemical investigation in rat pituitary indicates that EM66-IR is expressed in gonadotrophs, lactotrophs, thyrotrophs and melanotrophs but not corticotrophs. Moreover, some nerve fibers in the neural lobe of posterior pituitary contain EM66-immunoreactive signals. These results indicated the generation and potential bioactivities of this SgII-derived peptide in the nervous and endocrine system (Montero-Hadjadje et al., 2003). EM66 was also characterized in rat and jerboa. EM66-IR was found in the parvocellular, paraventricular, preoptic and arcuate nuclei, and the lateral hypothalamus in these two species, suggesting several neuroendocrine modulatory roles for EM66, such as regulation of feeding (Boutahricht et al., 2007). Food deprivation caused a significant increase in the number of EM66-containing neurons within the arcuate nucleus and the parvocellular aspect of the paraventricular nucleus in jerboa, suggesting an effect of EM66 in controlling food intake and/or the response to stress-relevant fasting (Boutahricht et al., 2005). Furthermore, human pheochromocytes (from chromaffin tumors) secrete a high level of EM66 (Guillemot et al., 2006b) as well as the CgA-derived WE-14 peptide (Anouar et al., 2006), supporting the roles of granins and their proteolytically

processed products as a circulating markers for the diagnosis of neuroendocrine tumors. In addition, PACAP stimulates EM66 secretion from bovine chromaffin cells through several signaling pathways including protein kinase A (PKA), PKC and MAPK (Guillemot et al., 2006a). However, other neuroendocrine activities of EM66 are unexplored and require further investigations.

1.7. Summarizing the endocrine functions of granin-derived peptides

Taken together, the granins and granin-dervied peptides exert important roles in the control of vertebrate nervous and endocrine systems. Some of their biological roles include regulation of growth and reproduction in vertebrates. A recent investigation of Snell dwarf mice indicates that they have elevated circulating levels of CgB and SgII, possibly linked to their low endogenous glucose generation and resistance to cancer (Alderman et al., 2009). Except for the chromogranin group (CgA and CgB), there is no clear evolutionary relationship among the other granin groups. However, some granin-derived peptides are highly conserved for example VS, β-granin and WE-14 derived from CgA; secretolytin, CCB, BAM-1745 and CgB_{1-41} generated from CgB and SN from SgII. The inhibitory effects of VS, PST and CgB_{1-41} on PTH secretion indicate that the Cg-derived peptides have negative effects on growth because PTH increases cartilage cell proliferation, development and gestation in mammals (Strewler, 2000). β-granin and PST suppress glucose absorption from blood by decreasing insulin secretion and increasing glucagon release. Moreover, SN might be associated with the obesity defect in human PWS disease (Horsthenke et al., 2003), whereas EM66 could affect growth through regulating food intake under the fasting stress in mammals (Boutahricht et al., 2007). The role of granins and their derived peptides in the control of

vertebrate reproduction remains largely unexplored. β-granin, WE-14 and SN are localized in rat gonadotrophs and correlate to gonadotropin production suggesting a potential autocrine and/or paracrine action on the secretion of LH and/or FSH. Best studied to date is the goldfish where it has been shown that SN stimulates LH release *in vivo* and *in vitro* (Blázquez et al., 1998a; Zhao et al., 2006a). Given that several granin-dervied peptides are moderately conserved in evolution, and can be localized to neuroendocrine brain regions and pituitary cells, it is reasonable to speculate that SN and other granin-related peptides have important roles in the control of vertebrate reproduction.

While there is considerable evidence that a variety of conserved granin-derived peptides exhibit multiple biological functions, this is largely confined to studies in laboratory rodents, and implications for neuroendocrine cancers and inflammation. Moreover, while there is good evidence for the existence of membrane receptors for some of the granin-derived peptides, these putative receptor proteins have not yet been isolated or characterized to any substantial degree. This remains as a major challenge in the field. Given the diversity of peptides possible both within a given granin family and between taxa, much remains to be discovered about the evolution and endocrine roles of the granins.

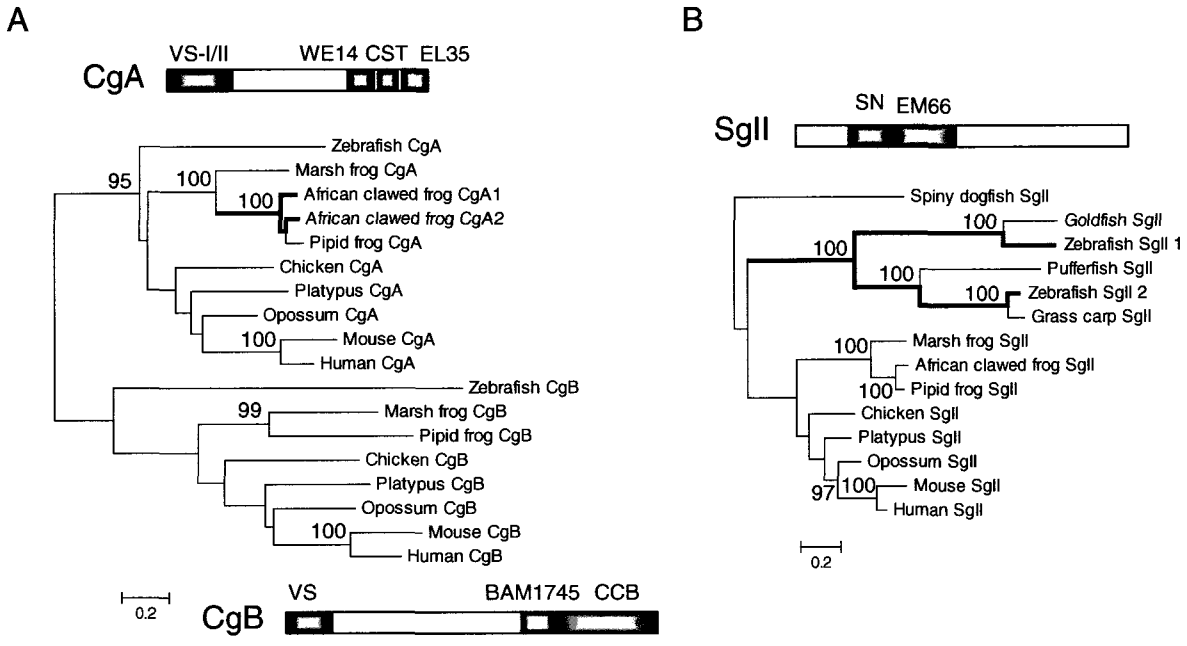

Figure 1.1. Phylogenetic trees for CgA/CgB (A) and SgII (B). All sequences are identified from PSI-Blast searching in NCBI NR database. An unrooted phylogenetic tree is reconstructed using PhyML program with 500 bootstrap analyses. Only bootstrap values higher than 95% are shown. The gene duplications in CgA and SgII are indicated. This figure also shows the conserved peptide fragments, whose sequences will be displayed in Figure 1.2.

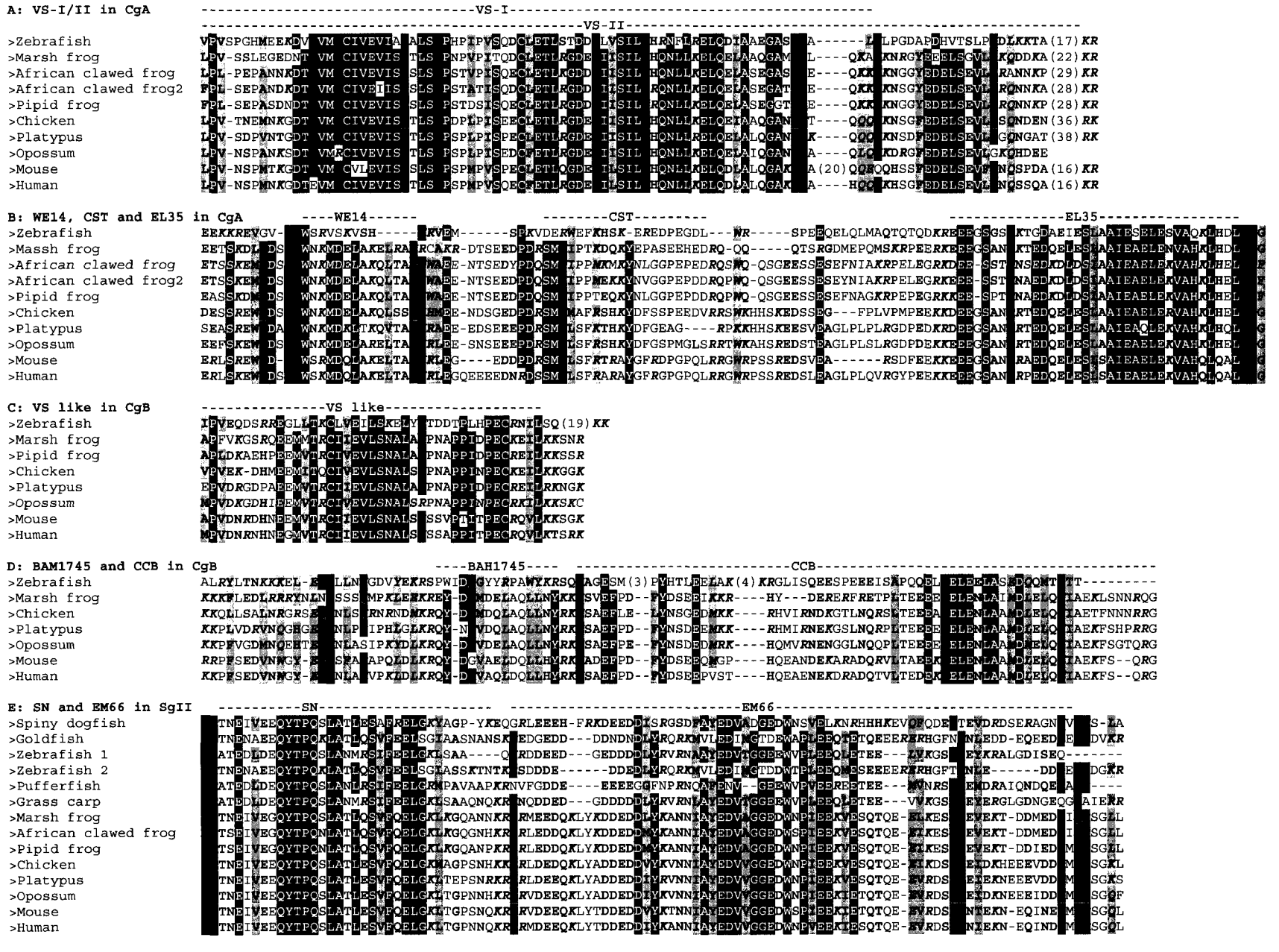

Figure 1.2. Conserved peptide fragments derived from CgA, CgB and SgII. The multiple alignment is built using Muscle program, and refined manually. All basic residues K or R are in red color to show the putative PC enzyme cleavage sites.

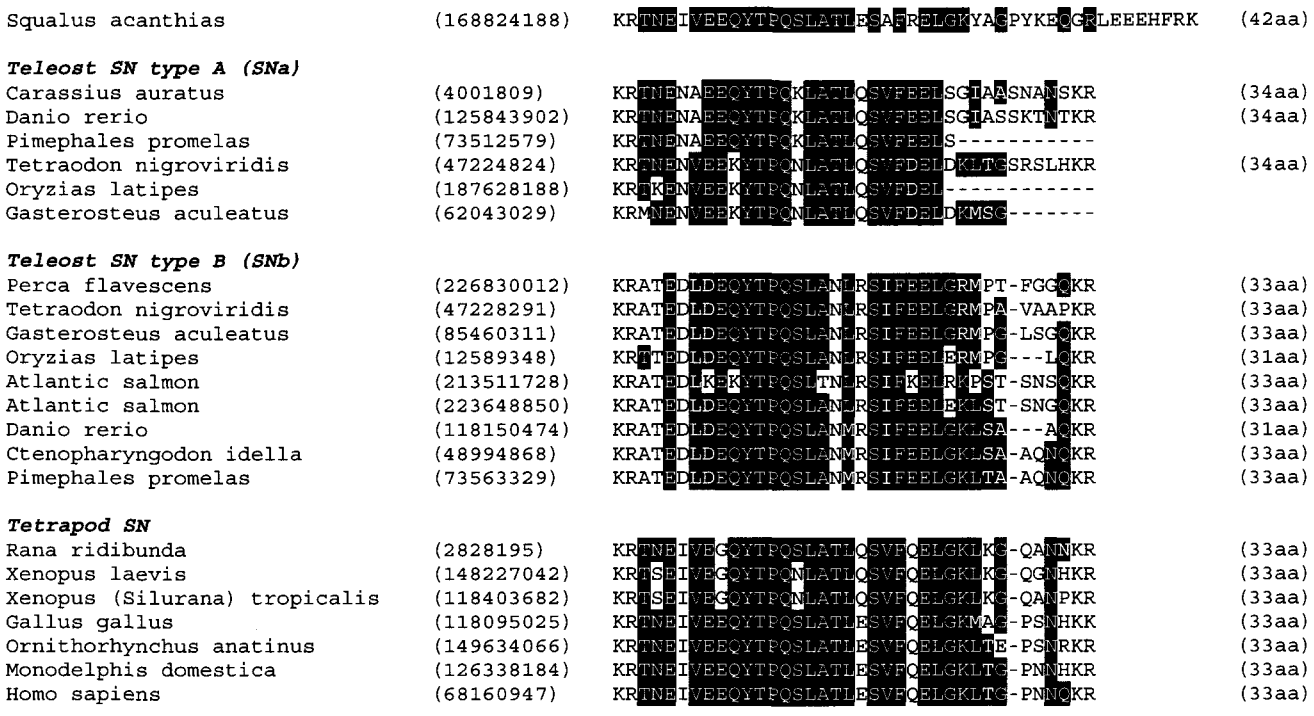

Figure 1.3. Conserved peptide fragments in the sequence of teleost fish SN type A & B (SNa & SNb), and tetrapod SN. The multiple alignments were built using the Muscle program and edited manually. All basic residues K or R are in red color to show the putative PC enzyme cleavage sites in the SgII precursor to generate SN peptide in various species.

Granin protein	Amino acid number *	Acidic residues (%) (g)	Isoelectric point	Calcium binding (a)	Coding Exons (j)	Species distribution (k)
CgA (human)	439 (a)	25	4.57-4.68 (a)	Yes	Multiple	Fish , frog , chicken , mammals
CgB (human)	657 (a)	24	5.2 (a)	Yes	Multiple	Fish , frog , chicken , mammals
SgII (human)	590 (b)	20	5.2 (h)	Yes	Single	Dogfish , fish , frog , chicken , mammals
SgIII (mouse)	468 (c)	19	5.1 (g)	ND	Multiple	Fish , frog , chicken , mammals
SgV (7B2) (human)	186 (d)	16	5.2 (g)	Yes	Multiple	Worm, fly, sea urchin, vertebrate
NESP55 (Sg VI) (bovine)	195 (e)	21	4.8 (i)	ND	Single	Mammals
VGF (human)	593 (a)	ND	ND (a)	ND	Multiple	Mammals
HISL-19	ND	ND	5.6 (g)	ND	ND	ND

Table 1.1. Characteristics of granin member proteins.
*, Amino acid residues are for mature protein without signal peptide.
a, Helle, 2004; b, Blázquez et al. 1998a ; c, Dopazo et al., 1993 ; d, Mbikay et al., 2001; e, Eder et al., 2004; f, O'Connor et al., 1984; g, Taupenot et al., 2003; h, Gerdes et al., 1989; i, Ischia et al., 1997; j, UCSC genome database; k, PSI-BLAST.

CHAPTER 2: The Secretogranin II-derived Peptide Secretoneurin Stimulates Luteinizing Hormone Secretion from Gonadotrophs in Goldfish

** Main contributors for this chapter:*

E Zhao: Original ideas, pituitary cell culture and treatment, radioimmunassay, peptide isolation and identification, western blotting analysis, data analysis and writing,

Anderson O. L. Wong: Immunohistochemical staining of goldfish pituitary,

Gustavo M. Somoza: Immuohistochemical staining of goldfish brain.

2.1. Abstract

Secretoneurin (SN) is a 33-34 amino acid neuropeptide derived from selective processing of the secretogranin-II (SgII) precursor protein, a member of the chromogranin family. Based on predicted amino acid sequences, we previously synthesized a putative goldfish (gf) SN and demonstrated its ability to stimulate LH release *in vivo*. However, it was not known if goldfish actually produced the free SN peptide or if SN directly stimulates LH release from isolated pituitary cells. Using a combination of reverse phase-HPLC and mass spectrometry analysis, we isolated for the first time a 34 amino acid free gfSN peptide from the whole brain. Moreover, western blot analysis indicated the existence of this peptide in goldfish pituitary. Immunocytochemical localization studies revealed the presence of SN-immunoreactivity in prolactin cells of rostral pars distalis of the anterior pituitary. Additionally, we found that magnocellular cells of the goldfish preoptic region are highly immunoreactive for SN. These neurons send heavily labeled projections that pass through the pituitary stalk and innervate the neurointermediate and anterior lobes. In static 12-hr incubation of dispersed pituitary cells, 1 and 10 nM gfSN respectively induced 2.5-fold ($p<0.001$) and 1.9-fold ($p<0.01$) increments of

LH release into the medium. This is similar to the levels of LH released by 100 nM concentration of gonadotropin-releasing hormone. Whereas we do not yet know the relative contribution of neural SN or pituitary SN to LH release, we propose that SN could act as a neuroendocrine and/or paracrine factor to regulate LH release from the anterior pituitary.

2.2. Introduction

Luteinizing hormone (LH) is an essential factor that stimulates gonadal maturation, steroid synthesis and gamete release in vertebrates. Brain regulation of pituitary LH release involves a complex interaction between principal stimulatory and inhibitory neuroendocrine systems, mainly including gonadotropin-releasing hormone (GnRH), catecholamines and amino acid neurotransmitters (Trudeau, 1997; Herbison, 2008). It is well established that GnRH is the primary regulator of pituitary LH synthesis and secretion. GnRH also stimulates the production and secretion of secretogranin II (SgII) gene products (Conn et al., 1992; Nicol et al., 2002; Samia et al., 2004), yet the potential roles of these peptides in the pituitary have received little attention.

Secretogranin II is a member of the chromogranin (Cg) family of acidic, heat-stable proteins that are widely distributed in secretory granules of a variety of endocrine cells and neurons (Huttner et al., 1991). The Cgs were first discovered in the bovine adrenal medulla by Blaschko et al. in 1967. Nine types of Cg precursor proteins and their diverse array of proteolytic products have been identified to date. They are known as CgA, CgB, SgII (CgC) (Winkler and Fischer-Colbrie, 1992), SgIII (Ottiger et al., 1990), SgIV (HISL-19) (Montero-Hadjadje et al., 2008), SgV (7B2) (Mbikay et al., 2001), SgVI (NESP55) (Montero-Hadjadje et al., 2008), SgVII (VGF, Helle, 2004) and proSAAS (Fricker et al., 2000). Some of these are

regulators in the secretory pathway and play roles in targeting release of peptide hormones and neurotransmitters. CgA was demonstrated to function as an "on/off" switch that controls dense-core secretory granule biogenesis and hormone sequestration in endocrine cells. The absence of CgA-driven granule biogenesis reduces the expression or storage of other granule components in the PC12 cell line (Kim et al., 2001). In the mouse anterior pituitary, SgII may co-aggregate with LH in secretory granules (Crawford et al., 2002). Moreover, Cg proteins serve as precursors of several biologically functional peptides.

Some years ago following a differential display screen of the goldfish pituitary our lab isolated the full length cDNA sequence encoding the first known fish chromogranin (Blázquez et al., 1998a). Unlike CgA and CgB, the SgII precursor protein is relatively poorly conserved in evolution. The comparison of vertebrate SgII sequences revealed that 6 of the 9 potential dibasic cleavage sites are common to fish and tetrapods (Blázquez et al., 1998a). The SgII precursor protein undergoes prohormone convertase (PC)-mediated proteolytic processing (Seidah et al., 1994; Wouters et al., 1998). In particular, PC1 and PC2 are capable of cleaving the SgII precursor at dibasic cleavage sites to generate a range of potential bioactive peptides (Hoflehner et al., 1995) of which the neuropeptide SN is best characterized (Fischer-Colbrie et al., 2005; Montero-Hadjadje et al., 2008). In addition, only the 33-34 amino acid SN sequence is relatively well conserved in fish (Samia et al., 2004; Blázquez et al., 1998a). The predicted goldfish SN (secretogranin $II_{214-248}$) amino acid sequence shares 59% identity to human SN and >75% similarity overall with other vertebrate SN sequences. However, two domains, in the middle of goldfish SN (QYTP and LATLEQSVFQEEL) are identical to those of mammals (Samia et al., 2004). Outside the SN segment, either between the N-terminal end and dibasic cleavage site 2 or between dibasic cleavage site 3 and the C-

terminal end the similarities between goldfish SgII and other known SgIIs become very low (10% identical and 40% similar in both cases) (Blázquez et al., 1998a). Recently, another SgII-derived 66-amino acid peptide, EM66, was identified. This sequence is conserved in tetrapods, as human and frog EM66 are 68% identical (Montero-Hadjadje et al., 2008). In contrast, the EM66 segment of tetrapod SgII is not conserved in goldfish (Blázquez et al., 1998a).

The SgII precursor protein is approximately 600 amino acids in length (range 588-624, depending on the species). It is a highly acidic and tyrosine-sulfated protein found in secretory granules of vertebrate neuroendocrine cells and vesicles of some neurons (Montero-Hadjadje et al., 2008; Rosa et al., 1985; Helle, 2004). SgII-immunoreactivity (IR) was detected in gonadotrophs, thyrotrophs and corticotrophs in human pituitaries using immunohistochemistry (Vallet et al., 1997). In rat brain, in situ hybridization studies indicate that the SgII gene is widely expressed (Mahata et al., 1991). Several SN-immunoreactive intermediate peptides derived from SgII processing have been identified by western blots of extracts from goldfish pituitary and brain (Zhao et al., 2006b).

More specifically, SN-IR was also detected in a wide variety of vertebrate neuroendocrine cells. High levels of SN-IR are found in human pituitary (Schmid et al., 1995). In a comprehensive neuroanatomical study, the highest density of SN-IR fibers and terminals was detected in rat hypothalamus, especially median eminence (Marksteiner et al., 1993a). The wide distribution of SN-IR and evolutionary conservation of the SN sequence suggests that it should have important but diverse biological activities. Indeed, SN has roles in neuroinflammation and neurotransmitter release (Fischer-Colbrie et al., 2005; Montero-Hadjadje., 2008; Helle, 2004). For example, local infusions of SN into rat substantia nigra and

neostriatum both up-regulate extracellular glutamate and GABA levels in a concentration-dependent manner (You et al., 1996). Moreover, SN enhances dopamine (DA) release from rat brain (Agneter et al., 1995). Our previous studies in goldfish indicated that intraperitoneal injection of SN increased serum LH level in goldfish treated with a DA antagonist (Blázquez et al., 1998a; Zhao et al., 2006a), also suggesting a role in neuroendocrine regulation.

The aim of the study in this chapter is to determine whether the free SN peptide is produced by goldfish. While several SN-IR fragments are detectable by western blot in goldfish pituitary and brain (Zhao et al., 2006b), their localization until now was unknown. Within the anterior pituitary (also called the pars distalis in teleost fish) where endocrine cells are highly regionalized, we detected SN-IR only in prolactin cells. We show that SN-IR is found in preoptic neuroendocrine neurons and fibres projecting to the neurointermediate lobe and anterior pituitary. Given that SN can directly stimulate LH release from disperse pituitary cells *in vitro*, we suggest that SN-IR products from both neural and endocrine origins may have a role in regulating reproduction.

2.3. Materials and Methods

2.3.1. Animals and tissue collection

Animals are handled according to protocols approved by the University of Ottawa Animal Care Committee. Goldfish (*Carassius auratus*) were purchased from Mount Parnell goldfish farms (Pennsylvania, USA). Their average length was 10-15 cm and average weight was approximately 20 g. The water temperature was maintained at 18 ^{0}C and the photoperiodic regime followed the light/dark cycle in Ottawa, Canada. Goldfish were anaesthetized using 3-aminobutyric acid ethyl ester in water, sacrificed by spinal transection,

and brains and pituitaries were then removed from the skull. Pituitaries were placed in ice-cold dispersion medium (M199 with Hanks' salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 0.3% bovine serum albumin (BSA), 100,000 U/l penicillin, 100 mg/l streptomycin, pH 7.2) for the pituitary cell dispersion and static culture. Tissue samples were also frozen on dry ice and stored at -80 ^{0}C for the peptide extraction.

2.3.2. Protein extraction

Frozen goldfish brains and pituitaries were homogenized in lysis buffer (150 mM sodium chloride, 25 mM Tris-HCl, 1% Triton X-100) containing complete Protease Inhibitor Cocktail (Roche Diagnostics GnbH, Germany). The homogenate was mixed at room temperature for 5 minutes and then centrifuged at 3,000 rpm for 30 minutes at 4 ^{0}C. The precipitate pellet was further extracted with lysis buffer twice and centrifuged. The three supernatants were pooled together for peptide separation by reverse-phased high-performance liquid chromatography (Varian Dynamax, Palo Alto, CA).

2.3.3. Reverse-phased high-performance liquid chromatography (RP-HPLC)

The supernatant from the goldfish brain extracts and synthetic goldfish secretoneurin (gfSN; 1 μg/μl) (Zhao et al., 2006a) neuropeptides were respectively loaded onto a C-4 semi-preparative column (Varian Dynamax 250 x 10.0 mm), which was eluted by using a linear gradient from 35% to 70% acetonitrile (ACN) in 0.1% (vol/vol) trifluoroacetic acid (TFA) in 65 min (minute) at a flow rate of 1.5 ml/min. The separation was monitored by UV absorbance at 214 nm. Under these conditions, free SN peptide isolated from goldfish brain and synthetic gfSN eluted from the column at identical retention times. The presence and

nature of free SN peptide isolated from goldfish brain were finally characterized by mass spectrometry, dot immunoblotting analysis, endoproteinase Lys-C digestion and C-terminal sequencing followed by mass spectrometry.

2.3.4. Mass spectrometry

Each mass spectrum was calibrated using a commercially available standard peptide mixture, CAL1 (Applied Biosystems, Foster City, CA, USA) as an external calibrant that consists of bradykynin [MW: 1060.2 Dalton (Da)], angiotensin I (MW: 1296.5 Da) and $ACTH_{7\text{-}38}$ (MW: 3659.8 Da). The SN-related chromatography fractions were concentrated by lyophilization (VIRTIS Freeze Mobile 8EL, the Virtis Company, Gandener NY) and identified by surface enhanced laser desorption ionization time of flight (SELDI-TOF) mass spectrometry (Ciphergen Biosystems, Fremont, CA) (Basak and Lotfipour, 2005). Purified RP-HPLC fraction (2 µl) was spotted on a gold chip plate and air-dried as described by the manufacturer (www.ciphergen.com). Energy absorbing matrix CHCA (α-cyano-4-hydroxycinnamic acid, CHCA; Fluka, Switzerland), freshly prepared by dissolving ~10 mg in 1 ml solvent containing of 500 µl of ACN and 500 µl of 0.6% (vol/vol) TFA/water, was spotted on the dried sample before mass spectrometry analysis. Masses were recorded in the range from 1,000 to 10,000 Da and shown as a plot of signal intensity *vs.* the mass-to-charge ratio (M/Z). Mass spectra of SN-related HPLC fractions were examined and compared with the signature signals corresponding to calculated molecular weight of the gfSN peptide (3,655.89 Da).

2.3.5. Dot immunoblotting analysis

Two microliters of each concentrated SN-related fractions (0.043 μg/μl) were spotted onto the nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was air dried at room temperature and soaked in blocking buffer, 20 mM Tris-HCl buffer (TBS, pH 7.5) containing 5% BSA and 0.05% Tween 20 for 45 min. After blocking non-specific binding sites, the membrane was incubated with our previously characterized rabbit anti-goldfish SN antibody (1:1,000 dilution in blocking buffer) (Zhao et al., 2006b) for 60 min. After the primary immunoreaction, the membrane was reacted with protein A-peroxidase (Sigma, Germany) at a dilution of 1:2000 at room temperature for 60 min. After washing, the membrane was covered with ECL plus western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) and then wrapped in plastic. Signals on the membranes were detected with the BioRad ChemiDoc Imaging System and quantified using the Quantity One (Bio-Rad, Italy) software package.

2.3.6. Endoproteinase Lys-C digestion

The endoproteinase Lys-C cleaves peptide bond at the C-terminal side of a lysine residue. In order to confirm the peptide separated from goldfish brain extract has the same lysine position as the SN peptide, the peptide isolated from goldfish brain (10 μl, 0.043 μg/μl) and synthetic SN (10μl, ~1 μg/μl) were incubated with 0.5μg of endoproteinase Lys-C (Roche Diagnostics GmbH, Germany) in 5μl double distilled water at 37 ^{0}C for 0, 1, 5, 24 hours. At each digest time, 2 μl of the digest was spotted on gold chip and then covered with 1 μl of CHCA matrix for mass spectrometry analysis.

2.3.7. C-terminal sequencing by carboxypeptidase Y (CPY) digestion

The SN-related peptide (0.3225 μg in 7.5 μl) isolated from goldfish brain extract by RP-HPLC was subjected to proteolysis by CPY (2 μl, 0.0019 units/μl, Sigma-Aldrich, Steinheim, Germany) in 10.5 μl of sodium citrate buffer (50 mM, pH 6.0). The digestion was performed in a shaker incubator (60 rpm rotation) at 37 ^{0}C. 2 μl aliquots of reaction mixture were taken after 2 min and spotted on a gold chip plate (Ciphergen Biosystems, Fremont, CA) covered with 1 μl CHCA matrix, and analyzed by SELDI-TOF mass spectrometry (Basak and Lotfipour, 2005).

2.3.8. Estimation of total SN-IR in goldfish brain and pituitary

A standard curve was established to estimate the quantities of SN-IR in goldfish brain and pituitary. Samples were assayed in triplicate in three independent experiments. Ten pituitaries and 5 whole brains were extracted in 2 ml and 12 ml, respectively. The average tissue wet weight was 134 mg for brain and 2.3 mg for pituitary. Synthetic gfSN at various amounts (20, 10, 5, 2 ng) and the goldfish brain and pituitary exacts were simultaneously analyzed by dot immunoblots and their immunoreative intensities were quantified.

2.3.9. Immunocytochemistry of the pituitary

Goldfish pituitaries were fixed in ice-cold Bouin's fixative for 15 hr, dehydrated with a graded series of ethanol and embedded in paraffin wax according to standard procedures. Pituitary sections of 5 μm thickness were prepared and pre-incubated in blocking solution (0.5% BSA, 0.5% gelatin and 2.5% normal goat serum) for 30 min. Subsequently, pituitary sections were incubated at 4 ^{0}C overnight with the antiserum for goldfish SN (1:12,000),

prolactin (PRL) (1:100,000), GH (1:500,000) and LH (1:60,000), respectively. The latter three antisera were generous gifts from Dr. R.E. Peter, University of Alberta, Alberta, Canada and have been extensively validated (Wong et al., 1998). On the following day, immunostaining for the respective hormones were performed using a Vectastatin ABC Kit (Vector Laboratories, Burlingame, CA) that is based on the avidin-biotin-peroxidase complex method with minor modifications (Wong et al., 1998). Parallel staining with similar dilutions of natural rabbit serum (NRS) were used as the negative control and the specificity of the SN immunostaining was further confirmed by preabsorption of the anti-SN antiserum with 1 M gfSN as has been reported for western blots of goldfish protein extracts (Zhao et al., 2006b). Colour photographs of pituitary sections were captured using the Leica DMDL compound microscrope with a Nikon Digital Sight DW-L1 Imaging system. This experiment was in collaboration with Dr. A.O.L. Wong in the University of Hong Kong.

2.3.10. Immunocytochemistry of the brain

Male and female goldfish were anesthetized with benzocaine and perifused with saline solution (0. 8% NaCl in PBS pH 7.4) followed by 50 ml Bouin's fixative. The brains were carefully dissected and fixed overnight in Bouin's fixtaive at 4 ^{0}C, submitted to routine histological procedures, embedded in paraffin, and processed according to standard procedures (Strobl-Mazzulla et al., 2005). In some cases, the pituitary was also processed along with the brains. Sections (6μm) were mounted on gelatin-coated slides and dried overnight at 20 ^{0}C. Following ethanol dehydration, sections were washed with PBS, and the endogenous peroxidase activity blocked for 30 minutes at room temperature with 0.3 % H_2O_2. The sections were washed and non-specific binding was blocked PBS containing 1% BSA

and 0.3% Triton X-100. Sections were then incubated with SN antiserum (1:2500) diluted in PBS overnight at 22 ^{0}C. A negative control was also performed pre-absorbing SN antiserum (1:2500) overnight (4 ^{0}C) with 2 μM synthetic gfSN. On the following day, sections were rinsed and immunostained for SN was the LSAB™-HRP kit (DAKO, Carpinteria, CA) based on the biotin-streptavidin-peroxidase complex. Briefly the sections were incubated with the biotinylated-secondary antibody for 35 min at room temperature, then washed with PBS and subsequently incubated with streptavidin-HRP for 30 min at room temperature. The sections were washed with PBS and developed using DAB (3-3′, diaminobenzidine, SIGMA, St. Louis, MO). Slides were finally washed with distilled water, dehydrated with ethanol and mounted with synthetic Canada balsam. Photographs of sections were captured using the Nikon Eclipse E7000 with a Nikon digital imaging system. This experiment was in collaboration with G. M. Somoza at the Instituto de Investigaciones Biotecnol□gicas-Instituto Tecnologico Chascomús, Argentina.

2.3.11. Static incubation of dispersed goldfish pituitary cells and neutralization experiment

A mixture of goldfish pituitaries from both males and females were dispersed using Typsin type II and DNase II enzymes as previously described (Chang et al., 1990). The cells were plated in 24-well culture plates at a density of ~2.5 x 10^5 cells/well (in December during the seasonal sexual redevelopment period of goldfish) or 6-well culture plates at a density of ~2.0 x 10^6 cells/well (in March at the beginning of the sexually mature pre-spawning period of goldfish). In the neutralization experiment, the cells were incubated in 24-well culture plates at a density of ~2.5 x 10^5 cells/well (in February at the end of the sexual redevelopment period of goldfish). After at least 12-hr plating period, cells were washed with testing medium

and pre-incubated for 1 hr to stabilize basal LH secretion. In previous studies, we showed that gfSN stimulated LH release from goldfish pituitary fragments *in vitro* over a relatively narrow response range (Zhao et al., 2006a), which is typical of SN in other systems. Depending on the stage of the reproductive cycle, doses of 100 to 1000 nM may cause down-regulating the LH response, perhaps because of SN receptor desensitization (Zhao et al., 2006a, and unpublished data). Therefore, to avoid a down-regulated response we used 1 or 10 nM gfSN in our experiments with dispersed cells. Media were then replaced with testing medium consisting of either synthetic salmon GnRH (sGnRH: Glu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2; 100 nM), synthetic gfSN peptide (1 and10 nM) or goldfish SN antibody (1:1,500 dilution in neutralization experiment) (Zhao et al., 2006b). Following a 12-hr static treatment, media samples were removed and stored in -20 ^{0}C for radioimmunoassay measurement of LH content.

2.3.12. Radioimmunoassay (RIA)

LH concentrations in incubation media were determined using a double antibody RIA, which was previously described by Zhao et al. (2006a). Briefly, 96-well Dynamex Immulon 4 HBX Flat Bottom Microtiter plates (Thermo Labsystems, Milford, MA) were filled with 100 µl rabbit anti-carp LH antibody (1:50,000), 50 µl of LH standards (0.19-100 ng/ml) or diluted sample (usually 1:100), and 25 µl ^{125}I-labeled LH (~18,000 CPM). All samples were assayed in triplicate. A group of 6 wells were filled with the same volumes of normal rabbit serum (1:100) and ^{125}I-labeled LH as the blank to determine non-specific binding. After 48 hr incubation at 4 ^{0}C, 50 µl of goat anti-rabbit IgG (Scottish Antibody Product Unit, Scotland, UK) at a dilution of 1:10 was added to each well and incubated for an additional 24 hr period

at 4 ^{0}C. Plates were centrifuged at 3,000 rpm and the supernatants were discarded. One hundred microliters of "Optiphase Supermix" (EG&G Wallac, Turku, Finland) scintillation fluid were then added to each well and incubated at room temperature for 4 hr. Plates counted in a Wallac Trilux Microbeta. WiaCalc software (version 2.60, EG&G Wallac, Turku, Finland) was utilized to estimate LH concentrations.

2.3.13. Western blot analysis of goldfish pituitary

Frozen goldfish pituitaries were homogenized in a buffer containing 20 mM Hepes, 200 mM sodium chloride, 0.1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium metavanadate, 10 mM β-glycerophosphate and protease inhibitor cocktail (1:100, Bioshop Canada, Burlington, ON), and then centrifuged at 12,000 rpm for 15 minutes at 4 ^{0}C. As described previously (Zhao et al., 2006b), total protein concentration of the supernatant was assayed by using the bicinchoninic acid method. Pituitary extract (100 μg of total protein) was separated by electrophoresis on a SDS-PAGE gel (15% bis-ascrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED). After electrophoresis, proteins were transferred onto a nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA). The membrane was air dried at room temperature and blocked in the 20 mM Tris-HCl buffer (TBS, pH 7.5) containing 5% BSA and 0.05% Tween 20. The membrane was then incubated with primary goldfish SN antibody (1:700 dilution in blocking buffer) for ~12 hours at 4 ^{0}C. After the primary immunoreaction, the membrane was reacted with protein A-peroxidase (Sigma, Germany) at a dilution of 1:1000 at room temperature for 60 min. After washing, the membrane was covered with ECL plus western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) and then wrapped in plastic. Signals on the membranes

were detected with the BioRad ChemiDoc Imaging System and quantified using the Quantity One (Bio-Rad, Italy) software package.

2.3.14. Data analysis

Results measured by LH-RIA were presented as mean ± S.E.M. The statistical analyses were performed using Sigma Stat version 3.5 software. LH medium levels were normally distributed. One-way Analysis of Variance (ANOVA) was chosen to assess difference between control and treatments, followed by Fisher LSD test. A difference with $p<0.05$ was considered to be significant.

2.4. Results

2.4.1. Isolation of free goldfish SN peptide in brain

Goldfish brain extracts were separated by RP-HPLC with a C-4 semi-preparative column. Four major HPLC fractions, labeled A, B, C and D were detected (Fig. 2.1A). As depicted in the enlarged elution profile (Fig. 2.1B), and according to the similar elution time to that of synthetic gfSN (Fig. 2.1A), a part of fraction C was collected and used to determine if it contained free gfSN. Two small peaks (fraction I and II) were isolated and collected from fraction C (Fig. 2.1B). Various concentrations of synthetic gfSN were blotted and served as a positive control for SN-IR in dot immunoblots with our polyclonal anti-SN antiserum (Fig. 2.1C, panel C-1). Background levels of the immunoreaction when only buffer was blotted were negligible (Blank). The fractions from the final RP-HPLC step were analyzed by dot immunoblots. Fraction II exhibited a SN-immunoreactive signal as did a crude goldfish brain extract in dot immunoblotting analysis, shown in Fig. 2.1C (panel C-2). This suggested the

presence of free gfSN peptide in fraction II obtained from goldfish brain extract. We used dot immunoblotting analysis rather than western blots because of low molecular size of gfSN peptide. SELDI-TOF mass spectrum of Fraction II showed peaks consistent with those observed in the mass spectrum of synthetic gfSN, its sodium adduct and oxidized form. The other fraction (# I) did not exhibit any peak of free gfSN peptide in the mass spectrum; instead it exhibited a peak of m/z 3454 due to deletion of "NS" dipeptide from the C-terminal sequence of the gfSN peptide.

In order to further purify the samples, Fraction II was pooled from several RP-HPLC runs and lyophilized. The obtained material was subjected to a second RP-HPLC run with C-4 semi-preparative column under identical gradient conditions. Four fractions named P1, P2, P3 and P4, respectively eluted at Rt (retention time) ~35.9, 36.8, 37.5 and 38.3 min (Fig. 2.2A) were collected and analyzed by SELDI-TOF mass spectrometry (Fig. 2.2B). Only fraction P2 exhibited a single major peak at m/z 3655.6 $(M + H)^+$ consistent with the calculated molecular weight of gfSN (3655.89 Da, average mass), with a minor peak at m/z 3677.7 $(M+Na)^+$ due to the formation of sodium adduct (calculated 3677.89 Da, average mass) (Fig. 2.2B). These data confirmed the identity of fraction P2 as free gfSN peptide. It was further established by a positive immunoreactive signal obtained in dot immunoblotting analysis (data not shown).

2.4.2. Further identification of brain SN-like peptide

2.4.2.1. Endoproteinase Lys-C digestion and mass spectra

In an attempt to identify Lys positions in the brain SN-like peptide, synthetic gfSN peptide and fraction-P2 eluate were both digested separately with endoproteinase Lys-C and then analyzed by SELDI-TOF mass spectrometry. The calculated molecular weights of two

expected Lys-C digest fragments from SN were m/z 1551 (SN_{1-13}: TNENAEEQYTPQK) and m/z 2122 (SN_{14-34}: LATLQSVFEELSGIAASNANS) (Fig. 2.3D). In the digestion of synthetic SN, mass spectra showed two major peaks at m/z 1577 and m/z 2147, which were labeled as (1) and (2) (Fig. 2.3A-SN). The peak at m/z 1577 (1.1) was sodium adduct of SN_{1-13} $(M+Na)^+$ along with a minor peak at m/z 1555 (1.2) consistent with SN_{1-13} [Fig. 2.3B-SN(1)]. Similarly, the other major peak at m/z 2147 (2.1) showed SN_{14-34} plus sodium ion $(M+Na)^+$, and two minor neighboring peaks at m/z 2169 (2.2) and m/z 2190 (2.3) respectively due to the formation of SN_{14-34} plus double sodium ions and SN_{14-34} plus triple sodium ions [Fig. 2.3C-SN(2)]. The mass spectrum of fraction-P2 Lys-C digests exhibited a similar pattern to that of synthetic SN (Fig. 2.3A), and showed peaks at m/z 1546 (1.1), 1569 (1.2), 2123 (2.0), 2146 (2.1), 2174 (2.2) and 2191(2.3), due to the fragments of SN_{1-13}, SN_{1-13} with single Na^+, SN_{14-34}, SN_{14-34} with single Na^+, SN_{14-34} with double Na^+ and SN_{14-34} with triple Na^+ ions [Fig. 2.3B-P2(1) and 2.3C-P2(2)].

The Lys-C enzyme specifically cleaves endo-proteolytically a peptide bond at the C-terminus of a Lys residue (Jenö et al., 1995) and the fact that gfSN contains only one Lys residue located near the mid-portion of the sequence allowed us to use this enzyme rather than more commonly used trypsin for our digestion experiment. The expected cleavage fragments and their molecular sizes following Lys-C digestion are depicted in Fig 2.3D. Our mass spectrum data indicated that fraction-P2 eluate and synthetic SN peptide are indeed identical. Further information about the identity of fraction-P2 eluate was obtained from co-RP-HPLC of the two samples that exhibited a single peak under various gradient conditions used (data not shown). The identity of fraction P2-elute was confirmed to be TNENAEEQYTPQKLATLQSVFEELSGIAASNANS by C-terminal sequencing using a CPY

digestion method coupled with mass spectrometry (Ocañal et al., 2005).

2.4.2.2. C-terminal sequencing

SELDI-TOF mass spectrum of crude digest of fraction-P2 with CPY showed that peaks at m/z 3655, 3568, 3454 and 3383 due to the undigested peptide and successive loss of one amino acid each time from the C-terminus. The differences in molecular sizes of the peaks namely 87, 114 and 71 Da respectively showed that the peptide contains the sequence ANS at the C-terminus as expected for synthetic gfSN. Our attempt to obtain subsequent sequence information of the fraction-P2 peptide was unsuccessful owing to a rapid proteolytic rate of CPY until the 14th cycle. Thus, several mass spectral peaks corresponding to molecular ions of $SN_{21\text{-}34}$, $SN_{20\text{-}34}$, $SN_{19\text{-}34}$, $SN_{18\text{-}34}$, $SN_{17\text{-}34}$, $SN_{16\text{-}34}$, $SN_{15\text{-}34}$, $SN_{14\text{-}34}$, $SN_{13\text{-}34}$, $SN_{12\text{-}34}$, $SN_{11\text{-}34}$, $SN_{10\text{-}34}$ and $SN_{9\text{-}34}$ fragments were noted at the lower mass range of the mass spectrum. These sequence data further provide necessary support to the identity of this brain-isolated peptide as gfSN.

2.4.3. Levels of total SN-IR in goldfish brain and pituitary

It is noted that on western blots the anti-SN antibody will cross-react with fragments of various lengths containing the SN sequence (Zhao et al., 2006b). Total SN-IR level was estimated using a standard curve generated from dot immunoblotting analysis of synthetic gfSN at various concentrations (2-20 ng, Fig. 2.4A). There was a strong linear relationship between SN concentration and immunoblotting intensity (R^2=0.99), and a formula was established for the following estimations of tissue SN-IR levels (Fig. 2.4B). Based on the equation, the levels of SN-IR in pituitary and whole brain were calculated to be 278 ± 38 and

65 ± 23 fmol/mg tissue respectively (Fig. 2.4C). Therefore, based on wet weight, the level of SN-IR in whole pituitary was approximately 4-times higher than of the level of SN-IR in the whole brain.

2.4.4. Immunocytochemical staining of SN in goldfish pituitary and brain sections

The goldfish pituitary is composed of the anterior and neurointermediate lobes. However, in contrast to mammals, the anterior pituitary is highly regionalized and is directly innervated by multiple neuroendocrine neuronal types that can directly regulate pituitary hormone secretion (Trudeau, 1997; Ball, 1981). Incubations with NRS or pre-absorption of the antiserum with gfSN comptelely abolished SN-IR in pituitary sections (Fig. 2.5A), confirming specificity of the reaction as we previously reported with western blot experiments (Zhao et al., 2006b). The anterior pituitary includes the rostral pars distalis (RPD) and proximal pars distalis (PPD) (Fig. 2.5B). SN-IR was located in the RPD and within nerve fibres in the neurointermediate lobe (NIL, Fig. 2.5B; Fig 2.6). The distribution of the major pituitary hormones is well described for goldfish and is confirmed here. Note that LH (Fig. 2.5D) and GH (Fig. 2.5F) cells are localized in the PPD. PRL-IR was identified exclusively in the RPD (Fig. 2.5E). The distribution pattern of SN-IR in the anterior pituitary was highly similar to that of PRL-IR (Fig. 2.5B versus 2.5E). In the NIL, SN immunostaining signals were found only in the nerve fibers (Fig. 2.5B), but not in neighboring pituitary cells. This is particularly clear at higher magnification of SN-IR cells in the RPD and SN-IR fibres in the NIL (Fig. 2.6). In adjacent sections, lactotrophs (Fig. 2.7C, D) could be identified that contained SN-IR (Fig. 2.7A, B).

Given the heavy staining in the fibres of the NIL, we speculated that neurons of the

preoptic area may be the origin of this immunoreactivity. Intense staining of cells bodies was indeed found in the magnocellular neurons of the nucleus preopticus (NPO) (Fig. 2.8A). This reaction was specific because it was blocked by preabsoption of the antiserum with gfSN (Fig. 2.8B). Heavily labeled fibres could be traced leaving the NPO laterally and posteriorly into the pituitary stalk, passing centrally to the NIL (Fig. 2.8C). Clearly labeled fibres could also be found entering the PPD (Fig. 2.8D) where the gonadotrophs and somatotrophs are located (Fig. 2.5D, F). This follows previously well-described tracts and pituitary innervation patterns established by retrograde tracing in goldfish (Anglade et al., 1993). The results on the distribution of SN-IR in fibres in the NIL and PPD in addition to endocrine cells in the RPD was confirmed in several independent experiments with both sexes following both the immunocytochemical protocols described for pituitary and subsequently that described for brain with different antibody concentrations and detection methods (not shown).

2.4.5. Western blot analysis of goldfish pituitary

While it is clear that free SN is produced in the brain, it was also important to determine if it was present in the pituitary. Goldfish pituitaries collected in May were separated by 15% SDS-PAGE gel and analyzed by western blots using the anti-SN antibody (Fig. 2.9A, Lane II). As a comparison, synthetic gfSN peptide was resolved in the same gel (Fig. 2.9A, Lane I). In the low molecular mass (<10,000 Da) region, anti-SN revealed a weak band in the pituitary extract as well as a strong band for synthetic gfSN peptide. The similar estimated masses of these two bands in western blot analysis indicate the existence of free gfSN peptide in goldfish pituitary. In addition to free SN peptide, three SN-IR signals were also detected at distinct molecular masses predicted as ~19.6, ~15 and ~12 kDa (Kilodalton),

respectively (Fig. 2.10A).

2.4.6 In vitro effect of gfSN on the LH secretion from pituitary cells and neutralizing action of goldfish SN antibody

Having determined that free SN is in the pituitary using immunocytochemistry and western blots, we performed experiments to determine whether synthetic gfSN has a direct stimulatory effect on LH release. Salmon GnRH (100 nM) and SN (1 and 10 nM) were administered for 12 hours in a static incubation experiment with dispersed pituitary cells from goldfish in the seasonal sexual redevelopment phase (Fig. 2.9B). Treatment with 100 nM sGnRH elevated LH levels by approximately 2.2-fold ($P<0.001$) compared with the control value. Similarly, exposure to 1 and 10 nM of SN, respectively induced 2.5-fold ($p<0.001$) and 1.9-fold ($p<0.01$) increments of LH secretion from dispersed goldfish pituitary cells. We performed another experiment to confirm the stimulatory actions of gfSN on LH using cells obtained from sexually mature animals just before the spawning season. We also maximized potential interactions amongst dispersed cells and plated them at a 4-fold higher density. In accordance with the previous experiment, sGnRH stimulated a 3.4-fold ($p<0.01$) and 10 nM gfSN stimulated a 2.7-fold ($p<0.05$) increase in LH levels (Fig. 2.9C). In the neutralization experiment, 100 nM sGnRH and 10 nM gfSN showed identical stimulatory effects on LH secretion to by 193% ($p<0.01$) and 159% ($p<0.05$) (Fig. 2.9D), respectively. Furthermore, 12-hour incubation with goldfish SN antibody at the dilution of 1:1,500 decreased LH level by 67% ($p<0.05$) in comparison with the control value (Fig. 2.9D).

2.5. Discussion

Here we demonstrated for the first time the presence of a 34 amino acid long free SN peptide derived from the SgII precursor in the brain and pituitary of a teleost species, the goldfish (*Carassius auratus*). The natural isolated peptide was fully characterized by comparative mass spectrometry with synthetic material. We also noted the presence of an oxidized form of SN_{34} in the goldfish brain. Whether this oxidation occurred during isolation or not cannot yet be fully ascertained. High SN-IR was detected in the NIL and in lactotrophs of rostral PD of the anterior pituitary. This is in agreement with previous studies of both male and female goldfish pituitaries indicating that levels of SgII mRNA (Samia et al., 2004) and levels of an immunoreactive SgII-derived protein containing SN (~57 kDa) were higher (Zhao et al., 2006b) in the PD than in the NIL. Moreover, the well-characterized NPO magnocellular cells sending projections to the posterior and anterior pituitary (Batten, 1986) were also SN-immunopositive. The result that SN stimulated LH release directly from dispersed goldfish pituitary cells strengthens our previous *in vivo* and *in vitro* findings (Blázquez et al., 1998a; Zhao et al., 2006a), indicating that SN is involved in the regulation of reproductive hormone secretion.

Widely distributed throughout the rat neuroendocrine system, SN-IR is highest in the hypothalamus while lower concentrations are shown in the hippocampus, caudate nucleus, thalamus and brainstem (Kirchmair et al., 1993). Free SN peptide was previously separated from rat brain extract by HPLC and characterized by radioimmunoassay (Leitner et al., 1996). Furthermore, reverse phase chromatographic analysis of human forebrain extract showed a single peak for SN-like IR, suggesting that SgII is processed to generate free SN peptide in human brain (Marksteiner et al., 1993b). In goldfish, the SgII precursor (69.6 kDa) and

intermediately-sized peptides (~57, ~30 and ~20 kDa) containing SN were previously detected using western blots of extracts from pituitary and brain (Zhao et al., 2006b). Based on the position of conserved dibasic cleavage sites in the SgII precursor and similarities between known SN sequences and predicted amino acid sequences of several species, we previously suggested that teleost SN is a 34 amino acid peptide, which is in contrast to the known 33 amino acid tetrapod SNs (Blázquez et al., 1998a; Samia et al., 2001). Here we determined that free goldfish SN peptide has indeed 34 amino acids. We utilized RP-HPLC to separate and isolate a SN-IR peptide from goldfish brain. The similarity of SELDI-TOF mass spectrometry profiles of the endoproteinase Lys-C digests of the isolated peptide and synthetic gfSN indicates that we isolated gfSN. To further analyze the isolated peptide, C-terminal sequencing by mass spectrometry was adopted. This method was demonstrated to sequence protonated peptides derived from proline-rich proteins that are difficult to sequence by standard collision-induced dissociation mass spectrometry (Fernandez Ocaña et al., 2005). Furthermore, C-terminal sequencing using CPY and SELDI-TOF mass spectrometry was used previously to another neuroendocrine factor, adrenocortiocotropic hormone ($ACTH_{1-39}$) (Cool and Hardiman, 2004). Thus, this approach was suitable for determining the sequence of gfSN, a proline-containing neuropeptide (Zhao et al., 2006b). Given that high SN-IR has been found in nerve fibres and terminals in the NIL and PPD, there is the potential that free gfSN of neuroendocrine origin is being released from nerve terminals in the pituitary.

The amount of total SN-IR level in the goldfish brain is 65.1 fmol/mg tissue weight, which is in the same range of SN-IR concentrations in rat brain (98.5 fmol/mg tissue weight, Leitner et al., 1996). However, goldfish pituitary was determined to contain 278 fmol/mg SN-IR (Fig. 2.4C), which was much lower than SN-IR levels found in rat pituitary (6,604 and

2,378 fmol/mg tissue weight, respectively in the anterior and posterior pituitary (Leitner et al., 1996). There are good indications that the fish pituitary produces multiple SN-IR fragments. High levels of SgII mRNA and SgII-derived proteins are found in the goldfish pituitary (Zhao et al., 2006b). In this study, western blot analysis using the same antiserum clearly demonstrated the presence of free gfSN peptide in the extract from goldfish pituitary. To predict the possible domains of detected SgII-derived peptides in the pituitary, we calculated the molecular masses of all potent fragments between putative dibasic cleavage sites in the goldfish SgII precursor sequence. The ~57 or ~30 kDa protein might respectively be derived following cleavages by a signal peptidase at the 34/35 ($GVQG^{34}\downarrow AT$) site and a PC-like protease at the 532/533 or 534/535 ($HGQ\mathbf{RR}^{532}\downarrow AR^{534}\downarrow LT$) sites, or at the 193/194 or 196/197 ($PDT\mathbf{RR}^{193}\downarrow QHR^{196}\downarrow KL$) and 459/460 ($PH\mathbf{RR}^{459}\downarrow PI$) sites (Zhao et al., 2006b; Fig. 2.10B (1)-(4)). The ~19.6, ~15 and ~12 kDa SN-IR peptides are likely derived from the individual proteolytic processing at the 193/194 or 196/197 ($PDT\mathbf{RR}^{193}\downarrow Q\mathbf{HR}^{196}\downarrow KL$) and 357/358 ($QEQQ^{357}\downarrow\mathbf{KR}$) sites, at the 193/194 or 196/197 ($PDT\mathbf{RR}^{193}\downarrow Q\mathbf{HR}^{196}\downarrow KL$) and 319/320 ($DV\mathbf{KR}^{319}\downarrow KI$) sites, and at the 214/215 ($PL\mathbf{KR}^{214}\downarrow TN$) and 317/318 ($DDDV^{317}\downarrow KR$) sites by the cleavage action of PC and/or CPY (Fig. 2.10B (5)-(7)). This ~12 kDa SgII-derived peptide might be processed by PC enzyme to directly generate free SN peptide ($gfSN_{1\text{-}34}$) in the pituitary (Fig. 2.10B (8), Fig. 2.9A). These results indicated the possibly complete process for yielding free SN peptide in the pituitary.

The distinct overlapping of SN-IR with that of PRL suggests that lactotrophs of the RPD could be an endocrine, possibly paracrine source of SN peptides within the PD of the goldfish pituitary. It has been known for several decades that rat gonadotrophs can affect lactotroph function by paracrine signals (Denef, 2008). More recently, Wong et al. (2005)

have shown that both LH and GH act as paracrine factors respectively regulating somatotrophs and gonadotrophs in the grass carp pituitary. We have not yet proven that the lactotrophs release free SN. However, SN is released from dispersed rat pituitary cells *in vitro* (Wei et al., 1995) and goldfish pituitary cells release small amounts of a SN-IR product (size unknown) detectable by dot blot of incubation medium (unpublished data).

One striking finding was the limited distribution of SN-IR within the anterior pituitary of goldfish. Previous reports on the distribution of SN-IR in the human pituitary (Schmid et al., 1995), and on the levels of SgII mRNA detected in fractionated semi-purified dispersed goldfish pituitary cell preparations led to our original hypothesis (Blázquez et al., 1998a) that SN was to be predominantly localized to gonadotrophs. Here we demonstrated the SN-IR is found in lactotrophs and not gonadotrophs. In mammals, SN is found in gonadotrophs, but also other major anterior pituitary cell types. While it is clear that most if not all pituitary cell types would express SgII, there is differential and specific prohormone convertase-mediated processing of the SgII precursor processing in each cell type. In the rat, for example, there is differential processing of SgII to produce the peptide manserin in corticotrphs only (Yajima et al., 2004). The rat SgII precursor also gives rise to a bioactive peptide, EM66, which is present in gonadotrophs, lactotrophs, thyrotrophs, and melanotrophs, but not corticotrophs (Montero-Hadjadje et al., 2003). In the goldfish anterior pituitary, SgII processing to SN is therefore limited to lactotrophs since no other cell types exhibited SN immunoreactivity.

We previously demonstrated that synthetic gfSN stimulates LH secretion from pituitary fragments *in vitro* (Zhao et al., 2006a). While an important preliminary observation, it was difficult to interpret these results because of the nature of the teleost pituitary. The neural lobe of the fish pituitary receives innervation from the magnocellular neuroendocrine

neurons as in mammals. However, the teleost pituitary complex is a highly derived organ and does not have a median eminence that is typical in tetrapods. Rather, the median eminence was lost at some time in evolution after the appearance of the bony fishes approximately 200 million years ago (Ball, 1981; Gorbman, 1995). Therefore, the anterior pituitary of teleosts is very heavily innervated by a multitude of neuropetide- and neurotransmitter-producing neurons. Indeed, nerve terminals containing diverse neuro-hormones known to affect LH and GH release in goldfish have been visualized by electron microscopy to make direct contact with endocrine cells (Trudeau, 1997; Batten, 1986). Goldfish pituitary fragment preparations therefore contain both endocrine cells and isolated nerve terminals. In this paradigm, SN could affect the release of a stimulatory or inhibitory factor from nerve terminals and/or directly stimulate LH release, which could partially explain our previous *in vivo* and *in vitro* LH release results. However, we now demonstrated that SN in the same dose range previously tested on goldfish pituitary fragments stimulates LH release directly from primary dispersed goldfish pituitary cells, eliminating any possibility of indirect effects on nerve terminals. As a positive control, we also showed that sGnRH stimulates LH release to a similar degree as SN in the same cell preparations. An important question remaining is whether co-treatments with one or several of the multiple stimulators of LH in goldfish, including SN, would have an additive or potentiating effects on GnRH-stimulated LH release.

For further investigating the neuroendocrine or paracrine action of gfSN, we performed a pilot neutralization experiment, showing the negative effect of gfSN antibody on the basic LH release *in vitro* during the sexual redevelopment period of goldfish. At that time, the animal displays a high serum LH level indicating the strong effects of various stimulators inside or outside pituitary, such as GnRH (Trudeau, 1997). Thus, we affirmed that

endogenous SN is truly involved in regulating LH secretion within the pituitary of gonadal mature goldfish. However, the neuroendocrine effect of brain-sourced SN still needs to be explored in the future.

Results from our recent study in collaboration with Dr. J.P. Chang at the University of Alberta suggest that elevation of $[Ca^{2+}]_i$ (intracellular Ca^{2+} concentration) is likely the signal transduction pathway mediating gfSN action (Zhao et al., 2009a). Despite the fact that the LH responses to 100 nM sGnRH and 10 nM gfSN are similar, the maximal amplitude of the $[Ca^{2+}]_i$ increase induced by gfSN is about half the size of that to sGnRH. Direct comparison of the magnitude of the Ca^{2+} responses (in minutes) to long-term stimulated LH release (in hours) cannot be made because of the following reasons: 1) the duration of the observations are vastly different; 2) signaling mechanisms in addition to Ca^{2+} are known to be involved in LH release; and, 3) the magnitude of the global Ca^{2+} signal is not linearly related to that of stimulated LH release in goldfish (Chang et al., 2000; Chang et al., 2009). The ability of gfSN to further increase $[Ca^{2+}]_i$ in the presence of a maximally stimulatory dose of 100 nM sGnRH (Johnson et al., 1999) suggest that the Ca^{2+} signaling mechanisms for, and/or the source of the Ca^{2+} mobilized by, these two neuropeptides are at least partly independent of one another. In goldfish gonadotrophs, multiple pharmacologically distinct intracellular Ca^{2+} stores are involved in ligand-specific signaling leading to LH secretion (Chang et al., 2000; Chang et al., 2009). It is possible that the differential use of Ca^{2+} stores and/or Ca^{2+} entry mechanisms is part of the basis for the additivity of the Ca^{2+} signals when gfSN is applied during sGnRH challenge. It is known the SN mechanism of action also involves protein kinase C and Jak/Stat signaling (Kähler et al., 2002; Shyu et al., 2008). Given that SN is likely to be an important endogenous neuroendocrine and/or paracrine stimulator of LH in

goldfish, it will be necessary to fully characterize the signaling mechanisms mediating gfSN action on LH release in future studies.

We conclude that SN is a neuroendocrine factor that can affect LH secretion in the pituitary. Free SN peptide was isolated and/or identified in brain and pituitary extracts. There is SN-IR in NPO magnocellular neurons and heavy projections entering the NIL and PPD of the pituitary indicating a neural source of SN. Moreover, SN-IR in lactotrophs of the rostral pars distalis indicates an endocrine source of SN. Nanomolar concentrations of synthetic gfSN stimulate LH release directly from dispersed cells *in vitro*. Immunoneutralization indicates that endogenous SN of pituitary origin has a stimulatory paracrine role. Brief application of SN induces moderate Ca^{2+} signals in individual identified gonadotrophs. We hypothesize that SN is acting on a membrane receptor but direct evidence is still lacking. This remains a significant challenge because development of specific SN agonists or antagonists, or identification of the putative cell surface SN receptor has not been reported for any species.

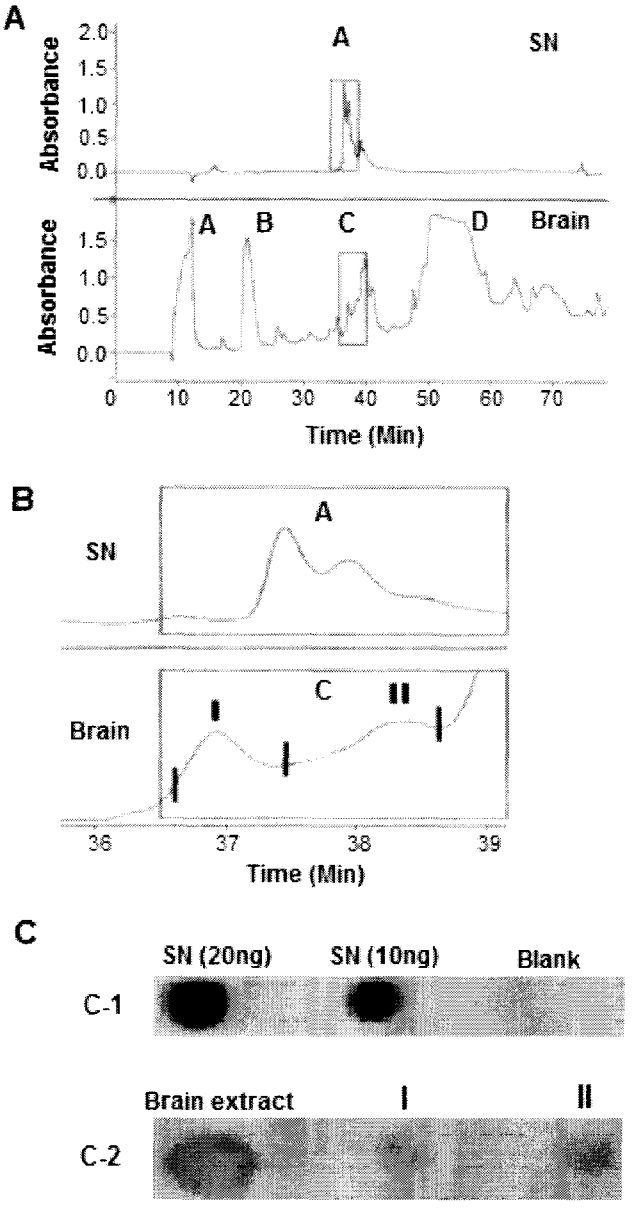

Figure 2.1. Separation and identification of SN-related peptides in goldfish brain. **A**, RP-HPLC elusion profile of synthesized SN peptide (100μg) and goldfish brain extracts. Peptides were separated by RP-HPLC on a C-4 semi-preparative column using a linear gradient from 35% to 70% ACN containing 0.1% TFA for 65 min at a flow rate of 1.5 ml/min. Fraction C separated from brain extract was collected because its elution time was similar to that of main fraction A from synthetic goldfish SN. **B**, Enlarged RP-HPLC elusion profiles of fraction A from synthetic gfSN and fraction C separated from brain extracts. Fraction I and II were separated within fraction C in brain extracts. **C**, Dot immunoblotting analysis of synthetic gfSN (panel C-1), crude whole brain extract and fraction II isolated by RP-HPLC (panel C-2). Anti-gfSN antiserum was used at a dilution of 1:1000. Blank indicates only buffer was blotted.

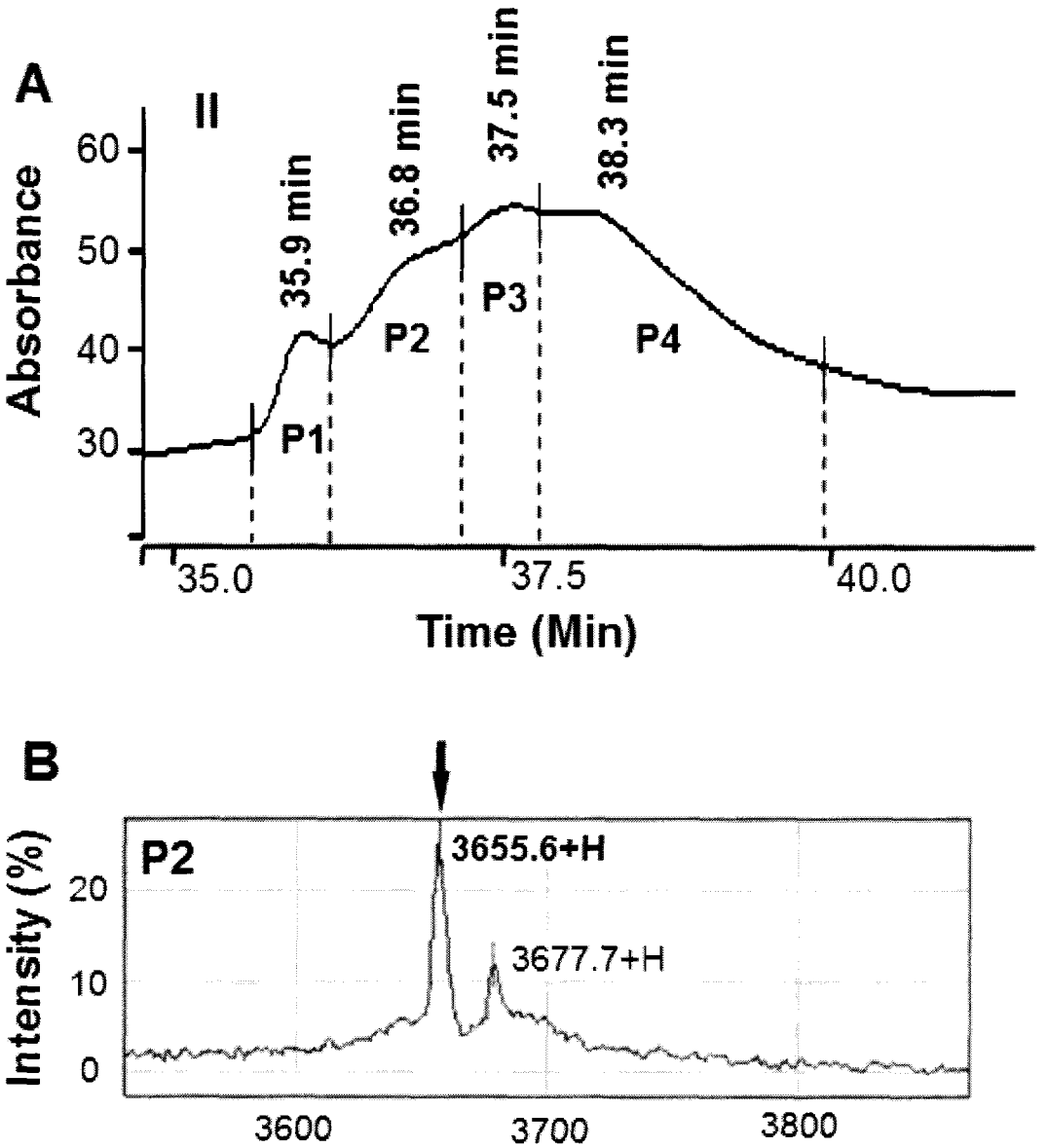

Figure 2.2. Further separation and purification of free SN peptides from fraction II. **A**, RP-HPLC elution profile of peptides generated from fraction II (Figure 2.1B). Fraction P1 through P4 peptides were separated using the protocol described in Figure 2.1A. Note that the retention time (Rt) was labeled on the top of each fraction. **B**, SELDI-TOF mass spectrometry analysis showing that faction P2 (m/z 3655.6) probably contains the free SN peptides.

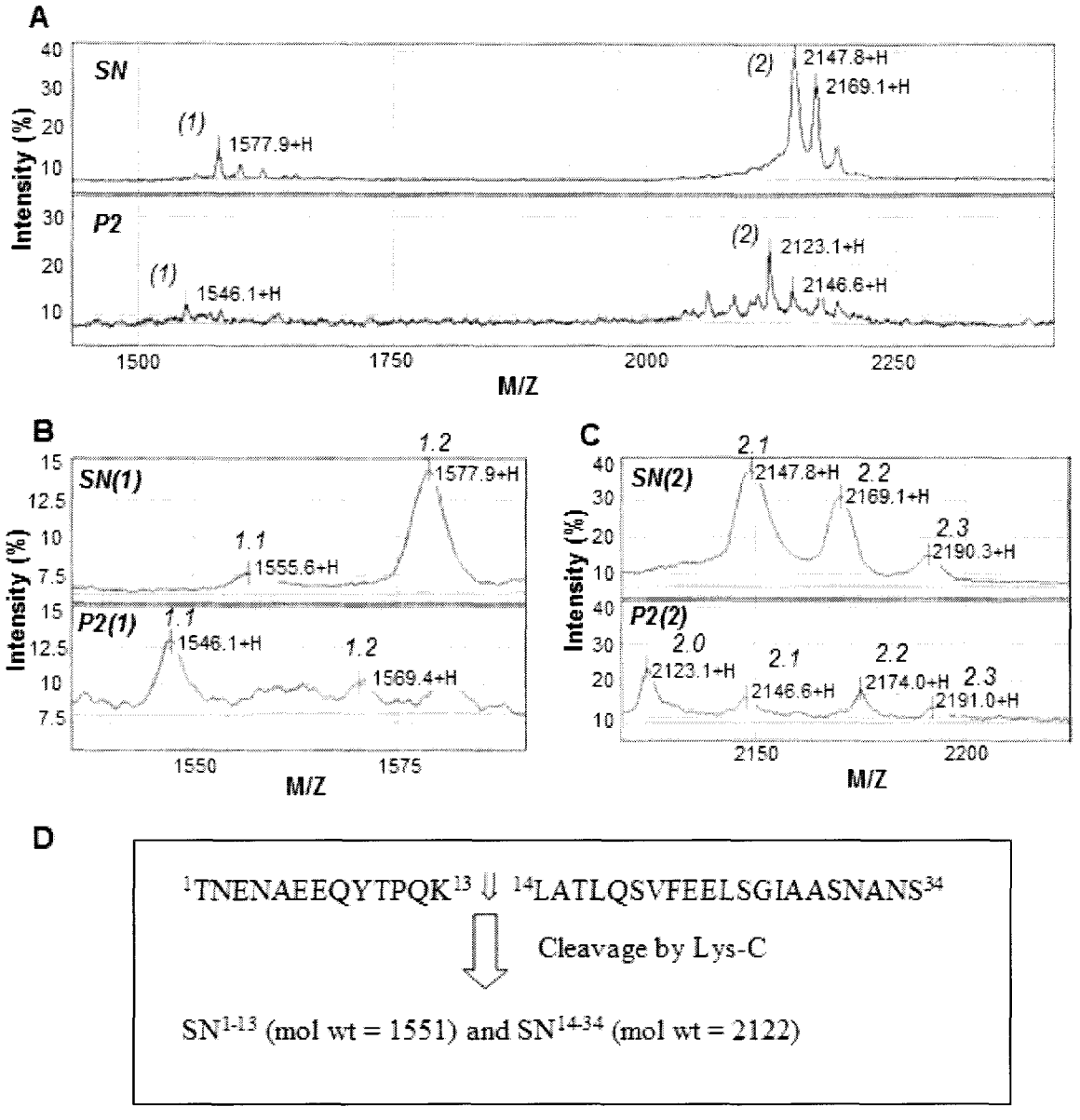

Figure 2.3. Endoproteinase Lys-C digestion of goldfish brain SN-like peptide obtained from fraction P2. **A**, SELDI-TOF mass spectra of synthesized SN and faction P2 identifying that Lys-C cleaved the peptides to two fragments, (1) and (2). **B**, Comparison of the enlarged mass spectra of fragment (1) and **C**, fragment (2) indicates that the Lys-C digestion pattern of synthetic SN and fraction P2 are similar. **D**, The expected cleavage fragments and their molecular sizes following Lys-C digestion are depicted.

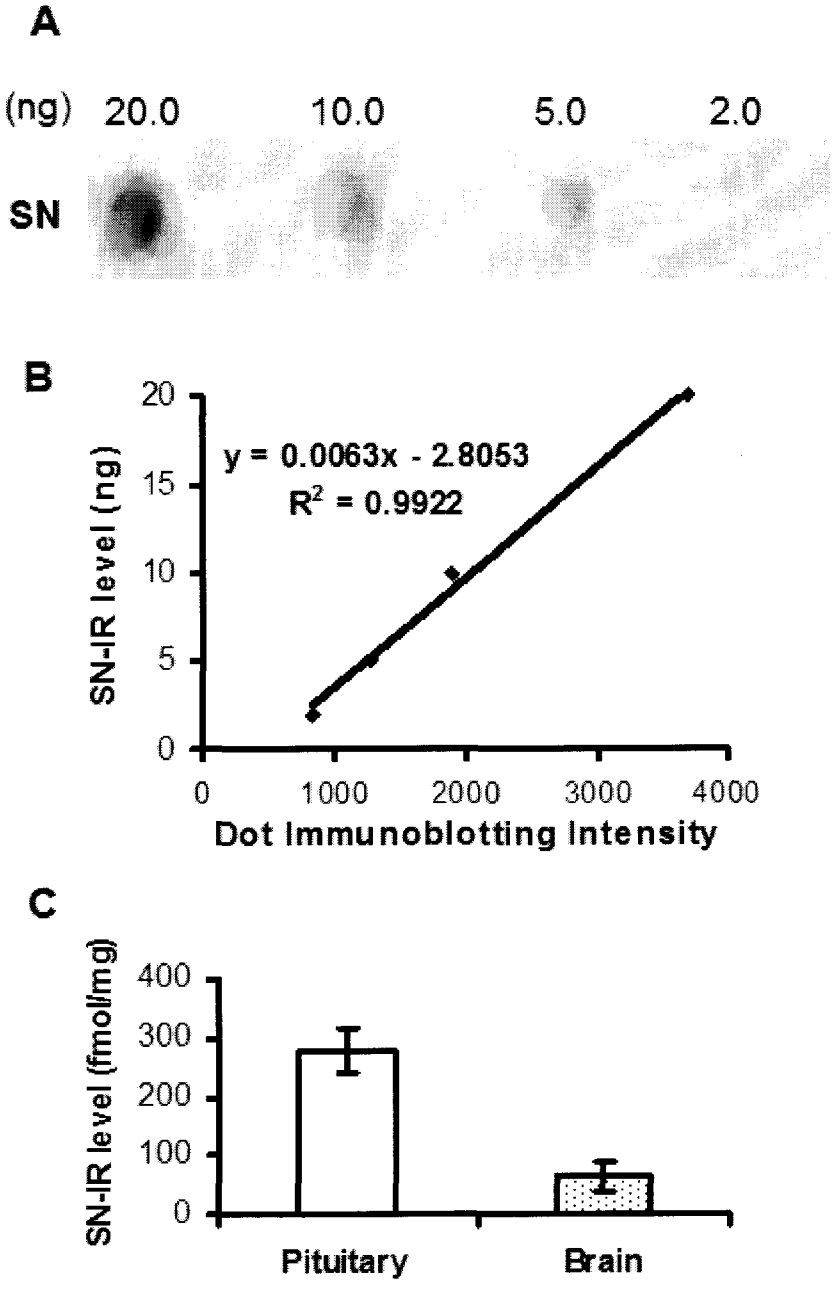

Figure 2.4. Estimation of SN-immunoreactive peptide concentrations. **A**, Dot immunoblotting analysis of synthetic goldfish SN using the anti-SN antiserum (dilution: 1:1000). **B**, A standard curve and a formula depicting the relationship between various SN levels (ng) and their intensity of SN-IR. In the formula, x represents SN-IR intensity, whereas y represents SN level (ng). **C**, Quantitation of total SN-IR in homogenates of whole pituitary and brain from goldfish. Data are means ± S.E.M. (n=3).

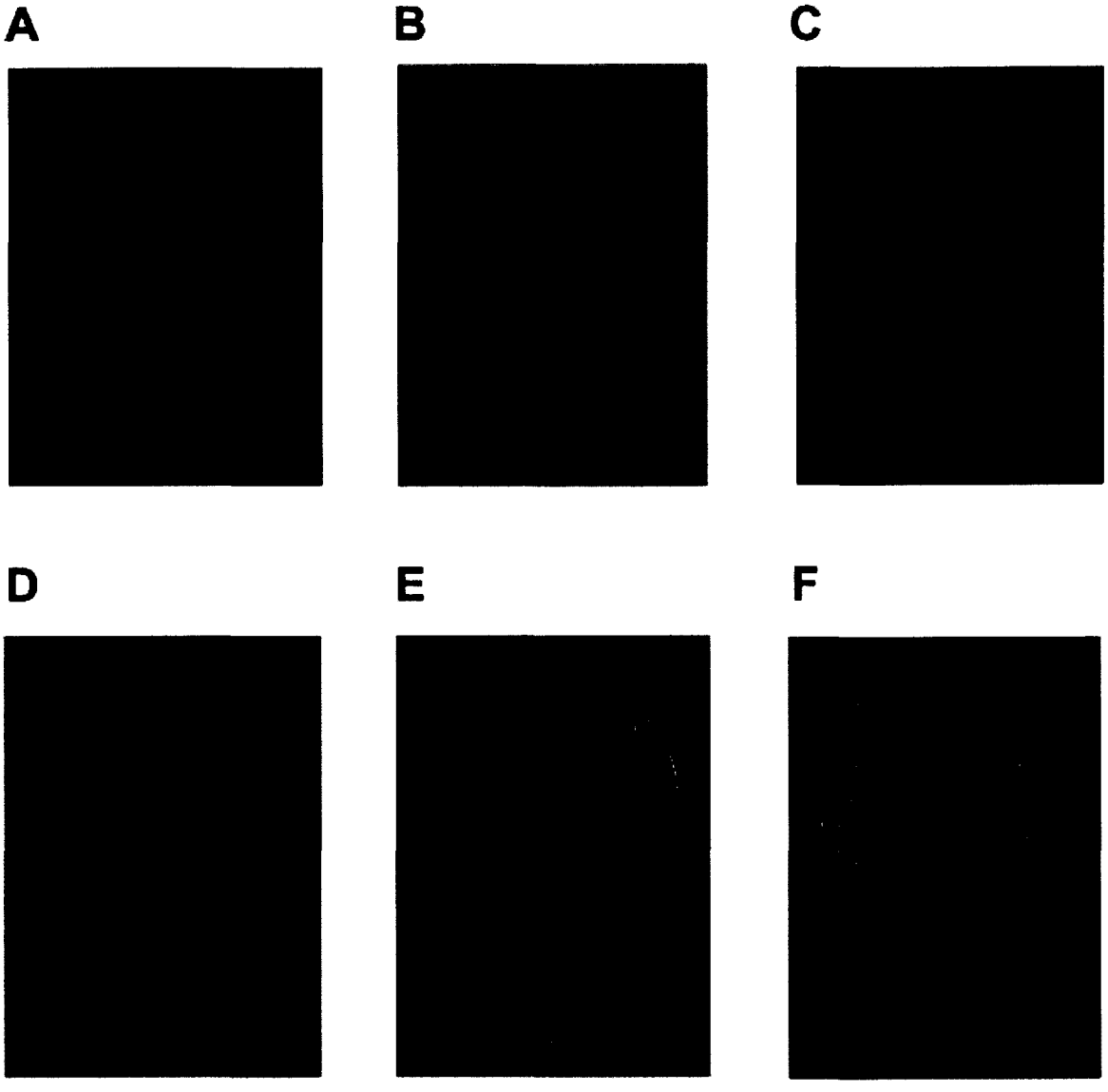

Figure 2.5. Immunocytochemical staining of goldfish pituitary sections. **A**, Background staining in control sections with normal rabbit serum (NRS; 1:12,000) was virtually non-detectable. **B**, Parasagittal section of goldfish pituitaries stained with SN antiserum (1: 12,000; middle panel). RPD: rostral pars distalis, PPD: proximal pars distalis, NIL: neurointermediate lobe. **C**, Preabsorption (Pabs) of the primary antiserum with synthetic goldfish SN (1 μM; overnight at 4 C) completely blocked the immunoreaction. **D**, Immunocytochemical staining of pituitary sections for LH-producing gonadotrophs (LH; 1:60,000). **E**, Immunocytochemical staining of pituitary sections for lactotrophs (PRL; 1:100,000). **F**, Immunocytochemical staining of pituitary sections for somatotrophs (GH; 1:500,000). SN- and PRL-IR overlapped in RPD whereas GH- and LH-IR were restricted to the PPD. SN-immunostaining in the NIL shows that SN-IR was found only in nerve fibers. This experiment was in collaboration with Dr. A.O.L. Wong in the University of Hong Kong.

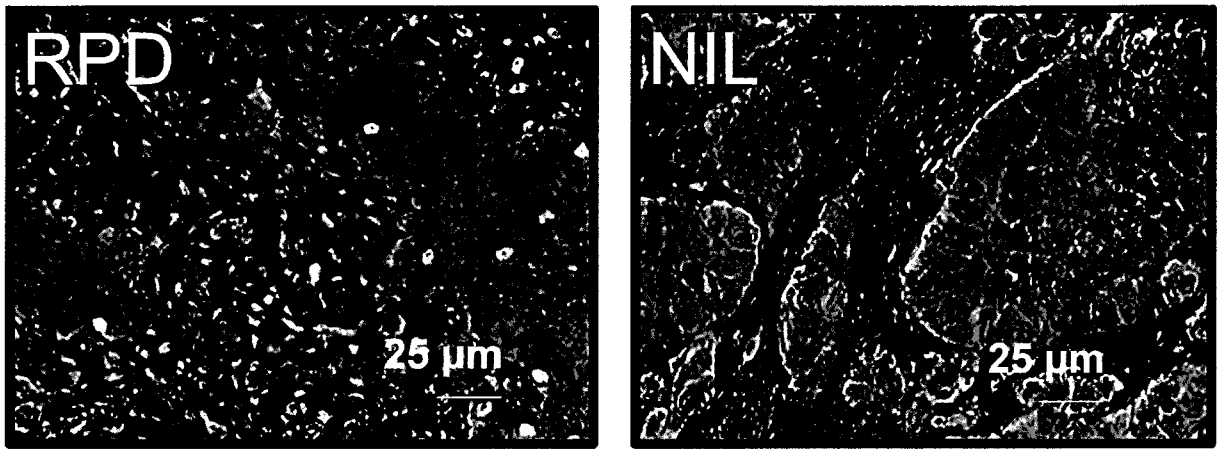

Figure 2.6. Immunocytochemical staining of goldfish pituitary sections for SN. **Left panel**; Magnified view of SN-IR in the RPD. Note the clear delineation between SN-IR cells in the RPD and the lack of SN-IR in the adjacent cells in the PPD. **Right panel**; Magnified view of SN-IR in fibres in the neurointermediate lobe. Note the lack of SN-IR in adjacent pituitary cells.

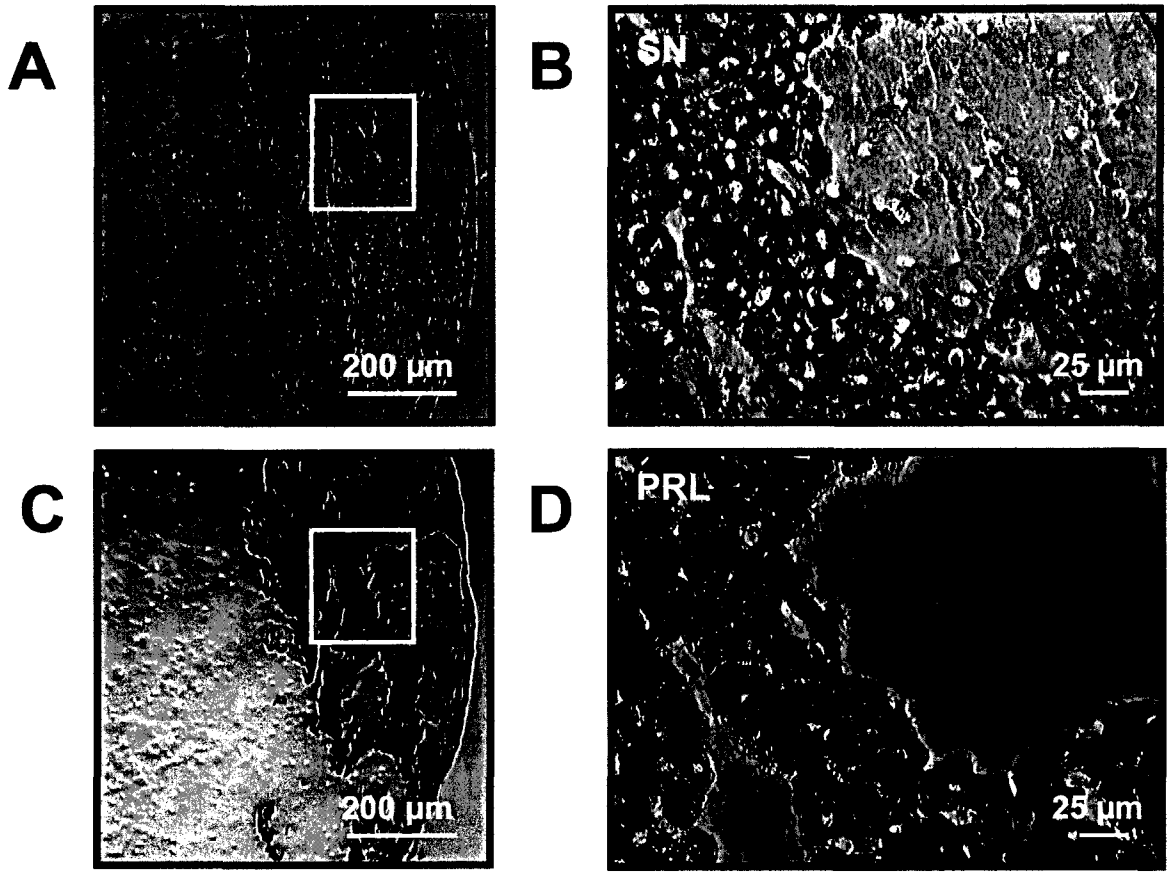

Figure 2.7. Immunocytochemical staining of adjacent goldfish pituitary sections for SN and PRL. **A**, SN-IR in the rostral pars distalis. The white box delineates the enlarged area in panel B. **B**, PRL in the rostral pars distalis. The white box delineates the enlarged area in panel D. **C**, Magnified view of cells exhibiting SN-IR in the rostral pars distalis. **D**, Magnified view of cell exhibiting PRL-IR in the rostral pars distalis. Note the similarity of distribution of the cells inmmunoreactive for SN and PRL. Antibody concentrations are the same as reported for Fig 2.5.

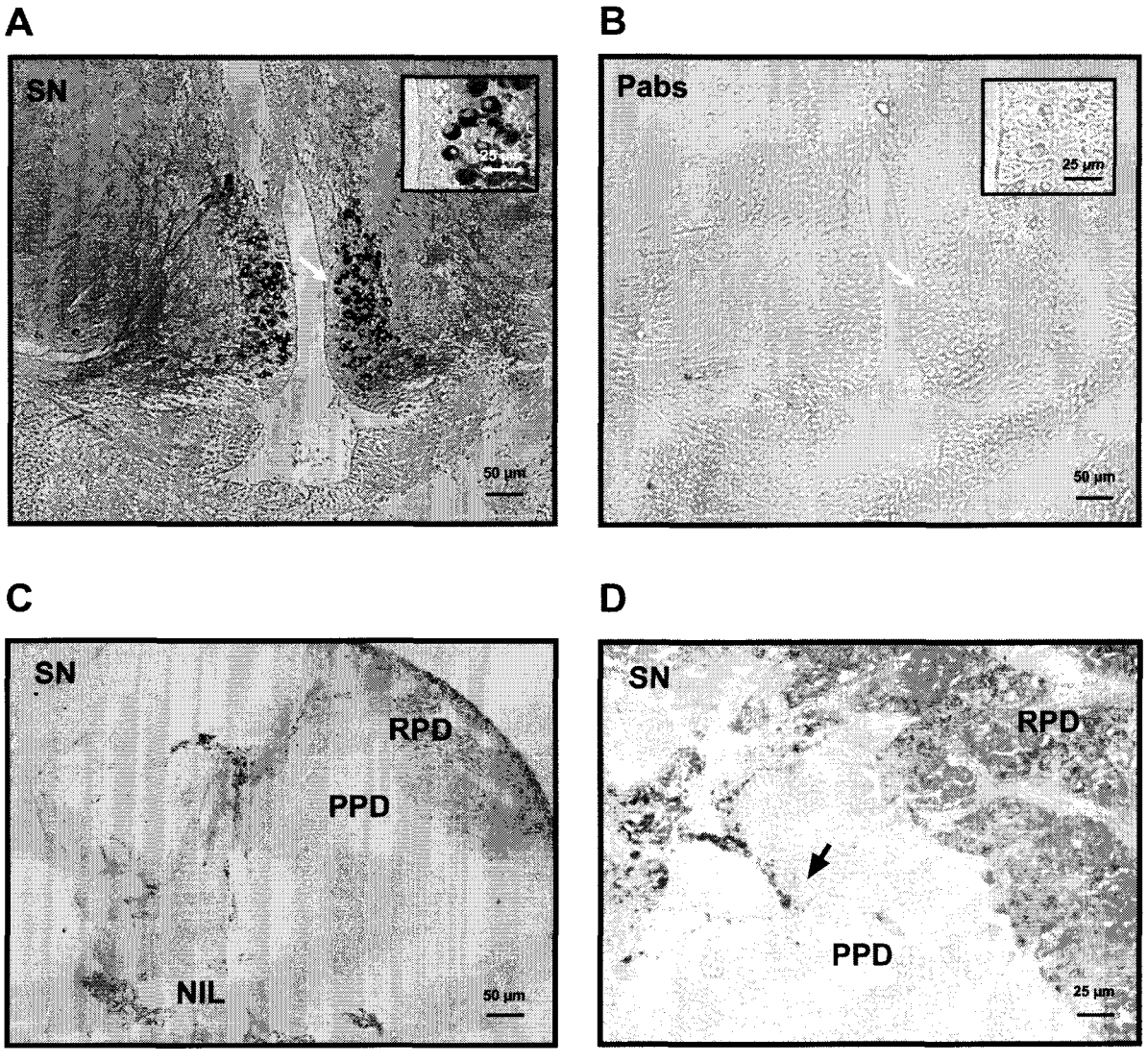

Figure 2.8. Immunocytochemical staining for SN in the preotic area of the female goldfish telencephalon and pituitary. **A**, Intense SN immunoreactivity (1:2,500) was observed in magocellular cells and projections The white arrow indicates the location of the magnified cell bodies in the inset photograph. Note the strong staining in the cytoplasm but not the nucleus. Similar results were found in males (not shown). **B**, Preabsorption (Pabs) of the primary antiserum with synthetic goldfish SN (2 µM; overnight at 4 ^{0}C) completely blocked the immunoreaction in an adjacent section. The white arrow indicates the location of the magnified cell bodies in the inset photograph. **C**, Immunocytochemical staining of a pituitary section for SN. Note the strong SN-IR in prolactin cells in the rostral pars distalis (RPD) and nerve fibres projecting to the proximal pars distalis (PPD) and neurointermediate lobe (NIL). **D**, Magnified view of fibres entering the PPD (black arrow).

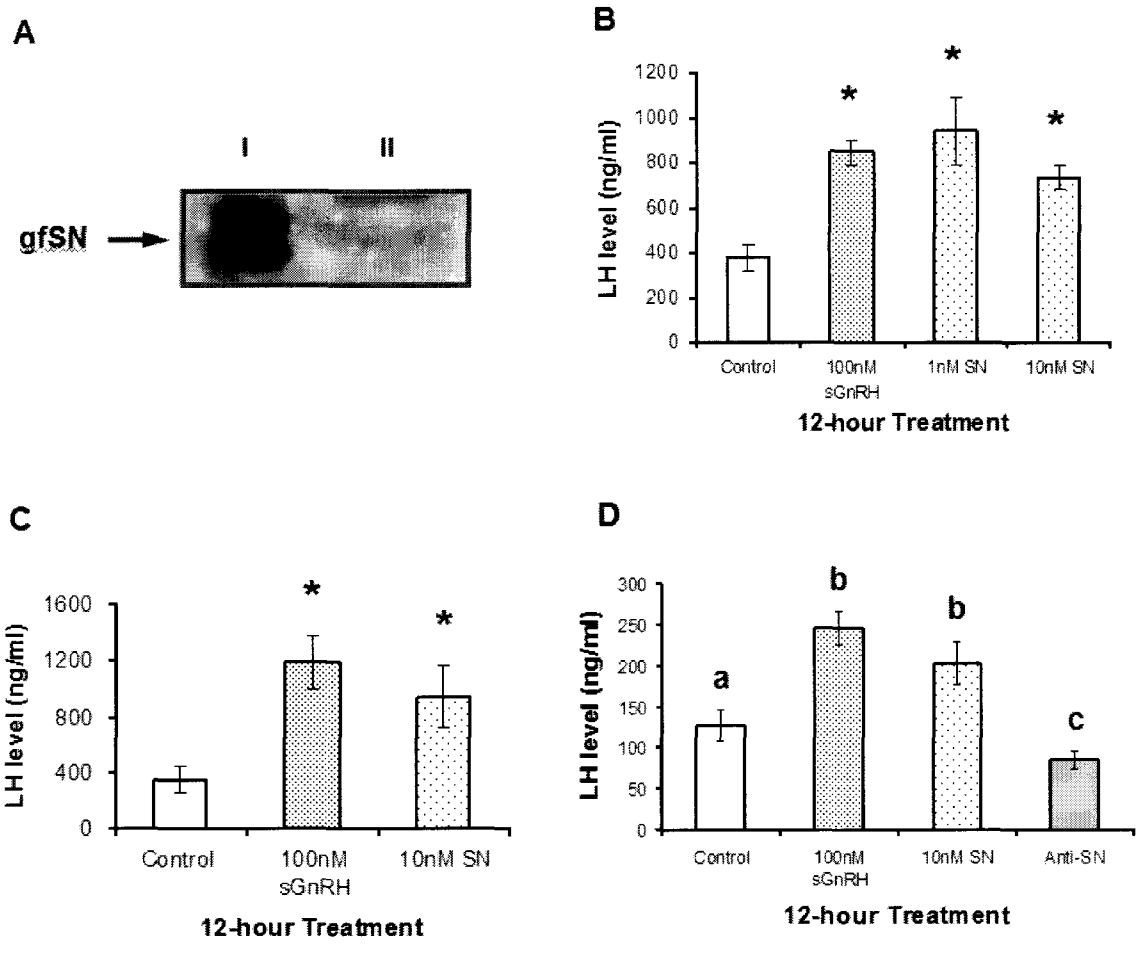

Figure 2.9. Detection and effects of SN in the pituitary. **A**, Western blotting analysis for free SN peptide in the goldfish pituitary. Pituitary total proteins (~100 μg, Lane II) were separated by 15% SDS-PAGE gel and detected using the anti-SN antiserum (dilution 1:700), in comparison with synthetic goldfish SN peptide (4 μg, Lane I). The arrow indicated that a small SN-IR peptide presented in the pituitary tissue, which had the same estimated molecular mass as synthetic gfSN. **B**, Effects of SN (1 and 10 nM) and sGnRH (100 nM) on LH release (ng/ml) from dispersed goldfish pituitary cells. Values were presented as mean ± SEM. The (*) indicated a significant difference from control group ($P<0.05$, Fisher LSD Method). This experiment was performed in December during the seasonal sexual redevelopment period of goldfish, using 24-well culture plates (~2.5 x 10^5 cells/well, n=9-10). **C**, Effects of SN (10 nM) and sGnRH (100 nM) on LH release (ng/ml) from dispersed goldfish pituitary cells. This experiment was performed in March at the beginning of the sexually mature pre-spawning period of goldfish, using 6-well culture plates (~2.0 x 10^6 cells/well, n=4). Values were presented as mean ± SEM. The (*) indicated a significant difference from control group ($P<0.05$, Fisher LSD Method). **D**, Neutralizing action of gfSN antibody (anti-SN, 1:1,500 dilution) on LH secretion (ng/ml) from dispersed goldfish pituitary cells. Data were indicated as mean ± SEM (n=12, 24-well incubation at the density of ~2.5 x 10^5 cells/well). The experiment was performed with sexual recovery goldfish. Different letters represent statistical differences ($P< 0.05$; Fisher LSD Method).

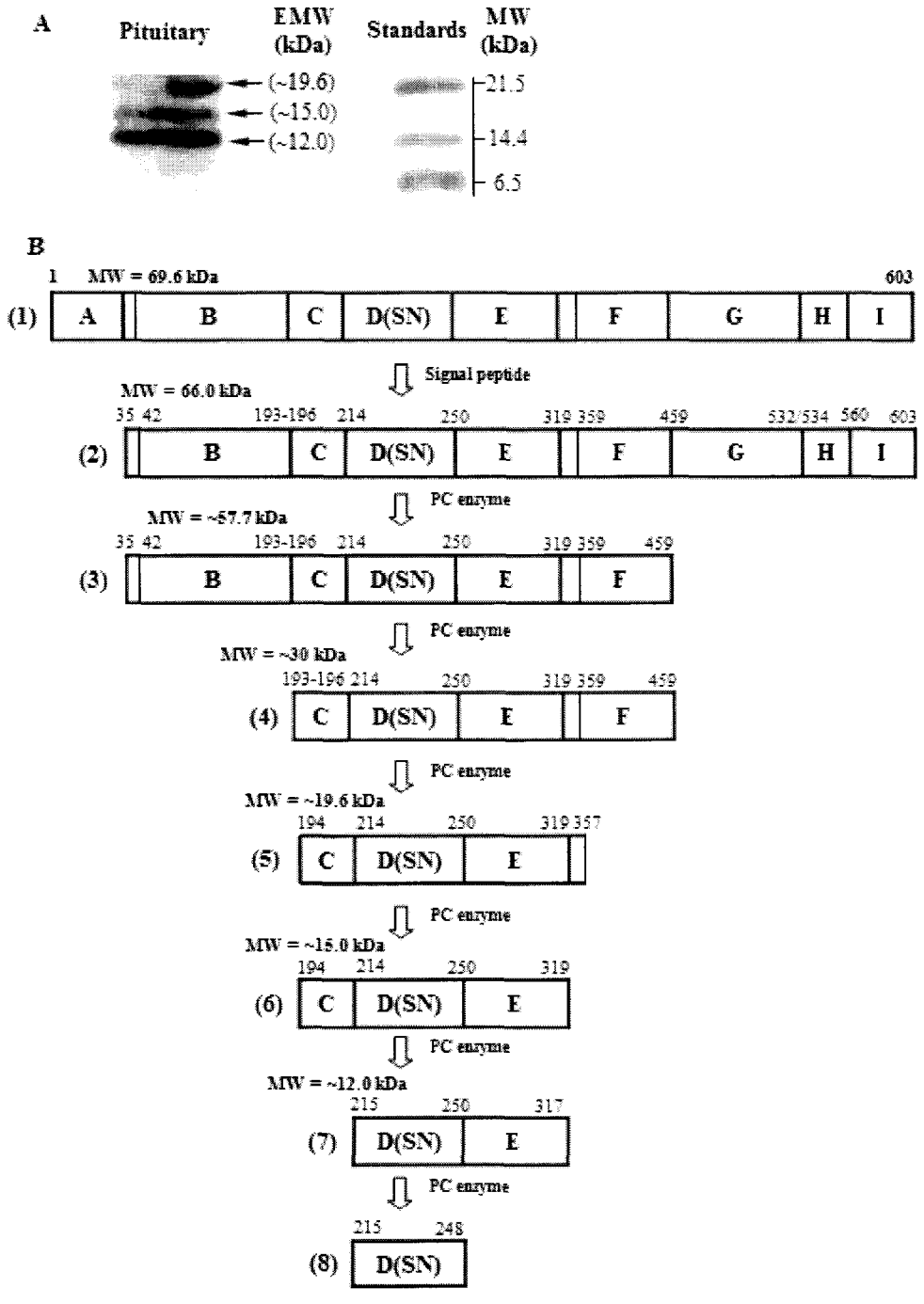

Figure 2.10. A, Estimation of apparent molecular weight (EMW) of SN-IR SgII derived proteins (<20 kDa) in goldfish pituitary. 15% SDS-PAGE separation followed by western blot analysis of a pituitary extract in May (~100 μg of total protein) using anti-SN antiserum (dilution: 1:1000). Results of pituitary protein and biotinylated SDS-PAGE standards are

shown. Arrows indicate three SN-IR proteins corresponding to ~19.6, ~15.0 and ~12.0 kDa, respectively. Molecular weights (MW) of standards are shown on the right. **B**, Proposed model for generation of free goldfish SN peptide from SgII as observed in pituitary. This model does not preclude other SN containing peptides, which we have not yet been detected. Abbreviations: PC, prohormone convertase-like protease; A, signal peptide; C, LF-19; D (SN), secretoneurin; H, LA-42. Note: we use the mammalian nomenclature for LF-19 and LA-42; however, they are poorly conserved in goldfish (Blázquez et al., 1998a).

CHAPTER 3: Secretoneurin (SN) is A Paracrine Factor of Goldfish Luteinizing Hormone Release under the Control of Gonadotropin-releasing Hormone

3.1. Abstract

Secretoneurin (SN) is a functional neuropeptide derived from the evolutionary conserved part of precursor protein secretogranin II. We previously found the SN-immunoreactivity (IR) in goldfish hypothalamic neurons and pituitary nerve fibers, and its luteinizing hormone (LH)-induced effect *in vivo* and *in vitro*, confirming that SN plays a regulatory role as a hypothalamic factor. In the present time-course study, SN (10 nM) shows a stimulatory effect to enhance LH production and secretion after the 6-hour static incubation of goldfish pituitary cells. Due to the existence of SN-IR in goldfish lactotrophs, endogenous SN might exert a paracrine effect on LH in the pituitary. In an immunoneutralization experiment, co-incubation with anti-SN antiserum (1:750) blocks the stimulatory effect of salmon GnRH (sGnRH) on LH increment by 64% ($p=0.004$) *in vitro*. Using western blot analysis, we demonstrate that sGnRH increases the expression of the major SgII-derived peptide (~57 kDa, with SN-IR) ($p=0.002$) and prolactin (PRL) ($p<0.05$) after 12 hours in the static culture of goldfish pituitary cells. Furthermore, there exists a significant correlation between the levels of these two proteins ($R=0.76$, $p=0.004$). Another ~30 kDa SgII-derived peptide containing SN is only observed in sGnRH-treated pituitary cells. Consistent with the western blot results, real-time RT-PCR analysis shows that a 12-hour treatment with sGnRH respectively induced 1.6- ($p=0.006$) and 1.7-fold ($p=0.01$) increments in SgII and PRL mRNA levels. SgII gene expression was also associated with PRL gene expression ($R=0.66$; $p=0.02$). Taken together, endogenous lactotroph-generated SN, under the control of hypothalamic

GnRH, exerts a paracine action on neighboring gonadotrophs to regulate LH release.

3.2. Introduction

Gonadotropin-releasing hormone (GnRH) is a 10-amino acid hypothalamic neuropeptide regulating reproduction in vertebrates (Trudeau 1997). Goldfish have two GnRH forms, [His^5, Trp^7, Tyr^8]-GnRH (chicken GnRH-II or cGnRH-II) and [Trp^7, Leu^8]-GnRH (salmon GnRH or sGnRH), controlling pituitary hormone release via activating GfA and GfB GnRH receptors, respectively (Chang et al., 1993; Peter et al., 1986; Illing et al., 1999). These two distinct GnRH receptors were demonstrated to show a similar pattern of gene expression in the proximal pars distalis (PPD) of the goldfish pituitary, indicating that both cGnRH-II and sGnRH are capable of stimulating LH secretion from the gonadotroph cells in the PPD (Illing et al., 1999). Gonadotropins (GTH) are classified as follicle stimulating hormone (FSH) and luteinizing hormone (LH). FSH and LH are glycoproteins composed of a common α subunit and a variable β subunit which is specific to the hormone (Schulz et al., 2001). Moreover, GnRH is a modulator to regulate other neuropeptide release from the pituitary. *In vitro* either cGnRH-II or sGnRH induces growth hormone (GH) secretion from goldfish pituitary cells (Chang et al., 1990). Salmon GnRH was also demonstrated to induce the release of prolactin (PRL) from the tilapia pituitary, but its effect is weaker than that of cGnRH-II (Weber et al., 1997). In the mouse LβT2 gonadotroph cell line, GnRH not only enhances LH release but also stimulates the secretion of secretogranin II (SgII), a granin protein (Nicol et al., 2002).

SgII is a tyrosine-sulfated granin subfamily distributed in cellular secretory granules of the nervous and endocrine systems (Blázquez et al., 1998a, Zhao et al., 2009b). Among

these small functional peptides derived from SgII upon processing, secretoneurin (SN) is the most conserved part of SgII precursor found in various vertebrate neuroendocrine cells (Kahler and Fischer-Colbrie, 2000; Natori and Huttner, 1994). Goldfish SN (gfSN, 34 amino acids) shares a relatively poor identity to mammalian SN. Only the middle part of the sequences shows some identity (Samia et al., 2001). The previous study in mammals revealed that SN is widely distributed in rat intestine, central nervous system, anterior pituitary, pancreas, and adrenal gland (Leitner et al., 1996). Chapter 2 also described both gfSN-IR and free gfSN peptide in the brain and pituitary, indicating the potential of generating SN in hypothalamic nucleus preopticus magnocellular cells and pituitary lactotrophs (PRL cells). The well conservation and wide distribution of SN-immunoreactive SgII-derived peptide suggests that SN exerts various essential and biological actions in the neuroendocrine system. As a neuropeptide, SN not only increases extracellular glutamate and γ-aminobutyric acid (GABA) levels but also potentiates dopamine release in the rat brain (You et al., 1996; Agneter et al., 1995). With regard to neuroendocrine systems controlling goldfish reproduction, our previous studies for the first time demonstrated that gfSN up-regulated LH serum level *in vivo* and showed a directly stimulatory effect on LH release and LHβ subunit gene expression within pituitary fragments *in vitro* (Blázquez et al., 1998a; Zhao et al., 2006a). Furthermore, gfSN showed the most efficient ability of enhancing LH secretion at a nanomolar concentration, which is similar to physiological level in the pituitary and brain (Chapter 2).

Although there is no report concerning the regulation of SN production and release, its precursor SgII, is also regulated by hormones and neurotransmitters in the vertebrate neuroendocrine system. Estrogen plays a direct role on rat pituitary cells to attenuate the SgII

mRNA steady-state level (Anouar and Duval, 1992). GnRH is capable of up-regulating SgII secretion from gonadotrophs in rat pituitary (Conn et al., 1992), and there exists a close correlation between the GnRH-induced release of SgII and LH in the mouse LβT2 gonadotropin cell line (Nicol et al., 2002). Consistent with its effect in rat, a GnRH agonist is able to enhance the SgII mRNA expression in the pars distalis (PD) of goldfish pituitary (Samia et al., 2004). The GABA metabolism inhibitor γ-vinyl-GABA can specifically stimulate SgII gene expression in goldfish gonadotrophs, concomitant with a decrease of LH cellular content and an increase in serum LH level, suggesting the inhibition of GABA on SgII production and a potential pathway mediating the GABA activity on LH secretion (Blázquez et al., 1998a).

In the study of the goldfish hypothalamo-pituitary-gonad axis, GnRH, GABA and dopamine are three main hypothalamic factors that regulate hormone pituitary release via direct innervation into the anterior pituitary (Blázquez et al., 1998b). Besides these brain-sourced neuropeptide and neurotransmitters, some endogenously secreted hormones in the pituitary also exert their biological action on neighboring pituitary cells to modulate their hormone release through a paracrine pathway. In carp pituitary, LH secreted from gonadotrophs was demonstrated to enhance the secretion and generation of GH in the close somatotrophs, whereas GH showed a feedback regulation on gonadotrophs to reduce LH secretion (Zhou et al., 2004). In addition to GTH, activin and inhibin act as two dimeric hormones that regulate LH in a paracrine manner. They have the stimulatory and inhibitory ability to act directly on gonadotrophs to increase LH secretion in goldfish pituitary (Ge et al., 1992). An immunohistochemistry investigation showed activin- and inhibin-IR in the goldfish pituitary, specially located in the somatotrophs, suggesting their potential generation with GH.

In brief, somatotroph-sourced activin and inhibin might play a regulatory role on neighboring gonadotroph to affect LH secretion in a paracrine manner (Ge and Peter, 1994). SN is a newly-identified neuropeptide in the pituitary. Considering that gfSN stimulates LH release *in vivo* and *in vitro* (Blázquez et al., 1998a; Zhao et al., 2006a) and its potential production in lactotrophs (Chapter 2), I hypothesized that lactoroph-sourced endogenous SN in the rostral PD (RPD) played a novel intrinsic paracrine modulatory role on regulating LH secretion from gonadotrophs within the PPD in goldfish pituitary.

In this chapter, I present a time course study of gfSN effect on the secretion and gene expression of various gonadotropin subunits in the static incubation of dispersed goldfish pituitary cells. When the action of gfSN-antibody on sGnRH-stimulating LH release is investigated, I hypothesize that lactotroph-secreted SN is a paracrine neuropeptide on regulating gonadotrophs, under GnRH control. For examining this hypothesis, I studied the modulation of sGnRH on SgII processing to generate SN and SgII production, sGnRH effect on the gene and protein expression of PRL in goldfish lactotrophs, and the correlation relationship between SgII and PRL mRNA modification.

3.3. Materials and Methods

3.3.1. Animals and tissue collection

Goldfish (*Carassius auratus*), bought from Mount Parnell goldfish farms (Pennsylvania, USA), are handled according to protocols approved by the University of Ottawa Animal Care Committee, which is described in Chapter 2. During the experiment, goldfish were anaesthetized using 3-aminobutyric acid ethyl ester in water, sacrificed by spinal transection, and pituitaries were removed from the skull and placed in ice-cold

dispersion medium (M199 with Hanks' salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 0.3% bovine serum albumin (BSA), 100,000 U/l penicillin, 100 mg/l streptomycin, pH 7.2) to obtain the dispersed pituitary cells. .

3.3.2. Static incubation of dispersed goldfish pituitary cells and SN immunoneutralization

A mixture of goldfish pituitaries from both males and females were dispersed using Typsin type II and DNase II enzymes, which was previously described (Chang et al., 1990). An approximate yield is 4 x 10^5 cells/pituitary. The cells were plated in 24-well culture plates at a density of ~2.5 x 10^5 cells/well (time-course study, in March at the beginning of the sexually mature pre-spawning period of goldfish) or 6-well culture plate at a density of ~5.0 x 10^6 cells/well (gene expression investigations after 6 and 12 hours, respectively in March at the beginning of the sexually mature pre-spawning period and in October during the seasonal sexual regressive period of goldfish; gene and protein expression studies for PRL and SgII after 12 hours, in January during the period of goldfish seasonal redevelopment), and then cultured in plating medium (M199 with Earle's salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 1% horse serum, 100,000 U/l penicillin, 100 mg/l streptomycin, pH 7.2) under the conditions of 5% CO_2, saturated humidity and 28 ^{0}C. After at least a 12-hour plating period, cells were washed with testing medium (M199 with Earle's salts, 25 mM HEPES, 26.2 mM sodium bicarbonate, 0.5% BSA, 100,000 U/l penicillin, 100 mg/l streptomycin, pH 7.2) and pre-incubated for 1 hour to stabilize basal LH secretion. In previous studies, we showed that 10 nM gfSN stimulated LH release *in vitro* after 12-hour static treatment (Chapter 2). To investigate gfSN effect on LH release at different time points, we adopted 3-, 6- and 12-hour static incubations in our experiments with dispersed cells. Media were then

changed to testing medium consisting of either synthetic sGnRH (Glu-His-Trp-Ser-Tyr-Gly-Trp-Leu-Pro-Gly-NH_2; 100 nM) or synthetic gfSN peptide (10 nM). In the SN immunoneutralization experiment (in October) pituitary cells in 24-well culture plates were pre-incubated for 2 hours with anti-SN antiserum (1:750) or normal rabbit serum (NRS at the dilution 1:750 as control). After 2 hours, highly concentrated gfSN (100 nM) and sGnRH (1 μM) were added in the culture medium to reach the final treatment level of gfSN (10 nM) and sGnRH (100 nM). Cells were then treated for 12 hours. During the experiment, the concentrations of anti-gfSN antiserum and NRS were always kept at the dilution of 1:750. Following various static treatments, media samples were collected and stored at -20 ^{0}C for radioimmunoassay measurement of LH content. Furthermore, total RNA and protein were isolated from the pituitary cells in 6-well plates for the further analysis.

3.3.3. Radioimmunoassay (RIA)

LH concentrations in incubation media were determined using a double antibody RIA that was previously described in Chapter 2.

3.3.4. Dot immunoblotting analysis

Four microliters of 100 μM gfSN and 100 μM sGnRH were spotted onto the nitrocellulose membrane (Bio-Rad Laboratories, Hercules, CA, USA) for dot immunoblotting analysis that I previously described in Chapter 2. Briefly, after blocking non-specific binding proteins, the membrane was incubated with anti-gfSN antiserum (Zhao et al., 2006b) at the dilution of 1:750 for 1 hour. After that, it was reacted with ECL rabbit IgG horseradish peroxidase (HRP) -linked whole antibody from donkey (dilution: 1:2,000, GE Healthcare,

Buckinghamshire, UK) for 50 minutes, and then detected by using ECL plus western blotting detection reagent (GE Healthcare, Buckinghamshire, UK). The peptide vehicle, water was adopted as the blank control.

3.3.5. RNA isolation, cDNA preparation and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

RNA was exacted using TRIzol reagent (Invitrogen, Burlington, ON, Canada) or RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany) following the company's standard protocol. Total RNA concentration was measured by the GeneQuant spectrophotomer (Pharmacia Biotech, Qakville, ON, Canada). Two micrograms of RNA was at first treated with 2 units of RQ1 RNase-Free DNase (Promega, Madison, WI) to remove genomic DNA, and then utilized to synthesize cDNA with 200 ng of random primers (Invitrogen, Carlsband, CA) and Superscript II RNase H^- reverse transcriptase (Invitrogen, Carlsband, CA). The original cDNA was diluted at the ratio of 0.1-0.01 for the PCR analysis. All primers were designed using Primer 3 (http://frodo.wi.mit.edu), verified by IDT OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer) and generated by Invitrogen. The amplifications of gonadotropin-relevant genes were carried out with 150-300 nM of primers (depending on specific genes) in the Mx3000 Mulitplex Quantitative PCR System (Stratagene, La Jolla, CA). The PCR parameters were adopted from Martyniuk et al. (2006) and briefly described as a 1st cycle of Taq enzyme activation at 95 ^{0}C for 10 minutes, followed by 40 cycles of 95 ^{0}C for 30 seconds and 58 ^{0}C for 5 seconds. A SYBR Green assay was used to measure the accumulation of PCR product for different genes. Results were analyzed by the Mx3000 Software Package. Standard curves relating initial template copy

number to fluorescence and amplification cycles were generated and utilized to calculate mRNA copy number in each sample. 18S ribosomal RNA was chosen as a housekeeping gene in the SYBR Green real-time RT-PCR assay due to its statistically unchanged gene expression under various static treatments *in vitro*. The PCR product of each gene was purified by QIAprep Sprin Miniprep Kit (Maryland, USA) and sequence to confirm the primer specificity. The primer sets utilized in this study are shown as following:

1. GTHα [D86552: forward primer 5'-TTGGATGTGAGGAGTGCAAA-3', reverse primer 5'-GGTAAGCCCTAGAAAAACAGCA-3'];

2. FSHβ [D88023; forward primer 5'- ATGCGCTTCGTTGTTATGGT-3', reverse primer 5'-AGCTGCCACATTCCTCACTT-3'];

3. LHβ [D88024; forward primer 5'- TGTGGAAAAGGAGGGCTGT-3', reverse primer 5'-CAGTGGAAAATGGGCTCTTG-3'];

4. 18S [AF047349; forward primer 5'-AAACGGCTACCACATCCAAG-3', reverse primer 5'- CACCAGATTTGCCCTCCA-3'];

5. SgII [AF046002; forward primer 5'- CCTCAGCCAGAGAACTCCAC-3', reverse primer 5'- ATGCCTCTATCCATCCGAGA-3'];

6. PRL [S82197; forward primer 5'- GTGGCTGTTCTGATGTGT-3', reverse primer 5'-CTGAAGAGAGGATGTGTG-3'].

3.3.6. Protein extraction and western blotting analysis of goldfish pituitary

Goldfish dispersed pituitary cells were homogenized in a buffer containing 20 mM Hepes, 200 mM sodium chloride, 0.1 mM EDTA, 10 mM sodium fluoride, 1 mM sodium metavanadate, 10 mM β-glycerophosphate and protease inhibitor cocktail (1:100, Bioshop

Canada, Burlington, ON), and then centrifuged at 12,000 rpm for 15 minutes at 4 ^{0}C. Total protein concentration of the supernatant was assayed by using a NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Wilmington, DE). As described previously (Zhao et al. 2006b), pituitary extract (~15 μg of total protein) was separated by electrophoresis on a 10% or 12% SDS-PAGE gel (10% or 12% bis-ascrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED) for the SN- or PRL-IR assay, respectively. After electrophoresis, proteins were transferred onto polyvinylidene difluoride (PVDF) membranes (immobilon-P, Millipore, Bedford, MA). The membrane was blocked in the 20 mM Tris-HCl buffer (TBS, pH 7.5) containing 5% fat-free milk and 0.05% Tween 20, and then incubated with primary goldfish SN antibody (Zhao et al. 2006b, 1:700 dilution in blocking buffer), carp anti-PRL antibody (a gift from Dr. A.O.L. Wong at the University of Hong Kong, 1:100,000 dilution in blocking buffer) and purified mouse anti-β-actin monoclonal antibody (Cedarlane, Hornby, ON, Canada; at the dilution of 1:240) for ~12 hours at 4 ^{0}C. After the primary immunoreaction, the membrane was reacted with protein A-peroxidase (Sigma, Germany) at a dilution of 1:2000 at room temperature for 90 min. After washing, the membrane was covered with ECL plus western blotting detection reagent (GE Healthcare, Buckinghamshire, UK) and then wrapped in plastic. Signals on the membranes were detected with the BioRad ChemiDoc Imaging System and quantified using the Quantity One (Bio-Rad, Segrate, Milan, Italy) software package.

3.3.7. Data analysis

Results were presented as mean ± S.E.M. The statistical analyses were performed using Sigma Stat version 3.5 software. For comparing multiple groups, one-way or two-way

Analysis of Variance (ANOVA) was chosen to assess difference between various treatments, followed by Fisher LSD test or Student-Newman-Keuls method. In the comparison of two groups, t-test was used to assess difference between control and treatment. A difference with $p<0.05$ was considered to be significant. Data were transformed to natural logarithms if they were not normally distributed. To determine correlation between a pair of variables, Spearman Rank Order Correlation method was adopted. If $p<0.05$, the correlation was considered as statistically significant.

3.4. Results

3.4.1. Time-course action of 10 nM gfSN on LH release from the pituitary cell

We performed a time-course study to explore the effect of gfSN on LH release from dispersed pituitary cells from sexually mature goldfish. Salmon GnRH (100 nM) and gfSN (10 nM, Chapter 2) were administrated for 3, 6 and 12 hours in a static incubation system (Fig. 3.1). No significant effect of sGnRH was observed after the first 3-hour incubation ($p=0.064$). However, the administration of 100 nM sGnRH enhanced LH levels by 3.4- and 3.1-fold respectively at the 6- and 12-hour time points in comparison with the corresponding time-matched control ($p<0.05$). Similar to the time-dependent pattern of sGnRH action on LH release, gfSN also did not affect LH release after the 3-hour incubation. In contrast, the 6- and 12-hour treatments of 10 nM gfSN induced significant increase of LH which were 2.7-fold and 2.0-fold higher than the corresponding time-matched controls ($p<0.05$).

3.4.2. Pituitary GTHα, FSHβ and LHβ gene expressions are increased by gfSN

RNA isolation, cDNA synthesis and real-time RT-PCR were adopted for determining

the gene expression of GTHα, FSHβ and LHβ in goldfish pituitary cells. After either 6- or 12-hour static incubation, the level of 18S ribosomal RNA was not modified by 10 nM gfSN ($p>0.05$). The mRNA levels of GTHα, FSHβ and LHβ were plotted as fold change with respect to the time-matched control values (Fig. 3.2). The 6-hour static treatment using 10 nM gfSN induced 1.25-, 1.34- and 1.27-fold changes of GTHα, FSHβ and LHβ mRNA ($p<0.05$) respectively using 18S ribosomal RNA as a correction factor (Fig. 3.2A). At the end of 12-hour incubation, the expression of these three genes were slightly increased under the treatment of 10 nM gfSN, however these were not statistically different from the corresponding control values ($p>0.05$) (Fig. 3.2B).

3.4.3. The anti-gfSN antibody reduced sGnRH stimulatory action on LH release

An SN-immunoneutralization experiment similar to the one previously described in Chapter 2 was performed to determine if there was an effect on the LH-stimulating ability of sGnRH and gfSN. We first utilized dot immunoblots to confirm the specific binding ability of anti-gfSN antibody against gfSN but not sGnRH. Synthetic gfSN and sGnRH at the same concentration (100 μM) were blotted and tested against the polyclonal anti-gfSN antibody. No signal was detected in the sGnRH position whereas synthetic gfSN as a positive control exhibited a strong SN-IR signal (Fig. 3.3A). Water served as a negative control (Blank) and showed a very low background level of the immuno-reaction. Consistent with the results in the previous study (Chapter 2), the administrations of sGnRH (100 nM) and gfSN (10 nM) respectively elevated the LH level by approximately 2.4- and 2.3-fold in NRS-treated group ($p<0.001$, Fig. 3.3B). However, in the co-exposure with the anti-gfSN antiserum (1:750), sGnRH only induced a 1.5-fold increment ($p=0.012$) of LH secretion, which was 64% lower

(p=0.004) than LH release induced by sGnRH in the presence of NRS (1:750). Furthermore, the anti-gfSN antiserum completely blocked the stimulatory effect of SN (p=0.001) and SN didn't affect LH release (p>0.05); its relative LH increase of approximately 1.1 was much less than the gfSN value in the NRS-treated group by 46% (p<0.001) (Fig. 3.3B).

3.4.4. sGnRH enhanced SgII processing and PRL protein expression

This experiment was performed to determine whether a 12-hour sGnRH (100 nM) exposure was able to affect SgII processing, namely SN production, in goldfish lactotrophs. β-actin protein expression, as an internal standard for loading control, was not significantly different between control and sGnRH-treated cells (Fig. 3.4A and 3.4B). Fig. 3.4A showed the level of SgII-derived peptides containing SN in western blots. The intensities of ~57 kDa SgII products in the sGnRH treatment were significantly higher than those of the control (Fig. 3.4A); sGnRH induced 2.6-fold increment (p=0.002) of ~57 kDa SN-IR peptide (Fig. 3.4C). Moreover, the other SgII-derived peptide (~30 kDa) was only detected in sGnRH-exposed cells (Lane G1-G3 in Fig. 3.4A) but not in the control group, suggesting that sGnRH increased SgII processing to generate SN. Simultaneously, PRL protein levels were also modified in response to the 12-hour sGnRH exposure (Fig. 3.4B); sGnRH significantly increased this protein expression by 1.9 fold (p<0.05) in comparison with the control (Fig. 3.4C). Furthermore, levels of the ~57 kDa SgII product were significantly correlated with the changes of PRL protein (R=0.76, p=0.004) as determined by western blot analysis.

3.4.5. sGnRH increased mRNA levels of SgII and PRL

The expressions of SgII and PRL following a 12-hour static incubation using 100 nM

sGnRH in goldfish pituitary cells were compared with the control. The mRNA level change was plotted as relative fold change above control values for these two genes (Fig. 3.5A). The housekeeping gene, 18S ribosomal RNA as control, was not significantly affected by sGnRH treatment ($p>0.05$). Treatment with sGnRH induced 1.6- ($p=0.006$) and 1.7-fold ($p=0.01$) increments in SgII and PRL mRNA levels, respectively (Fig. 3.5A). Moreover, SgII gene expression was significantly ($R=0.66$; $p=0.02$) correlated with PRL gene expression (Fig. 3.5B).

3.5. Discussion

Our previous data on time-course study in static incubation of goldfish pituitary fragments showed that 10 nM gfSN stimulated LH release *in vitro* at the end of 6-hour incubation rather than 3-hour (Zhao et al., 2006a). In the present study, we adopted the primary static incubation of dispersed goldfish pituitary cells for exploring gfSN activity. In this system, individual pituitary cells are more directly exposed to the treatment, avoiding the influence of hypothalamic neuropeptides and neurotransmitter residues in the nerve fibers within the pituitary fragments (Chang et al., 1990). We also demonstrated that 10 nM SN not only stimulated LH release after either a 6- or 12-hour incubation, but also up-regulated gene expression of GTHα, FSHβ and LHβ subunits at the end of a 6-hour exposure. An intriguing observation in this study is that the anti-gfSN antibody is capable of inhibiting the stimulatory effect of sGnRH on LH secretion from goldfish pituitary cells, suggesting a new paracrine pathway of lactotroph-sourced SN. To further confirm this speculation, we also demonstrated that sGnRH enhances SgII mRNA and SN-IR peptide levels, in parallel with PRL gene and protein expressions in goldfish pituitary cells, respectively.

First of all, we investigated the gene and protein expression of gonadotropin under time-dependent exposure of gfSN, as shown by real time RT-PCR and LH-RIA. In our previous reports, it was demonstrated that 10 nM gfSN was one of the most effective doses for controlling LH release in static incubation of goldfish pituitary fragments and dispersed cells (Zhao et al., 2006a; Chapter 2). In the present study, treatment with 100 nM sGnRH, a known and effective LH stimulator, enhanced LH release at the 6- and 12-hour time points, suggesting that static incubation of disperse goldfish pituitary cells is a useful system to study time-dependent LH secretion. Goldfish SN exhibited similar LH-stimulating effects to that of sGnRH in this time-course study. SN significantly stimulated LH release after the incubations of 6 and 12 hours but not 3 hours. These data confirmed that gfSN stimulates LH secretion *in vitro*.

In the study of LH production, Klausen et al. (2001) demonstrated that a 12-hour exposure to 100 nM sGnRH enhanced LHβ subunit gene transcription in the static incubation of dispersed goldfish pituitary cells. In the same system, we showed that a 6-hour treatment with gfSN (10 nM) significantly elevates LHβ mRNA level by 1.27 fold. This result is consistent with the previous finding concerning the stimulatory effect for a high dose of gfSN (500 nM) on the production of LH from goldfish pituitary fragments (Zhao et al., 2006b). Furthermore, we also detected that gfSN potentiated gene expression of the GTHα subunit after the 6-hour incubation, providing additional support for gfSN (10 nM) effect on LH production in pituitary cells. In addition, a 6-hour treatment with gfSN (10 nM) induced a 1.34-fold increase of FSHβ mRNA level in dispersed goldfish pituitary cell, indicating its potential role on the generation of another GTH, FSH. However, the action of gfSN on GTH-relative genes is related with the incubation time *in vitro*, because in the same experimental

system this peptide (10 nM) had no significant stimulatory effect on GTHα, FSHβ and LHβ subunit mRNA in goldfish pituitary cells after 12 hours. In brief, SN shows time-dependent differences in the increment of GTH subunit mRNA levels *in vitro*. Interestingly, we found that the time-dependent trend of small gfSN-evoked increment of GTH subunit gene expressions was similar to the previous data concerning the sGnRH modulatory action on GTH subunit mRNA in static culture of dispersed goldfish pituitary cells (Klausen et al., 2002). Twelve-hour but not 24-hour static treatment with sGnRH induced a small but significant 17-42% increments of GTHα, FSHβ and LHβ subunit mRNA levels in dispersed pituitary cells adopted from early-middle recrudescent goldfish (Klausen et al., 2001; 2002). Consistent with the stimulatory effect of sGnRH, only 6-hour SN-stimulated GTH subunit gene expression was able to increase by 25-34% with respect to the control. Although SN slightly potentiated LHβ gene expression, SN remarkably induced more than 2.0-fold increase of LH secretion from the dispersed pituitary cell. The discrepancy between the levels of gene transcription and protein generation might be explained as the possible involvement of translational regulation of protein production. This phenomenon was also reported in GnRH-induced LH production and release in mouse LβT2 pituitary cells (Nguyen et al., 2004). It was explained that the acute response to GnRH stimulation led to activation of translation initiation protein, including 4E-binding protein, eukaryotic initiation factor 4E and 4G via the signaling pathway of mitogen-activated protein kinase (MAPK) cascade in this cell line (Nguyen et al., 2004). Thus, SN might have a similar characteristic to modulate LH protein synthesis; the question whether SN activates the MAPK transduction or not will be investigated in Chapter 4 using the mouse LβT2 pituitary cell line.

Salmon GnRH and gfSN show the similar patterns on modulating LH release in the

dose-response (Chapter 2) and time-course studies (this chapter), indicating a relationship between these two neuropeptides on the regulation of goldfish GTH yield and secretion. To demonstrate this possibility, we performed an immunoneutralization to block the intrinsic SN secretion from goldfish pituitary cells during sGnRH stimulation. In the present study, our anti-gfSN antiserum was demonstrated to recognize the gfSN antigen but not sGnRH, suggesting this antibody only blocks the gfSN action and not sGnRH effect on LH release. We found that sGnRH still significantly up-regulated LH release under the co-incubation of the anti-gfSN antiserum. However, its relative fold increase with respect to the group-matched control significantly decreased in comparison with the sGnRH treatment without the anti-gfSN antibody co-incubation. These data revealed that anti-gfSN antibody suppressed the sGnRH effect on LH release, indicating the possible participation of SN in one of multiple signaling pathways to mediate GnRH action on LH. Several factors were previously shown to participate in GnRH regulatory effect in teleosts. GnRH stimulated GH release and production in goldfish (Marchant et al., 1989; Klausen et al., 2001), and GH showed a paracrine action in elevating LHβ mRNA expression in carp pituitary (Zhou et al., 2004), suggesting that the regulatory role of GnRH on LH in gonadotrophs possibly partially through an intrapituitary paracrine manner of somatotroph-sourced GH. Similar to our experimental design, De Paul and her co-workers (2000) co-incubated the dispersed rat lactotrophs with GnRH and an antagonist to the angiotensin II receptor. They reported a suppressive effect of this antagonist on PRL release stimulated by GnRH, confirming the paracrine role of gonadotroph-sourced angiostensin II in rat pituitary. Therefore, the observed inhibition of anti-gfSN antibody on sGnRH-stimulating LH secretion indicates a SN-associated pathway within the pituitary under the sGnRH control.

In our previous study, SN-IR signal was detected only in goldfish lactotrophs providing the evidence of potential SN production with PRL (Chapter 2). It was recently demonstrated that GnRH-stimulated PRL secretion is not mediated through LH or FSH because of the absence of gonadotropin receptors in ovine pituitary gland. This fact indicated a direct effect of GnRH on the lactotrophs in this species (Henderson et al., 2008). Although, up to now, there is still no report about the GnRH receptor on the lactotroph membrane in goldfish, Stefano et al. (1999) demonstrated for the first time specific GnRH binding sites on the membrane of PRL-expressed pituitary cells of the pejerrey (*Odontesthes bonariensis*) *in vitro*. In the RPD part of cichlid fish pituitary, GnRH receptor type IB IR had also been detected within the lactotrophs, suggesting GnRH modification on PRL generation (Parhar et al., 2002). The first PRL-releasing action of GnRH was observed in tilapia (*Oreochromis mossambicus*); three forms of GnRH (cGnRH-II, sGnRH and sea bream GnRH) stimulated PRL secretion from the RPD part of the pituitary (Weber et al., 1997). Moreover, 1 and 10 nM sGnRH were also capable of elevating PRL gene expression in primary pituitary cell cultures of female maturing masu salmon, indicating that sGnRH not only regulates PRL secretion but also modifies PRL production (Onuma et al., 2005). Our *in vitro* data are consistent with these previous observations and for the first time revealed that sGnRH increased the PRL protein and gene expression in goldfish pituitary.

SgII, the SN precursor, is also modulated by GnRH in the vertebrate neuroendocrine system. The phenomenon that GnRH stimulated SgII release *in vitro* was reported initially in female rat pituitary by Chanat and her/his colleagues in 1988. Consistent with this result, it was demonstrated that 10-1000 nM GnRH showed a time-dependent action to enhance SgII secretion from dispersed rat pituitary cells, which was similar to LH and FSH response to

GnRH (Conn et al., 1992). Furthermore, in an *in vivo* study, SgII and LH gene expression were down-regulated in rat pituitary by the exposure of a GnRH antagonist, suggesting that GnRH stimulatory effect on SgII production in relation to LH generation (Kakar et al., 1993). In the mouse pituitary cell (LβT2 cell line), the pulsatile stimulations of GnRH not only elicited SgII (with SN-IR), FSH and LH release but also up-regulated the mRNA levels of their relevant genes; whereas the GnRH-induced SgII release was associated with that of LH but not FSH secretion, indicating a granin-related secretion pathway (Nicol et al., 2002; 2004). Samia et al. (2004) reported that injection of sGnRH agonist induced an increment of SgII mRNA level in the PD part of goldfish pituitary *in vivo*. In agreement with the *in vivo* finding of Samia et al. (2004), our present study demonstrated that sGnRH remarkably elevated the SgII gene expression and protein levels of the major SgII product (a ~57 kDa SN-IR peptide) in the static culture of dispersed goldfish pituitary. These data provided the first *in vitro* evidence for a direct stimulatory action of GnRH on SgII production and its subsequent processing to generate free SN peptide in teleosts. Moreover, as previously reported in mammals that the secretion of SN-IR peptide showed a close correlation with the LH release in rat gonadotroph cells (Nicol et al., 2002; 2004), the modifications of gene and protein expression for SgII and its product are significantly correlated with changes of PRL mRNA and peptide level, suggesting a close association between GnRH-induced SN and PRL secretions. Considering the identification of free SN peptide in goldfish pituitary and its IR is detected in the lactotrophs (Chapter 2), we speculate that endogenous SN of the pituitary is generated within the lactotroph in association with PRL.

In conclusion, SN exerts a stimulatory effect on LH secretion from goldfish gonadotrophs in both neuroendocrine and paracrine manners (Fig. 3.6). Our previous finding

concerning free SN peptide isolated from the brain, SN-IR located in the hypothalamus and pituitary nerve fibres, and GnRH-mimicking effect *in vitro* and *in vivo* (Chapter 2; Zhao et al., 2006a; 2006b; Blázquez et al., 1998a) strongly supports the concept that brain-associated SN is a neuroendocrine factor to regulate LH release in the pituitary. In the present study, we observed the GnRH stimulation on goldfish lactotrophs to induce SgII generation and its processing to SN, in correlation with the PRL production. These results suggest that SN is secreted from lactotrophs, and are consistent with the high expression of SN-IR in lactotrophs (Chapter 2). Moreover, SN elevates the gene expression of not only LHβ but also GTHα and FSHβ subunits in physiological dose *in vitro*, but also shows a time-dependent effect on LH release from dispersed goldfish pituitary cells. Here, this chapter elucidates that the endogenously generated SN in lactotrophs, under hypothalamic GnRH control, plays a paracrine role on neighboring gonadotrophs to stimulate LH release and production. The previous and current data provide a framework for a better understanding of SN modulation of pituitary hormone secretion in the fish hypothalamo-pituitary-gonad axis. Further study should be focused on the mechanisms that mediate the SN effect, such as SN receptor and its relative second message signaling pathway. However, the mechanism study of SN effect in fish gonadotrophs is very difficult due to the limitation of experimental conditions for this species. Therefore, I adopted a mammalian gonadotropin cell line, mouse LβT2 cells, for signal transduction studies of this neuropeptide. This cell line has not only the common characteristics of normal mouse pituitary cells, such as GnRH-induced LH generation and release, but also the expression of SgII, the SN precursor (Nicol et al., 2002; 2004). Chapter 4 mainly describes the investigations concerning SN effect on LH release and its relevant second messenger signaling pathway in mouse LβT2 cells.

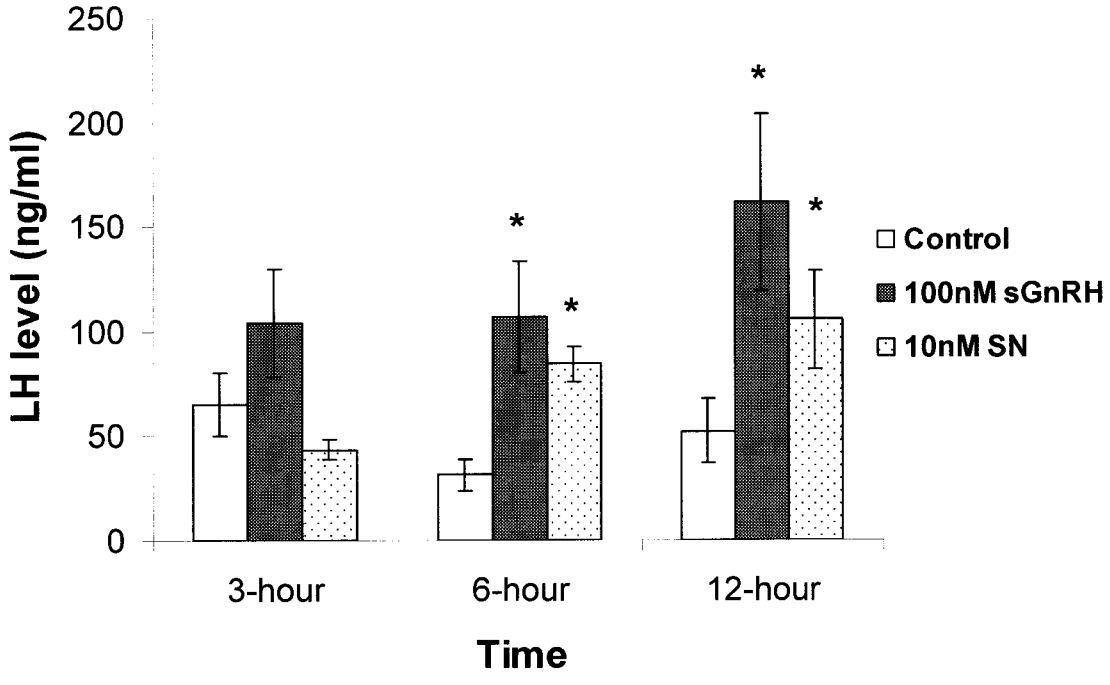

Figure 3.1. Time-dependent effect of 10 nM gfSN on LH release from the dispersed goldfish pituitary cells in the presence of 100 nM sGnRH after the 3-, 6- and 12-hour static incubations. Results presented means ± S.E.M. (standard error of the mean), n=8. This experiment was performed in March at the beginning of the sexually mature pre-spawning period of goldfish. One-way ANOVA was chosen to assess the difference between control and treatment at a given time point, followed by Fisher LSD test. When the data were non-parametric, the Kruskal-Wallis One-way ANOVA on Ranks followed by Student-Newman-Keuls method was adopted. The (*) mark indicates a significant difference from control at a given time point ($p<0.05$).

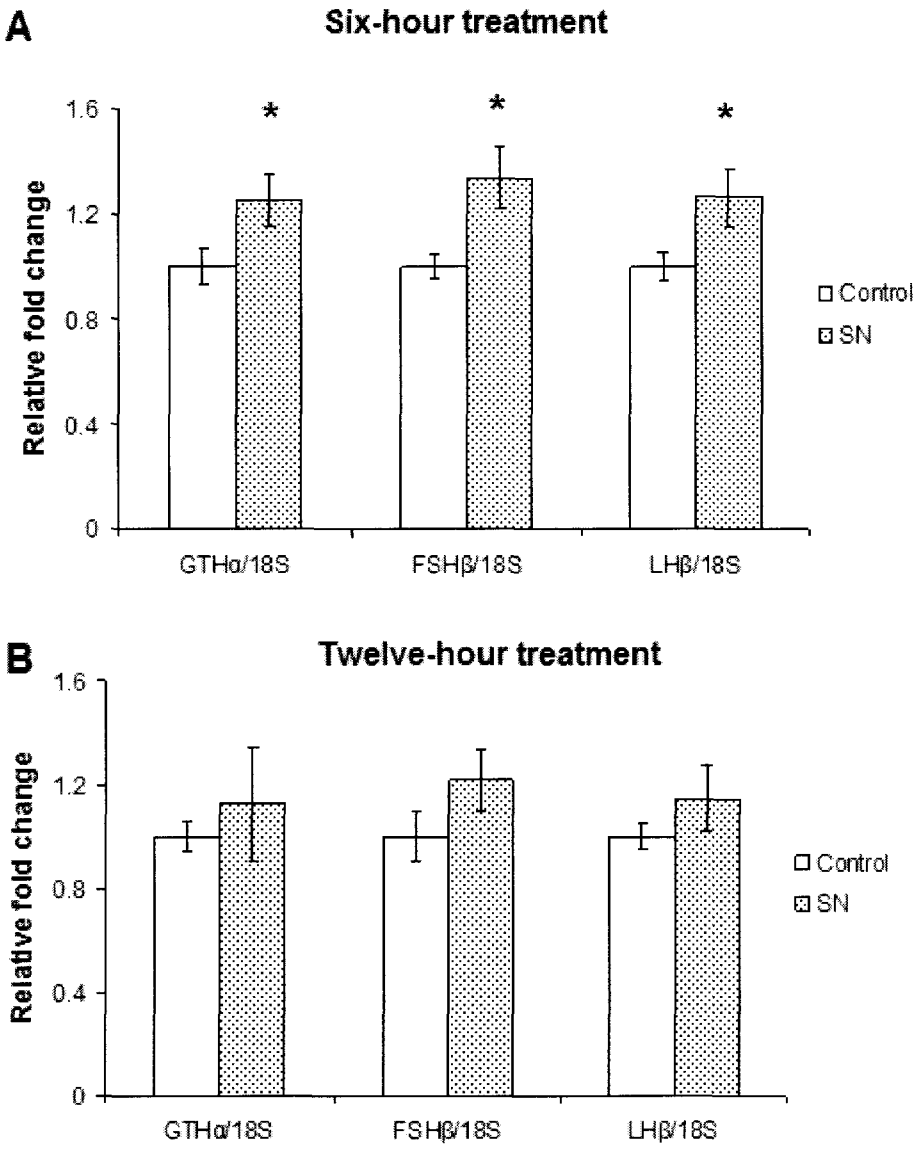

Figure 3.2. GTHα, FSHβ and LHβ subunit mRNA levels of dispersed goldfish pituitary cells after 6-hour (A) and 12-hour (B) static incubation with the treatment of gfSN (10 nM). The experiments of 6-hour and 12-hour were carried on in March and October, respectively. Relative fold changes (means ± S.E.M.) versus the time-matched control were measured by real-time RT-PCR in panel **A** (n = 9) and panel **B** (n = 3-4). 18S ribosomal RNA was used for RNA loading control. Asterisk (*) mark indicates a significant difference between gfSN-treated cells and control at the end of 6-hour incubation (t-test, $p<0.05$). When the data were non-parametric, their natural logarithms were adopted before the t-test analysis.

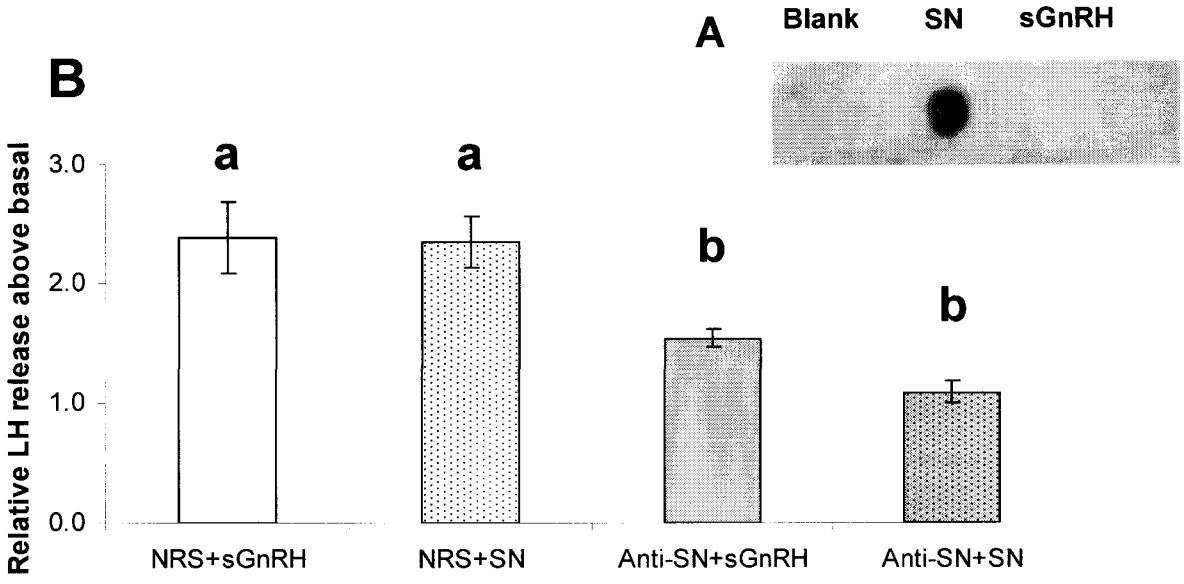

Figure 3.3. **A**, Dot immunoblotting analysis of gfSN (100 μM) and sGnRH (100 μM) by using anti-gfSN antiserum at the dilution of 1:750. Blank indicates that only water was blotted. **B**, Neutralizing action of anti-gfSN antiserum (1:750) on the stimulations of sGnRH (100 nM) and gfSN (10 nM) to elevate LH secretion from dispersed goldfish pituitary cells. Relative LH increase fold with respect to the group-matched control value was determined by RIA in the NRS and anti-gfSN groups (n=11). Data are presented as means ± S.E.M. The experiment was performed in October with sexually regressed goldfish. Two-way ANOVA followed by Fisher LSD test was used to assess the effects of neuropeptides and antiserum on LH release. Different letters represent statistical differences ($p<0.05$; Fisher LSD test).

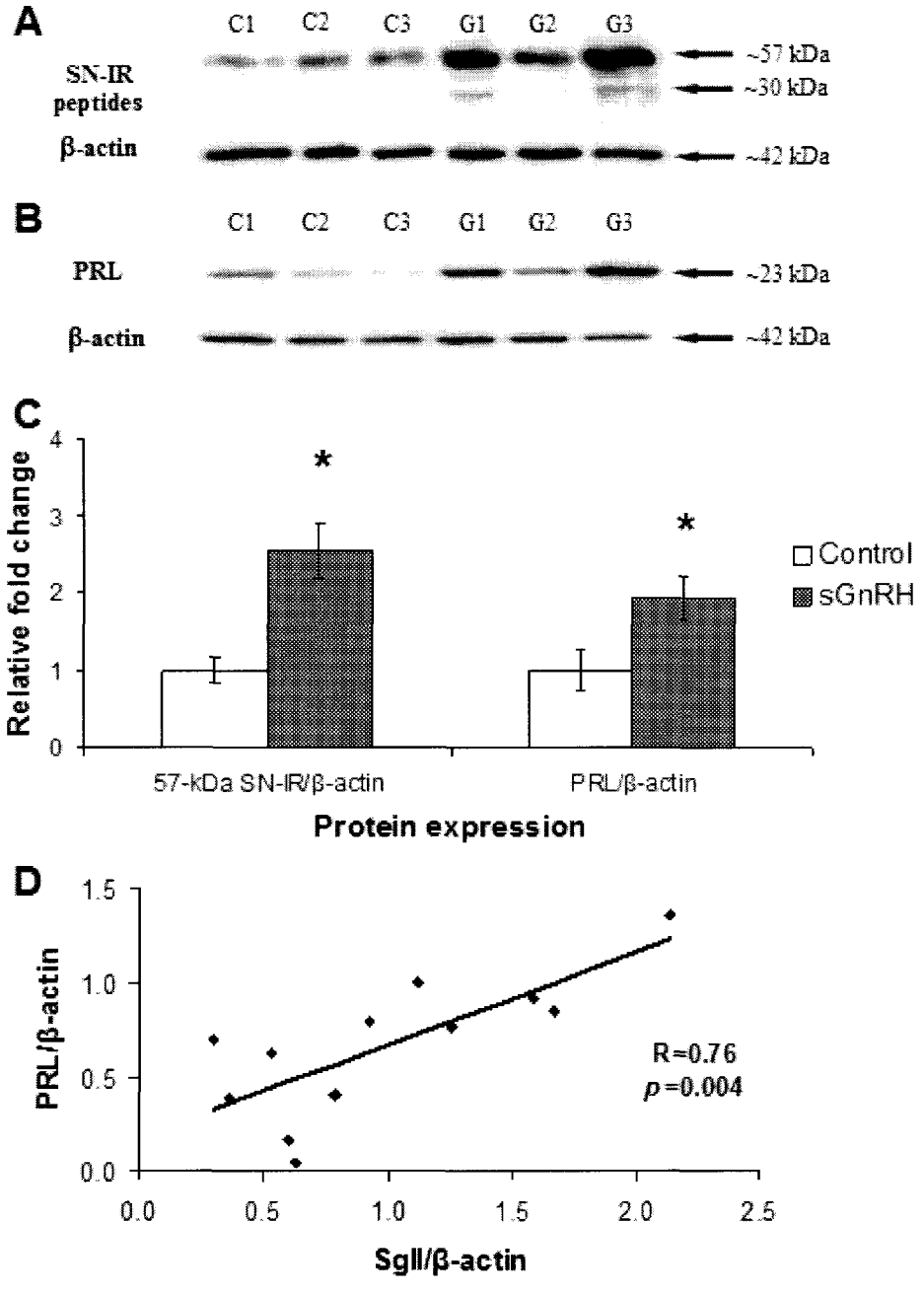

Figure 3.4. Effect of sGnRH (100 nM) on SN-IR SgII-derived peptides and PRL in dispersed goldfish pituitary cells after 12-hour static incubation. The pituitary cells were collected from goldfish in January during the sexual redevelopment period, and then cultured in 6-well incubation at the density of ~5 x 10^6 cells/well. Western blotting analyses of SN-IR peptide (**A**) and PRL (**B**) expressions were performed for the control (C1, C2 and C3) and sGnRH (G1, G2 and G3) groups. Total proteins isolated from the pituitary cells (~15μg) were separated by 10% and 12% SDS-PAGE gel respectively and detected using the anti-gfSN antiserum (dilution: 1:2000), anti-PRL antibody (dilution: 1:100,000) and anti-β-actin

antiserum (dilution: 1:240). Two SN-IR peptides, ~57kDa and ~ 30kDa, were indicated by arrows. β-actin was used to control the total protein loading. In panel **C**, data were presented as means ± S.E.M. of the relative increase fold above the control for ~57 kDa SN-IR peptide and PRL expression (n=6). The t-test was adopted to compare control and sGnRH treated pituitary cells for independent proteins ($p<0.05$). Asterisk (*) indicated a significant difference. Panel **D** showed correlation of the change in ~57kDa SgII product expression and PRL protein expression with and without sGnRH static treatment, normalized by β-actin level. The correlation between these 2 variables were statistically significant ($p<0.05$) by using the Spearman Rank Order Correlation method.

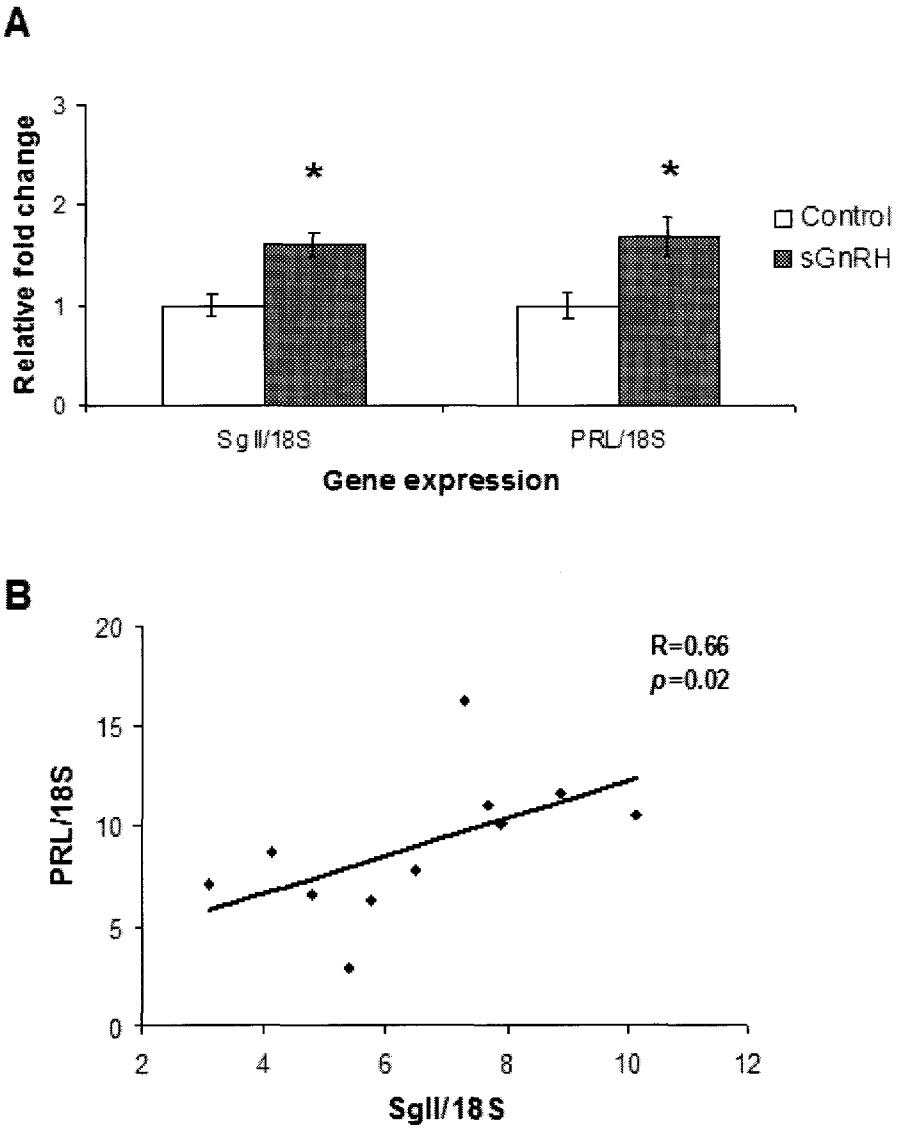

Figure 3.5. Effect of the 12-hour treatment with 100 nM sGnRH on SgII and PRL gene expressions in dispersed goldfish pituitary cells. **A**, relative fold changes (means ± S.E.M.) above the control values was determined by real-time RT-PCR in dispersed goldfish pituitary cells (n=7 for control, n=4 for sGnRH, 24-well incubation at the density of ~2.5 x 10^5 cell/well). 18S ribosomal RNA was adopted for loading normalization. This experiment was performed in the same season as Figure 3.4. *$p<0.05$ vs control group for individual genes (t-test). **B**, regression plot showing correlation between mRNA of SgII and PRL standardized with 18S ribosomal RNA. The correlation of SgII and PRL mRNA level was statistically significant ($p<0.05$, Spearman Rank Order Correlation method).

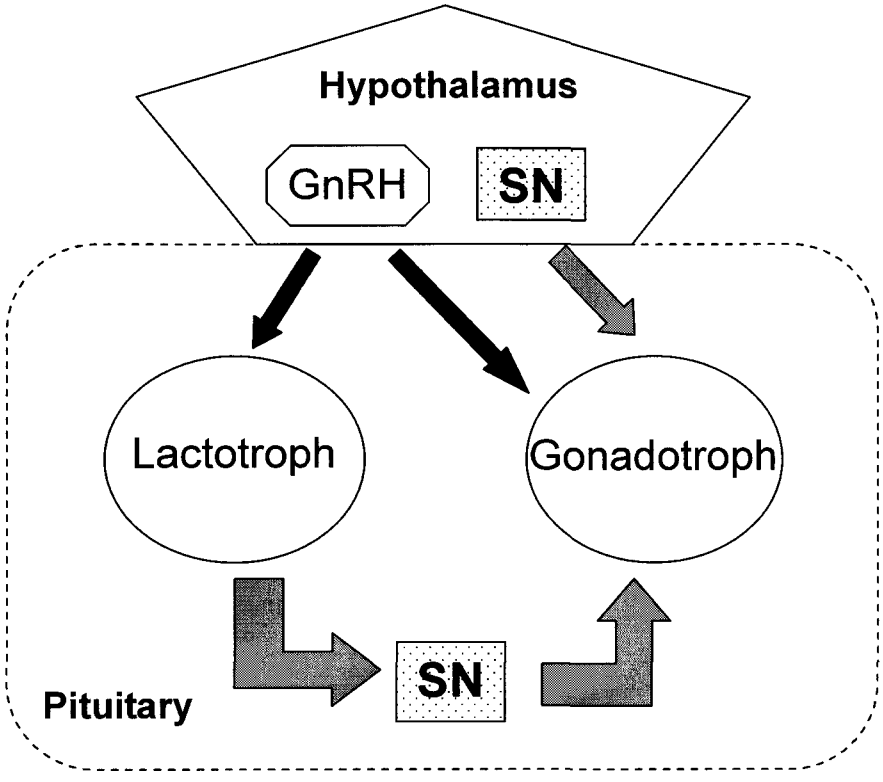

Figure 3.6. Paracrine and neuroendocrine model of SN actions on LH-secreting gonadotrophs in goldfish pituitary. Arrows indicated stimulatory effects. Please see the discussion for details.

CHAPTER 4: Secretoneurin Stimulates the Production and Release of Gonadotropin in the Mouse LβT2 Gonadotropin Cells

** Major contributors for this chapter:*

E Zhao: Original ideas, LβT2 cell culture and treatment, real-time RT-PCR, western blotting analysis, data analysis and writing,

Alan S. McNeilly: Mouse LH-specific radioimmunoassay for culture medium,

Andrew Chen: Synthesis of mouse secretoneurin.

4.1. Abstract

Secretoneurin (SN), a functional secretogranin-II (SgII)-derived peptide, exerts a regulatory action on goldfish gonadotrophs to stimulate luteinize hormone (LH) production and its release. However, the effects of SN on the pituitary of mammalian species and the underlying mechanisms remain poorly understood. To study functional activities of SN in mammals, we adopted the mouse LβT2 gonadotropin cell line that has characteristics consistent with the normal pituitary gonadotrophs. Using radioimmunoassay and real time RT-PCR investigation, we demonstrated that static treatment with SN induced a significant increment of LH release and production in LβT2 cells. Moreover, a ~71-kDa SgII precursor and three intermediate SgII-derived peptides containing SN were observed in LβT2 cells, suggesting the proteolytic processing of SgII to yield SN in mouse pituitary. In addition, we demonstrated that GnRH increased SgII mRNA level but decreased cellular SgII protein levels and its intermediate products, indicating that GnRH-induced SgII processing is faster than GnRH-evoked SgII synthesis for generating SN in mouse pituitary cells. We also reported that SN activated extracellular signal-regulated kinases (ERK) in either 10-minute

acute stimulation or 3-hour chronic treatment. The SN-induced ERK activation was significantly blocked by pharmacological inhibition of MAPK kinase (MEK) by 60%. Thus, we speculate that the mechanism of the SN-induced LH response might be mediated through ERK signaling pathway.

4.2. Introduction

The mouse LβT2 gonadotropin cell line was derived from pituitary tumors induced by the SV40 T-antigen oncogene (Tag) linked to the rat luteinizing hormone (LH) β subunit in transgenic mice (Alarid et al., 1996; Turgeon et al., 1996). This cell line exhibits functional characteristics consistent with those of normal pituitary gonadotrophs. Gonadotropin contains a common α-subunit (GTHα) linked with a hormone-specific β-subunit. Whereas GTHα subunit was detected in the LβT2 cells, gonadotropin releasing hormone (GnRH) mainly enhances LHβ production and LH secretion via a regulated pathway and changes in GnRH-receptors (Turgeon et al., 1996). LβT2 cells also express follicle stimulating hormone (FSH). The gene expression of FSHβ-subunit was demonstrated to be regulated by several neuroendocrine factors in the LβT2 cell line (Ooi et al., 2004). Besides the expression of LH and FSH, this cell line shows a close correlation between LH and secretogranin II (SgII) storage and secretion under the regulation of GnRH suggesting that SgII may play an important role on hormone regulation in mammalian pituitary (Nicol et al., 2002 and 2004).

GnRH is a hypothalamic decapeptide that regulates gonadotropin release from the pituitary. Turgeon and her colleagues (1996) demonstrated for the first time that GnRH pulse treatment not only induced a pronounced increment in LH release on successive days, but also up-regulated the gene expression of LHβ subunits and GnRH receptors in the mouse LβT2

cell line. With regards to the specific mechanism involved in the regulation of LH production by GnRH in the LβT2 gonadotroph cell line, several studies have shown that the GnRH stimulatory effect is mediated by activating the mitogen-activated protein kinase (MAPK) cascade to affect LHβ-subunit gene transcription (Ruf and Sealfon, 2004). Extracellular signal-regulated kinase (ERK), a classical MAP kinase, was expressed in LβT2 cells and activated by continuous GnRH pulse treatment for LH generation in a perfusion system (Kanasaki et al., 2005). The GnRH-induced LHβ gene transcription via ERK was also found in the static incubation of LβT2 gonadotrophs; moreover, the ERK activation was proved to be induced by protein kinase C (PKC), cyclic adenosine monophosphate (cAMP) and calcium ion (Ca^{2+})-dependent signaling pathways (Liu et al., 2002; 2003). In addition, it was reported that GnRH-induced LHβ gene transcription is blocked by the MAPK kinase (MEK) inhibitors, PD98059 and U0126 suggesting that GnRH receptor activation also modifies MEK, an upstream enzyme of ERK and a downstream kinase under PKC control in the LβT2 cell line (Yamada et al., 2004). GnRH not only modulates the intracellular signaling network but also regulates transcription factors for LHβ gene expression in LβT2 gonadotrophs (Ruf and Sealfon, 2004). Dorn and his co-workers (1999) reported that GnRH enhanced the mRNA level of early growth response-1 (Erg-1), but does not remarkably modify the gene encoding steroidogenic factor-1 (SF-1) in LβT2 cells. However, the synergistic interaction of these two transcription factors is necessary for GnRH activation of the LHβ promoter in this cell line. A recent experiment using a microarray approach to examine multiple gene expression modifications in perfused LβT2 cells also revealed that the expression of Erg-1 and Erg-2, important for inducing LHβ-subunit promoter, are stably up-regulated by a high-frequent pulsatile GnRH stimulation (Lawson et al., 2007).

Follicle stimulating hormone is the other crucial gonadotropin under the control of hypothalamic GnRH in the mammalian reproduction system. In LβT2 gonadotrophs, FSH secretion is not changed under GnRH treatment alone, but stimulated by concomitant GnRH and activin A in a dose-responsive manner (Graham et al., 1999). Using this mouse gonadotroph model, Pernasetti et al. (2001) reported for the first time that the gene expression of the FSHβ subunit is enhanced by activin and attenuated by follistatin, a powerful activin-binding protein. Moreover, follistatin inhibited the stimulatory effect of GnRH on the ovine FSHβ promoter after transfection into LβT2 pituitary cells, suggesting the involvement of endogenous activin in the GnRH regulation on FSHβ gene transcription (Pernasetti et al., 2001). Regarding the mechanisms of FSH yielding in the LβT2 gonadotrophs, Ca^{2+}, PKC and MAPK transduction signals were demonstrated to be important factors to mediate FSHβ-subunit gene transcription by GnRH stimulation (Vasilyew et al., 2002). Phosphoinositide 3-kinase (PI3-kinase) is an essential phosphorylating enzyme that contributes to cell survival. The research using the PI3-kinase inhibitor (LY294002) showed that PI3-kinase is also an important modulator to attenuate the promoter activities of FSHβ and GTHα subunits induced by GnRH in LβT2 cells (Mutiara et al., 2008).

As one of the major granin proteins, SgII was initially characterized in bovine anterior pituitary (Rosa and Zanini, 1981). It is a ~600 amino acid, very acidic, tyrosine-sulfated protein located in secretory granules of vertebrate neuroendocrine cells and vesicles of some neurons, relatively poorly conversed in evolution (Mahata et al., 1991; Natori and Huttner, 1994; Blázquez et al., 1998a). In human pituitary, SgII immunoreactivity (IR) was discovered within gonadotrophs, thyrotrophs and corticotrophs in human pituitaries using immunohistochemistry (Vallet et al., 1997). SgII-IR was detected in the secretory granules

and co-localized with LH in the gonadotropin cells of cow pituitary, indicating the co-package of granins and gonadotropins to form secretory granules (Bassetti et al., 1990). Two types of secretory granules were detected in rat gonadotrophs: a large-sized moderately electron-dense granule and a small-sized electron dense granule. The latter granule exclusively contained the immunoreactive signals of SgII and LH (Watanbe et al., 1991). Another study using GnRH antibody to block its regulation on mouse pituitary *in vivo* revealed that the absence of GnRH resulted in co-aggregating LH and SgII within secretory granules, suggesting the potential co-release of LH and SgII from gonadotrophs under GnRH control (Crawford et al., 2002). In addition to the pituitaries of cow, rat and mouse, SgII was found in the LH-positive granules from female sheep gonadotropin cells (Crawford and McNeilly, 2002). Taken together, the above immunocytochemical studies revealed a potential association of SgII and LH in secretory granules of mammalian gonadotrophs. Nicol et al. (2002) utilized radioimmunoassay (RIA) with specific mouse LH and human secretoneurin (SN, a well-conserved fragment in the middle segment of SgII sequence) antibodies to investigate protein release from mouse LβT2 gonadotropin cell line. They observed that pulsatile stimulation of GnRH resulted in the marked increments of both LH and SgII release together at each time point, suggesting a close correlation between the secretions of these two proteins from LβT2 cells under the control of hypothalamic GnRH. Also, this finding indicated that SgII and/or its derived peptides (SN containing peptides) might be the crucial factors to regulate LH secretion from gonadotrophs in mammals (Nicol et al., 2002). A further study of Nicol and her colleagues (2004) also demonstrated that LH, but not FSH, is co-released with SgII along with chromogranin A (CgA) in response to GnRH in LβT2 cells, indicating GnRH-stimulated LH secretion via a regulated granin-associated pathway.

The free SN is a 33-34 amino acid long peptide derived from the precursor protein SgII, exhibiting a high sequence similarity among mammalian species, such as mouse, pig, hamster, rat and human (Kähler and Fischer-Colbrie, 2000). The high conservation of mammalian SN sequence indicates several potential physiological activities for this peptide. Our previous studies in the goldfish model revealed that SN not only stimulates the secretion and production of LH *in vivo* (Blázquez et al., 1998a) and *in vitro* (Zhao et al., 2006a and 2009a; Chapter 2), but is also generated from the SgII proteolytic processing in the brain and pituitary (Zhao et al., 2006b; Chapter 2 and 3). In a rat pituitary prolactinoma cell line GH4C1, the transfections of two prohormone convertases, PC1 and PC2, resulted in the increment of SgII processing to generate two SN-containing intermediate-sized proteins (47.5 and 29.5 kDa) (Hoflehner et al., 1995). Based upon the early investigation of SN in the fish neuroendocrine system, I hypothesize that the well-conserved SN peptide plays a pronounced role in regulating LH secretion in mammals. The mouse LβT2 pituitary cell line also expressed two major granin proteins, SgII and CgA (Nicol et al., 2002; 2004). Thus my investigations in this chapter mainly focus on the biological effect of mouse SN (mSN) peptide in the regulation of LH release and gonadotropin subunit (LHβ and FSHβ) gene expression. The mechanism underlying hormone-like functions of SN is not clear. Our previous study showed that in comparison with GnRH, SN only induced a moderate increase of intracellular Ca^{2+} level in identified goldfish pituitary cells (Zhao et al., 2009a). We therefore speculate that SN-induced LH release might be partially mediated through a G-protein coupled signaling pathway. In addition, the MAPK cascade was previously reported as a signal transduction pathway for the SN-stimulated inflammatory response (Kirchmair et al., 2004). In the present study, we explore whether SN regulates LH generation via activating the

MAPK cascade. In order to clarify the potential for SN production in LβT2 cells, we set out to characterize SgII processing using western blot analysis with the anti-goldfish SN antibody (Zhao et al., 2006b and 2009b).

4.3. Material and Methods

4.3.1. Neuropeptides

Mammalian GnRH [mGnRH, (Des-Gly^{10}, D-Leu^{6}, Pro-NHEt^{9})-LHRH] was purchased from Bachem Bioscience Inc. (King of Prussia, PA, USA). Based upon potential cleavage sites and predicted sequence, 33-amino acid mSN was synthesized by us on Fmoc-PAL-PEG polystyrene solid support by using an automated peptide synthesizer (Pioneer, PE-PerSeptive Biosystems, Framingham, MA, USA), following HATU/DIEA-mediated Fmoc chemistry (Basak et al., 2007). The peptide product was purified by reversed phase-HPLC with a C_4 semi-preparative column (Varian Dynamax 250 x 10.0 mm) which was eluted by using a linear gradient from 35% to 70% acetonitrile (ACN) in 0.1% (vol/vol) trifluoroacetic acid (TFA) in 65 min at a flow rate of 1.5 ml/min. The fractions were detected by UV absorbance at 214 nm and collected for lyophilization. The elution of purified mSN peptide was confirmed by its observed molecular mass (3652 Da) that was recorded on a surface enhanced laser desorption ionization time of flight (SELDI-TOF) mass spectrometry (Ciphergen Biosystems, Fremont, CA).

4.3.2. Cell culture and static incubation of LβT2 gonadotrophs

The LβT2 gonadotroph cell line was generously provided by Dr. P. Mellon (University of California, San Diego, CA, USA). The culture method was described previously (Turgeon

et al., 1996). Briefly, LβT2 cells were maintained in Dulbecco's modified Eagle's Medium (DMEM containing 4.5 g/L glucose, 110 mg/L pyruvate, and 548 mg/L L-glutamine; Mediatech, Manassa, VA, USA) supplemented with 10% fetal bovine serum (Gibco, Grand Island, NY, USA) and 1% penicillin/streptomicin antibiotics (10,000 units/ml pencillini, 10,000 μg/ml streptomycin; Gibco, Grand Island, NY, USA) in a humidified 5% CO_2 atmosphere at 37 ^{0}C. Cells were passaged by using 10X trypsin-EDTA solution (Sigma-Aldrich, Ayrshire, UK) at the dilution of 1:10 in PBS once a week. Cells from passage 5-68 were utilized for the experiments.

Prior to each static incubation experiment, the cells were plated in 24-well culture plates at a density of ~2.5 x 10^5 cells/well or 6-well culture plates at a density of ~2.0 x 10^6 cells/well, and cultured in the 5% CO_2 air at 37 ^{0}C for 48 hours. At the beginning of experimentation, the medium was replaced with DMEM containing either 10 nM mGnRH or various dose of mSN (1-100 nM). After the individual static treatments of 3, 6 and/or 12 hours depending on the experiment, media were collected and stored at -20 ^{0}C for radioimmunoassay of LH secretion. Moreover, cells were removed from the plate bottom and kept at -80 ^{0}C for RNA or protein extraction. For the study of the signaling pathway, cells were starved in serum-free DMEM overnight in wet 5% CO_2 atmosphere at 37 ^{0}C at first before the static treatment of mGnRH (10 or 100 nM) and mSN (10 and/or 100 nM) for 10 minutes or 3 hours. For blocking the MAPK pathway, 20 μM PD98059 (a MEK inhibitor; Cell Signaling Technology, Danvers, MA, USA) was utilized to pre-treat the LβT2 cells for 1 hours prior to the 10-minute acute stimulation of mGnRH (100 nM) and mSN (100 nM). This dose of PD98059 was chosen because it was shown to be effective in other experiments with LβT2 cells (Liu et al., 2002).

4.3.3. Radioimmunoassay (RIA)

The LH level in the culture medium was assayed by mouse LH-specific RIA in Dr. A.S. McNeilly's lab as previously described (McNeilly et al., 1996; Nicol et al., 2002; 2004). The intra- and inter-assay coefficient of variance were <10% for LH-RIA.

4.3.4. RNA isolation, cDNA preparation and real-time reverse transcriptase polymerase chain reaction (RT-PCR) analysis

After a 6-hour static incubation with mGnRH and mSN, the LβT2 cells were carefully washed by 1X PBS. Total RNA was isolated by utilizing RNeasy Micro Kit (QIAGEN GmbH, Hilden, Germany) following the company's standard protocol. The performances of cDNA synthesis and real time RT-PCR were previously described in Chapter 3. Briefly, after sample RNA level was measured by NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Wilmington, DE), Aliquots of 2 μg DNA-free RNA was adopted to synthesize cDNA with 200 ng of random primers (Invitrogen, Carlsband, CA) and Superscript II RNase H^- reverse transcriptase (Invitrogen, Carlsband, CA). The PCR primers were designed using Primer 3 (http://frodo.wi.mit.edu), verified by IDT OligoAnalyzer 3.1 (http://www.idtdna.com/analyzer/Applications/OligoAnalyzer) and synthesized by Invitrogen. Based on the published sequences, the primer sets in the present investigation are: mouse LHβ (NM_008497: forward primer 5'-CTGTCAACGCAACTCTGG-3', reversed primer 5'-AGGGCTACAGGAAAGGAG-3'), mouse FSHβ (U12932: forward primer 5'-CAGTAGAGAAGGAAGAGTG-3', reversed primer 5'-ACTGGATATGTGTAGAGG-3'), mouse SgII (NM_009129, forward primer: 5'-CTACCCTGGAGTCTGTGTTC-3', reversed

primer 5'-TTGCTGTCTCTCACCTCTTC-3') and mouse CgA (NM_007693: forward primer 5'- AGCATCCAGTTCCCACTT-3', reversed primer 5'-AAGCCTCTGTCTTTCCATC-3'). We adopted 18S ribosomal RNA as a housekeeping gene, and its primers (forward: 5'-GATACCGTCGTAGTTCC-3', reversed 5'-ATCTGTCAATCCTGTCC-3') were designed according to the published sequence of mouse 18S (X00686). The amplifications of genes were performed with 150-300 nM of primers (depending on different genes) in the Mx3000 Mulitplex Quantitative PCR System (Stratagene, La Jolla, CA).

4.3.5. Intracellular protein extraction and western blot analysis

The LβT2 cells were removed from the 6-well culture plates and collected by centrifuging at 2000 rpm for 10 min. These cells were then sonicated in a homogenizing buffer described in Chapter 2 and 3. After centrifugation (12,000 rpm at 4 ^{0}C), a protein extract was obtained from the supernatant. The concentration of intracellular extract was measured by utilizing a NanoDrop ND-100 Spectrophotometer (Thermo Scientific, Wilmington, DE). In comparison the protocol in Chapter 2 and 3, after denaturation at 100 ^{0}C, equal amounts of the intracellular extracts (25-50 μg) were separated by SDS-PAGE in 10% gels and semi-dry electro-transferred to polyvinylidene difluoride (PVDF) membranes (immobilon-P, Millipore, Bedford, MA, USA). The membrane, which was blocked by 5% fat-free skim milk for 1 hour, was incubated in the primary antibodies at 4 ^{0}C for at least 12 hours. The primary antibodies used were anti-goldfish SN antiserum (Zhao et al., 2006b; 2009b) (dilution: 1:2000-1:4000), anti-actin antibody (A2066, Sigma, St Louis, MO, USA) (dilution: 1:500), phospho p44/42 MAP kinase (ERK1/2) (Thr202/Tyr204) antibody (9101S, Cell Signaling Technology, Danvers, MA, USA) (dilution: 1:600-1:1000) and p44/42 MAPK

(ERK1/2) rabbit antibody (9102, Cell Signaling Technology, Danvers, MA, USA) (dilution: 1:1000). Thereafter, the membrane was washed and incubated in ECL anti-rabbit IgG horseradish peroxidase-linked species-specific antibody from donkey (NA934, GE Healthcare, Buckinghamshire, UK) at a dilution of 1:4000 at room temperature for 90 min. In the end, the membrane with ECL Plus Western Blotting Detection Reagent (GE Healthcare, Buckinghamshire, UK) was detected with the BioRad ChemiDoc Imaging System and quantified using the Quantity One (Bio-Rad, Segrate, Milan, Italy) software package.

4.3.6. Data analysis

Data were expressed as means ± S.E.M. The statistical analyses were carried out using Sigma Stat version 3.5. The effects of mSN and mGnRH on LH release and mRNA levels of LHβ, FSHβ, SgII and CgA were examined by one-way Analysis of Variance (ANOVA) followed by the Fisher LSD test. When the values were non-parametric, data were natural log-transformed before one-way ANOVA. When examining the regulation of 10 nM mGnRH on SgII processing, we utilized either the t-test (for parametric data) or the Mann-Whitney Rank Sum test (for non-parametric data) to compare control and mGnRH-treated groups. The phospho-ERK1/2 and total ERK1/2 data were normalized by the average signal intensity for each membrane. The ratios between normalized phospho-ERK1/2 and total ERK1/2 levels were examined by one-way or two-way ANOVA followed by Fisher LSD test to determine specific difference among treatments. If the data were not parametric, the Kruskal-Wallis One Way Analysis of Variance on Ranks was used. Means were considered significantly different at $p<0.05$.

4.4. Results

4.4.1. Dose-response and time-course studies of SN effect on LH release

Various concentrations (1, 10 and 100 nM) of mSN were chosen for the time-course and dose-response study of LH secretion from LβT2 cells. The levels of medium LH increased from 3-hour to 12-hour in the control group as well as in the mGnRH and mSN treatments. As a traditional stimulator of LH secretion from gonadotrophs, 10 nM of mGnRH was adopted as a positive control in this experiment (Nicol et al., 2002). After 3-hour treatment, mGnRH induced increments of LH levels by 3.1 fold ($p<0.001$), in comparison with the control (Fig. 4.1). The LH levels after the exposure to 1 and 10 nM mSN were respectively 2.9- ($p<0.001$) and 2.6-fold ($p<0.01$) higher than the time-matched controls. After 3 hours, the LH level in the 100 nM mSN group was increased by 161% but this did not reach statistical significance ($p>0.05$). After a 6-hour incubation, LH increased by 3.4 fold ($p<0.001$) in response to mGnRH; in contrast, exposures to all mSN doses (1-100 nM) enhanced ($p<0.05$) LH levels in culture medium by 2.9-2.7 fold (Fig. 4.1). In contrast, the effects of mGnRH and mSN were no longer evident after 12 hours (Fig. 4.1).

4.4.2. Expression of LHβ, FSHβ, SgII and CgA in LβT2 cells after 6-hour static incubation

We have demonstrated that exposures to all doses of mSN (1-100 nM) enhance the LH secretion from LβT2 cells after 6 hours (Fig. 4.1). Thus, another experiment was carried out to explore the effects of the same concentrations of mSN on regulation of LHβ subunit mRNA levels at the same time point. The cellular 18S ribosomal RNA level were not significantly modified under any treatment (data not shown), and thus was chosen as a housekeeping gene to normalize the expression of LHβ-subunit and other genes. In the presence of 10 nM

mGnRH, the LHβ mRNA level was increased by 2.0 fold ($p<0.001$) in comparison with the control. This result was consistent with previous findings (Turgeon et al., 1996). Treatments of 1-100 nM mSN induced 1.8-1.6 fold ($p<0.05$) increments in LHβ gene expression (Fig. 4.2A). Furthermore, SN-induced LHβ mRNA level change exhibited a similar pattern to that of LH secretion shown in Fig. 4.1 after 6 hours. Mouse GnRH-induced LHβ mRNA and LH level modifications were slightly higher than any dose of mSN, by 116-126% and 117-125%, respectively. However, differences in the effects of mGnRH and any dose of mSN were not statistically significant ($p>0.05$).

Altered expression of FSHβ-subunit, SgII and CgA were detected in LβT2 cells following mGnRH (10 nM) and mSN (1, 10 and 100 nM) treatments. Six-hour exposures to mGnRH dramatically enhanced FSHβ mRNA levels by 9.9 fold ($p<0.001$), demonstrating a highly sensitive response of FSH production to GnRH stimulation. The effect of mSN on the FSHβ gene was weaker than that of mGnRH in LβT2 gonadotrophs. The treatments of 1 and 10 nM mSN significantly increased FSHβ gene transcription by 3.2 ($p<0.05$) and 2.1 fold ($p<0.05$), respectively, whereas 100 nM SN induced a non-significant ($p>0.05$) 1.9-fold increment of FSHβ mRNA level (Fig. 4.2B). In this experiment, GnRH was also demonstrated to up-regulate the SgII gene expression in comparison with the control by 2.4 fold ($p<0.001$) in LβT2 cells. However, SgII mRNA was not affected by any dose of mSN during the 6-hour static incubation of LβT2 cells (Fig. 4.2C). In comparison with SgII expression, CgA mRNA levels were not affected by mGnRH and were somewhat reduced by 1 nM (57%, $p<0.001$) and 10 nM (71%, $p<0.05$) SN in the 6-hour treated-cells (Fig. 4.2D).

4.4.3 Evaluation of anti-goldfish SN antiserum specificity in the detection of mouse SN (mSN)

Our anti-goldfish SN antiserum was generated against the 15-amino acid antigenic peptide YTPQKLATLQSVFEE that is the most conserved part of SN sequence between various species (Zhao et al., 2006b). The antigenic peptide shows 80% identity to the middle portion of mSN (Chapter 1), indicating the possibility of using the anti-goldfish antibody to examine the SN-IR in mice. In addition, our anti-goldfish SN antibody was also used to detect the SN-IR signals within the pituitary cells in human, rat and trout (Appendix I). In order to evaluate the specific binding ability of anti-goldfish SN antibody to the protein extracted from mouse LβT2 pituitary cells, we carried out western blot analysis using anti-goldfish SN antiserum pre-absorbed with its antigenic peptide, goldfish SN (Fig. 4.3B). We detected several SN-immunreactive protein bands when using the anti-goldfish SN antiserum (Fig. 4.3B-I), whereas no signal was observed in the membrane that was incubated in the SN antibody pre-absorbed with SN (Fig. 4.3B-II). These results confirmed that the rabbit anti-goldfish SN antiserum recognized mSN-associated proteins derived from mouse pituitary cells.

4.4.4. SgII processing to generate several SN-containing peptides in LβT2 cells.

Under a longer time ECL exposure than that of Fig. 4.3B, two strong high-molecular weight (MW) bands and two weak low-MW signals were observed in different samples (Fig. 4.3C). A standard formula ($y=116.65x^{-0.7378}$, $R^2=0.999$) was established for describing the relationship between apparent MW (y) and gel running distance (x) based on protein standards. The estimated MW of the 2 strong and 2 weak bands were ~71, ~46, ~42 and ~32 kDa (Fig. 4.3C). Fig. 4.3A showed the possible cleavage sites within the mouse SgII sequence

(617 amino acids, calculated MW: 70.6 kDa) reported previously (Blázquez et al., 1998a; Zhao et al., 2006b). The ~71-kDa band is corresponding to the size of the published unprocessed mouse SgII precursor. Other SN-IR signals were noticeably smaller than the calculated MW of the SgII precursor indicating that they are processing products derived from this granin.

4.4.5. Effects of 6- and 12-hour mGnRH treatments on SgII processing in mouse LβT2 pituitary cells

The SgII precursor (~71 kDa) and the 3 SN-IR SgII products (~46, ~42 and ~32 kDa) were further studied to determine whether the hypothalamic GnRH regulates SN generation in this mouse pituitary cell model. After a 6-hour static incubation, 10 nM mGnRH significantly decreased the levels of SgII, ~46- and ~32-kDa SN-IR polypeptides by 49% ($p=0.05$), 28% ($p<0.001$) and 37% ($p=0.05$), respectively, and also induced a 36% but statistically non-significant decrease ($p=0.505$) in the level of ~42-kDa SN-IR SgII-derived peptide (Fig. 4.4A and 4.4B). Twelve-hour treatment of mGnRH (10 nM) also modulated the levels of all SN-IR polypeptides. Mouse GnRH decreased the ~71-, ~46-, ~42- and ~32-kDa SN-containing proteins by 63% ($p<0.01$), 31% ($p<0.001$), 41% ($p<0.05$) and 49% ($p=0.05$), respectively (Fig. 4.4C and 4.4D). Note that β-actin served as an appropriate protein loading control because levels were not affected by mGnRH treatment (Fig. 4.4B and 4.4D).

4.4.6. mSN activates ERK in LβT2 cells

ERK is a classic MAP kinase involved in GnRH-stimulated LH production and release in mouse LβT2 cells (Ruf and Sealfon, 2004). In this experiment, western blot analysis was

adopted to detect the changes of active phospho-ERK1/2 and total inactive ERK1/2 levels under various treatments. At first, we examined the acute effect of SN on the activation of ERK1/2. Serum-starved LβT2 cells were treated with 100 nM mGnRH, 10 and 100 nM mSN for 10 minutes. As a positive control group, the treatment of 100 nM mGnRH increased the protein expression of activated ERK1/2 by 3.0 fold ($p<0.001$) (Fig. 4.5A), which is consistent with the data previously described (Liu et al., 2002). Similar to the action of mGnRH, 10 and 100nM of mSN were capable of inducing a 2.2- ($p<0.01$) and 4.6-fold ($p<0.001$) rapid increments of phospho-ERK1/2 level, respectively (Fig. 4.5A). The 100 nM mSN-stimulated phospho-ERK1/2 increment was significantly higher than that of 100 nM mGnRH by 155% ($p=0.001$) (Fig. 4.5A). When LβT2 cells were exposed to the low-dose treatment for a longer time (3 hours), 10 nM mSN also significantly increased the phospho-ERK1/2 levels by 142% ($p<0.05$) whereas 10 nM mGnRH down-regulated ERK1/2 expression by 45% ($p<0.05$) (Fig. 4.5B). To further define the signaling pathways involving in activation of ERK by mSN, the cells were pre-treated with PD98059 to pharmacologically inhibit MEK, the upstream kinase of ERK. We compared the relative change of activated ERK1/2 after a 10-minute stimulation with 100 nM mSN and 100 nM mGnRH. PD98059 significantly reduced both the mSN- and mGnRH-induced activation of ERK1/2 by 60% and 52% respectively (Fig. 4.5C).

4.5. Discussion

Our previous studies in goldfish demonstrated not only the generation of SN in the brain and pituitary, but also its neuroendocrine and paracrine effects to stimulate LH secretion and production in dispersed pituitary cells (Chapter 2 and 3). Furthermore, in collaboration with Dr. J.P. Chang's lab to investigate the SN-associated signaling pathway, we suggested

that the elevation of intracellular Ca^{2+} level is a potential transduction signal mediating SN action on goldfish LH cells. This SN-induced Ca^{2+} signaling mechanism was also demonstrated to be partly independent of GnRH-stimulated Ca^{2+} entry (Zhao et al., 2009a). However, the SN signaling pathway leading to LH secretion is still unclear in gonadotrophs. We therefore chose the LβT2 gonadotropin cell that has many of the common characteristics of normal mouse pituitary cells, and the signaling pathways to mediate GnRH-stimulated LH release are well studied in this cell line (Ruf and Sealfon, 2004). In agreement with our previous research in goldfish (Chapter 2 and 3), the present study revealed that SN increased not only LH secretion but also the transcription of both LHβ and FSHβ subunits in LβT2 cells, indicating a potential regulatory role of SN in mammalian reproduction. Using our anti-goldfish SN antiserum (Zhao et al., 2006b), SgII processing in mouse LβT2 gonadotrophs was found to be under the control of GnRH. We also explored the intracellular signaling pathway (MAPK cascade) mediating SN action in LβT2 cells.

Various studies in goldfish have shown that SN induces LH release and LHβ subunit gene expression in pituitary cells (Blázquez et al., 1998a; Zhao et al., 2006a; Chapter 2 and 3). However, the studies concerning the regulatory effect of SN are quite limited in the mammalian central nervous system or neuroendocrine system. In 1993, SN was demonstrated for the first time to dose-dependently increase dopamine outflow in the superfusion of rat striatal slices (Saria et al., 1993). An *in vivo* experiment showed that local infusion of SN into the substantia nigra and neostriatum of halothane-anaesthesized rats elevated the extracellular concentrations of dopamine, γ-aminobutyric acid and glutamate (You et al., 1996). Here, we adopted the mouse LβT2 gonadotrophs to study the time- and dose-dependent effects of SN on LH release. In this cell line, there is a close correlation between GnRH-induced LH release

and secreted SN-immunoreactive SgII-like proteins. A possible regulatory effect of SgII (or derived peptides) on LH secretion has been proposed previously (Nicol et al., 2002). To test this speculation, the direct stimulatory action of SN on LH release in mouse LβT2 gonadotrophs were determined using a static treatment of SN at the range of 1-100 nM for 3, 6 and 12 hours. We found that low doses of SN (1 and 10 nM) consistently stimulate LH secretion from mouse pituitary cells after 3- and 6-hour treatments. The effective concentrations of SN were similar to that found in *in vitro* studies using goldfish pituitary fragments. In the static incubation of pituitary fragments from sexually regressed or mature goldfish, 10 nM SN stimulated LH secretion after 3 and 6 hours (Zhao et al., 2006a). A similar effect of SN was also observed in primary cultures of dispersed goldfish pituitary cells. Six-hour static treatment of 10 nM SN significantly increased LH secretion from gonadotrophs (Chapter 2 and 3). In summary, we conclude that low physiological doses of SN are able to affect gonadotropin secretion from pituitary cells.

During the 3- and 6-hour static culture, the LβT2 cells were very sensitive to even the lowest dose of SN (1 nM) tested. However, the highest concentration of SN did not show any significant effect on LH release at the time point of 3 hour. Similar to the non-dose dependent effect of SN in LβT2 cells, a low dose pulse of 10 nM GnRH could induce more LH release than 50 and 200 nM GnRH (Nicol et al., 2002). Unlike LβT2 gonadotrophs which are tumour-derived, dispersed normal mouse pituitary cells show a classical LH response to increasing doses of GnRH after 4-hour static treatments over a wide range of GnRH concentrations (10 to 1000 nM; Abbot et al., 1986). This difference between LβT2 gonadotrophs and normal pituitary cells might be due to alteration of secretory pathways in the LβT2 cell line. A previous investigation revealed that 10 and 100 nM GnRH evoked a

similar increment in exocytosis as well as intracellular Ca^{2+} level within a single LβT2 cell (Thomas et al., 1996). The interpretation of this result was that only ~400 vesicles were found to be involved in fusion with the plasma membrane for further secretion. Thus, secretion under the long-period stimulation might be limited by the availability of secretory products (Thomas et al., 1996). The secretory limitation was also observed in long-term GnRH-evoked LH release: the LH-secretory response dramatically declined under the repeated stimulation of GnRH for several hours in LβT2 cells (Turgeon et al., 1996). In contrast, multiple administrations of GnRH on the perifused normal rat pituitary cells always induced dose-dependent LH release (Loughlin et al., 1981). Therefore, we speculated that the absence of dose-response to SN-induced LH release over the dose range tested might result from this limitation of secretory vesicles in mouse LβT2 cell line. Nevertheless, we showed for the first time that mSN stimulates LH release from these mouse cells in a manner similar to that which we have documented for the goldfish.

To further examine the SN regulatory activities on LH generation in LβT2 cells, we adopted real time RT-PCR to measure the mRNA level of LHβ subunit under the stimulation from various concentrations of SN. The 6-hour treatment was chosen due to the strongest LH secretory response to various doses of SN at that time point. The increasing doses of SN induced similar increment in LHβ gene expression within LβT2 gonadotrophs, consistent with the pattern of SN-induced LH release. The unvaried LH synthesis under increasing doses of SN treatments might be the other reason to explain the absence of a dose-dependent effect of SN on LH secretion in LβT2 cells. Interestingly, the levels of SN-induced LH release (2.6-2.8 fold increase) was higher than those of LHβ subunit mRNA (1.6-1.8 fold increment). We therefore speculated that SN might affect not only LHβ mRNA synthesis but also other

pathways associated with LH protein generation and secretion. This possibility has been reported in the previous observation about GnRH-stimulated LH release in this cell line. The acute GnRH-induced LH protein synthesis and secretion were mainly dependent upon the activation of translation initiation proteins, 4E-binding protein 1 and eukaryotic initiation factors 4E/4G, but not LHβ transcription. The stimulation of these proteins by GnRH was mediated by the MAPK/ERK pathways (Nguyen et al., 2004). The stimulatory effect of SN on the activation of the MAPK cascade in the inflammatory response (Kirchmair et al., 2004) and the mouse LβT2 cell line indicate the possibility of the multiple pathways mediating SN-induced LH release.

SN also plays a role in up-regulating the gene expression of FSHβ subunit. As a positive control, mGnRH dramatically increased the FSHβ subunit gene transcription in LβT2 cells after 6 hours. This result was consistent with the previous data that GnRH directly affects FSHβ transcription (Miller et al., 2002), affirming that the static incubation system is suitable for studying the regulation of FSHβ subunit gene. Several previous investigations in the LβT2 cell line showed that activin is an important factor to maintain and enhance GnRH-induced FSHβ mRNA production (Graham et al., 1999; Nicol et al., 2004). However, the approach of co-treatment with activin was not adopted in our experimental design, because endogenous activin is involved in an autocrine GnRH-dependent pathway of FSH regulation (Pernasetti et al., 2001). Interestingly, SN also induced statistically significant increments of FSHβ level in LβT2 cells. However, SN-induced FSHβ gene expression was much weaker than that of GnRH (9.9 fold). Due to the potential generation of SN within the LβT2 cell, we speculated that SN might be another autocrine factor to strengthen GnRH effects on FSH generation and secretion in the mammalian pituitary. In comparison with pulsatile GnRH

stimulation that increased the secretion of both SgII and CgA from perifused LβT2 cells (Nicol et al., 2002), we determined that static exposure to GnRH also increases the cellular level of SgII mRNA but has no remarkable effect on CgA gene expression. The GnRH-evoked SgII response was also detected in the static culture of goldfish pituitary cells (Chapter 3). However, Nicol et al. (2002) reported that the pulses of GnRH did not alter SgII mRNA level in estradiol and dexamethasone-treated LβT2 cells. The variation between our data and early observations might be attributed to differences in GnRH stimulation strategies and the different *in vitro* models adopted. Under the same condition as the GnRH treatment, SN was unable to elicit any changes in SgII mRNA levels in our experiment with LβT2 cells. In contrast, SN significantly decreases CgA mRNA level at the dose of 1 and 10 nM. The inhibitory action of SN on CgA suggests that these two peptides and/or their associated polypeptides might play opposite regulatory effects in gonadotrophs. The opposing activities of CgA-derived peptides versus SN have been observed in other systems. For example, during the formation of blood vessels, vasostatin-I (a functional CgA product) inhibits angiogenic parameters whereas SN activates not only angiogenesis but also vascularization (Helle, 2009).

In the present study, we also observed specific proteolytic processing of SgII to generate SN in mouse LβT2 gonadotrophs. To our knowledge, Nicol and her colleagues (2002) were the first to measure the intracellular and extracellular levels of total SgII-associated proteins and polypeptides in LβT2 cells using anti-human SN antibody in RIA. Herein, our investigations mainly focused on detecting SgII and its intermediate products potentially yielding SN in the cell line. The anti-goldfish SN antibody was used for detecting total SN-IR in mouse pituitary cells. Since this antibody had been generated against the most conserved sequence segment located in the middle of goldfish SN that shows a high sequence similarity

to that of mSN (Zhao et al., 2006b), we expected it to be highly effective and efficient. Our results also confirmed the specific binding ability of anti-goldfish SN antibody to mouse SN. Furthermore, the same anti-goldfish SN antiserum was successfully utilized to detect SN-IR signals in the pituitary of human and rat (Appendix I).

Several proteins and polypeptides ranging from 75 to 21 kDa molecular weights were previously reported to be derived from proteolytic processing of the precurser 86-kDa SgII proprotein in endocrine tissues and neurons of mammals (Fisher-Colbrie et al., 1995). A previous human immunological study showed that two SgII-derived proteins with molecular weights 46 and 31 kDa, were observed in gonadotrophs and non-functioning pituitary adenomas (Vallet et al., 1997). It is likely that the mammalian subtilisins called prohormone or proprotein convertases (PCs) presently called proprotein convertase Subtilisin Kexins (PCSKs) have the potential abilities to cleave SgII precursor protein at the carboxy terminal of evolutionary conserved dibasic amino acids (**KR**↓ or **RK**↓) to generate free SN peptide (Zhao et al., 2009b). Our previous studies of SN generation in goldfish revealed the formation of several SgII-derived proteins and polypeptides. These were found to exist in the pituitary, and include proteins of molecular weights of ~57, ~30, ~19.6, ~15 and ~12 kDa besides the free goldfish SN peptide (MW 3,655 Da) (Zhao et al., 2006b; Chapter 2). Using a similar strategy, we discovered four SN-IR signals with distinct molecular masses predicted as ~71, ~46, ~42 and ~32 kDa, respectively in mouse LβT2 gonadotrophs. Moreover, the ~71 and ~46 kDa proteins showed significantly higher levels than the ~42 and ~32 kDa polypeptides. To predict the possible domains of detected SgII-derived peptides in mouse LβT2 cells, we estimated the molecular masses of all potential fragments between the putative dibasic cleavage sites in the mouse SgII precursor sequence. The ~71 kDa protein is likely the SgII

precursor due to its nearly equal molecular mass to that of calculated mouse SN (70.6 kDa). The strong ~46 kDa protein detected might be derived from the cleavages by PC-like proteases at the 162/163 (**PERK^{162}↓LK**) site and the 568/569 (**VSKR^{568}↓IP**) sites of the SgII precursor. Thus, we speculated that SgII precursor is first cleaved to produce a ~46 kDa fragment with the C, D(SN), E, F and G segments being present within it at the first step of biosynthesis [Fig. 4.3D-(2)]. In the next processing step, a polypeptide with mass of 41.9 kDa was likely to be generated from cleavage at the 526/527 (**QLKR^{526}↓VP**) at the C-terminus of ~46.2 kDa residue to remove the fragment G, which was very similar to the observed ~42 kDa peptide. Concerning the ~32 kDa band, it is most likely derived from the proteolytic processing at the 181/182 (**PFKR^{181}↓TN**) and 492/493 (**VESR^{492}↓QA**) sites in the ~41.9 kDa SgII product. The theoretical molecular mass of this product is 34.9 kDa and it might consist of fragment D(SN), fragment E and a part of fragment F. Free SN peptide was not detectable in the 10% SDS-PAGE gel and western blots because of its small molecular size (MW 3,652 Da). We have shown for the first time the possible pattern of SgII processing in mouse LβT2 pituitary cells. Some of the resultant processing products have similar sizes to those reported for other mammalian SgIIs (Hoflehner et al., 1995). Almost all of SgII precursor protein is processed to generate free SN in normal rat pituitary (Leitner et al., 1996), and gonadotrophs-secreted SN-IR products (Nicol et al., 2002). Together the data suggest that SN acts via an autocrine loop to regulate mammalian gonadotrophs. This contrasts the SN-mediated lactotroph to gonadotroph paracrine pathway we discovered in goldfish.

It was demonstrated using RIA that GnRH pulse frequency decreased the cell content and increased the secretion of total SgII-related proteins, suggesting the stimulatory effect of GnRH on pituitary-sourced SN release (Nicol et al., 2002). Consistent with this previous

investigation, our quantitative luminescent image analysis of western blots showed that GnRH was able to decrease the levels of SgII and its derived proteins after 6- and 12-hours static culture of LβT2 cells. GnRH induces a remarkable increment of SgII gene expression indicating the potential for increases in SgII precursor protein production. A possible explanation of these conflicting data is that GnRH enhances SgII synthesis and its processing to generate SN at the same time. The speed of SgII processing is faster than SgII generation at some certain time points under the stimulation of GnRH, which results in decreasing SgII-derived proteins and increasing SN-IR peptide release from LβT2 cells (Nicol et al., 2002). In contrast to its effect in mouse LβT2 cells, GnRH not only increased SgII mRNA level *in vivo* (Samia et al., 2004) and *in vitro* (Chapter 3) in goldfish but also enhanced the SN-IR signals of major SgII-derived proteins within goldfish prolactin (PRL) cells after 12-hour static treatment (Chapter 3). The conflicting results of GnRH-evoked SgII-derived proteins in mouse gonadotrophs and goldfish PRL cells might be due to different species and various pituitary cell types. The other reasonable interpretation is that the amount of GnRH-induced SgII synthesis is more pronounced than that of SgII processing to yield SN in goldfish PRL cells, which leads to the accumulation of intracellular SgII-derived peptides. Further studies will be required to determine whether GnRH enhances the release of free SN peptides from either mouse LβT2 gonadotrophs or goldfish PRL cells.

The MAPK transduction is one of the essential GnRH-activated signaling pathways to induce early genes for gonadotropin production and release. The MAPK pathway mainly includes ERK, p38MAPK and c-Jun N-terminal kinase (JNK) (Ruf and Sealfon, 2004). The activated form of ERK is phosphorylated on a threonine and a tyrosine residue within the phosphorylation lip (Canagarajah et al., 1997). In the LβT2 cell line, GnRH was demonstrated

to strongly activate ERK and p38MAPK in a rapid manner but activation of JNK was weaker and slower (Liu et al., 2002). In agreement with the previous reports (Liu et al., 2002), our observations confirmed that the 10-minute acute GnRH stimulation caused the activation of ERK in LβT2 cells. Similar to the stimulatory action of GnRH, 10 and 100 nM SN also dramatically increase the ERK phosphorylation by 2.2 and 4.6 fold, respectively. However, chronic GnRH exposure has been shown to result in suppressing PKC- and cAMP-induced ERK activation in LβT2 cells (Liu et al., 2003). In another experiment of 3-hour static incubation, we found that a low dose of GnRH (10 nM) down-regulated ERK activation; however, 3-hour SN (10 nM) treatment still exerted a stimulatory effect on ERK phosphorylation but was weaker than that observed at 10-minutes. MEK, in association with GnRH-stimulated PKC and Raf, has been reported to stimulate activation of downstream ERK in LβT2 cells (Liu et al., 2002). Here, we demonstrated that the inhibition of MEK with PD98059 reduced not only GnRH-induced ERK phosphorylation by 52%, but also SN-stimulated ERK activation by 60%. This result indicates that SN signals through the ERK-dependent MAPK pathways. SN and GnRH might share similar signaling pathways to influence ERK phosphorylation via the Raf-MEK pathway in gonadotrophs. Regarding the signaling pathway to mediate GnRH in gonadotrophs, GnRH binding to its G-protein coupled receptor leads to the modification of Gq heterotrimeric G protein complex and then activation of phospholipase C (PLC) to produce (1,4,5) inositol trisphosphate (IP3) and diacylglycerol (DAG). IP3 is able to evoke the influx of Ca^{2+} through L-type calcium channels and the release from Ca^{2+} internal store. Then, Ca^{2+}-dependent and non- Ca^{2+}-dependent PKC are, respectively activated by the increment of intracellular Ca^{2+} level and DAG release (Ruf et al., 2003). The PKC is the most important signaling molecule to connect the G-protein coupled

signal transduction and growth factor-stimulated MAPK cascade for mediating GnRH-induced LH subunit gene transcription (Naor et al., 2000). The translocation of activated ERK to the nucleus results in the regulation for the transcriptions of LHβ and FSHβ-encoding genes (Naor et al., 2000). In previous reports, the activation of ERK caused by GnRH-induced PKC was found in rat pituitary cells (Weck et al., 1998) and mouse αT3-1 gonadotropin cell line (Sim et al., 1995). Liu and his colleagues (2002) demonstrated that GnRH activated ERK and two other MAPK family members, JNK and p38MAPK, via PKC-dependent signaling pathway in LβT2 cells. In the present investigation, we observed for the first time SN-stimulated ERK activation in LβT2 cells. SN-regulated migration and number of mouse endothelial progenitor cells in the inflammatory system is also via ERK activation (Kirchmair et al., 2004). In addition, elevation of intracellular Ca^{2+} level is a likely signal transduction mediating SN action on LH in goldfish pituitary cells (Zhao et al., 2009a). We therefore speculated that SN might regulate gonadotropin gene transcription through a multi-step signaling pathway in the pituitary as follows: G-protein→ Ca^{2+} → PKC→ RAF→ MEK→ MAPK (ERK). However, most of the mechanisms involved in the SN-induced activation of the MAPK cascade are still not clear and are under exploration. It will be essential to determine if the SN receptor is G-protein-coupled or growth factor-related. Other important questions remain as related to the importance of Ca^{2+} dependent or non-dependent PKC activation and other MAPK subfamilies (JNK and/or p38MAPK).

In conclusion, results from the present study provide the first evidence for a direct stimulatory action of SN on LH release and gonadotropin subunit (LHβ and FSHβ) generation in mammalian pituitary cells (mouse LβT2 gonadotrophs), which is consistent with the SN effect in goldfish pituitary cells. Furthermore, we have shown that SN activates ERK which is

likely involved in the stimulation of gonadotropin subunit transcription via a PKC-MAPK-dependent signaling pathway in a similar manner as observed for GnRH actions on LβT2 cells. Another intriguing finding of the present study is the observation of several SN-immunoreactive SgII-derived peptides within LβT2 cells suggesting SgII processing to generate SN and other short peptides in mammalian gonadotrophs with biological activity. The expressions of these SgII-derived peptides are also regulated by GnRH, indicating that endogenous SN might regulate LH within an autocrine loop under the control of hypothalamic hormones. The studies of SN production, biological activities and functional mechanisms in a mouse gonadotroph cell line not only support our previous finding concerning SN in goldfish pituitary (Chapter 2 and 3) but also further examine the hypothesis that SN is a newly-identified hormonal peptide that acts as a regulator of vertebrate reproduction.

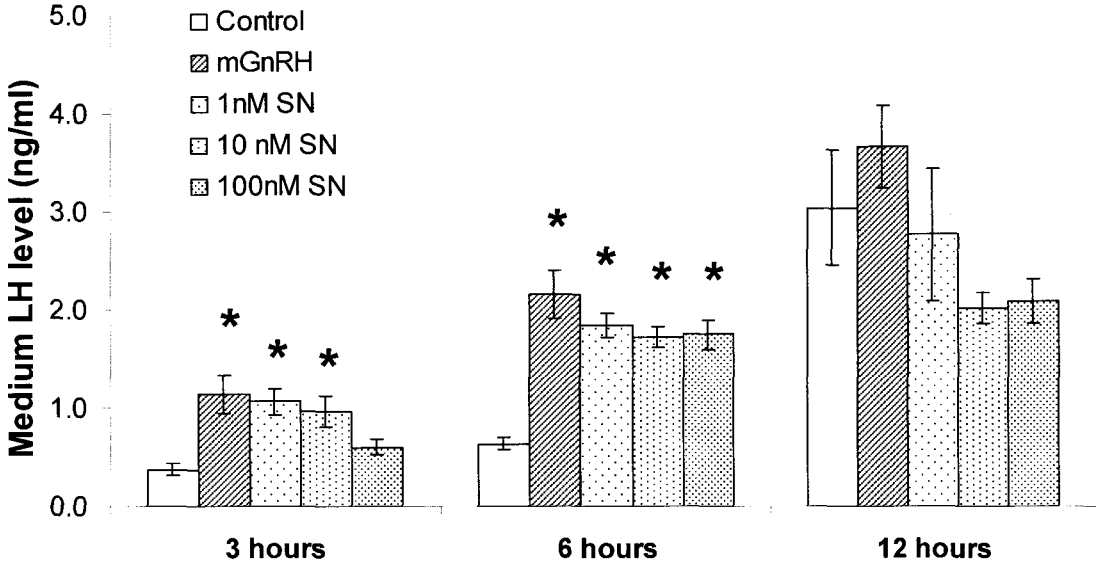

Figure 4.1. Time- and dose-dependent effects of various doses of mSN (1, 10 and 100 nM) in comparison to 10 nM mGnRH after the 3-, 6- and 12-hour static incubation on LH secretion from the mouse LβT2 pituitary cells. Results presented means ± S.E.M. (standard error of the mean), n=9-10. One-way ANOVA was chosen to assess the difference between control and treatment at a given time point, followed by Fisher LSD test. Natural logarithms were adopted if data were not normally distributed in statistics. The (*) indicates a significant difference from control at a given time point ($p<0.05$).

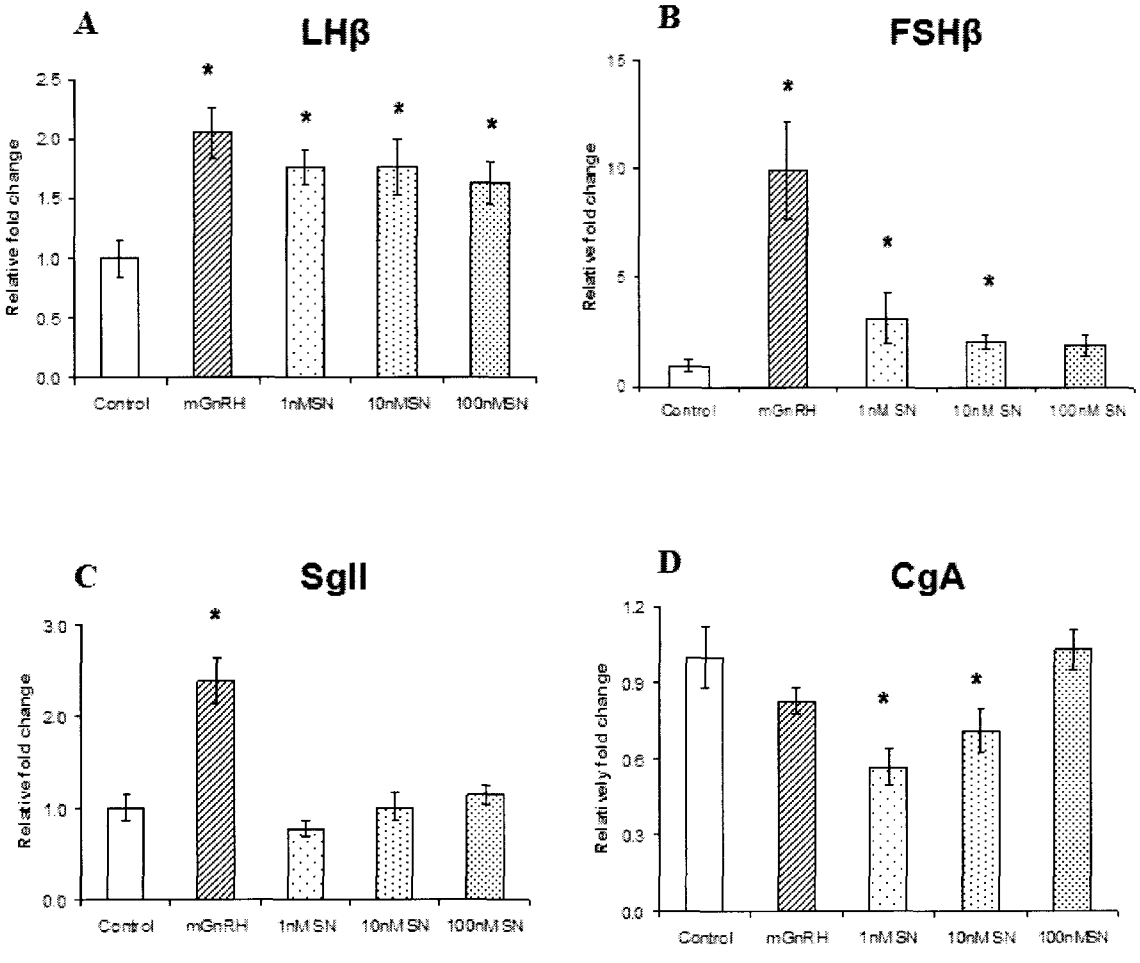

Figure 4.2. Assessment of the gene expression changes of LHβ (**A**), FSHβ (**B**), SgII (**C**) and CgA (**D**) in LβT2 cells after 6-hour static incubation with treatments of 10 nM mGnRH and 10 nM mSN. The mRNA level was measured by real time RT-PCR; 18S ribosomal RNA was used as an internal standard for sample loading control. One-way ANOVA was chosen to assess the difference between control and treatment, followed by Fisher LSD test. Natural logarithms were adopted if data were not normally distributed in statistics. Values expressed as mean folds of Control ± S.E.M. The (*) indicates a significant difference relative to control ($p<0.05$).

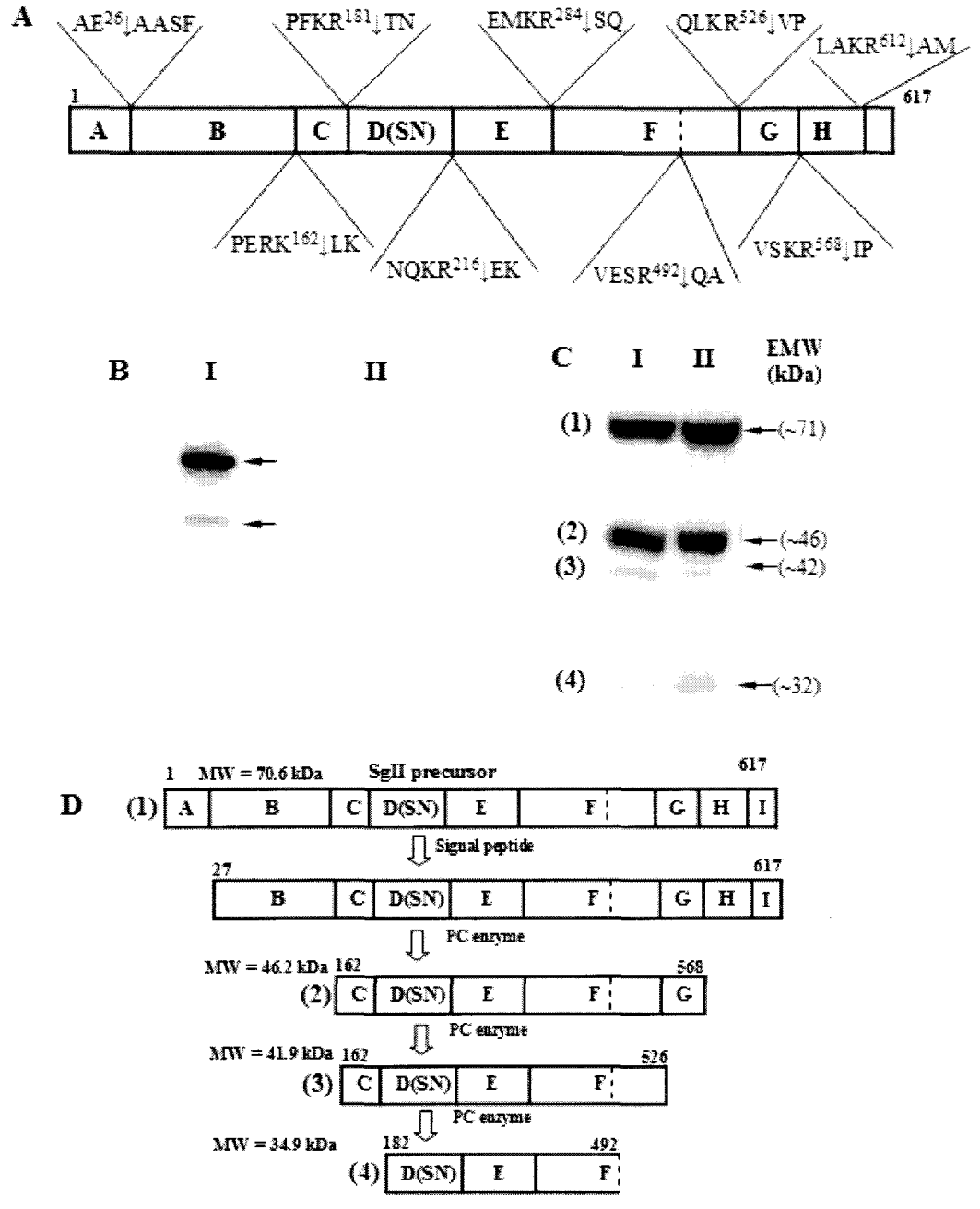

Figure 4.3. Potential SgII processing pathway to produce SN-containing peptides in mouse LβT2 gonadotropin cells. **A**, Potential cutting sites with dibasic amino acid residue in 617 amino acid-long mouse SgII. Possible cleavage sites and approximate position in the SgII precursor sequence are indicated based upon a previous study (Blázquez et al., 1998a). The arrows with numbers represent the amino acid positions immediately after putative cleavages sites. In panel **B** and **C**, western blotting analysis of the total proteins exacted from mouse LβT2 cells using anti-SN antibody (1:2000-1:4000). **B**, Thirty micrograms of protein extracts were separated by 10% SDS-PAGE gel and detected by anti-goldfish SN antiserum (Zhao et al., 2006b). (I) Detection using anti-SN antibody (1:4000); (II) Detection using anti-SN

antibody (1:4000) pre-absorbed by 1 μM goldfish SN. **C**, The loading samples of Lane I and II are taken from different culture wells. Arrows indicate 4 SN-IR signals that are ~71, ~46, ~42 and ~32 kDa. Their estimated molecular weights (EMW) are calculated from a standard curve and a formula depicting the relation between molecular weight (MW) of biotinylated protein standards and gel running distance. **D**, Proposed model for production of various processed forms derived of mouse SgII as observed in LβT2 cells. The model does not preclude other SN containing peptides. Abbreviations: PC, prohormone convertase-like protease; A, signal peptide; D (SN), secretoneurin.

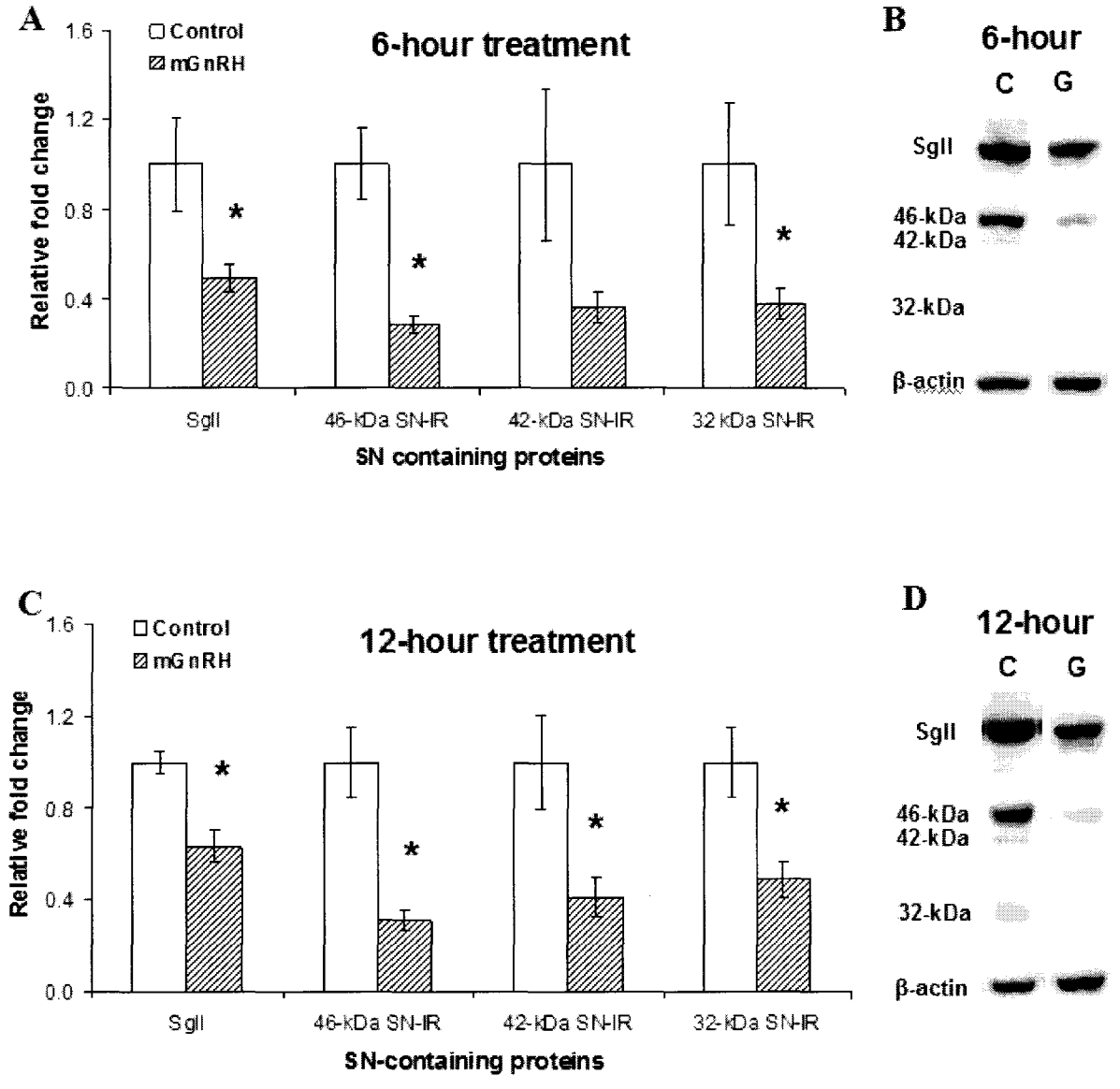

Figure 4.4. Effects of mGnRH on intracellular content of SgII and its derived peptides (containing SN sequence) in LβT2 cells. In panel **A** and **B**, levels of SN-IR proteins (~71, ~46, ~42 and ~32 kDa) were analyzed by western blots using anti-SN antibody (dilution 1:2000) after 6-hour treatment of 10 nM mGnRH. Panel **C** and **D** showed the expression changes of SN-IR proteins under 12-hour static incubation of mGnRH (10 nM). β-actin was measured as an internal standard for loading control. For Panel **A** and **C**, results were presented as mean ± S.E.M. of the relative expression of SN-IR; n=8 for both experiments. A t-test was used to compare control and mGnRH groups. The results of ~42 and ~46 kDa protein intensities after 12-hour treatment were parametric, and the rest data were non-parametric. When the data were non-parametric, Mann-Whitney Rank Sum test was used instead. The (*) indicated significant difference between treatment and control ($p<0.05$). Abbreviations: C, control; G, mGnRH.

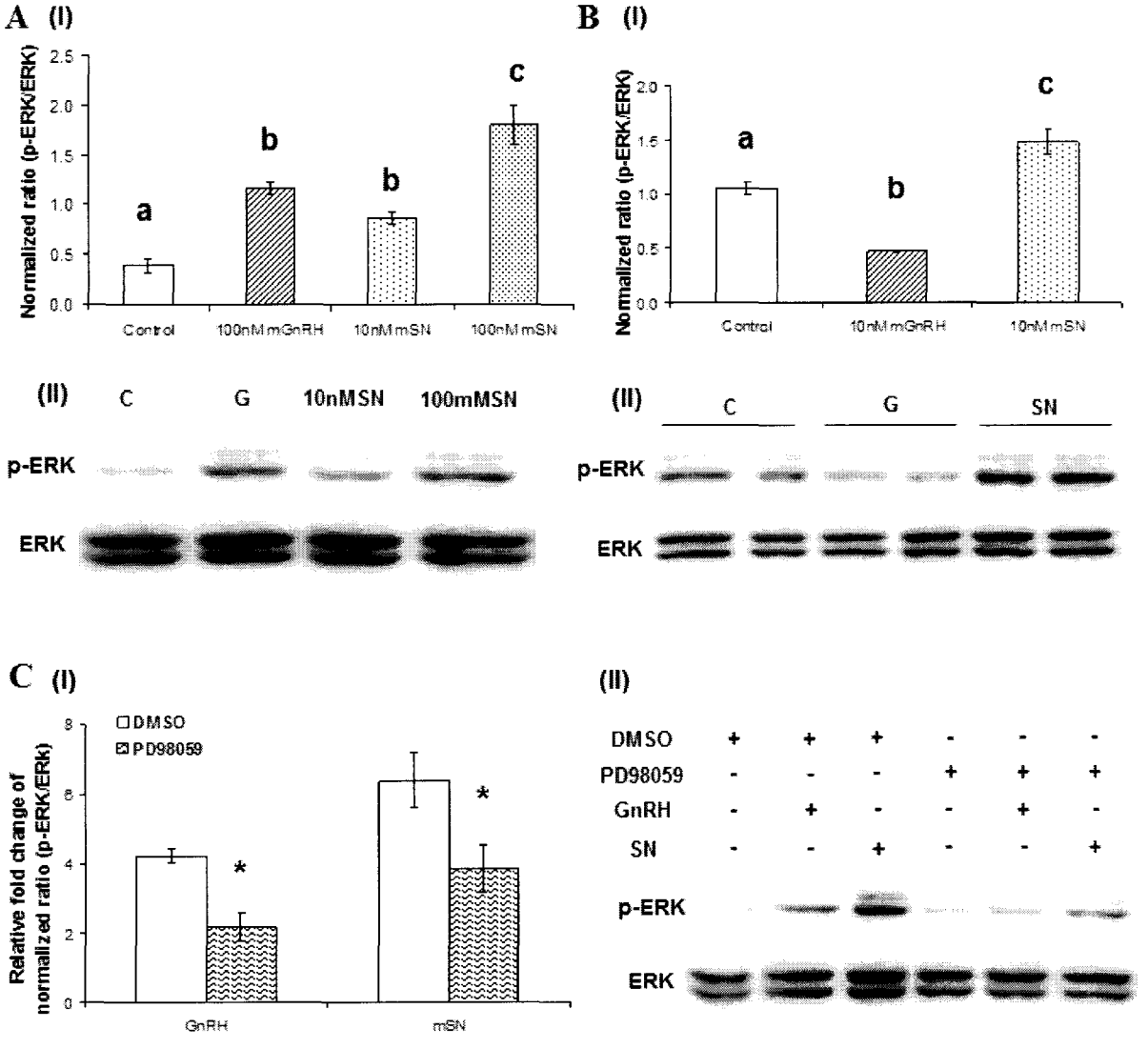

Figure 4.5. Action of mSN to stimulate ERK1/2 in LβT2 cells. **A**, Both mGnRH (100 nM) and mSN (10 and 100 nM) stimulated ERK phosphorylation. These cells were cultured in serum-free DMEM medium overnight and stimulated by mGnRH and mSN for 10 min. Their protein extract was firstly analyzed western blots with the antibody for phospho-ERK1/2 (panel II-top). After phospho-ERK1/2 blots were stripped, the membranes were immunoblotted again with the total ERK1/2 antibody (panel II-bottom). Abbreviations: C, control; G, 100 nM mGnRH; p-ERK, phospho-ERK1/2. In panel I, data were presented as mean ± S.E.M. of the normalized ratio between phospho-ERK1/2 and total ERK1/2, n=5. One-way ANOVA was chosen to assess the difference from control, followed by Fisher LSD test. Various letters indicated a statistically significant difference between treatment and control ($p<0.05$). **B**, Both mGnRH (10 nM) and mSN (10 nM) activated ERK after 3 hours. Cells were stimulated by mGnRH and mSN for 3 hours in the static incubation. Western blots were adopted to explore the expressions of phospho-ERK1/2 (panel II-top) and total ERK

(panel II-bottom). Abbreviations: C, control; G, 10 nM mGnRH; SN, 10nM mSN, p-ERK, phospho-ERK1/2. The normalized ratio of 3 hour-induced phospho-ERK/total ERK was shown as mean ± S.E.M. in panel I. Different letters represented statistical difference ($p<0.05$, Kruskal-Wallis One Way Analysis of Variance on Ranks followed by Student-Newman-Keuls Method, n=6). **C**, PD98059 (20 μM, a MEK inhibitor) attenuated ERK1/2 phosphorylation. LβT2 cells were cultured in serum-free medium overnight and pre-treated with 0.1% DMSO (dimethyl sulfoxide, the PD98059 vehicle) and 20 μM PD98059 for 1 hour, and then respectively stimulated by mGnRH (100 nM) and mSN (100 nM) for 10 minutes. Cellular exacts were separated by 10% SDS-PAGE gel and detected by phospho-ERK1/2 (p-ERK) and ERK1/2 antibodies. In panel I, relative change of normalized ratio (p-ERK/ERK) with respect to the group-matched control value was shown as means ± S.E.M. (n=6). *, $p<0.05$ vs. treatment without the MEK inhibtor PD98059. The statistical difference was analyzed by two-way ANOVA followed by Fisher LSD method. Panel II exhibited the changes of signal intensities for phospho-ERK1/2 (top) and total ERK1/2 (bottom).

CHAPTER 5: General Discussion and Conclusion

A hormone is a chemical synthesized by specific tissues or glands that can modify the activities of target tissues or organs via specific receptor signaling pathways (Randall et al., 1997). The general characteristics of a hormone include the following: the hormone has important effects on the body such as control of the reproductive cycle; it is biosynthesized, stored and secreted from a particular tissue; its biosynthesis and secretion is regulated physiologically, for example by other hormones; and the hormone interacts with one or several specific intracellular or cell membrane associated receptors leading to activation of signal transduction pathways and biological effects. In this thesis, I examined the hypothesis that SN is a newly-identified hormone that regulates vertebrate reproduction. In this chapter, I will place my discoveries within the context of what else is known about secreotoneurin (SN).

5.1. SN effect in the brain-pituitary-gonad axis

In previous studies, SN showed a stimulatory effect on several neurotransmitters in the brain. SN was demonstrated to elevate extracellular glutamate and γ-aminobutyric acid (GABA) in the the rat substantia nigra and neostriatum (You et al., 1996) and dopamine (DA) outflow from the striatum of the awake rat (Agneter et al., 1995). My PhD thesis mainly revealed SN function as a hormone regulator in the pituitary.

5.1.1. SN stimulates LH release

Our research team first demonstrated that SN rapidly stimulated *in vivo* LH release in goldfish pre-treated with the specific DA type 2 receptor antagonist domperidone (Blázquez et al., 1998a). It is also interesting that following treatments of GnRH and testosterone, SgII

gene expression is significantly correlated with that of LHβ subunit in the pars distalis of the goldfish pituitary, suggesting a close relationship between the generation of the SN precursor and LH secretion (Samia et al., 2004). In my MSc investigations, I observed that SN is able to increase LH release and LHβ subunit mRNA levels in goldfish pituitary fragments (Zhao et al., 2006a). Using another *in vitro* model of dispersed goldfish pituitary cells that avoids the influence of the hypothalamic hormones remaining in pituitary fragments, my PhD thesis confirms the direct stimulatory action of SN on LH secretion (Chapter 2) and gonadotropin subunit gene expression (Chapter 3). Immunoneutralization with goldfish SN antibody significantly reduced LH release suggesting a potential endogenous SN-induced LH response in goldfish pituitary cells (Chapter 2, Fig. 2.9D). The other immunoneutralization experiment also showed that SN is involved in the regulatory pathway of GnRH-evoked LH release from the pituitary (Chapter 3, Fig. 3.3). Critically missing are studies on SN actions on pituitary cells in other species, especially in mammals, although SN-immunoreacitivity (IR) was detected in mammalian pituitary and hypothalamus (Schmid et al., 1995; Marksteiner et al., 1993a). To further explore SN effect on mammalian pituitary, I adopted the mouse LβT2 pitutiary cell line that shares many characteristics with normal mouse gonadotrophs (Chapter 4). Consistent with its action in goldfish pituitary, SN is capable of up-regulating protein secretion and gene transcription of LH in mouse LβT2 cells (Chapter 4, Fig. 4.1 and Fig. 4.2). These data indicate an important reproductive neuroendocrine role for SN to regulate LH release.

5.1.2. SN and growth regulation

In my MSc study, I observed that a high dose of SN (500 nM) did not modify growth

hormone (GH) mRNA level after a 6-hour static incubation of goldfish pituitary fragment (Zhao et al., 2006a). Here, my PhD thesis also demonstrated that a physiological low dose of SN (10 nM) shows no effect on GH gene transcription and secretion when incubated with dispersed goldfish pituitary cells for 6 hours (Fig. 5.1). This suggests, at least in goldfish, that SN action is specific to gonadotrophs without actions on somatotrophs. While speculative, other anatomical and *in vitro* culture data are suggestive of developmental roles for SN. Immunohistochemical investigations revealed that high concentrations of SN-IR were detected in the rat amygdala, the hypothalamus and several brainstem areas during the early embryonic life (Leitner et al., 1997). Moreover, SN-IR levels determined by radioimmunoassay were higher in a rat embryonic brain than in adult adrenal gland (Leitner et al., 1997). In the human vagal/nucleus solitary complex, SN-IR and proprotein convertases (PC1 and PC2) responsible for SN generation, shows a high density during later fetal stages (Bitsche et al., 2006). Thus, the SN action on vertebrate growth is still not clear and further investigations are required.

5.2. SN-IR in neuroendocrine tissues

High levels of SN-IR were found in human pituitary (Schmid et al., 1995) and the highest density of SN-IR fibres and terminals was detected in rat hypothalamus, especially the median eminence (Marksteiner et al., 1993a). Moreover, in rat tumor GH4C1 lactotrophs, prolactin (PRL) and secretogranin II (SgII, SN precursor) were released together under stimulatory conditions *in vitro* (Hinkle et al., 1992). My PhD investigations about SN-IR in Chapter 2 focused on goldfish hypothalamus and pituitary lactotrophs. In goldfish hypothalamus, SN immunoreactive signals were observed in the magnocellular neurons of the

nucleus preopticus (NPO) and nerve fibres leaving from the NPO laterally (Chapter 2, Fig. 2.8A). Immunocytochemical studies in goldfish pituitary (Chapter 2, Fig. 2.5-2.7) indicate that SN-IR is discreetly localized in the PRL cells of the rostral pars distalis (RPD) and in the fibres of the neurointermediate lobe (NIL). These fibres innervating the pituitary are likely arising from multiple populations of hypothalamic neurosecretory neurons; however, it is clear that the magnocellular cells of the preoptic area are an important source of the SN-IR fibres terminating in the NIL (Chapter 2). In Chapter 3, I also found a close correlation of the protein expression between PRL and the major SN-immunoreactive SgII-derived peptides (~57 kDa) in the pituitary, confirming the SN-IR expresson in lactotrophs (PRL cells). Based upon these results, I therefore speculate that there is a classical neuroendocrine pathway (hypothalamic NPO-sourced SN-IR peptides innervating via NIL) and a separate paracrine pathway (lactotroph-generated SN-IR peptides) that can potentially mediate the regulation of pituitary hormones in goldfish.

5.3. Proteolytic processing of SgII precursor to yield SN in the brain and pituitary

Previous studies determined the pattern of SgII precursor processing to generate SN in goldfish brain and pituitary (Zhao et al., 2006b). Two SN-IR intermediate-size products (~57 kDa and ~30 kDa proteins) and one SN-containing SgII-derived peptide (~20 kDa) were respectively observed in goldfish pituitary and hypothalamus (Zhao et al., 2006b). My PhD studies further identify free SN peptide (3655 Da) in protein extracts from goldfish pituitary and brain confirming SN generation from neuroendocrine tissues in goldfish (Chapter 2). Moreover, I also found other intermediate-sized SgII products of low molecular mass (~19.6, ~15 and ~12 kDa) within goldfish pituitary tissues using the anti-SN antiserum (Chapter 2,

Fig. 2.10). These observations led to the proposed model for the generation of free goldfish SN peptide from its SgII precursor in goldfish pituitary (Chapter 2, Fig. 2.9A and 2.10). In addition, I demonstrated that GnRH enhances not only SgII mRNA level but also the generation of the major SgII-derived proteins (~57-kDa SN-IR intermediate-sized fragment), suggesting that SN generation might be controlled by GnRH, an essential hypothalamic hormone (Chapter 3). To further identify SgII processing in other teleosts, I also explored SN-IR in trout pituitary using western blot analysis with anti-goldfish SN antibody that was generated against a 15 amino acid conserved region (YTPQKLATLQSVFEE) within the goldfish SN sequence (Zhao et al., 2006b). In the previous studies for this species, Miot et al. (1998) characterized a SgII-like molecule in rainbow trout by using other antibodies against human and rat SgII, but these are hard to interpret given that the SgII precursor is not well conserved except in the SN segment (Chapter 1). Here, I observed that SgII processing to generate SN is also evident in a trout pituitary extract and the major SgII intermediate-size fragment (~57 kDa) is similar to that of goldfish pituitary (Appendix I, Fig. A2).

In comparison with teleosts, the proteolytic processing of SgII in various mammalian pituitary cells was also explored and discussed in this thesis. During my PhD study, I discovered the ~57-kDa SN-containing SgII-derived peptide in both human and rat pituitary tissues, consistent with that in goldfish. This result indicates that the first step of processing to the resultant mature protein is highly conserved during evolution (Appendix I, Fig. A1). Furthermore, Chapter 4 provided evidence about SgII processing generates SN in the mouse LβT2 gonadotropin cell line (a SV40 T-antigen oncogene-induced pituitary tumor cell) with the high expression of LH and SgII proteins (Nicol et al., 2002). The SgII precursor (~71 kDa) and several intermediate-size fragments (~46, ~42 and ~32 kDa) were identified in this cell

line by using anti-goldfish SN antiserum (Zhao et al., 2006b). Based on the molecular masses of these intermediate-sized products, I speculated a potential SN generation within the mouse pituitary adenoma cell (Chapter 4, Fig. 4.3). In addition, I demonstrated that GnRH shows a similar effect to stimulate SgII gene expression (Chapter 4, Fig. 4.2C) but decreases the protein level of SgII and its medium products for SN generation (Chapter 4, Fig. 4.4). Thus, I speculate that the velocity of SN generation from SgII is faster than that of SgII synthesis in mouse LβT2 cells.

Using PSI-Blast searching in the NCBI non-redundant, we revealed that SgII precursor has two paralogs in teleosts, including SgIIa and SgIIb. Goldfish SgII belongs to SgIIa whereas pufferfish and grass carp SgIIs belong to SgIIb (Chapter 1, Fig. 1.2). Two subtypes of SN, SNa and SNb, arose from SgIIa and SgIIb, were identified in some teleost species. Although the sequences of different teleost SN are conserved in comparison with those of tetropod SN, SNa shows a low identity to SNb in amino acid sequence (Chapter 1, Fig. 1.3), suggesting differing biological activities for these two teleost SN subtypes. This speculation remains to be tested.

5.4. Mechanisms to mediate SN regulatory effect on LH

Several encouraging investigations have demonstrated the regulatory effects of SN on immune cells (Kirchmair et al., 2004) or hormone secretion in the pituitary (Chapter 2 and 3). Moreover, SN significantly activates extracellular signal-regulated kinase (ERK) in mouse LβT2 gonadotrophs (Chapter 4, Fig. 4.5A). The pharmacological inhibitor of the upstream enzyme, MEK (MAPK kinase) decreases activation of ERK (Chapter 4, Fig. 4.5C). Nevertheless, the receptor remains enigmatic and the mechanism of SN action remains ill-

defined. Here, I briefly cover what is known about SN action and summarize the known players in signal transduction with the hope of providing the framework for future research on the mechanism of SN action to regulate the vertebrate gonadotroph. .

5.4.1. A SN receptor is detected in monocytes

Two independent research teams demonstrated specific SN binding sites on human blood monocytes and human monocytic cell line (human Mono Mac 6 cell) (Kong et al., 1998; Schneitler et al., 1998). They found high affinity binding with Kd values in the range of 7-14 nM. These two observations not only strongly supported the existence of SN receptors on human monocytes for mediating the function of SN chemotaxis, but also suggested the potential existence of SN receptors in other systems, such as neuroendocrine tissues.

5.4.2. Ca^{2+}, protein kinase C, MAPK, and SN receptor type

Schratzberger et al. (1996) reported that SN induced a pertussis toxin-sensitive increase in intracellular Ca^{2+} levels suggesting that the putative SN receptor was G-protein coupled in human monocytes. Moreover, an inhibitor of protein kinase C (PKC) was able to reduce SN-stimulated chemotaxis of monocytes (Schneitler et al., 1998). SN not only elevates the basal concentration of intracellular Ca^{2+}, but also activates PKC in human polymorphonuclear leukocytes (Kähler et al., 2002). Furthermore, SN can significantly enhance migration of human isolated peripheral blood natural killer cells. This SN-induced migration was blocked by the inhibitors of intracellular signaling enzymes, including phosphatidylinositol 3-kinase (PI3K) and PKC (Feistritzer et al., 2005). The experimental results concerning SN-induced elevation of intracellular Ca^{2+} and PKC activation supports the

hypothesis that the receptor for SN mediating its involvement in the inflammatory response is of GPCR (G protein-coupled receptor). However, Kong et al. (1998) reported that SN did not increase intracellular Ca^{2+} concentration in human monocytes which had high affinity to SN binding sites, suggesting some differences between cell types.

In contrast, there is also evidence for SN action via a non-GPCR growth factor-like signaling cascade. For example, SN stimulates migration, exerts anti-apoptotic effects and increases the number of mouse endothelial progenitor cells (Kirchmair et al., 2004). This major effect of SN is mediated through ERK and Akt signaling systems. These are respectively activated by the MAPK cascade and PI3-kinase, which argues for the existence of another type of SN receptor (Kirchmair et al., 2004; Shyu et al., 2008). It was also observed that SN exerted neuroprotective actions in models of stroke by activation of antiapoptic pathways involving Bcl-2 and Bcl-xl proteins, Jak2/Stat3 signal transduction and inhibition of caspase-3 activation (Shyu et al., 2008). In Chapter 4, it was shown that SN activates ERK, a major subfamily in the MAPK signaling pathway within mouse LβT2 cells. Taken together, SN has several signal transduction systems in different tissues suggesting that there may be several SN receptor types. Whether SN receptors on gonadotrophs are coupled with G-proteins is a critical question that requires further investigations.

5.4.3. Hypothesis about SN receptors on gonadotrophs to regulate LH

Although we have demonstrated that SN stimulates LH production and release in the pituitary, the study of its transduction mechanism is still limited. In mammalian monocytes and leukocytes, SN signaling involves intracellular Ca^{2+} (Ca^{2+}_i) (Schneitler et al., 1998; Kähler et al., 2002). Likewise, Ca^{2+} is an important signaling factor to mediate LH production

and secretion from goldfish gonadotrophs (Chang et al., 2000). Our recent investigation in collaboration with Dr. J.P. Chang at the University of Alberta demonstrated that SN induces a moderate increase of $Ca^{2+}{}_i$ concentration in identified goldfish gonadotrophs, suggesting that Ca^{2+} signaling system plays a role in SN-induced LH release (Zhao et al., 2009a). The maximal amplitude of the $Ca^{2+}{}_i$ increment induced by goldfish SN (10 nM) is about half that induced by a maximal dose of salmon GnRH (100 nM). Moreover, SN is able to further elevate $Ca^{2+}{}_i$ level in the presence of sGnRH, suggesting the differential use of Ca^{2+} stores and/or Ca^{2+} entry mechanisms for these two neuropeptides. In other words, the transduction pathway of SN stimulation is partially independent of the GnRH-induced G-protein coupled signaling system (Zhao et al., 2009a). In addition, PKC and ERK were demonstrated as the signal transduction molecules for SN-induced inflammatory response (Kirchmair et al., 2004; Feistritzer et al., 2005). These two molecules are the important post-receptor factors in both GnRH and epidermal growth factor (EGF) signaling in vertebrate gonadotroph cells (Chang et al., 2000; Ruf et al., 2003). There is also evidence that the ERK pathway is activated in fish gonadotrophs and is involved in the regulation of LHβ subunit transcription (Yaron et al., 2003). I furthermore observed that SN is able to up-regulate the activated ERK level in mouse LβT2 pituitary cell line (Chapter 4).

Based on these various data I present a hypothetical model for SN action in the vertebrate gonadotroph. The purpose of this cautious speculation is to provide the basic framework required for future studies on SN actions on the gonadotroph (Fig. 5.2). Although I have revealed that intracellular Ca^{2+} and ERK are the signaling molecules modulated by SN, the nature of the SN receptor remains speculative and could be either a GPCR or a growth factor receptor (GFR). Given the possibility of GnRH signaling pathway cross-talk, as has

been observed in vertebrate gonadotrophs (Chang et al., 2000; Ruf et al., 2003), it is possible that a putative GPCR for SN could invoke ERK-related signaling in addition to traditional Ca^{2+} and PKC-related processes. On the other hand, SN might bind to GFR-like receptor to activate MAPK signaling transductions for regulating LH gene transcription in gonadotrophs. Identification of the SN receptor and pharmacological manipulations of the likely signaling cascades within gonadotrophs must be undertaken. Only then we will be able to firmly establish SN as new-identified hormone in the neuroendocrine system.

5.5. Summarizing the major contributions of my PhD thesis on the SN study

SN is an important regulator to control hormone secretion in the pituitary. As the most conserved sequence derived from SgII, free SN peptide was isolated and identified in goldfish brain and pituitary (Chapter 2). Furthermore, SN-IR was widely distributed not only in PRL cells of the anterior pituitaries and nerve fibres of the posterior pituitary, but also in the NPO magnocellular neurons and nerve fibres in goldfish brain (Chapter 2). In addition, I also demonstrated that SgII production and processing to generate SN are correlation with the yielding of PRL under the control of GnRH (Chapter 3). Thus, these results in this thesis strongly support that brain- and PRL cell-sourced SN is able to stimulate LH release from goldfish pituitary in neuroendocrine and paracrine manners (shown as solid lines, Fig. 5.3). Using mouse LβT2 pituitary cell line, I confirmed the conserved SN-induced LH response and observed the potential GnRH-evoked SN production in mammalian gonadotrophs, indicating an autocrine manner of the SN effect in mammalian pituitary (Chapter 4). Furthermore, I clarified a part of the signaling pathway, which includes the MAPK cascade (Chapter 4). In brief, SN is generated and secreted from NPO in brain and lactotrophs or

gonadotrophs in pituitary; the pituitary-sourced SN production might be regulated by hypothalamic GnRH; this neuropeptide stimulates LH secretion thru the MAPK signal transduction pathway.

5.6. Next steps for the SN studies

Based on this thesis and our previous findings, I establish a neuroendocrine model to summarize the reported (solid line) and speculated/uncertain (dashed line) effects of the SN peptide in the pituitary (Fig. 5.3). In this model, the stimulatory activity of SN on LH is clearly identified in teleosts and mammals. Consequently, the long-term research of SN will focus on isolation and localization of SN receptors, identifying the SN receptor structure, and clarifying the signal transduction mechanisms mediating SN-induced LH secretion in gonadotrophs. Although SN was determined to elevate the FSHβ subunit gene expression in a mouse gonadotropin cell line, the role of SN on FSH protein synthesis and secretion has yet to be elucidated. In addition, the question whether SN shows any hormone-like activities in the regulation of PRL in lactotrophs is still not clear and needs to be elucidated in the future. Herein, this model (Fig. 5.3) might provide a direction for our further investigation to demonstrate SN as hormone.

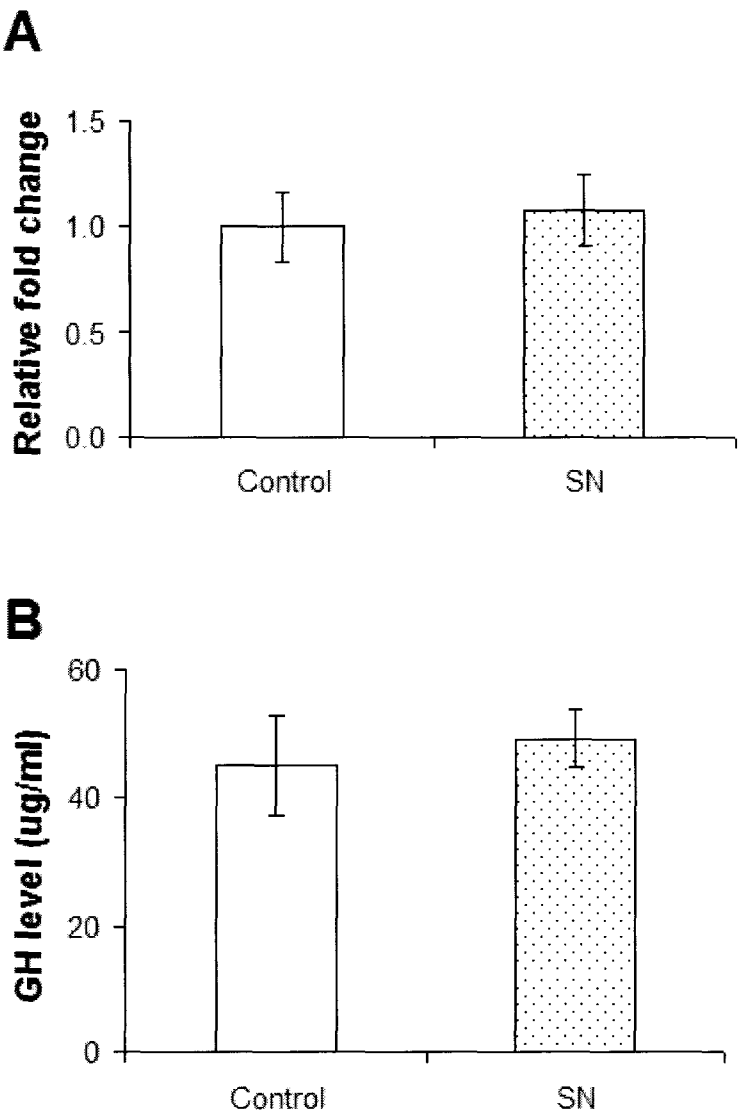

Figure 5.1. SN (10 nM) -induced GH gene expression (**A**) and GH release (**B**) from dispersed goldfish pituitary cells after 6-hour static incubation. 18S ribosomal RNA was adopted as an internal standard for real time RT-PCR. These experiments were performed in March at the beginning of the sexually mature pre-spawning period of goldfish. The data were presented as the means ± S.E.M. (n=8). The t-test indicated that the SN treatment group had similar values ($p>0.05$) to the control. The measurement of GH level was in collaboration with Dr. J.P. Chang at the University of Alberta.

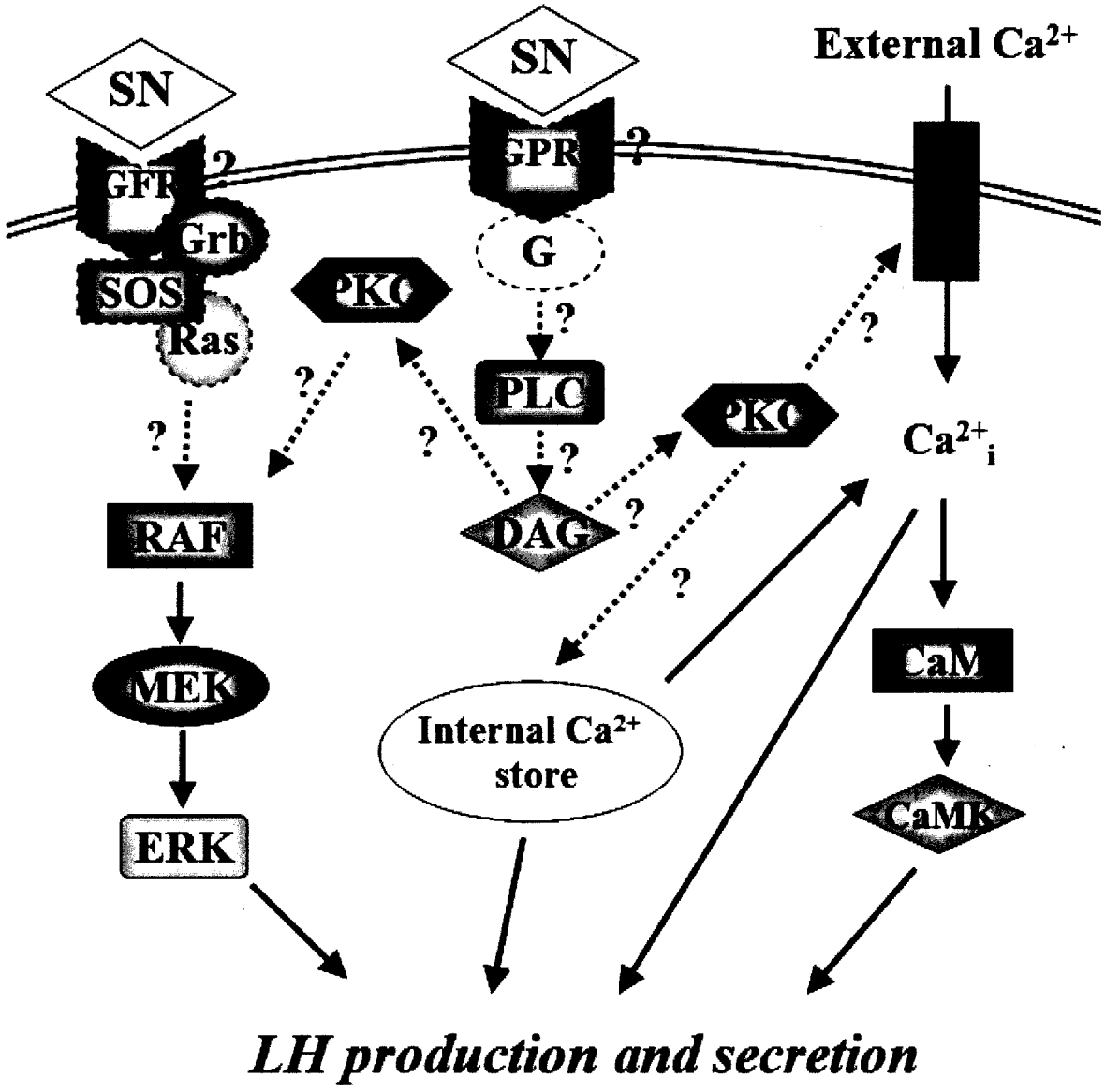

Figure 5.2. A hypothetical model of SN receptor inducing signaling pathways in the gonadotroph cell. Activation is indicated as arrows. Dashed lines with question marks represent speculations. The facts shown as real lines are mainly adapted from Chapter 4, Zhao et al., 2009a, Chang et al., 2000 and Ruf et al., 2003. Abbreviations: G, G protein; PLC, phospholipase C; DAG, diacylglycerol; VSCC, voltage-sensitive Ca^{2+} channels; CaM, calmodulin; CaMK, CaM kinase; MEK, mitogen/extracellular signal protein kinase; Grb, growth factor-bound protein; RAF, v-raf-1 murine leukemia viral oncogene; Ras, related RAS viral oncogene homolog; SOS, son of sevenless.

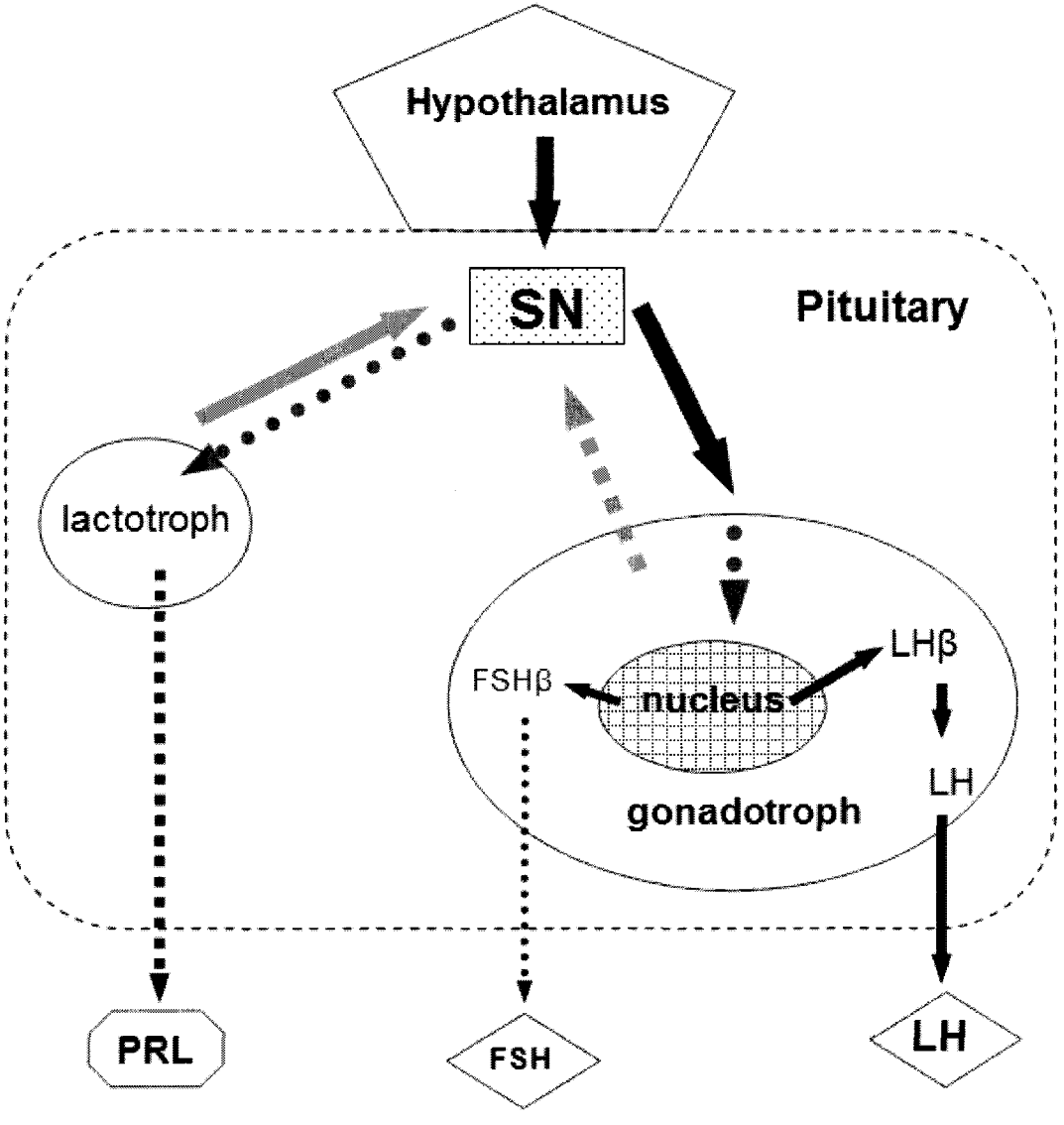

Figure 5.3. Neuroendocrine model of SN effects on the regulation of pituitary hormones and its generation in pituitary and brain. Testified results (solid line) and speculations (dashed line) are indicated. This model simplifies further SN studies in neuroendocrine system. The major data of this model are adopted from Chapter 2-4, Zhao et al., 2006a and Blázquez et al., 1998a.

References

Aardal, S., Helle, KB., Elsayed, S., Reed, R.K., Serck-Hanssen, G., 1993. Vasostatins, comprising the N-terminal domain of chromogranin A, suppress tension in isolated human blood vessel segments. J. Neuroendocrinol. 5, 405-412.

Abbot, S.D., Naik, S.I., Clayton, R.N., 1986. Dissociation between pituitary GnRH binding sites and LH response to GnRH in vitro. Mol. Cell. Endocrinol. 48, 191-197.

Agneter, E., Sitte, H.H., Stockl-Hiesleitner, S., Fischer-Colbrie, R., Winkler, H., Singer, E.A., 1995. Sustained dopamine release induced by secretoneurin in the striatum of the rat: a microdialysis study. J. Neurochem. 65, 622-625.

Ahrén, B., Lindskog, S., Tatemoto, K., Efendić, S., 1988. Pancreastatin inhibits insulin secretion and stimulates glucagon secretion in mice. Diabetes 37, 281-285.

Ait-Ali, D., Turquier, V., Alexandre, D., Grumolato, L., Jegou, S., Vaudry, H., Anouar, Y., 2002. Molecular characterization of frog chromogranin B reveals conservation of selective sequences encoding potential novel regulatory peptides. FEBS Lett. 511, 127-132.

Alarid, E.T., Windle, J.J., Whyte, D.B., Mellon, P.L., 1996. Immortalization of pituitary cells at discrete stages of development by directed oncogenesis in transgenic mice. Development. 122, 3319-3329.

Alderman, J.M., Flurkey, K., Brooks, N.L., Naik, S.B., Gutierrez, J.M., Srinivas, U., Ziara, K.B., Jing, L., Boysen, G., Bronson, R., Klebanov, S., Chen, X., Swenberg, J.A., Stridsberg, M., Parker, C.E., Harrison, D.E., Combs, T.P., 2009. Neuroendocrine inhibition of glucose production and resistance to cancer in dwarf mice. Exp. Gerontol. 44, 26-33.

Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., Lipman, D.J., 1997. Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. Nucleic. Acids. Res. 25, 3389-3402.

Amores, A., Force, A., Yan, Y.L., Joly, L., Amemiya, C., Fritz, A., Ho, R.K., Langeland, J., Prince, V., Wang, Y.L., Westerfield, M., Ekker., M., Postlethwait., J. H., 1998. Zebrafish hox clusters and vertebrate genome evolution. Science 282, 1711-1714.

Ang, C.W., Dotman, C.H., Winkler, H., Fischer-Colbrie, R., Sonnemans, M.A., Van Leeuwen, F.W., 1997. Specific expression of secretogranin II in magnocellular vasopressin neurons of the rat supraoptic and paraventricular nucleus in response to osmotic stimulation. Brain Res. 765, 13-20.

Angeletti, R.H., Mints, L., Aber, C., Russell, J., 1996. Determination of residues in

chromogranin A-(16-40) required for inhibition of parathyroid hormone secretion. Endocrinology 137, 2918-2922.

Anglade, I., Zandbergen, T., Kah, O., 1993. Origin of pituitary innervation in the goldfish. Cell Tissue Res. 273, 345-355.

Anouar, Y., Desmoucelles, C., Yon, L., Leprince, J., Breault, L., Gallo-Payet, N., Vaudry, H., 1998. Identification of a novel secretogranin II-derived peptide (SgII(187-252)) in adult and fetal human adrenal glands using antibodies raised against the human recombinant peptide. J. Clin. Endocrinol. Metab. 83, 2944-2951.

Anouar, Y., Duval, J., 1992. Direct estradiol down-regulation of secretogranin II and chromogranin A mRNA levels in rat pituitary cells. Mol. Cell. Endocrinol. 88, 97-104.

Anouar, Y., Jegou, S., Alexandre, D., Lihrmann, I., Conlon, J.M., Vaudry, H., 1996. Molecular cloning of frog SgII reveals the occurrence of several highly conserved potential regulatory peptides. FEBS Lett. 394, 295-299.

Anouar, Y., Yon, L., Desmoucelles, C., Leprince, J., Breault, L., Gallo-Payet, N., Vaudry, H., 1998. Identification of a novel secretogranin II-derived peptide in the adult and fetal human adrenal gland. Endocrinol. Res. 24, 731-736.

Anouar, Y., Yon, L., Guillemot, J., Thouennon, E., Barbier, L., Gimenez-Roqueplo, A.P., Bertherat, J., Lefebvre, H., Klein, M., Muresan, M., Grouzmann, E., Plouin, P.F., Vaudry, H., Elkahloun, A.G., 2006. Development of novel tools for the diagnosis and prognosis of pheochromocytoma using peptide marker immunoassay and gene expression profiling approaches. Ann. N. Y. Acad. Sci. 1073, 533-540.

Bailey, T.L., Elkan, C., 1994. Fitting a mixture model by expectation maximization to discover motifs in biopolymers. Proceedings of the Second International Conference on Intelligent Systems for Molecular Biology, pp. 28-36.

Ball, J.N., 1981. Hypothalamic control of the pars distalis in fishes, amphibians, and reptiles. Gen. Comp. Endocrinol. 44, 135-170.

Barkatullah, S.C., Pogue, K.M., Depreitere, J., Boutajangout, A., Liang, F., DePotter, W., Curry, W.J., 2001. Immunohistochemical localization of WE-14 in the developing porcine sympathoadrenal cell lineage. Histochem. Cell. Biol. 116, 255-262.

Barrenechea, M.A., López, J., Martínez, A., 1994. Regulatory peptides in gastric endocrine cells of the rainbow trout Oncorhynchus mykiss: general distribution and colocalizations. Tissue Cell 26, 309-321.

Basak, A., Boudreault, A., Chen, A., Chretien, M., Seidah, N.G., Lazure, C., 1995. Application of the multiple antigenic peptides (MAP) strategy to the production of prohormone convertases antibodies: synthesis, characterization and use of 8-branched immunogenic

peptides. J. Pept. Sci. 1, 385-395.

Basak, A., Lotfipour, F. 2005. Modulating furin activity with designed mini-PDX peptides: synthesis and *in vitro* kinetic evaluation. FEBS Lett. 579, 4813-4821.

Basak, A., Mitra, A., Basak, S., Pasko, C., Chrétien, M., Seaton, P., 2007. A fluorogenic peptide containing the processing site of human SARS corona virus S-protein: kinetic evaluation and NMR structure elucidation. Chembiochem. 8, 1029-1037.

Bassetti, M., Huttner, W.B., Zanini, A., Rosa, P., 1990. Co-localization of secretogranins/chromogranins with thyrotropin and luteinizing hormone in secretory granules of cow anterior pituitary. J. Histochem. Cytochem. 38, 1353-1363.

Batten, T.F., 1986. Ultrastructural characterization of neurosecretory fibres immunoreactive for vasotocin, isotocin, somatostatin, LHRH and CRF in the pituitary of a teleost fish, Poecilia latipinna. Cell Tissue Res. 244, 661-672.

Bauer, J.W., Kirchmair, R., Egger, C., Fischer-Colbrie, R., 1993. Histamine induces a gene-specific synthesis regulation of secretogranin II but not of chromogranin A and B in chromaffin cells in a calcium-dependent manner. J. Biol. Chem. 268, 1586-1589.

Bauer, S.H., Zhang, X.Y., Liang, F., De Potter, W.P., Claeys, M., Przybylski, M., 1997. Isolation and identification of intact chromogranin A and two N-terminal processing products, vasostatin I and II, from bovine adrenal medulla chromaffin granules by chromatographic and mass spectrometric methods. Neuropeptides 31, 273-280.

Benjannet, S., Leduc, R., Adrouche, N., Falgueyret, J.P., Marcinkiewicz, M., Seidah, N.G., Mbikay, M., Lazure, C., Chretien, M., 1987. Chromogranin B (secretogranin I), a putative precursor of two novel pituitary peptides through processing at paired basic residues. FEBS Lett. 224, 142-148.

Belloni, D., Scabini, S., Foglieni, C., Veschini, L., Giazzon, A., Colombo, B., Fulgenzi, A., Helle, K.B., Ferrero, M.E., Corti, A., Ferrero, E., 2007. The vasostatin-I fragment of chromogranin A inhibits VEGF-induced endothelial cell proliferation and migration. FASEB J. 21, 3052-3062.

Benedum, U.M., Lamouroux, A., Konecki, D.S., Rosa, P., Hille, A., Baeuerle, P.A., Frank, R., Lottspeich, F., Mallet, J., Huttner, W.B., 1987. The primary structure of human secretogranin I (chromogranin B): comparison with chromogranin A reveals homologous terminal domains and a large intervening variable region. EMBO J. 6, 1203-1211.

Bitsche, M., Schrott-Fischer, A., Hinterhoelzl, J., Fischer-Colbrie, R., Sergi, C., Glueckert, R., Humpel, C., Marksteiner, J., 2006. First localization and biochemical identification of chromogranin B- and secretoneurin-like immunoreactivity in the fetal human vagal/nucleus solitary complex. Regul. Pept. 134, 97-104.

Blaschko, H., Comline, R.S., Schneider, F.H., Silver, M., Smith, A.D., 1967. Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. Nature 215, 58-59.

Blázquez, M., Bosma, P.T., Chang, J.P., Docherty, K., Trudeau, V.L., 1998a. Gamma-aminobutyric acid up-regulates the expression of a novel secretogranin-II messenger ribonucleic acid in the goldfish pituitary. Endocrinology 139, 4870-4880.

Blázquez, M., Bosma, P.T., Fraser, E.J., Van Look, K.J.W., Trudeau, V.L., 1998b. Fish as models for the neuroendocrine regulation of reproduction and growth. Comp. Biochem. Physiol. C Pharmacol. Toxicol. Endocrinol. 119, 345-364.

Blázquez, M., Shennan, K.I., 2000. Basic mechanisms of secretion: sorting into the regulated secretory pathway. Biochem. Cell. Biol. 78, 181-191.

Boutahricht, M., Guillemot, J., Montero-Hadjadje, M., Barakat, Y., El Ouezzani, S., Alaoui, A., Yon, L., Vaudry, H., Anouar, Y., Magoul, R., 2007. Immunohistochemical distribution of the secretogranin II-derived peptide EM66 in the rat hypothalamus: a comparative study with jerboa. Neurosci. Lett. 414, 268-272.

Boutahricht, M., Guillemot, J., Montero-Hadjadje, M., Bellafqih, S., El Ouezzani, S., Alaoui, A., Yon, L., Vaudry, H., Anouar, Y., Magoul, R., 2005. Biochemical characterisation and immunohistochemical localisation of the secretogranin II-derived peptide EM66 in the hypothalamus of the jerboa (Jaculus orientalis): modulation by food deprivation. J. Neuroendocrinol. 17, 372-378.

Canagarajah, B.J., Khokhlatchev, A., Cobb, M.H., Goldsmith, E.J., 1997. Activation mechanism of the MAP kinase ERK2 by dual phosphorylation. Cell 90, 859-869.

Chanat, E., Cozzi, M.G., Sion, B., de Monti, M., Zanini, A., Duval, J., 1988. The gonadotrope polypeptide (GP 87) released from pituitary cells under luteinizing hormone-releasing hormone stimulation is a secretogranin II form. Biochimie. 70, 1361-1368.

Chang, J.P., Cook, H., Freedman, G.L., Wiggs, A.J., Somoza, G.M., de Leeuw, R., Peter, R.E., 1990. Use of a pituitary cell dispersion method and primary culture system for the studies of gonadotropin-releasing hormone action in the goldfish, *Carassius auratus*. I. Initial morphological, static, and cell column perifusion studies. Gen. Comp. Endocrinol. 77, 256-273.

Chang, J.P., Jobin, R.M., Wong, A.O.L., 1993. Intracellular mechanisms mediating gonadotropin and growth hormone release in the goldfish, Carassius auratus. Fish Physiol. Biochem. 11, 25-33.

Chang, J.P., Johnson, J.D., Sawisky, G.R., Grey, C.L., Mitchell, G., Booth, M., Volk, M.M., Parks, S.K., Thompson, E., Goss, G.G., Klausen, C., Habibi, H.R., 2009. Signal transduction in multifactorial neuroendocrine control of gonadotropin secretion and

synthesis in teleosts – studies on the goldfish model. Gen. Comp. Endocrinol. 161, 42-52.

Chang, J.P., Johnson, J.D., Van Goor, F., Wong, C.J.H., Yunker, W.K., Uretsky, A.D., Taylor, D., Jobin, R.M., Wong, A.O.L., Goldberg, J.I., 2000. Signal transduction mechanisms mediating secretion in goldfish gonadotropes and somatotropes. Biochem. Cell. Biol. 78, 139-153.

Chrétien, M., Seidah, N.G., Basak, A., Mbikay, M., 2008. Proprotein convertases as therapeutic targets. Expert. Opin. Ther. Targets. 12, 1289-1300.

Conn, P.M., Janovick, J.A., Braden, T.D., Maurer, R.A., Jennes, L., 1992. SIIp: a unique secretogranin/chromogranin of the pituitary released in response to gonadotropin-releasing hormone. Endocrinology 130, 3033-3040.

Cool, D.R., Hardiman, A., 2004. C-terminal sequencing of peptide hormones using carboxypeptidase Y and SELDI-TOF mass spectrometry. Biotechniques 36, 32-34.

Crawford, J.L., McNeilly, A.S., 2002. Co-localisation of gonadotrophins and granins in gonadotrophs at different stages of the oestrous cycle in sheep. J. Endocrinol. 174, 179-194.

Crawford, J.L., McNeilly, J.R., Nicol, L., McNeilly, A.S., 2002. Promotion of intragranular co-aggregation with LH by enhancement of secretogranin II storage resulted in increased intracellular granule storage in gonadotrophs of GnRH-deprived male mice. Reproduction 124, 267-277.

Cunningham, R.T., Pogue, K.M., Curry, W.J., Johnston, C.F., Buchanan, K.D., 1996. PC12 cells show immunoreactivity to a number of proteins and peptides, including vasostatin. Peptides 17, 1297-1301.

Curry, W.J., Barkatullah, S.C., Johansson, A.N., Quinn, J.G., Norlen, P., Connolly, C.K., McCollum, A.P., McVicar, C.M., 2002. WE-14, a chromogranin a-derived neuropeptide. Ann. N. Y. Acad. Sci. 971, 311-316.

Curry, W.J., Shaw, C., Johnston, C.F., Thim, L., Buchanan, K.D., 1992. Isolation and primary structure of a novel chromogranin A-derived peptide, WE-14, from a human midgut carcinoid tumour. FEBS Lett. 301, 319-321.

De Paul, A.L., Bonaterra, M., Aoki, A., Torres, A.I., 2000. Cellular and functional interactions between gonadotrophs and lactotrophs in pituitary cell cultures. Med. Electron. Microsc. 33, 231-240.

Deftos, L.J., Bjornsson, B.T., Burton, D.W., O'Connor, D.T., Copp, D.H., 1987. Chromogranin A is present in and released by fish endocrine tissue. Life Sci. 40, 2133-2136.

Denef, C., 2008. Paracrinicity: the story of 30 years of cellular pituitary crosstalk. J. Neuroendocrinol. 20, 1-70.

Díaz-Troya, S., Najib, S., Sánchez-Margalet, V., 2005. eNOS, nNOS, cGMP and protein kinase G mediate the inhibitory effect of pancreastatin, a chromogranin A-derived peptide, on growth and proliferation of hepatoma cells. Regul. Pept. 125, 41-46.

Dopazo, A., Lovenberg, T.W., Danielson, P.E., Ottiger, H.P., Sutcliffe, J.G., 1993. Primary structure of mouse secretogranin III and its absence from deficient mice. J. Mol. Neurosci. 4, 225-233.

Dorn, C., Ou, Q., Svaren, J., Crawford, P.A., Sadovsky, Y., 1999. Activation of luteinizing hormone beta gene by gonadotropin-releasing hormone requires the synergy of early growth response-1 and steroidogenic factor-1. J. Biol. Chem. 274, 13870-13876.

Drees, B.M., Hamilton J.W., 1992. Pancreastatin and bovine parathyroid cell secretion. Bone Miner 17, 335-346.

Drees, B.M., Hamilton, J.W., 1994. Processing of chromogranin A by bovine parathyroid secretory granules: production and secretion of N-terminal fragments. Endocrinology 134, 2057-2063.

Drees, B.M., Rouse, J., Johnson, J., Hamilton, J.W., 1991. Bovine parathyroid glands secrete a 26-kDa N-terminal fragment of chromogranin-A which inhibits parathyroid cell secretion. Endocrinology 129, 3381-3387.

Eder, U., Hukkanen, M., Leitner, B., Mur, E., Went, P., Kirchmair, R., Fischer-Colbrie, R., Polak, J.M., Winkler, H., 1997. The presence of secretoneurin in human synovium and synovial fluid. Neurosci. Lett. 224, 139-141.

Eder, S., Leierer, J., Klimaschewski, L., Wilhelm, A., Volknandt, W., Laslop, A., Fischer-Colbrie, R., 2004. Secretion and molecular forms of NESP55, a novel genomically imprinted neuroendocrine-specific protein from AtT-20 cells. Neurosignals 13, 298-307.

Efendić, S., Tatemoto, K., Mutt, V., Quan, C., Chang, D., Ostenson, C.G., 1987. Pancreastatin and islet hormone release. Proc Natl. Acad. Sci. U. S. A. 84, 7257-7260.

Fasciotto, B.H., Gorr, S.U., DeFranco, D.J., Levine, M.A., Cohn, D.V., 1989. Pancreastatin, a presumed product of chromogranin-A (secretory protein-I) processing, inhibits secretion from porcine parathyroid cells in culture. Endocrinology 125, 1617-1622.

Feistritzer, C., Mosheimer, B.A., Colleselli, D., Wiedermann, C.J., Kähler, C.M., 2005. Effects of the neuropeptide secretoneurin on natural killer cell migration and cytokine release. Regul. Pept. 126, 195-201.

Feldman, S.A., Eiden, L.E., 2003. The chromogranins: their roles in secretion from

neuroendocrine cells and as markers for neuroendocrine neoplasia. Endocr. Pathol. 14, 3-23.

Fernandez Ocaña, M., Jarvis, J., Parker, R., Bramley, P.M., Halket, J.M., Patel, R.K., Neubert, H., 2005. C-terminal sequencing by mass spectrometry: application to gelatine-derived proline-rich peptides. Proteomics 5, 1209-1216.

Fischer-Colbrie, R., Laslop, A., Kirchmair, R., 1995. Secretogranin II: Molecular properties, regulation of biosynthesis and processing to the neuropeptide Secretoneurin. Prog. Neurobiol. 46, 49-70.

Fischer-Colbrie, R., Kirchmair, R., Kahler, C.M., Wiedermann, C.J., Saria, A., 2005. Secretoneurin: a new player in angiogenesis and chemotaxis linking nerves, blood vessels and the immune system. Curr. Protein Pept. Sci. 6, 373-385.

Flanagan, T., Taylor, L., Poulter, L., Viveros, O.H., Diliberto, E.J. Jr., 1990. A novel 1745-dalton pyroglutamyl peptide derived from chromogranin B is in the bovine adrenomedullary chromaffin vesicle. Cell. Mol. Neurobiol. 10, 507-523.

Forsythe, P., Curry, W.J., Johnston, C.F., Harriott, P., MacMahon, J., Ennis, M., 1997. The modulatory effects of WE-14 on histamine release from rat peritoneal mast cells. Inflamm. Res. 46, 13-14.

Fricker, L.D., McKinzie, A.A., Sun J., Curran, E., Qian, Y., Yan, L., Patterson, S.D., Courchesne, P.L., Richards, B., Levin, N., Mzhavia, N., Devi, L.A., Douglass, J., 2000. Identification and characterization of proSAAS, a granin-like neuroendocrine peptide precursor that inhibits prohormone processing. J. Neurosci. 20, 639-648.

Funakoshi, A., Miyasaka, K., Kitani, K., Tatemoto, K., 1989. Effect of pancreastatin on pancreatic endocrine function in the conscious rat. Regul. Pept. 24, 225-231.

Funakoshi, A., Miyasaka, K., Nakamura, R., Kitani, K., Funakoshi, S., Tamamura, H., Fujii, N., Yajima, H., 1988. Bioactivity of synthetic human pancreastatin on exocrine pancreas. Biochem. Biophys. Res. Commun. 156, 1237-1242.

Ge, W., Chang, J.P., Peter, R.E., Vaughan, J., Rivier, J., Vale, W., 1992. Effects of porcine follicular fluid, inhibin-A, and activin-A on goldfish gonadotropin release in vitro. Endocrinology 131, 1922-1929.

Ge, W., Peter, R.E., 1994. Activin-like peptides in somatotrophs and activin stimulation of growth hormone release in goldfish. Gen. Comp. Endocrinol. 95, 213-221.

Gerdes, H.H., Rosa, P., Phillips, E., Baeuerle, P.A., Frank, R., Argos, P., Huttner, W.B., 1989. The primary structure of human secretogranin II, a widespread tyrosine-sulfated secretory granule protein that exhibits low pH- and calcium-induced aggregation. J. Biol. Chem. 264, 12009-12015.

Gleeson, C.M., Curry, W.J., Johnston, C.F., Buchanan, K.D., 1996. Occurrence of WE-14 and chromogranin A-derived peptides in tissues of the human and bovine gastro-entero-pancreatic system and in human neuroendocrine neoplasia. J. Endocrinol. 151, 409-420.

González-Yanes, C., Sánchez-Margalet, V., 2001. Pancreastatin, a chromogranin-A-derived peptide, inhibits insulin-stimulated glycogen synthesis by activating GSK-3 in rat adipocytes. Biochem. Biophys. Res. Commun. 289, 282-287.

González-Yanes, C., Sánchez-Margalet, V., 2003. Pancreastatin, a chromogranin A-derived peptide, inhibits leptin and enhances UCP-2 expression in isolated rat adipocytes. Cell. Mol. Life Sci. 60, 2749-2756.

Gorbman, A., 1995. Olfactory origins and evolution of the brain-pituitary endocrine system: facts and speculation. Gen. Comp. Endocrinol. 97, 171-178.

Graham, K.E., Nusser, K.D., Low, M.J., 1999. LbetaT2 gonadotroph cells secrete follicle stimulating hormone (FSH) in response to activin A. J. Endocrinol. 162, R1-5.

Grandy, D.K., Leduc, R., Makam, H., Flanagan, T., Diliberto E.J. Jr., Civelli, O., Viveros, O.H., 1992. Nucleotide and deduced amino acid sequence of bovine adrenal medulla chromogranin B (secretogranin I). Cell. Mol. Neurobiol. 12, 185-192.

Guillemot, J., Aït-Ali, D., Turquier, V., Montero-Hadjadje, M., Fournier, A., Vaudry, H., Anouar, Y., Yon, L., 2006a. Involvement of multiple signaling pathways in PACAP-induced EM66 secretion from chromaffin cells. Regul. Pept. 137, 79-88.

Guillemot, J., Anouar, Y., Montero-Hadjadje, M., Grouzmann, E., Grumolato, L., Roshmaninho-Salgado, J., Turquier, V., Duparc, C., Lefebvre, H., Plouin, P.F., Klein, M., Muresan, M., Chow, B.K., Vaudry, H., Yon, L., 2006b. Circulating EM66 is a highly sensitive marker for the diagnosis and follow-up of pheochromocytoma. Int. J. Cancer 118, 2003-2012.

Helle, K.B., 2004. The granin family of uniquely acidic proteins of the diffuse neuroendocrine system: comparative and functional aspects. Biol. Rev. Camb. Philos. Soc. 79, 769-794.

Helle, K.B., 2009. Regulatory peptides from chromogranin A and secretogranin II: Putative modulators of cells and tissues involved in inflammatory conditions. Regul. Pept. doi:10.1016/j.regpep.2009.09.009.

Henderson, H.L., Hodson, D.J., Gregory, S.J., Townsend, J., Tortonese, D.J., 2008. Gonadotropin-releasing hormone stimulates prolactin release from lactotrophs in photoperiodic species through a gonadotropin-independent mechanism. Biol. Reprod. 78, 370-377.

Hendy, G.N., Bevan, S., Mattei, M.G., Mouland, A.J., 1995. Chromogranin A. Clin. Invest. Med. 18, 47-65.

Herbison, A.E., 2008. Estrogen positive feedback to gonadotropin-releasing hormone (GnRH) neurons in the rodent: The case for the rostral periventricular area of the third ventricle (RP3V). Brain Res. Rev. 57, 277-287.

Herrero, C.J., Alés, E., Pintado, A.J., López, M.G., García-Palomero, E., Mahata, S.K., 2002. O'Connor DT, García AG, Montiel C. Modulatory mechanism of the endogenous peptide catestatin on neuronal nicotinic acetylcholine receptors and exocytosis. Neurosci. 22, 377-388.

Hertelendy, Z.I., Patel, D.G., Knittel, J.J., 1996. Pancreastatin inhibits insulin secretion in RINm5F cells through obstruction of G-protein mediated, calcium-directed exocytosis. Cell Calcium 19, 125-132.

Hinkle, P.M., Scammell, J.G., Shanshala, E.D. II., 1992. Prolactin and secretogranin-II, a marker for the regulated pathway, are secreted in parallel by pituitary GH4C1 cells. Endocrinology 130, 3503-3511.

Hoflehner, J., Eder, U., Laslop, A., Seidah, N.G., Fischer-Colbrie, R., Winkler, H., 1995. Processing of secretogranin II by prohormone convertases: importance of PC1 in generation of secretoneurin. FEBS Lett. 360, 294-298.

Holthuis, J.C., Martens, G.J., 1996. The neuroendocrine proteins secretogranin II and III are regionally conserved and coordinately expressed with proopiomelanocortin in Xenopus intermediate pituitary. J. Neurochem. 66, 2248-2256.

Horsthemke, B., Nazlican, H., Husing, J., Klein-Hitpass, L., Claussen, U., Michel, S., Lich, C., Gillessen-Kaesbach, G., Buiting, K., 2003. Somatic mosaicism for maternal uniparental disomy 15 in a girl with Prader-Willi syndrome: confirmation by cell cloning and identification of candidate downstream genes. Hum. Mol. Genet. 12, 2723-2732.

Huttner, W.B., Gerdes, H.H., Rosa, P., 1991. The granin (chromogranin/secretogranin) family. Trends Biochem. Sci. 16, 27-30.

Hutton, J.C., Hansen, F., Peshavaria, M., 1985. Beta-Granins: 21 kDa co-secreted peptides of the insulin granule closely related to adrenal medullary chromogranin A. FEBS Lett. 188, 336-340.

Hutton, J.C., Peshavaria, M., Johnston, C.F., Ravazzola, M., Orci, L., 1988. Immunolocalization of betagranin: a chromogranin A-related protein of the pancreatic B-cell. Endocrinology 122, 1014-1020.

Iguchi, H., Bannai, S., Takanashi, N., Tsukada, Y., 1992. Production of chromogranin A and B derived peptides in human small cell lung carcinoma cell lines. Eur. J. Cancer 28, 1458-1462.

Illing, N., Troskie, B.E., Nahorniak, C.S., Hapgood, J.P., Peter, R.E., Millar, R.P., 1999. Two

gonadotropin-releasing hormone receptor subtypes with distinct ligand selectivity and differential distribution in brain and pituitary in the goldfish (*Carassius auratus*). Proc. Natl. Acad. Sci. USA 96, 2526-2531.

Imbrogno, S., Angelone, T., Corti, A., Adamo, C., Helle, K.B., Tota, B., 2004. Influence of vasostatins, the chromogranin A-derived peptides, on the working heart of the eel (Anguilla anguilla): negative inotropy and mechanism of action. Gen. Comp. Endocrinol. 139, 20-28.

Ischia, R., Lovisetti-Scamihorn, P., Hogue-Angeletti, R., Wolkersdorfer, M., Winkler, H., Fischer-Colbrie, R., 1997. Molecular cloning and characterization of NESP55, a novel chromogranin-like precursor of a peptide with 5-HT1B receptor antagonist activity. J. Biol. Chem. 272, 11657-11662.

Jenö, P., Mini, T., Moes, S., Hintermann, E., Horst, M., 1995. Internal sequences from proteins digested in polyacrylamide gels. Anal. Biochem. 224, 75-82

Johnson, J.D., Van Goor, F., Wong, C.J.H., Goldberg, J.I., Chang, J.P., 1999. Two endogenous gonadotropin-releasing hormones generate dissimilar Ca^{2+} signals in identified goldfish gonadotropes. Gen. Comp. Endocrinol. 116, 178-191.

Jin, L., Zhang, S., Bayliss, J., Scheithauer, B., Qian, X., Kobayashi, I., Stridsberg, M., Lloyd, R.V., 2003. Chromogranin a processing in human pituitary adenomas and carcinomas: analysis with region-specific antibodies. Endocr. Pathol. 14, 37-48.

Kähler, C.M., Fischer-Colbrie, R., 2000. Secretoneurin--a novel link between the nervous and the immune system. Conservation of the sequence and functional aspects. Adv. Exp. Med. Biol. 482, 279-290.

Kähler, C.M., Kirchmair, R., Kaufmann, G., Kahler, S.T., Reinisch, N., Fischer-Colbrie, R., Hogue-Angeletti, R., Winkler, H., Wiedermann, C.J., 1997. Inhibition of proliferation and stimulation of migration of endothelial cells by secretoneurin in vitro. Arterioscler. Thromb. Vasc. Biol. 17, 932-939.

Kähler, C.M., Schratzberger, P., Kaufmann, G., Hochleitner, B., Bechter, O., Götsch, C., Wöll, E., Marschang, P., Herold, M., Wiedermann, C.J., 2002. Transendothelial migration of leukocytes and signalling mechanism in response to the neuropeptide secretoneurin. Regul. Pept. 105, 35-46.

Kakar, S.S., Wei, N., Mulchahey, J.J., LeBoeuf, R.D., Neill, J.D., 1993. Regulation of expression of secretogranin II mRNA in female rat pituitary and hypothalamus. Neuroendocrinology 57, 422-431.

Kanasaki, H., Bedecarrats, G.Y., Kam, K.Y., Xu, S., Kaiser, U.B., 2005. Gonadotropin-releasing hormone pulse frequency-dependent activation of extracellular signal-regulated kinase pathways in perifused LbetaT2 cells. Endocrinology. 146, 5503-5513.

Kandlhofer, S., Hoertnagl, B., Czech, T., Baumgartner, C., Maier, H., Novak, K., Sperk, G., 2000. Chromogranins in temporal lobe epilepsy. Epilepsia 41, S111-114.

Karlsson, E., Stridsberg, M., Sandler, S., 2000. Chromogranin-B regulation of IAPP and insulin secretion. Regul. Pept. 87, 33-39.

Kennedy, B.P., Mahata, S.K., O'Connor, D.T., Ziegler, M.G., 1998. Mechanism of cardiovascular actions of the chromogranin A fragment catestatin in vivo. Peptides 19, 1241-1248.

Kim, T., Tao-Cheng, J.H., Eiden, L.E., Loh, Y.P., 2001. Chromogranin A, an "on/off" switch controlling dense-core secretory granule biogenesis. Cell 106, 499-509.

Kirchmair, R., Egger, M., Walter, D.H., Eisterer, W., Niederwanger, A., Woell, E., Nagl, M., Pedrini, M., Murayama, T., Frauscher, S., Hanley, A., Silver, M., Brodmann, M., Sturm, W., Fischer-Colbrie, R., Losordo, D.W., Patsch, J.R., Schratzberger, P., 2004. Secretoneurin, an angiogenic neuropeptide, induces postnatal vasculogenesis. Circulation 110, 1121-1127.

Kirchmair, R., Hogue-Angeletti. R., Gutierrez, J., Fischer-Colbrie, R., Winkler, H., 1993. Secretoneurin--a neuropeptide generated in brain, adrenal medulla and other endocrine tissues by proteolytic processing of secretogranin II (chromogranin C). Neuroscience 53, 359-365.

Klausen, C., Chang, J.P., Habibi, H.R., 2001. The effect of gonadotropin-releasing hormone on growth hormone and gonadotropin subunit gene expression in the pituitary of goldfish, Carassius auratus. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 511-516.

Klausen, C., Chang, J.P., Habibi, H.R., 2002. Time- and dose-related effects of gonadotropin-releasing hormone on growth hormone and gonadotropin subunit gene expression in the goldfish pituitary. Can. J. Physiol. Pharmacol. 80, 915-924.

Klimaschewski, L., Benndorf, K., Kirchmair, R., Fischer-Colbrie, R., Heym, C., 1995. Secretoneurin-immunoreactivity in nerve terminals apposing identified preganglionic sympathetic neurons in the rat: colocalization with substance P and enkephalin. Chem. Neuroanat. 9, 55-63.

Kong, C., Gill, B.M., Rahimpour, R., Xu, L., Feldman, R.D., Xiao, Q., McDonald, T.J., Taupenot, L., Mahata, S.K., Singh, B., O'Connor, D.T., Kelvin, D.J., 1998. Secretoneurin and chemoattractant receptor interactions. J. Neuroimmunol. 88, 91-98.

Krüger, P.G., Mahata, S.K., Helle, K.B., 2003. Catestatin (CgA344-364) stimulates rat mast cell release of histamine in a manner comparable to mastoparan and other cationic charged neuropeptides. Regul. Pept. 114, 29-35.

Laslop, A., Weiss, C., Savaria, D., Eiter, C., Tooze, S.A., Seidah, N.G., Winkler, H., 1998. Proteolytic processing of chromogranin B and secretogranin II by prohormone convertases. J. Neurochem. 70, 374-383.

Lawson, M.A., Tsutsumi, R., Zhang, H., Talukdar, I., Butler, B.K., Santos, S.J., Mellon, P.L., Webster, N.J., 2007. Pulse sensitivity of the luteinizing hormone beta promoter is determined by a negative feedback loop Involving early growth response-1 and Ngfi-A binding protein 1 and 2. Mol. Endocrinol. 21, 1175-1191.

Leitner, B., Fischer-Colbrie, R., Scherzer, G., Winkler, H., 1996. Secretogranin II: relative amounts and processing to secretoneurin in various rat tissues. J. Neurochem. 66, 1312-1317.

Leitner, B., Kaufmann, W.A., Marksteiner, J., Hoflehner, J., Traurig, H., Saria, A., Fischer-Colbrie, R., Winkler, H., 1997. Ontogenic development of secretogranin II and of its processing to secretoneurin in rat brain. Brain. Res. Dev. Brain. Res. 100, 161-168.

Leitner, B., Schneitler, C., Klocker, H., Volknandt, W., Zimmermann, H., Winkler, H., Fischer-Colbrie, R., 1998. Formation and sequence analysis of secretoneurin, a neuropeptide derived from secretogranin II, in mammalian, bird, reptile, amphibian and fish brains. Neurosci. Lett. 248, 105-108.

Lewis, J.J., Zdon, M.J., Adrian, T.E., Modlin, I.M., 1988. Pancreatatin: a novel peptide inhibitor of parietal cell secretion. Surgery 104, 1031-1036.

Liu, F., Austin, D.A., Mellon, P.L., Olefsky, J.M., Webster, N.J., 2002. GnRH activates ERK1/2 leading to the induction of c-fos and LHbeta protein expression in LbetaT2 cells. Mol. Endocrinol. 16, 419-434.

Liu, F., Austin, D.A., Webster, N.J., 2003. Gonadotropin-releasing hormone-desensitized LbetaT2 gonadotrope cells are refractory to acute protein kinase C, cyclic AMP, and calcium-dependent signaling. Endocrinology. 144, 4354-4365.

Loughlin, J.S., Badger, T.M., Crowley, W.F. Jr., 1981. Perifused pituitary cultures: a model for LHRH regulation of LH secretion. Am. J. Physiol. 240, 591-596.

Lugardon, K., Raffner, R., Goumon, Y., Corti, A., Delmas, A., Bulet, P., Aunis, D., Metz-Boutigue, M.H., 2000. Antibacterial and antifungal activities of vasostatin-1, the N-terminal fragment of chromogranin A. J. Biol. Chem. 275, 10745-10753.

Mahata, S.K., Mahata, M., Marksteiner, J., Sperk, G., Fischer-Colbrie, R., Winkler, H., 1991. Distribution of mRNAs for chromogranin A and B and secretogranin II in rat brain. Eur. J. Neurosci. 3, 895-904.

Mahata, S.K., Mahata, M., Steiner, H.J., Fischer-Colbrie, R., Winkler, H., 1992. In situ hybridization: mRNA levels of secretogranin II, neuropeptides and carboxypeptidase H

in brains of salt-loaded and Brattleboro rats. Neuroscience 48, 669-680.

Mahata, S.K., Mahata, M., Wen, G., Wong, W.B., Mahapatra, N.R., Hamilton, B.A,, O'Connor, D.T., 2004. The catecholamine release-inhibitory "catestatin" fragment of chromogranin a: naturally occurring human variants with different potencies for multiple chromaffin cell nicotinic cholinergic responses. Mol. Pharmacol. 66, 1180-1191.

Marchant, T.A., Chang, J.P., Nahorniak, C.S., Peter, R.E., 1989. Evidence that gonadotropin-releasing hormone also functions as a growth hormone-releasing factor in the goldfish. Endocrinology 124, 2509-2518.

Marksteiner, J., Kirchmair, R., Mahata, S.K., Mahata, M., Fischer-Colbrie, R., Hogue-Angeletti, R., Saria, A., Winkler, H., 1993a. Distribution of secretoneurin, a peptide derived from secretogranin II, in rat brain: an immunocytochemical and radioimmunological study. Neuroscience 54, 923-944.

Marksteiner., J, Saria, A., Kirchmair, R., Pycha, R., Benesch, H., Fischer-Colbrie, R., Haring, C., Maier, H., Ransmayr, G., 1993b. Distribution of secretoneurin-like immunoreactivity in comparison with substance P- and enkephalin-like immunoreactivities in various human forebrain regions. Eur. J. Neurosci. 5, 1573-1585.

Marksteiner, J., Lechner, T., Kaufmann, W.A., Gurka, P., Humpei, C., Nowakowski, C., Maier, H., Jellinger, K.A., 2000. Distribution of chromogranin B-like immunoreactivity in the human hippocampus and its changes in Alzheimer's disease. Acta Neuropathol. 100, 205-212.

Marksteiner, J., Saria, A., Hinterhuber, H., 1994. Distribution of secretoneurin-like immunoreactivity in comparison with that of substance P in the human brain stem. J. Chem. Neuroanat. 7, 253-270.

Martyniuk, C.J., Xiong, H., Crump, K., Chiu, S., Sardana, R., Nadler, A., Gerrie, E.R., Xia, X., Trudeau, V.L., 2006. Gene expression profiling in the neuroendocrine brain of male goldfish (*Carassius auratus*) exposed to 17alpha-ethinylestradiol. Physiol. Genomics. 27, 328-336.

Mazza, R., Mannarino, C., Imbrogno, S., Barbieri, S.F., Adamo, C., Angelone, T., Corti, A., Tota, B., 2007. Crucial role of cytoskeleton reorganization in the negative inotropic effect of chromogranin A-derived peptides in eel and frog hearts. Regul. Pept. 138, 145-151.

Mbikay, M., Seidah, N.G., Chrétien, M., 2001. Neuroendocrine secretory protein 7B2: structure, expression and functions. Biochem. J. 357, 329-342.

McNeilly, J.R., Brown, P., Mullins, J., Clark, A.J., McNeilly, A.S., 1996. Characterization of the ovine LH beta-subunit gene: the promoter is regulated by GnRH and gonadal steroids in transgenic mice. J. Endocrinol. 151, 481-489.

Miller, W.L., Shafiee-Kermani, F., Strahl, B.D., Huang, H.J., 2002. The nature of FSH induction by GnRH. Trends. Endocrinol. Metab. 13, 257-263.

McVicar, C.M., Cunningham, R.T., McClure, N., Curry, W.J., 2003. Chromogranin A proteolysis to generate beta-granin and WE-14 in the adenohypophysis during the rat oestrous cycle. Regul. Pept. 115, 1-10.

Miot, S., Le Goff, P., Duval, J., 1998. Evidence for the presence of a secretogranin II-related protein in rainbow trout pituitary. Ann. N. Y. Acad. Sci. 839, 508-509.

Montero-Hadjadje, M., Pelletier, G., Yon, L., Li, S., Guillemot, J., Magoul, R., Tillet, Y., Vaudry, H., Anouar, Y., 2003. Biochemical characterization and immunocytochemical localization of EM66, a novel peptide derived from secretogranin II, in the rat pituitary and adrenal glands. J. Histochem. Cytochem. 51, 1083-1095.

Montero-Hadjadje, M., Vaingankar, S., Elias, S., Tostivint, H., Mahata, S.K., Anouar, Y., 2008. Chromogranins A and B and secretogranin II: evolutionary and functional aspects. Acta Physiol. (Oxf.) 192, 309-324.

Montero-Hadjadje, M., Vaudry, H., Turquier, V., Leprince, J., Do Rego, J.L., Yon, L., Gallo-Payet, N., Plouin, P.F., Anouar, Y., 2002. Localization and characterization of evolutionarily conserved chromogranin A-derived peptides in the rat and human pituitary and adrenal glands. Cell Tissue Res. 310, 223-236.

Mutiara, S., Kanasaki, H., Harada, T., Oride, A., Miyazaki, K., 2008. The involvement of phosphatidylinositol 3-kinase in gonadotropin-releasing hormone-induced gonadotropin alpha- and FSHbeta-subunit genes expression in clonal gonadotroph LbetaT2 cells. Mol. Cell. Endocrinol. 283, 1-11.

Naor, Z., Benard, O., Seger, R., 2000. Activation of MAPK cascades by G-protein-coupled receptors: the case of gonadotropin-releasing hormone receptor. Trends. Endocrinol. Metab. 11, 91-99.

Natori, S., Huttner, W.B., 1994. Peptides derived from the granins (chromogranins/secretogranins). Biochimie. 76, 277-282.

Nicol, L., McNeilly, J.R., Stridsberg, M., Crawford, J.L., McNeilly, A.S., 2002. Influence of steroids and GnRH on biosynthesis and secretion of secretogranin II and chromogranin A in relation to LH release in LbetaT2 gonadotroph cells. J. Endocrinol. 174, 473-483.

Nicol, L., McNeilly, J.R., Stridsberg, M., McNeilly, A.S., 2004. Differential secretion of gonadotrophins: investigation of the role of secretogranin II and chromogranin A in the release of LH and FSH in LbetaT2 cells. J. Mol. Endocrinol. 32, 467-480.

Nguyen, K.A., Santos, S.J., Kreidel, M.K., Diaz, A.L., Rey, R., Lawson, M.A., 2004. Acute regulation of translation initiation by gonadotropin-releasing hormone in the

gonadotrope cell line LbetaT2. Mol. Endocrinol. 18, 1301-1312.

Nielsen, E., Welinder, B.S., Madsen, O.D., 1991. Chromogranin-B, a putative precursor of eight novel rat glucagonoma peptides through processing at mono-, di-, or tribasic residues. Endocrinology 129, 3147-3156.

Nobels, F.R., Kwekkeboom, D.J., Coopmans, W., Schoenmakers, C.H., Lindemans, J., De Herder, W.W., Krenning, E.P., Bouillon, R., Lamberts, S.W., 1997. Chromogranin A as serum marker for neuroendocrine neoplasia: comparison with neuron-specific enolase and the alpha-subunit of glycoprotein hormones. J. Clin. Endocr. Metab. 82, 2622-2628.

O'Connor, D.T., Cadman, P.E., Smiley, C., Salem, R.M., Rao, F., Smith, J., Funk, S.D., Mahata, S.K., Mahata, M., Wen, G., Taupenot, L., Gonzalez-Yanes, C., Harper, K.L., Henry, R.R., Sanchez-Margalet, V., 2005. Pancreastatin: multiple actions on human intermediary metabolism in vivo, variation in disease, and naturally occurring functional genetic polymorphism. J. Clin. Endocrinol. Metab. 90, 5414-5425.

O'Connor, D.T., Frigon, R.P., Sokoloff, R.L., 1984. Human chromogranin A. Purification and characterization from catecholamine storage vesicles of human pheochromocytoma. Hypertension 6, 2-12.

Ocañal, M.F., Jarvis, J., Parker, R., Bramley, P.M., Halket, J.M., Patell, R.K.P., Neubert, H., 2005. C-terminal sequencing by mass spectrometry: Application to gelatine-derived proline-rich peptides. Proteomics 5, 1209-1216.

Onuma, T., Ando, H., Koide, N., Okada, H., Urano, A., 2005. Effects of salmon GnRH and sex steroid · hormones on expression of genes encoding growth hormone/prolactin/somatolactin family hormones and a pituitary-specific transcription factor in masu salmon pituitary cells in vitro. Gen. Comp. Endocrinol. 143, 129-141.

Ooi, G.T., Tawadros, N., Escalona, R.M., 2004. Pituitary cell lines and their endocrine applications. Mol. Cell. Endocrinol. 228, 1-21.

Ottiger, H.P., Battenberg, E.F., Tsou, A.P., Bloom, F.E., Sutcliffe, J.G., 1990. 1B1075: a brain- and pituitary-specific mRNA that encodes a novel chromogranin/secretogranin-like component of intracellular vesicles. J. Neurosci. 10, 3135-3147.

Parhar, I.S., Soga, T., Sakuma, Y., Millar, R.P., 2002. Spatio-temporal expression of gonadotropin-releasing hormone receptor subtypes in gonadotropes, somatotropes and lactotropes in the cichlid fish. J. Neuroendocrinol. 14, 657-665.

Peinado, J.R., Vazquez-Martinez, R., Cruz-García, D., Ruiz-Navarro, A., Anouar, Y., Tonon, M.C., Vaudry, H., Gracia-Navarro, F., Castano, J.P., Malagón, M.M., 2006. Differential expression and processing of chromogranin A and secretogranin II in relation to the secretory status of endocrine cells. Endocrinology 147, 1408-1418.

Peiró, E., Miralles, P., Silvestre, R.A., Villanueva, M.L., Marco, J., 1989. Pancreastatin inhibits insulin secretion as induced by glucagon, vasoactive intestinal peptide, gastric inhibitory peptide, and 8-cholecystokinin in the perfused rat pancreas. Metabolism 38, 679-682.

Pernasetti, F., Vasilyev, V.V., Rosenberg, S.B., Bailey, J.S., Huang, H.J., Miller, W.L., Mellon, P.L., 2001. Cell-specific transcriptional regulation of follicle-stimulating hormone-beta by activin and gonadotropin-releasing hormone in the LbetaT2 pituitary gonadotrope cell model. Endocrinology. 142, 2284-2295.

Peter, R.E., Chang, J.P., Nahorniak, C.S., Omeljaniuk, R.J., Sokolowska, M., Shih, S.H., Billard, R., 1986. Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog. Horm. Res. 42, 513-548.

Portela-Gomes, G.M., Grimelius, L., Stridsberg, M., Bresaola, E., Viale, G., Pelosi, G., 2005. Expression of amino acid sequences of the chromogranin A molecule and synaptic vesicle protein 2 in neuroendocrine tumors of the lung. Virchows. Arch. 446, 604-612.

Possenti R., Eldridge J.D., Paterson B.M., Grasso A., Levi A., 1989. A protein induced by NGF in PC12 cells is stored in secretory vesicles and released through the regulated pathway. EMBO J. 8, 2217-2223.

Preece, N.E., Nguyen, M., Mahata, M., Mahata, S.K., Mahapatra, N.R., Tsigelny, I., O'Connor, D.T., 2004. Conformational preferences and activities of peptides from the catecholamine release-inhibitory (catestatin) region of chromogranin A. Regul. Pept. 118, 75-87.

Randall, D., Burggren, W., French, K., 1997. Eckert Animal Physiology: Mechanisms and Adaptations (Fourth Edition). pp. 302-303.

Rao, F., Wen, G., Gayen, J.R., Das, M., Vaingankar, S.M., Rana, B.K., Mahata, M., Kennedy, B.P., Salem, R.M., Stridsberg, M., Abel, K., Smith, D.W., Eskin, E., Schork, N.J., Hamilton, B.A., Ziegler, M.G., Mahata, S.K., O'Connor, D.T., 2007. Catecholamine release-inhibitory peptide catestatin (chromogranin A(352-372)): naturally occurring amino acid variant Gly364Ser causes profound changes in human autonomic activity and alters risk for hypertension. Circulation 115, 2271-2281.

Reinecke, M., Höög, A., Ostenson, C.G., Efendic, S., Grimelius, L., Falkmer, S., 1991. Phylogenetic aspects of pancreastatin- and chromogranin-like immunoreactive cells in the gastro-entero-pancreatic neuroendocrine system of vertebrates. Gen. Comp. Endocrinol. 83,167-182.

Reinecke, M., Maake, C., 1993. A phylogenetic survey of pancreastatin and chromogranin immunoreactivity in chromaffin (TH-, DBH-, and PNMT-immunoreactive) cells of the adrenal organ of vertebrates. Gen. Comp. Endocrinol. 90, 251-265.

Reinecke, M., Müller, C., Segner, H., 1997. An immunohistochemical analysis of the ontogeny, distribution and coexistence of 12 regulatory peptides and serotonin in endocrine cells and nerve fibers of the digestive tract of the turbot, Scophthalmus maximus (Teleostei). Anat. Embryol. (Berl.) 195, 87-101.

Reinisch, N., Kirchmair, R., Kahler, C.M., Hogue-Angeletti, R., Fischer-Colbrie, R., Winkler, H., Wiedermann, C.J., 1993. Attraction of human monocytes by the neuropeptide secretoneurin. FEBS Lett. 334, 41-44.

Rieker, S., Fischer-Colbrie, R., Eiden, L., Winkler, H., 1988. Phylogenetic distribution of peptides related to chromogranins A and B. J. Neurochem. 50, 1066-1073.

Rosa, P., Fumagalli, G., Zanini, A., Huttner, W.B., 1985. The major tyrosine-sulfated protein of the bovine anterior pituitary is a secretory protein present in gonadotrophs, thyrotrophs, mammotrophs, and corticotrophs. J. Cell. Biol. 100, 928-937.

Rosa, P., Zanini, A., 1981. Characterization of adenohypophysial polypeptides bytwo-dimensional gel electrophoresis: II. Sulfated and glycosylated polypeptides. Mol. Cell. Endocrinol. 24, 181-193.

Ruf, F., Fink, M.Y., Sealfon, S.C., 2003. Structure of the GnRH receptor-stimulated signaling network: insights from genomics. Front. Neuroendocrinol. 24, 181-199.

Ruf, F., Sealfon, S.C., 2004. Genomics view of gonadotrope signaling circuits. Trends. Endocrinol. Metab. 15, 331-338.

Russell, J., Gee, P., Liu, SM., Angeletti, R.H., 1994. Inhibition of parathyroid hormone secretion by amino-terminal chromogranin peptides. Endocrinology 135, 337-342.

Salvi, E., Buffa, R., Renda, T.G., 1995. Ontogeny, distribution and amine/peptide colocalization of chromogranin A- and B-immunoreactive cells in the chicken gizzard and antrum. Anat. Embryol. (Berl.) 192, 547-555.

Salzet, M., Verger-Bocquet, M., 2002. Cellular localization of a chromogranin B-like derived peptides in leeches. Neuro. Endocrinol. Lett. 23, 209-212.

Samia, M., Lariviere, K.E., Basak, A., Trudeau, V.L., 2001. Distribution of Secretogranin-II (SgII) mRNA in the Goldfish. Processings of the XIV International Congress of Comparative Endocrinology, pp. 655-661.

Samia, M., Lariviere, K.E., Rochon, M.H., Hibbert, B.M., Basak, A., Trudeau, V.L., 2004. Seasonal cyclicity of secretogranin-II expression and its modulation by sex steroids and GnRH in the female goldfish pituitary. Gen. Comp. Endocrinol. 139, 198-205.

Sánchez-Margalet, V., 1999. Modulation of insulin receptor signalling by pancreastatin in HTC hepatoma cells. Diabetologia 42, 317-325.

Sánchez-Margalet, V., Goberna, R., 1993. Pancreastatin decreases plasma epinephrine levels in surgical stress in the rat. Peptides 14, 797-799.

Sánchez-Margalet, V., González-Yanes, C., 1998. Pancreastatin inhibits insulin action in rat adipocytes. Am. J. Physiol. 275, E1055-1060.

Sánchez-Margalet, V., Lobón, J.A., González, A., Fernández-Soto, M.L., Escobar-Jiménez, F., Goberna, R., 1998. Increased plasma pancreastatin-like levels in gestational diabetes: correlation with catecholamine levels. Diabetes Care 21, 1951-1954.

Sánchez-Margalet, V., Valle, M., Lobón, J.A., Escobar-Jiménez, F., Pérez-Cano, R., Goberna, R., 1995. Plasma pancreastatin-like immunoreactivity correlates with plasma norepinephrine levels in essential hypertension. Neuropeptides 29, 97-101.

Saria, A., Troger, J., Kirchmair, R., Fischer-Colbrie, R., Hogue-Angeletti, R., Winkler, H., 1993. Secretoneurin releases dopamine from rat striatal slices: a biological effect of a peptide derived from secretogranin II (chromogranin C). Neuroscience 54, 1-4.

Sato, F., Hasegawa, T., Katayama, Y., Iwanaga, T., Yanaihara, N., Kanno, T., Ishida, N., 2000. Molecular cloning of equine chromogranin A and its expression in endocrine and exocrine tissues. J. Vet. Med. Sci. 62, 953-959.

Schmid, K.W., Kunk, B., Kirchmair, R., Totsch, M., Bocker, W., Fischer-Colbrie, R., 1995. Immunohistochemical detection of secretoneurin, a novel neuropeptide endoproteolytically processed from secretogranin II, in normal human endocrine and neuronal tissues. Histochem. J. 27, 473-481.

Schmid, G.M., Meda, P., Caille, D., Wargent, E., O'Dowd, J., Hochstrasser, D.F., Cawthorne, M.A., Sanchez, J.C., 2007. Inhibition of insulin secretion by betagranin, an N-terminal chromogranin A fragment. J. Biol. Chem. 282, 12717-12724.

Schneitler, C., Kähler, C., Wiedermann, C.J., Hogue-Angeletti, R., Fischer-Colbrie, R., 1998. Specific binding of a 125I-secretoneurin analogue to a human monocytic cell line. J. Neuroimmunol. 86, 87-91.

Schratzberger, P., Wöll, E., Reinisch, N., Kähler, C.M., Wiedermann, C.J., 1996. Secretoneurin-induced in vitro chemotaxis of human monocytes is inhibited by pertussis toxin and an inhibitor of protein kinase C. Neurosci. Lett. 214, 208-210.

Schulz, R.W., Vischer, H.F., Cavaco, J.E., Santos, E.M., Tyler, C.R., Goos, H.J., Bogerd, J., 2001. Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp. Biochem. Physiol. B Biochem. Mol. Biol. 129, 407-417.

Schuster-Bockler, B., Bateman, A., 2005. Visualizing profile-profile alignment: pairwise HMM logos. Bioinformatics 21, 2912-2913.

Schwarzer, C., Marksteiner, J., Kroesen, S., Kohl, C., Sperk, G., Winkler, H., 1997. Secretoneurin: a market in rat hippocampal pathways. J. Comp. Neurol. 377, 29-40.

Seidah, N.G., Chretien, M., 1999. Proprotein and prohormone convertases: a family of subtilases generating diverse bioactive polypeptides. Brain Res. 848, 45-62.

Seidah, N.G., Chretien, M., Day, R., 1994. The family of subtilisin/kexin like pro-protein and pro-hormone convertases: divergent or shared functions. Biochimie. 76, 197-209.

Seidah, N.G., Mayer, G., Zaid, A., Rousselet, E., Nassoury, N., Poirier, S., Essalmani, R., Prat, A., 2008. The activation and physiological functions of the proprotein convertases. Int. J. Biochem. Cell. Biol. 40, 1111-1125.

Shyu, W.C., Lin, S.Z., Chiang, M.F., Chen, D.C., Su, C.Y., Wang, H.J., Liu, R.S., Tsai, C.H., Li, H., 2008. Secretoneurin promotes neuroprotection and neuronal plasticity via the Jak2/Stat3 pathway in murine models of stroke. J. Clin. Invest. 118, 133-148.

Sim, P.J., Wolbers, W.B., Mitchell, R., 1995. Activation of MAP kinase by the LHRH receptor through a dual mechanism involving protein kinase C and a pertussis toxin-sensitive G protein. Mol. Cell. Endocrinol. 112, 257-263.

Simonneaux, V., Vuillez, P., Eder, U., Miguez, J.M., Pevet, P., Fischer-Colbrie, R., 1997. Secretoneurin: a new neuropeptide in the rodent pineal gland. Cell. Tissue Res. 288, 427-434.

Stefano, A.V., Vissio, P.G., Paz, D.A., Somoza, G.M., Maggese, M.C., Barrantes, G.E., 1999. Colocalization of GnRH binding sites with gonadotropin-, somatotropin-, somatolactin-, and prolactin-expressing pituitary cells of the pejerrey, Odontesthes bonariensis, in vitro. Gen. Comp. Endocrinol. 116, 133-139.

Strewler, G.J., 2000. The parathyroid hormone-related protein. Endocrinol. Metab. Clin. North Am. 29, 629-645.

Stridsberg, M., Oberg, K., Li, Q., Engstrom, U., Lundqvist, G., 1995. Measurements of chromogranin A, chromogranin B (secretogranin I), chromogranin C (secretogranin II) and pancreastatin in plasma and urine from patients with carcinoid tumours and endocrine pancreatic tumours. J. Endocrinol. 144, 49-59.

Strobl-Mazzulla, P.H., Moncaut, N.P., López, G.C., Miranda, L.A., Canario, A.V., Somoza, G.M., 2005. Brain aromatase from pejerrey fish (Odontesthes bonariensis): cDNA cloning, tissue expression, and immunohistochemical localization. Gen. Comp. Endocrinol. 143, 21-32.

Strub, J.M., Garcia-Sablone, P., Lonning, K., Taupenot, L., Hubert, P., Van Dorsselaer, A., Aunis, D., Metz-Boutigue, M.H., 1995. Processing of chromogranin B in bovine adrenal medulla. Identification of secretolytin, the endogenous C-terminal fragment of residues

614-626 with antibacterial activity. Eur. J. Biochem. 229, 356-368.

Strub, J.M., Hubert, P., Nullans, G., Aunis, D., Metz-Boutigue, M.H., 1996. Antibacterial activity of secretolytin, a chromogranin B-derived peptide (614-626), is correlated with peptide structure. FEBS Lett. 379, 273-278.

Takeuchi, T., Hosaka, M., 2008. Sorting mechanism of peptide hormones and biogenesis mechanism of secretory granules by secretogranin III, a cholesterol-binding protein, in endocrine cells. Curr. Diabetes Rev. 4, 31-38.

Tatemoto, K., Efendić, S., Mutt, V., Makk, G., Feistner, G.J., Barchas, J.D., 1986. Pancreastatin, a novel pancreatic peptide that inhibits insulin secretion. Nature 324, 476-478.

Taupenot, L., Harper, K.L., O'Connor, D.T., 2003. The chromogranin-secretogranin family. N. Engl. J. Med. 348, 1134-1149.

Taupenot, L., Mahata, S.K., Mahata, M., Parmer, R.J., O'Connor, D.T., 2000. Interaction of the catecholamine release-inhibitory peptide catestatin (human chromogranin A(352-372)) with the chromaffin cell surface and Torpedo electroplax: implications for nicotinic cholinergic antagonism. Regul. Pept. 95, 9-17.

Thomas, P., Mellon, P.L., Turgeon, J., Waring, D.W., 1996. The L beta T2 clonal gonadotrope: a model for single cell studies of endocrine cell secretion. Endocrinology. 137, 2979-2989.

Tota, B., Angelone, T., Mazza, R., Cerra, M.C., 2008. The chromogranin A-derived vasostatins: new players in the endocrine heart. Curr. Med. Chem. 15, 1444-1451.

Tota, B., Quintieri, A.M., Di Felice, V., Cerra, M.C., 2007. New biological aspects of chromogranin A-derived peptides: focus on vasostatins. Comp. Biochem. Physiol. A. Mol. Integr. Physiol. 147, 11-18.

Trandaburu, T., Ali, S.S., 1998. Granin proteins (chromogranin A and secretogranin II C23-3 and C26-3) in the intestine of amphibians. Anat. Anz. 180, 523-528.

Trandaburu, T., Ali, S.S., Trandaburu, I., 1999a, Granin proteins (chromogranin A and secretogranin II C23-3 and C26-3) in the endocrine pancreas of amphibians. Anat. Anz. 181, 585-592.

Trandaburu, T., Ali, S.S., Trandaburu, I., 1999b. Granin proteins (chromogranin A and secretogranin II C23-3 and C26-3) in the intestine of reptiles. Anat. Anz. 181, 261-268.

Trudeau, V.L., 1997. Neuroendocrine regulation of gonadotropin II release and gonadal growth in the goldfish, Carassius auratus. Rev. Reprod. 2, 55-68.

Tsunashima, K., Wolkersdorfer, M., Schwarzer, C., Sperk, G., Fischer-Colbrie, R., 1997. Limbic seizures induce neuropeptide and chromogranin mRNA expression in rat adrenal medulla. Brain Res. Mol. Brain Res. 5, 42-48.

Turgeon, J.L., Kimura, Y., Waring, D.W., Mellon, P.L., 1996. Steroid and pulsatile gonadotropin-releasing hormone (GnRH) regulation of luteinizing hormone and GnRH receptor in a novel gonadotrope cell line. Mol. Endocrinol. 10, 439-450.

Turquier, V., Vaudry, H., Jégou, S., Anouar, Y., 1999. Frog chromogranin A messenger ribonucleic acid encodes three highly conserved peptides. Coordinate regulation of proopiomelanocortin and chromogranin A gene expression in the pars intermedia of the pituitary during background color adaptation. Endocrinology 140, 4104-4112.

Turquier, V., Yon, L., Grumolato, L., Alexandre, D., Fournier, A., Vaudry, H., Anouar, Y., 2001. Pituitary adenylate cyclase-activating polypeptide stimulates secretoneurin release and secretogranin II gene transcription in bovine adrenochromaffin cells through multiple signaling pathways and increased binding of pre-existing activator protein-1-like transcription factors. Mol. Pharmacol. 60, 42-52.

Udupi, V., Lee, H.M., Kurosky, A., Greeley, G.H. Jr., 1999. Prohormone convertase-1 is essential for conversion of chromogranin A to pancreastatin. Regul. Pept. 83, 123-127.

Uehara, M., Yaoi, Y., Suzuki, M., Takata, K., Tanaka, S., 2001. Differential localization of prohormone convertases PC1 and PC2 in two distinct types of secretory granules in rat pituitary gonadotrophs. Cell Tissue Res. 304, 43-49.

Vallet, V.S., Li, J.Y., Duval, J., 1997. Secretogranin II (SgII) distribution and processing studies in human normal and adenomatous anterior pituitaries using new polyclonal antibodies. Regul. Pept. 68, 155-163.

van Horssen, A.M., Martens, G.J., 1999. Biosynthesis of secretogranin II in Xenopus intermediate pituitary. Mol. Cell. Endocrinol. 147, 57-64.

Vasilyev, V.V., Pernasetti, F., Rosenberg, S.B., Barsoum, M.J., Austin, D.A., Webster, N.J., Mellon, P.L., 2002. Transcriptional activation of the ovine follicle-stimulating hormone-beta gene by gonadotropin-releasing hormone involves multiple signal transduction pathways. Endocrinology 143, 1651-1659.

Vaudry, H., Conlon, J.M., 1991. Identification of a peptide arising from the specific post-translation processing of secretogranin II. FEBS Lett. 284, 31-33.

Vieau, D., Rojas-Miranda, A., Verley, J.M., Lenne, F., Bertagna, X., 1991. The secretory granule peptides 7B2 and CCB are sensitive biochemical markers of neuro-endocrine bronchial tumours in man. Clin. Endocrinol. (Oxf.) 35, 319-325.

Wand, G.S., Takiyyuddin, M., O'Connor, D.T., Levine, M.A., 1991. A proposed role for

chromogranin A as a glucocorticoid-responsive autocrine inhibitor of proopiomelanocortin secretion. Endocrinology 128, 1345-1351.

Wang, Z., Vandenberghe, I., Depreitere, J., Devreese, B., Clerens, S., Nouwen, E.J., Van Beeumen, J., De Potter, W., 2001. Identification and characterization of novel chromogranin B-derived peptides from porcine chromaffin granules by liquid chromatography/electrospray tandem MS. Eur. J. Biochem. 268, 235-242.

Watanabe, T., Uchiyama, Y., Grube, D., 1991. Topology of chromogranin A and secretogranin II in the rat anterior pituitary: potential marker proteins for distinct secretory pathways in gonadotrophs. Histochemistry 96, 285-293.

Weck, J., Fallest, P.C., Pitt, L.K., Shupnik, M.A., 1998. Differential gonadotropin-releasing hormone stimulation of rat luteinizing hormone subunit gene transcription by calcium influx and mitogen-activated protein kinase-signaling pathways. Mol. Endocrinol. 12, 451-457.

Wei, N., Kakar, S.S., Neill, J.D., 1995. Measurement of secretogranin II release from individual adenohypophysial gonadotropes. Am. J. Physiol. 268, E145-152.

Weiler, R., Marksteiner, J., Bellmann, R., Wohlfarter, T., Schober, M., Fischer-Colbrie, R., Sperk, G., Winkler, H., 1990. Chromogranins in rat brain: characterization, topographical distribution and regulation of synthesis. Brain Res. 532, 87-94.

Winkler, H., Fischer-Colbrie, R., 1992. The chromogranins A and B: The first 25 years and future perspectives. Neuroscience 18, 261-290.

Wong, A.O., Ng, S., Lee, E.K., Leung, R.C., Ho, W.K., 1998. Somatostatin inhibits (d-Arg6, Pro9-NEt) salmon gonadotropin-releasing hormone- and dopamine D1-stimulated growth hormone release from perifused pituitary cells of chinese grass carp, ctenopharyngodon idellus. Gen. Comp. Endocrinol. 110, 29-45.

Wong, A.O., Zhou, H., Jiang, Y., Ko, W.K., 2005. Feedback regulation of growth hormone synthesis and secretion in fish and the emerging concept of intrapituitary feedback loop. Comp. Biochem. Physiol. A Mol. Integr. Physiol. 144, 284-305.

Wouters, S., Leruth, M., Decroly, E., Vandenbranden, M., Creemers, J.W., van de Loo, J.W., Ruysschaert, J.M., Courtoy, P.J., 1998. Furin and proprotein convertase 7 (PC7)/lymphoma PC endogenously expressed in rat liver can be resolved into distinct post-Golgi compartments. Biochem. J. 336, 311-316.

Wu, H.J., Rozansky, D.J., Parmer, R.J., Gill, B.M., O'Connor, D.T., 1991. Structure and function of the chromogranin A gene. Clues to evolution and tissue-specific expression. J. Biol. Chem. 266, 13130-13134.

Yajima, A., Ikeda, M., Miyazaki, K., Maeshima, T., Narita, N., Narita, M., 2004. Manserin, a

novel peptide from secretogranin II in the neuroendocrine system. Neuroreport 15, 1755-1759.

Yamada, Y., Yamamoto, H., Yonehara, T., Kanasaki, H., Nakanishi, H., Miyamoto, E., Miyazaki, K., 2004. Differential activation of the luteinizing hormone beta-subunit promoter by activin and gonadotropin-releasing hormone: a role for the mitogen-Yaron, Z., Gur, G., Melamed, P., Rosenfeld, H., Elizur, A., Levavi-Sivan, B., 2003. Regulation of fish gonadotropins. Int. Rev. Cytol. 225, 131-185.

You, Z.B., Saria, A., Fischer-Colbrie, R., Terenius, L., Goiny, M., Herrera-Marschitz, M., 1996. Effects of secretogranin II-derived peptides on the release of neurotransmitters monitored in the basal ganglia of the rat with in vivo microdialysis. Naunyn Schmiedebergs Arch. Pharmacol. 354, 717-724.

Zhang, J.X., Fasciotto, B.H., Darling, D.S., Cohn, D.V., 1994. Pancreastatin, a chromogranin A-derived peptide, inhibits transcription of the parathyroid hormone and chromogranin A genes and decreases the stability of the respective messenger ribonucleic acids in parathyroid cells in culture. Endocrinology 134, 1310-1316.

Zhao, E., Basak, A., Trudeau, V.L., 2006a. Secretoneurin stimulates goldfish pituitary luteinizing hormone production. Neuropeptides 40, 275-282.

Zhao, E., Basak, A., Crump, K., Trudeau, V.L., 2006b. Proteolytic processing and differential distribution of secretogranin-II in goldfish. Gen. Comp. Endocrinol. 146, 100-107.

Zhao, E., Basak, A., Wong, A.O.L., Ko, W., Chen, A., López, G.C., Canosa, L.F., Somoza, G.M., Trudeau, V.L., 2009a. The secretogranin II-derived peptide secretoneurin stimulates luteinizing hormone secretion from gonadotrophs. Endocrinology 150: 2273-2282.

Zhao, E., Zhang, D., Basak, A., Trudeau, V.L., 2009b. New insights into granin-derived peptides: evolution and endocrine roles. Gen. Comp. Endocrinol. 164, 161-174.

Zhao, E., Hu, H., Trudeau, V.L., 2009c. Secretoneurin as a hormone regulator in the pituitary. Regulatory Peptides. doi:10.1016/j.regpep.2009.11.019.

Zhou, H., Wang, X., Ko, W.K., Wong, A.O., 2004. Evidence for a novel intrapituitary autocrine/paracrine feedback loop regulating growth hormone synthesis and secretion in grass carp pituitary cells by functional interactions between gonadotrophs and somatotrophs. Endocrinology 145, 5548-5559.

APPENDIX I: Recognizing Secretoneurin (SN)-immunoreactivies in Pituitaries of Several Species Using Anti-goldfish SN Antibody

1. Introduction

The polyclonal antibody of goldfish SN was first generated using a multiple antigenic peptides (MAP) strategy (Basak et al., 1995). A 15-amino acid peptide sequence, YTPQKLATLQSVFEE, which is located in the middle region of goldfish SN (gfSN), was chosen as the antigenic peptide. Four copies of the peptide sequence were incorporated onto a polylysine MAP core to form a 4-branched immunogenic peptide called SN15-MAP (MW: ~14 kDa) which were used to induce the generation of polyclonal antibodies against SN in rabbits (Zhao et al., 2006b). The entire SN molecule is the most highly conserved part derived from secretogranin II (SgII) sequence, especially in mammalian species (Kähler and Fischer-Colbrie, 2000). Between mammals and goldfish, two stretches in the middle of SN including the sequences QYTP and LATLEQSVFQEEL are 100% conserved. Moreover, the 15-amino acid middle sequence of gfSN used for generating SN antiserum shares 87% and 80% identity to those of mammalian SN and trout SN, respectively (Chapter 1, Fig. 1.2 and 1.3). In the present study, I try to investigate the specific binding ability of anti-goldfish SN antiserum to SN-associated peptide in human, rat and trout pituitary tissues.

2. Materials and Methods

2.1. Animal pituitary tissues

Human and rat pituitary samples were generously provided by Dr. M. Chretien in OHRI, Ottawa. Trout pituitary tissues were kindly contributed by Dr. T. Moon in Biology

Department at the University of Ottawa.

2.2. Western blot analysis

These pituitary tissues were cut to small pieces on dry ice, and then homogenized in a buffer with protease inhibitor. Total protein extract of pituitaries was collected from the supernatant after centrifuging at 12,000 rpm for 15 minutes at 4 ^{0}C. These pituitary extracts were separated by 10% SDS-PAGE gels followed by western blots using anti-goldfish SN antiserum (Zhao et al., 2006b). The approach was previously described in Chapter 2.

3. Results

3.1. SgII-derived peptides in human and rat pituitaries

Using the anti-gfSN antibody, I tested its utility to detect SN-IR in the pituitaries of human, rat and goldfish (Fig. A1). The specificity of this antiserum was determined by pre-incubation with the gfSN peptide which blocked the western blot immunoreaction (Fig. A1B). A strong band of high molecular weight (~57 kDa) was detected in all 3 species. The precursor protein is ~69.6-71.0 kDa and the first processing site for cleavage of the 27-34 amino acid signal peptide is conserved. Detection of the resultant mature peptide indicates that the first step of processing to the resultant ~57 kDa mature peptide is also highly conserved (Zhao et al., 2009b). Moreover, another weak SN-IR signal in the region of low molecular mass was only found in the rat pituitary (Fig. A1A), indicating species differences in the degree of processing of the SgII precursor, supporting previous conclusions (Fischer-Colbrie et al., 1995; van Horssen and Martens, 1999).

3.2. SgII products in trout pituitary

I used our anti-goldfish SN antiserum for western blot analysis of trout tissues, and found that SN-IR was also evident in a trout pituitary extract which was similar in size to that of the major goldfish SgII intermediated-size product (~57 kDa) (Fig. A2A). Pre-incubation of synthetic gfSN peptide completely blocked the western blotting reaction indicating specific IR (Fig. A2B). These data confirmed that the first step of SgII processing to remove signal peptide and produce the ~57 kDa product well-conserved in fish and mammals (Zhao et al., 2009b). The subsequent processing steps to produce free SN appear to vary between species, raising the distinct possibility of species-specific fragments with potential biological activities.

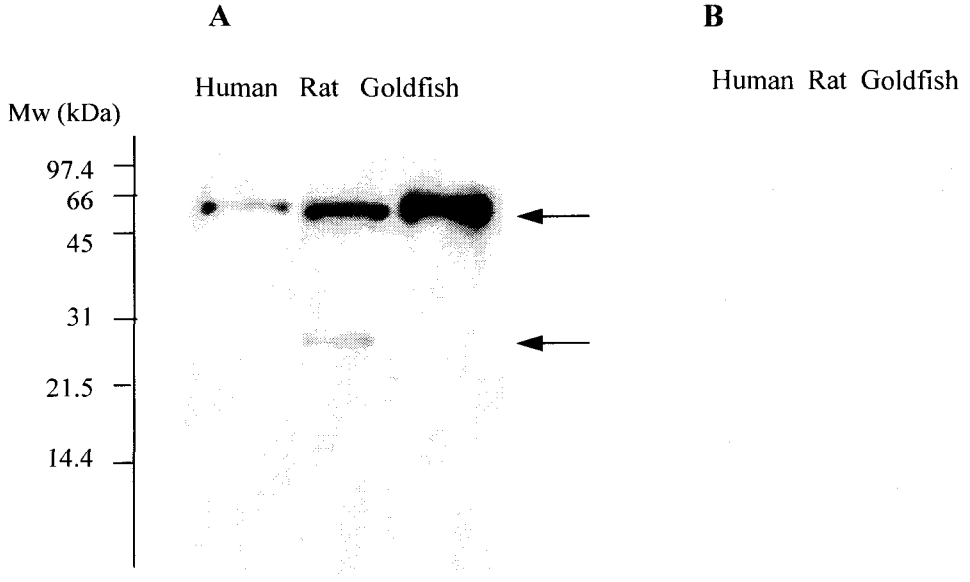

Figure A1. Western blot analysis of pituitary tissue from human, rat and goldfish. Pituitary total proteins (~100 μg) of different species were separated by 10% SDS-PAGE and detected using gfSN antiserum (Zhao et al., 2006b). **A**, gfSN antiserum (dilution: 1:2000); **B**, gfSN antiserum (dilution 1:2000) pre-absorbed with 1 μM gfSN. Arrows indicate the SN-IR Sg-II derived peptides. Relative placement of the molecular mass standards is shown on the left.

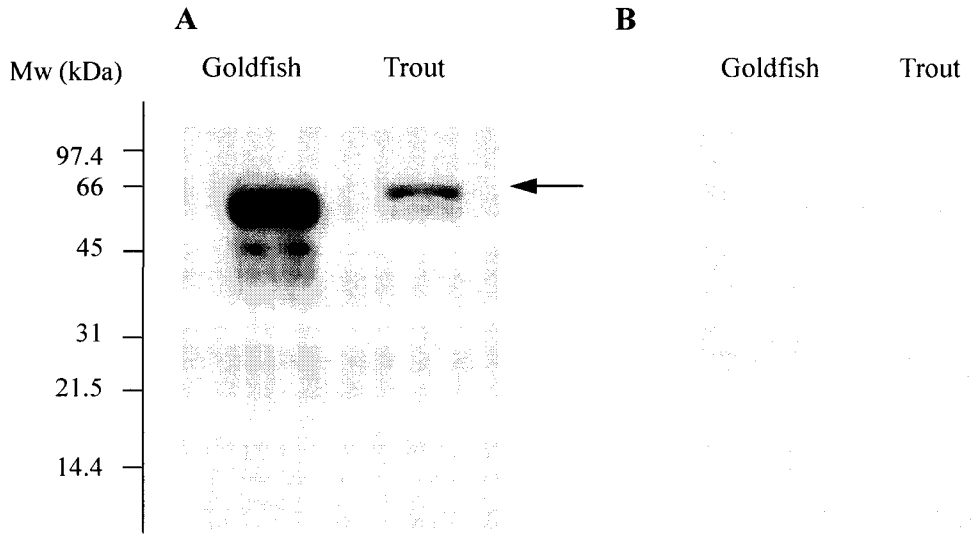

Figure A2. Western blot analysis of goldfish and trout pituitaries. Pituitary total protein (~50 μg) was extracted, separated by 10% SDS-PAGE and analyzed using gfSN antibody (Zhao et al., 2006b). **A**, gfSN antibody (dilution 1:4,000), **B**, gfSN antibody (1:4,000) pre-absorbed with 1μM goldfish SN. Arrow shows SN-immunoreactive SgII-derived peptides in the trout pituitary. Relative placement of the molecular weight standards is indicated on the left.

APPENDIX II: Manuscripts Published During My PhD Study

Zhao, E., Hu, H., and Trudeau, V.L. (2009) Secretoneurin as a hormone regulator in the pituitary. Regulatory Peptides. doi:10.1016/j.regpep.2009.11.019.

Zhao, E., Zhang, D., Basak, A., and Trudeau, V.L. (2009) New insights into granin-derived peptides: evolution and endocrine roles. General and Comparative Endocrinology. 146: 100-107.

Zhao, E., Basak, A., Wong, A.O.L., Ko, W., Chen, A., López, G.C., Grey, C.L., Canosa, L.F., Somoza, G.M., Chang, J.P., and Trudeau, V.L. (2009) The secretogranin II-derived peptide secretoneurin stimulates luteinizing hormone secretion from gonadotrophs. Endocrinology. 150: 2273-2282.

Mennigen, J.A., Martyniuk, C.J., Crump, K., Xiong, H., **Zhao, E.**, Popesku, J., Anisman, H., Cossins, A.R., Xia X., and Trudeau, V.L. (2008) Effects of fluoxetine on the reproductive axis of female goldfish (*Carassius auratus*). Physiological Genomics. 35: 273-282.