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ROLE AND PRODUCTION OF SECRETONEURIN (SN) IN THE GOLDFISH PITUITARY

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Abstract

Secretoneurin (SN) is a 33-34 amino acid neuropeptide derived from secretogranin II (SgII), a protein belonging to the chromogranin family. In static incubation studies, SN (10-500 nM) had a direct effect on pituitary fragments to increase luteinizing hormone (LH) release after 3-hour treatment. SN also induced a specific 2.6-fold increment of LHβ subunit messenger RNA levels after 6-hour treatment. Using a western blot analysis with a polyclonal rabbit anti-SN antibody, two intermediate proteins (~57 kDa and ~30 kDa) likely processed from the 69.6-kDa SgII precursor were detected in the goldfish pituitary. Levels of the ~57-kDa SN-immunoreactive protein were ~5-fold higher in the *pars distalis* than the neurointermediate lobe. In summary, SN has a direct stimulatory action on LH release and synthesis. High production of SN-containing proteins in the *pars distalis* of the pituitary suggests the existence of a local SN-mediated mechanism to regulate LH in goldfish.

Résumé

La sécrétoneurine (SN) est un neuropeptide de 33-34 acides aminés dérivé de la sécrétogranine II (SgII) de la famille des chromogranines. Trois heures d'incubation statique avec SN (500 nM) affecte des fragments d'hypophyse en augmentant la sécrétion de LH ($p<0.05$). Un traitement de six heures avec SN (500 nM) augmente de 2.6 fois des niveaux d'ARN messager de la sous-unité β de l'hormone lutéinisante (LH). Deux protéines intermédiaires (d'environ 57 et 30 kDa) contenant SN et provenant possiblement du clivage du précurseur SgII de 69.6 kDa ont été détectées dans l'hypophyse du poisson rouge à l'aide d'une analyse de type western utilisant un antisérum anti-SN polyclonal. La protéine de 57 kDa était ~5 fois plus abondante dans le *pars distalis* comparé au lobe neurointermédiaire. En résumé, SN stimule la synthèse et la sécrétion de LH. Les hauts niveaux de production de SN dans le *pars distalis* de l'hypophyse suggèrent un rôle pour SN dans la régulation de LH chez le poisson rouge.

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List of abbreviations

Ca^{2+}	calcium ion
cAMP	3', 5' cyclic adenosine monophosphate
cDNA	complimentary deoxyribonucleic acid
Cg	chromogranin
CgA	chromogranin A
CgB	chromogranin B
CgC	chromogranin C
cGnRH-II	chicken gonadotropin-releasing hormone-II
D_1 receptor	dopamine type 1 receptor
D_2 receptor	dopamine type 2 receptor
DA	dopamine
DAG	diacylgylcerol
DOM	domperidone
E_2	estradiol
FSH	follicle stimulating hormone
GABA	γ-aminobutyric acid
GVG	γ-vinyl-GABA
GH	growth hormone
GnRH	gonadotropin-releasing hormone
GTH	gonadotropin
IR	immunoreactivity
kDa	kilodalton

LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
MAP	multiple antigenic peptides
mRNA	messenger ribonucleic acid
MW	molecular weight
NE	norepinephrine
NIL	neurointermediate lobe
PC	prohormone convertase
PD	*pars distalis*
PKC	protein kinase C
RIA	radioimmunoassay
RNA	ribonucleic acid
RT-PCR	reverse transcription-polymerase chain reaction
sGnRH	salmon gonadotropin-releasing hormone
sGnRHa	salmon gonadotropin-releasing hormone analog
SgII	secretogranin II
SN	secretoneurin
SN15	the middle 15-amino acid sequence of SN (YTPQKLATLQSVFEE)
SN-IR	secretoneurin immunoreactivity
T	testosterone
TGN	trans-Golgi network

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Chapter 1: General introduction

1.1 Overview of my project

Brain regulation of vertebrate reproduction involves a complex interaction between neuroendocrine systems controlling pituitary hormone release. In particular, the neurotransmitters, including γ-aminobutyric acid (GABA) and the catecholamine dopamine (DA), and the neuropeptide gonadotropin-releasing hormone (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 gonadotropin (GTH) release from the pituitary (Peter *et al.*, 1986; Chang *et al.*, 1993; Trudeau, 1997). In mammals, GTHs are classified as follicle stimulating hormone (FSH) and luteinizing hormone (LH) that in fish are also referred to as GTH-I and GTH-II respectively. Both FSH and LH are released into the general circulation and stimulate sex steroid production, e.g. estradiol (E_2) and testosterone (T), and also control ovulation in females or sperm production in males. Another important neurotransmitter is GABA, which stimulates LH release by enhancing GnRH effect while inhibiting DA function in goldfish (*Carassius auratus*). The interaction between these stimulating and inhibiting factors is believed to drive rhythms in LH release and the seasonal cycle of reproduction (Trudeau, 1997).

Growth hormone (GH), another anterior pituitary hormone, not only regulates growth but also plays an important role in reproduction by enhancing gonadal steroid production. Treatment with recombinant salmon GH prevents gonadal regression and induces T and E_2 release in hypophysectomized killifish (Singh *et al.*, 1988). In goldfish ovary, GH induces a stimulatory effect on E_2 production (Van Der Kraak and Wade, 1994). The regulation of GH release is a complex process and involves many

neuropeptides and neurotransmitters. It is important to note that GnRH and DA stimulate GH release whereas GABA inhibits GH secretion in goldfish (Trudeau *et al.*, 2000a).

In the course of their studies on the action of GABA in the goldfish pituitary, Blazquez *et al.* (1998a) demonstrated that GABA increases gene expression of secretogranin II (SgII), a member of chromogranin (Cg) family. The chromogranins, a family of acidic, heat-stable proteins, have been widely investigated since one of their members, chromogranin A, was first discovered in 1967 (Winkler and Fischer-Colbrie, 1992). It includes four proteins that are chromogranin A (CgA), chromogranin B (CgB), SgII (Natori and Huttner, 1994; Rosa and Gerdes, 1994) and 7B2 (Mbikay *et al.*, 2001). Secretoneurin (SN) is one fragment derived from SgII, functioning as a neuropeptide in rat brain (Kirchmair *et al.*, 1993). Goldfish SN consists of 34 amino acids in contrast with SN in mammals and amphibians, which is 33-amino acid long (Blazquez *et al.*, 1998a; Fischer-Colbrie *et al.*, 1995). SN is conserved in the evolution of vertebrates (Kahler and Fischer-Colbrie, 2000) and widely distributed in vertebrate brain and endocrine tissues (Marksteiner *et al.*, 1993).

The overall goal of this Master's research project is to determine the bioactivity and the regulation of SN in the goldfish pituitary. We intended to determine SN effect on LH release from goldfish pituitaries in static and perifusion incubation. We also set out to explore the SN effect on LHβ mRNA level of goldfish pituitaries *in vitro*. In order to detect SN generation in the goldfish pituitary, western blot analysis was used to find SgII processing there. In the next step, we attempted to determine the levels of SgII-derived product expression following treatments with GnRH, domperidone (DOM) and GABA. According to previous studies of the neuroendocine axis in goldfish and SN in

vertebrates, we hypothesize that SN can affect the release of LH from the goldfish pituitary *in vitro*, and this effect shows a dose-response relationship. Furthermore, SN might be produced from SgII in the goldfish pituitary; this SgII processing should be regulated by some neurotransmitters and neuropeptides *in vitro*, such as GABA and GnRH.

1.2 The hypothalamo-pituitary-gonad axis in goldfish

The pituitary gland in goldfish consists of several different hormone-secreting cell types. FSH and LH are released from gonadotrophs in the anterior pituitary and the latter is the best studied in goldfish. GH is released from somatotrophs which are located close to gonadotrophs in the *pars distalis* (PD) of goldfish pituitary. GnRH is a key endogenous neuropeptide secreted from the pituitary, the preoptic-anterior hypothalamic (P-AH) region and other parts in the goldfish brain (Yu *et al.*, 1991). It exists as two forms in goldfish, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II), both of which stimulate LH release (Trudeau, 1997). Unlike mammals, teleosts have no functional hypothalamo-hypophyseal portal blood system, thus neurons synthesizing neuropeptides and neurotransmitters can directly innervate the anterior pituitary to regulate the release of LH and GH (Blazquez *et al.*, 1998b). It has been reported that a number of neurotransmitters, neuropeptides and hormones are involved in regulating the GnRH function to up-regulate secretion of LH and GH (Figure 1.1). For example, T, a male sex steroid, can potentiate GnRH-stimulated LH secretion from the goldfish pituitary *in vivo* (Trudeau *et al.*, 1993a) and *in vitro* (Lo and Chang, 1998). GABA may stimulate LH release in goldfish by decreasing dopaminergic inhibition *in vitro* (Trudeau

et al., 1993b). Neuropeptide Y stimulates LH secretion and enhancing GnRH release as well (Peng *et al.*, 1993). In the goldfish anterior pituitary, DA up-regulates GH release through a unique D_1 receptor (Wong *et al.*, 1994a) that is dependent on the function of voltage-sensitive Ca^{2+} channels (Wong *et al.*, 1994b). On the other hand, DA, functioning as a negative regulating factor, may reduce GnRH stimulation of GTH release *in vitro* or *in vivo* via a D_2 receptor-mediated mechanism (Omeljaniuk *et al.*, 1989a and 1989b).

1.2.1 Gonadotropin-releasing hormone (GnRH)

GnRH, originally named luteinizing hormone-releasing hormone (LHRH), is known and named for its function as the final common hypothalamic signal peptide to regulate reproduction in vertebrates. It was first characterized and synthesized from mammals in 1971 (Amoss *et al.*, 1971; Matsuo *et al.*, 1971) and from teleosts in 1983 (Sherwood *et al.*, 1983). Fourteen distinct molecular forms of GnRH all of which are 10 amino acids long have been isolated and sequenced in many vertebrates and some protochorates (Sherwood *et al.*, 1986a; Sherwood *et al.*, 1986b; Sherwood and Whittier, 1988; Okubo and Aida, 2001). Six of these 10 amino acids are identical among the different GnRHs, suggesting that GnRH was highly conserved during the evolution of vertebrates (Okubo *et al.*, 2000). Goldfish has two GnRH forms, [Trp^7, Leu^8]-GnRH (sGnRH) and [His^5, Trp^7, Tyr^8]-GnRH (cGnRH-II) (Chang *et al.*, 1993; Chang and Jobin, 1994).

cGnRH-II is the most widespread form and has been found in following species: chicken (Miyanoto *et al.*, 1984), alligator (Lovejoy *et al.*, 1991a), frog (Conlon *et al.*, 1993), sea bream (Powell *et al.*, 1994), tilapia (Weber *et al.*, 1997), catfish (Bogerd *et al.*,

1992), dogfish (Lovejoy *et al.*, 1992) and ratfish (Lovejoy *et al.*, 1991b). As a hypothalamic neuropeptide, cGnRH-II stimulates the synthesis and release of LH from the anterior pituitary to regulate the goldfish reproductive system (Trudeau, 1997). *In vitro*, cGnRH-II can induce GH secretion from goldfish pituitary cells (Chang *et al.*, 1990a) and prolactin secretion from the rostral *pars distalis* of tilapia pituitary (Weber *et al.*, 1997). Furthermore, it may induce some putative behavior and reproduction effects as a neuro-modulator because of the wide distribution of cGnRH-II in midbrain neurons of all vertebrates including primates (Lescheid *et al.*, 1997). Additionally, cGnRH-II expression in fish embryos (White and Fernald, 1998) and fetal monkeys (Quanbeck *et al.*, 1997) suggests that this peptide may have as yet undiscovered functional roles during embryonic development.

In addition to goldfish brain, sGnRH has been isolated and sequenced as a peptide from salmon brain (Sherwood *et al.*, 1983) and tilapia brain (Weber *et al.*, 1997). Like cGnRH-II, sGnRH enhances the synthesis and release of LH and GH from the pituitary in most teleosts. Furthermore, the immunocytochemical distribution of sGnRH as well as cGnRH-II in the brain of goldfish suggests functions as the neuromodulators in various central nervous regions (Kim *et al.*, 1995). sGnRH is also demonstrated to induce the release of prolactin from the tilapia pituitary *in vitro*, but its effect is weaker than that of cGnRH-II (Weber *et al.*, 1997).

1.2.2 Mechanism of sGnRH and cGnRH-II action in goldfish

Both GnRHs are capable of stimulating LH and GH release through a G-protein coupled membrane receptor (Chang *et al.*, 1993; Leung and Peng, 1996). After GnRH

receptors are stimulated by either sGnRH or cGnRH-II in goldfish, phospholipase C (PLC) is activated. Then, the second messenger diacylgylcerol (DAG) is produced and DAG may activate the extracellular Ca^{2+} ($[Ca^{2+}]_0$) entry and phospholipid-dependent protein kinase C (PKC) together; $[Ca^{2+}]_0$ then enters the cells by passing through voltage-sensitive Ca^{2+} channels. $[Ca^{2+}]_0$ entry and PKC participate in the transduction mechanisms mediating the LH release response to GnRH (Jobin *et al.*, 1996). Furthermore, sGnRH signaling is more dependent on the entry and mobilization of Ca^{2+} than is cGnRH-II. In addition, sGnRH can mobilize arachidonic acid through phospholipase A_2 to stimulate LH release in goldfish (Figure 1.2) (Chang *et al.*, 1993; Chang and Jobin, 1994). Similarly, GH release induced by sGnRH and cGnRH-II is dependent on PKC, $[Ca^{2+}]_0$ and voltage-sensitive Ca^{2+} channels (Chang *et al.*, 1993).

1.2.3 Dopamine (DA)

The catecholamine dopamine is one of the most widespread and essential neurotransmitters in the vertebrate central nervous system and is regarded as the main inhibitor of LH secretion in fish pituitary. DA has a direct effect on gonadotrophs in the teleost pituitary to inhibit LH release induced by GnRH (Peter *et al.*, 1986). In anestrous ewes, a DA receptor agonist is capable of inhibiting LH pulse frequency (Havern *et al.*, 1994). In goldfish, DA inhibits LH secretion from the pituitary by a DA type-2 like receptor mechanism (Omeljaniuk *et al.*, 1987). DA, on the other hand, acts indirectly on the release of LH through inhibiting GnRH secretion and function. Two mechanisms of DA effects are demonstrated to regulate GnRH level in the goldfish pituitary. Firstly, DA may inhibit the GnRH production via binding the D_1 receptor in the hypothalamus.

Secondly, it attenuates GnRH release from nerve terminals mediated by the D_2 receptor in the goldfish pituitary (Trudeau *et al.*, 1997). These two mechanisms indicate that DA induces strong inhibitory effects on LH secretion indirectly and directly in fish pituitary.

DA also stimulates the secretion of GH from somatotrophs mediated by the D_1 receptor in the goldfish pituitary. This process is dependent on cAMP whereas the GH response to GnRH involves PKC activation and extracellular Ca^{2+} entry through voltage-sensitive Ca^{2+} channels. This suggests that DA and GnRH both initiate the GH release, but their mechanisms are independent of each other. In the other words, they have different receptors and intracellular signaling pathways (Wong *et al.*, 1994b).

1.2.4 γ-Aminobutyric acid (GABA)

GABA and its precursor glutamate are respectively the main inhibitory and stimulatory amino acid neurotransmitters regulating neurotransmission. Glutamate is demonstrated to stimulate LH release by a combined action on N-methyl-D-aspartic acid and S-α-amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid receptors. It is transformed to GABA in a single enzymatic step catabolized by the enzyme glutamic acid decarboxylase (Trudeau *et al.*, 2000b). Most data in mammals indicate that GABA has a potent inhibitory effect on LH secretion. However, GABA shows a stimulatory effect on some aspects of the neuroendocrine axis in vertebrates. For example, GABA may increase the GnRH-induced LH release mediated by the $GABA_B$ receptor located in the medial basal hypothalamus of male sheep (Jackson and Kuehl, 2002). In experiments on rat pituitary cells, GABA stimulated LH secretion via binding non-classical $GABA_A$ receptors, which was independent of GnRH action (Virmani *et al.*, 1990).

In goldfish and catfish where GABAergic neurons innervate the pituitary directly, GABA may regulate pituitary function differently than it does in mammals (Kah *et al.*, 1987; Trudeau *et al.*, 2000b). It has been demonstrated that GABA promotes an obvious and dominant stimulatory effect on LH release from the pituitary to regulate reproductive function in goldfish (Kah *et al.*, 1992). Also, GABA is reported to enhance the basal GnRH-induced FSH and LH release from dispersed pituitary cells in female rainbow trout *in vitro* (Mananos *et al.*, 1999). In goldfish, GABA stimulates the pituitary LH release by dual stimulatory effects on the $GABA_A$ and $GABA_B$ receptors (Trudeau *et al.*, 1993b). GABA can also enhance LH release via inhibiting DA function in the preoptic-hypothalamus (Trudeau *et al.*, 1993b) and enhancing GnRH release from the neurons innervating the goldfish pituitary (Kah *et al.*, 1992). The stimulatory effect of GABA on LH release is dependent on goldfish sexual maturity and is affected by administration of sex steroids (Kah *et al.*, 1992; Trudeau *et al.*, 1993b). Furthermore, GABA acts to increase LHβ subunit mRNA levels in the goldfish pituitary (Trudeau *et al.*, 2000b).

In addition to its effects on LH secretion, GABA regulates the release of GH from the pituitary. In mammals, GABA induces a dose-dependent increase in GH secretion from the pituitary of neonatal rat *in vitro* (Acs *et al.*, 1987). However, GABA shows an inhibitory effect on GH secretion from the goldfish pituitary, and this GABAergic control of GH is modulated by some sex steroids (Trudeau *et al.*, 2000a).

1.3 Chromogranin (Cg) family

The chromogranins, also named the secretogranins, are a family of acidic, heat-stable proteins widely distributed in secretory granules of a variety of endocrine cells and

neurons (Huttner *et al.*, 1991). They were first discovered in the bovine adrenal medulla by Blaschko *et al.* in 1967. At present, four types of Cg proteins have been demonstrated to exist with their proteolytic products: CgA, CgB, SgII (chromogranin C) (Winkler and Fischer-Colbrie, 1992) and 7B2 (Mbikay *et al.*, 2001). These protein family members have been isolated and sequenced not only in mammals, such as human and bovine, but also amphibian and reptile (Trandaburu *et al.*, 1999a and 1999b). It is reported that CgA and CgB have relatively highly homologous amino acid sequences or similar gene expressions among different vertebrate species. This indicates that these two granins may share well-conserved characters in the vertebrate evolution (Sato *et al.*, 2000; Ait-Ali *et al.*, 2002). CgA and CgB are well studied as regulators in the secretory process and play roles in targeting peptide hormones and neurotransmitters. CgA is demonstrated to function 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 the PC12 endocrine cell line (Kim *et al.*, 2001). Unlike CgA and CgB, SgII shows very different sequences among the vertebrates, especially between mammalian and non-mammalian species, suggesting the poor conservation of SgII during evolution. Only the middle sequence of SN is conserved well during the evolution of vertebrates (Blazquez *et al.*, 1998a). Thus, the SN peptide may possess the biological activity of SgII in the neuroendorine system, and is an attractive peptide for functional studies in the goldfish pituitary.

1.3.1 Chromogranin A (Cg A)

CgA is a key member of the Cg family that is co-released with catecholamine in storage granules of the adrenal gland (Hendy *et al.*, 1995). Human CgA is a 439-residue protein preceded by an 18-residue signal peptide. CgA can be processed into several small peptides, such as pancreastatin which perhaps plays an important role in homestasis and pathological suppression of blood insulin level (Konecki *et al.*, 1987). The CgA gene comprises 8 exons and 7 introns spanning approximately 11 kilobases. 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 are found 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 *et al.*, 1998, 1999a and 1999b), and the gizzard of chicken (Salvi *et al.*, 1995). Regarding the study of the CgA nucleotide sequence in vertebrates, the C-terminal 314-430 amino acid region and N-terminal 177-amino acid region are over 80% homologous among mammals (Sato *et al.*, 2000). In addition, it is reported that 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). Therefore, some parts of CgA are considered to be highly conserved.

CgA is widely distributed in vertebrate neuroendocrine and endocrine cells. It exists in adrenal medulla, pituitary, spinal cord, liver, thyroid gland, striated muscle, lung, spleen, kidney, parotid gland and sublingual gland. In addition, there is a significant amount of CgA in the brain regions, such as cerebrum and cerebellum (Sato *et al.*, 2000),

with the highest concentrations of CgA found in the hypothalamus, amygdala/piriform cortex and hippocampus of rats (Weiler *et al.*, 1990). CgA is also highly expressed in patients with neuroendocrine tumors, such as nonfunctioning tumors of the endocrine pancreas and medullary thyroid carcinomas (Nobels *et al.*, 1997).

Furthermore, it is reported that CgA and its derived peptides play essential functional roles in the regulation of hormone release. Chromostatin, a functional 20-residue peptide derived from CgA, inhibits catecholamine release from the chromaffin cells in the adult bovine adrenal medulla *in vitro* (Galindo *et al.*, 1992). CgA is also the precursor of biological autocrine and paracrine peptides involved in the regulation of parathyroid cell function and secretion. It has been demonstrated that the CgA N-terminal fragment (26 kDa) and two CgA-derived peptides, pancreastatin and parastatatin, inhibit the parathyroid hormone and co-stored CgA secretion from parathyroid cells (Drees *et al.*, 1991; Cohn *et al.*, 1995). CgA displays an autocrine inhibition on the secretion of proopiomelanocortin-derived peptide in mouse corticotropic cells in a concentration-dependent manner (Wand *et al.*, 1991). In addition, CgA suppresses the human exocrine pancreas from releasing cholecystokinin-induced amylase (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 system in vertebrates.

1.3.2 Chromogranin B (Cg B)

CgB is an essential tyrosine-sulfated secretory 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). CgB has been found in several species of arthropods, fish, amphibians, birds and mammals using one and two-dimensional electrophoresis followed by immunoblotting. CgB has a well conserved molecular size, isoelectric points and immunological epitopes (Rieker *et al.*, 1988). For example, the entire CgB amino acid sequence from frogs shows a 35%-40% overall identity as compared to that of mammals. The N- and C-terminal domains of CgB are 57%-65% identity between frogs and mammals (Ait-Ali *et al.*, 2002).

Like other members of the granin family, CgB is distributed in the endocrine and nervous systems. It is reported that a bovine CgB is found to be present in adrenal medulla chromaffin granules. This granin reveals 67%, 58% and 58% identity to CgB of human, rat and mouse respectively (Grandy *et al.*, 1992). Furthermore, CgB is stored in terminals of mossy nerve fibers in the human hippocampus (Kandlhofer *et al.*, 2000) and is significantly down-regulated in Alzheimer's disease (Marksteiner *et al.*, 2000). Clinically, CgB is measured in the plasma and urine of patients with endocrine pancreatic tumors and the multiple endocrine neoplasia 1 syndrome. This granin is detected in the serum from 88% of patients and the urine from 15% of patients with these diseases (Stridsberg *et al.*, 1995).

Similar to the biological action of CgA, CgB is involved in regulating the release of some peptide hormones. It is reported that a CgB antibody increases the insulin synthesis in the endocrine pancreas. This suggests that this granin, released from islet, may have an autocrine inhibitory effect on insulin secretion (Karlsson *et al.*, 2000).

1.3.3 Secretogranin II (SgII)

SgII, also known as CgC, was initially characterized in the bovine anterior pituitary (Rosa and Zanini, 1981). It is a ~600-amino acid, very acidic, tyrosine-sulfated protein expressed in secretory granules of cells in the vertebrate neuroendocrine system (Mahata *et al.*, 1991; Natori and Huttner, 1994; Blazquez *et al.*, 1998a) and is typically processed to small peptides within the secretory vesicles (Fischer-Colbrie *et al.*, 1995). Although the primary sequence of mammalian SgII reveals a 79-87% identity between species (Fischer-Colbrie *et al.*, 1995), the SgII sequences of some non-mammalian vertebrates appear to be very different from the mammalian SgII suggesting a low degree of conservation (Blazquez *et al.*, 1998a). Furthermore, SgII is distributed in the endocrine cells (Rosa and Zanini, 1983) and expressed in some endocrine tumors with other granins (Umemura *et al.*, 2001). Some small peptides derived from SgII play important roles in regulating the nervous and endocrine systems. Among these small peptides, SN, a 33-34 amino acids long peptide, exhibits a stimulatory effect on goldfish LH release *in vivo* when the D_2 receptor is inhibited with DOM (Blazquez *et al.*, 1998a).

1.3.3.1 Distribution and processing of SgII

SgII is distributed widely in neurons and endocrine cells of vertebrates. A SgII fragment, Sg-IIC26-3, is detected throughout the frog intestine (Trandaburu *et al.*, 1998) and stored in the snake enteric serotonin cells (Trandaburu *et al.*, 1999b). Another SgII fragment, SgIIC23-3, not only exists within enteric serotonin cells in lizards and snakes but also is co-stored with neurotensin in the endocrine cells of lizards gut and throughout snake organs (Trandaburu *et al.*, 1999b). Concerning the distribution in mammals, SgII

immunoreactivity (IR) is found in gonadotrophs, thyrotrophs and corticotrophs in human pituitaries using immunohistochemistry (Vallet *et al.*, 1997). SgII mRNA is expressed in some parts of the 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 is also observed in a subpopulation of vasopressinergic magnocellular neurons in rat supraoptic and paraventricular nucleus under osmotic stimulation (Ang *et al.*, 1997). In teleosts, SgII mRNA has been detected in the pituitary using semi-quantitative PCR and Northern blots (Blazquez *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.

Some small functional peptides can be produced from SgII *in vivo*. SN is the main peptide generated from SgII in various rat tissues, including intestine, central nervous system, anterior pituitary, pancreas, and adrenal gland (Leitner *et al.*, 1996). Furthermore, a conserved 66-amino acid SgII product (EM66) is detected in the adult and fetal human adrenal gland (Anouar *et al.*, 1998). The group of enzymes involved in the processing of bovine SgII is called prohormone convertase (PC) including furin/PACE, PC1/PC3, PC2, PACE4, PC4, PC5/PC6 and PC7/LPC (Seidah *et al.*, 1994; Wouters *et al.*, 1998). Only PC1 and PC2 are capable of cleaving the SgII precursor to generate intermediated-sized fragments. PC1 is the only enzyme that catalyzes tissue-specific processing of SgII to yield the neuropeptide SN in neurons (Hoflehner *et al.*, 1995). PC2 is reported to have a pronounced ability to produce free peptides but apparently not to generate SN (Hoflehner *et al.*, 1995; Laslop *et al.*, 1998).

1.3.3.2 Regulation of SgII synthesis and release

SgII synthesis and release are regulated by various factors in neuroendocrine systems. The chronic application of vasopressin induces an increase of SgII synthesis in rat brain (Mahata *et al.*, 1992). In bovine chromaffin cells, histamine can specifically up-regulate SgII mRNA levels by 4 folds without any significant effect on CgA and CgB (Bauer *et al.*, 1993). The neuropeptide adenylate cyclase-activating polypeptide causes an up to 60-fold increment of SgII mRNA level by a rapid and long-lasting stimulation of bovine adrenochromaffin cells (Turquier *et al.*, 2001). Co-localizing with LH in the rat anterior pituitary (Watanabe *et al.*, 1991), the SgII synthesis and release may be regulated by hormones and neurotransmitters along the vertebrate hypothalamo-pituitary-gonad axis. It has been demonstrated that a direct E_2 effect on rat pituitary cells can attenuate the SgII mRNA steady-state level (Anouar and Duval, 1992). GnRH is capable of up-regulating SgII secretion from gonadotrophs in the rat pituitary, and it was recently reported that there was a close correlation between the GnRH-induced release of SgII and LH in the LbetaT2 gonadotroph cell line (Conn *et al.*, 1992; Nicol *et al.*, 2002). The first fish that SgII has been cloned from is the common goldfish (Blazquez *et al.*, 1998a). The GABA metabolism inhibitor γ-vingl-GABA can specifically stimulate SgII gene expression in goldfish gonadotrophs, concomitant with a decrease of LH cellular content and an increase of serum LH level (Blazquez *et al.*, 1998a). Likewise, a GnRH agonist up-regulates the SgII mRNA expression in the PD of goldfish pituitary by decreasing dopaminergic inhibition (Samia, 2002).

1.3.3.3 SN, a biologically active SgII-derived peptide

SN is a 33-34 amino acid neuropeptide derived from the SgII precursor (Kahler and Fischer-Colbrie, 2000), and is the most conserved part of SgII during the polygeny of vertebrates, especially in mammals. However, goldfish SN shares a relatively poor identity to mammal SN, and two sequences, QYTP and LATLEQSVFQEEL, in the middle of 34 amino acid SN peptide are identical to those of mammals (Samia *et al.*, 2001). It is possible that these two stretches determine the biological activity of SN.

1.3.3.3.1 SN is conserved among species:

SN is expressed not merely in various mammalian species but in birds, reptiles, amphibians and fish as well (Table 1.1) (Anouar *et al.*, 1996; Blazquez *et al.*, 1998a; Leitner *et al.*, 1998). The entire SN molecule is highly conserved between mammalian species, such as human, pig, hamster, rat etc. (Kahler and Fischer-Colbrie, 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 to the 33-amino acid homologues in mammals. Only two stretches in the middle of SN including the sequences QYTP and LATLEQSVFQEEL are 100% conserved, whereas its C-terminal end varies considerably between goldfish and mammals (Samia *et al.*, 2001).

1.3.3.3.2 Distribution of SN:

It has been reported that SN is widely distributed in various tissues of some vertebrates. In rat gastrointestinal tracts and pancreas, approximate 20-120 fmol/mg of

secretoneurin immunoreactivity (SN-IR) has been detected, in contrast to 2-5 fmol/mg of SN-IR in the aortic arch, thyroid gland, trachea and the urinary bladder (Leitner *et al.*, 1996). SN 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 high concentration of SN in human anterior and posterior pituitary (Schmid *et al.*, 1995). The highest levels of this peptide are measured in rat hypothalamus, especially median eminence (Marksteiner *et al.*, 1993). Furthermore, SN was observed recently in the rodent central and sympathetic nervous systems; its IR is detected in parenchyma of the rat pineal gland and along the pineal stalk (Simonneaux *et al.*, 1997). SN-like IR is also widely distributed in neurons of the rat hippocampal formation with a preferential localization in excitatory pathways (Schwarzer *et al.*, 1997). The wide distribution of SN suggests that it may have diverse and important biological functions in central nervous systems and endocrine systems.

1.3.3.3.3 Functions and mechanism of action of SN:

SN exhibits many biological activities in both immune and neuroendocrine systems. Firstly, SN is an important peptide in the immune system and involved in inflammatory responses. SN is a potent attractor of human monocytes and significantly augments migration and locomotion of monocytes in response to injury (Reinisch *et al.*, 1993). *In vitro*, SN stimulates not only lung fibroblast chemotaxis but also specific migration, growth and DNA synthesis of rat cultured arterial smooth muscle cells (Kahler *et al.*, 1997b; Kahler and Fischer-Colbrie, 2000). Furthermore, SN might induce

monocytes to migrate mediated by PKC activation and Ca^{2+} release, perhaps via a G-protein related mechanism (Schratzberger *et al.*, 1996b; Wiedermann, 2000). In 1996, it was first reported that SN might affect human neutrophil migration via activating a priming-type receptor *in vitro* (Schratzberger *et al.*, 1996a). In human eosinophils, SN action may be mediated by an independent and specific binding receptor signaling mechanism without interference from other chemotaxis (Dunzendorfer *et al.*, 1998). Furthermore, there are specific high affinity binding sites for SN on a human monocytic cell line, suggesting that SN has a potential functional plasma membrane receptor (Schneitler *et al.*, 1998). However, mechanisms of SN immuno-activities are still unclear to us up to date and require further investigation.

Secondly, SN, functioning as a neuropeptide, has a complex relationship with other essential neuropeptides and steroid hormones. Local infusions of SN into the rat substantia nigra and neostriatum both cause an increase of extracellular glutamate and GABA levels in a concentration-dependent manner (You *et al.*, 1996). Furthermore, SN has a stimulating effect on DA release *in vivo* (Agneter *et al.*, 1995; You *et al.*, 1996). Rat SN is capable of enhancing DA outflow in a concentration-dependent way without marked effects on the outflow of the metabolites, 3, 4-dihydroxyphenylacetic acid and homovanillic acid in the awake rat. Human SN and its 15-amino acid C-terminal sequence can also increase DA outflow *in vivo* (Agneter *et al.*, 1995). *In vitro*, SN calcium-dependently up-regulate the DA release from rat striatal slices in a dose-related manner (Saria *et al.*, 1993). Injection of SN alone *in vivo* does not affect serum LH level in sexually regressed goldfish, whereas SN can rapidly induce a great increment of LH serum concentration when the D_2 receptor is blocked (Blazquez *et al.*, 1998a). To our

knowledge, there has been no report of a SN receptor in the neuroendocrine system. The mechanism of its action is also unclear.

1.4 Objectives of my thesis research

There are two main objectives of the thesis research. We have studied the bioactivity and the regulation of SN in goldfish. With regard to the activity of SN, we set out to explore the possible direct effects of SN on LH release from the pituitary *in vitro*. The only known endocrine effect of SN is the stimulation of LH secretion *in vivo* (Blazquez *et al.*, 1998a). Furthermore, the fish anterior pituitary is unique because it receives direct innervations from neurons of GnRH, DA, GABA and others. Thus, pituitary fragments, containing numerous nerve terminals plus gonadotrophs and somatotrophs, show complex and mutual regulating effects in the reproductive system, such as autocrine, paracrine and neuroendocrine. We studied the effects of synthetic goldfish SN on LH release from goldfish pituitary fragments in static and perifusion incubations. We also set out to explore the dose-response relationship between SN treatment and LH release. Furthermore, to determine possible effects on LH synthesis, we measured LHβ mRNA level in pituitary fragments after the SN treatment in static incubation. GH and SgII are also expressed in the pituitary; therefore we also studied the effect of SN on these two genes.

Secondly, we set out to study the production, regulation and distribution of SgII-derived peptides in goldfish. Given that SgII-derived peptides were detected in the goldfish pituitary, we attempted to measure variations of SgII product levels following

treatments of GnRH, DOM and GABA. We expected to find SN-like peptides generated from SgII in the goldfish pituitary and perhaps other tissues.

1.5 Organization of my thesis

My thesis includes five chapters: the general introduction (Chapter 1), three individual studies concerning SN (Chapter 2-4), and the general discussion with major conclusions (Chapter 5). As a series of chapters, some of which are intended for publication, are in manuscript format, with a necessary but minimal repetition in the introduction of each independent data chapter.

Table 1.1: Clustal W alignment sequences of SN from various species showing the identities with human SN. A consensus sequence and only amino acids differing from human SN are shown. Adapted from Samia *et al.*, 2001.

Species	Sequence	Identity(%)
human	TNEIVEEQYTPQSLATLESVFQEL.GKLTGPN.NQ	100
hamster	----------.-------.--	100
pig	----------.-------.--	100
rabbit	----------.-------.--	100
mouse	----------.------s.--	97.0
rat	----------.------s.--	97.0
bovine	----------n----------.-------.s-	93.9
chicken	----------.--ma--s.-h	87.9
guinea-pig	----i------ln----------.----r--.s-	84.8
xenopus	-s----g-----n----q------.---k-qg.-h	75.8
rana	------g----------q------.---k-qa.-n	81.8
goldfish	---na-------k----q---e--s-.iaas-a-s	67.8
Consensus	t e e qytp latl*svf*el g	

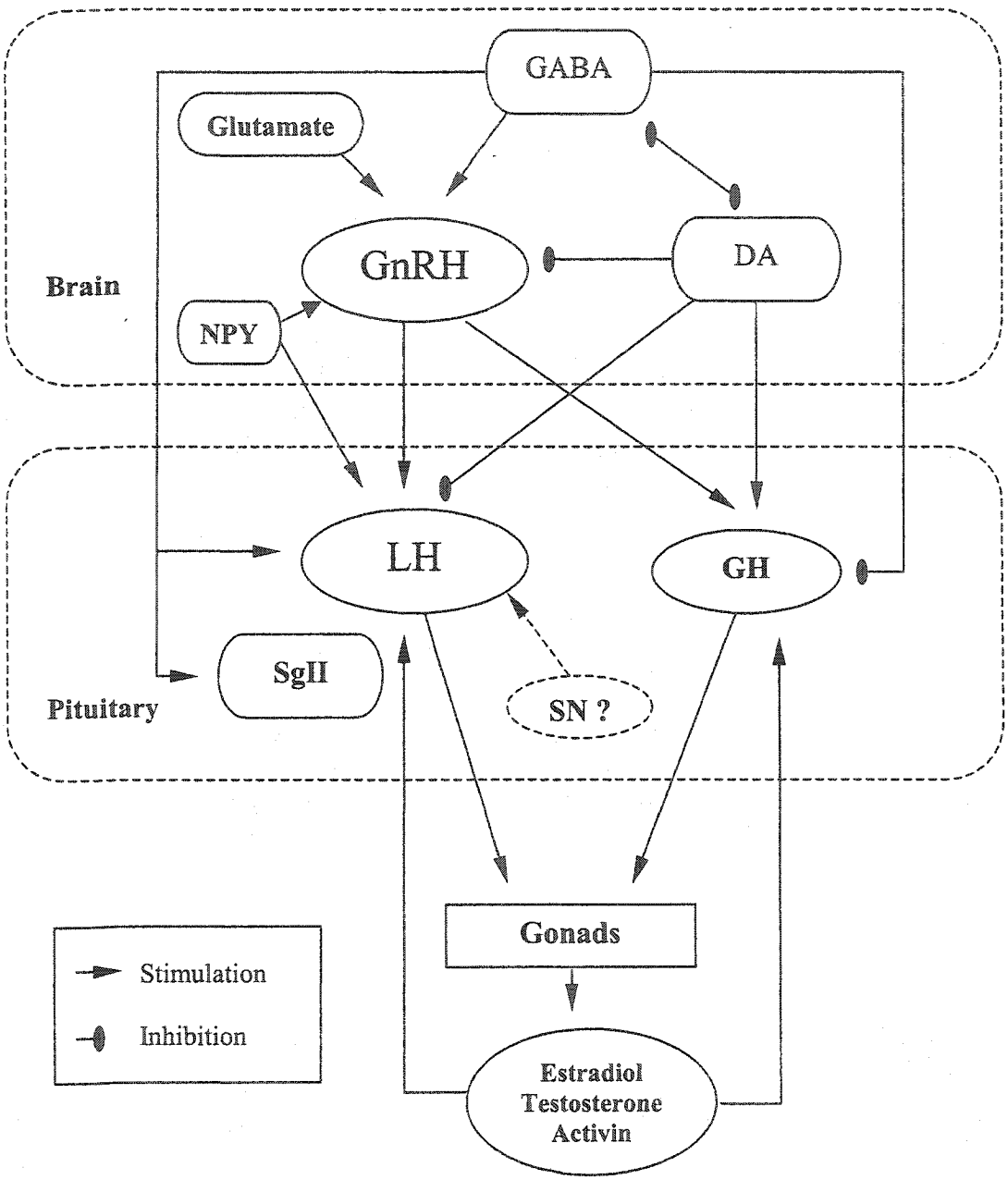


Figure 1.1: Neuroendocrine model of integrated differential regulation of LH and GH release in goldfish. Stimulatory (arrow) and inhibitory (ellipse) effects are indicated. Sex steroid having similar effects on LH and GH are grouped functionally but it does not mean that they are secreted from the same neurons. Abbreviations: GABA, γ-aminobutyric acid; DA, dopamine; GnRH, gonadotropin-releasing hormone; NPY, neuropeptide Y; LH, luteinizing hormone; GH, growth hormone; SgII, secretogranin II; SN, secretoneurin. This model is modified from Trudeau, 1997 and Blazquez *et al.*, 1998b.

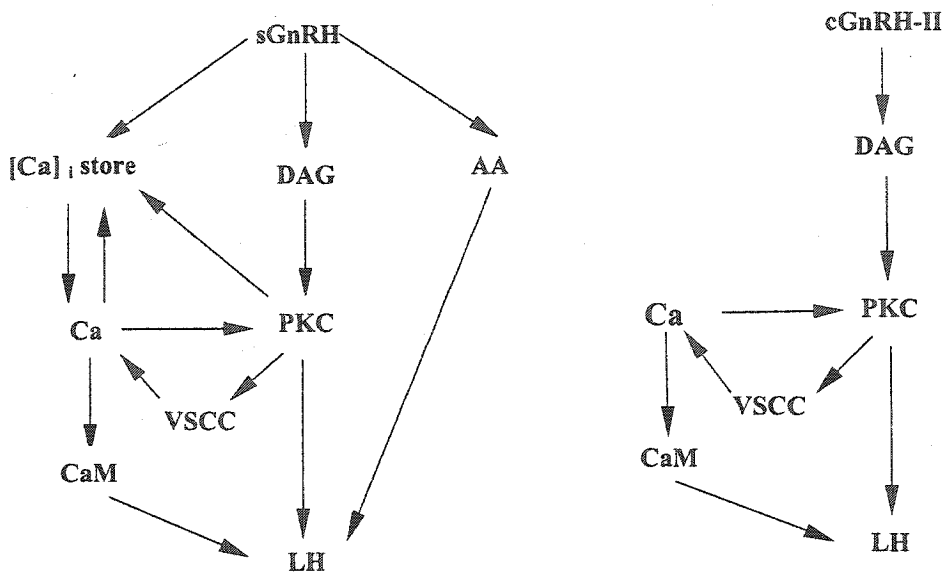


Figure 1.2: Models of major intracellular transduction pathways mediating sGnRH and cGnRH-II stimulated LH release in goldfish. Abbreviations: sGnRH, salmon gonadotropin-releasing hormone; cGnRH-II, chicken gonadotropin-releasing hormone II; DAG, diacylglycerol; PKC, phospholipid-dependent protein kinase C; AA, arachidonic acid; VSCC, voltage-sensitive Ca^{2+} channels; LH, luteinizing hormone; Ca, calcium ion; $[Ca]_i$ store, intracellular calcium store; CaM, calmodulin. Adapted from Chang and Jobin, 1994.

Chapter 2: Effects of secretoneurin on luteinizing hormone production by the goldfish pituitary *in vitro*

2.1 Introduction

The goldfish is a widely used model for studying the neuroendocrine regulation of the reproductive system. It has a special hypothalamo-pituitary-gonad axis where neurons can directly innervate the pituitary to release neuropeptides and neurotransmitters (Blazquez *et al.*, 1998b). In the goldfish anterior pituitary, luteinizing hormone (LH) released from gonadotrophs regulates gonadal steroidogenesis, gametogenesis and ovulation/ sperm release (Trudeau, 1997) while growth hormone (GH) produced from the somatotrophs not only regulates body growth but also potentiates LH-induced steroid production in carp (Van Der Kraak *et al.*, 1994). The fish gonadotropins are heterodimeric glycoproteins composed of a common α-subunit and a hormone-specific β-subunit. Thus, β-subunit is validated as a standard for use in immunoassay, receptor-binding assays and physiological studies (Schulz *et al.*, 2001). In brain, gonadotropin-releasing hormone (GnRH, both sGnRH and cGnRH-II in goldfish) and dopamine (DA) are respectively the main stimulator and inhibitor of LH secretion from the anterior pituitary (Peter *et al.*, 1986; Chang *et al.*, 1993; Trudeau, 1997). Concerning the regulation of GH release, GnRH and DA are capable of stimulating GH release from somatotrophs in the goldfish pituitary (Blazquez *et al.*, 1998b).

Recently, secretogranin II (SgII), a main member of chromogranin family of secretory vesicle proteins was sequenced in the goldfish (Blazquez *et al.*, 1998a). SgII was first characterized in the bovine and rat anterior pituitary (Rosa and Zanin, 1981). In rat pituitary, SgII is co-localized with LH and released from the individual

adenohypophysial gonadotrophs *in vitro*, which is detected using immunohistochemistry. Also, GnRH causes a potent dose-response related increase of SgII secretion from cultured rat pituitary cells (Wei *et al.*, 1995). In contrast to GnRH effects, treatment with the GABA drug γ-vinyl-GABA *in vivo* promotes a 5-fold increment of SgII mRNA levels in the regressed goldfish pituitary, concomitant with the increase of LH secretion. This suggests that SgII or related processed peptides may be co-stored and secreted with LH in the goldfish pituitary (Blazquez *et al.*, 1998a). Currently, it is reported that treatment with sGnRH analog together with a dopaminergic inhibition *in vivo* can stimulate SgII mRNA expression in the *pars distalis* (PD) of goldfish pituitary concomitant with an increase of serum LH level (Samia, 2002). SgII is also distributed in other endocrine and nervous systems in different vertebrate species. SgII not merely is co-stored with histamine in gastric cells of fish and lizard (D'Este and Renda, 1995), but also shows a wide distribution in the different regions of rat brain; for example, SgII mRNA is significantly expressed in several regions of hippocampus, amygdala, thalamic nuclei and pyriform cortex (Mahata *et al.*, 1991).

Although SgII occurs in various cells of vertebrate endocrine and nervous systems, its function remains unclear. There are two major possible functions of SgII. Firstly, SgII plays an intracellular role in packaging and sorting of regulatory peptides and/or hormones into secretory granules with which they coexist (Rosa *et al.*, 1985a; Rosa *et al.*, 1985b). Secondly, SgII perhaps has an extracellular bioactivity to control the local hormones and/or peptides release after its secretion at exocytosis (Huttner *et al.*, 1991). SgII undergoes proteolytic processing to generate some small peptides mediated by the prohormone convertases PC1 and PC2 in mammals (Hoflehner *et al.*, 1995), suggesting

that the SgII-derived peptides maybe play some roles in the regulation of neuroendocrine systems. Secretoneurin (SN) is one of the most important peptides arising from the proteolytic cleavage of SgII.

As a fragment potentially derived from SgII, goldfish SN consists of 34 amino acids and may regulate LH release from the goldfish pituitary *in vivo* (Blazquez *et al.*, 1998a). SN is widely distributed in various tissues of the brain and endocrine systems. There exists a high concentration of this peptide in human anterior and posterior pituitary (Schmid *et al.*, 1995) and rat hypothalamus (Marksteiner *et al.*, 1993). This attracts us to explore its function in the goldfish pituitary. It is reported that PC1 is the only prohormone convertase involved in cleaving SgII precursor at dibasic amino acid sites to generate the fragment of SN in mammals (Hoflehner *et al.*, 1995). This processing occurs to a high degree in brain, intestine, endocrine pancreas, thyroid gland and posterior pituitary of rats (Leitner *et al.*, 1996). Factors known to regulate the production and secretion of SN include histamine and pituitary adenylate cyclase-activating polypeptide (Bauer *et al.*, 1993; Turquier *et al.*, 2001).

Although SN appears to be conserved in frogs and mammals, goldfish SN shares less than 70% identity as those in mammals. Only two sequences, QYTP and LATLEQSVFQEEL, in the middle of 34-amino acid SN peptide are 100% conserved (Samia *et al.*, 2001). This suggests that this well conserved 15-amino acid sequence of SN peptide may determine potential biological functions for SN, which attracts us to study the role of SN regulating the neuroendocrine system.

With regards to its biological activities, SN not only plays an important role in the immune system and inflammatory response, but also is a new neuropeptide in nervous

and endocrine systems. It has been reported that SN is present in human osteoarthritic joint and its level decreases in the rheumatoid joint, suggesting it is involved in the inflammation (Eder *et al.*, 1997). As a potent attractor, SN can significantly increase migration and locomotion of blood monocytes in response to injury (Reinisch *et al.*, 1993). Furthermore, SN consistently triggers the migration of endothelial cells via the combination of chemotactic and antiproliferative effects, suggesting SN acts as a regulatory peptide of vascular cell functions (Kahler *et al.*, 1997a).

Regarding the function of SN in central nervous and neuroendocrine systems, it was demonstrated that rat SN and its 15-amino acid C-terminal sequence stimulated DA release in the striatum of the awake rat, implying that SN has a strong effect on DA secretion *in vivo* (Agneter *et al.*, 1995). Preganglionic sympathetic neurons projecting to the superior cervical ganglion contain both substance P and SN, which indicates a role for SN in sympathetic nerve activity (Klimaschewski *et al.*, 1995). Although SN is not well studied in teleosts compared to mammals, *in vivo* injection of SN increased serum LH level in goldfish treated with a DA antagonist (Blazquez *et al.*, 1998a). Therefore, it is intriguing for us to explore the neuroendocrine effect of SN on the goldfish pituitary *in vitro*.

Static incubation of goldfish pituitary cells is an *in vitro* method frequently used to study the chronic effects of GnRH and other regulatory factors on LH and GH release. In 1990(a), Chang *et al.* applied the primary static culture to explore GnRH action on dispersed goldfish pituitary cells. Using static incubation, DA was shown to stimulate GH release via a DA type 1 receptor. This effect was mediated through the adenylate cyclase-cAMP-PKC pathway and Ca^{2+} entry (Wong *et al.*, 1994a; Wong *et al.*, 1994c).

Furthermore, static incubation of dispersed cells was the main method used to investigate the effects of norepinephrine (NE) and NE agonists and antagonists (Chang *et al.*, 1991). Concerning our study of SN action, we choose static incubation of goldfish pituitary fragments instead of dispersed cells, because there is no report about direct SN effects on the regulation of goldfish neuroendocrine system *in vitro*. In our pituitary preparation, neuroendocrine nerve terminals innervating the fish anterior pituitary remain in the fragments in close proximity to the endocrine cells, thus approximating the *in vivo* condition.

Previous studies indicated that SN stimulated LH secretion from the goldfish pituitary *in vivo* (Blazquez *et al.*, 1998a). We hypothesize that SN can regulate the LH release from the goldfish pituitary *in vitro*, in a dose-related manner. In order to test this hypothesis, we first set out to study LH release from the cultured goldfish pituitary fragments in treatment with a high dose of SN. Based on these preliminary data, we then determined whether or not SN could induce the pituitary LH secretion in a dose-related manner. The other aspect of our studies was to determine how goldfish pituitary LHβ mRNA levels changed under static treatment with SN *in vitro*. In contrast, variations of pituitary GH and SgII gene expression were also investigated to find other potential actions of SN in goldfish.

2.2 Materials and Methods:

2.2.1 Experimental animals and pituitary tissue collection

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 goldfish were kept indoors in 70-l tanks supplied with flowing, aerated and dechlorinated tap water. The water temperature was maintained at 18 °C and the photoperiodic regime followed the day and night cycle in Ottawa. Goldfish were acclimated for at least 2 weeks before they were used in experiments. Goldfish were anaesthetized using 3-aminobutyric acid ethyl ester and dissected; the pituitary was then removed from the skull. Pituitaries were collected in culture medium (M199 with 2.2 g/l sodium bicarbonate, 5.96 g/l Hepes, 10 mg/l nystatin and 0.1% bovine serum albumin, pH 7.2) on ice.

2.2.2 Neuropeptides

Two main neuropeptides were utilized in the treatment of goldfish *in vitro*: the salmon GnRH analog (sGnRHa; [Des-Gly^{10}, D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}]-LHRH, Peninsula Laboratories) and goldfish SN which we previously synthesized (Blazquez *et al.*, 1998a).

2.2.3 Static incubation of goldfish pituitary fragments

Short-term tissue culture experiments were performed. Each pituitary was chopped to 4 pieces with a razor blade, and fragments of 3-5 pituitaries were incubated in a 35×10 mm petri dish with 3 ml of culture medium. Prior to the incubation, the pituitary fragments were washed and pre-incubated at room temperature (20 °C) for 1 hour. Following pre-incubation, experimental incubations of 6 or 12 hours were set up at room temperature. Treatments included the control (culture medium), 100 nM sGnRHa and SN at various concentrations. The test media were removed from the petri dishes and replaced with the fresh media at the 1st, 3rd and 6th or 12th hours. The test media

samples were stored at -20 °C. They were diluted by a factor of 100 or greater in assay diluent (25 mM sodium barbitone, 40 mM sodium acetate, 0.25 mM thimerosal, 0.5% bovine serum albumin, pH 8.6) and their LH levels were measured by the specific radioimmunoassay (RIA). Pituitary fragments were collected in 15-ml plastic tubes, frozen on dry ice and stored at -80 °C for the Northern blotting analysis.

2.2.4 LH radioimmunoassay

LH concentrations in incubation media were determined by using RIA adapted from Peter *et al.*, 1987. Ninety-six well Dynex Immulon 4 HBX Flat Bottom Microtiter plates (Thermo Labsystems) were filled with 100 µl rabbit anti-carp LH antibody at a dilution of 1:50,000 in diluent with 1:100 normal rabbit serum for each well. A group of 6 wells were filled with normal rabbit serum at a dilution of 1:100 in diluent as blanks to determine non-specific binding. Next, LH was reconstituted at the concentration of 0 (100% binding), 0.19, 0.39, 0.79, 1.57, 3.13, 6.25, 12.5, 25, 50 and 100 ng/ml to establish a standard curve. Then, 50µl of standards (LH in diluent), samples or blanks (diluent only) and 25 µl diluent containing ~18,000 CPM of I^{125} labeled LH were incubated with LH antibody at a final dilution of 1:100,000 or normal rabbit serum at a final dilution of 1:200 at 4 °C for 48 hours. All assays were done in triplicates.

Forty-eight hours later, 50 µl of anti-rabbit IgG (Scottish Antibody Product Unit) at a dilution of 1:10 in diluent was added to each well and incubated for an additional 24-hour period at 4 °C. Next, the plates were centrifuged at 3,000 rpm at 4 °C for 30 minutes. 100 µl of diluent was used to rinse the residual radioactivity from each well. Finally, 100 µl of "Optiphase Supermix" (Wallac) scintillation fluid was added to each well. After 4-

hour incubation, the plates were counted in a Wallac Trilux Microbeta; WiaCalc software was utilized to evaluate the concentration of each sample assay.

2.2.5 RNA isolation

RNA was isolated from goldfish pituitary fragments using the acid guanidinium thiocyanate (GITC)-phenol-chloroform extraction (Chomczynski and Sacchi, 1987). The frozen tissue sample from the static incubation (fragments of 5 pituitaries) with 1-1.5 ml solution D [per 100 ml: 73 ml of DEPC (diethylpyrrocarbonate) treated water, 62.5 g of solid GITC, 4.4 ml of 0.75 M sodium citrate, pH 7.0, 6.6 ml of 10% sarkosyl and 0.3 ml of 2-mercaptoethanol] was sonicated for less than 1 minute on ice. 0.1-0.15 ml of 2 M sodium acetate, pH 4.0 and 0.5-0.75 ml of phenol (saturated with DEPC treated water) were added and vortexed. Then, 0.2-0.3 ml of chloroform was added and the solution was mixed by vortexing. The whole mixture was transferred to 2-ml micro-centrifuge tubes and centrifuged at 12,000 g for 20 minutes. The supernatant was transferred to another tube and 0.15-0.2 ml of high salt buffer (1.2 M sodium chloride, 0.8 M sodium citrate) and 0.6 ml of isopropanol were then added. The whole mixture was incubated at room temperature for 10 minutes and centrifuged at 12,000 g for 15 minutes, and it was then incubated at -20 °C for 20 minutes and centrifuged again at 12,000 g for 15 minutes. The supernatant was removed and the RNA pellet was washed with 2x 80% ethanol with 10-minute centrifugation at 12,000 g after each wash. The RNA pellet was then dried in air for 10 minutes and suspended in 50 µl or less of DEPC treated water. 2 µl were diluted in 98 µl of 0.1X TE (1 mM Tris-Base pH 7.4, 0.1 mM EDTA pH 8.0) for measuring the RNA concentration, and the rest was flash frozen in liquid nitrogen and stored at -80 °C.

A GeneQuant DNA/RNA spectrophotometer (Pharmacia Biotech) was utilized to determine the concentration and purity of the RNA; the purity of the RNA was indicated by the ratio of absorbance at 260 and 280nm (A_{260}/A_{280}). As a reference, the ratio of pure RNA was 2.0. If the concentration of the RNA was low, it was precipitated with 1/10 volumes of 3 M sodium acetate, pH 5.5 and 2.5 volumes of 95% ethanol at -20 °C for 30 minutes, washed with 80% ethanol 3 times, dried and suspended.

2.2.6 Gel electrophoresis and Northern blotting

Total RNA was separated on a denaturing agarose gel by electrophoresis. The gel was made of 1% agarose in 1X MOPS (morpholinopropanesulfonic acid; per liter: 4.8 g of MOPS, 4.1 g of sodium acetate, 2.9 g of EDTA, pH adjusted to 7.0 with 10 N sodium hydroxide) and 1.75% formaldehyde. 10 µg of RNA were taken from the stock and then 2.25 volumes of RNA loading buffer (per 7 ml: 4 ml of formamide, 0.8 ml of 10X MOPS, 0.4 ml of glycerol, 1.4 ml of formaldehyde, 0.01 ml of 0.5 M EDTA, pH 8.0, 0.4 ml of DEPC treated water, 20 mg of bromophenol blue, 3.5 µl of 10 mg/ml ethidium bromide) were added. The mixture was incubated at 65 °C for 20 minutes, quickly chilled on ice and loaded on the gel. The gel was run in the buffer of 1X MOPS at 100 volts for approximately 6 hours. The gel was then washed twice with double distilled water (ddH_2O) for 20 minutes and once in 20X SSC (17.53% sodium chloride, 8.82% sodium citrate, pH adjusted to 7.0) for 20 minutes at room temperature. A piece of Hybond N+ nylon membrane (Amersham) was cut to the same size of the gel, which was washed with ddH_2O and soaked in 2X SSC for 10 minutes. The 24-hour capillary transfer was set up to transfer the RNA from the gel to the membrane. After the transfer, the RNA was cross-linked to the membrane by exposure to ultraviolet light for 5 minutes. Before

hybridization, the membrane was incubated in 0.1X SSC and 0.1% SDS (sodium dodecyl sulfate) at 65 °C for 20 minutes, and it was then pre-hybridized in the hybridization solution (10% dextran sulfate, 1% SDS, 6X SSC, 100 μg/ml sheared herring sperm genomic DNA, 5X Denhardt's solution; 10 ml of solution per 100 cm^2 of membrane) at 65 °C for at least 3 hours. For preparing the radio-labeled probe, 25 ng of cDNA template was diluted in 45 μl of 1X TE (10 mM Tris pH 8.0, 1.0 mM EDTA pH 8.0), denatured at 100 °C for 5 minutes and then chilled on ice. The DNA mixed with the Redi Prime II kit (Amersham) and 50 μCi of radioactive α^{32}P-dCTP at 37 °C for 15 minutes to synthesize the probe. The probe was filtered by Sephadex G50 spin column (Amersham) and denatured at 100 °C. It was then added to the hybridization buffer and reacted with the membrane overnight at 65 °C. The membrane was washed in the solution of 0.1% SDS with different concentration of SSC (0.1X to 1X) at 65 °C for 20 minutes. The wash times depended on the type of hybridization probe and the membrane background level. The membrane was then wrapped by plastic sheet and exposed to an Imaging Screen-K (BIO-RAD) for 1 hour or overnight. The K-screen was scanned using the Molecular Imager FX (BIO-RAD); the Quantity One (BIO-RAD) software was utilized to quantify each band. To control for RNA loading, every membrane was probed with goldfish 18S ribosomal RNA and β-actin mRNA under similar conditions. The LHβ [AB015596; forward primer 5'-GAGCATGCGAGAGTTAGGCG-3', reverse primer 5'-CGGACAAGGGACAGTATCGC-3'], GH [AF069398; forward primer 5'-ATGGCTAGAGCATTAGTCCTG–3', reverse primer 5'-AGGGTCCAGGTTGAATCC AG-3'], SgII [AF046002; forward primer 5'-TTCTTACCACGCTACAACAGACC-3', reverse primer 5'-GACATCATCGTCTTCTTCGTCCT-3'], 18S [AF047349; forward

primer 5'-GAGCCTGAGAAACGGCTACC-3', reverse primer 5'-GTATTCAGCGGC GACAGG-3'] and β-actin [AB039726; forward primer 5'-ATTTAGGTGACACTATAGGATGGTGGGAATGGGTCA-3', reverse primer 5'-TAATACGACTCACTATAGGGACAGCACAGCCTGGAT-3'] cDNA templates were amplified by reverse transcription-polymerase chain reaction (RT-PCR) from goldfish total RNA and cloned into the PCRII-TOPO vector using the Invitrogen TOPO-TA cloning kit. The sizes of the LHβ, GH, SgII, 18S and β-actin cDNA templates were respectively 462, 580, 471, 551 and 304 base pairs. Generations of the cDNA templates were performed by Suzanne Chiu, Marwan Samia, Benjamin Hibbert and Katherine Larivere.

2.2.7 Statistical analysis

The relative expression is the ratio of mRNA level and 18S ribosomal RNA or β-actin mRNA level averaged over a given Northern blot membrane. The statistical analyses were performed using Sigma Stat version 2.03 software. One-way Analysis of Variance (ANOVA) was chosen to assess the difference from control at a given time period, followed by Fisher LSD test. When the data were non-parametric, the Kruskal-Wallis one-way ANOVA on Ranks followed by Student-Neuman-Keuls or Dunn's method was used as appropriate. A difference with $p<0.05$ was considered to be significant. The Q-test was used on 1 occasion to determine whether or not a suspected outlier datum point could be statistically rejected (Sokal and Rohlf, 1981).

2.3 Results

2.3.1 Effects of SN on LH release from goldfish pituitary fragments

In the control group, distinct patterns of LH levels were observed at 1st, 3rd and 12th hour (Figure 2.1). The highest concentration of LH occurs after 12-hour incubation while the lowest was detected at the 3rd hour. Treatments with 100 nM sGnRHa elevated the medium LH levels by approximately 171% at the 3rd hour and by 210% at the 12th hour, both of which were significantly different ($p<0.05$) from the time-matched control. In contrast, no effect of sGnRHa was evident at the 1st hour. Incubation with 500 nM SN did not affect LH levels at the 1st and 12th hours. However, the addition of 500 nM SN for 3 hours did increase in the medium LH-level by 141% compared to the time-matched control ($p<0.05$).

2.3.2 Dose-related effect of SN on LH release in the static incubation

Different concentrations (10 nM, 100 nM and 1000 nM) of SN were administered for 6 hours in a static incubation experiment in October (Figure 2.2). The levels of medium LH decreased from 1-hour to 6-hour incubation in the control group. No significant effect of sGnRHa was observed at the 1st and 3rd hours. The LH levels at 6-hours of exposure to 100 nM sGnRHa were 3.9-fold higher than that of the control ($p<0.05$). After 3- and 6-hour incubations with 10 nM SN, LH levels were 2.5- and 5-fold higher than the control ($p<0.05$). In contrast, there were no statistically significant differences in LH levels following incubations with 100 or 1000 nM SN at any time point.

Different levels (1 nM, 10 nM and 100 nM) of SN were chosen for a second time and dose-response study in April when the goldfish pituitary is known to be highly

responsive to GnRH (Figure 2.3). In the control group, the highest medium level of LH occurred at the 1st hour, and decreased at the 3rd and 6th hour, similar to previous experiments. The addition of 100 nM sGnRHa enhanced ($p<0.05$) LH levels by 1.9-, 4.5- and 2.6-fold respectively at the 1st, 3rd and 6th hour (Figure 2.3), demonstrating high sensitivity to GnRH. This was in contrast to the results in October (Figure 2.2) when GnRH responsiveness was lower. Exposure to 1 nM SN did not affect LH levels at any time point. However treatment with 10 nM SN induced 2.3-, 2.5- and 2.2-fold increase in LH release respectively at the 1st, 3rd and 6th hour. Exposure to 100 nM SN also affected LH release but the effects were less evident than those seen after the 10 nM SN treatments; 100 nM SN had no effect at the 1st hour, induced a statistically significant ($p<0.05$) 1.8-fold increase after 3 hours and a 2-fold but statistically non-significant increase ($p=0.06$) after 6 hours.

2.3.3 Pituitary LHβ mRNA expression after the static incubation

The mRNAs were isolated from goldfish pituitary fragments after 6-hour static incubation. The 18S ribosomal RNA and β-actin mRNA levels of pituitary fragments were not modified by any treatment. Treatment with 100 nM sGnRHa increased the LHβ mRNA levels by 1.6-fold using 18S ribosomal RNA as a loading control, but this was not different from the control values. In contrast, treatment with 500 nM SN induced a 2.6-fold increment ($p<0.05$) in LHβ gene expression in the goldfish pituitary *in vitro*, using either 18S ribosomal RNA or β-actin mRNA as internal standards (Figure 2.4).

2.3.4 *Pituitary GH and SgII mRNA expressions after the static incubation*

Treatments showed similar patterns of mRNA expression to those of the control (Figure 2.5). The pituitary GH and SgII gene expressions were not modified by the 100 nM sGnRHa treatment *in vitro*. Treatment with 500 nM SN also caused no significant variation in the GH and SgII mRNA levels from control after 6-hour static incubation.

2.4 Discussion

SN was previously demonstrated to be widely distributed in the rat central nervous and neuroendocrine system, and this neuropeptide appears to have essential biological activity in the mammalian central nervous system, like stimulating the DA release (Agneter *et al.*, 1995). However, the biological function of SN in other vertebrate groups is largely unexplored. This study demonstrates that SN modulates not only the release of LH from the goldfish pituitary but also LHβ gene expression *in vitro*, providing evidence that SN has a potential role in the regulation of fish reproduction.

Several different *in vitro* methods have been used to study the LH regulation in the goldfish, such as static incubation and perifusion experiment with dispersed pituitary cells. In our static incubation experiments, relatively large pituitary fragments were cultured in culture medium and directly stimulated by various concentrations of SN. In every experiment, treatment with sGnRHa, a proven LH stimulator, consistently stimulated LH release from goldfish pituitary fragments. This indicates that goldfish pituitary fragment in static incubation is a useful experimental model to study LH secretion *in vitro*. Additionally, the chosen incubation time was normally less than 12 hours because it has been recently demonstrated that apoptosis of gonadotrophs occurs in

the goldfish pituitary after 6-hour static incubation *in vitro* (Therriault, 2003). Furthermore, it became very difficult to isolate adequate amounts of total RNA from goldfish pituitary fragments if the incubation time increased to more than 6 hours (data not presented).

Three-hour treatment with 500 nM SN promoted a significant increase of LH levels by approximately 141%, which was an encouraging preliminary observation. This supports previous *in vivo* data showing that SN induces a robust increase of LH serum levels when goldfish were pretreated with the specific D_2 receptor antagonist domperidone (DOM) to inhibit DA action in the pituitary (Blazquez *et al.*, 1998a). Different from the physiological environment of LH secretion under a tonic and potent DA suppression *in vivo*, dissection of the pituitary gland cuts the neuroendocrine neurons innervating the PD of the pituitary thereby reducing DA inhibition on gonadotrophs. Therefore, the data of SN action *in vitro* and *in vivo* suggests that this SgII-derived peptide has a biologically significant effect on the regulation of the neuroendocrine system. Similarly, peptides derived from other chromogranins are reported to regulate endocrine cell function. For example, chromostatin, a 20-residue peptide derived from chromogranin A, is capable of suppressing catecholamine release from the chromaffin cells in the adult bovine adrenal medulla *in vitro* (Galindo *et al.*, 1992). Furthermore, some biological activities of SN have been found in the regulation of the mammalian neuroendocrine system. Studies in the superfusion of rat striatal slices demonstrate that SN dose-dependently increases DA outflow *in vitro* (Saria *et al.*, 1993). Thus, it can be concluded that SN is an important functional peptide derived from SgII in goldfish and has pronounced stimulatory effects on LH release from the pituitary *in vivo* and *in vitro*,

which may contribute to a positive feedback control of gonadotroph function. SgII and SN are produced by the pituitary and are markers for the regulated cellular section pathway (Conn *et al.*, 1992). Upon stimulation of the gonadotroph by GnRH or other releasing factors, SN could act on gonadotrophs by a paracrine or autocrine mechanism to further increase LH release.

In order to further understand mechanisms of SN action, we studied the time and dose-response relationship of LH release. 10 nM SN consistently stimulated LH release from the pituitary of sexually regressed or mature goldfish after 3- and 6-hour static incubation, whereas low (1 nM) and high (1000 nM) concentrations of SN had no significant effect in these two experiments (Figure 2.2 and Figure 2.3). On the other hand, 100 nM SN showed a stimulating effect on LH release in April when the pituitary gland is sensitive to LH-releasing factors. The LH dose-response curves to SN were 'bell-shaped' indicating this peptide stimulates LH secretion over a narrow concentration range. Level of SN in the rat anterior pituitary is approximately 6.6 nM (Leitner *et al.* 1996), which is similar to the concentrations of SN stimulating LH release in static culture of goldfish pituitary fragments.

The basis of the 'bell-shaped' dose-response curve is not understood, however three inferences below are possible. Firstly, a high level of SN may produce a toxic effect in the pituitary. Secondly, a high concentration of SN might potentiate inhibitory functions, for eample, release of DA from remaining nerve terminals in the pituitary fragments to decrease LH release. In this regard, it is interesting to note that SN can dose-dependently increase the outflow of DA from rat striatal slices *in vitro* (Saria *et al.*, 1993). Thirdly, excessive amount of this peptide might have a negative feedback effect on the

SgII processing and SN secretion in the pituitary, which may also influence LH release. In addition, 'bell-shaped' dose-response curves have been observed for other regulators of LH release from the goldfish pituitary. It is not higher (micromolar) but lower dose (nanomolar) of NE that stimulates LH secretion in the goldfish gonadotrophs (Chang *et al.*, 1991). Although we still have difficulties in understanding SN action, the similarities in the results from the two experiments (Figure 2.2 and Figure 2.3) indicate that the goldfish gonadotroph may have a 'bell-shaped' dose-response characteristic to SN.

Another possible explanation for the lack of effects for high dose of SN is the fact that many biological agents and drugs display hormetic dose-response relationships where a modest stimulation of response is observed at low doses and inhibition of response is found at high ones (Calabrese and Baldwin, 2003a and 2003b). It is not unique that a dual regulatory influence of a hypothalamic factor on the release of a pituitary hormone depends on concentration. For example, DA administration produces a dose-related biphasic response pattern for the release of prolactin from rat lactotrophs with a low dose being stimulatory and a high dose being inhibitory (Kineman *et al.*, 1994). Furthermore, somatostatin is well-known to up-regulate the basal secretion of GH from cultured porcine somatotrophs at low but physiologically relevant dose but inhibiting growth hormone releasing factor induced GH release at a high dose *in vitro* (Ramirez *et al.*, 1998). In a recent microarray analysis study, Coser *et al.* (2003) demonstrated 'bell-shaped' dose responses to estradiol (E_2). Whereas very low levels of E_2 suppressed more genes than it induced, higher concentrations induced more genes than it suppressed. This is probably the first study to suggest that non-classical 'bell-shaped' dose-response comes from a molecular basis. Hormetic responses of some peptides may

be explained by the down-regulation of their relevant receptors. Thyrotropin-releasing hormone (TRH) receptors are highly sensitive to down-regulation by TRH in the chicken pituitary (Harvey and Baidwan, 1990), while TRH induces GH secretion possibly mediated by TRH receptors in chicken (Harvey and Baidwan, 1989). It is also reported that TRH causes a down-regulation of TRH-receptor mRNA in rat pituitary GH3 cell line while also increasing TRH-receptor mRNA degradation (Fujimoto *et al.*, 1992). In an investigation of GnRH autoregulation of GnRH receptor expression in human granulosa-luteal cells, low doses and short-term treatments of GnRH up-regulate its receptor whereas high doses or long-term treatments down-regulate receptors (Peng *et al.*, 1994). Biphasic autoregulation of receptors perhaps provides an explanation of the hormesis-like dose-response relationship of SN on LH release. Further studies are necessary to define this dose-response and to confirm the existence of SN receptors in the goldfish pituitary. To date, the putative SN receptor has not been cloned and sequenced in any species, although monocytes have been shown to bind iodinated human SN with high affinity in the nanomolar range (Schneitler *et al.*, 1998).

SN also had time-dependent effects on LH release in static incubation. The data out of the three experiments suggest that 3-hour static treatment was optimal for LH release. Seasonal control of reproduction is important in many teleosts in temperate climates. A strong LH release from the pituitary will result in the maximal annual LH levels to induce spermiation or ovulation in the sexually mature goldfish (Blazquez *et al.*, 1998b). Seasonal variation in the pituitary gland responsiveness is maintained *in vitro* (Habibi *et al.*, 1992; Johnson and Chang, 2002). This was also seen in the present experiments where a 1-hour treatment with SN or sGnRHa caused a significant

stimulation on LH release from the sexually mature goldfish pituitary (Figure 2.3), whereas a 1-hour exposure to either SN or sGnRHs didn't affect LH release from pituitaries of sexually regressed goldfish (Figure 2.2). Likewise, a 3-hour exposure to 100 nM SN increased LH release from pituitaries of sexually mature but not sexually regressed goldfish.

SN may also have a role in stimulating LH production in addition to effects on LH release. A 6-hour static treatment with 500 nM SN induced a 2.6-fold increase in LHβ subunit mRNA levels in pituitary fragments, regardless of whether LHβ mRNA levels were expressed relative to 18S ribosomal RNA or β-actin mRNA. In contrast, treatment with 100 nM sGnRHa for 6 hours stimulated only a modest and statistically non-significant 1.6-fold increase in LHβ/18S mRNA expression ratio. It was reported previously that GnRH increased cellular levels of mRNA of LHβ-subunit in monolayer cultures of rat pituitary between 6 and 24 hours (Andrews *et al.*, 1988). In the static incubation of dispersed goldfish pituitary cells and fragments, 12-hour treatment with 100 nM sGnRH is required to significantly elevate LHβ subunit mRNA expression (Klausen *et al.*, 2001). The explanation of the difference in our results and those from the previous studies is that the incubation time we applied (6 hours) is much shorter (at least 12 hours) so that sGnRHa is unlikely to affect LHβ expression within 6 hours. Thus, SN is capable of more rapidly stimulating LHβ subunit expression than a high dose of a potent sGnRH analogue.

The effects of SN on LHβ appear to be specific because in the same pituitary fragments SN had no effect on the GH and SgII mRNA expression relative to both 18S ribosomal RNA and β-actin mRNAs (Figure 2.5). Furthermore, sGnRHa did not alter GH

and SgII mRNA levels significantly following 6-hour static incubation. However, it is reported previously that sGnRHa can increase GH mRNA levels *in vitro* in the goldfish pituitary, which occurs after 12-hour treatment (Klausen *et al.*, 2001). In addition, the former study *in vivo* in our laboratory indicated that treatments with sGnRHa plus DOM cause an increase in GH mRNA expression in the goldfish pituitary after 24 hours (Samia, 2002). The variation between the former and present study might be related to shorter exposure periods and the contrasting *in vivo* and *in vitro* models used. In brief, SN might not regulate the SgII and GH gene expression in the goldfish pituitary *in vitro*. However, this requires further investigation.

In summary, data from the present study provides the first evidence for a direct stimulatory action of SN on LH release and LHβ subunit mRNA expression in the vertebrate pituitary *in vitro*, supporting the hypothesis that SN has a reproductive role. Moreover, SN had a 'bell-shaped' dose-related manner on the regulation of LH release from the goldfish pituitary *in vitro*. SN significantly stimulated LH secretion in a narrow concentration range of 10-100 nM, perhaps indicating the presence of a sensitive SN receptor on gonadotrophs in the PD of goldfish pituitary. This investigation provides the basic data for further studies about the roles of SN in the physiological modulation of LH release, the location and structure of SN receptors, as well as the signal transduction mechanisms mediating SN effects.

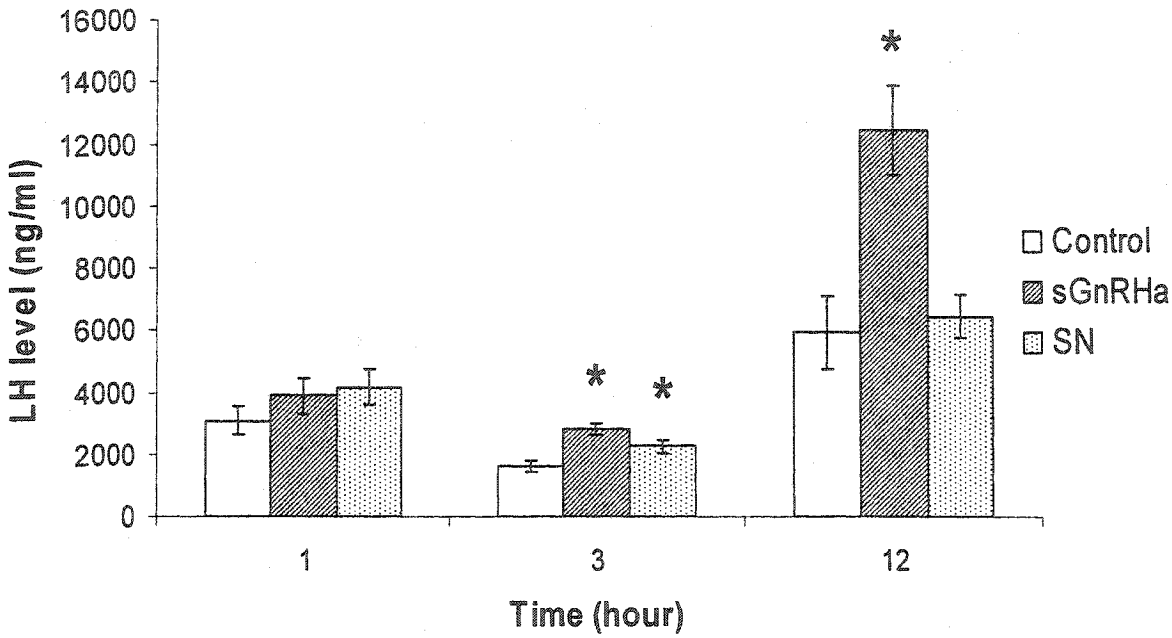


Figure 2.1: Comparisons of LH level response to control, 100 nM sGnRHa and 500 nM SN in the static incubation of goldfish pituitary fragments. Results presented (mean ± SE) were pooled data from 5 separate experiments (n=14 for control and sGnRHa and n=19 for SN) that were performed between November and February. One-way ANOVA was chosen to assess the difference from control at a given time period, followed by Fisher LSD test. When the data were non-parametric, the Kruskal-Wallis One-way ANOVA on Ranks followed by Dunn's method was used. The (*) indicates a significant difference from control at a given time point ($p < 0.05$).

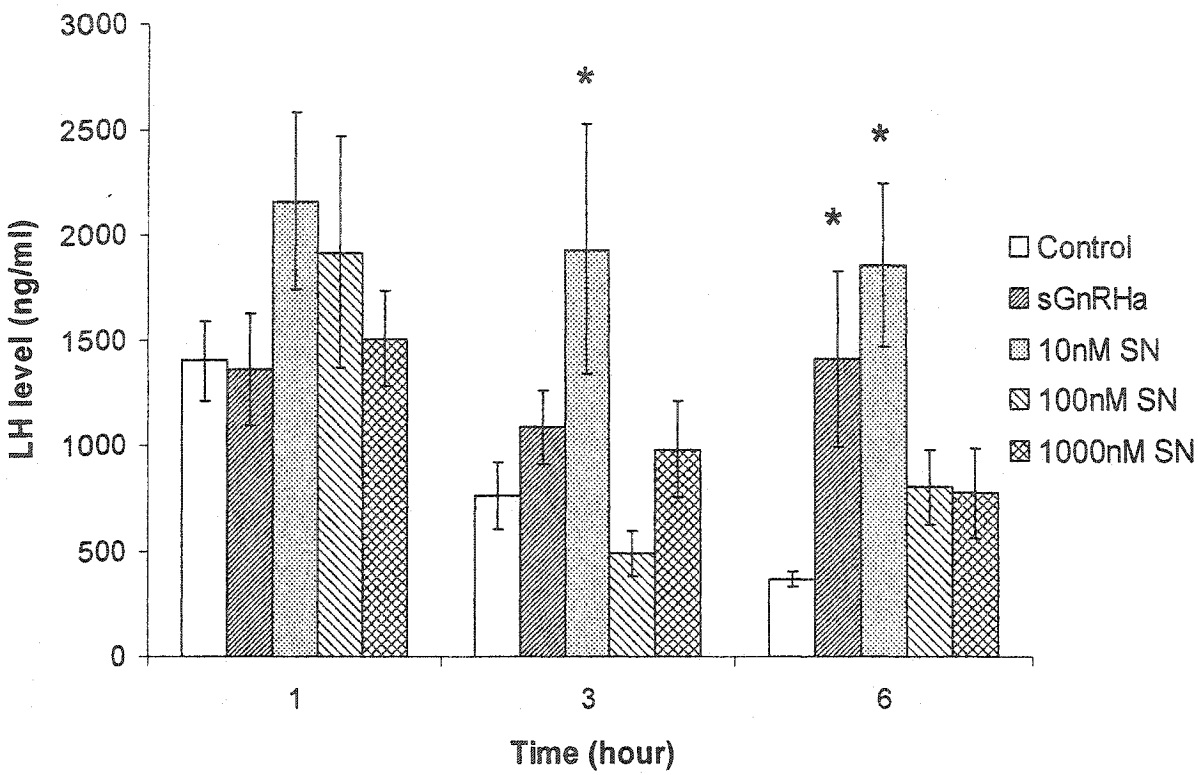


Figure 2.2: Time and dose-dependent actions of SN (10, 100 and 1000 nM) on LH release from the goldfish pituitary fragments in the presence of 100 nM sGnRHa. Results presented mean ± SE, n=6 for control, sGnRH, 100 nM SN and 1000 nM SN; n=5 for 10 nM SN. This experiment was performed in October when goldfish were at the beginning of seasonal sexual redevelopment. One-way ANOVA was chosen to assess the difference from control a given time period, followed by Fisher LSD test. The (*) indicates a significant difference from control at a given time point ($p < 0.05$).

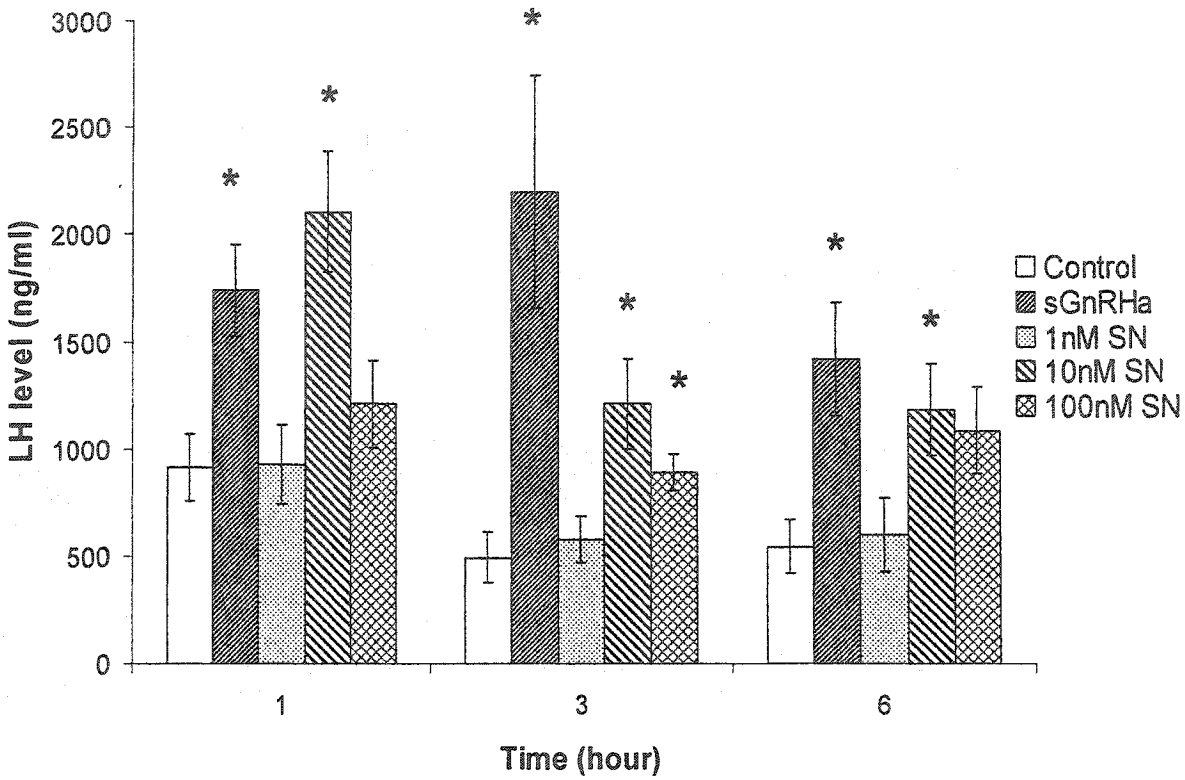
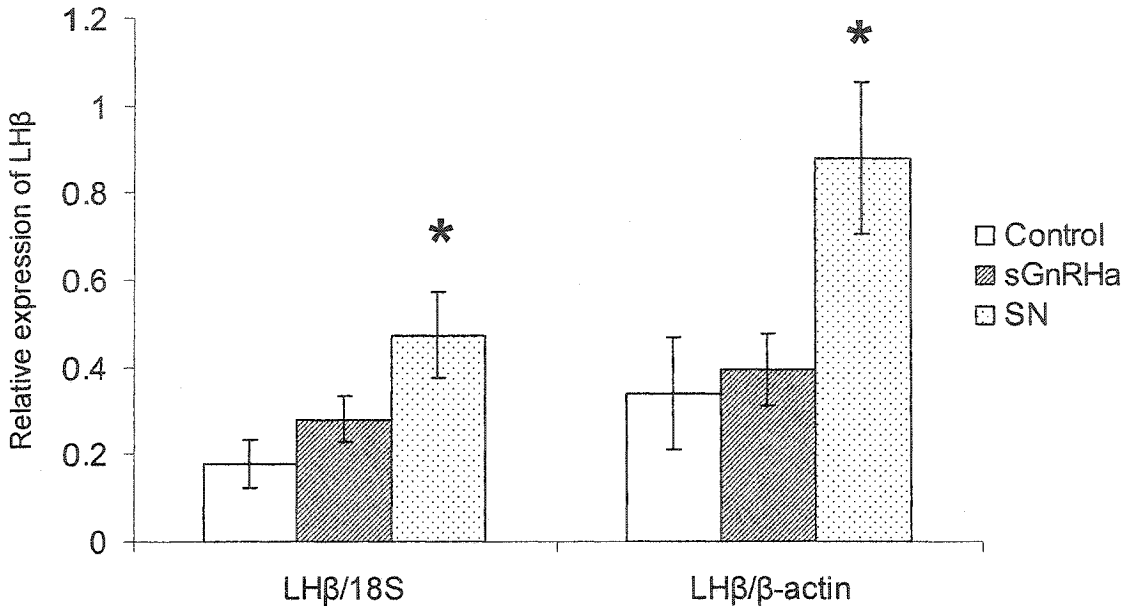


Figure 2.3: Time and dose-dependent actions of SN (1, 10 and 100 nM) on LH release from the goldfish pituitary fragments in the presence of 100 nM sGnRHa. Results presented mean ± SE, n=9. This experiment was performed in April when goldfish were in sexually mature pre-spawning goldfish. One-way ANOVA was chosen to assess the difference from control at a given time period, 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 used. The (*) indicates a significant difference from control at a given time point ($p < 0.05$).

A



B

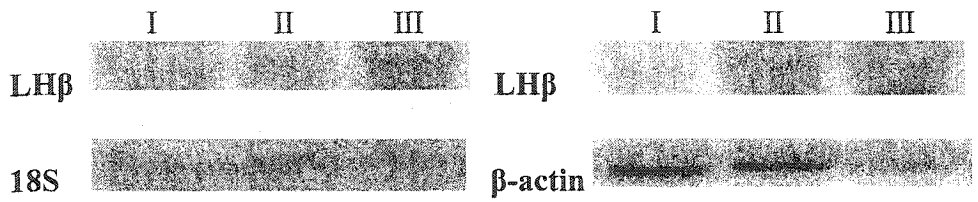


Figure 2.4: A: LHβ mRNA levels of goldfish pituitary fragments after 6-hour static incubation with treatments of 100 nM sGnRHa or 500 nM SN. The mRNA levels were measured by Northern blots; 18S ribosomal RNA and β-actin mRNA were used as internal standards for RNA loading control. Results represented mean ± SE, n=5 for control (I), n=3 for sGnRHa (II), and n=4 for SN (III). This experiment was performed in June when goldfish were in the sexual mature period. One-way ANOVA was chosen to assess the difference from control at a given time period, followed by Fisher LSD test. The (*) indicates a significant difference from control at a given time point ($p < 0.05$). **B:** Representative photomicrographs of Northern blots for LHβ mRNA, 18S ribosomal RNA and β-actin mRNA.

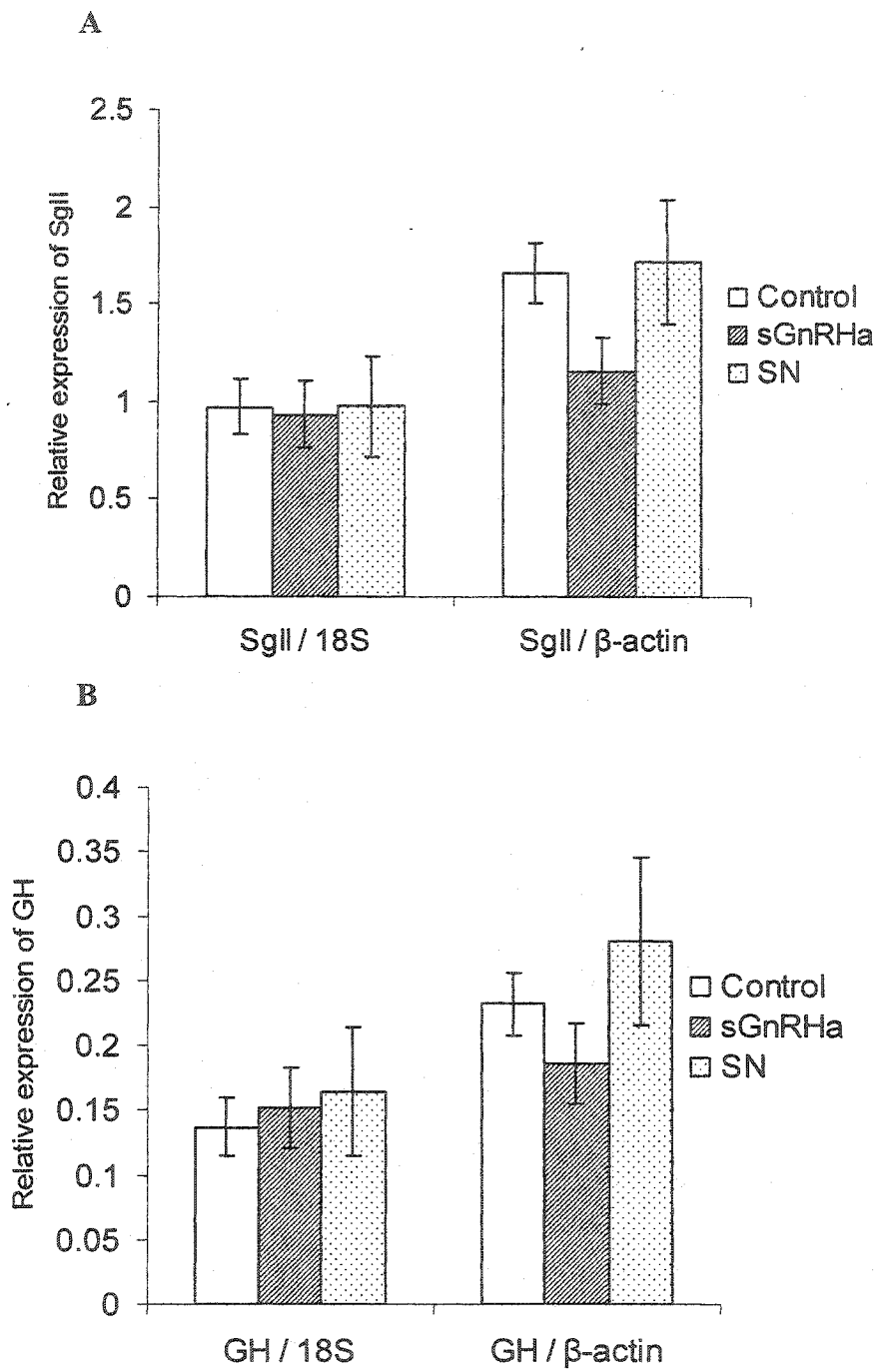


Figure 2.5: SgII and GH mRNA levels of goldfish pituitary fragments after 6-hour static incubation with treatments of 100 nM sGnRHa or 500 nM SN. The mRNA levels were measured by Northern blots; 18S ribosomal RNA and β-actin mRNA were used as internal standards for RNA loading control. Results represented mean ± SE, n=5 for control and sGnRHa, and n=4 for SN. This experiment was performed in June when goldfish were in sexually mature period. One-way ANOVA was chosen to assess the difference from control at a given time period, followed by Fisher LSD test. The statistical analysis showed the treatment groups with similar values ($p>0.05$).

Chapter 3: Effects of secretoneurin on pituitary luteinizing hormone release in an *in vitro* perifusion system

3.1 Introduction

As described in Chapter 2, secretoneurin (SN) induced a stimulatory effect on goldfish pituitary fragments to release and synthesize luteinizing hormone (LH) in static incubation. In order to study this function *in vitro* from another aspect, we designed a perifusion experiment to explore the acute time and dose-course action of SN on LH secretion.

Perifusion of goldfish pituitary cells and fragments is widely utilized in the study of acute effects of gonadotropin-releasing hormone (GnRH) and others regulatory factors on the LH and growth hormone (GH) release. Since 1984, Chang *et al.* investigated the direct dopamine (DA) inhibition of spontaneous LH release, the DA blockade of GnRH actions, and the norepinephrine stimulation of LH secretion in a column perifusion system of dispersed goldfish pituitary cells and fragments. Trypsin-dispersed goldfish pituitary cells in perifusion have been used to investigate the dose-related stimulatory actions of GnRH on LH and GH secretion (Chang *et al.*, 1990a). Furthermore, the study of perifused goldfish pituitary fragments *in vitro* suggests that serotonin is capable of inducing a dose-related release of LH and an inhibition of GH release (Somoza and Peter, 1991). Data from goldfish pituitary cell column studies have also demonstrated that activations of DA type 1 and type 2 receptors specifically inhibit LH release and stimulate GH release, respectively (Chang *et al.*, 1990b). Furthermore, results from cell perifusion experiment and static incubation indicate that calmodulin-dependent mechanisms are involved in mediating the long-term, but not the acute, LH response to

GnRH, whereas extracellular Ca^{2+} mediates the acute LH response to salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II) in the goldfish pituitary (Jobin *et al.*, 1996). In brief, the perifusion system is an effective model to study the acute neuroendocrine action of hormones and neuropeptides; it is always utilized together with static incubation to explore the short- and long- term regulation on LH and/or GH release in the goldfish pituitary. In the present study, we set out to explore the SN effect on LH secretion from goldfish pituitary fragments in perifusion to compare with the results from static incubation.

3.2 Materials and Methods

3.2.1 Perifusion of tissue fragments

The SF-12 suprafusion system (Biomedical Research & Development Laboratories) was used. With its multi-channel design, up to 12 groups of tissues were perifused simultaneously, and each channel was completely isolated from the others. Goldfish were sacrificed, and pituitaries were dissected and kept at 4 °C. After an extensive series of pilot studies to establish incubation condition were used, pools of 3 pituitaries were chopped to a diameter of ~0.1 mm by the McIlwain Tissue Chopper (Mickle Laboratory Engineering). Each pool of chopped pituitaries was suspended in 100 µl of culture medium (M199 with 2.2 g/l sodium bicarbonate, 5.96 g/l Hepes, 10 mg/l nystatin and 0.1% bovine serum albumin, pH 7.2) with 100 µl of autoclaved pre-swollen Cytodex-I beads (Pharmacia), and then loaded into a column of the temperature-controlled perifusion system. Pre-incubation lasted for overnight in culture medium at 20 °C and the speed of perifusate flow was 0.1 ml/minute. Before the experiment, the flow

was switched to 1 ml/minute and the perifusion machine began to collect an effluent every 5 minutes after 2-hour per-incubation perifusion with culture media. SN and salmon GnRH analog (sGnRHa) were applied to treat the tissues as 5-minute pulses. In this system, interval between any 5-minute pulses of treatment was ~60 minutes. Level of LH in the perifusate from all channels was measured by the specific LH radioimmunoassay (RIA), and all samples were assayed in triplicate (See Chapter 2).

3.3 Results

3.3.1 LH responses to 5-minute pulses of SN

Two groups of data from the valid failed perifusion experiments were shown in Figure 3.1 before the suprafusion system was improved. Their basal LH secretion was unstable during perifusion. In Figure 3.1A, the basal LH secretion decreased to 0 ng/ml in the midway of the experiment, and different doses of SN did not cause any change in LH level. Treatment with 500 nM SN induced a 2.2-fold increase of LH level in another perifusion (Figure 3.1B), whereas the basal LH level was very high (approximately 20 ng/ml) and very unstable. After the suprafusion system was improved, one group of data chosen from the results of goldfish pituitary perifusions was presented in Figure 3.2. The basal LH release varied in the range of 0-4 ng/ml. Under the stimulation of 1000 nM, 10 nM and 1 nM SN, the basal LH release was not altered immediately; the obvious response happened at least 20 minutes after the SN treatment and the LH levels increased to more than 4 ng/ml. Treatment with 100 nM SN caused a striking increase of the LH secretion both in terms of the duration and magnitude (response duration was shorten from more than 20 minutes to 0 minute and the magnitude rose to ~2.5 folds of the basal

LH level). In addition, the basal LH secretion was diminished to nearly 0 ng/ml at the end of the 5-hour perifusion challenge.

3.3.2 LH responses to 5-minute pulses of sGnRHa

Treatment with 100 nM sGnRHa in two failed experiments (Figure 3.1) up-regulated the LH level by 124% (A) and 125% (B) respectively although the basal secretion was unstable or of a high level. In Figure 3.2, exposure to 1000 nM sGnRHa induced weak increments of LH values at the 3rd and 5th fraction following the treatment, whereas the basal LH level was approximately 0 ng/ml.

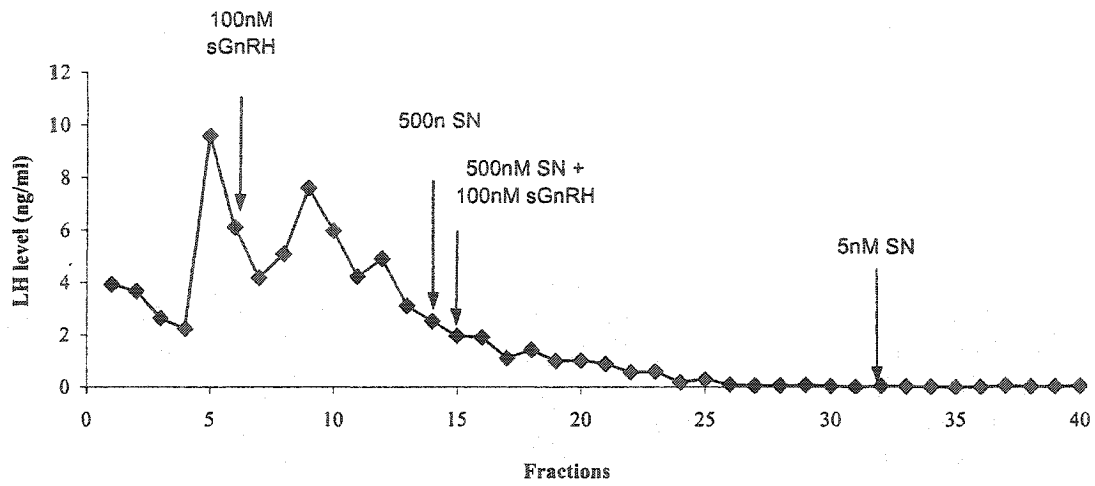
3.4 Discussion

Although other perifusion systems were reported to apply in goldfish neuroendocrinology studies for a long time (Murthy *et al.*, 1993; Chang *et al.*, 2001), it was the first time to establish this system in our laboratory. From the data in Figure 3.1, it is clear that the baseline of LH release curve was very unstable. Furthermore, it is likely that the tissues were dying because the positive sGnRHa stimulus was largely ineffective. According to our knowledge and experiences, any influence from perifusion environment, such as slight shaking, led to the unstable basal LH secretion and poor health of cells. In order to solve the problem, we did much work to avoid shaking columns that contained pituitary fragments, such as separating the pump and incubation columns from the main suprafusion machine, moving the whole system to a firm table in a quiet room and changing direction of medium flow. Thus, the baseline of LH release

(Figure 3.2) actually improved to some extent compared to the previous results (Figure 3.1) although it was still not acceptable.

In Figure 3.2, the 5-minute pulse treatment with SN appeared to increase goldfish pituitary fragments to release LH, confirming results from the static incubation study (Chapter 2). The obvious response happened at least 20 minutes after treatments with 1000, 10 and 1 nM SN. Thus, we suppose that SN acute stimulation of LH release may be a postponed effect which is different from the known rapid actions of GnRH; or there really exist some weak acute effects of this peptide which are hidden by the unstable baseline. Furthermore, pituitary fragments released more LH in response to low dose of SN (10 and 100 nM) than did pituitary fragments in the treatment with high dose of SN (1000 nM). In contrast, sGnRHa action was not consistent and the basal LH secretion rate was diminished to approximately 0 ng/ml at the end of perifusion, suggesting a poor health of the pituitary fragments when the incubation conditions were still not optimal. The perfusion system must be improved before we can confirm acute dose-related effects of SN on LH.

A.



B.

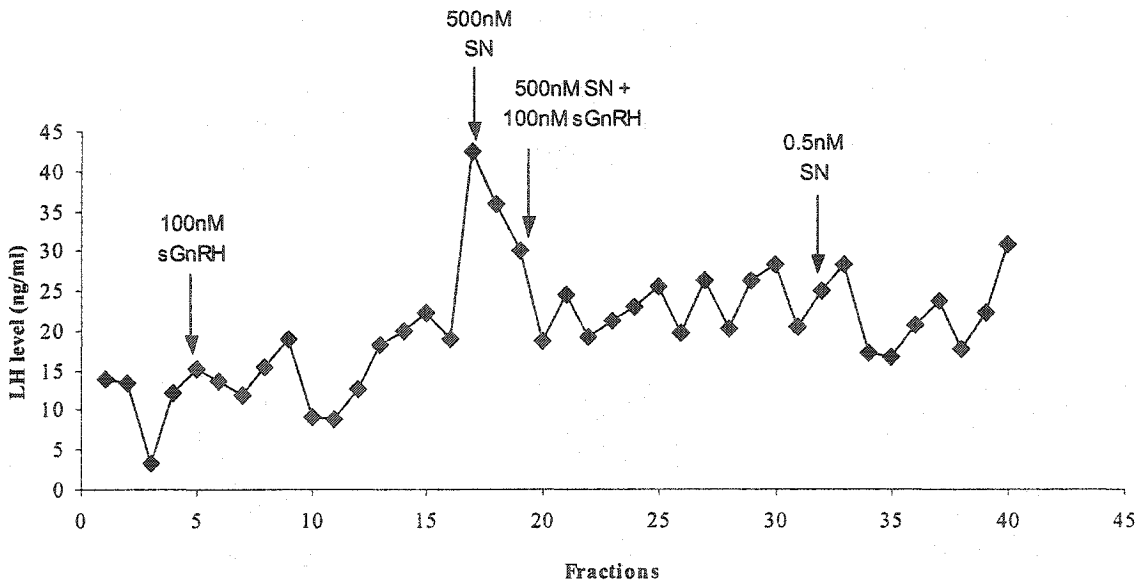


Figure 3.1: Perifusion experiments of goldfish pituitary fragments for studying effects of different doses of SN and 100 nM sGnRHa induced LH release before the suprafusion system was improved. Five-minute fractions were collected. The arrows indicate the applications of the SN and sGnRHa pulses. **A** and **B** are two perifusion columns as examples from the experiments performed in September and July respectively.

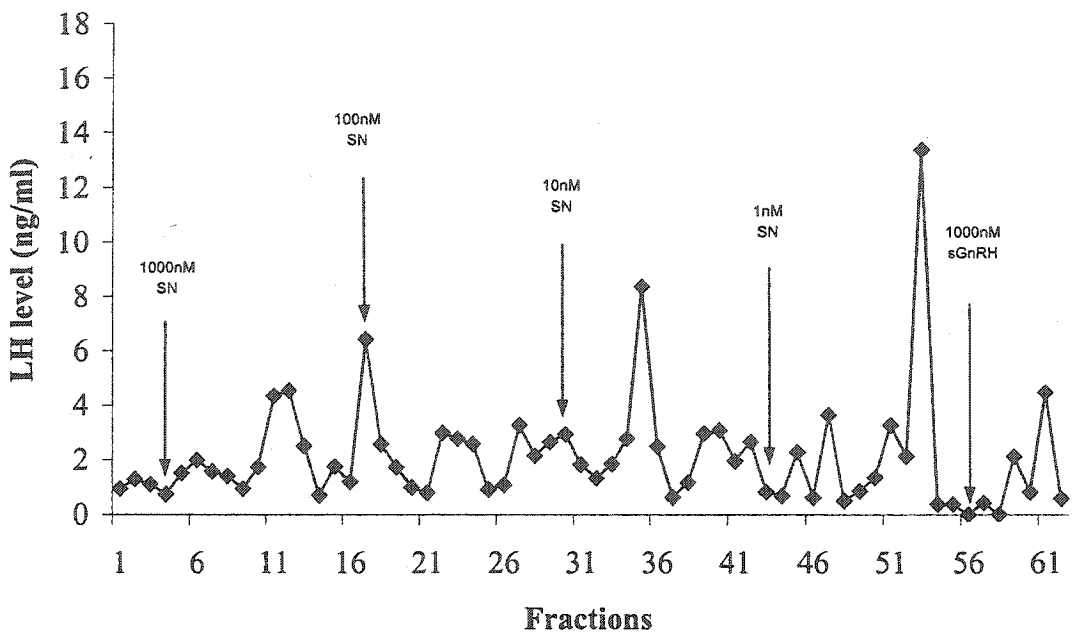


Figure 3.2: Effects of 1000, 100, 10, 1 nM SN and 1000 nM sGnRHa induced LH release in perifusion of goldfish pituitary fragments after the suprafusion system was improved. Five-minute fractions were collected. The arrows indicate the applications of SN and sGnRHa pulses. Shown is one perifusion column as an example from the experiments performed in June.

Chapter 4: Proteolytic processing and differential distribution of secretogranin II in goldfish

4.1 Introduction

Secretogranin II (SgII) is a member of chromogranin family. It is a sulfated highly acidic, heat stable protein expressed mainly in secretory granules of neuroendocrine cells and in vesicles of some neurons (Rosa *et al.*, 1985b; Mahata *et al.*, 1991). It was first found to be present in the bovine anterior pituitary (Rosa.and Zanni, 1981), and has been localized in gonadotrophs, thyrotrophs and corticotrophs of human pituitaries (Vallet *et al.*, 1997). The biological action of this granin is not completely understood. However, SgII is specifically stored in secretory granules of the regulated secretion pathway (Rosa *et al.*, 1985b). There, SgII serves as a precursor protein which can be proteolytically processed to small biologically functional peptides in human pituitary (Vallet *et al.*, 1997). Secretoneurin (SN), a small 33-34 amino acid peptide is one of the products derived from SgII. This neuropeptide is shown to have many physiological functions that include regulation of the immune, nervous and endocrine systems. For example, this peptide can up-regulate the outflow of dopamine (DA) from rat striatal slices perifused *in vitro* in a dose-dependent manner (Saria *et al.*, 1993). Furthermore, rat SN can increase DA outflow from the striatum of the awake rat in a concentration-related manner *in vivo* (Agneter *et al.*, 1995).

In several tissues, SgII molecule can be proteolytically processed in large dense core vesicles of neurons or secretory granules of endocrine cells. Evidence has been provided in previous studies to support the SgII processing in the pituitary. An N-terminal fragment of the SgII molecule is identified in porcine pituitary, suggesting a

possible proteolytic cleavage of SgII precursor molecule (Lazure *et al.*, 1991). In human normal and adenomatous anterior pituitaries, the 97-kDa SgII polypeptide is cleaved into two protein fragments of intermediate sizes, 46 kDa and 31 kDa, as determined by western blot analysis (Vallet *et al.*, 1997). Furthermore, previous studies also have proven the processing of SgII in the nervous system. High-performance liquid chromatography (HPLC) shows a high immunoreactive expression of SN in human brain and cerebrospinal fluid, implying a predominant SgII processing to SN there (Kirchmair *et al.*, 1993). Likewise, the SgII processing occurs in other tissues of rats, notably in amygdala, hypothalamus, olfactory bulb and several brainstem nuclei (Leitner *et al.*, 1997). It has also been demonstrated that SgII is completely processed to SN in both terminal and proximal parts of rat peripheral nerves, as detected by radioimmunoassay (RIA) and cytofluorimetric-scanning (Li *et al.*, 1999).

Prohormone convertases (PCs) are implicated in cleaving the SgII precursor at dibasic amino acid cleavage sites to generate intermediate-sized fragments (Hoflehner *et al.*, 1995). Although at least 7 processing enzymes (furin/PACE, PC1/PC3, PC2, PACE4, PC4, PC5/PC6 and PC7/LPC) are known in mammals (Seidah *et al.*, 1994; Wouters *et al.*, 1998), only PC1 and PC2 appear to be important in SgII processing (Hoflehner *et al.*, 1995). In an immunohistochemical localization study of rat anterior pituitary, PC1 and PC2 are expressed in the lactotrophs and show major localization along the secretory pathway. Furthermore, PC1 immunoreactivity (IR) is prominent in the Golgi apparatus, whereas PC2 IR is concentrated in the rough endoplasmic reticulum (RER) (Muller *et al.*, 1998). With regard to mechanisms of the proteolytic processing in prolactin cells, sulfated SgII is firstly transported to a distal compartment of the trans-Golgi network

(TGN) where it is cleaved and packaged in the secretory granules (Muller *et al.*, 1997). This suggests that PC1 is maybe the major enzyme involved in the cleavage of SgII in the TGN rather than other endocrine convertase PC2. However, immunofluorescence studies demonstrate that PC2 and SgII are co-localized in small, dense secretory granules of rat pituitary gonadotrophs, indicating that PC2 but not PC1 may be necessary to process SgII in the granules (Uehara *et al.*, 2001). In addition, PC1 shows to process the SgII precursor to small fragments, including SN in the GH4C1 pituitary prolactinoma cell line. PC2 enzyme expressed in neurons also processes SgII to other small peptides but not to SN (Hoflehner *et al.*, 1995).

A number of factors are able to regulate these two PCs (PC1 and PC2) or directly influence the SgII processing. It has been demonstrated that forskolin and depolarization increase PC1, but not PC2 in cultures of rat hippocampal slices, suggesting that PC1 is more important for the SN production (Bauer *et al.*, 2000). Additionally, brefeldin A suppresses SgII processing and peptide secretion via blocking anterograde transport of sulfated proteins in the TGN and inhibiting the formation of secretory vesicles and granules there (Muller *et al.*, 1997).

Following *in vitro* processing of PCs in adrenal medulla *in vivo*, 86-kDa mammal SgII is cleaved to several polypeptides of intermediate size ranging from 75 kDa to 21 kDa (Fischer-Colbrie *et al.*, 1995). Furthermore, western blot analysis reveals that the processing of human SgII (97 kDa) in human anterior pituitary yields two low molecular weight proteins, 46 kDa and 31 kDa, both of which arose from the C-terminal part of the precursor molecule (Vallet *et al.*, 1997). In addition to its processing in mammals, frog SgII (84 kDa) is processed to the protein fragments of 69, 54, 34, 21 and 15 kDa in

Xenopus intermediate pituitary *in vitro*, among which 21-kDa polypeptide is the major SgII-derived product and contains the SN sequence (Van Horssen and Martens, 1999).

Immunohistochemical studies indicate that SN-immunoreactivity (SN-IR) shows high levels in rat pituitary and adrenal gland, and also can be detected in the central nervous system, different regions of the gastrointestinal tract and the pancreas (Leitner *et al.*, 1996). The wide distribution of SgII and its differential processing towards SN suggests that SN is an essential peptide derived from SgII and plays roles in various biological events. Recently, SgII-derived 66-amino acid peptide, EM66, has been localized and characterized in human adrenal gland. EM66 is highly conserved in mammals and appears early during ontogenesis of human adrenal gland (Anouar *et al.*, 1998). EM66 immunoreactivity (EM66-IR) is observed not only in gonadotrophs, lactotrophs, thyrotrophs, and melanotrophs of the rat pituitary but also in nerve terminals of the neural lobe, and exclusively in epinephrine-producing cells of the adrenal glands. This characteristic of cell-specific distribution also suggests that EM66 may play a role in the endocrine system (Montero-Hadjadje *et al.*, 2003). EM66 is a potential marker present in human tumoral chromaffin tissue, and difference in the EM66 level between benign and malignant pheochromocytomas may be used to characterize these tumors (Yon *et al.*, 2003).

The regulation of SgII biosynthesis has been studied in mammals. SgII is localized in neuroendocrine secretory granules. The biosynthesis and release of SgII may therefore be influenced by other co-stored components like hormones, neurotransmitters and neuropeptides. Some hormones are reported to regulate the generation and secretion of SgII in mammals. Ovariectomy can induce a 2-fold increase in the mRNA and protein

levels of SgII in the female rat pituitary (Anouar *et al.*, 1991). Treatment with a gonadotropin-releasing hormone (GnRH) antagonist *in vivo* suppresses pituitary SgII mRNA levels in the ovariectomized female rat (Kakar *et al.*, 1993), implying that GnRH has a stimulatory effect on the SgII production. In addition, estradiol (E_2) is capable of down-regulating increased SgII mRNA levels in rat pituitary *in vivo* (Anouar *et al.*, 1991; Karhar *et al.*, 1993). Likewise, *in vitro* studies indicate that E_2 has a direct inhibitory effect on SgII expression in rat pituitary (Anouar and Duval, 1992).

In the present study, we set out to characterize SgII processed in goldfish. Considering previous studies in mammals (Leitner *et al.*, 1996), we propose a hypothesis that the SgII-derived products containing SN are expressed in the goldfish pituitary as well as in other tissues. Samia (2002) examined the roles of GnRH and sex steroids to regulate pituitary SgII mRNA expression *in vivo*. Treatment with a salmon GnRH agonist plus domperidone (DOM, a D_2 receptor antagonist) increased SgII mRNA levels in the goldfish pituitary. Sex steroids (testosterone, E_2 and progesterone) when given alone had no significant effects on the SgII expression in the goldfish pituitary, whereas the suppressive effects of E_2 on SgII mRNA exists in rats (Anouar *et al.*, 1991; Karkar *et al.*, 1993). However, testosterone in goldfish does block the stimulatory effect of GnRH on pituitary SgII gene expression (Samia, 2002). It is also reported that γ-aminobutyric acid (GABA) metabolism inhibitor γ-vinyl-GABA (GVG) stimulates SgII gene expression in goldfish pituitary gonadotrophs *in vivo* (Blazquez *et al.*, 1998a). Since GnRH and GABA play important roles in the regulation of SgII mRNA expression in goldfish gonadotrophs, we studied the effects of these two neuroendocrine factors on SgII processing in the goldfish pituitary.

4.2 Materials and Methods

4.2.1 Production of antibody to goldfish SN

A new 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, was chosen as the antigenic peptide. This selection is determined,based on its secondary structure, antigenicity and hydrophility calculations as done by using Expasy software (www. expasy.ch). 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). Four female New Zealand White rabbits (approximately 2.5 kg weight) were used to generate polyclonal antibodies against SN. SN15-MAP (1 mg) was suspended by vortexing in a few drops (approximately 0.1 ml) of pure glacial acetic acid. This suspension was treated within 0.9 ml of sterile distilled water and centrifuged to precipitate any undissolved SN15-MAP. The clear top supernatant was then mixed with Freund's complete adjuvant for the first injection (1 mg of SN15-MAP per rabbit). Later on, additional SN15-MAP (0.5 mg) (1:1) in Freund's incomplete adjuvant was injected for all subsequent injections. Animals were injected (0.25 ml x 2 intramuscular; 0.1 ml x 5 subcutaneously) every 4 weeks. Blood sample (3 ml) was collected after every 10 days following the third injection. This was continued for approximately 6 months. A preliminary evaluation of antibody responses using western blot analysis indicated that highest titre was obtained for rabbit 42 (sample 42, October 10th, 2000) and this SN antiserum was subsequently used in all other experiments. Generation of SN15-MAP and

production of its antiserum were performed as the collaboration between the laboratories of Dr. Ajoy Basak (OHRI) and Dr. Vance Trudeau (University of Ottawa).

4.2.2 Evaluation of SN antibody specificity

The SN antibody at dilution of 1:4000 was incubated with 1 μM SN15-MAP or goldfish SN (Blazquez *et al.*, 1998a) diluted in the blocking buffer [1X TBS (0.3% Tris-Base, 0.8% sodium chloride, 0.02% potassium chloride, pH adjusted to 8) with 5% fat-free milk powder and 0.05% (v/v) Tween 20] at 4 °C overnight. To determine specificity of the reaction, the SN antibody was pre-absorbed by 1 μM SN15-MAP or SN and compared to normal antiserum in a western blot analysis.

4.2.3 Tissue homogenization and protein assay

Frozen tissue samples were homogenized in 3X reducing sample buffer [RSB, 0.15 M Tris-HCl pH 6.8, 24% (v/v) glycerol, 4.8% SDS, 0.06% bromophenol blue] on ice for 2 minutes. The bicinchoninic acid (BCA) method (Smith *et al.*, 1985) was utilized to assay total protein concentration. A 96-well polystyrene plate (Corning) was filled with 10 μl of sample and 200 μl of BCA protein assay reagent [2% copper (II) sulfate, 98% bicinchoninic acid] for each well and then incubated at 37 °C for 20 minutes. Finally, the plate was read at the wavelength of 560 nm in a Spectra Max Plus Microplate Spectrophotometer (Molecular Devices) and the absorbance of protein samples was evaluated. Prior to the calculation of protein concentration, the standard bovine serum albumin solutions were assayed using the BCA method; Microsoft Excel was utilized to establish the standard curve and the formula for the relationship between protein

concentration and absorbance (Figure 4.3). The total protein level of each tissue sample was estimated using the standard formula.

4.2.4 SDS-PAGE gel electrophoresis

The total protein (100 μg) was separated using electrophoresis on a SDS-PAGE gel (10% bis-ascrylamide, 0.375 M Tris-HCl pH 8.8, 0.1% SDS, 0.1% ammonium persulfate, 0.04% TEMED). A stacking gel (5% bis-acrylamide, 0.0625 M Tris-HCl pH 6.8, 0.1% SDS, 0.1% of ammonium persulfate, 0.1% TEMED) was placed at the top of the SDS-PAGE gel for loading the samples. 100 μg of total protein were removed from the stock homogenates and 0.12 volumes of 2-mercaptoethanol was added to reduce disulphide bonds. The mixture was boiled for 5 minutes and chilled on ice. After loading, the gels were run in 1X running buffer (0.3% Tris-Base, 1.44% glycine, 0.1% SDS, pH adjusted to 8.3) at 85 volts for ~10 minutes until the blue front passed the stacking gel. Then, voltage was increased to 133 volts and the gel was run for another ~80 minutes. An Immobilon-P transfer membrane (Millipore) was cut to the same size as the SDS-PAGE gel, washed with methanol and soaked in the 1X Blot Buffer [per 1 liter: 200 ml of methanol, 100 ml of 10X blot buffer stock (1.01% Tris-Base, 4.8% glycine, pH adjusted to 8.3), 700 ml of ddH_2O]. The separated protein bands were transferred from the gel to the membrane using the Semi-dry transfer cell (BIO-RAD) at 20 volts for ~70 minutes. The membrane was ready for western blot analysis.

4.2.5 Western blot analysis

Following the transfer or stripping of specific protein-band, the membrane was incubated in the blocking buffer at room temperature (20 °C) for 70 minutes. The part of the membrane containing the standard ladder (Biotinylated SDS-PAGE Standards, Broad Range) was separated from the protein samples. The ladder was then treated with ECL Streptavidin-Horseradish Peroxidase Conjugate (Amersham, 1:4000) diluted in 1X TBS-T [1X TBS with 0.05% (v/v) Tween 20] at room temperature for 45 minutes. The membrane with protein samples was incubated with primary antibody [1:2000 or 1:4000 dilution of SN antibody or 1:150 dilution of purified mouse anti-actin monoclonal antibody (CLT9001, Cedarlane)] diluted in blocking buffer at 4 °C overnight. On the following day, membranes were reacted with protein A-peroxidase (Sigma) at a dilution of 1:2000 or 1:4000 at room temperature for 1-1.5 hours. The membranes with ladders and protein samples were washed with 1X TBS-T and 1X TBS. They were covered with ECL Plus Western Blotting Detection Reagent (Amersham) and then wrapped in plastic sheet. Signals on the membranes were detected with a Chemi Doc (Bio-RAD) and quantified using the Quantity One (Bio-Rad) software package. In order to strip the blots, the membrane with protein samples was washed with stripping buffer (100 mM 2-mercaptoethanol, 2% SDS, 62.5 mM Tris-HCl pH 6.7) at 50 °C for 30 minutes and rinsed with 1X TBS-T twice for 10 minutes. Membranes were stored in 1X TBS at 4 °C until probed with the β-actin antibody (CLT 9001, Cedarlane), which served as a protein loading control.

4.2.6 Dissection of various goldfish tissues

Goldfish were anaesthetized using 3-aminobutyric acid ethyl ester, and the spinal cord transected for all dissections. The pituitary, interrenal, ovary, hypothalamus, cerebellum and telencephalon were rapidly dissected and frozen in dry ice. For some studies, the *pars distalis* (PD, which contains mostly gonadotrophs and somatotrophs) and the neurointermediate lobe (NIL, which contains various other endocrine cells) of the pituitary were separated using a dissection microscope and a fine blade. All tissues were stored at -80 °C, for later use in western blot analysis or reverse transcription-polymerase chain reaction (RT-PCR).

4.2.7 γ-Vinyl-GABA treatment in vivo

Goldfish were anaesthetized using 3-aminobutyric acid ethyl ester and then weighed. GVG (γ-vinyl-GABA, Hoeschst Marion Roussel, 300 μg per g body weight), a specific inhibitor of GABA transaminase, was injected into the intraperitoneal cavity. 0.6% saline (1 μl per g body weight) was vehicle and served as the control. After recovering in fresh water, the goldfish were moved back to their tanks. Twenty-four hours after injection, each goldfish was dissected and the pituitary was removed and stored frozen at -80 °C for western blot analysis.

4.2.8 sGnRHa time-course treatment in vivo

After anesthesia, salmon gonadotropin-releasing hormone analog (sGnRHa, [Des-Gly^{10}, D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}]-LHRH, Peninsula Laboratories, 50 ng per g body weight) and DOM (a specific dopamine D_2 antagonist, gift of Janssen Pharmaceutica, Belgium, 5

μg per g body weight) were injected into the intraperitoneal cavity of goldfish using 0.6 % saline (5 μl per g body weight) and dimethyl sulfoxide (DMSO, 1 μl per g body weight) as the vehicles, respectively. Pituitaries were collected 0, 24, 48 and 72 hours after injection. The 0 hour treatment indicates that dissection was performed immediately after injection. Pituitaries from goldfish without any treatment were collected as an additional control. All tissues were stored at -80 °C for western blot analysis.

4.2.9 Statistical analysis

The relative expression of SN containing proteins is the ratio of SN-IR and β-actin-IR averaged over a given western blot membrane. The statistical analyses were performed using Sigma Stat 2.03 software. One-way or two-way Analysis of Variance (ANOVA) was chosen to assess the difference between the control and treatment, followed by Fisher LSD test. A difference with $p<0.05$ was considered to be significant. The Q-test was used on 2 occasions to determine whether or not a suspected outlier datum point could be statistically rejected (Sokal and Rohlf, 1981).

4.2.10 RT-PCR distribution analysis of SgII in various goldfish tissues

Total RNA was obtained from frozen tissues using TRIzol reagent as described by the manufacturer (Invitrogen). Isolated RNA was re-suspended in DEPC treated water and stored in -80 °C. Complementary DNA (cDNA) was obtained from 2 μg of total RNA using Superscript II RNase H^- reverse transcriptase and oligo dT primer as described by the manufacturer (Invitrogen). 100 ng of total RNA were reversed and transcribed to cDNA and subjected to PCR amplification. Primers used for SgII were 5'-

TTCTTACCACGCTACAACAGT-3' and 5'-TCATCATCTTCGCCATCCTC-3', and for 18S 5'-GAGCCTGAGAAACGGCTACC-3' and 5'-GTATTCAGCGGCGACAGG-3'. The thermocycle program for both SgII and 18S included a denaturation step at 94 °C (5 minutes), 30 cycles for 94 °C (1 minute), 54 °C (2 minutes), 72 °C (2 minutes) and a final elongation step at 72 °C (10 minutes). These conditions were previously used for a radioactive semi quantitative PCR reaction (Blazquez *et al.*, 1998a). The cDNA was separated on an agarose gel by electrophoresis. The gel was made of 1% agarose in 0.5 X Tris-borate-EDTA (TBE, 45 mM Tris-borate, 1 mM EDTA) with 0.05% ethidium bromide. 25 µl of PCR product mixed with 5 µl of loading buffer (0.25% bromophenol blue, 0.25% xylene cyanol FF, 30% glycerol in water) was loaded on the agarose gel for each sample, and 7 µl of P0X (digested with Hae III) DNA ladder was loaded as a standard. The gel was run in 0.5 X TBE at 90 volts for approximately 60 minutes. The gel was then detected with a Gel Doc (Bio-RAD). The RT-PCR analysis of goldfish SgII distribution was performed by Kate Werry in our laboratory.

4.3 Results

4.3.1 Evaluation of anti-SN antiserum specificity

In order to determine the specificity of the anti-SN activity, we performed two western blot experiments using anti-SN antisera pre-incubated with SN15-MAP (Figure 4.1) and goldfish SN (Figure 4.2). In Figure 4.1, the normal SN-antiserum showed a number of intense protein bands along the lanes containing pituitary proteins at different dilutions and also the lane containing the original SN15-MAP antigen. No SN-IR bands were observed in lanes containing pituitary proteins when the SN antibody had been pre-

absorbed with SN15-MAP (Figure 4.1) or goldfish SN (Figure 4.2). However, a faint residual band was observed in the SN15-MAP lane when the antiserum was blocked with SN15-MAP. This is in contrast to the total blockage of all IR by pre-incubation with the full length (34 amino acids) synthetic goldfish SN.

4.3.2 SgII processing in the goldfish pituitary and various forms of SgII-derived peptides

Three samples of goldfish pituitaries collected between March and June were analyzed by western blots, using anti-SN antiserum. Results are reported in Figure 4.3 and Figure 4.4. A strong band, consistent with the size of a known SgII-derived peptide observed in human pituitary (Vallet *et al.*, 1997) was detected in each pituitary sample. Furthermore, a weak SN-IR band of lower molecular weight was observed with a long-time exposure in the goldfish pituitary. A standard curve establishing the relationship between apparent protein molecule weight and gel running distance (GRD) was established (Figure 4.3). The molecular weights of the strong band (GRD, 16 mm) and the weak band (GRD, 34 mm) were respectively ~57 kDa and ~30 kDa. Both of these bands are smaller than the calculated molecular weight of the goldfish SgII precursor which is estimated to be ~69.9 kDa based on amino acid sequence (Blazquez *et al.*, 1998a).

4.3.3 Distribution of SgII mRNA expression and its processing

SgII mRNA expression was studied in different tissues using RT-PCR analysis. The SgII mRNA levels in the pituitary as well as the regions of brain were much higher than those in the peripheral tissues. Goldfish had the highest SgII level in the pituitary

gland. The same samples were analyzed by western blots using the anti-SN antiserum to find the potential SgII processing in different tissues of goldfish (Figure 4.5B). As previously described (Figure 4.4), the anti-SN antiserum revealed the presence of two SgII-derived polypeptides (~57 kDa and ~30 kDa) with SN-IR in the pituitary. A faint band of low molecule weight (~20 kDa) was detected in the hypothalamus whereas no signal was found in the other tissues.

4.3.4 Effects of sGnRHa and GVG on the SgII processing

The major SN-IR SgII-derived protein (~57 kDa) was further investigated to determine if SgII processing was affected by neuroendocrine factors. Figure 4.6 shows the time-course effects of sGnRHa plus DOM on the levels of this protein expression in the pituitary of sexually mature goldfish. An one-way ANOVA indicated sGnRHa plus DOM *in vivo* did not significantly affect levels of this SgII-derived protein at any time after the treatment.

Figure 4.7 shows the levels of the SgII-derived protein containing SN (~57 kDa) in the PD and NIL of female and male goldfish injected with GVG *in vivo*. There was a higher level (~5-fold) of this SgII product in the PD than in the NIL in the control sexually recrudescent female and male goldfish. The level of the SgII product decreased in the NIL of female fish by 67.7% and increased the NIL of male fish by 2.8 folds after GVG injection. However, treatments with GVG did not cause any statistically significant change in the levels of the ~57 kDa SgII-product in the PD or NIL of female and male goldfish. We were unable to reliably quantify the smaller ~30-kDa SN-IR protein because in some instances it was at the limit of detection in western blot analysis.

4.4 Discussion

In this study, we demonstrate for the first time SgII processing in the goldfish pituitary and potential distribution of a SN-IR product in the hypothalamus. Furthermore, we have studied the regulatory effects of sGnRHa and GABA on this SgII processing in the PD and NIL of goldfish pituitary. This study revealed that levels of a major SgII-derived protein containing SN (~57 kDa) were higher in the PD than in the NIL of goldfish pituitary. This is in agreement with results from a previous study indicating that SgII mRNA levels are consistently higher in the PD than in the NIL of goldfish (Samia, 2002).

Before we could study SgII processing in goldfish, a new SN polyclonal antibody was generated for western blot analysis of pituitary proteins. In evaluations with the pre-absorption of SN15-MAP and goldfish SN, no signal band was detected in the treatment compared to the control, confirming that anti-SN antiserum can specifically bind to peptides containing the middle 15-amino acid sequence of goldfish SN, YTPQKLATLQSVFEE. As previously reported in mammals (Vallet *et al.*, 1997), specific human SgII antisera were applied to explore processing and distribution in the human normal anterior pituitaries and pituitary adenomas. Likewise, in the present study, we explored the SgII processing and its distribution in the goldfish pituitary and hypothalamus using the anti-SN antiserum.

SN is a critical peptide derived from SgII in many neuronal and endocrine tissues, and shows several biological activities (Kahler and Fischer-Colbrie, 2000). Moreover, the middle 15-amino acid sequence of SN (SN15) is the most conserved part of SgII during the evolution in vertebrates (Samia *et al.*, 2001). Thus, the antiserum generated from

SN15-MAP, can detect proteins containing whole SN sequences. This makes it very useful for determining the pattern of processing of 603-amino acid goldfish SgII precursor in the goldfish pituitary (Blazquez *et al.*, 1998a). Using this anti-SN antiserum, two SN-IR intermediate proteins, ~57 kDa and ~30 kDa, were detected in potential goldfish SgII processing. The ~57-kDa SgII-derived protein was expressed in higher level than the ~30-kDa protein. This suggests that a partial endogenous proteolytic processing of SgII to intermediate polypeptides was detectable using western blot analysis of the goldfish pituitary protein. Similarly, it has been reported that several SgII-immunoreactive proteins of intermediate sizes ranging from 75 kDa to 21 kDa are generated by the processing of 86-kDa SgII in endocrine tissues and neurons of mammals (Fisher-Colbrie *et al.*, 1995). Concerning SgII processing in human anterior pituitary, two SN-IR proteins derived from the 97-kDa precursor, 46 kDa and 31 kDa, are found in the gonadotroph cell using western blot analysis (Vallet *et al.*, 1997). This is partially in agreement with the sizes of SgII-derived peptide we detected in the goldfish pituitary. SgII processing in teleosts has not been previously reported. To our knowledge, this is the first study showing that SgII was cleaved to yield several intermediate peptides which perhaps may be further processed ultimately to the biologically active SN *in vivo*.

In order to understand possible amino acid sequence of the SgII-derived peptides in the pituitary, we calculated the molecular weights of all potential fragments between putative dibasic cleavage sites in goldfish SgII sequence as described by Blazquez *et al.* (1998a). Six out of 9 dibasic cleavage sites are conserved between fish and tetrapods. According to the molecular weights of two processing peptides (~57 kDa and ~30 kDa), the possible SN-containing combinations of potential fragments from SgII precursor were

calculated and are presented in Figure 4.8 and Figure 4.9. A polypeptide composed of B, C, D, E and F fragments will consist of ~496-amino acids with an estimated molecular weight of approximately 57.7 kDa. This suggests that the ~57-kDa band detected by western blot analysis may be derived following cleavages by a signal peptidase at GVQG$\overset{34}{\blacktriangledown}$AT and a PC-like protease at HGQRR$\overset{532}{\blacktriangledown}AR\overset{534}{\blacktriangledown}$LT. Likewise, another 283-amino acid polypeptide composed of B, C, D and E fragments, with an estimated molecular weight of 33 kDa, may explain the formation of the observed protein band with a weak signal and with a lower molecular weight (~30 kDa). Thus, ~30 kDa band is possibly generated following cleavages at signal peptidase mediated 34 site and PC-like protease mediated 319 position. The agreement between the results obtained from western blot analysis of goldfish pituitaries and calculated data from the SgII amino acid sequence analyses demonstrates the potential for some degree of SgII processing to produce SN in the goldfish pituitary. It also reveals that a cleavage may occur at the 532 or 534-dibasic pair, giving the N-terminal part of the molecule that contains SN sequence, therefore corresponding to the ~57-kDa protein. The most abundant ~57-kDa protein detected by our anti-SN antisera suggests that SgII-products containing SN are mostly stored in secretory granules in this form. A small amount of the N-terminal ~57 kDa protein, is then probably processed at the site of KR319-basic pair producing another small N-terminal part of the molecule whose size is approximately ~30 kDa. It is also possible that the ~30-kDa polypeptide directly originates from SgII precursor that is cleaved at the KR319-basic pair. In summary, we propose a hypothesis about the whole SgII processing in the goldfish pituitary that (Figure 4.9): PC-like proteases cleave the whole SgII molecule of 69.6 kDa (A-H fragments) to generate the ~57-kDa protein with B, C, D, E

and F fragments at the first step. The ~57-kDa protein is then further processed to the ~30-kDa protein by removing the F fragment. Apart from the ~30-kDa protein, other potential polypeptides containing SN were not detected by our present western blot analysis. A similar SgII processing has been reported in the human pituitary (Vallet *et al.*, 1997), where the details of such a cleavage also remain ill-defined. We did not expect to detect the 34-amino acid SN peptide because it is too small (3.7 kDa) and would run off the gel very quickly. Future studies will be carried out to detect this small peptide under different conditions of gel preparations.

The present study on the distribution of SgII and its processing has yielded two surprise findings: (a) the expression of SgII was detected in ovary and interrenal by RT-PCR but no SN-IR products were observed using western blot analysis; (b) the goldfish SgII precursor is processed in the pituitary and to some degree in the hypothalamus. In agreement with the previous studies, SgII mRNA was detected in these goldfish organs using RT-PCR in the present study. Because RT-PCR is a more sensitive method to detect mRNA rather than Northern blot analysis, the SgII mRNA expressions in ovary and interrenal were observed in the present study of goldfish which were not detected earlier by Northern blots (Samia, 2002). Concerning the distribution of SgII-processing products in mammals, the highest concentration of SN-IR has been found in the rat pituitary and brain (Leitner *et al.*, 1996). Likewise, the present study demonstrated that SgII processing occurred in the goldfish pituitary and most of the two major intermediate products are ~57-kDa and ~30-kDa proteins. Furthermore, only a weak ~20-kDa SN-IR peptide was detected in the hypothalamus (Figure 4.5) indicating a different SgII cleavage pathway in this tissue compared to the pituitary (Figure 4.9). No SN-IR was

observed in tissues other than the pituitary and hypothalamus in goldfish, although SgII mRNA was found to be present there.

It is intriguing that some tissues express SgII mRNA yet no SN-IR was detectable. There are several explanations for this observation. Firstly, perhaps there is no translation of SgII mRNA occurring in some of these goldfish tissues. Secondly, SgII products could have been rapidly degraded or secreted immediately after the translation of SgII mRNA. Thirdly, SgII might be processed extensively to yield small peptides that remain undetected under the gel conditions used in the present study. This possibility agrees with a study in rats where only free SN peptide is detected in the brain, whereas larger SN-IR molecules are only found in the anterior pituitary (Leitner *et al.*, 1996). Finally, the SgII precursor may yield other peptides not containing the SN sequence. For example, SgII could be processed to peptides similar to mammalian EM66, which are found in rat pituitary and adrenal gland (Montero-Hadjadje *et al.*, 2003). To test this hypothesis, attempts could be made to detect smaller SN-IR peptides using higher density SDS-PAGE gels or HPLC coupled to a specific SN RIA. To detect other SgII-derived peptides not containing SN, new antisera would have to be generated, as has been done for other parts of SgII (Vallet *et al.*, 1997). Further studies along these lines will be of crucial importance to understand the mechanism and biochemical significance of the SgII processing in goldfish.

GnRH and GABA are two important factors to regulate the hormone release from the goldfish pituitary, such as luteinizing hormone (LH) and growth hormone (Trudeau, 1997). Concerning the regulation of SgII synthesis in goldfish, treatment with sGnRHa plus DOM *in vivo* increases levels of SgII mRNA in the PD concomitant with LH release

(Samia, 2002). Likewise, treatment with GVG *in vivo* specifically up-regulates SgII mRNA expression in gonadotrophs, concomitant with an increase in the serum LH level and a decrease in the LH cell content (Blazquez *et al.*, 1998a). In our study of SgII processing, we found that a ~57-kDa protein was the predominant intermediate SgII product that is highly expressed in the goldfish pituitary (Figure 4.3 and Figure 4.4). Co-treatment with sGnRHa and DOM didn't cause any statistical change in levels of the ~57-kDa protein in sexually mature fish *in vivo*. This suggests that GnRH might not influence the SgII process in the goldfish pituitary, which is different from the strong stimulatory effect of GnRH on SgII mRNA expression (Samia, 2002). It is also possible that after translation of SgII mRNA there is a rapid export and processing of newly synthesized SgII products following GnRH stimulation. If this is the case, no dramatic changes in SN-IR will be expected. Similarly, the GABA drug, GVG, was without any effect on levels of the SN-IR ~57-kDa SgII-product in the PD of the pituitary. However, there was a trend for increased levels of ~57-kDa peptide in the male NIL after GVG injection. In the present study, we have shown that the PD, where gonadotroph cells are located, had a statistically higher level of the ~57-kDa SgII-derived protein than the NIL in female and male fish, which is consistent with the recent report concerning SgII mRNA level in different parts of goldfish pituitaries (Samia, 2002). This observation supports the idea that the PD is the critical part of goldfish pituitary for the SgII synthesis and processing. Furthermore, this result agrees with a previous study of human anterior pituitary where SgII and its derived proteins are detected in gonadotrophs (Vallet *et al.*, 1997).

In summary, using a new anti-SN antiserum we demonstrated the existence of two SgII-derived proteins of intermediate sizes containing SN sequence in the goldfish

pituitary. Based on their molecular sizes, namely ~57 kDa and ~30 kDa, these two proteins are possibly composed of 496 and 283 amino acids containing the N-terminal fragment of full length SgII molecule. Detection of a ~20 kDa protein in the hypothalamus and the widespread expression of SgII mRNA but not SN-IR in various organs suggest differential processing of SgII in the brain in a tissue-dependent manner. Although *in vivo* treatment with sGnRHa plus DOM or GVG showed no obvious effects on SgII processing, we did notice that the PD produced more of the ~57-kDa protein than did the NIL. **High production of SgII-derived proteins in the PD supports the hypothesis that goldfish SN is important for PD function, namely LH release.** It has previously been demonstrated that SN can stimulate LH release from PD fragments *in vitro* (Chapter 2) or *in vivo* in DOM treated goldfish (Blazquez *et al.*, 1998a). It is possible that there is local production and action of SN-IR peptides within the PD, which would represent a novel intrapituitary control mechanism linking the regulated secretory pathway to hormone release. It is equally possible that SN is produced in the hypothalamus or other tissues, and affects LH release by more classical neuroendocrine routes.

Future research on the distribution of SgII processing should concentrate on detection of the free SN peptide and other functional products from SgII in the goldfish pituitary as well as other organs. Because LH secretion and reproduction are on a seasonal cycle in the adult goldfish (Blazquez *et al.*, 1998b), it will be important to study the potential effects of neuroendocrine factors on the regulation of SgII processing in different sexual periods. The specific enzymes responsible for cleavages of SgII leading to the formation of ~57, ~30 and ~20 kDa fragments, have not been identified in the

present study. However, further studies using specific PC-inhibitors may confirm the role of PCs in these proteolytic events - a notion that has been already established for Cg processing in most mammals so far studied.

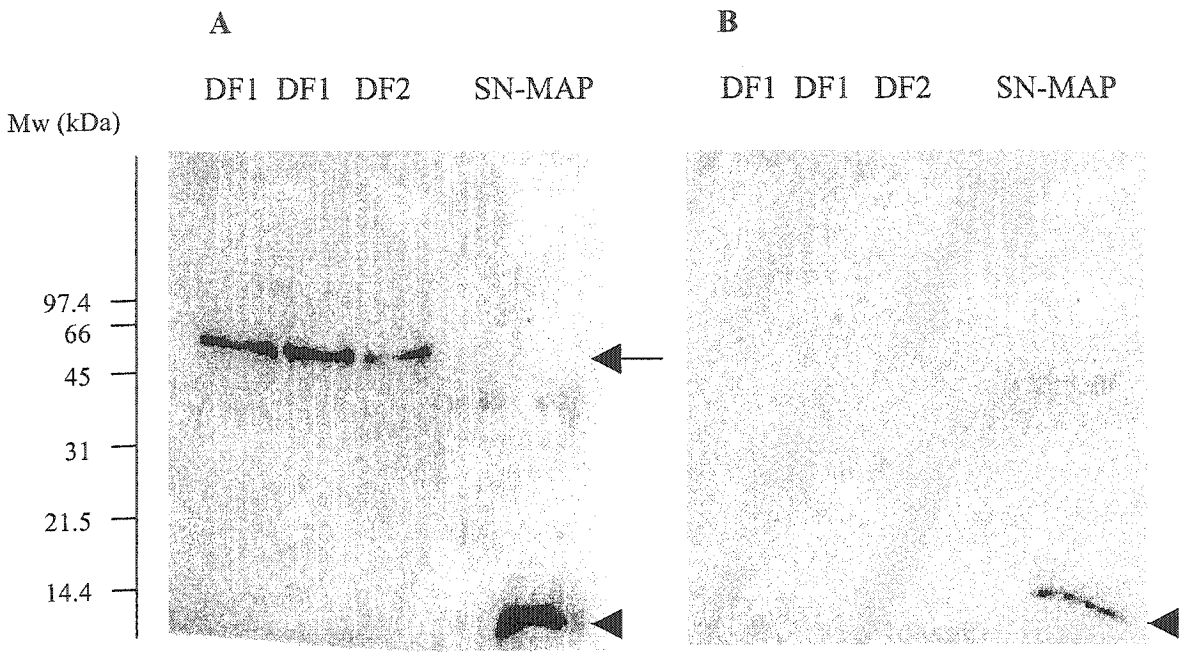


Figure 4.1: Western blot analysis for evaluation of anti-SN antiserum (#42) specificity using pre-absorbed SN15-MAP. Various volumes of pituitary proteins and 0.3 μg of SN15-MAP were detected by western blot analysis. 3 pituitaries were homogenized in 300 μl of RSB to make the stock. 15 μl and 7.5 μl of the stock were respectively loaded in the columns of DF1 and DF2. **A,** anti-SN antiserum (dilution: 1:4000); **B,** anti-SN antiserum (dilution 1:4000) pre-absorbed with 1 μM SN15-MAP. Arrow indicates the SN-IR Sg-II derived peptide, and arrowhead the SN15-MAP band (A) or the residual SN15-MAP band (B). Relative placement of the molecular weight standards is shown on the left. Abbreviation: DF1, dilution factor zero; DF2, dilution factor 2.

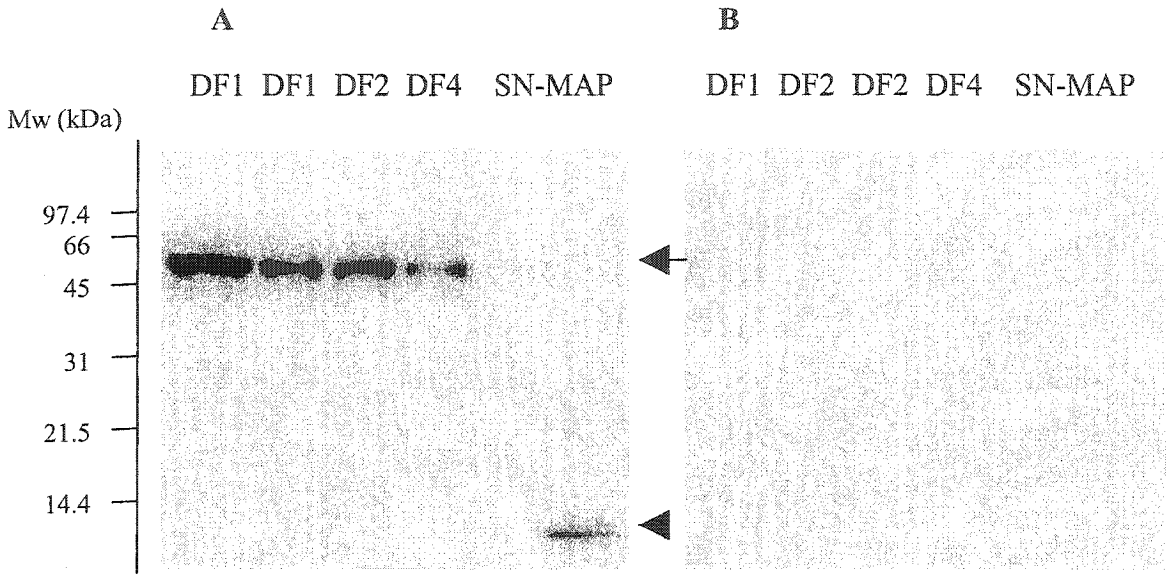


Figure 4.2: Western blot analysis for evaluation of anti-SN antiserum (#42) specificity using goldfish SN peptide. Various volumes of pituitary proteins and 0.3 μg of SN15-MAP were detected by western blot analysis. 3 pituitaries were homogenized in 300 μl of RSB to make the stock. 15 μl, 7.5 μl and 3.75 μl of the stock were respectively loaded in the columns of DF1, DF2 and DF4. **A,** anti-SN antiserum (dilution: 1:4000); **B,** anti-SN antiserum (dilution 1:4000) pre-absorbed with 1 μM goldfish SN. Arrow indicates the SN-IR Sg-II derived peptide and arrowhead the SN15-MAP band. Relative placement of the molecular weight standards is shown on the left. Abbreviation: DF1, dilution factor zero; DF2, dilution factor 2; DF4, dilution factor 4.

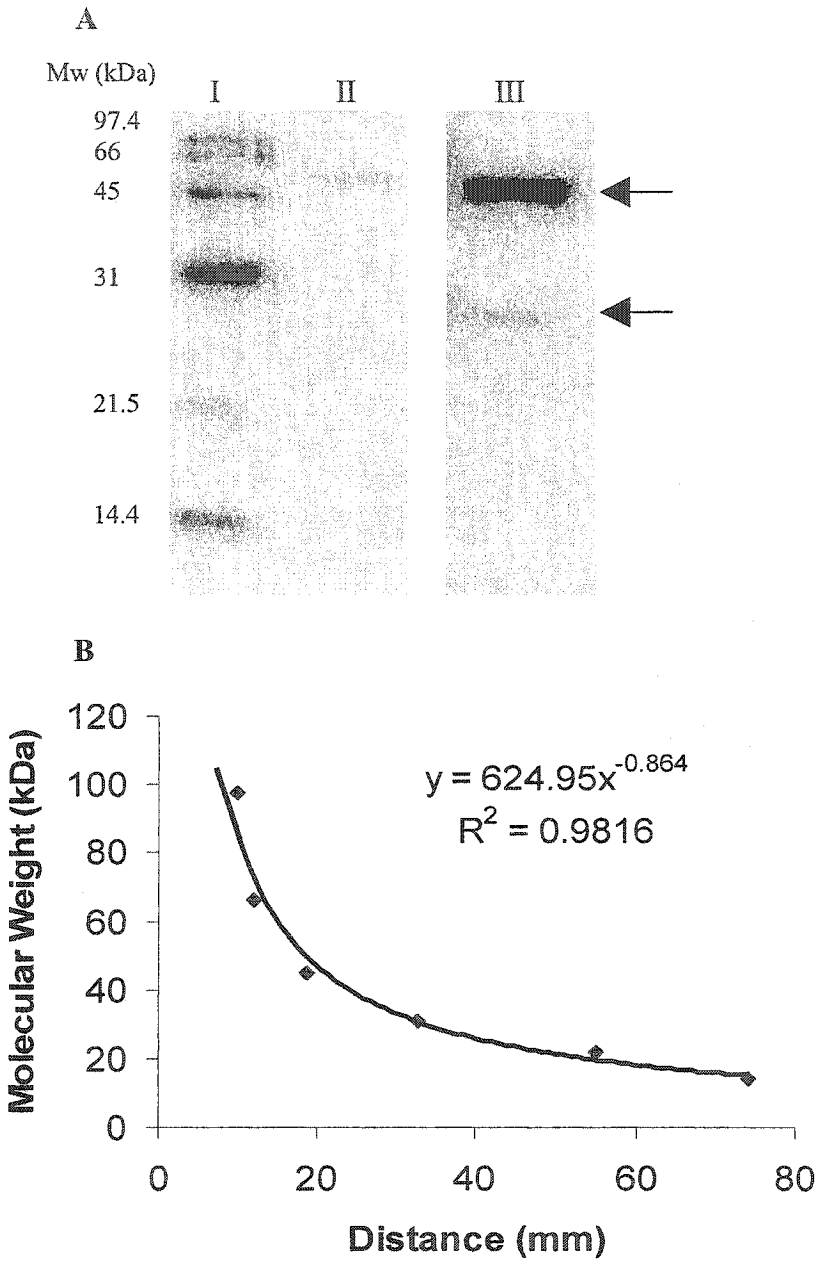


Figure 4.3: Estimation of apparent molecular weight of SN-IR SgII-derived proteins in the goldfish pituitary. **A,** western blot analysis of a pituitary sample in June (~100 μg of protein) using the anti-SN antiserum (dilution: 1:4000). Results of biotinylated SDS-PAGE standards (I) and pituitary protein detections (II, 17-second exposure; III, 200-second exposure) are shown. Arrows indicate two SN-IR SgII products whose gel running distances are 16 and 34 mm, corresponding to ~57 kDa and ~30 kDa respectively. Molecular weight standards are shown on the left. **B,** a standard curve and a formula depicting the relationship between molecular weight of biotinylated protein standards and gel running distance. In the formula, x presents gel running distance (mm), and y presents molecular weight (kDa).

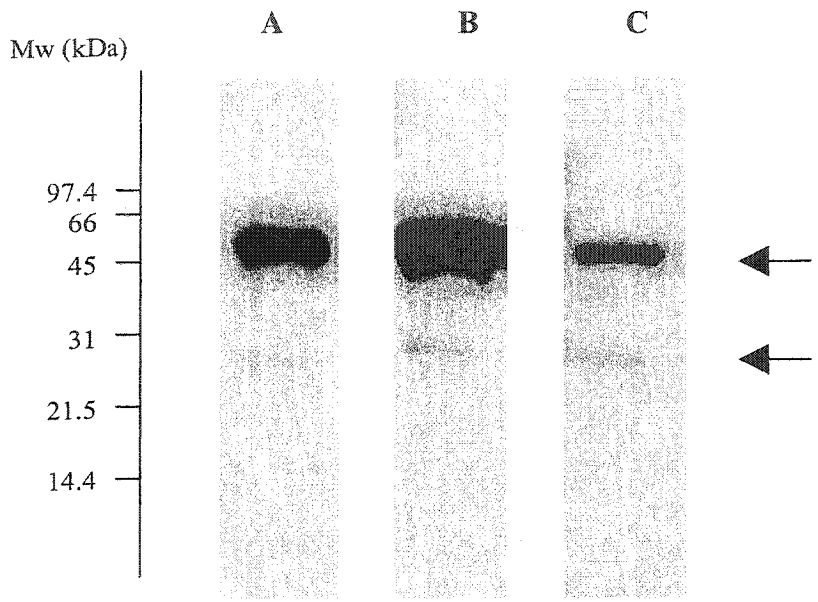


Figure 4.4: Western blot analysis for SN-IR SgII-derived peptides in the goldfish pituitary. Pituitary total proteins (~100 μg) were separated by 10% SDS-PAGE and detected using the anti-SN antiserum (dilution: 1:4000). **A**, **B** and **C**, respectively indicating the goldfish pituitaries collected in March, April and June, were taken from independent gels. Arrows indicates two SN-IR SgII products, ~57 kDa and ~30 kDa. Relative placement of the molecular weight standards is shown on the left.

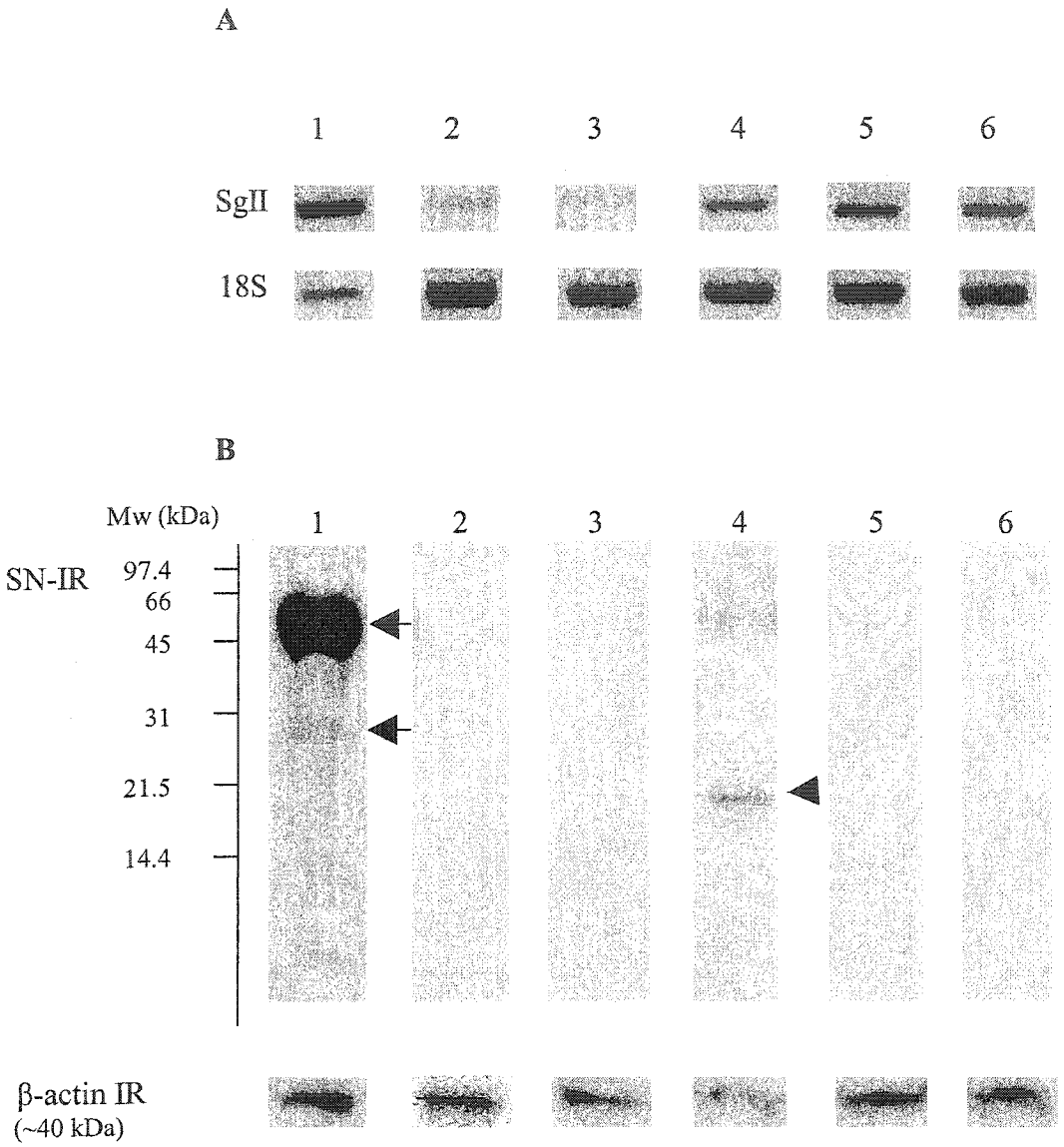


Figure 4.5: Distribution of SgII mRNA and SN-IR peptides in goldfish. **A,** RT-PCR analysis of SgII mRNA expression in tissues of whole pituitary (1), interrenal (2), ovary (3), hypothalamus (4), cerebellum (5) and telencephalon (6). PCR products were analyzed on an agarose gel and stained with ethidium bromide. 18S ribosomal RNA was used as a loading control. **B,** western blot analysis of various tissues (1-6) in goldfish. Proteins (~100 μg) were separated by 10% SDS-PAGE and detected using the anti-SN antiserum (dilution: 1:4000). β-actin IR was used as a protein loading control. Arrows and arrowhead indicate the SN-IR Sg-II derived peptides in the pituitary (~57 kDa and ~30 kDa) and in the hypothalamus (~20 kDa), respectively. Relative placement of the molecular weight standards is shown on the left.

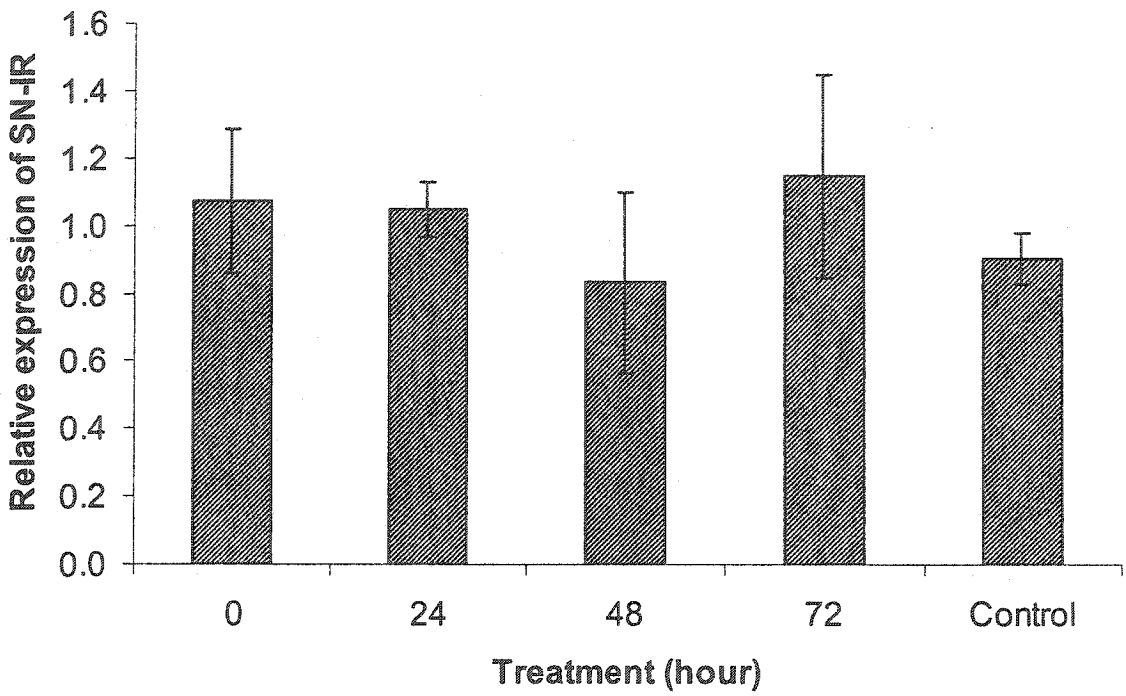


Figure 4.6: Time-course effects of sGnRHa and DOM on SgII processing *in vivo* in the goldfish pituitary. Mixed sex regressed fish were intraperitoneal injected with sGnRHa (0.05 μg/g body weight, BW) in 0.6% saline vehicle (1 μl/g BW) and DOM (5 μg/g BW) in DMSO vehicle (1 μl/g BW). The control did not receive any treatments. Pituitary samples were collected 0, 24, 48 and 72 hours after injections, and levels of the major SN-IR SgII-derived proteins (~57 kDa) were analyzed by western blot analysis using the anti-SN antiserum (dilution: 1:2000). β-actin IR was used as an internal standard for protein loading control. Samples were pools of 5 pituitaries. Results are presented as the mean ± SEM (n=5) of the relative expression of SN-IR. One-way ANOVA indicated that the treatment groups had similar values ($p>0.05$) to 0-hour or control.

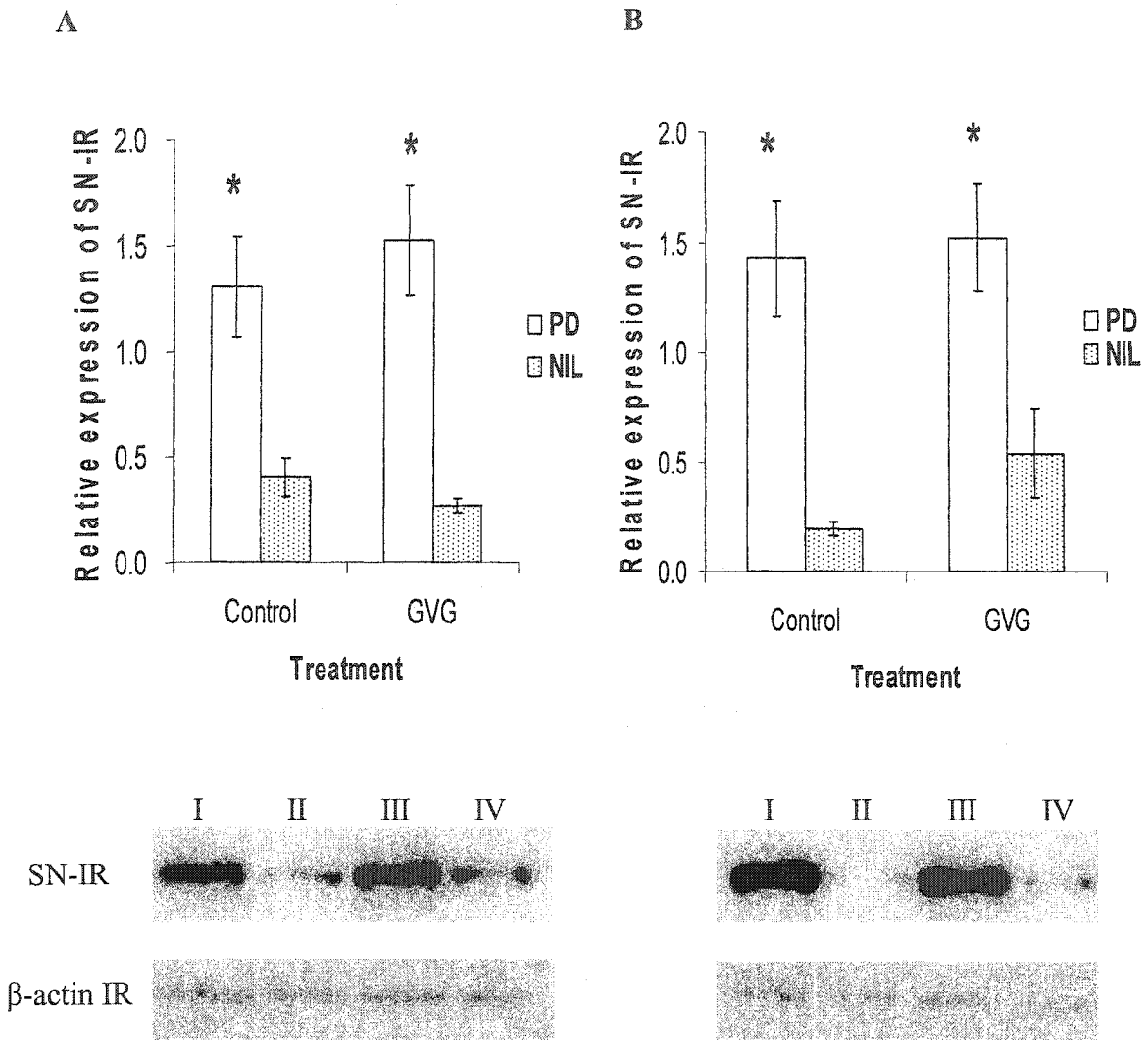


Figure 4.7: Effects of GVG on SN-IR SgII-derived peptides *in vivo* in the goldfish pituitary. Sexually recrudescent female (A) and male fish (B) were intraperitoneal injected with GVG (300 μg/g body weight, BW) in 0.6% saline vehicle (1 μl/g BW). Controls received an equivalent volume of 0.6% saline. The PD and NIL of pituitary were collected 24 hours after injections, and levels of the major SN-IR SgII-derived proteins (~57 kDa) were analyzed by western blot analysis using the anti-SN antiserum (dilution: 1:2000). β-actin IR was used as a protein loading control. Samples were pools of PD or NIL from 5 pituitaries. Results are presented as the mean ± SEM of the relative expression of SN-IR **A,** n=7 for the PD of control (I) and GVG treatment (III), n=6 for the NIL of control (II) and n=4 for the NIL of GVG treatment (IV); **B,** n=4 for the NIL of control (II), n=5 for the PD of control (I) & GVG treatment (III) and the NIL of GVG treatment (IV). The (*) indicated significant different between tissues ($p<0.05$). Similar values were shown between treatments ($p>0.05$).

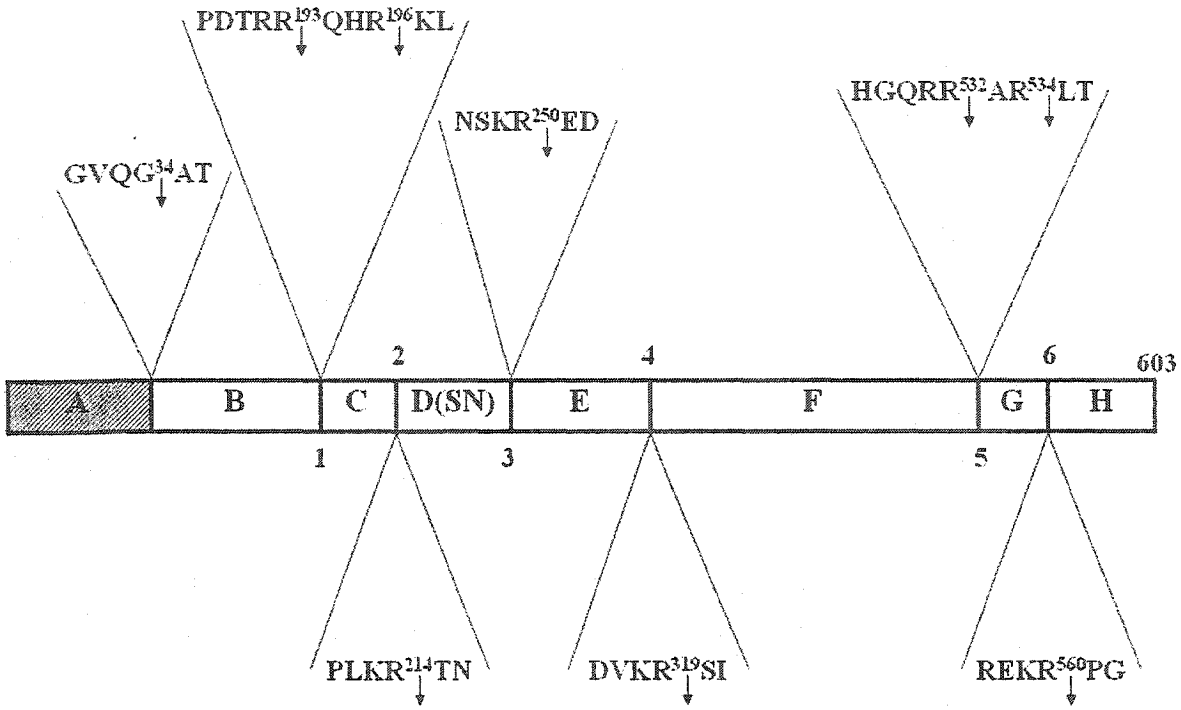


Figure 4.8: Potential cleavage sites with dibasic amino acid residues in goldfish SgII (603 amino acids). The six possible dibasic cleavage sites and sequences are indicated by numbers on the top or bottom of SgII precursor molecule. The arrows with numbers indicate the amino acid positions immediately after putative cleavages sites. Abbreviations: A, signal peptide; C, LF-19; D (SN), secretoneurin; H, LA-42. Note: we use the mammalian nomenclature for these peptides; however, they are poorly conserved in goldfish (Blazquez *et al.*, 1998a).

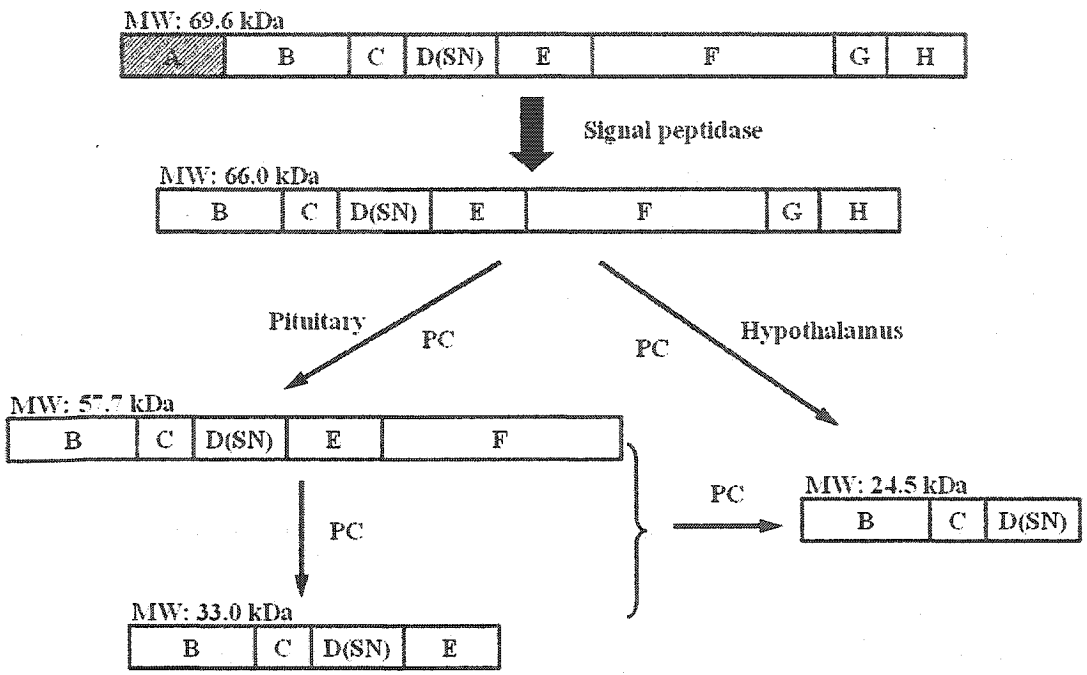


Figure 4.9: Proposed model for generation of various processed forms of goldfish SgII as observed in pituitary and hypothalamus. This model does not preclude other SN containing peptides, which we have not yet been detected. Abbreviations: PC, prohormone convertase-like protease; MW, molecular weight; A, signal peptide; C, LF-19; D (SN), secretoneurin; H, LA-42. Note: we use the mammalian nomenclature for these peptides; however, they are poorly conserved in goldfish (Blazquez *et al.* 1998a).

Chapter 5: General discussion and major conclusions

Secretoneurin (SN) is a recently discovered functional peptide derived from secretogranin II (SgII), a protein belonging to the family of chromogranins (Fischer-Colbrie *et al.*, 1995). Concerning its biological activity, SN stimulates luteinizing hormone (LH) release in goldfish pre-injected with a dopamine (DA) antagonist (Blazquez *et al.*, 1998a). This attracted us to further study the direct stimulation of SN on the LH release from the goldfish pituitary *in vitro*. It is now clear that SN exhibits direct stimulation of LH release as well as to specifically up-regulate LHβ mRNA expression in static incubation of goldfish pituitary fragments. The other aim of our studies was to determine the site of production of SgII-derived peptides containing SN. Our observations indicated that SgII processing occurred in the goldfish pituitary. Furthermore, the main intermediate protein containing a SN-immunoreactivity (SN-IR) sequence was ~57 kDa and showed higher levels in the *pars distalis* (PD) than in the neurointermediate lobe (NIL).

The first objective of the study was to explore the potential function of SN in the regulation of goldfish reproduction. We hypothesized that SN would have a direct dose-related effect on LH release *in vitro*. Goldfish pituitary fragments in static incubation were chosen as the experimental model *in vitro* to explore the chronic effects of SN on the LH secretion. A surprising finding was shown in the first static incubation: treatment with a high dose of SN caused an increase of LH secretion from the pituitary at the 3rd hour but not at the 12th hour. Salmon gonadotropin-releasing hormone analog (sGnRHa) displayed a similar stimulatory effect on LH release after 3-hour incubation, and this persisted for up to 12 hours, consistent with a previous study using dispersed pituitary

cells (Chang *et al.*, 1990a). 'Bell-shaped' dose-response curves of SN were obtained from two different experiments, suggesting that middle (10 nM) of the dose-response range was more effective to induce LH secretion *in vitro*. In addition, 100 nM SN showed an effect on up-regulation of LH release. Therefore, SN significantly stimulated LH secretion from the goldfish pituitary in the range of 10-100 nM. According to SN-IR measurements in various rat tissues (Leitner *et al.*, 1996), this LH level is close to a physiological level of SN in the pituitary. Although details in the specific dose-related manner of SN are still unclear, the hormetic dose-response concept seems to provide a possible explanation (Calabrese and Baldwin, 2003b). Furthermore, the time-course study revealed that a 3-hour static treatment was optimal for LH release. Another observation indicates that there might be seasonal change in the pituitary responsiveness to SN, as with other LH releasing hormone (Trudeau, 1997).

The gonadotropins are heterodimeric glycoprotein composed of a common α-subunit and a hormone-specific β-subunit (Schulz *et al.*, 2001). In order to explore SN regulation of LH synthesis, we measured levels of the LHβ subunit mRNA after 6-hour static incubation using Northern blots. Treatment with SN caused a significant increase of LHβ gene expression in the goldfish pituitary, suggesting that SN can modulate LH synthesis in addition to its secretion. However, SN had no effect on gene expression of pituitary GH and SgII in static incubation, demonstrating specificity of SN action on LHβ subunit mRNA.

The perifusion system was the other effective method *in vitro* to investigate the effect of various regulatory factors in the goldfish pituitary which has been applied in neuroendocrine studies (Murthy *et al.*, 1993; Chang *et al.*, 2001). It was evident that 5-

minute pulse of SN mediated a rise on the LH secretion in perifusion experiment, although the basal LH secretion was not very stable in our aspect. In comparing the action of gonadotropin-releasing hormone (GnRH) on LH release, the SN stimulation appeared a postponed effect that occurred at least 20 minutes after treatment. However, responses were variable and have to be confirmed. Future research should focus on SN dose-response characteristics on the LH gene expression and release after the perifusion system is improved considerably.

To study production of SN containing peptides, a new SN polyclonal antibody generated in our laboratory was characterized for western blot analysis. Several intermediate peptides from 75 kDa and 21 kDa are demonstrated to generate from 86-kDa mammalian SgII in endocrine tissue and neurons (Fischer-Colbrie *et al.*, 1995), especially in the pituitary (Vallet *et al.*, 1997). We hypothesized that SgII-derived protein containing SN sequence would be produced in the goldfish pituitary. Two SgII products, ~57 kDa and ~30 kDa, were found to be present in the pituitary using the anti-SN antiserum. The level of ~57-kDa protein was expressed much higher than that of ~30-kDa protein, which indicates the former is the predominant intermediate polypeptide derived from SgII processing in the goldfish pituitary. According to the amino acid sequence and possible dibasic cleavage sites of goldfish SgII described previously (Blazquez *et al.*, 1998a), these two SN-IR intermediate proteins are perhaps composed of 496 and 283 amino acids derived from the N-terminal part of goldfish SgII molecule.

SgII mRNA are produced in the goldfish pituitary and brain (Samia, 2002). In our RT-PCR experiment, SgII mRNA expression was detected in the goldfish brain and peripheral organs, like ovary and interrenal, with the highest level being in the pituitary.

Furthermore, tissue distributions of SgII processing were established, and the SN-IR polypeptides were only observed in the pituitary (~57 kDa and ~30 kDa) and hypothalamus (~20 kDa). This finding is in agreement with results concerning rat tissues that the highest concentration of SN-IR is found in the pituitary (Leitner *et al.*, 1996). The possible main explanations for the absence of SgII products containing SN in the other organs might be that, SgII is directly cleaved to produce the free SN peptide which is too small to detect in our western blot analysis. Alternatively, these tissues may yield different SgII-derived peptides not containing the SN sequence.

GnRH and γ-aminobutyric acid (GABA) are two stimulators of SgII mRNA expression in the goldfish pituitary *in vivo* (Blazquez *et al.*, 1998a; Samia, 2002). We studied levels of the major ~57-kDa SgII-derived protein in the PD and the NIL after treatment with the GABA drug GVG or sGnRHa plus a DA antagonist. Although neither of these two treatments was capable of altering the ~57-kDa protein level significantly, a surprising finding was the striking high level of this SgII product in the PD compared to the NIL regardless of sex. This result is consistent with the recent data concerning the levels of SgII mRNA expression in the goldfish pituitary (Samia, 2002).

The present research has demonstrated for the first time in any vertebrate a direct stimulatory effect of SN on LH release *in vitro*. Furthermore, the studies of SN-IR have identified and explored the SgII processing in goldfish pituitary. In order to confirm its distribution in goldfish, a free SN peptide should be detected in the pituitary as well as other tissues. This would be best performed using HPLC with specific SN RIA (Leitner *et al.*, 1996). Seasonal variation of reproduction might influence the SgII processing to generate SN in fish, which is worth investigating in the future. In order to clarify

mechanism of SN production in goldfish, the specific enzymes responsible for cleavages of SgII leading to the formation of ~57, ~30 and ~20 kDa fragments have to be identified in future studies. If the function, production and distribution of SN are clearly described, our studies will concentrate on answering the question whether or not SN is a new hormone in the neuroendocrine system of goldfish and/or other vertebrates. Consequently, the long-term research of SN will focus on the isolation and localization of SN receptors, and then the structure of SN receptors as well as the signal transduction mechanisms mediating SN effects in the pituitary or other potential target tissues.

References

Acs Z, Szabo B, Kapocs G, Makara GB 1987 Gamma-aminobutyric acid stimulates pituitary growth hormone secretion in the neonatal rat. A superfusion study. Endocrinology 120:1790-1798

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

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

Amoss M, Burgus R, Blackwell P, Vale W, Fellows R and Guillemin R 1971 Purification, amino acid composition and N-terminus of the hypothalamic luteinizing hormone releasing factor (LRF) of ovine origin. Biochem Biophys Res Commun 44:205-210

Andrews WV, Maurer RA, Conn PM 1988 Stimulation of rat luteinizing hormone-β messenger RNA levels by gonadotropin releasing hormone: apparent role for protein kinase. C J Biol Chem 263:13755–13761

Ang CW, Dotman CH, Winkler H, Fischer-Colbrie R, Sonnemans MA, Van Leeuwen FW 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

Anouar Y, Benie T, De Monti M, Counis R, Duval J 1991 Estradiol negatively regulates secretogranin II and chromogranin A messenger ribonucleic acid levels in the female rat pituitary but not in the adrenal. Endocrinology 129:2393-2399

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 JM, 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

Basak A, Boudreault A, Chen A, Chretien M, Seidah NG, 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

Bauer JW, 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 R, Hoflehner J, Doblinger A, Kapeller I, Laslop A 2000 Regulation of the biosynthesis and processing of chromogranins in organotypic slices: influence of depolarization, forskolin and differentiating factors. Eur J Neurosci 12:2746-2756

Benedum UM, Lamouroux A, Konecki DS, Rosa P, Hille A, Baeuerle PA, Frank R, Lottspeich F, Mallet J, Huttner WB 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

Blaschko H, Comline RS, Schneider FH, Silver M, Smith AD 1967 Secretion of a chromaffin granule protein, chromogranin, from the adrenal gland after splanchnic stimulation. Nature 215:58-59

Blazquez M, Bosma PT, Chang JP, Docherty K, Trudeau VL 1998a Gamma-aminobutyric acid up-regulates the expression of a novel secretogranin-II messenger ribonucleic acid in the goldfish pituitary. Endocrinology 139:4870-4880

Blazquez M, Bosma PT, Fraser EJ, Van Look KJW, Trudeau VL 1998b Fish as models for the neuroendocrine regulation of reproduction and growth. Comp Biochem Physiol C Pharmacol Toxicol Endocrinol 119:345-364

Bogerd J, Li KW, Janssen-Dommerholt C, Goos H 1992 Two gonadotropin-releasing hormones from African catfish (*Clarias gariepinus*). Biochem Biophys Res Commun 187:127-134

Calabrese EJ, Baldwin LA 2003a Toxicology rethinks its central belief. Nature 421:691-692

Calabrese EJ, Baldwin LA 2003b Peptides and hormesis. Crit Rev Toxicol 33:355-405

Chang JP, Cook H, Freedman GL, Wiggs AJ, Somoza GM, de Leeuw R, Peter RE 1990a 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 JP, Jobin RM, Wong AOL 1993 Intracellular mechanisms mediating gonadotropin and growth hormone release in the goldfish, *Carassius auratus*. Fish Physiol Biochem 11:25-33

Chang JP, Jobin RM 1994 Regulation of gonadotropin in vertebrates: a comparison of GnRH mechanisms of action. Processings of the XII International Congress of Comparative Endocrinology 41-51

Chang JP, MacKenzie DS, Gould DR, Peter RE 1984 Effects of dopamine and norepinephrine on in vitro spontaneous and gonadotropin-releasing hormone-induced gonadotropin release by dispersed cells or fragments of the goldfish pituitary. Life Sci 35:2027-2033

Chang JP, Van Goor F, Acharya S 1991 Influences of norepinephrine, and adrenergic agonists and antagonists on gonadotropin secretion from dispersed pituitary cells of goldfish, *Carassius auratus*. Neuroendocrinology 54:202-210

Chang JP, Wirachowsky NR, Kwong P, Johnson JD 2001 PACAP stimulation of gonadotropin-II secretion in goldfish pituitary cells: mechanisms of action and interaction with gonadotropin releasing hormone signaling. J Neuroendocrinology 11:1-12

Chang JP, Yu KL, Wong AO, Peter RE 1990b Differential actions of dopamine receptor subtypes on gonadotropin and growth hormone release in vitro in goldfish. Neuroendocrinology. 51:664-674

Chomczynski P, Sacchi N 1987 Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal Biochem 1621:156-159

Cohn DV, Fasciotto BH, Reese BK, Zhang JX 1995 Chromogranin A: a novel regulator of parathyroid gland secretion. J Nutr 125:2015S-2019S

Conlon JM, Collin F, Chiang YC, Sower SA and Vaudry H 1993 Two molecular forms of gonadotropin-releasing hormone from the brain of the frog, *Rana ridibunda*: Purification, characterization, and distribution. Endocrinology 132:2117-2120

Conn PM, Janovick JA, Braden TD, Maurer RA, Jennes L 1992 SIIp: a unique secretogranin/chromogranin of the pituitary released in response to gonadotropin-releasing hormone. Endocrinology 130:3033-3040

Coser KR, Chesnes J, Hur J, Ray S, Isselbacher KJ, Shioda T 2003 Global analysis of ligand sensitivity of estrogen inducible and suppressible genes in MCF7/BUS breast cancer cells by DNA microarray. Proc Natl Acad Sci U S A 100:13994-13999

D'Este L, Renda T 1995 Phylogenetic study on distribution and chromogranin/secretogranin content of histamine immunoreactive elements in the gut. Ital J Anat Embryol 100:403-410

Deftos LJ, Bjornsson BT, Burton DW, O'Connor DT, Copp DH 1987 Chromogranin A is present in and released by fish endocrine tissue. Life Sci 40:2133-2136

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

Dunzendorfer S, Schratzberger P, Reinisch N, Kahler CM, Wiedermann CJ 1998 Secretoneurin, a novel neuropeptide, is a potent chemoattractant for human eosinophils. Blood 91:1527-1532

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

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

Fujimoto J, Narayanan CS, Benjamin JE, Heinflink M, Gershengorn MC 1992 Mechanism of regulation of thyrotropin-releasing hormone receptor messenger ribonucleic acid in stably transfected rat pituitary cells. Endocrinology 130:1879-1884

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 Commun156:1237-1242

Galindo E, Zwiller J, Bader MF, Aunis D 1992 Chromostatin inhibits catecholamine secretion in adrenal chromaffin cells by activating a protein phosphatase. Proc Natl Acad Sci U S A 89:7398-7402

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

Habibi HR, Peter RE, Nahorniak CS, Milton RC, Millar RP 1992 Activity of vertebrate gonadotropin-releasing hormones and analogs with variant amino acid residues in positions 5, 7 and 8 in the goldfish pituitary. Regul Pept 37:271-284

Harvey S, Baidwan JS 1989 Thyrotrophin-releasing hormone (TRH)-induced growth hormone secretion in fowl: binding of TRH to pituitary membranes. J Mol Endocrinol 3:23-32

Harvey S, Baidwan JS 1990 Desensitization of thyrotrophin-releasing hormone (TRH)-induced growth hormone secretion in chickens: coincident down-regulation of TRH binding to pituitary membranes. J Mol Endocrinol 4:223-230

Havern RL, Whisnant CS, Goodman RL 1994 Dopaminergic structures in the ovine hypothalamus mediating estradiol negative feedback in anestrous ewes. Endocrinology 134:1905-1914

Hendy GN, Bevan S, Mattei MG, Mouland AJ 1995 Chromogranin A. Clin Invest Med 18:47-65

Hoflehner J, Eder U, Laslop A, Seidah NG, 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

Huttner WB, Gerdes HH, Rosa P 1991 The granin (chromogranin/secretogranin) family. Trends Biochem Sci 16:27-30

Jackson GL, Kuehl D 2002 The GABA(B) antagonist CGP 52432 attenuates the stimulatory effect of the GABA(B) agonist SKF 97541 on luteinizing hormone secretion in the male sheep. Exp Biol Med (Maywood) 227:315-320

Jobin RM, Neumann CM, Chang JP 1996 Roles of calcium and calmodulin in the mediation of acute and sustained GnRH-stimulated gonadotropin secretion from dispersed goldfish pituitary cells. Gen Comp Endocrinol 101:91-106

Johnson JD, Chang JP 2002 Agonist-specific and sexual stage-dependent inhibition of gonadotropin-releasing hormone-stimulated gonadotropin and growth hormone release by ryanodine: relationship to sexual stage-dependent caffeine-sensitive hormone release. J Neuroendocrinol 14:144-155

Kah O, Dubourg P, Martinoli MG, Rabhi M, Gonnet F, Geffard M, Calas A 1987 Central GABAergic innervation of the pituitary in goldfish: a radioautographic and immunocytochemical study at the electron microscope level. Gen Comp Endocrinol 67:324-332

Kah O, Trudeau VL, Sloley BD, Chang JP, Dubourg P, Yu KL, Peter RE 1992 Influence of GABA on gonadotropin release in the goldfish. Neuroendocrinology 55:396-404

Kahler CM, 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

Kahler CM, Kirchmair R, Kaufmann G, Kahler ST, Reinisch N, Fischer-Colbrie R, Hogue-Angeletti R, Winkler H, Wiedermann CJ 1997a Inhibition of proliferation and stimulation of migration of endothelial cells by secretoneurin in vitro. Arterioscler Thromb Vasc Biol 17:932-939

Kahler CM, Schratzberger P, Wiedermann CJ 1997b Response of vascular smooth muscle cells to the neuropeptide secretoneurin. A functional role for migration and proliferation in vitro. Arterioscler Thromb Vasc Biol 17:2029-2035

Kakar SS, Wei N, Mulchahey JJ, LeBoeuf RD, Neill JD 1993 Regulation of expression of secretogranin II mRNA in female rat pituitary and hypothalamus. Neuroendocrinology 57:422-431

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

Kim M-H, Oka Y, Amano M, Kobayashi M, Okuzawa K, Hasegawa Y, Kawashma S, Suzuki Y, Aida K 1995 Immunocytochemical localization of sGnRH and cGnRH-II in the brain of goldfish, *Carassius auratus*. J Comp Neurol 356:72-82

Kim T, Tao-Cheng JH, Eiden LE, Loh YP 2001 Chromogranin A, an "on/off" switch controlling dense-core secretory granule biogenesis. Cell 106:499-509

Kineman RD, Gettys TW, Frawley LS 1994 Paradoxical effects of dopamine (DA): Gi alpha 3 mediates DA inhibition of PRL release while masking its PRL-releasing activity. Endocrinology 135:790-793

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 JP, Habibi HR 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

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

Konecki DS, Benedum UM, Gerdes HH, Huttner WB 1987 The primary structure of human chromogranin A and pancreastatin. J Biol Chem 262:17026-17030

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

Lazure C, Benjannet S, Seidah NG, Chretien M 1991 Processed forms of neuroendocrine proteins 7B2 and secretogranin II are found in porcine pituitary extracts. Int J Pept Protein Res 38:392-400

Lescheid DW, Terasawa E, Abler LA, Urbanski HF, Warby CM, Millar RP, Sherwood NM 1997 A second form of gonadotropin-releasing hormone (GnRH) with characteristics of chicken GnRH-II is present in the primate brain. Endocrinology 138:5618-5629

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 WA, 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

Leung PC, Peng C 1996 Gonadotropin-releasing hormone receptor: gene structure, expression and regulation. Biol Signals 5:63-69

Lewis JJ, Zdon MJ, Adrian TE, Modlin IM 1988 Pancreatatin: a novel peptide inhibitor of parietal cell secretion. Surgery 104:1031-1036

Li JY, Leitner B, Lovisetti-Scamihorn P, Winkler H, Dahlstrom A 1999 Proteolytic processing, axonal transport and differential distribution of chromogranins A and B, and secretogranin II (secretoneurin) in rat sciatic nerve and spinal cord. Eur J Neurosci 11:528-544

Lo A, Chang JP 1998 In vitro application of testosterone potentiates gonadotropin-releasing hormone-stimulated gonadotropin-II secretion from cultured goldfish pituitary cells. Gen Comp Endocrinol 111:334-346

Lovejoy DA, Fischer WH, Ngamvongchon S, Craig AG, Nahorniak CS, Peter RE, Rivier JE, Sherwood NM 1992 Distinct sequence of gonadotropin-releasing hormone (GnRH) in dogfish brain provides insight into GnRH evolution. Proc Natl Acad Sci U S A 89:6373-6377

Lovejoy DA, Fischer WH, Parker DB, McRory JE, Park M, Lance V, Swanson P, Rivier JE, Sherwood NM 1991a Primary structure of two forms of gonadotropin-releasing hormone from brains of the American alligator (Alligator mississippiensis). Regul Pept 33:105-116

Lovejoy DA, Sherwood NM, Fischer WH, Jackson BC, Rivier JE, Lee T 1991b Primary structure of gonadotropin-releasing hormone from the brain of a holocephalan (ratfish: *Hydrolagus colliei*). Gen Comp Endocrinol 82:152-161

Mahata SK, 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 SK, Mahata M, Steiner HJ, 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

Mananos EL, Anglade I, Chyb J, Saligaut C, Breton B, Kah O 1999 Involvement of gamma-aminobutyric acid in the control of GTH-1 and GTH-2 secretion in male and female rainbow trout. Neuroendocrinology 69:269-280

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

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

Matsuo H, Baba Y, Nair RMG, Arimura A, Schally AV 1971 Structure of the porcine LH- and FSH-releasing hormone. I. The proposed amino acid sequence. Biochem Biophys Res Commun 43:1334-1339

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

Miyanoto K, Hasegawa Y, Nomura M, Igarashi M, Kangawa K, Matsuo H 1984 Identification of the second gonadotropin-releasing hormone in chicken hypothalamus: Evidence that gonadotropin secretion is probably controlled by two distinct gonadotropin-releasing hormones in avian species. Pro Natl Acad Sci U S A 81:3874-3878

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

Muller L, Barret A, Picart R, Tougard C 1997 Proteolytic processing of sulfated

secretogranin II in the trans-Golgi network of GH3B6 prolactin cells. J Biol Chem 272:3669-3673

Muller L, Picart R, Barret A, Seidah NG, Tougard C 1998 Immunocytochemical localization of the prohormone convertases PC1 and PC2 in rat prolactin cells. J Histochem Cytochem 46:101-108

Murthy CK, Nahorniak CS, Rivier JE, Peter RE 1993 In vitro characterization of gonadotropin-releasing hormone antagonists in goldfish, *Carassius auratus*. Endocrinology 133:1633-1644

Natori S, Huttner WB 1994 Peptides derived from the granin (Chromogranins / secretogranins). Biochimie 76:277-282

Nobels FR, Kwekkeboom DJ, Coopmans W, Schoenmakers CH, Lindemans J, De Herder WW, Krenning EP, Bouillon R, Lamberts SW 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

Nicol L, McNeilly JR, Stridsberg M, Crawford JL, McNeilly AS 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

Okubo K, Aida K 2001 Gonadotropin-releasing hormones (GnRHs) in a primitive teleost, the arowana: phylogenetic evidence that three paralogous lineages of GnRH occurred prior to the emergence of teleosts. Gen Comp Endocrinol 124:125-133

Okubo K, Amano M, Yoshiura Y, Suetake H, and Aida K 2000 A novel form of gonadotropin-releasing hormone in the medaka, *Oryzias latipes*. Biochem Biophys Res Commun 276:298-303

Omeljaniuk RJ, Habibi HR, Peter RE 1989a Alterations in pituitary GnRH and dopamine receptors associated with the seasonal variation and regulation of gonadotropin release in the goldfish (*Carassius auratus*). Gen Comp Endocrinol 74:392-399

Omeljaniuk RJ, Tonon MC, Peter RE 1989b Dopamine inhibition of gonadotropin and alpha-melanocyte-stimulating hormone release in vitro from the pituitary of the goldfish (*Carassius auratus*). Gen Comp Endocrinol 74:451-467

Omeljaniuk RJ, Shih SH, Peter RE 1987 In-vivo evaluation of dopamine receptor-mediated inhibition of gonadotropin secretion from the pituitary gland of the goldfish, *Carassius auratus*. J Endocrinol 114:449-458

Peng C, Chang JP, Yu KL, Wong AO, Van Goor F, Peter RE, Rivier JE 1993 Neuropeptide-Y stimulates growth hormone and gonadotropin-II secretion in the goldfish

pituitary: involvement of both presynaptic and pituitary cell actions. Endocrinology 132: 1820-1829

Peng C, Fan NC, Ligier M, Vaananen J, Leung PC 1994 Expression and regulation of gonadotropin-releasing hormone (GnRH) and GnRH receptor messenger ribonucleic acids in human granulosa-luteal cells. Endocrinology 135:1740-1746

Peter RE, Chang JP, Nahorniak CS, Omeljaniuk RJ, Sokolowska M, Shih SH, Billard R 1986 Interactions of catecholamines and GnRH in regulation of gonadotropin secretion in teleost fish. Recent Prog Horm Res 42:513-548

Peter RE, Nahorniak CS, Shih S, King JA, and Millar RP 1987 Activity of position-8-substituted analogs of mammalian gonadotropin-releasing hormone (mGnRH) and chicken and lamprey gonadotropin-releasing hormones in goldfish. Gen Comp Endocrinol 65:385-393

Powell JF, Zohar Y, Elizur A, Park M, Fischer WH, Craig AG, Rivier JE, Lovejoy DA and Sherwood NM 1994 Three forms of gonadotropin-releasing hormone characterized from brains of one species. Proc Natl Acad Sci U S A 91:12081-12085

Quanbeck C, Sherwood N, Millar R and Terasawa E 1997 Two populations of luteinizing hormone-releasing neurons in the forebrain of the rhesus macaque during embryonic development. J Comp Neurol 380:293-309

Ramirez JL, Castano JP, Gracia-Navarro F 1998 Somatostatin at low doses stimulates growth hormone release from intact cultures of porcine pituitary cells. Horm Metab Res 30:175-177

Reinisch N, Kirchmair R, Kahler CM, Hogue-Angeletti R, Fischer-Colbrie R, Winkler H, Wiedermann CJ 1993 Attraction of human monocytes by the neuropeptide secretoneurin. FEBS 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 WB 1985a 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, Gerdes HH 1994 The granin protein family: Markers for neuroendocrine cells and tools for the diagnosis of neuroendocrine tumors. J Endocr Invest 17:207-225

Rosa P, Hille A, Lee RW, Zanini A, De Camilli P, Huttner WB 1985b Secretogranins I and II: two tyrosine-sulfated secretory proteins common to a variety of cells secreting peptides by the regulated pathway. J Cell Biol 101:1999-2011

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

Rosa P, Zanini A 1983 Purification of a sulfated secretory protein from the adenohypophysis. Immunochemical evidence that similar macromolecules are present in other glands. Eur J Cell Biol 31:94-98

Salvi E, Buffa R, Renda TG 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

Samia M 2002 Neuroendocrine regulation of secretogranin-II (SgII) expression in the goldfish pituitary. MSc thesis submitted to University of Ottawa

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

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 KW, 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

Schneitler C, Kahler C, Wiedermann CJ, 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, Reinisch N, Kahler CM, Wiedermann CJ 1996a Deactivation of chemotaxis of human neutrophils by priming with secretogranin II-derived secretoneurin. Regul Pept 63:65-71

Schratzberger P, Woll E, Reinisch N, Kahler CM, Wiedermann CJ 1996b 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 RW, Vischer HF, Cavaco JE, Santos EM, Tyler CR, Goos HJ, Bogerd J 2001 Gonadotropins, their receptors, and the regulation of testicular functions in fish. Comp Biochem Physiol B Biochem Mol Biol 129:407-417

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 NG, 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

Sherwood NM, Eiden L, Brownstein M, Rivier J, Vale W 1983 Characterization of a teleost gonadotropin-releasing hormone. Proc Natl Acad Sci U S A 80:2794-2798

Sherwood NM, Sower SA, Marshak DR, Fraser BA, Brownstein MJ 1986a Primary structure of gonadotropin-releasing hormone from lamprey brain. J Biol Chem 261:4812-4819

Sherwood NM, Whittier JM 1988 Gonadotropin-releasing hormone from brains of reptiles: turtles (*Pseudemys scripta*) and snakes (*Thamnophis sirtalis parietalis*). Gen Comp Endocrinol 69:319-327

Sherwood NM, Zoeller RT, Moore FL 1986b Multiple forms of gonadotropin-releasing hormone in amphibian brains. Gen Comp Endocrinol 61:313-322

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

Singh H, Griffith RW, Takahashi A, Kawauchi H, Thomas P, Stegeman JJ 1988 Regulation of gonadal steroidogenesis in Fundulus heteroclitus by recombinant salmon growth hormone and purified salmon prolactin. Gen Comp Endocrinol 72:144-153

Smith PK, Krohn RI, Hermanson GT, Mallia AK, Gartner FH, Provenzano MD, Fujimoto EK, Goeke NM, Olson BJ, Klenk DC 1985 Measurement of protein using bicinchoninic acid. Anal Biochem 150:76-85

Sokal RR, Rohlf FJ 1981 Biometry. The principles and practice of statistics in biological research (second edition) p774

Somoza GM, Peter RE 1991 Effects of serotonin on gonadotropin and growth hormone release from in vitro perifused goldfish pituitary fragments. Gen Comp Endocrinol 82:103-110

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

Therriault P 2003 Apoptosis in the adult goldfish pituitary. MSc thesis submitted to University of Ottawa

Trandaburu T, Ali SS 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 SS, 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 SS, 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 VL 1997 Neuroendocrine regulation of gonadotropin II release and gonadal growth in the goldfish, *Carassius auratus*. Rev Reprod 2:55-68

Trudeau VL, Kah O, Chang JP, Sloley BD, Dubourg P, Fraser J, Peter RE 2000a The inhibitory effects of γ-Aminobutyric Acid (GABA) on growth hormone secretion in the goldfish are modulated by sex steroids. J Exp Biol 203:1477-1485

Trudeau VL, Spanswick D, Fraser EJ, Lariviere K, Crump D, Chiu S, MacMillan M, and Schulz RW 2000b The role of amino acid neurotransmitters in the regulation of pituitary gonadotropin release in fish. Biochem Cell Biol 78:241-259

Trudeau VL, Murthy CK, Habibi HR, Sloley BD, Peter RE 1993a Effects of sex steroid treatments on gonadotropin-releasing hormone-stimulated gonadotropin secretion from the goldfish pituitary. Biol Reprod 48:300-307

Trudeau VL, Sloley BD, Peter RE 1993b GABA stimulation of gonadotropin-II release in goldfish: involvement of $GABA_A$ receptors, dopamine, and sex steroids. Am J Physiol 265:348-355

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

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

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

Umemura S, Iwasaka T, Osamura RY 2001 Expression of chromogranin/secretogranin mRNA in spontaneous mammary tumors in aging Fischer-344 rats. Pathol Int 51:667-670

Vallet VS, Li JY, 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 Der Kraak G, Wade MG 1994 A comparison of signal transduction pathways mediating gonadotropin actions in vertebrates. Processings of the XII International Congress of Comparative Endocrinology 59-63

Van Horssen AM, Martens GJ 1999 Biosynthesis of secretogranin II in Xenopus intermediate pituitary. Mol Cell Endocrinol 147:57-64

Virmani MA, Stojilkovic SS, Catt KJ 1990 Stimulation of luteinizing hormone release by gamma-aminobutyric acid (GABA) agonists: mediation by GABAA-type receptors and activation of chloride and voltage-sensitive calcium channels. Endocrinology 126:2499-2505

Wand GS, Takiyyuddin M, O'Connor DT, Levine MA 1991 A proposed role for chromogranin A as a glucocorticoid-responsive autocrine inhibitor of proopiomelanocortin secretion. Endocrinology 128:1345-1351

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

Weber GM, Powell JEE, Park M, Fischer WH, Craig AG, Rivier JE, Nanakorn U, Parhar ID, Ngamvongchon S, Grau EG, Sherwood NM 1997 Evidence that gonadotropin-releasing hormone (GnRH) functions as a proclactin-releasing factor in a teleost fish (*Oreochromis mossambicus*) and primary structures for three native GnRH molecules. J Endocrinol 155:121-132

Wei N, Kakar SS, Neill JD 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

White RB, Fernald RD 1998 Ontogeny of gonadotropin-releasing hormone (GnRH) gene expression reveals a distinct origin for GnRH-containing neurons in the midbrain. Gen Comp Endocrinol 112:322-329

Wiedermann CJ 2000 Secretoneurin: a functional neuropeptide in health and disease. Peptides 21:1289-1298

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

Wong AO, Van Goor F, Chang JP 1994a Entry of extracellular calcium mediates dopamine D_1-stimulated growth hormone release from goldfish pituitary cells. Gen Comp Endocrinol 94:316-328

Wong AO, Van Goor F, Jobin RM, Neumann CM, Chang JP 1994b Interactions of cyclic adenosine 3',5'-monophosphate, protein kinase-C, and calcium in dopamine- and gonadotropin-releasing hormone-stimulated growth hormone release in the goldfish. Endocrinology 135:1593-1604

Wong AO, Van Der Kraak G, Chang JP 1994c Cyclic 3',5'-adenosine monophosphate mediates dopamine D1-stimulated growth hormone release from goldfish pituitary cells. Neuroendocrinology 60:410-417

Wouters S, Leruth M, Decroly E, Vandenbranden M, Creemers JW, van de Loo JW, Ruysschaert JM, Courtoy PJ 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 HJ, Rozansky DJ, Parmer RJ, Gill BM, O'Connor DT 1991 Structure and function of the chromogranin A gene. Clues to evolution and tissue-specific expression. J Biol Chem 266:13130-13134

Yon L, Guillemot J, Montero-Hadjadje M, Grumolato L, Leprince J, Lefebvre H, Contesse V, Plouin PF, Vaudry H, Anouar Y 2003 Identification of the secretogranin II-derived peptide EM66 in pheochromocytomas as a potential marker for discriminating benign versus malignant tumors. J Clin Endocrinol Metab 88:2579-2585

You ZB, 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

Yu KL, Rosenblum PM, Peter RE 1991 In vitro release of gonadotropin-releasing hormone from the brain preoptic-anterior hypothalamic region and pituitary of female goldfish. Gen Comp Endocrinol 81:256-267