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Neuroendocrine regulation of secretogranin-II (SgII) expression in the goldfish pituitary

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Table of contents

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List of abbreviations

Arg	arginine
cDNA	complimentary deoxyribonucleic acid
CNS	central nervous system
DA	dopamine
DEPC	diethyl pyrrocarbonate
DOM	domperidone
E2	estradiol
ER	endoplasmic reticulum
FSH	follicle stimulating hormone
GABA	gamma-aminobutyric acid
GAD	glutamic acid decarboxylase
GH	growth hormone
GnRH	gonadotropin-releasing hormone
sGnRHa	salmon gonadotropin-releasing hormone analog
GSI	gonado-somatic index
GTH	gonadotropin
GVG	gamma-vinyl-GABA
ISG	immature secretory granule
LH	luteinizing hormone
LHRH	luteinizing hormone-releasing hormone
Lys	lysine
mRNA	messenger ribonucleic acid
MSG	mature secretory granule
MSH	melanocyte stimulating hormone
NIL	neurointermediate lobe
NPY	neuropeptide Y
PACAP	pituitary adenylate cyclase-activating polypeptide
PC	prohormone convertase
PD	pars distalis
PI	pars intermedia
POMC	pro-opiomelanocortin
PPD	proximal pars distalis
RIA	radioimmunoassay
RNA	ribonucleic acid
RPD	rostral pars distalis
SgII	secretogranin-II
SN	secretoneurin
T	testosterone
TGN	trans-Golgi network

Abstract

Secretogranin-II (SgII) is a secretory protein involved in the regulated secretory pathway of endocrine and neural cells. Preliminary evidence has suggested a role for it in reproductive function. The objectives of our studies were to elucidate how SgII expression is regulated within the goldfish reproductive system in an attempt to shed some light on its possible actions.

Goldfish are a seasonally reproducing species with an annual cycle. The pituitary SgII expression levels fluctuate with the reproductive cycle. Sex steroids are not directly responsible for the cyclicity of SgII expression, nor do they regulate it at any time of the year. On the other hand, gonadotropin-releasing hormone (GnRH), the principal stimulatory factor of the reproductive system upregulates SgII expression. Testosterone suppresses the stimulatory effect of GnRH at certain stages of the reproductive cycle. Many other studies are necessary to fully comprehend the regulation of SgII within the reproductive system and whether it has any concrete functions therein.

Résumé

La sécrétogranine-II (SgII) est une protéine de sécrétion trouvée dans les cellules endocriniennes et nerveuses. Des donnés préliminaires ont suggérés un rôle pour la SgII dans la reproduction. Les objectifs de ma recherche étaient de déterminer les mécanismes de modification de l'expression génique de la SgII dans le système reproductif, ce qui pourrait donner des indices sur son mode d'action.

Le poisson rouge est une espèce à reproduction cyclique annuelle. Les nivaux d'expression génique hypophysaire de la SgII fluctuent avec le cycle reproductif. Les stéroïdes sexuels ne sont ni responsables de la cyclicité de l'expression génique de la SgII, ni de son modification en auccun moment de l'année. Par contre, la gonadolibérine (GnRH), le stimulateur principal du système reproductif, stimule l'expression génique de la SgII. La téstostérone supprime cet effet stimulateur de la GnRH a certains stades du cycle reproductif. Plusieurs autres études seront nécéssaires afin de pleinement comprendre comment la SgII est controllée dans le système reproductif, et si elle y joue un rôle concret.

Chapter 1. General Introduction

Complex neuroendocrine regulatory mechanisms have evolved to control reproductive cycles in the vertebrates. The hypothalamus releases the decapeptide gonadotropin-releasing hormone which stimulates the synthesis and release of gonadotropins from the pituitary gland. The gonadotropins act on the gonads to stimulate development, gametogenesis, and sex steroid production. The sex steroids feedback to the hypothalamus, pituitary and other higher brain nuclei. Other non-steroid feedback loops also exist, some of which are of a paracrine or autocrine nature.

Secretogranin-II (SgII) is a secretory protein which belongs to the chromogranin family. It has a widespread distribution within the nervous and endocrine systems of vertebrates where it is proteolytically processed to a high degree into smaller bioactive peptides, the most important of which is secretoneurin (SN). In goldfish, SgII was recently shown to play a role within the reproductive system (Blazquez *et al.*, 1998). Previous studies (Fischer-Colbrie *et al.*, 1995 ; Balzquez *et al.*, 1998) have presented two distinct hypotheses of how SgII regulates gonadotropins. It could modulate the intracellular packaging of gonadotropic hormones by acting as a secretory protein or it indirectly regulates their release via SN acting on the gonadotrophs through autocrine or paracrine feedback. The objectives of my research were to examine how SgII is regulated within the goldfish reproductive system (figure 1), possibly shedding some light into its function.

1. The reproductive axis

Two gonadotropin molecules exist within the vertebrates: follicle stimulating hormone

(FSH) and luteinizing hormone (LH). These are respectively homologous to gonadotropin-I (GTH-I) and gonadotropin-II (GTH-II) in teleosts. Both gonadotropins are glycoproteins made of a common α subunit and a variable β subunit which infers specificity to the hormone (Schulz *et al.*, 2001). Gonadotropins are the most important factors stimulating sexual maturation and reproduction. Many neuroendocrine systems interact to control gonadotropin synthesis and release (Trudeau, 1997).

The common goldfish, *Carassius auratus*, is a widely used model for the study of reproductive endocrinology. It has two gonadotropins, GTH-I and GTH-II. GTH-II appears to be functionally more important than GTH-I. Its synthesis and release are stimulated by such factors as GnRH and GABA and inhibited by dopamine, the main inhibitory factor of the system. Sex steroids also play a very important role in GTH-II regulation by modulating different neuroendocrine factors. The modes of action of some of these factors are discussed below. For a comprehensive review of the system, refer to Trudeau (1997).

Goldfish are a seasonally reproducing teleost species. Their reproductive cycle is annual; spawning occurs in the spring (April to May) following which their gonad size decreases to the sexually regressed stage. During late fall and winter, gonadal recrudescence occurs, reaching a maximum size prior to spawning (Peng *et al.*, 1993). Different environmental factors have been shown to modulate the seasonal cycle including water temperature and photoperiod (Razani *et al.*, 1988). The levels of many reproductive hormones such as gonadotropin and sex steroids vary with season, and the seasonal variation of sex steroids modulates different neuroendocrine systems.

During gonadal recrudescence in both males and females, serum gonadotropin-II (GTH-

II) levels increase and reach a peak in April (Sohn *et al.*, 1999). Seasonal increases in GTH-II release stimulate gonadal development and sex steroid production. The sex steroids, testosterone (T) and estradiol (E2), positively feed back at the pituitary to enhance gonadotropin releasing hormone (GnRH) responsiveness and GTH-II release, further promoting gonadal development (Trudeau, 1997). GTH-II levels remain elevated during spawning season, then they decrease to reach their lowest levels around November. The seasonal cyclicity of sex steroids directly correlates with that of GTH-II. Serum steroid levels increase during late fall and winter and peak at spawning (Sohn *et al.*, 1999).

1.1 Gonadotropin-releasing hormone (GnRH)

The decapeptide gonadotropin-releasing hormone is the primary stimulator of gonadotropin release in vertebrates. The primary structures of sixteen different GnRH molecules have been elucidated and are evolutionary highly conserved (Dubois *et al.*, 2001). GnRH neurons originate in the olfactory placode and migrate to the basal forebrain (hypothalamus, pre-optic area) during development (Dellovade *et al*, 1998). In mammals, GnRH is delivered to the pituitary via a portal blood system whereas in teleosts, GnRH neurons in the pre-optic area and hypothalamus directly innervate the pituitary (Dubois *et al.*, 2001). GnRH stimulation of gonadotropin release occurs in a pulsatile fashion; this pulsatility is an intrinsic feature of the GnRH neuronal network and depends on Ca^{2+} signaling. GnRH modulates its own secretion via an autocrine feedback loop (Van Goor *et al.*, 2000).

The goldfish contains two forms of GnRH, salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II) (Debananda and Habibi, 1998). Recent studies have shown that most modern teleost

species have three forms of GnRH, the third form being expressed in the olfactory bulb and rostral telencephalon (Dubois *et al.*, 2001). A third form has not yet been characterized in goldfish. In addition to stimulating gonadotropin release, goldfish GnRHs also stimulate the release of growth hormone (GH) from the pituitary which enhances the effects of GTH-II on the gonads by stimulating gonadal growth and potentiating GTH-II-stimulated steroidogenesis (Chang *et al.*, 1996). In the goldfish, two GnRH receptor subtypes have been cloned (Illing *et al.*, 1999). These receptors share a 71% identity at the amino acid level, but they have marked ligand selectivity and are differentially expressed in the brain, ovary and liver (Illing *et al.*, 1999). Both receptors bind cGnRH-II more potently than sGnRH. cGnRH-II and sGnRH have different intracellular signal transduction pathways, with cGnRH-II being more dependent on extracellular calcium entry than sGnRH, and sGnRH being the only form that mobilizes arachidonic acid (Chang *et al.*, 1993).

GnRH-stimulated GTH-II release is subject to desensitization (Khakoo *et al.*, 1994). Treatment with a single form of GnRH causes a more dramatic desensitization than treatment with a combination of both forms. GnRH not only stimulates GTH-II and GH release, but it also increases their expression (Khakoo *et al.*, 1994 ; Klausen *et al.*, 2001). Treatment with GnRH causes increases in mRNA levels of the α, GTH-I β and GTH-IIβ subunits of gonadotropin as well as GH in both sexually regressed and sexually mature goldfish. This same effect has been reported in other teleost fish species (Melamed *et al.*, 1998). In mammals GnRH upreglates the expression of both LH and FSH subunit mRNA (Kaiser, 1998).

1.2 Dopamine

The catecholamine dopamine (DA) is a very widespread and important neurotransmitter

in vertebrates, along with being the precursor for norepinephrine and epinephrine. DA has been reported to have both stimulatory and inhibitory roles on different systems within the mammalian CNS, and its actions are modulated by sex steroids and other neuroendocrine factors. In ewes pre-treated with estradiol, dopaminergic neurons in the ventromedial hypothalamus were reported to stimulate GnRH and LH release (Anderson *et al.*, 2001). In contrast, another study in ewes reported that median eminence dopaminergic activity inhibited LH release (Bertrand *et al.*, 1998). In rats, the preoptic anterior-hypothalamic dopaminergic system was reported to regulate the release of LH, and this regulation is modulated by the cholinergic system (Cruz *et al.*, 2001). DA acts via a family of five receptors (D1-D5) belonging to the G-protein coupled receptor superfamily (Lachowicz and Sibley, 1997). These receptors regulate a variety of signaling events including adenylate cyclase, phospholipase and various ion channels.

In teleost fishes, dopamine in the principal inhibitory factor of reproductive function. In the goldfish, DA was shown to act directly on gonadotrophs to inhibit spontaneous GTH-II release through the D2 receptor, and indirectly on pre-optic GnRH neurons to inhibit GnRH-stimulated GTH-II release through the D1 receptor *in vitro* and *in vivo* (Chang *et al.*, 1990). Inhibition of GTH-II release by DA has been observed in several other fish species. In the African catfish, DA inhibited both spontaneous and stimulated GTH-II release (de Leeuw *et al.*, 1986). In the rainbow trout, DA inhibited GTH-II release *in vivo*. Its inhibitory actions are modulated by seasonality and the sexual maturity stage of the fish (Saligaut *et al.*, 1992) as well as by estradiol (Linard *et al.*, 1995 ; Saligaut *et al.*, 1998). DA can act upon reproduction via other neuroendocrine systems such as the GABA system, and its actions are modulated by sex steroids and other factors (Trudeau, 1997).

1.3 Gamma-aminobutyric acid (GABA)

The amino acid GABA is one of the most abundant neurotransmitters in the vertebrate central nervous system. It is synthesized principally from glutamate in a single enzymatic step catalyzed by the enzyme glutamic acid decarboxylase (GAD), and is considered to be the major inhibitory neurotransmitter in the vertebrate brain (Trudeau *et al.*, 2000). However, there is increasing evidence that GABA also has stimulatory actions. It plays a robust excitatory role in developing mouse hypothalamic neurons (Gao and van den Pol, 2001), as well as in the rat supra-chiasmatic hypothalamic nucleus, where it regulates diurnal rhythms (Wagner *et al.*, 1997). *In vitro*, GABA was shown to stimulate GnRH release from immortalized rat neurons (Hales *et al.*, 1994).

GABA is found in the major neuroendocrine brain regions of the goldfish hypothalamus and telencephalon where it stimulates GTH-II release form the pituitary (Trudeau *et al.*, 1993a ; Blazquez *et al.*, 1998). This stimulatory effect is a result of stimulation of GnRH release, potentiation of GnRH action, direct action on GTH-II cells and inhibition of dopamine release (Trudeau *et al.*, 2000). The GABAergic control of GTH-II release depends on the sexual maturity stage of the fish and is modulated by sex steroids (Kah *et al.*, 1992). GABA preferentially regulates sGnRH neurons (Sloley *et al.*, 1994), and a $GABA_A$-like receptor seems to be involved (Trudeau *et al.*, 1993a). In addition to stimulating GTH-II release, GABA increases GTH-IIβ mRNA levels in the goldfish pituitary (Blazquez *et al.*, 1998), demonstrating an effect on both synthesis and release of GTH-II.

1.4 Sex steroids

Sex steroids are produced from the gonads upon stimulation with gonadotropins via

specific receptors. Two major types of receptors have been characterized in many mammalian and piscine species: an FSH specific receptor localized to Sertoli cells which stimulates the production of estrogens and an LH specific receptor localized to Lydig cells which stimulates the production of androgens (Schulz *et al.*, 2001). Sex steroids feedback to the pituitary and brain to regulate gonadotropin synthesis and release (Huggard *et al.*, 1996a ; Trudeau *et al.*, 1993b). The effects of sex steroid feedback vary with season and sexual stage, keeping the reproductive axis under tight control (Trudeau, 1997).

In mammals, steroids have been shown to exert both stimulatory and inhibitory effects at the level of the pituitary and the brain. They can act via a classical negative feedback loop to inhibit gonadotropin secretion. This is thought to be achieved by impairing GnRH pulsatility (Van Goor *et al.*, 2000). Other studies have shown that both estrogen and testosterone stimulate gonadotropin synthesis and release from the rat pituitary (Gharib *et al.*, 1990 ; Miller and Wu, 1981). In various teleost species, sex steroids have also been shown to play both stimulatory and inhibitory functions on gonadotropin synthesis and release (Habibi and Huggard, 1998). These contrasting roles are likely due to variations in pituitary sensitivity to sex steroids at various times of the year. Sex steroids also regulate gonadotropin release by modulating such factors as GABA, dopamine, GnRH and others.

1.4.1 Testosterone (T)

There is evidence supporting the role of testosterone in both positive and negative feedback on GTH-II synthesis and release. In both male and female goldfish, testosterone enhances pituitary GnRH responsiveness. This effect is seen in both sexually regressed and

sexually mature fish (Trudeau *et al.*, 1993b). Testosterone also enhances the stimulatory effects of GABA and taurine on GTH-II release in both sexes (Trudeau *et al.*, 1993c). In sexually regressed goldfish, T enhances the inhibition of GTH-II release by dopamine (Trudeau, 1997).

Testosterone not only regulates GTH-II release, it has also been shown to play a role in regulating GTH-IIβ mRNA levels in the pituitary. In early recrudescent fish, T was shown to decrease GTH-Iβ mRNA levels in both males and females, and to increase GTH-IIβ mRNA levels in females (Sohn *et al.*, 1998). In sexually immature goldfish, T treatments at pharmacological doses were shown to modulate GTH-IIβ mRNA levels in a time-dependent fashion. They first caused a decrease in mRNA levels at 12 and 24 hours then an increase at 72 and 96 hours after treatment (Huggard *et al.*, 1996a). In sexually mature fish, T causes a dose-dependent increase in GTH-IIβ expression (Habibi and Huggard, 1998).

1.4.2 Estradiol (E2)

Estradiol has been shown to have both positive and negative feedback roles on gonadotropin synthesis and release. It enhances pituitary GnRH responsiveness in sexually regressed but not in sexually mature goldfish (Trudeau *et al.*, 1991a). In sexually recrudescent female goldfish E2 was also shown to enhance pituitary GnRH-stimulated GTH-II release, whereas in post-spawning fish this effect was not seen. E2 decreases GABA-stimulated GTH-II release (Trudeau *et al.*, 1993b). As its serum concentrations increase during gonadal development, E2 exerts a negative feedback effect by reducing pituitary GABA responsiveness (Trudeau, 1997). E2 also exerts a negative feedback effect by enhancing dopamine inhibition of GTH-II release in sexually regressed goldfish (Trudeau *et at*, 1993d).

Estradiol regulates GTH-II mRNA levels. In recrudescent goldfish, E2 causes a decrease in GTH-Iβ mRNA and an increase in GTH-IIβ mRNA levels in both males and females. In sexually mature goldfish, E2 increases GTH-IIβ mRNA levels only in male fish (Sohn *et al.*, 1998).

Both T and E2 can regulate reproductive function by modulating different neuroendocrine systems. They upregulate the expression of neuropeptide Y mRNA in the hypothalamus (Peng *et al.*, 1993), which in turn stimulates GTH-II release from the pituitary. Other sex steroids are also involved in regulating reproduction. In sexually regressed goldfish for example, progesterone potentiates the positive effect of E2 on GnRH-stimulated GTH-II release (Trudeau *et al.*, 1991b). All the above-mentioned factors along with many others act to maintain a functional reproductive system.

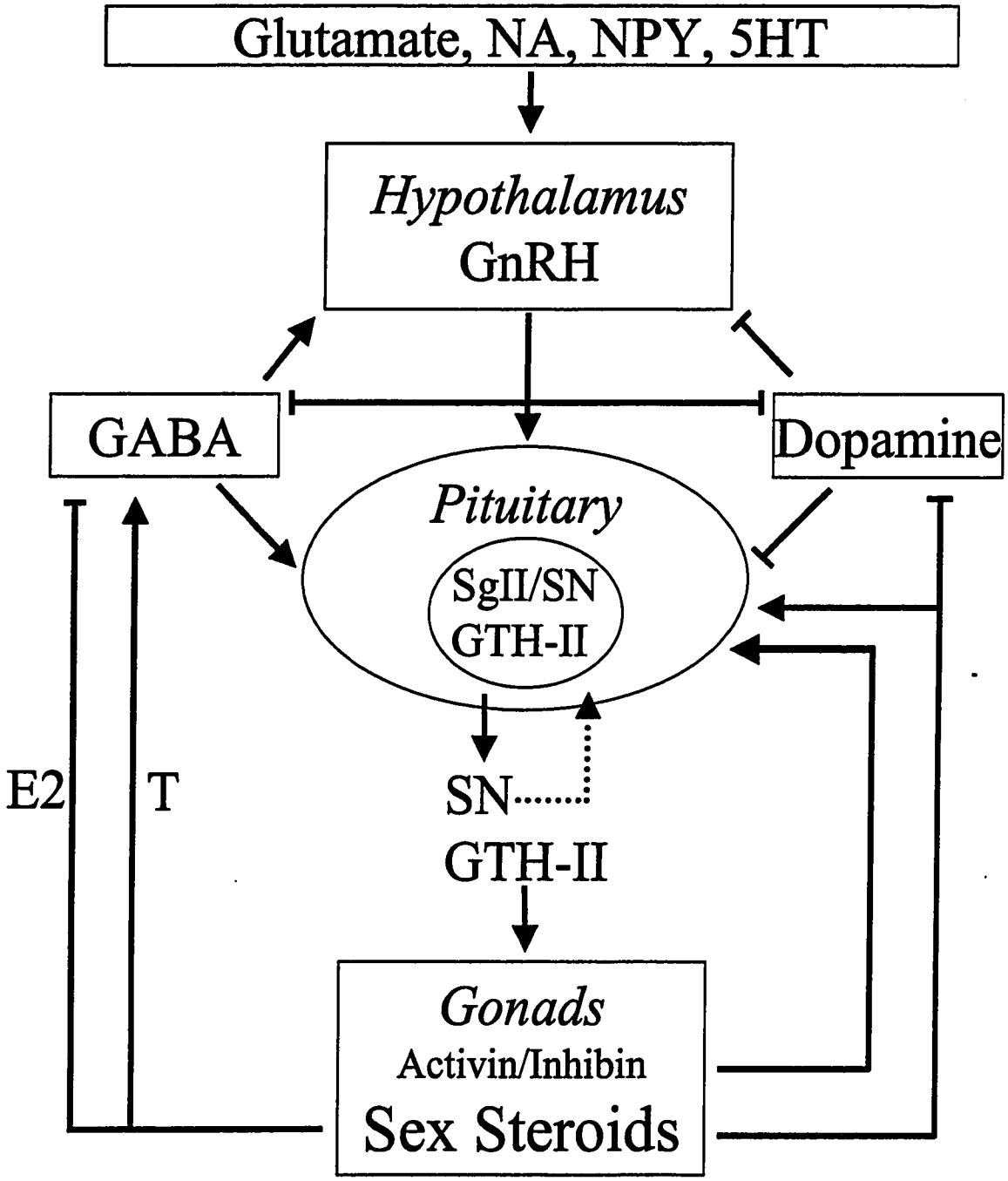

Figure 1. Theoretical model of the goldfish reproductive system. Notice the many different factors involved in the regulation and control of the system. This model has been adapted from Trudeau, 1997. NA: noradrenaline, NPY: neuropeptide Y, 5HT: serotonin, GnRH: gonadotropin-releasing hormone, SgII: secretogranin-II, SN: secretoneurin, GTH-II: gonadotropin-II, T: testosterone, E2: estradiol, GABA: gamma-aminobutyric acid.

2. Secretogranin-II (SgII)

Secretogranin-II (SgII), also known as chromogranin C, is a 67 kDa, acidic, heat stable secretory protein that belongs to the chromogranin family (Natori and Huttner, 1994). The family also includes chromogranins A, B, and 7B2 which have a very widespread distribution in the endocrine and nervous systems where they are found in large dense-core secretory vesicles (Mahata *et al.*, 1991). These proteins share many biochemical properties which include having an NH_2-terminal signal peptide, being hydrophilic and containing a large proportion of acidic amino acids, containing numerous potential cleavage sites for prohormone convertases, binding Ca^{2+} and aggregating under conditions characteristic of the *trans*-Golgi network (TGN). Because of these properties, the granins seem to play a role in prohormone sorting into the regulated secretory pathway (Fischer-Colbrie *et al.*, 1995). SgII was first characterized in 1981 in the rat pituitary gland by Rosa and Zanini (1981). It seems to have a special relevance for the brain where it is present in higher concentrations than the other chromogranins (Marksteiner *et al.*, 1993). The highest concentration of SgII in mammals was found in the evolutionarily older parts of the brain, the hypothalamus, and in particular the median eminence. It has also been detected in the pineal gland, adrenal medulla (Simonneaux *et al.*, 1997), pancreas, stomach, gastrointestinal tract, thyroid gland and gonads (both testes and ovaries) (Fischer-Colbrie *et al.*, 1995). SgII has been used as a model protein in many studies concerning the sorting and packaging of prohormones and the biogenesis of secretory granules. Although these studies have provided insight into many characteristics of the regulated secretory pathway, the role of SgII itself is still unclear (Park *et al.*, 2002). In the rat pituitary, SgII was found to colocalize in gonadotrophs with luteinizing hormone (LH), and its secretion is strongly stimulated by

gonadotropin-releasing hormone (GnRH) in a dose related fashion (Cozzi and Zanini, 1986).

2.1 Involvement of SgII in the Regulated Secretory Pathway

Neurons and endocrine tissues exhibit two distinct pathways for secreting their products, namely the constitutive pathway and the regulated pathway. The proteins that are secreted through either of these pathways share a common biosynthetic origin. They are synthesized on the rough endoplasmic reticulum (ER) and are translocated into the lumen of the ER by virtue of an NH_2-terminal signal peptide (Shennan, 1996). The signal peptide is then cleaved by signal peptidase, and the protein folds into its tertiary structure. The protein is then translocated into the *cis*-Golgi and through individual Golgi stacks into the TGN. Within the TGN, the proteins destined for packaging in lysosomes are sorted by virtue of a mannose-6-phosphate receptor which interacts with a mannose-6-phosphate residue present on lysosomal proteins (Shennan, 1996). The proteins destined for regulated secretory pathway are actively sorted, whereas the proteins destined to the constitutive pathway are sorted by default. The constitutively sorted proteins are exported from the TGN in small vesicles towards the cell membrane, and they are believed to represent the tonic release of hormones that is characteristic of nervous and endocrine tissues (Carnell and Moore, 1994).

There has been many models proposed to explain how proteins are segregated to the regulated pathway. One model proposes that the sorting might occur by a receptor mediated process analogous to the sorting of lysosomal proteins. To support this model, a secondary structure termed an N-terminal hydrophobic peak has been identified in some secretory proteins that follow the regulated pathway (Shennan, 1996). Another model proposes that some proteins,

targeted to the regulated pathway, have in common pairs of cysteine residues that could form disulfide bridges and hence hairpin loops at the N-terminal. This disulfide loop has been shown to be sufficient in targeting proteins to the regulated pathway as in the case of pro-opiomelanocortin (POMC) (Shennan, 1996). A third model proposes that the proteins destined to the regulated pathway are segregated by aggregation. As these proteins move further in the TGN, they encounter lower pH and higher Ca^{2+} concentrations, so they aggregate together excluding the proteins destined to the constitutive pathway. The proteins destined to the regulated pathway are segregated into small immature secretory granules (ISG). These granules coalesce together to form mature secretory granules (MSG) which are transported to the site of release. There the MSG's await a chemical or an electrical signal after which they fuse to the cell membrane and release their contents into the extracellular fluid.

It is hypothesized that SgII is segregated into the dense core vesicles of the regulated pathway following the third model. This hypothesis stems from the observation that like chromogranin A, SgII aggregates in a milieu of low pH and high calcium (Fischer-Colbrie *et al.*, 1995). In astrocytes lacking large secretory vesicles, SgII is constitutively secreted (Fischer-Colbrie *et al.*, 1993). This raises the question whether SgII is segregated into the regulated pathway because it plays a physiological role, or whether it is simply a molecular chaperone involved in the segregation of other hormones and neuropeptides. The fact that only SgII-derived peptides but not the precursor, which is proteolytically processed to about 90%, are secreted *in vivo*, and the fact that SgII colocalizes with LH in secretory granules of rat gonadotrophs (Cozzi and Zanini, 1986) supports the second role. However stimulated secretion of SgII from primary

cell cultures and cell lines is well established. Further studies are required to elucidate the role of SgII in secretion.

2.2 Regulation of SgII synthesis and release

Synthesis of SgII is regulated by different factors. In rats, salt loading induced up to a 7-fold increase in SgII mRNA levels in different parts of the brain (Mahata *et al.*, 1992). Estrogens also regulate SgII synthesis. Ovariectomy induces a 2 to 3-fold increase in SgII mRNA and protein in the pituitary of female rats, and estrogen substitution partially reduces these levels (Kakar *et al.*, 1993). A GnRH antagonist is also capable of reducing the SgII levels in the ovariectomized rats. Recently, SgII mRNA levels were shown to increase in the rat brain following treatment with phenylcyclidine, a glutamatergic NMDA receptor antagonist, suggesting a role for glutamate in SgII regulation (Marksteiner *et al.*, 2001). *In vitro* studies have shown an increase of SgII mRNA levels and release in bovine adrenochromaffin cells upon treatment with pituitary adenylate cyclase-activating polypeptide (PACAP) (Turquier *et al.*, 2001). Other factors such as catecholamines, histamine and reserpine are known to regulate SgII synthesis and release in chromafin cells through very complex regulatory mechanisms. In 1998 the only known SgII gene in a fish was cloned from the common goldfish (Blazquez *et al.*, 1998). Two distinct mRNA transcripts of approximately 2650 and 2950 nucleotides were detected. It was hypothesized that these two transcripts are a result of alternate splicing (Blazquez *et al.*, 1998). In the goldfish, GABA caused an increase in SgII mRNA in the gonadotrophs concomitant with a decrease in GTH-II cell content and enhanced GTH-II release *in vivo* (Blazquez *et al.*, 1998). Therefore, SgII has been shown to be involved in different

systems, and its regulation to be controlled by different neuroendocrine factors.

2.3 Processing of SgII and Evolutionary Conservation of Secretoneurin (SN)

In the rat and bovine brains, SgII is proteolytically processed to a high degree (approximately 90%) into smaller bioactive peptides (Hoflehner *et al.*, 1995). This processing occurs by cleavage at pairs of basic amino acids (Lys-Arg or Arg-Arg) by endopeptidases that belong to the prohormone convertase (PC) family, specifically PC1/3 and PC2 (Troger *et al.*, 1994). A recent immunocytochemical study in the rat anterior pituitary showed that SgII is colocalized specifically with PC2 in small dense granules (Uehara *et al.*, 2001). Other studies have reported detecting SgII-derived peptides in many peripheral organs. This suggests that SgII is processed in the periphery as well as in the CNS. The proteolytic processing occurs after the proteins aggregate in the TGN but before the final condensation occurs in the mature secretory granules (Natori and Huttner, 1994). The degree of processing depends on many parameters including the expression of SgII, the expression of the prohormone convertases and the biochemical conditions in the secretory granules (Natori and Huttner, 1994). The most important of these bioactive peptides is the 33-amino acid secretoneurin (SN) which is suggested to have many functions within the central nervous system.

SgII has been cloned and sequenced from several mammals, a bird, 2 frogs and the goldfish. Alignment of the full length deduced amino acid SgII sequence from the goldfish with the human sequence showed only a 44% similarity (Blazquez *et al.*, 1998). When the putative secretoneurin amino acid sequences were aligned, a high degree of conservation was observed (table 1). Mammalian sequences were more than 85% identical, amphibian sequences were more

than 75% identical to the human sequence, and the goldfish sequence was 68% identical to the human sequence (Samia *et al.*, 2001). Unlike all other known SN sequences, the goldfish sequence is 34 amino acids in length. The high degree of conservation of SN sequences across species suggests important biological roles for SN. The putative dibasic cleavage sites where SgII processing occurs are also highly conserved across the known sequences, and they occur at similar positions in the different species.

2.4 Distribution and Function of Secretoneurin (SN)

SN is composed of 33 amino acids representing amino acids 154 to 186 of the mammalian SgII. It is widely distributed in the brain with the highest levels being found in the hypothalamus followed by the hippocampus, caudate nucleus, thalamus and brainstem (Kirchmair *et al.*, 1993). At the level of the periphery, SN was detected in female rat uterus (Collins *et al.*, 2000), enteric and sensory ganglia (Dun *et al.*, 1997), pituitary gland where it colocalizes with PC1 and PC2, endocrine pancreas, intestine and thyroid gland (Leitner *et al.*, 1996). It was also detected in the porcine spleen (Liang *et al.*, 1999) and in human synovium and synovial fluid (Eder *et al.*, 1997), enteric nervous system, carotid body, retina and various tumors (Wiedermann, 2000). This widespread distribution suggests multiple roles for SN within the CNS and periphery.

Secretoneurin exerts its effects by binding to specific high-affinity receptors which have kinetically and functionally been characterized to be G-protein linked (Wiedermann, 2000). Cellular responses to SN include activation of G-protein and protein kinases and increase in intracellular calcium levels. Among its functions within the CNS, SN has been shown to

stimulate dopamine release from rat striatal slices *in vitro*. This dopamine release is dose-dependent, occurs via exocytosis since it is calcium dependent and is subject to desensitization (Reinisch *et al.*, 1993). In cultured rat pinealocytes, SN significantly inhibited serotonin release and, to a lower extent, melatonin release (Simonneaux *et al.*, 1997). SN also seems to function as a neuropeptide and evidence has been shown that it is secreted from neurons in the hypothalamus. This secretion depends on calcium influx via L-type calcium channels and can be inhibited by nifedipine, a specific L-type calcium channel blocker (Egger *et al.*, 1994). In the goldfish, SN injections have been shown to stimulate GTH-II release from the pituitary when used in combination with domperidone, a dopamine antagonist, suggesting a possible role in reproduction (Blazquez *et al.*, 1998).

Multiple functions for SN have also been reported in the periphery. It has been shown to trigger selective migration of human blood monocytes *in vitro* and *in vivo* (Kahler *et al.*, 1997), to stimulate the migration of endothelial cells, and to exert a potent reversible inhibitory effect on endothelial cell growth and proliferation under low serum conditions (Liang *et al.*, 1999). These data suggest that SN might act as a regulatory factor of vascular cell functions. There is also evidence that SN is present and released from nerve terminals of the porcine spleen upon nerve stimulation, suggesting that it may modulate adrenergic neurotransmission and may also play a role in the neuroimmune communication (Kahler *et al.*, 1997). The presence of SN in the human synovium and its absence from muscles of patients with fibromylagia, a muscular disorder, suggest that it might be involved in rheumatic disorders (Eder *et al.*, 1997). Its presence in various types of endocrine tumors seems to suggest a possible role, but none has been yet characterized (Wiedermann, 2000). The novel neuropeptide SN has different functions in

multiple central and peripheral systems, and further exploration of these functions and possible others is necessary.

Table 1. Clustal W alignment of SN sequences showing the % identities with human SN. A consensus sequence is shown. Adapted from Samia *et al.*, 2001. See also appendix A.

human	TNEIVEEQYTPQSLATLESVFQEL.GKLTGPN.NQ	% Identity
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	

3. Teleost pituitary

The teleost pituitary is composed of two parts, the adenohypophysis and the neurohypophysis. The adenohypophysis is made of endocrine tissue and is divided into three lobes, the rostral pars distalis (RPD), the proximal pars distalis (PPD) and the pars intermedia (PI) (Quesada *et al.*, 1988). The RPD contains primarily prolactin cells, thyrotrophs and corticotrophs (Yan and Thomas, 1991). GH cells are found at the dorsal PPD, whereas gonadotrophs are concentrated in the ventral PPD and extend posteriorly to form a border around the PI (Garcia-Hernandez *et al.*, 1996). In some species, gonadotrophs are also found in the fingers of the PPD that project into the PI (Calman *et al.*, 2001) The PI contains mostly MSH cells and in some species somatolactin was also detected (Rodriguez-Gomez *et al.*, 2001). The neurohypophysis is mainly composed arginine vasotocin, isotocin or mesotocin axons and nerve terminals originating from neurons in the hypothalamus and pre-optic area (Rendon *et al.*, 1997).

A functional hypothalamo-hypophyseal portal blood system is lacking in teleosts, and the adenohypophysis is directly innervated by pre-optic hypothalamic neurons of the anterior neurohypophysis (Batten *et al.*, 1999). Pituitary cell types are highly homogeneous and regionalized (Yan and Thomas, 1991). The RPD and PPD are considered to compose the anterior pituitary, and the PI and neurohypophysis the posterior pituitary.

4. Experimental objectives

Various studies have suggested a role for SgII in reproduction. Cozzi and Zanini (1986) demonstrated that SgII in co-localized with LH in rat gonadotrophs and that its secretion is stimulated by GnRH. Blazquez *et al.* (1998) showed that GABA can upregulate SgII mRNA and

stimulate GTH-II release from the goldfish pituitary, and that the SgII-derived peptide SN can stimulate GTH-II release. Samia *et al.* (2001) detected the presence of SgII mRNA at all levels of the goldfish reproductive axis, i.e., the hypothalamus, pituitary and ovaries. These studies led to the hypothesis that SgII is involved in goldfish reproduction. The objectives of my research were to study the regulation of SgII in the goldfish pituitary which might help in elucidating its role within the reproductive system.

The first series of experiments presented in chapter 3 aimed at studying the regulation of SgII by sex steroids and the annual reproductive cycle of the goldfish. The second set of experiments looked at the regulation of SgII by GnRH and dopamine, the main stimulatory and inhibitory factors of the reproductive system respectively (chapter 4). Finally, knowing that sex steroids modulate the actions of different neuroendocrine factors, the third series of experiments presented in chapter 5 aimed at studying the regulatory effects of GnRH and the modulatory effects on testosterone on SgII expression.

Chapter 2. Materials and Methods

Experimental animals

10-15 cm common goldfish (*Carassius auratus*) of either sex were obtained from Mount Parnell goldfish farms (Pennsylvania, USA) and transferred to the fish-holding facility of the University of Ottawa. The sexes were separated and the fish held indoors in 70-l tanks supplied with flowing, aerated and dechlorinated tap water. The photoperiodic regime mimicked the natural light/dark cycle of Ottawa, and the water temperature was kept at 18°C. The fish were fed *ad libitum* daily with commercial goldfish flakes. They were acclimated for at least two weeks before any experiments were performed.

Hormone treatments

Sex steroids

The fish were anaesthetized in 3-aminobutyric acid ethyl ester (Sigma) until opercular movement stopped. Each fish was then weighed, and a 3.0mm incision was made in its side, approximately 3.0mm above the midpoint of the ventral fin. A solid silastic pellet containing testosterone, estradiol, progesterone (100mg of steroid/g of elastomere) or no steroid (blank) was then introduced through the incision into the peritoneum of the fish. The fish were then left to recover in 20-1 water containers before they were moved back into their tanks. The pellets were left for five days, and the fish received a steroid dose of 100 μg/g body weight producing steroid levels in the physiological range (~5-10ng/ml of serum) (Trudeau *et al.*, 1991a).

GnRH

The fish were anaesthetized and weighed as described above. Each fish was injected intra-peritoneally with 10ng/g body weight of salmon GnRH analog (sGnRHa ; [Des-Gly^{10}, D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}]-LHRH ; Bachem) dissolved in 0.6% NaCl solution (4µl/g body weight). Control fish were injected with an equivalent volume of vehicle.

Tissue collection

The fish were anaesthetized and weighed. A blood sample was collected from each fish using a 1.0ml syringe with a 26 gauge needle through a caudal puncture for determining serum GTH-II concentrations. Then the fish was euthanized with a spinal incision. The skull was opened, and the brain dissected out carefully. The areas of interest were then separated from the rest of the brain. The pituitary was in turn dissected out of the skull. The gonads were then dissected out and weighed in order to determine the gonado-somatic index (GSI ; gonad weight/body weight), an indication of the sexual maturity stage of the fish (Trudeau *et al.*, 1991a). Any peripheral tissues that were needed were dissected out at this point. All tissues were collected in 15ml plastic tubes, instantly frozen on dry ice, and stored at -80°C. In some experiments, the anterior pituitary (Pars distalis, PD) was separated from the posterior pituitary (neurointermediate lobe, NIL) in an attempt to study regional effects and the specific responses of gonadotrophs which are primarily found in the PD. Due to the distribution of cell populations in the teleost pituitary and the presence of gonadotrophs on the border of the pars intermedia (see introduction), contamination of the NIL pool with gonadotrophs and possibly somatotrophs is likely to have occurred.

RNA purification

The RNA was purified using the acid guanidinum thiocyanate (GITC)-phenol-chloroform extraction method first described by Chomcyzynski and Sacchi (1987). Frozen tissue samples (4-6 pituitaries) were homogenized for 1 min in 1.0ml of solution D (per 100ml: 73ml of DEPC (diethyl pyrrocarbonate) treated water, 62.5g of solid GITC, 4.4ml of 0.75M sodium citrate, pH 7.0, 6.6ml of 10% sarkosyl and 0.3ml of β-mercaptoethanol) until a homogenous liquid was obtained. 0.1ml of 2M sodium acetate, pH 4.0 was added and mixed by vortexing. 0.5ml of Phenol saturated with DEPC treated water was then added and the solution was vortexed. Finally 0.2ml chloroform was added, the mixture was vortexed again, transferred to 2.0ml microcentrifuge tubes and centrifuged at 12000G for 20 min. The supernatant was then transferred to another tube, 0.6ml of isopropanol was added, the mixture was vortexed and then centrifuged at 14000G for 15 min. The supernatant was discarded and the RNA pellet was washed twice with 80% ethanol for 10 min. The samples ware centrifuged for 10 min at 14000G following each wash. The RNA pellets were then air dried for 10 min and suspended in 50µl of DEPC treated water. 2.0µl were then removed for determination of the RNA concentration, and the rest was flash frozen in liquid nitrogen and stored at -80°C. The concentration of the RNA was determined using a Pharmacia Gene Quant DNA/RNA calculator spectrophotometer and an indication of the purity of the RNA was given by the ratio of absorbencies at 260 and 280nm (A_{260}/A_{280}), with pure RNA having a ratio of 2.0.

Gel electrophoresis and Northern blotting

The RNA was separated on denaturing agarose gels by electrophoresis. The gels were made of 1.2% agarose in 1X MOPS (morpholinopropanesulfonic acid; per 1L of 10X MOPS: 41.8g MOPS, 4.1g sodium acetate, 2.9g disodium EDTA, pH adjusted to 7.0 with 10N NaOH) with 5% formaldehyde (37% solution). 20µg of RNA were then removed from the stock solution, and the

total volume of the RNA sample was adjusted to 20µl with DEPC treated water. 40µl of loading buffer (per 7ml: 4ml formamide, 0.8ml of 10X MOPS, 0.4ml glycerol, 1.4ml formaldehyde, 0.1ml of 0.5M EDTA, pH 8.0, 0.4ml DEPC treated water, 20mg bromophenol blue, 3.5:1 of 10mg/ml ethidium bromide) were then added. The samples were heated at 65°C for 20 min, chilled on ice and loaded on the gel. The gel was run in 1X MOPS buffer at 95 volts for four hours. It was then washed twice in five volumes of water for 20 min each wash and once with 20X SSC for 20 min. A piece of Hybond N+ nylon membrane (Amersham) was cut to the same size as the gel, wetted in ddH_2O and washed for 10 min in 2X SSC. Then the RNA was transferred from the gel onto the membrane by capillary action for 24 hours. When the transfer was finished, the membrane was treated with ultra violet light for five minutes in order to covalently bond the RNA to the membrane. For hybridization, the membrane was washed in 0.1X SSC and 0.1% SDS for 20 min at 65°C. Hybridizaton solution (10% dextran sulfate, 1% SDS, 6X SSC, 100µg/ml salmon sperm DNA, 5X Denhardt's solution) was added (10 ml of solution per 100 cm^2 of membrane) and the membrane was prehybridized at 65°C for 3-4 hours. For preparing the probe, 25ng of template DNA was diltued in 45µl of 1X TE (10mM Tris, 1.0mM EDTA). The DNA was then boiled for five minutes and chilled on ice. A radioactive (α^{32}P-dCTP) probe was then prepared using the Redi Prime II kit (Amersham). The probe was centrifuged through a Sephadex G50 spin column to remove the free radioactive nucleotides. The probe was then boiled for 5 min, chilled on ice and added to the prehybridizing membranes. The probe was left to hybridize overnight at 65°C. The next morning, the membrane was washed at 65°C with solutions of 0.1% SDS and varying concentrations of SSC (0.1X to 2.0X), placed on filter paper soaked with ddH_2O, wrapped in Saran wrap, and exposed to a Kodak Phosphor screen overnight. The phosphor screen was then scanned with a BioRad phosphor-imager and the signal intensity quantified using the BioRad "Quantity One" software.

Radioimmunoassay

Serum gonadotropin-II (GTH-II) concentrations were determined using radioimmunoassay (RIA) adapted from Peter *et al.* (1987). The assay was adapted to the solid phase format following the protocol by Merali *et al* (1998). 96-well Dynex Immulon 4 polypropylene plates were coated with 100µl of 1.0µg/ml protein A/G (Calbiochem) in 0.1M $NaHCO_3$ pH9 solution at 4°C overnight. The plates were then washed twice for 2min at room temperature (RT) with wash buffer (25mM Na barbitone, 40mM Na acetate, 0.25mM thimerosal, 0.5% BSA, 0.1% Tween-20, pH 8.6) and once for 20min at RT with diluent (25mM Na barbitone, 40mM Na acetate, 0.25mM thimerosal, 0.5% BSA, pH8.6). The plates were then treated at 4°C overnight with 50µl of rabbit anti-carp GTH-II antiserum at a dilution of 1:60,000 in diluent. A series of six wells were treated with normal rabbit serum at a dilution of 1:60,000,

and these were used to determine non-specific binding. The next day, the plates were then washed three times at RT with wash buffer. The reaction mixtures were then prepared. A standard curve using GTH-II concentrations of 0 (100% binding), 0.19, 0.39, 0.79, 1.57, 3.13, 6.25, 12.5, 25, 50 and 100ng/ml was prepared (25µl of diluent, 50µl of standard in diluent and 25µl of I^{125}-GTH-II (~20,000CPM)). The serum sample reactions used 10µl of serum(with 65µl of diluent and 25µl of I^{125}-GTH-II). All reactions were done in triplicates. The plates were then incubated at 4°C for 48 hours. They were then washed three times at RT with wash buffer, and 100µl of "Optiphase Supermix" (Wallac) scintillation fluid was added to each well. After a four-hour incubation, the plates were counted in a Wallac Trilux Microbeta counter, and the assay evaluated using WiaCalc software.

Seasonality and sex steroid experiment (Chapter 3)

Tissue Distribution

Central and peripheral tissues from sexually recrudescent male and female goldfish were collected in February in order to determine where SgII is expressed. The PD was separated from the NIL.

Sex steroid treatments

Fish from both sexes were treated with sex steroids in February (sexually recrudescent), May (sexually mature) and November (sexually regressed). These three times of the year represent the major stages of the goldfish reproductive cycle. Six pools of 6 fish were used per treatment per sex per time of year. Whole pituitaries were analyzed in these experiments enabling us to study the response of the full system.

Statistical analysis

1-way and 2-way analysis of variance (ANOVA) models followed by Bonferroni pairwise comparison tests were used to compare among sex steroid treatments and among seasons. Two-group t-tests were used to compare between sexes. The software used for statistical analysis is SYSTAT 9.

GnRH and Domperidone experiment (Chapter 4)

GnRH and domperidone treatments

Sexually recrudescent (February) female goldfish (GSI = 5.6±0.8) were treated with salmon GnRH analog (sGnRHa ; [Des-Gly^{10}, D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}]-LHRH) (10ng/g body weight) and domperidone (1.0μg/g body weight ; gift of Janssen Pharmaceutica, Belgium), a specific dopamine D2 receptor antagonist which affects GTH-II but not GH cells in 0.6% NaCl vehicle (4μl/g body weight). Control fish were treated with an equivalent volume of vehicle. Six

pools of 6 fish were used per treatment. The chemicals were injected intraperitoneally under 3-aminobutyric acid ethyl ester anaesthesia. 24 hours later, the pituitaries were dissected out of the fish, and the PD was separated from the NIL

Statistical analysis

Two-group t-tests were used to compare between treatments and between tissues. Bonferroni chi-square correlation test was used to compare expression levels of different genes. The statistical software used was SYSTAT 9.

Testosterone and GnRH experiments (Chapter 5)

Testosterone and GnRH treatments

Sexually recrudescent female (GSI = 3.6 ± 0.1, December) and sexually mature male (GSI = 3.3 ± 0.1, May) goldfish were treated with either 100μg/g of body weight (5-10ng/ml of serum) of testosterone or a blank implant for five days. On the fifth day, the fish were injected intraperitoneally under 3-aminobenzoic acid ethyl ester anaesthesia with either salmon GnRH analog (sGnRHa ; [Des-Gly^{10}, D-Arg^{6}, Trp^{7}, Leu^{8}, Pro^{9}]-LHRH) in 0.6% NaCl vehicle (10ng/g body weight in 4μl/g body weight) or vehicle only for control fish. Twenty four hours later, the pituitaries were dissected out of the fish. The PD was separated from the NIL. Six pools of 6-8 fish were obtained for each treatment.

Statistical analysis

Two-way analysis of variance (ANOVA) models followed by Bonferroni pairwise comparison tests were used to compare among treatments. Two-group t-tests were used to compare between tissues. Bonferroni chi-square correlation test was used to compare expression levels of different genes. The statistical software used was SYSTAT 9.

Chapter 3. Effects of seasonality and sex steroids on secretogranin-II (SgII) expression in the goldfish pituitary.

Results

Tissue distribution

SgII mRNA levels were studied in males and females in order to determine tissue distribution and levels of expression (figure 1). In the majority of brain parts as well as in the pituitary gland, females had higher levels of SgII mRNA than males. In the peripheral tissues, the levels were generally very low, and they were similar in males and females. It was also observed that the large and small transcripts of SgII co-vary in all tissues in males and females. The highest SgII mRNA levels were found in the pituitary.

Gonado-somatic index and Gonadotropin-II levels

In both males and females, the gonado-somatic index (GSI) was highest in May, lowest in November and at a median level in February. The GSI in females was always higher than in males (figure 2). Serum gonadotropin-II (GTH-II) levels were not significantly different among sex steroid treatments regardless of seasonal stage or sex (table1). Therefore, serum GTH-II values in the various sex steroid treatments were pooled and differences among seasons and between sexes determined. GTH-II levels were highest in the sexually mature fish in May and lowest in the sexually regressed fish in November (figure 3). In February, serum GTH-II levels in females were not significantly different from the levels in May. In males, the levels in February were in between the levels in November and May. Serum GTH-II levels were higher in females than in males in February only.

Secretogranin-II expression in the pituitary

Table 2 shows the effects of sex steroids on the levels of secretogranin-II (SgII) mRNA in the pituitary. An initial 2-way ANOVA showed that sex steroids did not affect SgII expression, so that variable dropped out of the model and we compared among seasons using a 1-way ANOVA (figure 3). Significant seasonal variation in SgII mRNA levels was observed in both sexes, with levels being highest in February. SgII mRNA levels were similar and low in May and November. Sex differences in pituitary SgII mRNA levels were only observed in February, when females had much higher levels than males.

Discussion

In order to determine expression patterns of SgII in goldfish, a tissue distribution comparing SgII levels in different central and peripheral tissues and between males and females was done. The fish were in the sexually recrudescent stage. As has been previously reported in mammals (Fischer-Colbrie *et al.*, 1995) and in goldfish (Samia *et al.*, 2001), SgII mRNA levels were highest in the pituitary gland, present in low levels in all parts of the brain, and absent from most peripheral tissues. SgII mRNA was not detected in the ovaries which conflicts with our previous report. When the reproductive system is stimulated with GnRH, ovarian SgII expression decreases (Samia *et al.*, 2001), and this could explain why no SgII was detected in the ovaries of the sexually recrudescent fish used for the tissue distribution. No SgII mRNA was detected in the testes which agrees with what we previously reported. When males were compared to females, lower SgII mRNA levels were observed in all tissues. This suggests that there are sexual differences in SgII distribution and expression between males and females, and further studies are required to elucidate what is at the basis of this sexual dimorphism. The pituitary was

separated into the PD and NIL for this study. The PD is mostly composed of endocrine tissue and the NIL is mostly, but not exclusively, composed of neural tissue. The cell populations in the two divisions are very different, however both express SgII at similar levels. Since both parts of the pituitary expressed SgII, and the expression levels were higher in both the PD and the NIL than in any other tissue examined, our subsequent study focused on the effects of sex steroids and season on SgII expression in the whole pituitary.

Over the experimental period, the fish underwent similar changes in GSI to those previously reported (Razani *et al.*, 1988 ; Sohn *et al.*, 1999), and GSI was used as an indication of the sexual stage of the fish. When we compared GTH-II levels among sex steroid treatments, we did not observe any significant effect. This has previously been reported by Trudeau *et al.* (1991a), where sex steroids did not directly affect basal GTH-II release but rather they potentiated the effect of GnRH on GTH-II. Sex steroids also function by modulating the levels and effects of other neuroendocrine factors such as GABA and dopamine that in turn control GTH-II, rather than directly affecting GTH-II release.

Serum GTH-II levels varied seasonally as has been previously reported (Sohn *et al.*, 1999 ; Trudeau *et al.*, 1991a). In both males and females, Sohn *et al* (1999) reported low levels in February (~5-6 ng/ml) and November (~5-6 ng/ml), and high levels in May (~34 ng/ml in females and ~11 ng/ml in males). Our results showed that in females, there were high levels of serum GTH-II in both February (~56 ng/ml) and May (~58 ng/ml), and lower levels in November (~30ng/ml). In males, GTH-II levels were lowest in November (~26 ng/ml), highest in May (~54 ng/ml) and at a median level in February (~37 ng/ml). Similar seasonal variation patterns were reported by Trudeau *et al.* (1991a). The GTH-II concentrations that we measured were 5 to 10

times higher than the previous reports; this difference is likely due to differences in assays. We used a solid phase single antibody assay whereas other studies used double antibody assays. Nevertheless, the overall patterns of GTH-II are similar in these various studies.

A 2-way ANOVA was used to compare SgII expression levels in the pituitary among sex steroid treatments and seasons. There was no direct effect of sex steroids on SgII expression. However, we hypothesize that sex steroids might regulate SgII expression indirectly by modulating neuroendocrine factors such as GnRH and dopamine via feedback loops. Significant seasonal changes in SgII mRNA levels were observed, with the highest levels being in February. The two SgII transcripts co-varied in all cases. SgII expression levels fluctuated with the sexual cycle. Sex steroids were not responsible for modulating SgII expression at any time of the year.

Pituitary SgII expression levels in February and November correlate with the serum GTH-II levels that we observed. The correlation breaks down in sexually mature fish (May) when GTH-II levels are high whereas SgII mRNA levels are low. In February, when the fish are sexually recrudescent and GTH-II expression and release are high, the high SgII levels could be indicative of increased demand on secretory proteins for GTH-II production and release. The same effect would be expected in May. Trudeau *et al.* (1991a) reported increases in pituitary GTH-II content, basal GTH-II release and pituitary GnRH responsiveness starting in November and attaining a maximum in May. This might explain why SgII expression is low in May; GTH-II cell content is at maximum, so SgII is no longer required for packaging and release, and its production decreases. In November, when the fish are either sexually regressed or only in the very early stages of sexual recrudescence, and the whole reproductive system is shut down, GTH-II expression (Sohn *et al.*, 1999) and release are low and so are SgII mRNA levels.

The only sex difference in SgII expression observed was in February when females had higher mRNA levels than males. This confirms what was observed in our preliminary examination of SgII mRNA levels (figure 1). Sexually recrudescent female goldfish express SgII at significantly higher levels than males. SgII levels in February correlate directly with serum GTH-II levels. This data suggests that SgII somehow regulates GTH-II production and release, possibly through SN autocrine or paracrine feedback.

Sex steroids play a very important role as feedback regulators of the goldfish reproductive system, but they do not seem to directly regulate GTH-II release and pituitary SgII expression, regardless of the sex and the sexual maturity stage of the fish. They have been shown to indirectly regulate GTH-II release through feedback loops via different neuroendocrine factors, and they might act through similar mechanisms to regulate SgII. Whereas sex steroids had no effect on SgII expression, its mRNA levels varied significantly with sexual stage. SgII correlation with pituitary GTH-II content (Trudeau *et al.*, 1991a) and serum concentrations suggests a role for SgII in GTH-II production and release. SgII expression is sexually dimorphic in sexually recrudescent goldfish but not at other stages which might be due to different dynamics in reproductive cyclicity of males and females. Based on the results presented, seasonal variations in SgII expression are not likely mediated by sex steroids. Therefore, they could be regulated by other neuroendocrine factors, and the subsequent chapters aim to identify and study those factors.

Table 1. Serum gonadotropin-II (GTH-II) concentrations (ng/ml) at three different times of the year and three different steroid treatments (100μg/g body weight). Levels were measured by specific radioimmunoassay. Means ± SEM are shown, n = 10.

Season	Males				Females			
	Control	Testosterone	Estradiol	Progesterone	Control	Testosterone	Estradiol	Progesterone
Feb	41 ± 5	38 ± 4	36 ± 4	30 ± 5	63 ± 14	65 ± 10	49 ± 11	47 ± 6
May	53 ± 9	66 ± 8	55 ± 11	40 ± 5	44 ± 10	70 ± 9	61 ± 4	52 ± 4
Nov	28 ± 3	24 ± 3	23 ± 2	29 ± 2	28 ± 2	28 ± 6	28 ± 5	37 ± 7

Table 2. Levels of the two secretogranin-II (SgII) transcripts in the pituitary at three different times of the year and three different steroid treatments (100µg/g body weight). Levels were determined by Northern blot and 18S ribosomal RNA was used as internal control. Means ± SEM are shown, n = 6 RNA pools.

Fragment	Season	Males				Females			
		Control	Testosterone	Estradiol	Progesterone	Control	Testosterone	Estradiol	Progesterone
Large	Feb	2.7 ±0.2	2.8 ±0.5	2.8 ±0.5	2.8 ±0.6	7.9 ±2.3	6.1 ±0.8	8.5 ±2.3	6.2 ±1.3
	May	2.2 ±1.1	0.5 ±0.3	1.0 ±0.4	0.9 ±0.2	0.5 ±0.2	0.5 ±0.1	0.5 ±0.1	0.5 ±0.1
	Nov	0.7 ±0.2	0.6 ±0.2	0.5 ±0.1	0.8 ±0.3	5.9 ±5.2	0.7 ±0.1	1.6 ±0.5	1.2 ±0.4
Small	Feb	2.5 ±0.2	2.8 ±0.5	2.8 ±0.5	2.6 ±0.6	7.2 ±2.3	5.7 ±0.7	7.6 ±1.9	5.8 ±1.1
	May	2.2 ±1.1	0.6 ±0.3	1.0 ±0.4	0.9 ±0.2	0.5 ±0.2	0.5 ±0.1	0.5 ±0.1	0.5 ±0.1
	Nov	0.7 ±0.2	0.5 ±0.2	0.5 ±0.1	1.0 ±0.4	4.6 ±3.8	0.7 ±0.1	1.7 ±0.6	1.1 ±0.3

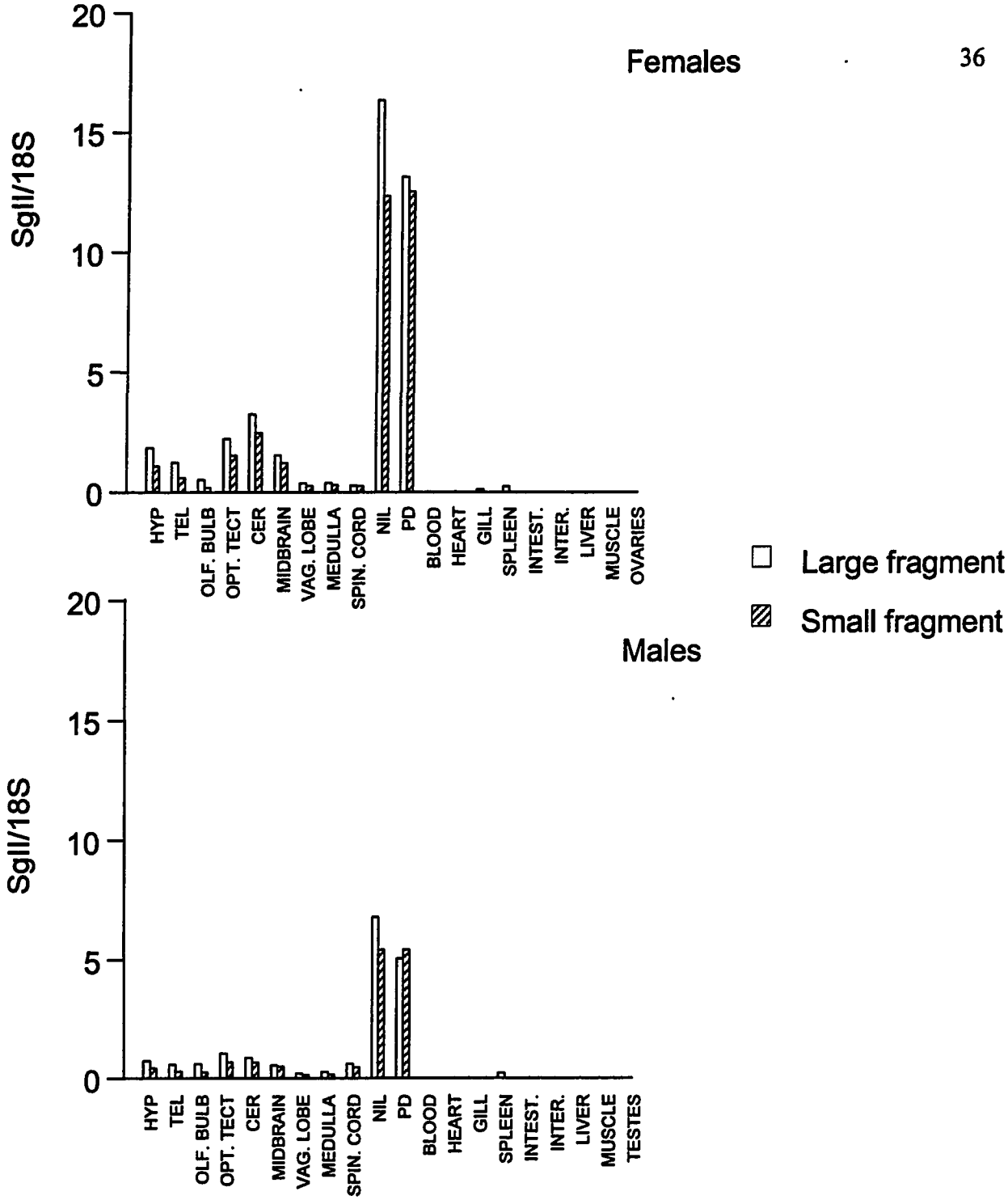

Figure 1. Tissue distribution of secretogranin-II (SgII) in female and male goldfish. The tissue sampling was done in February when the fish were in a sexually recrudescent stage. Bars represent one pool of eight fish, 18S ribosomal RNA was used as internal control to correct for loading. Notice the co-variation of the large and small transcripts and the higher levels of SgII in females in contrast to males. HYP:hypothalamus; Tel:telencephalon; OLF.BULB: olfactory bulb; CER:cerebellum; VAG.LOBE: vagal lobe; SPIN.CORD: spinal cord; NIL: neurointermediate lobe of the pituitary; PD: pars distalis of the pituitary; INTEST.: intestine; INTER.: interrenal.

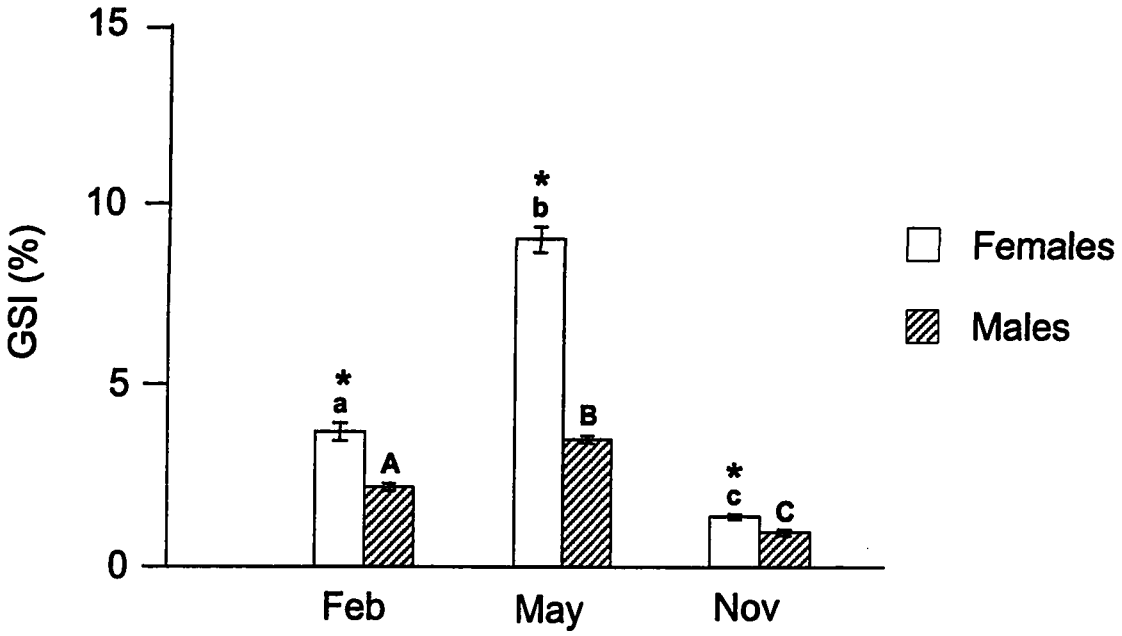

Figure 2. Gonadal somatic index (GSI) of male and female goldfish at three different times of the year representing sexual recrudescence (February), maturity (May) and regression (November). Bars represent means ± SEM, n = 140. Different upper case and lower case letters indicate significant difference among seasons ($P<0.05$). * indicates significant difference between sexes ($P<0.05$).

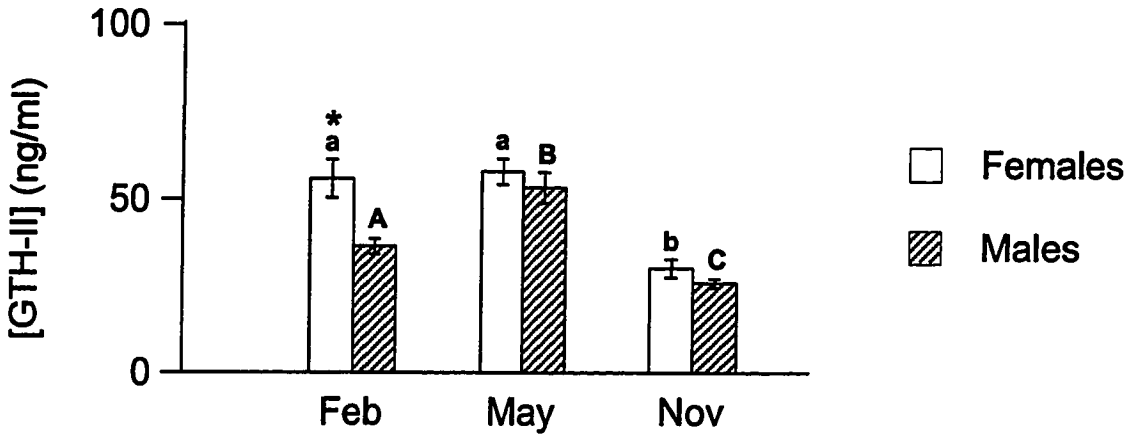

Figure 3. Serum gonadotropin-II (GTH-II) concentrations of male and female goldfish at three different times of the year representing sexual recrudescence (February), maturity (May) and regression (November). Bars represent means ± SEM, n = 40. Different upper and lower case letters indicate significant difference among seasons ($P<0.05$). * indicates significant difference between sexes ($P<0.05$).

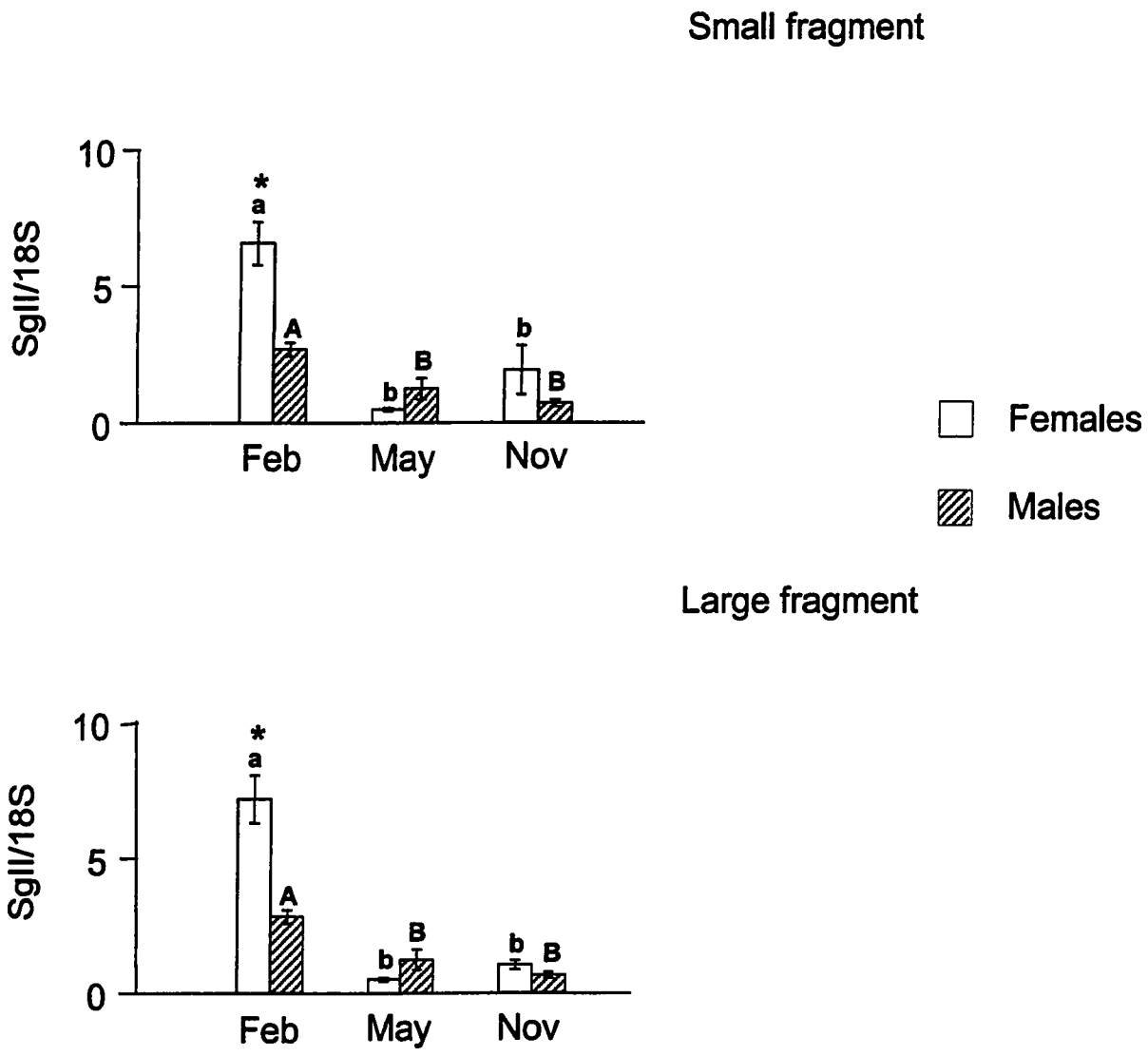

Figure 4. mRNA levels of secretogranin-II (SgII) in the pituitary of male and female goldfish at three different times of the year representing sexual recrudescence (February), maturity (May) and regression (November). mRNA levels were measured by Northern blot analysis and 18S ribosomal RNA was used as an internal control. Bars represent means ± SEM, n = 24 (6 pools of 6 pituitaries). Different upper and lower case letters indicate significant difference among seasons ($P<0.05$). * indicates significant difference between sexes ($P<0.05$).

Chapter 4. Regulation of secretogranin-II (SgII) expression by gonadotropin-releasing hormone (GnRH) and the dopamine antagonist domperidone in the goldfish pituitary.

Results

Serum gonadotropin-II levels

Serum gonadotropin-II (GTH-II) concentrations in the control fish were relatively high. These levels are to be expected considering the advanced sexual maturity of the fish (GSI = 5.6 ± 0.8%).Upon treatment with sGnRHa and domperidone (DOM), there was a 150% increase in serum GTH-II levels (figure 1), so the treatment stimulated the release of GTH-II from the pituitary.

Gene expression levels

In this experiment, SgII, GTH-II and GH expression levels were studied in the PD and NIL separately. Figure 2 shows the mRNA levels in the neurointermediate lobe (NIL) and pars distalis (PD) of GTH-II and growth hormone (GH). In the control group, there was a significantly higher level of GH mRNA in the PD than in the NIL. Treatment with sGnRHa and DOM caused a significant increase in GTH-II and GH mRNA levels in the NIL but not in the PD. Figure 3 shows secretogranin-II (SgII) mRNA levels in the NIL and PD. Treatment with sGnRHa and DOM caused a significant increase of both the large and small SgII mRNA fragments in the NIL but not in the PD. After the treatment, SgII mRNA levels in the NIL became significantly higher than the levels in the PD.

Correlations between the expression levels of SgII with GTH-II and GH

SgII gene expression was correlated with that of GTH-II and GH (figure 4). GTH-II levels significantly correlate with those of SgII with a correlation factor R = 0.82. However, GH levels do not correlate with those of SgII; correlation factor R = -0.06.

Discussion

The purpose of this study was to determine whether SgII expression and GTH-II expression and release were correlated. Sexually recrudescent female goldfish were treated with sGnRHa and the dopamine antagonist domperidone (DOM); this treatment stimulated GTH-II release. This observation is in agreement with many previously published studies that have shown a stimulatory effect of GnRH on pituitary GTH-II release and an inhibitory effect of dopamine which can be diminished by specific antagonists (Trudeau *et al.*, 1993d ; Chang *et al.*, 1990).

The goldfish pituitary is divided into the neurointermediate lobe (NIL) which corresponds to the neural lobe and the pars intermedia of the pituitary and the pars distalis (PD) which corresponds to the anterior pituitary. The PD contains GTH-II, GH, prolactin, thyrotropin and adrenocorticotropin secreting cells, whereas the neural lobe contains arginine vasotocin and isotocin terminals originating from neurons in the hypothalamus and pre-optic area (Yan and Thomas, 1991). The pars intermedia contains MSH cells and other endocrine cell types. Basal GTH-II and GH levels would be expected to be much higher in the PD than in the NIL. In this experiment, this was the case for GH mRNA levels but not for GTH-II mRNA levels (figure 2). This could be explained by the fact that it is impossible to obtain pure NIL or PD. Moreover, GTH-II cells in the teleost pituitary are concentrated in the ventral PPD and extend posteriorly

to form a border around the pars intermedia (Garcia-Hernandez *et al.*, 1996). Therefore, the presence of high levels of GTH-II in the NIL is probably due to contamination. When the pituitary was stimulated with GnRH and DOM, an increase in GTH-II and GH mRNA levels was expected in the PD. However, we observed a significant increase in GTH-II and GH expression in the NIL but not in the PD, which suggests that the NIL pool was contaminated with both GTH-II and GH producing cells. These cells are strongly responsive to GnRH stimulation during sexual recrudescence. No previous studies have examined the regional difference in responsiveness to GnRH in the goldfish pituitary, so what we observed here is a likely novel effect.

As we reported in the previous chapter basal SgII mRNA levels are slightly, but not significantly, higher in the NIL than in the PD. Moreover, the mRNA levels of the large fragment and the small fragment are highly correlated, so we shall for convenience discuss them together. Upon treatment with GnRH and DOM we observed a statistically significant increase in SgII expression in the NIL, but not in the PD. Since SgII is a secretory protein it is found throughout the pituitary gland, but only the SgII-producing cell populations that were present in the NIL fragment responded to GnRH and DOM stimulation. We hypothesized that the SgII-producing cell populations that responded to GnRH and DOM stimulation were the same as those that produce GTH-II and/or GH. To test this hypothesis, SgII mRNA levels were compared to GTH-II or GH mRNA levels (figure 4).

SgII and GTH-II expression levels were significantly highly correlated, whereas SgII and GH mRNA levels were not significantly correlated. This provides evidence that the increased SgII mRNA levels are likely in the GTH-II producing cell populations and not in the GH-

producing cell populations. Moreover, in goldfish, injection of the GABA metabolism inhibitor gamma-vinyl GABA (GVG) stimulates GTH-II release and SgII expression in gonadotrophs but not somatotrophs (Blazquez *et al.*, 1998). These data together suggest that SgII might colocalize with GTH-II in the goldfish pituitary, which agrees with what has previously been reported in rats (Cozzi and Zanini, 1986). It further suggests that SgII or SgII-related peptides are involved in goldfish reproduction.

In conclusion, this study demonstrated that there are GTH-II and GH cell populations which are highly responsive to GnRH and DOM. GTH-II-producing cell populations are localized at the ventral PD and extend posteriorly to form a border around the pars intermedia (dorsal NIL) as has been observed in various teleost species (Yan and Thomas, 1991). Our observations give evidence for the existence of populations or differential GTH-II and GH cell abundance within the pituitary. The cell populations respond to GnRH and DOM stimulation with increased GTH-II and GH expression levels. The results suggest that the GTH-II-producing cell populations also express SgII, and that SgII expression within those cells in regulated by GnRH and DOM. Finally, this study provides data that supports the hypothesis that SgII is involved in reproduction, and that the expression of SgII and GTH-II are somehow linked.

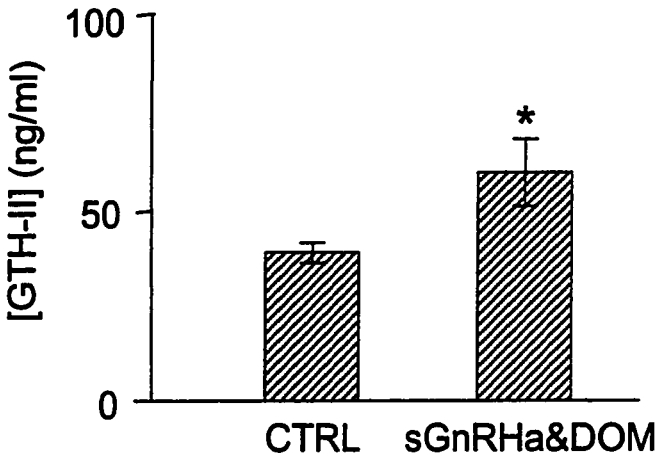

Figure 1. Serum gonadotropin-II (GTH-II) concentrations in control female goldfish (CTRL) and in fish treated for 24 hours with salmon gonadotropin-releasing hormone analog (sGnRHa) and the dopamine antagonist domperidone (DOM). Data are presented as mean ± SEM, n=17. * indicates statistical significance ($P<0.05$).

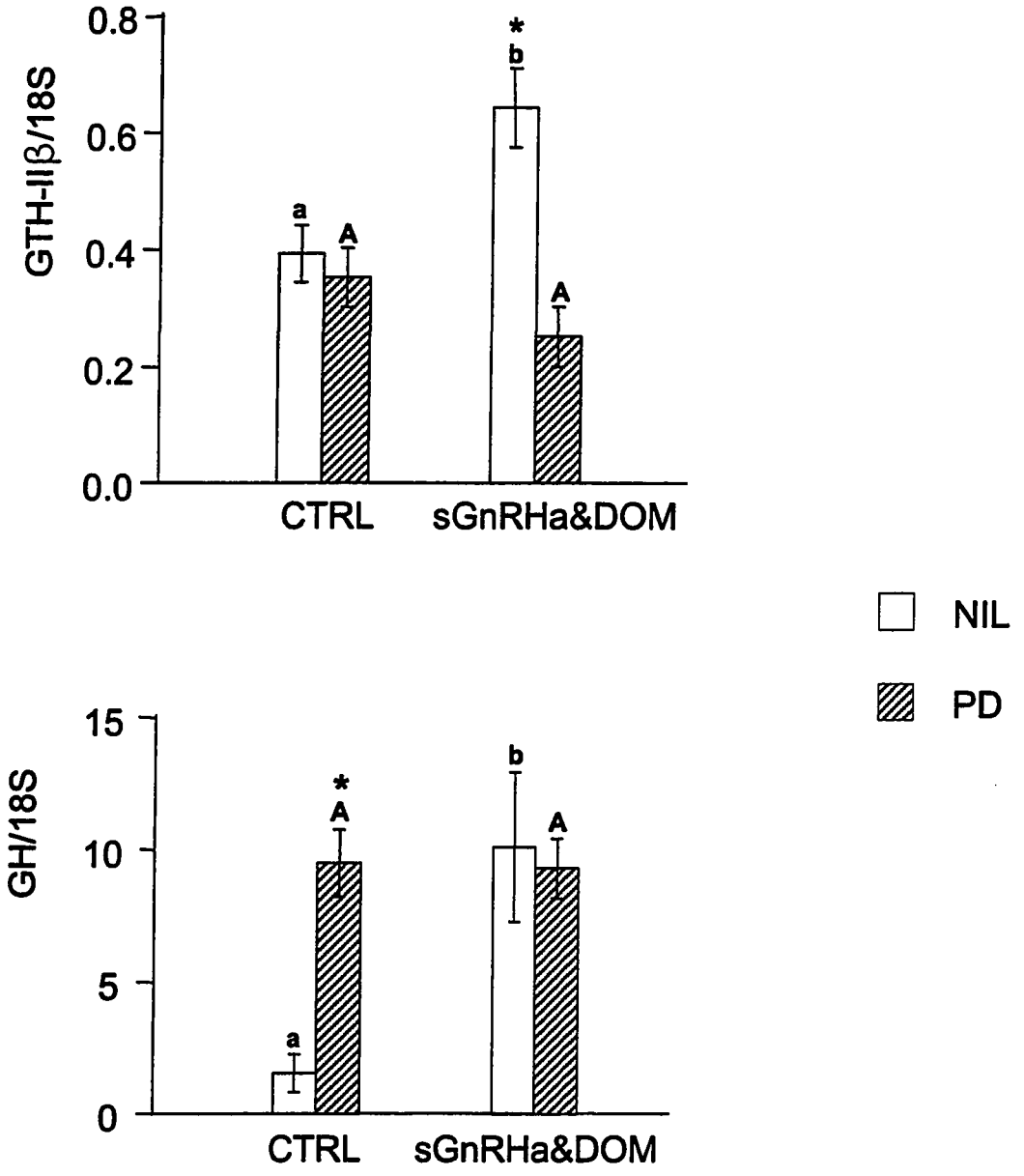

Figure 2. mRNA levels of gonadotropin-IIβ (GTH-IIβ) subunit and growth hormone (GH) in the neurointermediate lobe (NIL) and pars distalis (PD) of the goldfish pituitary. The fish were treated with salmon gonadotropin-releasing hormone analog (sGnRHa) and the dopamine antagonist domperidone (DOM). mRNA levels were measured by Northern blot analysis and 18S ribosomal RNA was used as an internal control. Data are presented as mean ± SEM, n = 6 pools of 6 fish. Different upper and lower case letters indicate significant difference between treatments ($P<0.05$). * indicates significant difference between tissues ($P<0.05$).

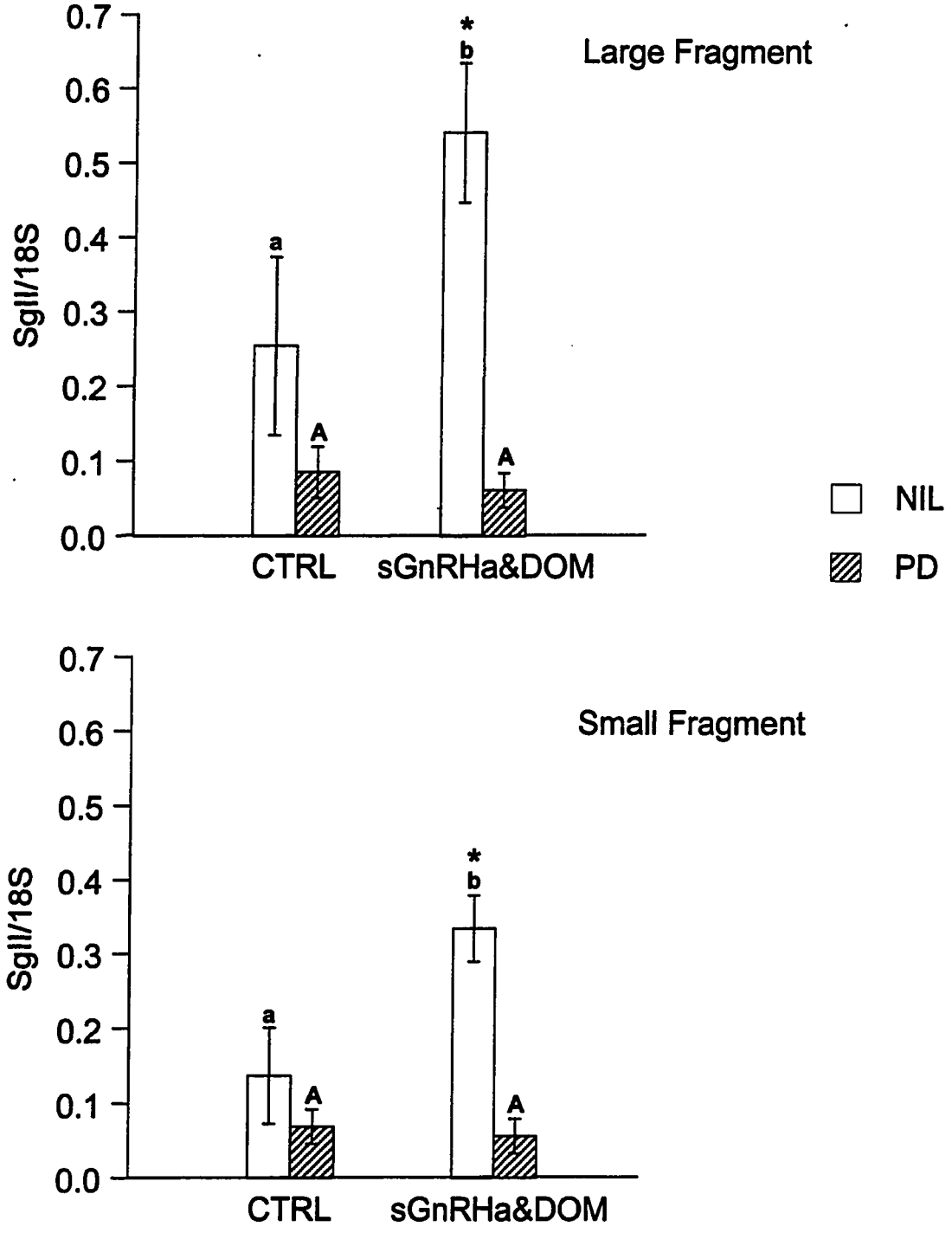

Figure 3. mRNA levels of secretogranin-II (SgII) in the neurointermediate lobe (NIL) and pars distalis (PD) of the goldfish pituitary. The fish were treated with salmon gonadotropin-releasing hormone analog (sGnRHa) and the dopamine antagonist domperidone (DOM). mRNA levels were measured by Northern blot analysis and 18S ribosomal RNA was used as an internal control. Data are presented as mean ± SEM, n = 6 pools of 6 fish. Different upper and lower case letters indicate significant difference between treatments ($P<0.05$). * indicates significant difference between tissues ($P<0.05$).

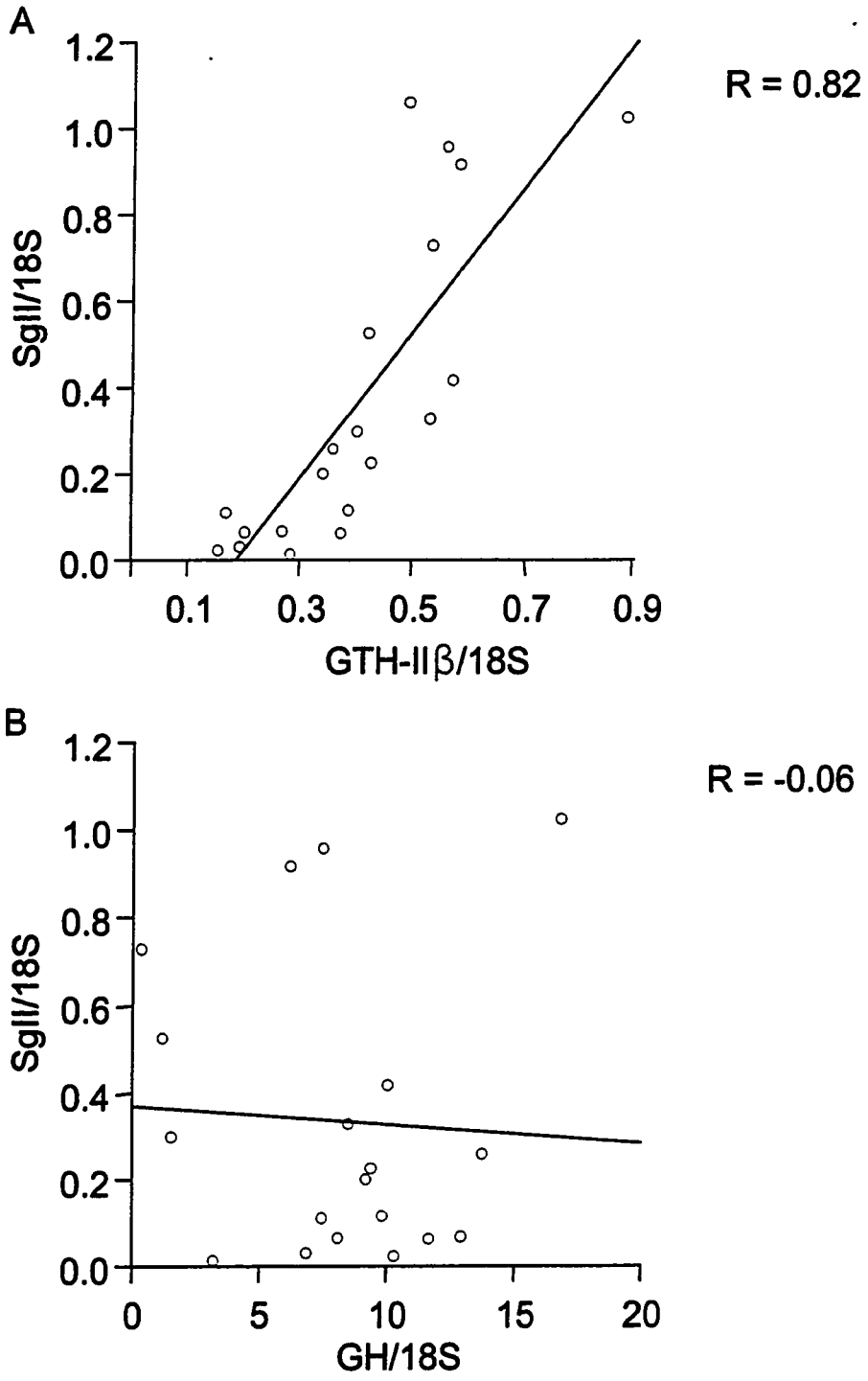

Figure 4. Correlation between expression levels of total secretogranin-II (SgII ; large+small) and gonadotropin-IIβ (GTH-IIβ ; pannel A) or growth hormone (GH ; pannel B) standardized with 18S ribosomal RNA. The correlation between SgII and GTH-IIβ is statistically significant ($P<0.05$) whereas the correlation between SgII and GH is not ($P>0.05$; Bonferroni chi-square test).

Chapter 5. Regulation of secretogranin-II (SgII) gene expression by testosterone (T) and gonadotropin-releasing hormone (GnRH) in the goldfish pituitary.

Results

Serum gonadotropin-II levels

In the sexually recrudescent females (GSI = 3.6 ± 0.1), GnRH stimulated a significant increase in serum GTH-II levels and so did the combined GnRH and T treatment. Testosterone alone did not affect GTH-II release, and it did not potentiate the GnRH stimulation. In the sexually mature males (GSI = 3.3 ± 0.1), GnRH significantly stimulated GTH-II release. Although testosterone on its own did not affect serum GTH-II levels, it potentiated the GnRH effect (figure 1).

Pituitary gonadotropin-II (GTH-II) and growth hormone (GH) expression

GnRH treatment stimulated an increase in GTH-II expression in the PD but not in the NIL of the sexually recrudescent females. Neither the testosterone treatment nor the combined GnRH and T treatment caused any change in GTH-II mRNA levels. None of the treatments caused any significant changes in GH mRNA levels in either part of the pituitary in the females (figure 2). In the sexually mature males, none of the treatments caused any significant changes in either GTH-II or GH mRNA levels in the NIL and PD (figure 4). Both GTH-II and GH mRNA levels were consistently higher in the PD than in the NIL in both males and females, but they were still present in the NIL. This is probably due to contamination during dissection and the presence of highly active GTH-II and GH-producing cells at the border of the two parts.

Pituitary secretogranin-II (SgII) expression

Messenger RNA levels of both the large and small SgII fragments increased significantly with GnRH treatment in the PD but not the NIL of the sexually recrudescent females (figure 3). In the sexually mature males, SgII mRNA levels of both fragments were consistently significantly higher in the PD than in the NIL. None of the treatments caused any significant changes in SgII mRNA levels in the PD and NIL of mature males (figure 5). GTH-II and SgII expression patterns are significantly ($P<0.01$) correlated in both the sexually recrudescent females ($R = 0.78$) and the sexually mature males ($R = 0.97$).

Discussion

GnRH treatment stimulated an increase in serum GTH-II levels in the sexually recrudescent female goldfish. This effect agrees with previous reports (Trudeau *et al.*, 1993b). Pre-treating the fish with testosterone did not potentiate the stimulatory effect of GnRH on GTH-II release. This contrasts with previous reports where testosterone pre-treatment potentiated GnRH stimulation, causing a 2 to 4-fold increase in serum GTH-II levels (Trudeau *et al.*, 1991a). In the sexually mature males, GnRH stimulated GTH-II release and this stimulation was potentiated by pre-treatment with testosterone. These observations agree with the observations of Trudeau *et al.* (1991a), but the fold increase reported in that study is much higher than what we observe, which might be a result of time course differences.

In the females, GnRH treatment significantly increased GTH-IIβ subunit mRNA levels in the PD of the pituitary. This effect of GnRH on GTH-II mRNA levels has been previously reported by Klausen *et al.* (2001) for whole pituitary. GnRH in therefore capable of regulating GTH-II synthesis and release. Unlike previous reports by Huggard *et al.* (1996a), testosterone

alone did not cause an increase in GTH-II expression. This contrast is likely due to the fact that their fish were not in the same sexual maturity stage, and they administered testosterone by injection and at different doses. Our data show that pre-treating the fish with testosterone for five days abolished the GnRH stimulation of GTH-II mRNA increase. This observation is similar to previous reports by Habibi and Huggard (1998). Testosterone in capable of potentiating GnRH-stimulated GTH-II release but inhibits GnRH-stimulated increases in GTH-II expression. None of these effects were observed in the NIL. This is to be expected since the PD is the part containing the majority of the GTH-II-synthesizing cells. In contrast to previous reports of GnRH treatment causing an increase in pituitary GH mRNA content (Klausen *et al.*, 2001), we did not observe any significant effect of GnRH on GH expression. However, Klausen *et al.* (2001) used fish of mixed sex and did not report the sexual stage, which might explain the different observations. Testosterone did not cause any significant changes in GH mRNA levels. This observation is in contrast with previously published data where T was shown to stimulate an increase in GH expression both *in vitro* and *in vivo* (Huggard *et al.*, 1996b). This is likely due to the fact that they administered testosterone by injections and at non-physiological doses. The combined GnRH and T treatment did not cause any changes in GH mRNA levels in the pituitary.

Secretogranin-II mRNA levels of both the large and small transcripts increased in response to GnRH treatment in the PD but not the NIL of the sexually recrudescent females. Since the PD is the part which contains the GnRH-responsive cell populations, the tissue difference is to be expected. This contrasts with our previous study where GnRH and domperidone treatment caused an increase in SgII expression levels in the NIL. This could possibly be explained by the fact that in the previous study the fish were treated with

domperidone, a dopamine antagonist, and that could have altered the responsiveness of the different cell populations. Another possible explanation is contamination during dissection where some highly responsive cells ended up in the NIL pool. Testosterone treatment alone did not alter SgII mRNA levels. This agrees with our previous study where we observed that sex steroids did not modulate SgII expression in any sexual stage in either sex. Pre-treating the fish with testosterone abolished the GnRH stimulation of SgII expression. In sexually recrudescent goldfish, GnRH stimulates the expression of both GTH-II and SgII, and this stimulation is abolished by testosterone pre-treatment.

In the sexually mature males, none of the treatments caused any changes in mRNA levels of GTH-II and GH. This is in contrast with previous reports where GnRH caused increases in both GTH-II and GH mRNA levels (Klausen *et al.*, 2001) and testosterone caused increases in GH levels (Huggard *et al.*, 1996b). Our data agree with studies by Sohn *et al.* (1998) where testosterone did not cause increases in GTH-II mRNA levels in sexually mature male fish. As previously discussed, Huggard *et al.* (1996b) used pharmacological doses, and Klausen *et al.* (2001) did not separate males from females and did not report the sexual stage of the fish used. These factors might explain the different observations. Another possible explanation for the lack of effect is the fact that the fish are fully mature and all pituitary hormones are at peak levels, so no further stimulation of synthesis is possible. SgII levels were also not affected by any of the treatments. The levels of both the small and large fragments were significantly higher in the PD than in the NIL. This is in contrast to our previous studies where we observed similar levels in both tissues or slightly higher levels in the NIL. This observation suggests that the PD is at peak activity, and no further stimulation of SgII expression is possible. This agrees with what was

observed for GTH-II and GH, so in fully mature animals, GnRH can stimulate release of previously synthesized GTH-II, but cannot stimulate increases in GTH-IIβ mRNA levels in the pituitary. Similarly to what was observed in the previous chapter, SgII expression significantly correlated with GTH-II expression in both the sexually recrudescent females and the sexually mature males. This suggests that these two genes might be co-localized in the same cells, and that their expression is somehow linked.

In conclusion, in sexually recrudescent female fish, GnRH stimulates the expression of GTH-II, GH and SgII. At physiological levels, testosterone alone does not affect any of these genes, and it abolishes the stimulatory effects of GnRH on GTH-IIβ and SgII expression. This is the first study to report modulatory effects of testosterone on SgII expression. These observations hold for the PD which contains the majority of GTH-II and GH-producing cells. The NIL pool, which contains a small proportion of GTH-II and GH cells (approximately 20% of total as determined by mRNA levels in this study) due to the nature of pituitary cell distribution, does not show similar expression patterns. In sexually mature fish, the GnRH stimulatory effect is completely abolished either because pituitary hormones are at peak levels and further stimulation is not possible, or because testosterone levels in these fish are elevated and they abolish the GnRH effect. Unlike its potentiating effect on GnRH-stimulated GTH-II release, testosterone plays a predominantly inhibitory role on GnRH-stimulated gene expression in the pituitary.

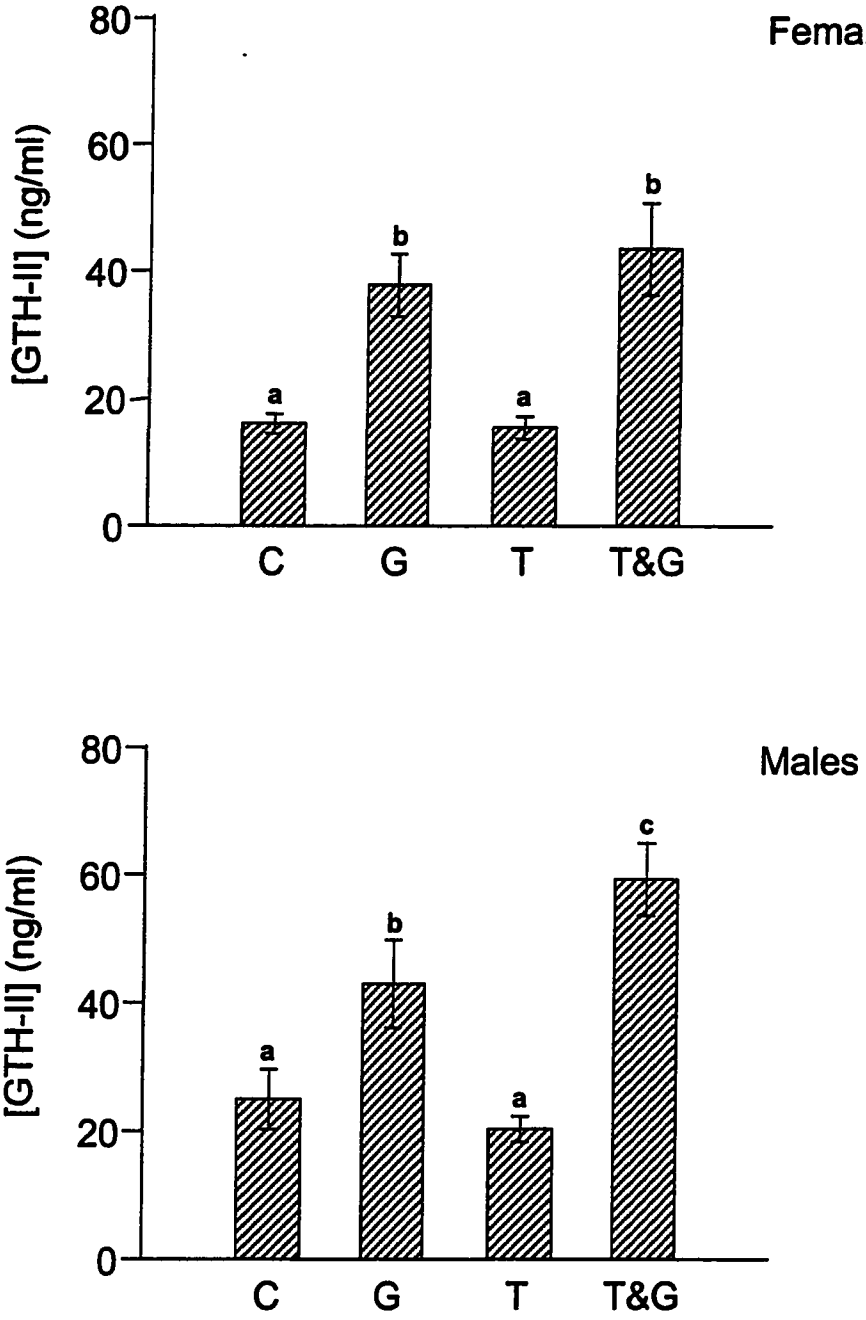

Figure 1. Serum gonadotropin-II (GTH-II) concentrations for sexually recrudescent females (GSI = 3.6 ± 0.1) and sexually mature males (GSI = 3.3 ± 0.1) treated with salmon gonadotropin-releasing hormone analog (sGnRHa ; G), testosterone (T), a combination of both (T&G) or sham treated (C). GTH-II levels were measured using a specific radioimmunoassay. Bars represent means ± SEM, n = 12 to 15. Different letters indicate significant difference ($P<0.05$).

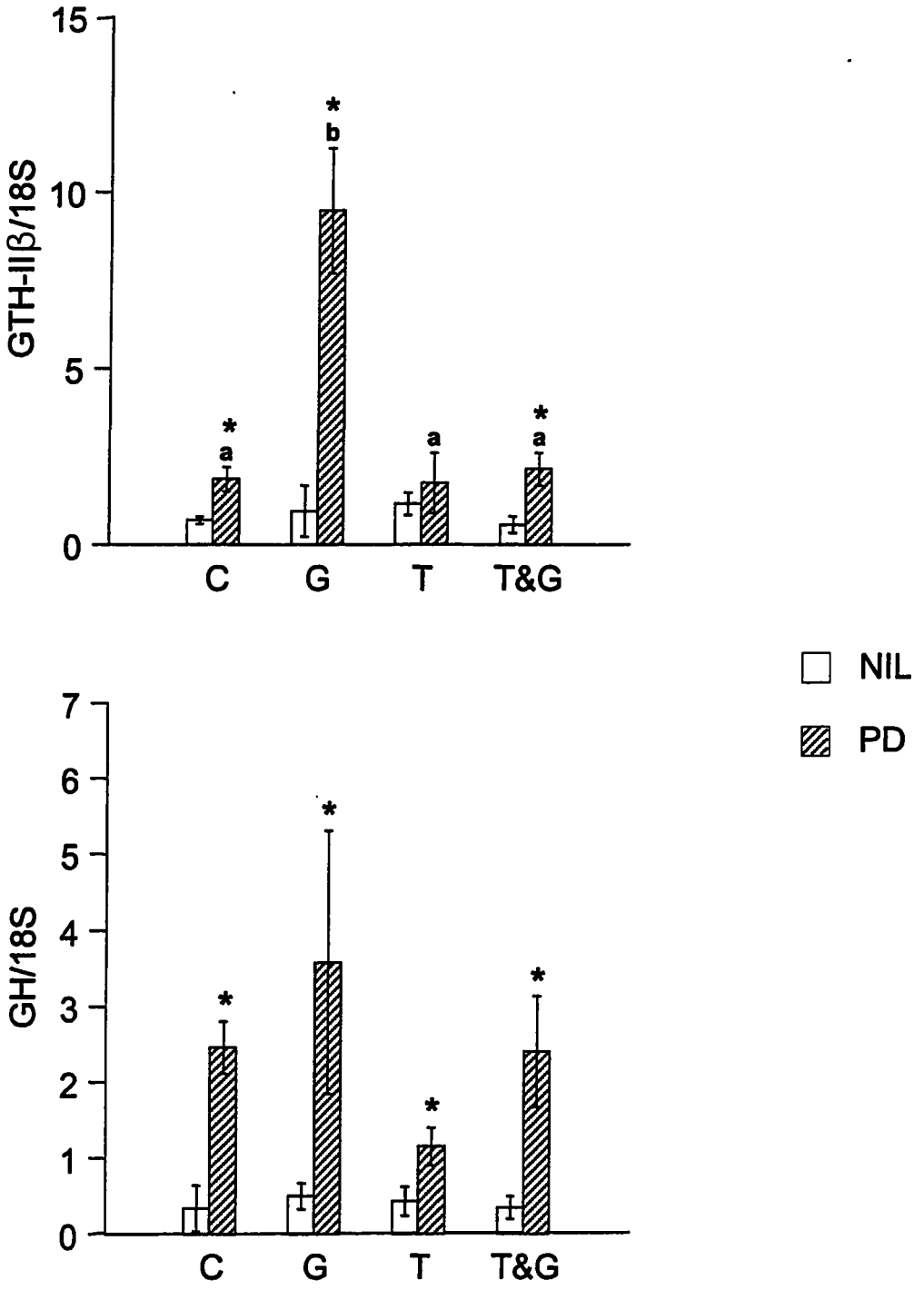

Figure 2. Gonadotropin-IIβ (GTH-IIβ) subunit and GH mRNA levels in the neurointermediate lobe (NIL) and pars distalis (PD) of sexually recrudescent female goldfish (GSI = 3.6 ± 0.1) treated with salmon gonadotropin-releasing hormone analog (sGnRHa ; G), testosterone (T), a combination of both (T&G) or untreated (C). mRNA levels were measured by Northern blot and 18S ribosomal RNA was used as an internal standard. Bars represent means ± SEM, n = 6 pools of 6 fish. Different letters indicate significant difference among treatments ($P<0.05$) and * indicates significant difference between NIL and PD ($P<0.05$).

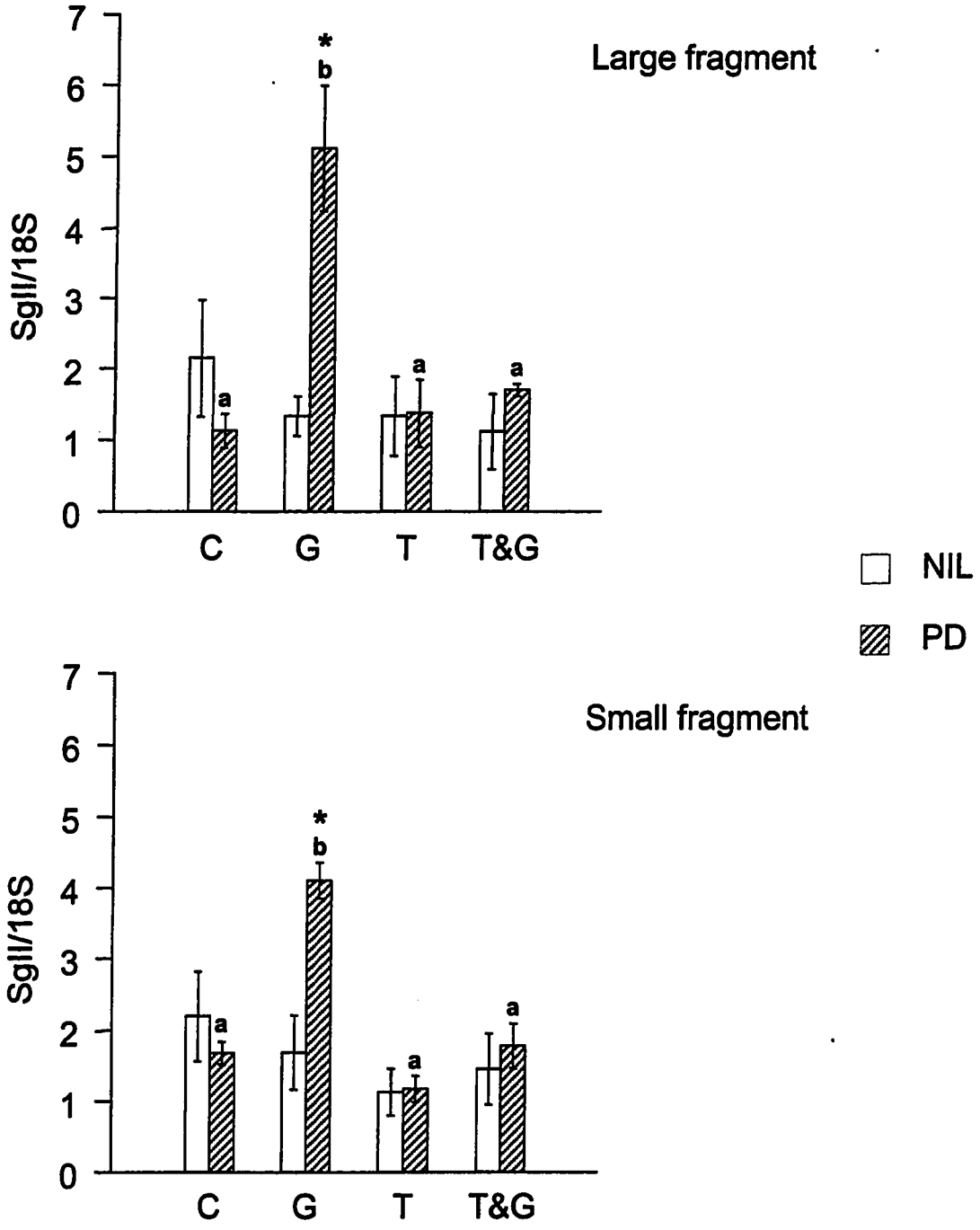

Figure 3. Secretogranin-II (SgII) mRNA levels in the neurointermediate lobe (NIL) and pars distalis (PD) of sexually recrudescent female goldfish (GSI = 3.6 ± 0.1) treated with salmon gonadotropin-releasing hormone analog (sGnRHa ; G), testosterone (T), a combination of both (T&G) or untreated (C). mRNA levels were measured by Northern blot and 18S ribosomal RNA was used as an internal standard. Bars represent means ± SEM, n = 6 pools of 6 fish. Different letters indicate significant difference among treatments ($P<0.05$) and * indicates significant difference between NIL and PD ($P<0.05$).

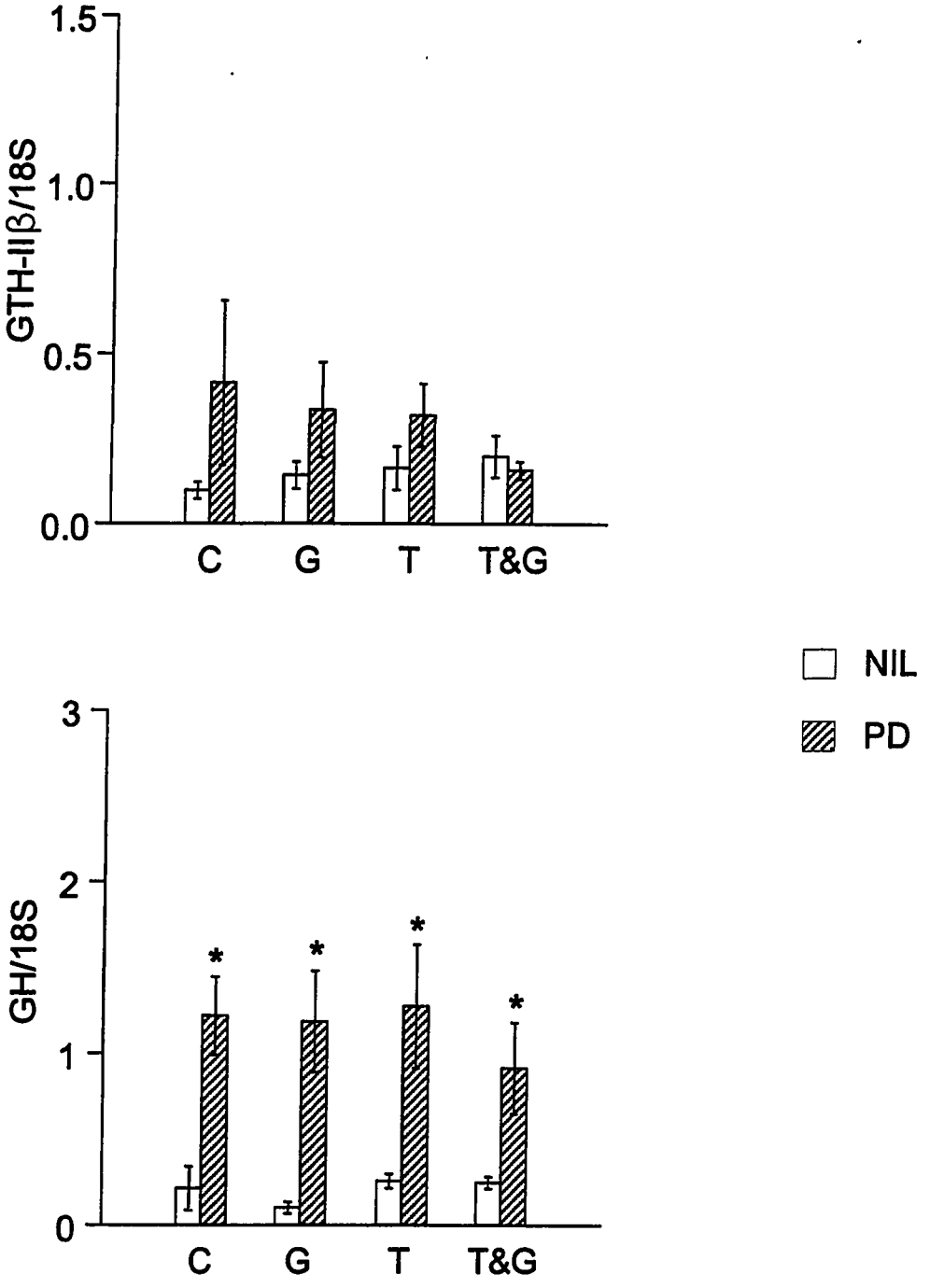

Figure 4. Gonadotropin-IIβ (GTH-IIβ) subunit and GH mRNA levels in the neurointermediate lobe (NIL) and pars distalis (PD) of sexually mature male goldfish (GSI = 3.3 ± 0.1) treated with salmon gonadotropin-releasing hormone analog (sGnRHa ; G), testosterone (T), a combination of both (T&G) or untreated (C). mRNA levels were measured by Northern blot and 18S ribosomal RNA was used as an internal standard. Bars represent means ± SEM, n = 6 pools of 6 fish. * indicates significant difference between NIL and PD ($P<0.05$).

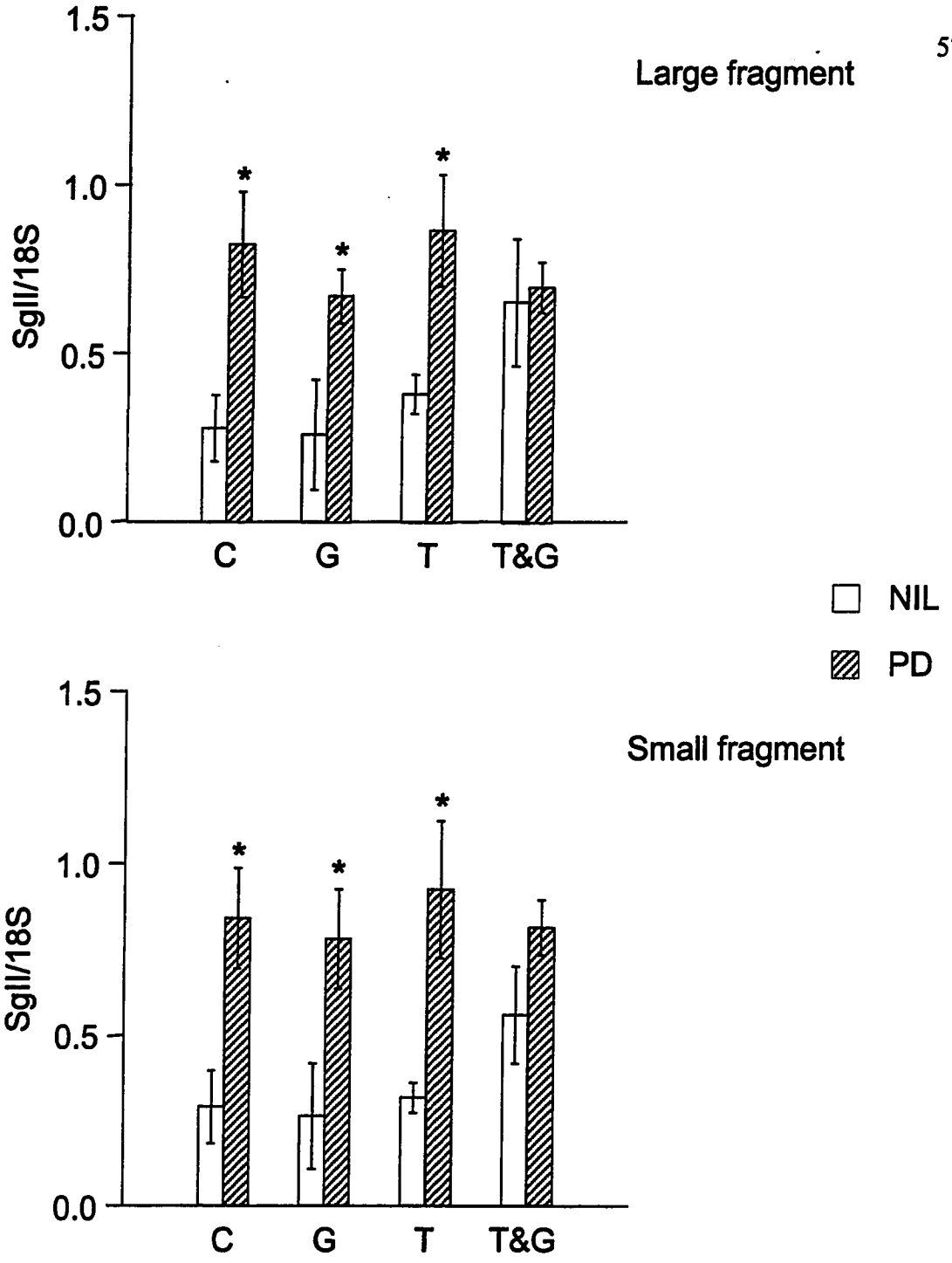

Figure 5. Secretogranin-II (SgII) mRNA levels in the neurointermediate lobe (NIL) and pars distalis (PD) of sexually mature male goldfish (GSI = 3.3 ± 0.1) treated with salmon gonadotropin-releasing hormone analog (sGnRHa ; G), testosterone (T), a combination of both (T&G) or untreated (C). mRNA levels were measured by Northern blot and 18S ribosomal RNA was used as an internal standard. Bars represent means ± SEM, n = 6 pools of 6 fish. * indicates significant difference between NIL and PD ($P<0.05$).

Chapter 6. General Discussion

Secretogranin-II (SgII) is a secretory protein that belongs to the chromogranin family. In secretory granules, it can be proteolytically processed by prohormone convertases into smaller peptides. The most important of these peptides is secretoneurin (SN) which is bioactive and has been shown to play multiple roles in the central and peripheral nervous systems. In the goldfish pituitary, SgII expression is up-regulated by GABA, and this increase in mRNA is concomitant with a reduction in pituitary GTH-II content. When goldfish are treated with SN in combination with the dopamine antagonist domperidone, there is a strong stimulation of GTH-II release (Blazquez *et al.*, 1998). These observations suggest that SgII and the SgII-derived peptide SN play a role in reproduction. The objectives of our research were to study the regulation of pituitary SgII. Establishing how this gene is regulated within the pituitary might shed some light on its possible functions in reproduction.

My first study (chapter 3) looked at the regulation of SgII by sex steroids and how its expression varies seasonally with the different sexual stages of male and female goldfish. Tissue distributions of SgII mRNA were established and the highest levels were observed in the pituitary. Our subsequent studies focused on its regulation in the pituitary. SgII expression was found to vary seasonally, with the highest levels being in the sexually recrudescent fish in February, and lower levels in sexually regressed and mature fish. We also observed that SgII expression is not affected by sex steroids, regardless of the sexual stage and the sex of the fish. Sex steroids did not alter basal GTH-II release confirming previous data (Trudeau *et al.*, 1991a). Since sex steroids are known to potentiate and modulate the effects of other factors on GTH-II

release and expression, we hypothesized that other neuroendocrine factors might play a role in regulating the seasonal variations in pituitary SgII mRNA levels.

Gonadotropin-releasing hormone (GnRH) is the principal stimulator of GTH-II release (Trudeau,1997) and of GTH-II and GH expression (Klausen *et al.*, 2001). We hypothesized that GnRH might regulate pituitary SgII. Dopamine is the principal inhibitory factor of the reproductive system. It has been reported to inhibit both basal and GnRH-stimulated GTH-II release (Chang *et al.*, 1990). By treating sexually recrudescent female fish with a GnRH agonist and a dopamine antagonist, we maximally stimulated the reproductive system, mimicking conditions that would induce ovulation in sexually mature fish (Peter *et al.*, 1986). We studied GTH-IIβ, GH and SgII mRNA levels in the pars distalis (PD), which contains the majority of GTH-II and GH-producing cells, separate from the neurointermediate lobe (NIL) (chapter 4). The treatment with GnRH and domperidone stimulated GTH-II release as expected. It also stimulated GTH-IIβ, GH and SgII expression in the NIL fragment but not in the PD. In this experiment, the levels of GTH-II and GH mRNA in the NIL fragment were higher than what would be predicted from conventional wisdom. It is generally accepted that GTH-II and GH cells are confined to the PD proper. Due to the heterogenous nature of the teleost pituitary, separation of PD and NIL produces an NIL fragment with some PD cells, specifically the cells that are found at the border of the two fragments. The most surprising find is that, in the sexually recrudescent females we used, there appears to be a sub-population of highly active and responsive GTH-II and GH cells at the ventral PD, dorsal NIL border. This preliminary study suggests that sub-populations of PD cells may be highly heterogenous and exhibit diverse secretory activity. This heterogeneity and regionalization of cell populations within the pars distalis has been reported in several fish

species including Atlantic croaker, spotted seafront, red drum (Yan and Thomas, 1991), Mediterranean yellowtail (Garcia-Hernandez *et al.*, 1996), bluefin tuna (Rodriguez-Gomez *et al.*, 2001) and Senegalese sole (Rendon *et al.*, 1997). GnRH is a potent stimulator of SgII expression, thus, GnRH may contribute to the regulation and seasonal changes in SgII mRNA we observed..

Testosterone has been reported to potentiate GnRH stimulation of GTH-II release (Trudeau *et al.*, 1993a) as well as to stimulate GTH-II (Huggard *et al.*, 1996a) and GH (Huggard *et al.*, 1996b) expression in the goldfish pituitary. It has also been reported to modulate the production and effects of other neuroendocrine factors. The aim of our third study (chapter 5) was to determine if testosterone could modulate the effects of GnRH on SgII expression in the pituitary. In sexually recrudescent females, GnRH stimulated GTH-II release, and it caused an increase in GTH-IIβ and SgII mRNA levels in the PD. Testosterone on its own did not cause any changes in the expression of either gene. Pre-treating the fish with testosterone abolished the stimulatory effect of GnRH on the expression of GTH-IIβ and SgII but not on GTH-II release. Therefore, in sexually recrudescent female fish, testosterone does modulate GnRH effects on GTH-II and SgII gene expression in an inhibitory manner; *in vivo* this might be part of a negative feedback loop. When the same experiment was repeated with sexually mature males, GnRH stimulated GTH-II release, and this stimulation was potentiated by testosterone. GnRH had no effect on gene expression of SgII and GTH-II, but higher levels of SgII were observed in the PD than in the NIL. This observation could be explained by the fact that at sexual maturity, GTH-II synthesis and content are at peak levels in the PD and no further production is possible. Another explanation is that the sensitivity of the pituitary to testosterone is very high and T is inhibiting the stimulatory effect of GnRH.

GnRH was shown to stimulate SgII expression in different tissues at different instances. Whereas in the second study (chapter 4) the stimulation of gene expression by GnRH was observed in the NIL, in the third study (chapter 5) it was observed in the PD. Since the dissections for both experiments were carried out in the same way, the major variables are the presence of domperidone, the dopamine antagonist and the different sexual stages. This suggests that dopamine and seasonal cyclicity can modulate the response of different cell populations to GnRH, and this is the first time that such a cell population difference is observed.

SgII expression varies with season and with sexual maturity stages of the goldfish. GnRH is capable of upregulating the expression of GTH-II, GH and SgII genes, and might be responsible for the seasonal variation in SgII expression. The stimulatory effect of GnRH is regionalized. The region or cell populations that respond to GnRH seem to be determined by dopamine and possibly by other factors as well. Sex steroids on their own do not alter SgII gene expression in the pituitary, but testosterone has been shown to inhibit the stimulatory effect of GnRH on GTH-II and SgII expression. SgII and GTH-II expression levels were highly correlated in two contrasting experiments, where the fish were treated with either T or DOM and injected with GnRH, which suggests that they are co-localized and/or co-regulated. SgII might play an intracellular role in GTH-II packaging and release as has been shown for the other chromogranins in other hormonal systems. However, Blazquez *et al.* (1998) showed that SN is capable of stimulating GTH-II release, so the up-regulation of SgII by GnRH could be an intermediate step for the production of SN which further stimulates GTH-II release and sexual maturation.

Our studies have identified and explored novel regulatory mechanisms of pituitary SgII expression, but several question remain unanswered. A very important issue to be analyzed is the different GTH-II and GH-producing cell populations that exist within the pituitary, and how their responsiveness is modulated by different factors. Neuroendocrine factors other than GnRH might play important roles in regulating SgII in the pituitary, and these roles must be explored. With respect to function, studies on SN are already underway to determine its role in regulating pituitary GTH-II synthesis and release. More importantly, a study on SgII where it is knocked out or knocked down is essential in order to differentiate whether it plays an intracellular role in hormone packaging and release, or whether its actions are solely via SN autocrine or paracrine feedback.

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Appendix A

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DISTRIBUTION OF SECRETOGRANIN-II (SgII) mRNA IN THE GOLDFISH

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SUMMARY

Secretogranin-II (SgII) is a large acidic protein found in the endocrine and nervous systems. SgII is proteolytically processed into smaller bioactive peptides, the most important of which is the conserved 33-34 amino acid secretoneurin (SN) sequence. Previous studies in the goldfish have shown that SN stimulates the release of gonadotropin-II (GTH-II) from the pituitary. Our study now determined the distribution of SgII mRNA in the goldfish, and tested whether its expression is regulated in the ovary. SgII mRNA was highest in the pituitary and the central nervous system and low in peripheral tissues. Injection of a gonadotropin-releasing hormone (GnRH) agonist plus a dopamine antagonist to upregulate pituitary GTH-II release decreased ovarian SgII mRNA levels. SgII is expressed throughout the brain-pituitary-ovarian axis and may have important roles in reproduction.

INTRODUCTION

Secretogranin-II (SgII), also known as chromogranin C, is a large (~600 a.a.), acidic and heat stable secretory protein that belongs to the chromogranin family (1). The family also includes chromogranins A, B, and 7B2 which have a very widespread distribution in the endocrine and nervous systems where they are found in large dense-core secretory vesicles. These 4 proteins are structurally unrelated, but they share many biochemical properties. The granins seem to play a role in prohormone sorting into the regulated secretory pathway and normally undergo only a limited proteolysis despite the presence of multiple pairs of basic residues. SgII was first characterized in 1981 in the rat pituitary gland by Rosa and Zanini (2). SgII seems to have a special relevance for the brain where it is present in higher concentrations than the other chromogranins. The highest concentration of SgII in the mammalian brain was found in the evolutionarily older parts, for example the hypothalamus, and in particular the median eminence (3). It has also been detected in varying quantities in the adrenal medulla, pineal gland (4), pancreas, stomach, gastrointestinal tract, thyroid gland, gonads and in many endocrine tumors (3).

SgII is proteolytically processed to a high degree (~90%) into smaller bioactive peptides. This processing occurs at the carboxy terminal of a pair of basic amino acids (Lys-Arg or Arg-Arg) and is mediated by endopeptidases that belong to the prohormone convertase (PC) family, specifically PC1/3 and PC2 (3). Demonstrated roles for secretoneurin (SN), a peptide processed

from SgII, include stimulation of dopamine release from the rat striatum, stimulation of chemotaxis of human monocytes, inhibition of serotonin and melatonin release from the rat pineal gland and stimulation of GTH-II release from the goldfish pituitary (6).

In goldfish, the hypothalamic decapeptide GnRH and the catecholamine dopamine are the principle neurohormones respectively stimulating and inhibiting the release of pituitary GTH-II (i.e., fish luteinizing hormone; 7). GTH-II in turn stimulates ovarian and testicular development and sex steroid prodution. Our study determined the distribution of SgII mRNA in central and peripheral goldfish tissues, and tested whether its expression is regulated in the ovary.

MATERIALS AND METHODS

SgII expression in the different tissues was studied using Northern hybridization following the methods of Blazquez et al. (8) except that 18S ribosomal RNA was used as the internal control. Sexually mature female goldfish were co-injected with salmon GnRH (sGnRHa; 10 ng/g Bwt) and the dopamine antagonist domperidone (DOM; 1ìg/g Bwt) in 0.6% NaCl to increase serum GTH-II levels (7). Twenty-four hours after injections the ovaries were dissected and SgII mRNA levels quantified.

RESULTS

Two SgII transcripts of 2975 and 2650 b.p. were detected, in agreement with our previous results (8). The 2 transcripts are co-regulated and were therefore quantified together. Figure 1 shows the distribution of SgII mRNA in pooled samples from 3 male and 3 female sexually regressed goldfish. Testes and ovaries were analyzed separately. The highest levels of SgII mRNA were found in the pituitary gland followed by the CNS. SgII was also detected in the ovaries and the interrenal. In contrast, SgII was not detectable in the testes and other tissues. A second distribution study in sexually mature animals (data not shown) showed similar results for pituitary, CNS and testes. However, only very low levels of SgII mRNA were found in the mature ovary, suggesting that SgII might be differentially expressed during the reproductive cycle. Additionally, SgII was not detected in muscle or intestine (not shown).

One potential regulator of ovarian SgII is GTH-II. Serum GTH-II levels vary seasonally and are highest in the breeding season and lowest in sexually regressed animals (7). Co-treatment with GnRHa and domperidone (DOM) to increase serum GTH-II decreased SgII expression in the ovary ($p<0.0001$).

Overall, the SgII precursor protein is not highly conserved between goldfish and the tetrapods (8). However, sequence alignments have shown that putative dibasic cleavage sites flanking the SN peptide segment of the SgII precursor are conserved. Figure 4 shows alignments of all known SN sequences. Within tetrapods, SN is 33 a.a. long and >75% similar in sequence. In goldfish, SN is 34 a.a. long and only 68% identical to human SN. A central fragment between positions 8-24 is highly conserved, including a tyrosine at position 9. Within this domain, glutamic acid (E) at position 18 in mammals and the chicken is conservatively replaced with glutamine (Q) in frogs and goldfish. Another E to Q conversion is noted at position 23 where goldfish contains E rather than Q as in all other species. Preliminary modeling studies of human and goldfish SN using HyperChem software suggest some interesting differences in the predicted

3-D structure upon energy minimization. Whereas human SN appears to possess a predominantly sheet-like structure, goldfish SN contains a predominantly turn-like structures at both the N- and C-terminals. The central domain (see the consensus sequence; Fig. 3) is predicted to be a highly conserved sheet-like linear structure with a minor component of helical structure. These observed structural differences may be related to the mutations observed at the N- and C- termini of goldfish SN compared to human SN and may explain some differences in bioactivity between the two SNs.

CONCLUSIONS

In the goldfish, the widespread central and peripheral distribution of SgII mRNA as determined using Northern blot analysis suggests diverse roles for SgII-derived bioactive peptides. Similar to mammals, SgII mRNA levels were highest in the goldfish pituitary. In contrast to mammals, however, the goldfish expresses 2 SgII transcripts, presumably because of alternate splicing events (7). Levels in the CNS were low but detectable. SgII levels were low in the goldfish interrenal (kidney plus adrenal) which is in contrast to mammals and frogs where high adrenal SgII levels have been found. The most striking difference is that SgII was not detected in the goldfish intestine, whereas in rats the largest pool of SgII is found the gastrointestinal tract (3).

We noted that SgII mRNA levels were detectable in the sexually regressed but not sexually mature ovary, suggesting that SgII is regulated during the seasonal reproductive cycle. To examine one possible hormonal factor, we induced pituitary GTH-II secretion by co-injection of GnRHa and DOM in sexually mature females. We originally hypothesized that increasing GTH-II in this manner would up-regulate ovarian SgII. Surprisingly, we found that SgII mRNA levels were decreased by 2-fold in the treated fish. This suggests that GTH-II negatively regulates SgII during the breeding season, which may help to explain why lower levels of SgII are found at this time of year. While it in known that SN is bioactive and stimulates pituitary GTH-II release in the goldfish (7), the roles of SN and other SgII-derived peptides in the ovary have not been studied in any species.

The highly conserved nature of the SN fragment of SgII suggests that it is a biologically important peptide. The presence of SgII mRNA at all three levels of the goldfish hypothalamo-pituitary-ovarian axis suggests a role for SgII-derived peptides in female reproduction. (Funded by NSERC to VLT).

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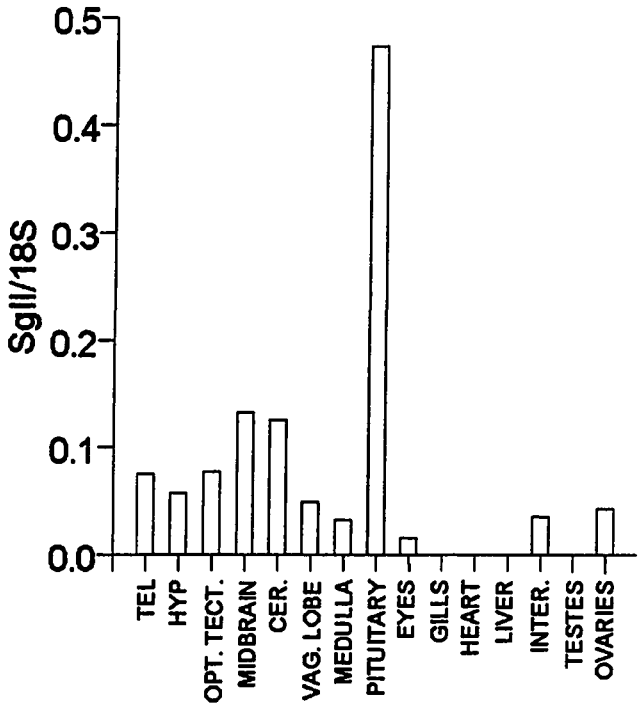

Figure 1. Secretogranin-II mRNA levels in the various tissues of sexually regressed goldfish. mRNA levels were measured using Northern hybridization, and expressed relative to 18S ribosomal RNA. TEL: telencephalon, HYP: hypothalamus, OPT TECT: optic tectum, CER: cerebellum, VAG LOBE: vagal lobe, INTER: interrenal.

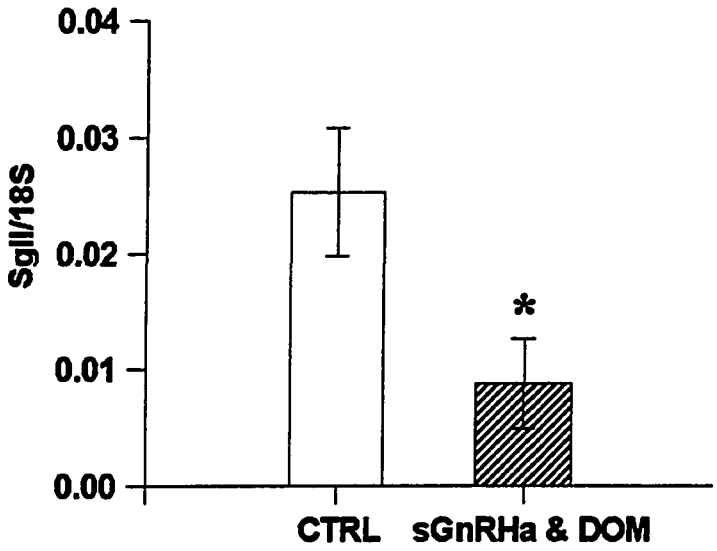

Figure 2. Secretogranin-II mRNA levels in the ovaries of goldfish treated with salmon gonadotropin-releasing hormone agonist (sGnRHa) and the dopamine antagonist domperidone (DOM). Data are means ± S.E.M (n=6; $p<0.0001$; t-test). GSI ~6.0%.

```
human       TNEIVEEQYTPQSLATLESVFQEL.GKLTGPN.NQ      % Identity
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 3. Clustal W alignment of SN sequences showing the % identities with human SN. A consensus sequence is shown. The * indicates the placement of the E-Q conversions at positions 18 and 24.

Appendix B

Goldfish gonadotropin-IIβ cDNA

Forward primer: GAGCATGCGAGAGTTAGGCG Tm = 63.0°C

Reverse primer: CGGACAAGGGACAGTATCGC Tm = 63.0°C

Product size: 462 base pairs

GenBank accession number: AB015596

Goldfish secretogranin-II cDNA

Forward primer: TTCTTACCACGCTACAACAGACC Tm = 60.5°C

Reverse primer: GACATCATCGTCTTCTTCGTCCT Tm = 62.0°C

Product size: 471 base pairs

GenBank accession number: AF046002

Goldfish 18S ribosomal RNA cDNA

Forward primer: GAGCCTGAGAAACGGCTACC Tm = 59.5°C

Reverse primer: GTATTCAGCGGCGACAGG Tm = 60.5°C

Product size: 551 base pairs

GenBank accession: AF047349

Goldfish growth hormone cDNA

Forward primer: ATGGCTAGAGCATTAGTCCTG Tm = 57.3°C

Reverse primer: AGGGTCCAGGTTGAATCCAG Tm = 60.1°C

Product size: 580 base pairs

GenBank accession: AF069398