The Role of Secretogranin-IIa and its derived peptide Secretoneurin a in Feeding Regulation in Female Goldfish

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Abstract

Secretoneurin (SN) is a 31-43 amino acid, functional peptide derived by proteolytic processing from the middle domain of the ~600 amino acid secretogranin-II (SgII) precursor. In teleosts there are 2 forms arising from 2 different genes, SgIIa and SgIIb. In turn, there are both SNa and SNb in teleost. Secretoneurin is a well-conserved peptide during evolution from fish to mammals and widely distributed in secretory granules of endocrine cells and neurons. Secretoneurin plays important roles in different biological processes, for example controlling vertebrate reproduction by stimulating luteinizing hormone release from the pituitary. A potential new role of SN in feeding in goldfish is the subject of the research presented in this thesis. Firstly, we looked at the distribution of SgIIa mRNA in various female goldfish tissues using both RT-PCR and Q-PCR techniques in order to determine which tissue expresses SgIIa mRNA and in which level. We found that SgIIa mRNA was detected in different amounts in all tissues examined. The main tissues of interest were hypothalamus, telencephalon and gut, they all expressed SgIIa. Secondly, we examined the effect of acute (26 h), short (3 days), medium (7 days) and long (14 days) fasting and periprandial changes on SgIIa mRNA level in hypothalamus, telencephalon and gut using Q-PCR method. The results showed that SgIIa mRNA increases under the effect of acute and short fasting, however, medium and long fasting did not affect SgIIa mRNA. Thirdly, we examined the effect of brain injection of goldfish SNa on food intake and locomotor behavior and the expression of some feeding neuropeptides such as neuropeptide Y, orexin, chocystokinin and cocaine- and amphetamine-regulated transcript I after treatment. Injection of SNa in the third brain ventricle increased food intake and fish activity. Associated with this was an increase in NPY and decrease in CARTI mRNA levels in hypothalamus. The increase in SgIIa mRNA following fasting and the increase of food intake as a result of SNa treatment suggest a novel role for SNa in feeding processes.

Résumé

La sécrétoneurine (SN) est un peptide fonctionnel d'environ 31 à 43 acides aminés qui est obtenu après la protéolyse du précurseur sécrétogranin-II (SgII). La protéolyse a lieu dans le domaine central du précurseur SgII qui est composé d'environ 600 acides aminés. Il existe deux formes du peptide SN, nommées SNa et SNb et qui sont situées sur deux gènes différents appelés SgIIa et SgIIb, chez les télestoéens. Le peptide SN a bien été conservé au cours de l'évolution chez les poissons et les mammifères et nous pouvons le retrouver notamment dans les granules de sécrétions des cellules endocrines et les neurones. Il joue un rôle important dans diverses activités cellulaires telles que le contrôle de la reproduction chez les vertébrés en stimulant la sécrétion de l'hormone lutéinisante du pituitaire. Cette thèse propose un nouveau rôle du peptide SN dans les mécanismes d'alimentation chez les poissons rouges. Premièrement, nous avons observé la distribution de l'ARNm de SgIIa dans divers tissus biologiques chez les femelles des poissons rouges en utilisant des techniques telles que la RT-PCR et la Q-PCR. Ces techniques ont été utilisées pour identifier les tissus biologiques qui contiennent l'ARNm de SgIIa et pour mesurer leur niveau d'expression. Les résultats démontrent que la SgIIa est exprimée dans chacun des tissus biologiques mais à différents niveaux d'expression. Les tissus biologiques d'intérêt qui ont été choisis pour cette recherche sont l'hypothalamus, le télencéphale et les intestins du poisson rouge. Deuxièmement, nous avons observé les effets aigus (26hrs), à court-terme (3 jours), à mi-terme (7 jours) ainsi qu'à long-terme (14 jours) du jeûne chez les femelles des poissons rouges. Nous avons aussi noté les changements d'expression de l'ARNm de SgIIa avant et après le repas dans l'hypothalamus, le télencéphale et les intestins en utilisant la technique Q-PCR. En effet, les niveaux d'expression de l'ARNm de SgIIa ont augmenté sous l'effet aigu et à court-terme du jeûne mais les niveaux n'ont pas changé sous l'effet mi-terme et long-terme. Troisièmement, nous avons observé l'alimentation et la motricité globale des poissons rouges suite à des injections cervicales du peptide SNa. De plus, nous avons observé l'expression de certains neuropeptides reliés à l'alimentation tels que le neuropeptide Y, l'orexine, la chocystokinine et les facteurs de transcription de la cocaïne et des amphétamines suite à l'injection. L'injection du SNa dans la troisième ventricule du cerveau a augmenté la prise de nourriture ainsi que l'activité physique chez les poissons rouges. De plus, une augmentation du neuropeptide Y ainsi qu'une diminution du niveau ARNm de CARTI ont été observées dans l'hypothalamus. Les

résultats ci-haut mentionnés et spécifiquement l'augmentation de l'ARNm de SgIIa suite au jeûne ainsi que l'augmentation de la prise de nourriture observée suite à l'injection cervicale de SNa suggèrent la possibilité d'un nouveau rôle de SNa dans les mécanismes d'alimentation chez les télestoéens.

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Table of Contents

Abbreviations

ANOVA	Analysis of variance
BW	Body weight
CART	Cocaine-and amphtamine regulated transcript peptide
CCK	Cholecystokinin
CgA	Chromogranin A
CgC	Chromogranin C
CgB	Chromogranin B
CRH	Corticotropin-releasing hormone
GAL	Galanin
GH	Growth hormone
GnRH	Gonadotropin-releasing hormone
ICV	Intracerebroventricular
IP	Intraperitoneal
LH	Luteinizing hormone
mRNA	Messenger ribonucleic acid
MS-222	3-aminobenzoic acide ethyl ester
NIL	Neurointermedial lobe
NPY	Neuropeptide Y
NPY-IR	Neuropeptide Y immunoreactivity
OX A	Orexin A
OX B	Orexin B
PCR	Polymerase chain reaction
PCs	Prohormone convertases
Q-PCR	Quantitative polymerase chain reaction
RPD	Rostral pars distalis
RT-PCR	Real time polymerase chain reaction
SEM	Standard error of the mean
SgII	Secretogranin II
SgII-IR	Secretogranin II immunoreactivity
SgIIa	Secretogranin IIa
SgIII	Secretogranin III
SN	Secretoneurin
SN-IR	Secretonurin immunireactivity
SNa	Secretonurin a
TRH	Thyrotropoin-releasing hormone

Figures

Thesis rational and hypothesis

This thesis investigates a new potential role of secretogranin-IIa and its derived peptide secretoneurin a in feeding processes in the female goldfish (*Carassius auratus*). The general introduction (Chapter 1) includes a detailed review of the granin family of proteins including secretogranin-II, a large precursor that can be processed to the peptide secretoneurin, the roles of several other neuropeptides relevant to this study and the control of food intake are also reviewed. The major hypothesis is that secretoneurin a is regulated by nutritional signals and works as orexigenic factor to stimulate food intake in female goldfish. The first hypothesis was that fasting would up-regulate secretogranin-IIa mRNA level in hypothalamus, telencephalon and gut (Chapter 2). To test this hypothesis, fish were submitted to acute, short, medium and long fasting and also examined the periprandial changes in secretogranin-IIa mRNA and other feeding neuropeptides such as neuropeptide Y, cholcystokinin and ghrelin. The second hypothesis was that treatment with secretoneurin a would increase food intake, activity and the expression of some feeding neuropeptides e.g. neuropeptide Y, orexin, cholcystokinin and cocaineand amphetamine-regulated transcript I (Chapter 3). To test this hypothesis, goldfish injected with secretoneurin a directly into the brain and then the food intake and locomotor behavior were measured. Also, the mRNA level for 2 appetite stimulators (neuropeptide Y and orexin) and 2 appetite inhibitors (cholcystokinin and cocaine-and amphetamine-regulated transcript I) at 2 h and 5 h post treatment were measured to be able to determine if SNa may affect feeding processes. Finally, the general discussion (Chapter 4) focuses on the most important findings in this thesis and indicates key future areas of investigation. This research helps to define a novel role for SNa in the control of food intake. Chapter 2 and 3 were organized to have their own materials and methods, results, discussions, which is easier to follow.

Chapter 1

General introduction

1.1 Granin family

The granins are a group of proteins that have special characteristics of acidity and heat-stability and are distributed in wide range of secretory granules cells of endocrine, neuroendocrine, neuronal and immune systems (Huttner et al., 1991). The granin proteins (CgA, CgB and SgII) play essential roles in the regulated secretory pathway that is responsible for secretory granule formation and delivering peptides, hormones and neurotransmitters. The mechanism by which granins are leading the regulated secretory pathway to the sorting and packaging of proteins in secretory granules is initiated in the trans-Golgi network (TGN) through multiple important steps. This starts with the biosynthesis of the granin proteins in the rough endoplasmic reticulum, then crossing the Golgi apparatus to the secretory vesicles through TGN. This transportation occurs under low pH level and high calcium ion concentrations. During the sorting and packaging in the TGN, granins form aggregates leading to the formation of the secretory granules. Granin proteins are processed by multiple prohormone convertases (PC) in the secretory vesicles. Then the secretory vesicles fuse with the cell membrane. Upon stimulation, the final step is the release of secretory granule contents (, i.e. secretion of hormones) from the cell (Bartolomucci et al., 2011; Courel et al., 1020; Fischer-Colbrie et al., 1995; Ozawa & Takata, 1995).

Granin proteins share many common characteristics such as hydrophobicity, having high proportion of acidic amino acids, binding calcium and forming complexes at low pH level, sorting and aggregation of secretory products in the Golgi network and forming of secretory granules (Ozawa & Takata, 1995). Granin proteins are also characterized by numerous pairs of basic amino acids which are possible cleavage sites for enzymes such as prohormone convertaes (PCs) enzymes PC1/3 and PC2 to generate smaller peptides that might control some endocrine, autocrine and paracrine processes by proteolytic processing (Helle, 2004; Huttner et al., 1991). The granin family is composed of two groups, the chromogranin group (Cg) distinct with disulfide-bonded loop at the N-terminus and the secretogranin group (Sg), where the disulfide loop is absent (Zhao et al., 2009b). The Cg family is divided into two subgroups including chromogranin A (CgA) and chromogranin B (CgB) while the Sg family consists of seven members that named as secretogranin II (SgII), secretogranin III (SgIII), secretogranin IV (SgIV), secretograninV (SgV), secretogranin VI (SgVI), secretogranin VII (Sg VII), and proSAAS (Zhao et al.,

2009b). Chromogranin proteins have relatively a high similarity of DNA and amino acid sequences and are considered well conserved in evolution between different vertebrate species. On the other hand, secretogranins are not as well conserved compared to the chromogranins. A short domain in the middle of SgII known as secretonurin shows a high similarity among different vertebrate species (Zhao et al., 2009b). The first peptides found to be generated from the granin family are pancreastatin from CgA, CgB1-14 from CgB and secretoneurin from SgII (Helle, 2004). The specific distribution of granins make them useful biomarkers for diagnosing and prognosting for some diseases such as neuroendocrine tumors (Portela-Gomes et al., 2010), hypertension, neurodegenerative disease (e.g., Alzheimer's disease) and neuropsychiatric disease (e.g., schizophrenia) (Bartolomucci et al., 2011). For instance, SgII has been detected in different types of endocrine tumors (e.g., pituitary adenoma, pheochromocytoma, ganglioneuroma, neuroblastoma, medullary thyroid carcinoma) (Conlon, 2010).

1.1.1 Secretogranin-II (SgII)

Secretogranin II, also known as chromogranin C, is a well studied member of the granin family, originally isolated and characterized in the bovine anterior pituitary (Rosa & Zanini, 1981) and the rat PC12 cell line (Helle, 2010). Secretogranin II is approximately 600 amino acids long (~67 kDa molecular weight) acidic, tyrosine-sulfated protein processed to small peptides in the secretory vesicles of neurons or may participate in the sorting and packaging of hormones and neuropeptides into secretory granules and dense-core vesicle (Miyazaki et al., 2011). The primary sequence of mammalian SgII reveals a 79–87% identity between species, however, the SgII sequences of some non-mammalian vertebrates has a low degree of conservation compared to the mammalian SgII (Montero-Hadjadje et al., 2008). In comparison to CgA and CgB, SgII has a low amino acid sequence similarity (e.g., overall 44% identity between goldfish SgII and other known SgII molecules) between mammalian and non-mammalian vertebrate only the short domain in the middle of SgII sequence called SN shows a relatively high similarity (59% identity between goldfish SN and human SN) among different vertebrate species (Zhao et al., 2009b). Secretogranin II is expressed in adrenochromaffin cells and high amounts of SgII are found in specific adrenal disorders such as ganglioneuromas in human (Anouar et al., 1998). It is also expressed in brain in the hypothalamus and pituitary of the frog *Rana ridibunda* (Montero-Hadjadje et al.,

2008). The SgII gene (SCG2), located on human chromosome 2q35-2q36 comprises two exons and encodes a transcript of 2476 bp (Bartolomucci et al., 2011). The SgII precursor protein is proteolytically processed to small peptides by cleavage at specific pairs of basic amino acids (Lys–Arg or Arg–Arg) by prohormone convertases (PCs) in particular PC1 and PC2 have the ability to cleave the SgII precursor to generate bioactive peptides (Hoflehner et al., 1995). Three peptides have been characterized in mammalian SgII, which are SN, EM66, and the 40 amino acid peptide (Fischer-Colbrie et al., 1995). Secretoneurin is considered as the best-characterized peptide derived from SgII precursor. Secretoneurin length depends on the vertebrate species in which is found and ranges from 31 to 43 amino acids (Trudeau et al., 2012) and is moderately conserved in evolution. About 59% of goldfish SN sequence is identical to human SN and the similarity to other vertebrate is about 75% (Blazquez et al., 1998; Trudeau et al., 2012; Zhao et al., 2009b). The EM66 peptide has been identified in adult and fetal human adrenal gland (Anouar et al., 1998). EM66 peptide is well conserved in tetrapods, human and frogs that exhibits 68– 70% sequence identity whereas it is not conserved in fish (Blazquez et al., 1998 ; Anouar et al., 1998). Detailed description for SN and EM66 will be discussed later in this chapter.

The goldfish *(Carassius auratus)* was the first teleost species for which SgII cDNA was cloned and sequenced (Blazquez et al., 1998). In goldfish, two different SgII mRNA of approximately 2975 bp and 2650 bp were detected in the pituitary gland (Blazquez et al., 1998). The existence of two SgII isoforms in several teleost were demonstrated by gene cloning and genome researches and have been named SgIIa and SgIIb by (Zhao et al., 2010b). The origins of the two type of SgII in teleost emerged because of gene duplication process. Both SgIIa and SgIIb are found in some species, for example the known goldfish SgII belongs to the SgIIa family whereas the known pufferfish and grass carp SgIIs belong to the SgIIb family. However, zebrafish expresses the both subtypes of SgII. Also, the peptides derived from both types of SgIIs are named "a" or "b" to correspond to the precursor protein (Zhao et al., 2010b). So far nothing is known about the roles and functions of SgIIb. The SgIIa mRNA was detected in different goldfish tissues using reverse transcriptase polymerase chain reaction RT-PCR including pituitary gland, hypothalamus, telencephalon, cerebellum, ovary and interrenal in different amounts (Zhao et al., 2006a). Moreover, SgII mRNA observed in the central and medial amygdaloid nuclei in fetal rat brain (Saria et al., 1997). Secretogranin II-immunoreactivity (SgII-IR) was detected widely in endocrine tumors (e.g., pituitary

adenoma, pheochromocytoma, ganglioneuroma, neuroblastoma, medullary thyroid carcinoma) (Conlon, 2010 ; Fischer-Colbrie et al., 1995). In adult rat brain, the distribution of SgII protein is somehow similar to SgII mRNA distribution, they are both found to be expressed in the olfactory bulb, hypothalamus, dentate gyrus and inferior colliculus, hippocampus and cerebellum (Miyazaki et al., 2011). In the rat anterior pituitary, it was demonstrated that CgB and SgII are co-localized with prolactin in the secretory granules of mammotrophs (Ozawa & Takata, 1995). In human pituitary, SgII found to be localized with gonadotrophs, thyrotrophs, and corticotrophs (Vallet et al., 1997). Secretogranin II-immunoreactivity (SgII-IR) was detected in the secretory granules co-localized with luteinizing hormone (LH) in bovine gonadotrophs, indicating the co-packaging of granins and gonadotropins to form secretory granules (Zhao et al., 2009b). There are some factors that stimulate SgII production and processing, for example, treatment with the gamma aminobutyric acid (GABA) metabolism inhibitor γ-vinyl-GABA up-regulates SgII mRNA in the goldfish pituitary (Blazquez et al., 1998). Also, treatment with gonadotropin-releasing hormone (GnRH) *in vivo* increases SgII mRNA expression in goldfish pituitary (Samia et al., 2004). In addition, treatment with mouse GnRH-A up-regulated SgII mRNA level in mouse LβT2 cells *in vitro* after 6-h static incubation (Zhao et al., 2011).

1.1.2 Secretoneurin

Secretoneurin is a functional peptide derived by endoproteolysis of the SgII precursor (Zhao et al., 2009b). The prohormone convertases (PCs) are a group of endoproteolytic enzymes that have the ability to cleave the SgII precursor at dibasic amino acid pairs to generate the SN peptide (Hoflehner et al., 1995). From the PCs family members (PC1/PC3, PC2, PACE 4, PC4, PC5/PC6 and furin/PACE), PC1 and PC2, are co-localized with SN in the bovine posterior pituitary secretory vesicles (Hoflehner et al., 1995). Secretoneurin exists from lower vertebrate sea lamprey to higher vertebrate human and its size ranges from 31 to 43 amino acids. In mammals, birds, frogs and tetrapods, SN is 33 amino acids long; in goldfish and zebrafish the SNa peptide is a 34 amino acid long (Blazquez et al., 1998; Zhao et al., 2009b); in zebrafish SNb is a 31 amino acids long; in lamprey and shark, SN is 43 and 41 amino acids long, respectively (Trudeau et al., 2012). Secretoneurin is moderately conserved in evolution (Trudeau et al., 2012). It is highly conserved in mammalian species, such as human, pig, hamster and rat (Fischer-Colbrie et al., 1995). The full 33 aminao acid sequence in

human is TNEIVEEQYTPQSLATLESVFQELGKLTGPNNQ (Trudeau et al., 2012). On the other hand, SN is somewhat conserved in some non-mammalian species such as amphibian. Recently, 2 forms of teleost SN has been determined arising from gene duplication named by (Zhao et al., 2010b) as SNa and SNb. In teleosts, the two segments ''QYTP" and ''LATLEQSVFQEL" that are located in the middle of SNa peptide are similar to mammalian SN. In contrast, two stretches in the middle of teleost SNb ''EQYTPQSLA'' and ''FE(Q)ELG'' are moderately conserved (Trudeau et al., 2012). This YTPQ-X-LA-X7-EL signature is identical in all known SNs sequences, including lamprey to human forms (Trudeau et al., 2012).

1.1.3 Distribution and multiple functions of secretoneurin

Secretoneurin is distributed in the central nervous system and peripheral tissues in different species. In mammals, SN has been detected in various regions of rat brain at different levels. The highest level of SN-IR was detected in the hypothalamus, particularly, in the median eminence. Secretoneurin-immunoreactive fibers were found in several brain areas, for example, the lateral septum, the medial parts of the amygdala, some medial thalamic nuclei, hypothalamus, habenula, nucleus interpeduncularis, locus coeruleus, nucleus tractus solitarii, the substantiae gelatinosae of the caudal trigeminal nucleus and of the spinal cord (Marksteiner et al., 1993). In fetal rat brain SN-IR presents in the anterior hypothalamus, medial portion of nucleus accumbens and ventral pallidum (Saria et al., 1997). Moreover, another study conducted by Leitner et al., 1996 demonstrated SN-IR in both brain and peripheral rat tissues including pituitary, intestine, endocrine pancreas and thyroid gland with the highest amount being detected in the intestine (Leitner et al., 1996). Also, SN is found in human synovial fluid (Eder et al., 1997). In goldfish, SN-IR fibers were found in the telencephalon, ventral and lateral parts of the ventral telencephalon, periventricular preoptic nucleus, pituitary, and the ventrocaudal aspect of the nucleus of the lateral recess. It has been shown that SN-IR was detected in the magnocellular and parvocellular cells of the preoptic nucleus projecting to the neural lobe of goldfish pituitary (Canosa et al., 2011). Secretoneurin has been identified in different types of neuroendocrine tumors such as thyroid carcinomas, carcinoids of the lung, duodenum and appendix, pancreatic and rectal neuroendocrine tumors (Portela-Gomes et al., 2010). Free SN peptide (3655 Da) was identified using western blotting analysis in goldfish pituitary and brain (Zhao et al., 2009a). A study

done by Zhao et al, 2006 using the western blot method showed the existence of two SgII precursor (57 and 30 kDa) in goldfish pituitary and (20 kDa) in hypothalamus containing the SN sequence. The SgII precursor (57 kDa) was found at higher levels in the rostal pars distalis (RPD) of the the anterior pituitary, than in the neurointermediate lobe (NIL) (Zhao et al., 2006a). Also by the same researchers, immunocytochemical studies indicate that SN-IR is localized in the prolactin cells of the rostal pars distalis (RPD) in the anterior pituitary and in the fibres of the neurointermediate lobe (NIL) (Zhao et al., 2009a). Also, it has been found that SN-IR is co-localized with isotocin in goldfish preoptic area (Canosa et al., 2011).

The neuropeptide SN is involved in a wide range of biological process. Secretoneurin plays a considerable role in response to inflammation. In rats, SN promotes the chemotactic attraction of monocytes, leukocytes and eosinophils and stimulates their migration and proliferation in response to injury (Taupenot et al., 2003). Also, SN contributes to neurogenic inflammation due to its co-localization with tachykinins in sensory fibers (Fischer-Colbrie et al., 1995). Moreover, SN promotes the process of angiogenesis, vasculogenesis and arteriogenesis through stimulating the migration of vascular smooth muscle cells. This regulatory role of SN on vascular cells has been used in clinical applications for SN gene therapy in a mouse hindlimb ischemia model to improve tissue integrity and recovering period (Bartolomucci et al., 2011; Kirchmair et al., 2004; Schgoer et al., 2009). Secretoneurin stimulates LH release from goldfish pituitary both *in vivo* and *in vitro*. *In vivo*, intraperitoneal injection (IP) of SN increased LH release in goldfish treated with dopamine antagonist domperidone (Blazquez et al., 1998). Also, incubation of goldfish pituitary fragments with goldfish SN caused an increase in LH level (Zhao et al., 2006b). *In vitro*, 6 and 12 h treatments of dispersed goldfish pituitary cells with goldfish SN induced significant increases on LH release from pituitary cells and also caused an increase in pituitary gonadotropin-α (GTHα), follicle-stimulating hormone-β (FSHβ) and luteinizing hormone-β (LHβ) mRNA level. Moreover, incubation of salmon GnRH on goldfish pituitary cells caused an increment on SgII and prolactin mRNA levels in goldfish pituitary (Zhao et al., 2010a). Mouse SN induced a significant increment of LH release in *vitro* from the LβT2 mouse gonadotroph cell line. Also, incubation with mouse SN caused an increase in luteinizing hormone-β (LHβ) mRNA level. In addition, treatment of LβT2 cells with GnRH increased SgII mRNA levels and SN-IR in the culture medium (Zhao et al., 2011).

Because of the wide range of SN distribution and its neuroendocrine functions in vertebrates, it may be classified as a new hormone but since the SN receptor has not yet been identified, this designation cannot be used (Trudeau et al., 2012).

1.1.4 EM66

EM66 is a 66 amino acid peptide generated from mammalian SgII by cleavage at dibasic amino acid sites (Anouar et al., 1998). It was named EM66 because there are glutamyl (E) and methioninyl (M) residues in the N and C terminals of the sequence. EM66 was detected in adult and fetal human adrenal gland (Anouar et al., 1998), gonadotroph and lactotroph cells of the rat pituitary and in adrenergic cells of the rat adrenal medulla (Montero-Hadjadje et al., 2003). EM66 is well conserved in human and tetrapods, but is not conserved in fish (Zhao et al., 2009b). The distribution of EM66 has been studied in some mammals. In rats and jerboas, EM66 immunoreactivity was found in several hypothalamic regions, including the preoptic area, the suprachiasmatic, supraoptic, parvocellular paraventricular arcuate nuclei (pPVN) and the lateral hypothalamus, which suggests several neuroendocrine roles for EM66, such as controlling food intake process (Boutahricht et al., 2007; Boutahricht et al., 2005). In jerboa, food deprivation caused a significant increase in EM66 neurons in the arcuate nucleus as well as the parvocellular paraventricular nucleus, suggesting an effect of EM66 in controlling feeding behavior and the response to stress associated with fasting (Boutahricht et al., 2005). Double immunohistochemical labeling indicates the existence of two different EM66 neuronal populations co-localized with corticotropin releasing hormone (CRH) and thyrotropin releasing hormone (TRH) of the rat pPVN as well as in the median eminence (El Yamani et al., 2013).

1.2 Control of food intake

In all vertebrates, the regulation of food intake and body weight is a complex process influenced by many factors such as the availability of nutrients, environmental cues, physiological factors, neurotransmitters and neuropeptides (Erlanson-Albertsson, 2005). In vertebrates, the hypothalamus works as an integration center to regulate feeding behavior and also the brain stem plays an important role in feeding regulation (Matsuda, 2009; Volkoff & Peter, 2006). Food intake is controlled by interaction between satiety and hunger signals that are released from the brain, particularly the

hypothalamus, and from peripheral tissues such as the digestive tract, liver, pancreas and adipose tissues which act on feeding centers in the brain to regulate dietary intake (Volkoff et al., 2009). Central hormones that release from the brain act directly on feeding centers in the brain, whereas peripheral hormones that release from various tissues outside the brain transfer information to feeding centers either through the vagus nerve or by crossing the blood-brain barrier and acting on receptors (Volkoff et al., 2009). Early studies showed that the hypothalamus is involved in the control of food intake in both fish and mammals. In mammals, hypothalamic nuclei such as the ventromedial nucleus (VMN), dorsomedial nucleus (DMN), paraventricular nucleus (PVN), arcuate nucleus and the lateral hypothalamus are involved in the control of feeding behavior. The lateral hypothalamus area works as a 'feeding center' whereas the ventromedial nucleus (VMN) as a 'satiety center' (Matsuda et al., 2011). In fish, a number of feeding peptides have been isolated or their cDNA sequences cloned and sequenced from brain and other peripheral tissues. These include orexigenic factors: neuropeptide Y (NPY), orexins (OX), melanin concentrating hormone (MCH), ghrelin, galanin and agouti-related protein (AgRP); and anorexigenic factors: cholecystokinin (CCK), corticotropin-releasing factor (CRF), bombesin, pro-opio-melanocorticoid (POMC), cocaine-and amphetamine-regulated transcript (CART) and leptin (Fig A) (Lin et al., 2000; Volkoff et al., 2005).

A brief summary about the most important peptides that regulate appetite released from brain and gut and relevant to this study are described below.

1.2.1 Appetite stimulators

1.2.1.1 Neuropeptide Y (NPY)

Neuropeptide Y a 36-amino-acid peptide, belongs to the neuropeptide Y family that consists of neuropeptide Y (NPY), peptide YY (PYY), pancreatic polypeptide (PP), and peptide Y (PY). NPY family members bind to the family of G-protein-coupled receptors that are composed of Y1, Y2, Y4, Y5, and Y6 (Volkoff et al., 2005). The NPY Y1 and Y5 receptors are essential for NPY function (Lin et al., 2000). In mammals, NPY is involved in many physiological functions such as cardiovascular control, anxiety and sexual behavior. It is considered as the most stimulatory factor of appetite and food intake (Matsuda et al., 2011; (Volkoff et al., 2009). In mammals, NPY is present in central nervous system particularly in hypothalamus; a great density of NPY neurons

observed in the arcuate nucleus, projecting to the paraventricular nucleus which are both important areas in the regulation of feeding (Volkoff et al., 2005). In fish, immunological and chromatographic studies showed for the first time the presence of NPY in goldfish brain (Kah et al., 1989). In goldfish, NPY-immunoreactive (NPY-IR) neurons are detected in several areas in the brain including the ventromedial-posterior hypothalamus and hypothalamic inferior lobes which are involved in the control of feeding behavior in fish (Pontet et al., 1989). In goldfish brain, NPY mRNA is expressed in the nucleus entopeduncularis of the ventral telencephalon, preoptic area, olfactory bulbs, hypothalamus and optic tectum (Narnaware & Peter, 2001; Narnaware et al., 2000; Peng et al., 1994). Several studies have shown that NPY is involved in the regulation of food intake in teleost. Food deprivation of goldfish for 24 h and 72 h resulted in increased NPY mRNA levels in hypothalamus and telencephalon (Narnaware & Peter, 2001). Moreover, two weeks fasting induced a significant increase in NPY mRNA in Brazilian flounder and winter skate brain (Campos et al., 2010; MacDonald & Volkoff, 2009b; Volkoff et al., 2009). Neuropeptide Y can act as both a short and/or longterm hunger factor in fish. In both goldfish and Atlantic cod, NPY mRNA levels showed periprandial changes around mealtime (Narnaware & Peter, 2001; Volkoff et al., 2009). Central administration of mammalian or fish NPY induced an increase in food intake in various species in teleost including goldfish, trout and catfish suggesting that NPY regulates feeding behavior in fish (Volkoff et al., 2009). In goldfish brain, intracerebroventricular (ICV) administration of goldfish NPY caused an increase in food intake. In addition, central injections of mammalian or fish NPY or either Y1 or Y5 receptor agonist caused a dose-dependent increase in food intake in goldfish (Narnaware & Peter, 2001). These results indicate that NPY is an important stimulator factor of food intake and feeding behavior in fish (Volkoff et al., 2005). In fish as in mammals, NPY interacts with several appetite regulators such as leptin, orexins, CART and galanin. Brain treatment with leptin induced a decrease in NPY mRNA level in goldfish hypothalamus and telencephalon (Volkoff et al., 2003). A study demonstrated by (Volkoff & Peter, 2001b) in goldfish showed that there is a significant interaction between NPY, orexin and galanin signaling pathways in the regulation of food intake. NPY mRNA showed a significant increase in the telencephalon and hypothalamus following injection with orexin A (Volkoff & Peter, 2001b). In addition, ICV co-injection with NPY and orexin A induced an increase in food intake compared to fish treated with NPY or OX A alone (Volkoff & Peter, 2001b). In goldfish, NPY stimulates the releasing and production of growth

hormone (GH) in both in *vivo* and in *vitro* (Narnaware & Peter, 2001).

1.2.1.2 Orexins/hypocretins

Orexins (also called hypocretins) consist of two peptides, orexin A (hypocretin-1) a 33-amino-acid peptide, and orexin-B (hypocretin-2), a 28-amino- acid peptide generated by cleavage of 130 amino acid pre-pro-orexin precursor by proteolytic processing (Lin et al., 2000). Orexin peptides are produced in the lateral and posterior hypothalamus in the brain (Erlanson-Albertsson, 2005). In mammals, orexins act through two G-protein coupled receptors, the orexin 1 and orexin 2 receptors (OX 1R and OX 2R) to stimulate food intake (Volkoff et al., 2005). In mammals, orexin neuronal cell bodies have been detected in the lateral hypothalamus, which is considered as the "feeding center" whereas fibers are found in the cerebral cortex, hippocampus, limbic system and brainstem (Matsuda et al., 2011). In goldfish, orexin cell bodies were detected in different brain regions including periventricular hypothalamus, anterior tuberal nucleus and lateral hypothalamus (Huesa et al., 2005). In mammals, orexin regulates several physiological activities such as feeding, reproduction, cardiovascular function and controlling of sleep and wake cycle and this function has also detected in fish too (Volkoff, 2012; Volkoff et al., 2005). In fish, orexins appear to have a role in the regulation of food intake and locomotor behavior. In goldfish brain, 7 days fasting induced a significant increase in orexin mRNA and orexin-immunoreactive cells (orexin-IR) in the hypothalamus (Nakamachi et al., 2006). Moreover, 10 days fasting induced an increase in orexin mRNA levels in cave fish brain, and also a periprandial changes in brain orexin expression was seen 1 h prior to a scheduled meal time (Wall & Volkoff, 2013). Brain administration of human orexins A and B increased food intake (Volkoff et al., 1999). Moreover, ICV injection of orexin A in goldfish brain resulted in increasing in food intake and locomotor activity in treated fish (Nakamachi et al., 2006; Volkoff et al., 2003). In fish as in mammals, orexin interacts with a number of appetite regulators such as NPY, TRH, CART, and leptin. Co-injection with NPY and orexin A resulted in a synergistic increase in food intake compared to fish treated with NPY or orexin A alone (Volkoff & Peter, 2001b). In goldfish, co-treatment with CART and orexin A caused an inhibition in feeding caused by the effect of orexin A (Volkoff & Peter, 2000). Brain administration with Leptin inhibited the orexigenic effect of orexin A in food intake stimulation in goldfish (Volkoff et al., 2003). The mRNA levels of hypothalamic pre-pro-orexin and orexin receptors significantly increased in goldfish injected with

thyrotropin-releasing hormone (TRH) (Abbott & Volkoff, 2011).

1.2.1.3 Ghrelin

Ghrelin is a 28-amino-acid peptide generated from pre-pro-ghrelin precursor isolated first from mammalian stomach (Kang et al., 2011). Ghrelin is synthesized mainly in the stomach and also in the brain (Volkoff et al., 2009). Ghrelin mRNA presents abundantly in the stomach and intestine and in lower level in other organs such as brain (hypothalamus), pituitary, heart, lung, pancreas, kidney, and placenta (Kang et al., 2011). Ghrelin has been identified in several fish species including goldfish, tilapia, Japanese eel, rainbow trout, Atlantic cod and channel catfish (Kang et al., 2011; Volkoff et al., 2005). Ghrelin is involved in the control of feeding in fish. Fasting demonstrated a significant increase in ghrelin mRNA in goldfish gut (Unniappan et al., 2004). Also, short and long starvation induced an increase in ghrelin mRNA level in both brain and gut of zebra fish and grass carp (Amole & Unniappan, 2009; Feng et al., 2013). Both central and peripheral treatments of either goldfish or human ghrelin stimulate food intake in goldfish (Unniappan et al., 2004; Unniappan et al., 2002). Also, brain injection with goldfish ghrelin increased food consumption in goldfish (Miura et al., 2007). It has been shown that ghrelin interacts with other feeding regulators such as NPY, orexin, nesfatin-1 and MCH. ICV injection with orexin-A induced a high increase in ghrelin mRNA in goldfish brain (Miura et al., 2007). Moreover, treatment with NPY Y1 and orexin A receptor antagonist inhibited the orexigenic effect of ghrelin in stimulating food intake (Miura et al., 2006). In goldfish, ICV injection of MCH decreased ghrelin mRNA expression in the brain (Shimakura et al., 2008). Furthermore, It has been found that nesfatin-1 (a novel anorexigenic peptide generated from nucleobindin-2 precursor) is co-localized with ghrelin in the hypothalamus and enteroendocrine cells of the intestine (J-loop) in goldfish (Kerbel & Unniappan, 2012). ICV administration of goldfish nesfatin-1 suppressed the expression of preproghrelin mRNA and ghrelin receptor GHS-R 1a-1 in goldfish brain (Kerbel & Unniappan, 2012).

1.2.2 Appetite inhibitors

1.2.2.1 Cocaine-and amphetamine-regulated transcript (CART)

Cocaine-and amphetamine-regulated transcript peptide was originally isolated as an mRNA from rat brain under the effect of cocaine and amphetamine stimulation

(Douglass et al., 1995). In mammals, two forms of CART are generated from pre-pro-CART precursor; pro-CART I encoding for 102 amino acid long peptide and pro-CART II for 89 amino acid long peptide (Lin et al., 2000). Two different forms of CART has been isolated from goldfish brain which are pro-CART I a 117-amino acid, and pro-CART II a 120-amino acid (Volkoff & Peter, 2001a). Both forms are detected in central and peripheral tissues such as brain, gonads, pituitary gland, eye and kidney (Volkoff & Peter, 2001a). In mammals, CART is present in the arcuate and paraventricular nucleus in the hypothalamus, which are known to be involved in the control of feeding behavior (Volkoff & Peter, 2001a). In fish, CART mRNA sequences have been identified in goldfish, Atlantic cod, medaka, zebra fish, puffer fish and winter skate (Kehoe & Volkoff, 2007; MacDonald & Volkoff, 2009b; Murashita & Kurokawa, 2011; Volkoff & Peter, 2001a). It has been shown that CART involve in various biological functions including controlling of feeding behavior and stress response (Volkoff & Peter, 2001a). Food deprivation in goldfish, medaka and Atlantic code induced a decreases in CART mRNA level in fish brain, and also a periprandial change in CART mRNA has been seen suggesting that CART plays an important role in the regulation of food intake (Kehoe & Volkoff, 2007; Volkoff & Peter, 2001a; Murashita & Kurokawa, 2011). In channel catfish brain, CART mRNA level decreased significantly after 30 days fasting (Kobayashi et al., 2008). In goldfish, central injection of human CART caused a significant decrease in food intake (Volkoff & Peter, 2000). In fish, CART is shown to be interacting with a number of appetite regulator peptides such as TRH, NPY, orexin and leptin. In goldfish, the mRNA levels of hypothalamic CART I significantly increased when fish injected with TRH (Abbott & Volkoff, 2011). Also, brain administration of human CART reduced both NPY and orexin A mRNA levels in goldfish brain (Volkoff & Peter, 2000). Moreover, central administration of the adipocyte hormone leptin induced an increase in CART I mRNA level in hypothalamus, optic tectum, and olfactory bulbs and enriched the inhibition in food intake in goldfish (Volkoff & Peter, 2001b). In channel catfish, ICV administration of leptin induced a significant increase in CART immunoreactive neurons in the entopeduncular nucleus (Subhedar et al., 2011).

1.2.2.2 Cholecystokinin (CCK)

Cholecystokinin (CCK) is generated from a 115 amino acid pre-pro-

cholecystokinin as a CCK and gastrin peptides. The structure Trp–Met–Asp–Phe–NH2 is important for CCK and gastrin family for its biological activities. Cholecystokinin is synthesized mainly in the endocrine cells of gastrointestinal tract (GI) and also present in central tissues (Lin et al., 2000). Cholecystokinin has several forms; the sulfated cholecystokinin (CCK-8) is the most active form. Cholecystokinin binds to two receptor subtypes, CCK-1 in the gastrointestinal tract and CCK-2 in the brain (Sam et al., 2012). Cholecystokinin has been isolated in several fish species including goldfish, rainbow trout, yellowtail, winter skate, winter flounder and grass carp (Feng et al., 2013; MacDonald & Volkoff, 2009a, 2009b; Peyon et al., 1998). In fish as in mammals, CCK is released when food is in the stomach suggesting that CCK is controlling digestion and feeding functions such as enzymatic secretion and gall bladder contraction (Himick & Peter, 1994). In goldfish, CCK mRNA is expressed in the brain including hypothalamus and peripheral tissues such as pituitary, ovary, kidney, gill and gastrointestinal tract (Peyon et al, 1998). Cholecystokinin/gastrin-immunoreactive fibers found to be distributed in the forebrain, midbrain and hindbrain of goldfish, in the somatotrophs and gonadotrophs of the anterior pituitary and endocrine cells of the anterior gut (Himick & Peter, 1994; Peyon et al, 1998). It has ben reported that CCK is involving in feeding regulation in fish, fasting induced a down-regulation in gut CCK mRNA level in different fish species including goldfish, winter skate, cunner, grass carp and white sea bream (Babichuk & Volkoff, 2013; Feng et al., 2013; MacDonald & Volkoff, 2009b; Micale et al., 2012). Moreover, CCK mRNA levels showed a postprandial increase after scheduled mealtime in goldfish brain (Peyon et al., 1999). In goldfish, both central and peripheral treatment of CCK-8S decreased food intake (Himick & Peter, 1994). Moreover, IP treatment with CCK induced a decrease in food intake in cavefish (Penney & Volkoff, 2014). Cholecystokinin interacts with other feeding factors such as growth hormone (GH), nesfatin-1, amylin, and leptin. Cholecystokinin stimulates the secretion and release of growth hormone that both IP and ICV administration of CCK in goldfish caused an increase in serum GH levels (Canosa & Peter, 2004; Himick & Peter, 1994). In addition, ICV administration of goldfish nesfatin-1 inhibited food intake and the expression of CCK mRNA (Kerbel & Unniappan, 2012). Fish co-treated ICV with amylin and CCK-8 had a lower food intake than fish treated with either CCK-8 or amylin alone indicating the inhibitory role of CCK in food intake (Thavanathan & Volkoff, 2006). An increase in CCK expression level has been detected in goldfish hypothalamus

after brain administration of leptin (Volkoff et al., 2003).

1.3 The correlation between food intake and reproduction

There is a possible link between the two peptides derived from the SgII precursor, secretoneurin and EM66 and food intake. It has been established that SN has paracrine and neuroendocrine roles to regulate luteinizing hormone (LH) release from goldfish and mouse pituitary, indicating its role in reproduction (Zhao et al., 2006b; Zhao et al., 2010a; Zhao et al., 2011). Also, goldfish SN-IR and free SN peptide have been detected in the brain and pituitary, indicating that SN is produced in magnocellular cells of preoptic nucleus and in pituitary lactotrophs (prolactin cells) in the RPD (Zhao et al., 2010a). The same researchers also found that SgII mRNA presents in pituitary, brain tissue including hypothalamus, telencephalon and cerebellum whereas, SN-IR was only detected in pituitary, telencephalon and hypothalamus (Zhao et al., 2006a). Thus, hypothalamus is a common area that presents SN in mammals and teleost and also an important area to control food intake since it has the hunger and satiety centers (Volkoff et al., 2009). In mammals, SN has been detected in various regions of rat brain with the highest level of SN-IR detected in the hypothalamus, particularly, in the lateral hypothalamus and median eminence. SN-IR fibers were found in several brain areas, for example, the lateral septum, the medial parts of the amygdala, some medial thalamic nuclei, the hypothalamus, habenula, nucleus interpeduncularis, locus coeruleus, nucleus tractus solitarii, the substantiae gelatinosae of the caudal trigeminal nucleus and of the spinal cord (Marksteiner et al., 1993). Moreover, *in situ* hybridization and immunohistochemistry studies reveled that SgII is expressed in mouse hypothalamus specifically lateral hypothalamus, paraventricular and arcute nucleases (Hotta et al., 2009). In goldfish, SN-IR fibers were found in the dorsal part of the dorsal telencephalon, ventral and lateral parts of the ventral telencephalon, periventricular preoptic nucleus, pituitary, and the ventrocaudal aspect of the nucleus of the lateral recess (Canosa et al., 2011). The distribution of SN in various hypothalamic areas that controls both feeding and reproduction gives support to my hypothesis that SNa can regulate feeding in addition to reproduction. Also, Boutahricht et al., 2005 speculated that the peptide EM66, also generated from SgII precursor processing, has a role related to feeding in mammals (Boutahricht et al., 2005). Food deprivation caused a significant increment in EM66-IR neurons in the arcuate nucleus and parvocellular paraventricular nucleus in the jerboa, a

desert rodent (Boutahricht et al., 2005). In rats and jerboas, EM66 IR is found in numerous hypothalamic regions, including the preoptic area, the suprachiasmatic, supraoptic, parvocellular paraventricular and arcuate nuclei, and the lateral hypothalamus which are hypothalamic regions that control feeding in mammals (Boutahricht et al., 2005; Boutahricht et al., 2007). However, there have been no reports directly testing the hypothesis that EM66 might control feeding, likely because the EM66 peptide within the SgII precursor is not well-conserved in evolution (Zhao et al., 2009b), which significantly reduces the potential importance of this peptide. In addition, SN-IR was detected in several goldfish brain nuclei that also produce some feeding neuropeptides such as neuropeptide Y (NPY), melanocyte-stimulating hormone (MSH), melanin-concentrating hormone (MCH), and orexin (OX) (Canosa et al., 2011). There is only a study to date has shown that ICV injection of SNa increased food intake and locomotor behavior in goldfish (Trudeau et al., 2012). In mammals, 24 h fasting increased SgII mRNA level in mice hypothalamus (Hotta et al., 2009). Moreover, it has been shown that SgII binds to SgIII to form secretory vesicles containing several feeding neuropeptides such as NPY, OX and POMC using 293 human embryonic kidney cell line (Hotta et al., 2009). There is also increasing evidence for cross-talk and differential roles of traditional reproductive and traditional feeding peptides to be involved in both processes (Volkoff et al., 2009). An interaction between orexin and gonadotropin-releasing hormone (GnRH) has been shown in goldfish (Volkoff et al., 2010). Central administration of orexin stimulated food intake and blocked spawning behavior, and therefore decreased GnRH mRNA levels in the brain (Hoskins et al., 2008). In contrast, ICV injection of chicken GnRH II induced a decrease in food consumption (Matsuda, 2009).

From the above information, we suspected that there is a close link between SgIIa and its derived peptide SNa and food intake. This led us to investigate if SgIIa and SNa are related to feeding processes in the goldfish. Therefore, based on this data and information, we hypothesize that SNa is regulated by nutritional signals and works as orexigenic factor to stimulate food intake in female goldfish. Two main objectives are our targets of investigation in this thesis. The first objective was to determine the effects of fasting at different time point and periprandial changes on gene expression of SgII-a and other feeding peptides in tissues that are known to be involved in feeding regulation such as hypothalamus, telencephalon and gut (Chapter 2). The second objective was to determine the effects of SNa brain injection on food intake, locomotor behavior and expression of some feeding neuropeptides such as NPY, OX, CCK-8 and CARTI in

hypothalamus and telencephalon (Chapter 3).

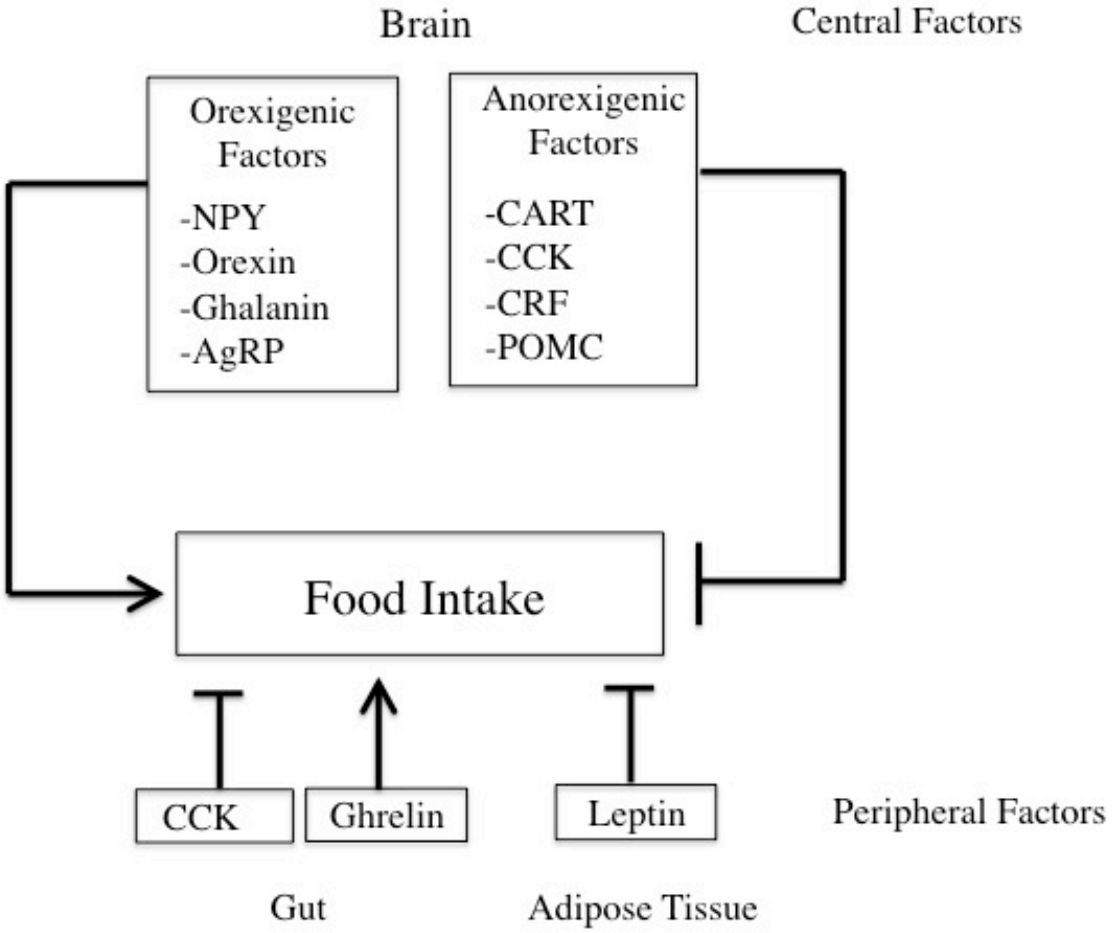

Fig A. A proposed model showing the central and peripheral factors that are known to regulate food intake in fish. The central factors in preoptic and hypothalamic areas are neuropeptide Y (NPY), orexin, ghalanin, agoti related peptide (AgRP), cocaine and amphetamine regulated transcript (CART), cholecystokinin (CCK), corticotropin releasing factor (CRF), and pro-opiomelanocortin (POMC). The peripheral factors are cholecystokinin (CCK), ghrelin and leptin.

CHAPTER 2

The effect of fasting on the expression of SgIIa and feeding peptides

Rational and objectives

A new potential role of SgII-a in feeding processes in the female goldfish is examined in this chapter. The main objective of the research described in this chapter is to determine the effect of fasting at different periods of time at 26 h (acute), 3 days (short), 7 days (medium) and 14 days (long) on the expression of SgII-a and other feeding neuropeptides such as neuropeptide Y (NPY), cholcystokinin (CCK-8) and ghrelin. Additionally, periprandial changes in these genes were examined 3 h before meal time, at meal time and 3 h after meal time in both fed and non fed fish. Three major tissues that are involved in feeding regulation that were examined are hypothalamus, telencephalon and gut.

2. Materials and methods

2.1 Experimental animals

For all experiments female goldfish (*Carassius auratus*) were used as an experimental model. Fish were ranging from 20 to 30 g in weight and purchased from a commercial supplier Aleong's Internation Inc. (Mississauga, ON, Canada). Fish were acclimated in the University of Ottawa Aquatic Care Facility. All tanks received dechloraminated City of Ottawa tap water at 18◦C and fish were held under a natural simulated photoperiod. In all fasting experiments, fish were fed a 1% wet body weight (BW) ration once a day at a regularly scheduled time, with commercially prepared Martin Proficient Classic Floating Trout Grower 3 mm pellets (Martin Mills Inc., Travistock, ON, Canada) containing 44% crude protein, 0.45% sodium, 10% crude fat, 3.5% crude fiber, 0.9% calcium, 0.97% phosphorous, 6800 IU/kg Vitamin A, 2100 IU/kg Vitamin D3, 320 IU/kg Vitamin E. Fish were anaesthetized with 3-aminobenzoic acid ethylester (MS-222; Syndel Laboratories, Vancouver, BC, Canada) for dissection procedures then euthanized with a spinal incision. All experiments were approved by the University of Ottawa Protocol Review Committee and followed standard Canadian Council on Animal Care guidelines on the use of animals in research.

2.2 Experimental design

2.2.1 Distribution of SgIIa mRNA in female goldfish tissues

SgIIa mRNA levels were studied in female goldfish in order to determine tissue distribution and levels of expression. Tissue sampling was done in January 2012. Thirteen tissues were collected from female goldfish (n=6). These were hypothalamus, telencephalon, pituitary gland, eye, gill, heart, liver, spleen, gall bladder, gut, interrenal, ovary and muscle. Tissues were rapidly dissected and frozen on dry ice. All tissues were stored at -80 °C, for later for in RNA isolation, reverse transcriptase RT-PCR and quantitative Q-PCR.

2.2.2 The effects of acute fasting on gene expression

The experiment was conducted in May 2012 (fish were in spawning condition). The experimental group was exposed to acute fasting for 26 h. Prior to the experiment, two groups of weight-matched (ranging from 20 to 30 g) female goldfish were distributed into 2 70-L tanks (10 fish per tank). Fish were acclimatied to the previous conditions (see section 2.1) before starting the experiment. All groups were trained for a daily scheduled feeding to be fed (1% BW ration) once a day at (10:00h) for approximately 2 weeks before starting the experiment. On the day of the experiment, a control (fed) group was fed at the feeding time (10:00h) then 2 h post feeding time the sampling was done, the second group was not fed at the feeding time and then sampled 2 h later (26 h food deprived).

2.2.3 The effect of short, medium and long term fasting on gene expression

The experiment was conducted in July 2011 (fish was in gonadal regression stage). Prior the experiment fish were distributed into six tanks (24 fish per tank). Fish were acclimatized to the previous conditions (section 2.1) before starting the experiment. All groups were trained for a daily scheduled feeding to be fed (1% BW ration) once a day at (10:00h) for approximately 2 weeks before starting the experiment. One experimental group exposed to short term fasting for 3 days with a control group being fed at feeding time. The other experimental group was fasted for 7 days (medium fasting) with a control group being fed. The last experimental group was fasting for 14 days (long-term fasting) with a control group being fed at the scheduled feeding time. All the six groups were killed at the end of food deprivation periods (3, 7 and 14 days). For gene expression studies, tissue samples from 3 fish for hypothalamus, telencephalon and gut were pooled together to ensure sufficient yield of RNA (n=6).

2.2.4 Periprandial changes in gene expression

The experiment was conducted in October 2012 (fish were in gonadal regression stage). Four groups of weight-matched female goldfish (n=10/group) were acclimated for 2 weeks to tank conditions (section 2.1) and fed daily at a scheduled time (12:00) a 1% BW ration for 2 weeks. On experimental day, a group of fish was killed 3 hours before the scheduled feeding time (3 h prior to feeding (-3h), upon commencement of feeding time (0 h), unfed group was killed 3 h after feeding time (+3 h) and a fed group was killed 3h after the scheduled feeding time. For all fasting experiments, hypothalamus, telencephalon and gut samples were collected and stored at -80°C for later use in RNA isolation and quantitative Q-PCR. For gene expression studies, SgIIa mRNA, NPY mRNA, CCK-8 mRNA and preproghrelin mRNA were measured using Q-PCR.

2.3 RNA Isolation

For tissue distribution study, total RNA from brain and body tissues (n=6) were isolated using Qiagen RNeasy Micro Kit for hypothalamus, telencenphalon, pituitary gland, Qiagen RNeasy Plus Mini kit for liver, gill, spleen, ovary, interenal, gut and combined protocol of TRIzol (Invetrogen) and Qiagen RNeasy Mini kit for eye, heart and TRIzol reagent (Invetrogen) for muscle as described in the manufacturer's protocol. For fasting study, total RNA was isolated using Qiagen RNeasy Micro Kit for hypothalamus, Telencenphalon and Qiagen RNeasy Plus Mini kit for gut as described in the manufacturer's protocol (Qiagen, Mississauga, ON, Canada). In all cases, an RNase-free DNase treatment was performed during the extractions to remove genomic DNA. Samples were homogenized using the stainless steel beads before using the RNeasy kit. The isolated RNA was re-suspended in RNase-free water. Total RNA quantity was measured and the ratio of absorbance at 260/ 280 nm and the ratio of absorbance at 260/230 nm were determined using a Spectrophotometer (NanoDrop-1000, Technologies, Inc.). Only RNA with a 260/280 ratio of >1.9 was used for cDNA synthesis. Samples were stored at -80 °C for further analysis.

2.4 Complementary DNA (cDNA) synthesis

Complementary DNA (cDNA) was obtained from 1-5µg of total RNA using 200 ng random hexamer primers (Invitrogen) and superscript II reverse transcriptase (Invitrogen). Samples were stored at -20°C to further use for Q-PCR.

2.5 Reverse transcriptase RT-PCR for tissue distribution study

The synthesized cDNA was used to amplify the desired gene SgIIa and the loading control β-actin using Taq DNA polymerase (Invitrogen) and the primers (Table A, appendix II). The PCR cycling conditions were: 2 min at 95°C; 40 cycles of 45 s at 95°C, 30 s at 56°C, 45 s at 72°C and a final extension of 10 min at 72°C. PCR products were separated on 1% agarose gel electrophoresis stained with (2.5 µl) ethidium bromide and visualized using the Alpha Innotech System (Alpha Imager EC., Inc). No other bands were observed.

2.6 Gene expression analysis by Q-PCR

For tissue distribution study, quantification using quantitative Q-PCR was conducted using gene-specific primers for SgIIa and ribosomal 18S primers served as internal control to compare mRNA quantity in all tissues. For fasting studies, the expression of SgIIa mRNA, NPY mRNA, CCK-8 mRNA, preproghrelin mRNA, ribosomal 18S and β-actin was measured using Q-PCR. In all studies, Q-PCR were performed using SYBR Green I chemistry. Each sample was run in duplicate in optically clear 96-well plates with a final volume of 25 µl. Each PCR reaction contained 1-5µg of RNA transformed to cDNA, 2.5 to 3.5 µM (optimized for each gene) MgCl2 (Qiagen), 1X Q-PCR buffer (Qiagen), 0.20 µM dNTPs (Invitrogen), 0.10 µM ROX reference dye (Stratagene), 0.25 X SYBR Green I Dye (Molecular Probes) and 1.25 U HotStarTaq (Qiagen). The optimized concentration for each primer (Table A, Appendix II) was added to the reaction. The cycling parameters were: enzyme activation step at 95 ° C for 10 minutes followed by 40 cycles of 95 °C denaturation step for 30 s, an annealing temperature of (58-63)°C for 1 min and an extension temperature of 72°C for 1 min and finally, a temperature-determining dissociation step was performed at the end of the amplification phase to ensure the presence of a single amplified product. Standard curves for each gene and tissue were generated using serial dilutions (six 5-fold dilution of cDNA mix from all samples starting at 1:10 cDNA stock). The standard curve was performed for each plate. For all genes, assays were performed using Mx3000P system (Stratagene, La Jolla, CA). For each gene, the threshold was calculated using the MxPro-Mx3000P v4.10 software (Stratagene, 2007). Efficiency of relative standard curve of the amplification was 100 ± 10 %, a slope between –3.1 to -3.6 and an $r^2 > 0.985$ according to the manufacturer's instructions.

2.7 Statistical analysis

For fasting studies, statistics were performed using SPSS 21 software (SPSS Inc., Chicago, IL, USA). Data were tested for normality (Shapiro-Wilk's test) and homogeneity of variance (Levene's test). Transformation was made for some groups that were not normally distributed to meet parametric assumptions. In order to compare the groups (fed-fasted) to each other for acute, short, medium and long fasting, statistical significance was tested using independent samples T-test to evaluate the significant differences between groups. For the periprandial study, statistical significance was tested with one-way analysis of variance (ANOVA) followed by Dunnett 2-sided t-test to evaluate significant differences between each group against the control group (0 h). Statistical significance for the test was set at $p < 0.05$. Data is expressed as mean ± standard error of the mean (SEM).

For fasting studies, we attempted to use ribosomal 18S and β-actin as reference genes, however, both were significantly changed by fasting. For this reason, mRNA levels were normalized using a data driven normalization algorithm (NORMA-Gene) method (Heckmann et al., 2011), which we have validated previously for studies where these so-called housekeeping genes are dramatically changing (e,g., tadpole development; Lanctôt et al., 2013). The method requires that normalization is performed using five genes for each tissue. For hypothalamus and telencephalon SgIIa, NPY, CCK-8, β-actin and 18S were assessed, and for gut SgIIa, CCK-8, preproghrelin, β-actin and 18S were assessed. The algorithm estimates a normalization factor by calculating mean expression values for each replicate for all genes. For each sample, duplicate data is obtained and averaged prior to normalization, then the fold change in normalized expression relative to the control was calculated. Biological replicates were averaged to obtain mean fold change gene expression ± standard error of mean (SEM). This procedure was run in Excel (Microsoft Inc.) according to author instructions (Heckmann et al., 2011).

2.8 Results

2.8.1 Distribution of SgIIa mRNA in female goldfish tissues

A fragment of (123bp) from SgIIa was amplified from each tissue using reverse transcriptase RT-PCR. SgIIa mRNA is detected in all central and peripheral tissues examined (Fig. 1A). SgIIa mRNA has highest levels in pituitary gland followed by brain tissues (hypothalamus and telencephalon) then peripheral tissues (spleen, eye, interrenal,

gills, gut, gall bladder, heart, liver, ovary and muscles) (Fig. 1B). The levels of 18S are shown for comparison, and because they were expectedly variable (as much as 3 times) between the diverse tissues. Therefore, we did not normalize SgIIa mRNA levels in the tissue distribution study, and the data are expressed per 1 μg total RNA used for cDNA synthesis.

2.8.2 The effect of acute fasting (26h) on gene expression

In hypothalamus, SgIIa mRNA was not affected by 26 h fasting whereas a 1.25 fold increase in SgIIa mRNA level was noted in the fasted fish in the telencephalon (p=0.000). A 1.5 fold increase was detected in SgIIa mRNA in gut (p=0.013) following fasting (Fig. 2.1). There was no significant effect of fasting on NPY mRNA levels in either hypothalamus or telencephalon (Fig. 2.1). In hypothalamus and telencephalon, fasting did not affect CCK-8 mRNA levels while a significant increase of ~5 fold has been detected in the fasted group in gut (p=0.000) (Fig. 2.1). Preproghrelin transcript expression was significantly increased (1.5 fold increase) in the gut of fasted fish (p=0.000) (Fig. 2.1).

2.8.3 The effect of short, medium and long fasting on gene expression

Fasting for 3 days (short term fasting period) induced a significant 1.25 fold increase in SgIIa mRNA levels in telencephalon (p=0.016) but not in the hypothalamus or gut. In marked contrast, fasting for seven days (medium term fasting period) caused a significant decrease of about 50% in SgIIa mRNA in hypothalamus (p=0.009) but not in the telencephalon or gut. Long term fasting for 14 days had no effect on SgIIa mRNA in hypothalamus, telencephalon or gut (Fig. 2.2 A). Short and medium fasting had no effect on either NPY mRNA levels in hypothalamus or telencephalon, whereas long term fasting for 14 days induced a significant increase about 2.5 fold in hypothalamus NPY mRNA (p=0.041) but not in telencephalon (Fig. 2.2 B). Short term fasting resulted in a significant decrease on CCK-8 mRNA 0.25% in hypothalamus (p=0.010) but not in the telencephalon or gut while there was no effect of either medium or long term fasting on hypothalamus, telencephalon or gut on CCK-8 mRNA (Fig. 2.2 C). Preproghrelin expression did not change in the short, medium or long-term fasting period in the gut (Fig. 2.2 D).

2.8.4 The effect of periprandial changes on gene expression

We were also interested in the possibility that rapid periprandial changes in gene expression may reveal involvement of SgIIa in the feeding response. In this experiment, 3 h after feeding time in the non-fed group is equivalent to the 27 h fasted group, whereas 3 h after meal time in fed fish is equivalent to the control group that fed at 27 h. In the telencephalon, SgIIa mRNA levels were increased 1.25 fold at 3 h after the scheduled feeding time in unfed group (p=0.014) compared with scheduled feeding time (0 h), with no significant differences between 21 h (3 h before scheduled time) or 27 h (3 h after feeding time in fed group) and 0 h. No differences were observed in the hypothalamus or gut SgIIa mRNA levels (Fig. 2.3). There were no significant differences between groups in both hypothalamus and telencephalon NPY mRNA levels (Fig. 2.3). In telencephalon, CCK-8 mRNA exhibited a significant ~ 1.25 fold increase at 27 h (3h after feeding time in non-fed group) (p=0.016) compared to the scheduled feeding time 0 h whereas no differences were detected between other groups. In gut, a significant decrease of 50% in CCK-8 mRNA 3 h after feeding time in fed group (27 h) was observed (p=0.005); no changes were detected between the other groups. No differences between groups in CCK-8 expression were detected in the hypothalamus (Fig. 2.3). In gut, no change in preproghrelin mRNA was noted for any group compared to the scheduled feeding time (Fig. 2.3).

2.9 Discussion

In order to determine if SgIIa is expressed in tissues that are involved in feeding processes, a tissue distribution study was performed in female goldfish. A previous study conducted by (Zhao et al., 2006a) showed the distribution of SgII mRNA in central and some peripheral goldfish tissues. Those RT-PCR experiments indicated that SgIIa mRNA levels were higher in the brain including hypothalamus, cerebellum and telencephalon than in internal and ovary, indicating that SgIIa mRNA levels are higher in central tissues than peripheral tissues. We expanded the study examining more tissues to investigate if the SgII mRNA is expressed in other tissues that are involved in feeding. The reverse transcriptase RT-PCR experiment showed that SgIIa mRNA is present in both central and peripheral tissues (some tissues are involved in feeding such as gut). More precise data was obtained by using Q-PCR. The highest levels of SgIIa mRNA are detected in pituitary gland followed by brain tissues (hypothalamus and telencephalon). Moderate levels were detected in spleen, eye, interrenal, gills and gut. The lowest levels

were detected in gall bladder, heart, liver, ovary and muscles. Since SgIIa is expressed differentially very high in some tissues and very low in others, the data was partitioned into three smaller graphs to more clearly show the difference between the levels. This result indicates that SgIIa mRNA is present highly in hypothalamus and telencephalon which are the two areas of the brain involved in the regulation of feeding in fish (Volkoff et al., 2005) and present moderately to lowly in the gut which is an important peripheral organ that controls feeding (Erlanson-Albertsson, 2005).

Our results show for the first time the effect of fasting on SgIIa mRNA levels in hypothalamus, telencephalon and gut. In hypothalamus, acute fasting for 26 h, short fasting for 3 days and long fasting for 14 days had no effect on SgIIa mRNA while medium fasting for 7 days induced a significant decrease in SgIIa transcript level. Also, no change was observed in SgIIa mRNA 3 h before or after scheduled feeding time. This result with the fasting result are matched together because 3 h after feeding time in unfed group is equivalent to fasting for 27 h, and fasting for 26 h did not display any change on SgIIa mRNA. In mice, a ~20% increase (~1.25-fold) was seen in SgII mRNA level in hypothalamus after 24 h fasting (Hotta et al., 2009). In our opinion, the decrease in SgIIa mRNA level in hypothalamus seems to be not because of the fasting effect. This decrease in SgIIa mRNA in hypothalamus seems to be a secondary effect that such a long fasting period might affect SgIIa producing neurons in hypothalamus that are not related to feeding. These hypothalamic could be related to reproductive control (Canosa et al., 2011; Trudeau et al., 2012), and perhaps an indication that such long fasting periods may cause a decline in reproductive function. Fasting for 26 h and 3 days induced a significant increase about 1.25 and 1.5 fold in SgIIa mRNA level in goldfish telencephalon, however, medium and long fasting had no effect on SgIIa mRNA. A 1.25 fold increase in SgIIa mRNA was seen 3 h after scheduled feeding time in the unfed group (27 h fasting) which confirmed the results for the acute fasting, as exactly the same patterns were seen. In gut, acute fasting for 26 h induced a significant 1.5 fold increase in SgIIa mRNA whereas short, medium and long fasting did not affect SgIIa transcript levels. Also, no periprandial changes in SgIIa mRNA were observed in gut. This time, 3 h after feeding time (27 h fasting) did not show any change on SgIIa mRNA, which is not matching the result for acute fasting, but same pattern was seen. This difference may be due to the effect of reproductive stages. Fish in the acute fasting study were in the spawning stage (May), whereas in the periprandial study, fish were in the gonadal regression stage (July). In the future it will be important to take seasonality into

consideration in experimental design and interpretation of results. In summary, SgIIa mRNA is affected by acute and short fasting and that was proven by the periprandial change especially in telencephalon area. The increase in SgIIa mRNA under the effect of fasting seems to be small, although changes are statistically significant, and similar in magnitude to fasting-induced changes observed for other orexigenic factors such as NPY and OX. For example, in Brazilian flounder, the effect of fasting was about 2 fold increase in NPY mRNA as determined using Q-PCR (Campos et al., 2010). In goldfish, Northern blot analysis showed a 2 and 1.5 fold increase respectively in NPY mRNA levels in hypothalamus and telencephalon after 3 days fasting (Narnaware & Peter, 2001) and a 1.5 fold increase in OX mRNA was observed in goldfish after 7 days fasting using a semiquantitative RT-PCR analysis method (Nakamachi et al., 2006).

We found that acute, short and medium fasting had no effect on NPY mRNA in hypothalamus, however, fasting for 14 day induced a significant increase in NPY mRNA level. No periprandial change was observed in hypothalamic NPY mRNA either 3 h before or after scheduled feeding time. In telencephalon, neither acute, short, medium and long fasting nor periprandial change were detected in NPY transcript levels. A previous study on goldfish using mixed-sex groups showed a periprandial increase on NPY expression level at 3 h before feeding time. However, no significant differences in NPY transcript levels between fed and unfed fish at 3 h after feeding time was observed (Narnaware et al., 2000) which agrees with our result of a change in NPY mRNA at 3 h after feeding time. Also, in Atlantic cod forebrain a decrease in NPY mRNA was seen at 2 h before feeding time (Kehoe & Volkoff, 2007). In goldfish, food deprivation for 72 h induced a significant increase in NPY mRNA level in hypothalamus and telencephalon (Narnaware & Peter, 2001; Narnaware et al., 2000) but this period of fasting did not alter NPY mRNA in our study. We found that there was no significant change in NPY mRNA at 3 h before or after meal time or after short fasting (for 3 days), which is equivalent to 72 h. This might be due to sex differences because that study (Narnaware & Peter, 2001) used a mix of both male and female while our study used only female goldfish. In Brazilian flounder, 2 weeks of fasting induced a significant increase in NPY mRNA level in the whole brain (Campos et al., 2010). Similar period of fasting induces an increase in NPY expression in telencephalon in winter skate (MacDonald & Volkoff, 2009b). Our results corroborate with these studies showing that NPY mRNA level increased after fasting for 14 days. Moreover, 3 weeks of food deprivation elevated an increase in NPY gene expression in the preoptic area of salmon (Silverstein et al., 1998). Together, these

findings suggest that a long term fasting period is needed to increase NPY expression in the teleost brain. However, in cunner, 3 weeks of fasting induces a decrease in NPY mRNA level, perhaps reflecting the different seasonal feeding strategy of this fish (Babichuk & Volkoff, 2013). It has also been reported that one week fasting did not alter NPY mRNA level in Atlantic cod (Kehoe & Volkoff, 2007), which is quite similar to our finding that 7 days of fasting (medium fasting) did not affect brain NPY mRNA in female goldfish. It has been reported that NPY is involved in food intake regulation as an appetite stimulator and central injection of NPY caused an increase in food intake in goldfish (Lopez-Patino et al., 1999; Narnaware et al., 2000). In brief, NPY mRNA seems to respond to long term fasting in goldfish, which has been observed for other fish species. Even though there are data supporting effects of acute and short fasting on NPY transcript level (Narnaware & Peter, 2001; Narnaware et al., 2000), there are numerous differences in experimental design between studies that may contribute to the variable responses. These include reproductive stage, sex of the fish, time of sampling, and diet.

In the current study, our result showed that fasting for 26 h, 7 and 14 days did not alter CCK-8 mRNA level whereas short fasting for 3 days induced a significant decrease in CCK-8 transcription level in hypothalamus. No periprandial change was observed in hypothalamus before or after meal time. In telencephalon, fasting for 26 h, 3 days, 7 days and 14 days did not induce any change on CCK-8 mRNA whereas an increase at 3 h after feeding time was observed in unfed fish. In gut, a significant increase was seen in CCK-8 mRNA after 26 h fasting while 3, 7 and 14 days fasting did not change CCK-8 transcription level. A decrease in CCK-8 mRNA at 3 h after feeding time in fed fish was noted in the gut. Similar decreases in CCK-8 mRNA in goldfish hypothalamus has been seen in other species such as winter skate after 2 week fasting (MacDonald & Volkoff, 2009b), grass carp after 15 days of fasting (Feng et al., 2013), after 3 weeks of fasting in cunner (Babichuk & Volkoff, 2013), yellowtail after 72 h fasting (Murashita et al., 2009). In cave fish and Atlantic salmon, 6 and 10 days of fasting respectively had no effect on CCK mRNA level in the brain (Murashita et al., 2009; Wall & Volkoff, 2013). Our results indicated a periprandial increase at 3 h after feeding time in unfed fish (27 h) in telencephalon. This increment has been detected at 2 h following the meal time in hypothalamus in goldfish (Peyon et al., 1999), 4 h post feeding in channel catfish brain (Peterson et al., 2012) and in yellowtail, 3 h post feeding (Murashita et al., 2006). Our finding showed that fasting for 26 h induced a significant increase in CCK-8 mRNA in the gut. This finding is not in agreement with previous studies on gut expression of CCK

because the expression of anorexigenic factors tend to decrease during fasting periods. Previous studies in some fish species showed that fasting down-regulated CCK transcript level in gut. In cunner, 7 days fasting decreased CCK mRNA (Babichuk & Volkoff, 2013); in grass carp, fasting for 2, 7 and 15 days induced a significant decrease in CCK level (Feng et al., 2013); in white sea bream 72 h of fasting decreased CCK level significantly in all intestine regions (anterior, posterior and hind gut) (Micale et al., 2012); in winter flounder, 2 weeks of fasting elevated significant decrease in CCK (MacDonald & Volkoff, 2009a). It has been shown that central and peripheral injection with CCK blocks food intake in goldfish (Himick & Peter, 1994; Thavanathan & Volkoff, 2006). However, 2 weeks of fasting increased CCK mRNA level significantly in winter skate gut. This increment was explained by possible effects of enzymatic or acidic secretion on CCK mRNA in the elasmobranch intestine (MacDonald & Volkoff, 2009b). Same reason might elucidate the increment of CCK-8 in goldfish gut beside some other reasons such as the gut part that used for the analysis (the first part of the gut was used for the analysis in the current study), the sex of the fish, feeding time, reproductive stage or due to other species-specific differences. Another reason could explain this increment that is fasting could change gut morphology, which affects CCK-8 secretion. It has been observed in teleost gastrointestinal tract the metabolic zonation phenomena and 5 days of fasting elevated a significant increase in enzyme activity in tilapia (Mommsen et al., 2003). In short, CCK-8 mRNA increased dramatically in goldfish gut following 26 h fasting. This observation was unexpected given the role of CCK as an appetite suppresser in other fish species as indicated above. It will be important to determine the effects of fasting on CCK protein production and release and to relate this to CCK mRNA levels to determine the true effect of fasting.

The result of the current study demonstrated that acute fasting for 26 h induced a significant increase in preproghrelin mRNA in female goldfish gut whereas short, medium and long fasting did not alter preproghrelin expression. A previous study on goldfish demonstrated that 7 days of starvation induced a significant increase in preproghrelin mRNA in gut (Unniappan et al., 2004). Also, in zebrafish, 3,7,14 days of food deprivation induced a significant increase in preproghrelin mRNA in brain and gut (Amole & Unniappan, 2009). In Grass carp, fasting for 2, 5, 7 and 15 days significantly increased ghrelin mRNA level in both brain and gut (Feng et al., 2013). In Atlantic salmon, 2 days of fasting induced a significant increase in ghrelin plasma level in the stomach whereas 14 days fasting did not cause any effect (Hevroy et al., 2011). However,

one day fasting did not alter stomach ghrelin expression in Schizothorax davidi (Zhou et al., 2014). Fasting for 4, 8 and 4 weeks in Tilapia and 4 weeks in Atlantic cod did not alter ghrelin mRNA level in the stomach (Fox et al., 2009; Xu & Volkoff, 2009). Those results suggest that the physiological role of ghrelin in feeding process is variable from species to species because some of them are responding to a short term fasting while the other to the long term starvation. The role of ghrelin in controlling food intake through increasing the food consumption has been demonstrated by the ICV and IP injections in goldfish (Matsuda et al., 2006; Miura et al., 2007). Also our result showed that no periprandial change was seen in preproghrelin mRNA before or after meal time in the gut. This result is not in agreement with a previous result conducted on goldfish showed a significantly postprandial decrease 3 h post feeding in both fed and unfed fish (Unniappan et al., 2004). That might be due to sex difference because our model was only female whereas Unniappan et al., 2004 used mixed-sex groups. Also, the sampling time could be an issue of this difference, a circadian variation in feeding may exist because we sampled at 12:00 h while in the Unniappan et al., 2004 study the sampling was done at 15:00 h. Shortly, preproghrelin mRNA expression seems to respond to both short and long term starvation and its role in stimulating appetite tend to be species-specific regulation.

In summary, our results show for the first time an effect of fasting on SgIIa mRNA in hypothalamus, telencephalon and gut in female goldfish. The most responsive tissue to fasting was the telencephalon. The response of SgIIa mRNA to acute and short term fasting in telencephalon, and to the acute fasting in gut suggests that SgIIa or derived peptides could somehow work as a short term hunger factor. These observations are in partial agreement with the original hypothesis that fasting would up-regulate SgIIa mRNA level in hypothalamus, telencephalon and gut. It has been known that SgII plays an important role in the regulation of secretory vesicles biogenesis (Courel et al., 2010) and is also known to be a good marker for endocrine secretory vesicles (Helle, 2004). The changes in SgIIa may also be indicative of other neuroendocrine responses to fasting. Thus, SgIIa or a peptide derived from SgIIa precursor protein processing might be a brain-gut hormone that can controls food intake in goldfish. Secretoneurin is known to be the most conserved peptide derived from SgII precursor (Zhao et al., 2009b). Testing the effect of SN treatment on food intake is the important next step addressed in Chapter 3.

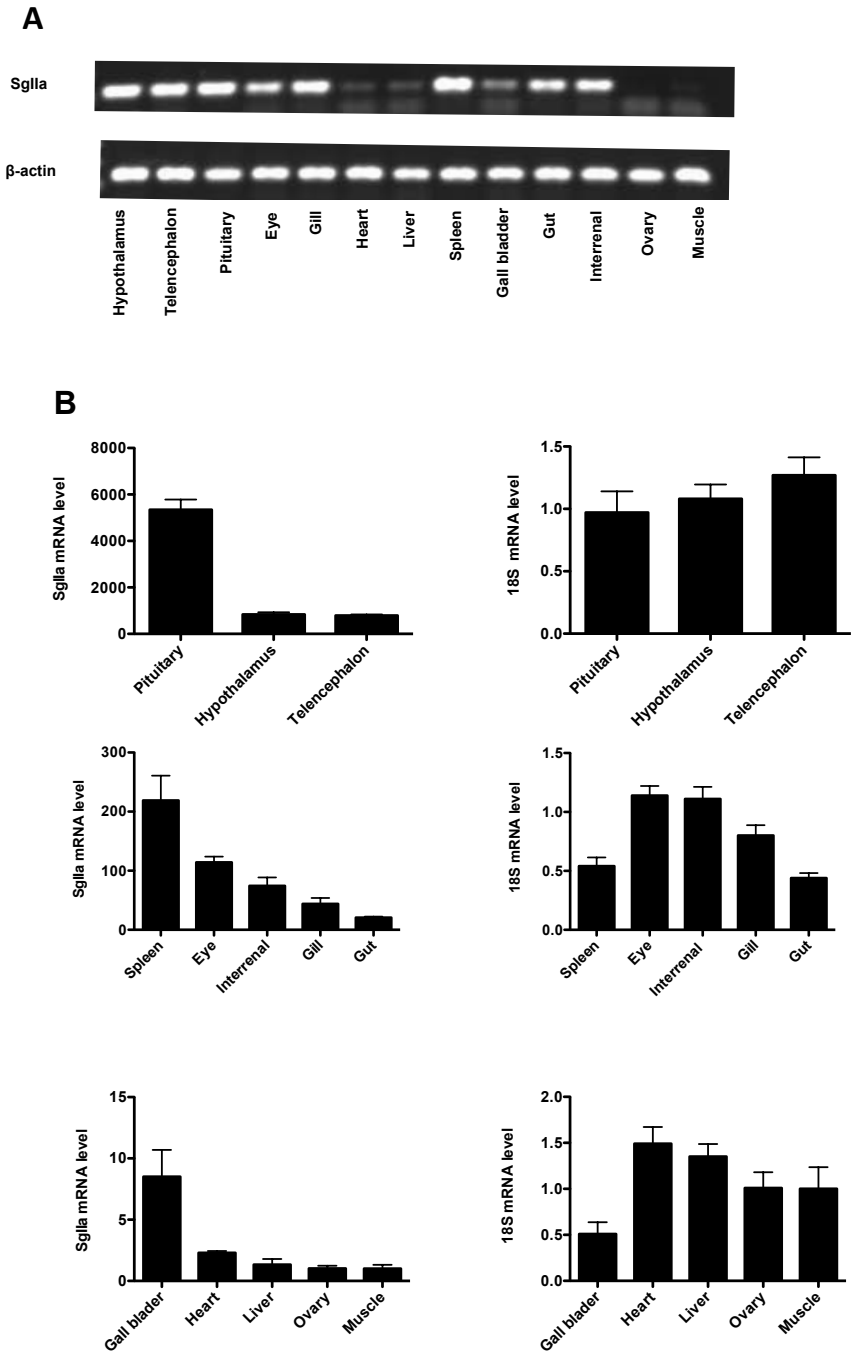

Fig 1. (A) The cDNA amplicon products by reverse transcriptase RT-PCR, SgIIa upper line and beta-actin lower line (n=6 fish). **(B)** Quantitative data was obtained by using Quantitative Q-PCR showed that SgIIa is expressed in different level pituitary > hypothalamus > telencephalon > spleen > eye > interrenal > gill > gut > gall bladder > heart > liver > ovary > muscle (n=6 fish). Right panel shows ribosomal 18S mRNA, left panel shows SgIIa mRNA. The levels of 18S are shown for comparison because they were expectedly variable (a much as 3 times) between the diverse tissues. Therefore, SgIIa mRNA is not normalized and the data are expressed per 1 μg total RNA used for cDNA synthesis.

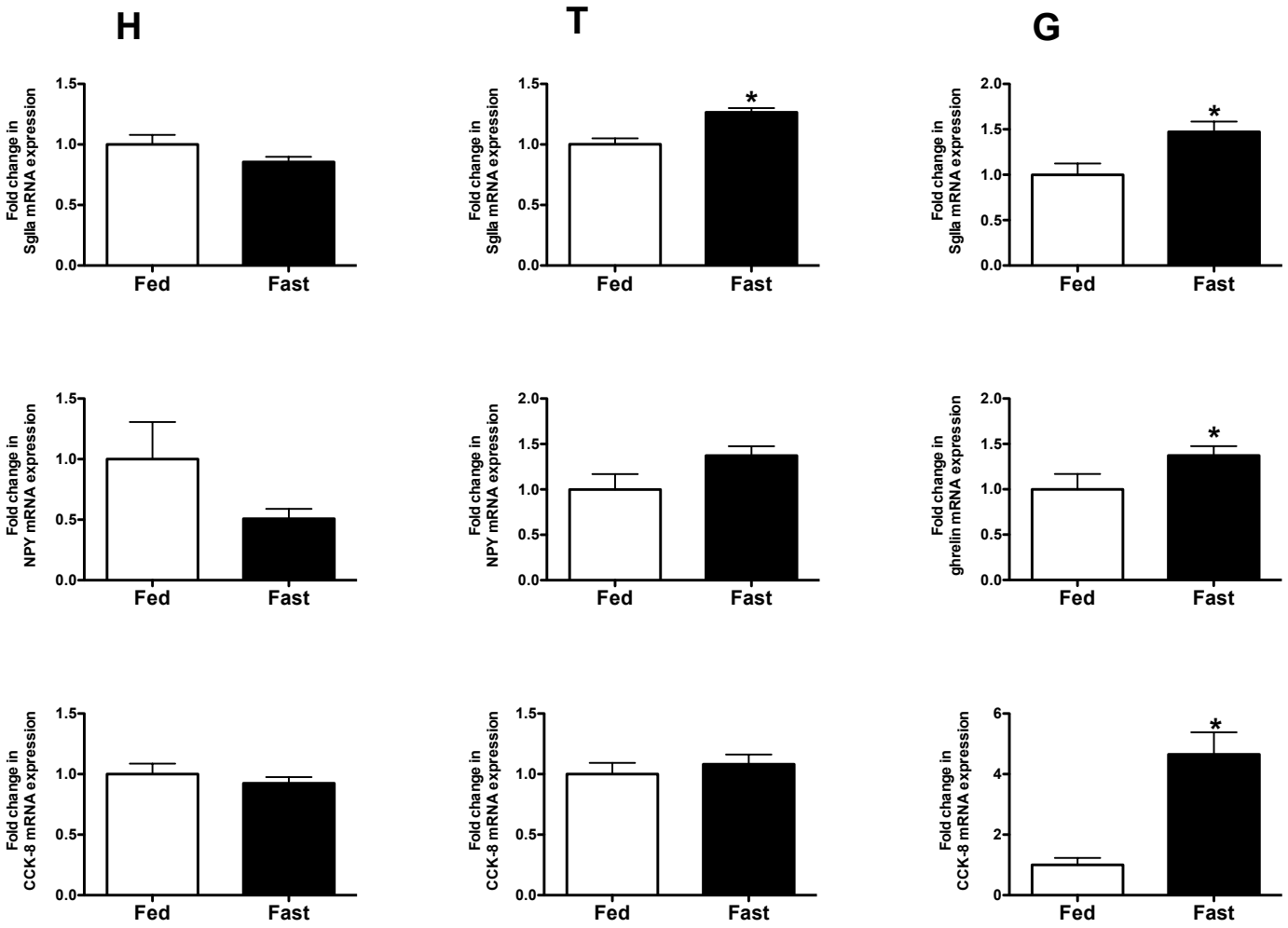

Fig 2.1. Levels of mRNA in hypothalamus (H), telencephalon (T) and gut (G) for SgIIa, NPY, CCK-8 and preproghrelin (ghrelin for short) in fed and 26 h acutely fasted female goldfish (n = 6-10 fish per group). Data are presented as means ± SEM. Stars indicate significant differences between the fed and fasted groups by independent samples T test.

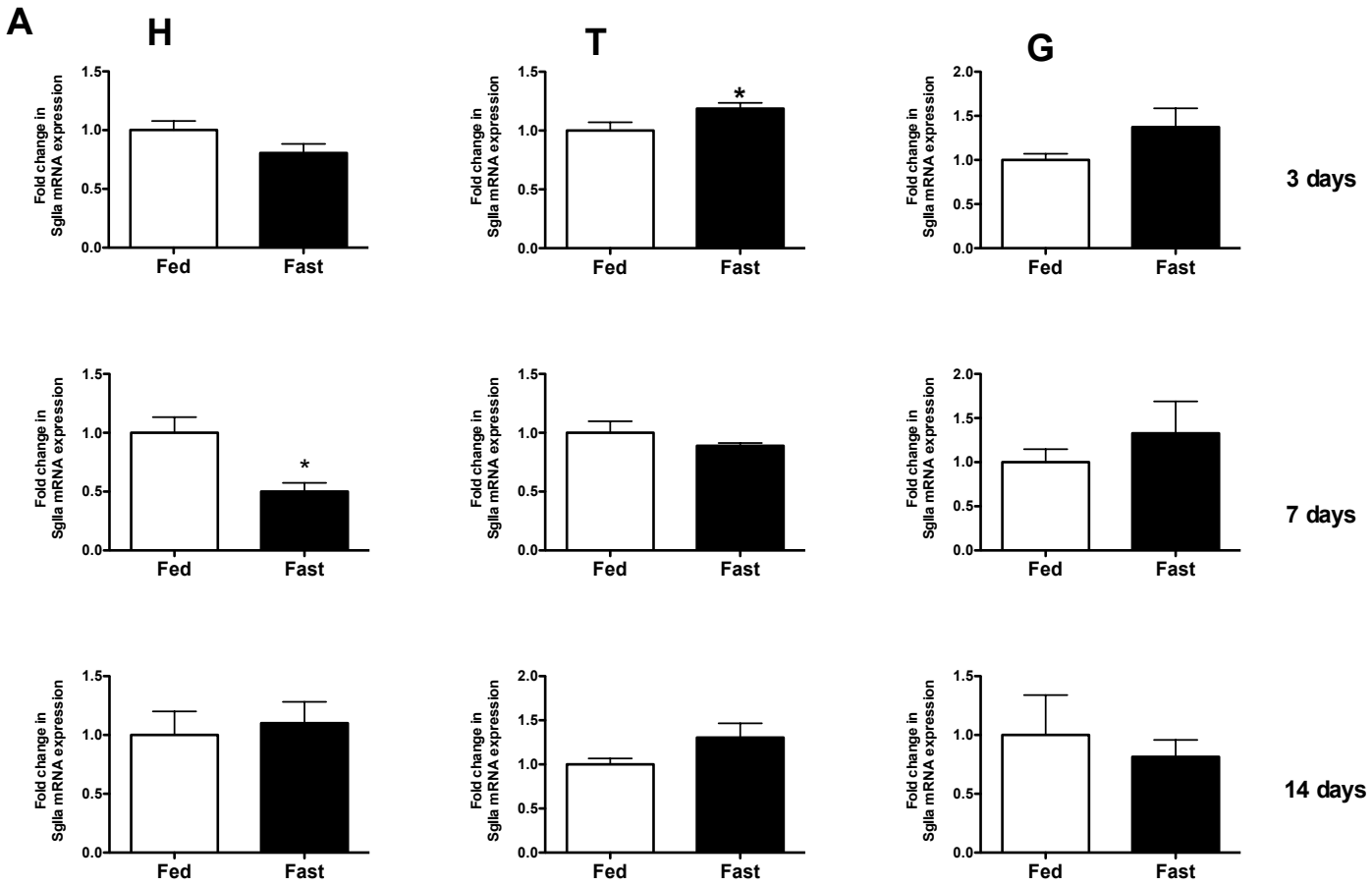

Fig 2.2. (A) Levels of mRNA in hypothalamus (H), telencephalon (T) and gut (G) for SgIIa in fed and fasted female goldfish (n=6 fish per group) after short (3 days), medium (7 days) and long fasting (14 days). Data are presented as means ± SEM. Stars indicate significant differences between the fed and fasted groups by independent samples T test.

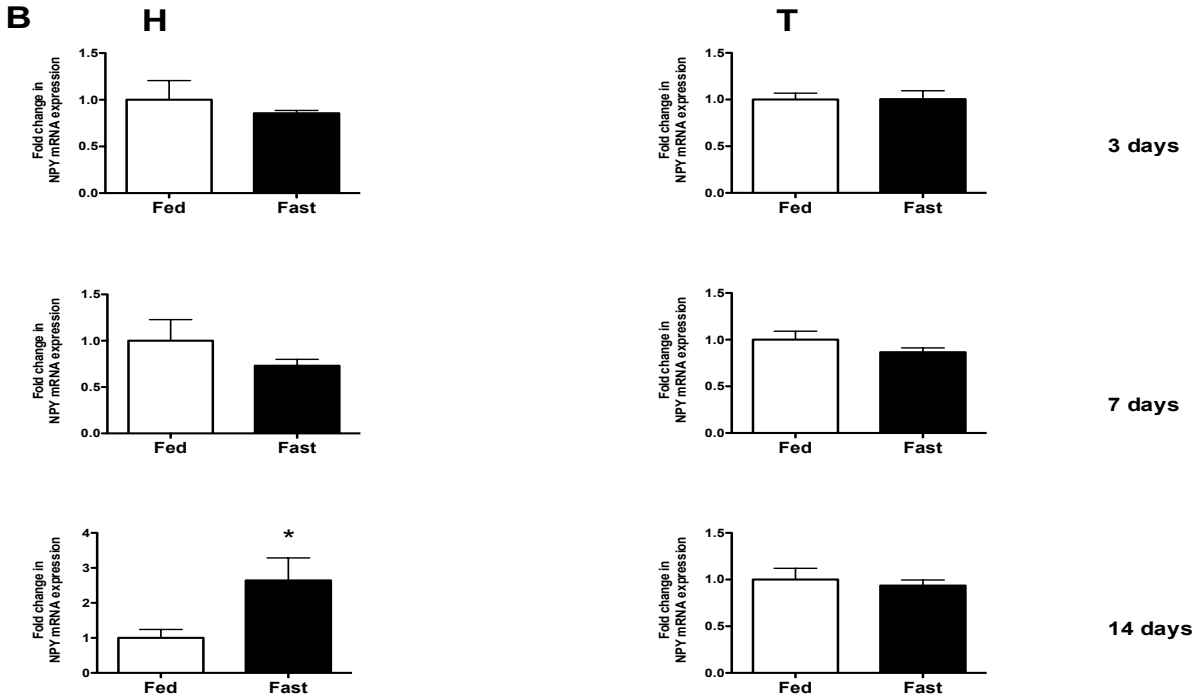

Fig 2.2. (B) Levels of mRNA in hypothalamus (H), telencephalon (T) and gut (G) for NPY in fed and fasted female goldfish (n=6 fish per group) after short (3 days), medium (7 days) and long fasting (14 days). Data are presented as means ± SEM. Stars indicate significant differences between the fed and fasted groups by independent samples T test.

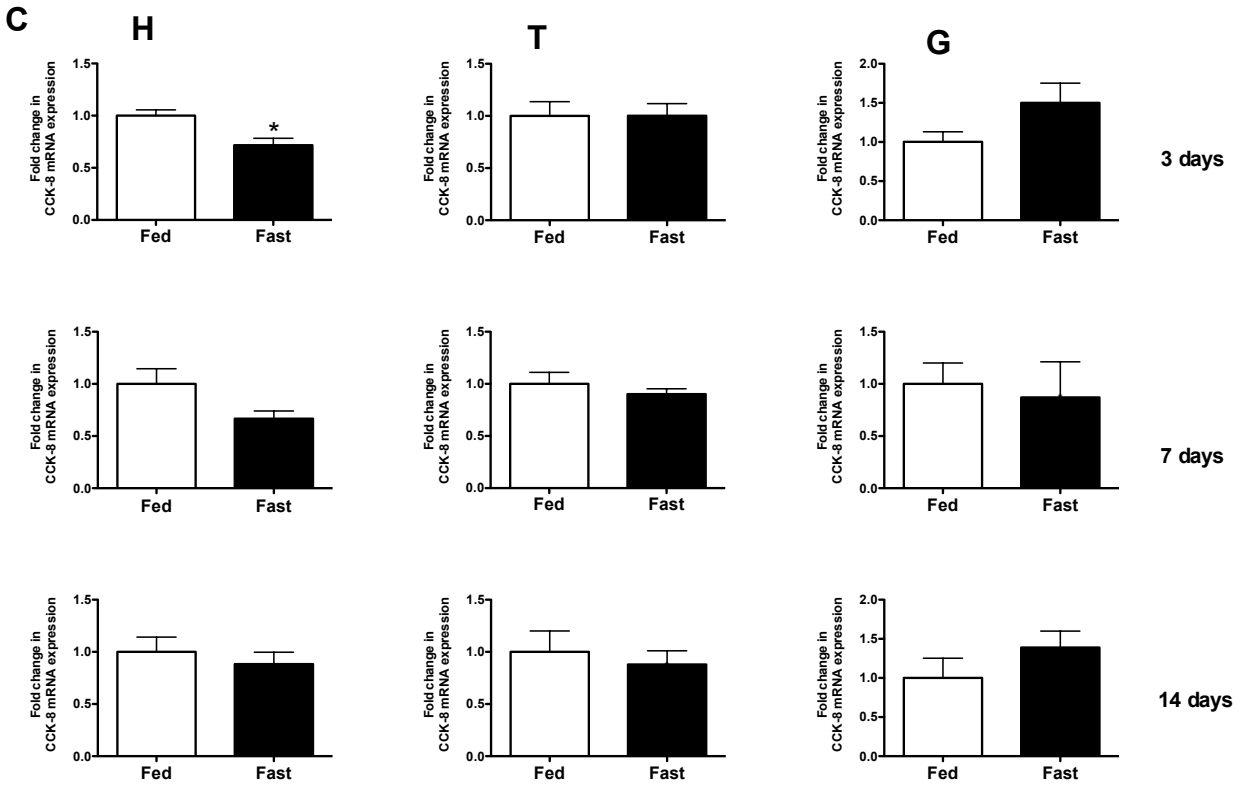

Fig 2.2. (C) Levels of mRNA in hypothalamus (H), telencephalon (T) and gut (G) for CCK-8 in fed and fasted female goldfish (n=6 fish per group) after short (3 days), medium (7 days) and long fasting (14 days). Data are presented as means ± SEM. Stars indicate significant differences between the fed and fasted groups by independent samples T test.

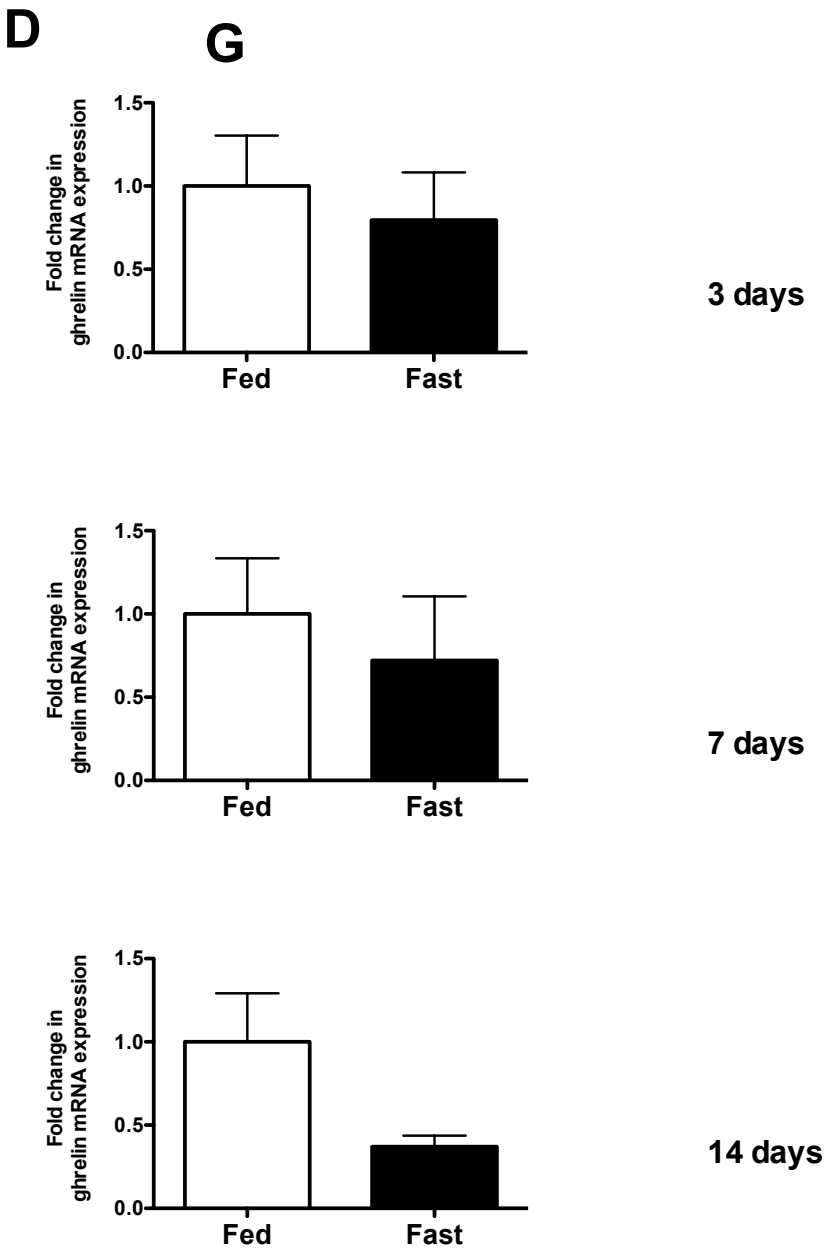

Fig 2.2. (D) Levels of mRNA in hypothalamus (H), telencephalon (T) and gut (G) for preproghrelin (ghrelin for short) in fed and fasted female goldfish (n=6 fish per group) after short (3 days), medium (7 days) and long fasting (14 days). Data are presented as means ± SEM. Stars indicate significant differences between the fed and fasted groups by independent samples T test.

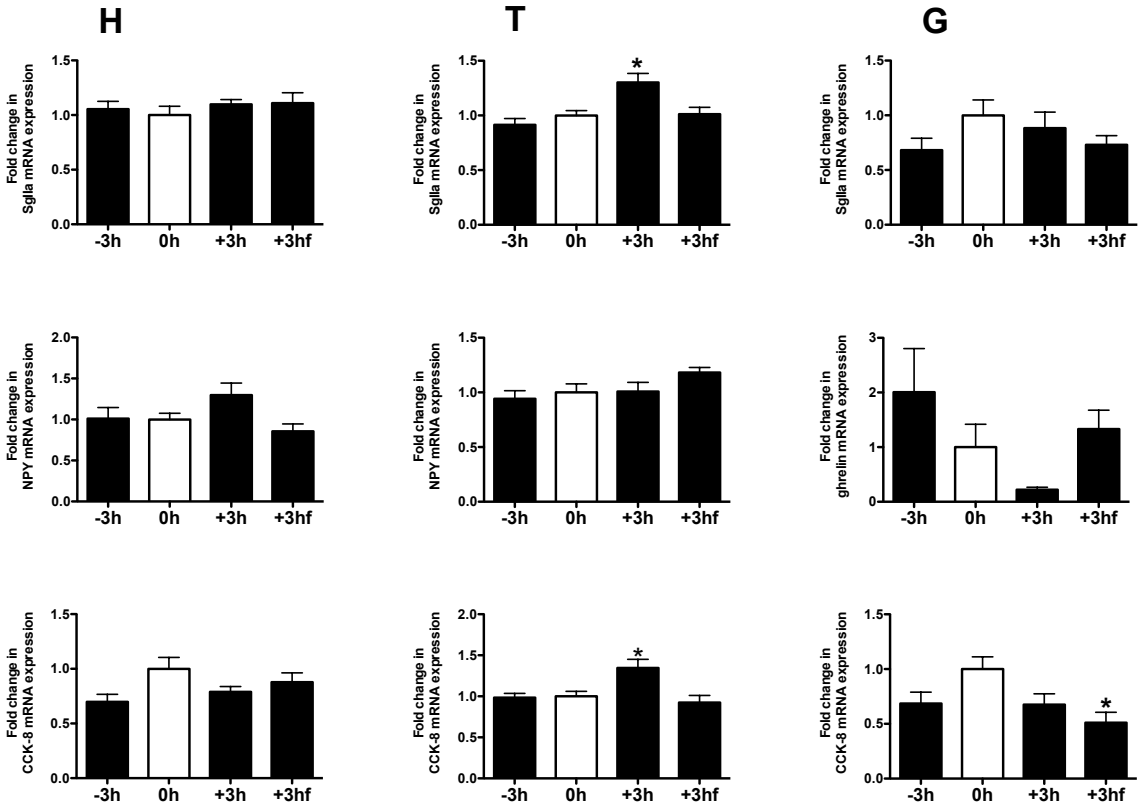

Fig 2.3. Periprandial changes in mRNA levels of SgIIa, NPY, CCK-8 and preproghrelin (ghrelin for short) in hypothalamus (H), telencephalon (T) and gut (G) in female goldfish. Fish were fed daily at (0 h) for 2 weeks. On the experimental day, group of fish was killed 3 h prior feeding time (-3 h), at feeding time (0 h) and 3 h after feeding time (+3 h) for unfed fish and (+3 h f) for fed fish (n=7-10 fish per group). Data are presented as means ± SEM. Stars indicate significant differences between groups using 1-way ANOVA followed by Dunnett t (2-sided) (comparing each group to 0 h group).

CHAPTER 3

The effect of intracerebroventricular injection of secretoneurin a on food intake and expression of feeding peptides in the brain of female goldfish

Rational and objectives

The role of secretoneurin a (derived from the SgII-a protein) in feeding regulation is examined in this chapter. The main objectives of the resesrch described in this chapter are to determine the effect of intracerebroventricular (ICV) injection of SNa on food intake and locomotor behavior. The effect of ICV injection of SNa on the expression of 2 feeding stimulatory factors (neuropeptide Y (NPY) and orexin (OX)) and 2 feeding inhibitory factors (chocystokinin (CCK-8) and cocaine-and amphetamine-regulated transcript I (CARTI)) in hypothalamus and telencephalon was also determined to gain insight into possible mechanisms of action of SNa.

3. Materials and methods

3.1 Experimental animals

For all feeding experiments female goldfish (*Carassius auratus*) were used as an experimental model ranging from 25 to 30 g in weight (for conditions refer to chapter 2, section 2.1). Forty four fish were fed to satiety 1% BW ration once a day at regularly scheduled time (10:00 h), with commercially prepared goldfish food natural color enhancing 3mm sinking pellets (New Life International, Inc., Florida, USA) containing 36% crude protein, 5% crude fat, 6% crude fiber, 10% moisture, 8% Ash, 8000 IU/kg Vitamin A, 2500 IU/kg Vitamin D, 200 IU/kg Vitamin E. Fish were acclimated for approximately 3 weeks before the start of an experiment. Twenty-four hours prior to the experiment, two fish were moved into an observation tank.

3.2 Intracerebroventricular (ICV) injection with SNa

Fish were injected directly into the brain third ventricle following the procedure described by (Peter & Gill, 1975) and a detailed protocol generously provided by Dr. Hélène Volkoff (Memorial University of Newfoundland). Goldfish SNa (TNENAEEQYTPQKLATLQSVFEELSGIAASNANS) was synthesized previously by Dr. A. Basak (Zhao et al., 2009b). The SNa stock solutions were made in water and stored at -20 C until use. Aliquots were thawed then diluted to the required concentration in physiological saline immediately prior to injection. The vehicle was physiological

saline (0.6% NaCl) with adjusted pH=6.5 (using 1N HCl), to control for the acidity of SN (Blazquez et al., 1998). In brief, a flap was cut in the skull of a fish using a modeler's drill apparatus (model 28-515; Proxxon, Niersbach, Germany) with a small circular saw. The bone flap was then folded to the right side, and 2 µl of the test solution (ether saline or SN) were injected using a 5 µl Hamilton micro syringe (Model 75N, 5 µl, SYR 26S/2/3) under the dissecting microscope into the brain third ventricle, according to coordinates (+1.0, M, D 1.2) from the goldfish forebrain stereotaxic atlas (Peter & Gill, 1975). Following injection, the skull flap was attached into its place using tissue adhesive (VetBond, 3M) then the fish were returned to their tanks, and normally recovered from anesthesia within 5 minutes. In both studies, two doses of SN were used (0.2 and 1 ng/g BW) and compared to the saline vehicle.

3.3 Observational experiments

Two fish were placed in an observation tank (40.5 cm length, 20 cm width and 24 cm depth filled with 14-L water at 18◦C) to avoid stress due to isolation and to allow for an accurate observation of feeding and locomotor behaviors. Observations were made for each individual fish. An approximate 3% BW ration of pellets per fish was administered at 15 min post-injection for a 60 minutes period that was divided into four 15 minutes time blocks. Behavioral observations and measurement of food consumption started when fish were offered pellets into the tank and lasted 1 h. Food intake (also known as a complete feeding acts) was measured by counting the number of pellets eaten by each fish per hour then converted to milligrams of food consumed/wet body weight/time feeding based on the mean pellet weight (22 mg) (the mean was calculated for 100 pellets). Incomplete feeding acts monitored and quantified were: the number of times a fish bumped a pellet with a closed mouth, engulfed a pellet and then spitted it out or the number of times a fish skimmed the bottom of the tank. Total feeding acts was assessed by adding the number of complete feeding acts to incomplete feeding acts. Locomotor behavior (fish activity) was measured by counting the number of non-feeding acts (bumping to any object in the tank (air stone), tank wall or tank mate and also by counting the number of swimming acts when fish crossed the line that was drawn on the middle of the tank. Total acts were quantified by adding the number of feeding acts to the number of non-feeding acts. Five hours (for first study) and two hours (for second study) beyond the observation period, fish were anaesthetized with 3-aminobenzoic acid

ethylester (MS-222; Syndel Laboratories, Vancouver, BC, Canada) for dissection procedures. Hypothalamus and telencephalon were dissected and immediately placed on dry ice and stored at -80 °C until RNA extractions were performed. All experiments were approved by the University of Ottawa Protocol Review Committee and followed standard Canadian Council on Animal Care guidelines on the use of animals in research. The first study was conducted in May 2013 (treatment with SN for 5 hours) while the second one (treatment with SN for 2 hours) was performed in October 2013. Food intake and locomotor behavior were assessed only for the first experiment, whereas gene expression analysis was conducted for both experiments.

3.4 Gene expression analysis.

3.4.1 RNA isolation and cDNA synthesis

Total RNA was isolated using Qiagen RNeasy Micro Kit following the manufacturers' protocol (for details refer to Chapter 2, section 2.3). Complementary DNA (cDNA) was obtained from 1µg for hypothalamus and 2 µg for telencephalon of total RNA using 200 ng random hexamer primers (Invitrogen) and superscript II reverse transcriptase (Invitrogen).

3.4.2 Gene expression analysis by Q- PCR

The levels of NPY mRNA, CCK-8 mRNA, pre-pro-orexin mRNA, CART1 mRNA and ribosomal 18S mRNA was measured using quantitative Q-PCR. Each sample was run in duplicate in optically clear 96-well plates with a final volume of 25 µl. Each PCR reaction contained 1µg for hypothalamus and telencephalon of RNA transformed to cDNA, 12.5 µl Maxima SYBR green qPCR master mix (2x) (Thermo scientific), 10 nM ROX solution and an optimized concentration for each primer (Table A, Appendix II) was added to the reaction. The cycling parameters were as: activation step at 95 ° C for 10 min followed by 40 cycles of 95 ° C for 30 s, 60°C for 1 min for CARTI, CCK-8 and pre-pro-orexin and 58 °C for 18S and NPY, 72°C for 1 min. Standard curves for each gene and tissue were generated using serial dilutions (six 5-fold dilution of cDNA mix from all samples starting at (1:10 cDNA stock) and (1:100) for sample dilution. The standard curve was duplicated for each plate. For all genes, assays were performed using Mx3000P system (Stratagene, La Jolla, CA). For each gene, the threshold was calculated using the MxPro-Mx3000P v4.10 software (Stratagene, 2007).

Efficiency of relative standard curve of the amplification was 100±10 %, a slope between–3.1 to -3.6 and an $r^2 > 0.985$ according to the manufacturer's instructions.

3.4.3 Statistical analysis

Statistics were performed using SPSS 21 software (SPSS Inc., Chicago, IL, USA). Data were tested for normality (Shapiro-Wilk's test) and homogeneity of variance (Levene's test). Statistical analyses for food intake, incomplete feeding acts, total feeding acts, locomotor behavior and total acts were conducted using one–way analysis of variance (ANOVA) followed by Dunnett's t-test (2-sided) to evaluate significant differences between groups, comparing all groups to the control group. Dunnett's T3 multiple comparison test was used for total feeding acts because this dataset did not meet the assumption for homogeneity of variance. Statistical differences between treatment groups were assessed using one–way analysis of variance (ANOVA) followed by Dunnett's t-test (2-sided) to evaluate significant differences between all groups against the control group. Significance was considered at $p < 0.05$. Data is expressed as mean ± standard error of the mean (SEM). In expression studies, all samples are normalized following data driven normalization algorithm (NORMA-Gene) method (Heckmann et al., 2011). Details of the normalization method using five genes (e.g., NPY, pre-pro-orexin, CCK-8, CARTI and 18S) for each tissue can be found in Chapter 2, section 2.6.

3.5 Results

3.5.1 Effect of ICV treatment on food intake and fish activity

Fish injected with both doses of SNa exhibited a significantly higher food intake than the control fish (p=0.0001) (Fig 3.1 A). Similar results were obtained for total feeding acts for both doses of SN (p=0.0001 for SN 1 ng/g and p=0.002 for SN 0.2 ng/g) (Fig 3.1 C), total acts (p=0.0001) (Fig 3.1 D) and locomotor behavior (p=0.0001) (Fig 3.1 E). For incomplete feeding acts, fish injected with 0.2 ng/g SNa had similar levels of activity compared to the control group whereas the 1 ng/g SNa injected group displayed higher incomplete feeding acts than the control group (p=0.046) (Fig 3.1 B).

3.5.2 Effect of ICV treatment on NPY, OX, CCK-8 and CARTI expression

In this study, we measured the mRNA levels for NPY, pre-pro-orexin, CCK-8 and CART1 in hypothalamus and telencephalon in fish injected with saline or SN. In the first

experiment, 5 hours post-injection with SNa, there were no significant differences between treatment and control fish in NPY, pre-pro-orexin and CCK-8 mRNA in both hypothalamus and telencephalon. The only observed change was in CARTI mRNA level**.** The high dose of SNa (1ng/g) caused a significant decrease in CARTI mRNA in hypothalamus but not telencephalon (p=0.010) (Fig 3.2). Given the robust effects of SNa on feeding activity, we were surprised by the lack of effects on expression of peptide mRNAs implication in the stimulation of feeding. We hypothesized that perhaps the late sampling time (5 h) may have been too long post-injection to observe effects. Therefore, we repeated the injections and sampled at 2 h for gene expression analysis. Two hours after SNa injection exhibited a significant increase (p=0.038) in NPY mRNA level in fish injected with high dose of SNa (1ng/g) in hypothalamus. However, both doses had no effect on NPY mRNA in levels of telencephalon. There were no effects of either SNa doses on pre-pro-orexin, CCK-8 and CARTI mRNA in hypothalamus or telencephalon (Fig 3.3).

3.6 Discussion

In Chapter 2, we reported that SgIIa mRNA level (the precursor of SNa) is increasing under the acute and short term fasting which gave us an idea that SNa could be involved in feeding processes. Based on the results of Chapter 2, we hypothesized that treatment with SNa would increase food intake in treated fish. For the subsequent step, we decided to examine the effect of SNa treatment through direct injection in the brain on food intake and expression of some feeding peptides. Our results showed that central injection of SNa at either 0.2 or 1 ng/g increased food consumption, total feeding act, locomotor behavior and total acts whereas, only 1ng/g increased incomplete feeding acts in female goldfish. This result is in agreement with a preliminary study suggesting that 1ng/g SNa increased food intake and fish activity in mixed-sex groups of goldfish (Trudeau et al., 2011). Here we demonstrate that fish injected with SNa at 0.2 and 1 ng/g had a greater food intake (actual number of pellets eaten by each fish) and total feeding acts (eating the pellets, spitting, bumping or skimming the bottom of the tank) than fish injected with saline. This robust response indicated that 1 ng/g SNa increased the amount of food consumed by more that 2 times in one hour. Higher activity (swimming, bumping to any object in the tank or tank mates) was seen in fish treated with SNa 0.2 and 1 ng/g compared to vehicle-injected fish. These observations suggest that SN a rapidly increases fish appetite. Most feeding happened in the first 15 minutes after

addition of food to the tank. This increase in feeding may be related to an increase in general activity as indicated by the increase in non-feeding acts.

The SNa treatment had no effect on NPY mRNA in hypothalamus or telencephalon at 5 hours post administration. Neuropeptide Y is considered one of the more potent orexigenic factors in mammals and fish, and is widely distributed in the hypothalamic area of fish brain (Volkoff et al., 2005). Previous studies demonstrated that ICV injection of nanogram quantities of NPY induced a significant increase in food intake in goldfish after 30 minutes of treatment (Narnaware et al., 2000). The effect of SNa treatment was not seen on NPY mRNA at 5 hours post treatment and that might be due to the long time between treatment and measurement of the gene expression. In contrast, the high does of SNa (1 ng/g) did increase NPY mRNA by 140% 2 h post-injection. In goldfish forebrain, current neuroanatomical evidence suggests that one possible site of interaction between SN-IR fibers (Canosa et al., 2011) and NPY cells bodies would be at the level of nucleus entopeduncular (NE) (Pontet et al., 1989). However, we did not observe effects of ICV injections of SNa on NPY mRNA in the telencephalon. On the other hand, there are very few NPY-IR cell bodies in the goldfish hypothalamus (Pontet et al., 1989), yet there are numerous SN-IR fibers (Canosa et al., 2011). In the posterior periventricular nucleus (NPPv), there is a clear cluster of NPY-IR cell bodies (Pontet et al., 1989) and both SN-IR cell bodies and fibers are also localized to this area (Canosa et al., 2011). Given that we are injecting into the third ventricle (V3), it is possible that SNa could be affecting this NPY cell population, which could explain the increase of NPY mRNA. It may be possible to block the SNa using an NPY antagonist, thereby proving that SNa regulated NPY neurons.

Pre-pro-orexin mRNA level did not exhibit any changes following SN treatment either at 5h or 2 h after SN treatment. It has been known that orexins stimulate appetite and control the locomotor behavior and arousal in fish (Volkoff, 2012). Orexin is widely distributed in different goldfish brain areas and expressed highly in the hypothalamus (Huesa et al., 2005). Brain administration of orexin A and B increases food intake in goldfish after 60 minutes (Nakamachi et al., 2006; Volkoff et al., 1999). It has also been shown in goldfish that IP treatment with amphetamine induced a decrease in hypothalamic orexin after 75 minutes (Volkoff, 2013). Our results indicate that SNa treatment increased both food intake and fish activity, but not pre-pro-orexin mRNA level in hypothalamus or telencephalon. This result of gene expression analysis on pre-pro-orexin was surprising to us given the known role of orexin in stimulating feeding and

locomotor behaviors in fish. It may be that the timing of sampling is too short or too long to observe a potential effect on pre-pro-orexin. Further experiments are required before it can be concluded that SNa treatment is not affecting orexin neurons in either hypothalamus or telencephalon.

We also report that SNa treatment did not affect CCK-8 mRNA in hypothalamus or telencephalon. In goldfish, CCK-8 brain injection decreases food intake at 30 and 60 minutes (Himick & Peter, 1994; Thavanathan & Volkoff, 2006). In cavefish, IP treatment with orexin had no effect on CCK-8 mRNA (Penney & Volkoff, 2014). This result somehow agreed with our result if we suspect that SNa is an appetite stimulator since SNa treatment induced an increase in food intake taking in our mind the difference between the injection types.

Treatment with 1ng/g SNa decreased CARTI mRNA level at 5 h post injection in hypothalamus but not telencephalon. In contrast, the SNa treatment did not affect hypothalamus or telencephalon CARTI mRNA at 2 h post treatment. The decrease of CARTI mRNA following SNa injection is in agreement with the anorexigenic role of CARTI as an appetite suppressor. Previous studies have shown that brain injection with recombinant human CART fragments (62-76) and (22-102) in goldfish induced a significant decrease in food consumption at 60 minutes (Volkoff & Peter, 2000). Indeed, SN and CART are both widely expressed in fish brain including the hypothalamus. Neuroanatomical studies report CART-IR cell bodies in the nucleus of the posterior recess (NRP) in catfish hypothalamus (Singru et al., 2007) and both SN-IR cell bodies and fibers are found in the goldfish posterior recess (NRP) (Canosa et al., 2011). These data indicate possibilities for SN-CART interactions. However, specific co-localizations studies must be performed in goldfish to confirm this proposal.

In summary, treatment with SNa increased food intake in female goldfish. From the current and the past results (Trudeau et al., 2012) we suspected that SNa could act as an orexigenic factor to stimulate feeding in goldfish. Possible interactions between SNa and NPY or CARTI neurons could occur in goldfish brain as previous neuroanatomical studies suggest. However, further studies are needed to examine the co-localization between SNa and NPY and CARTI.

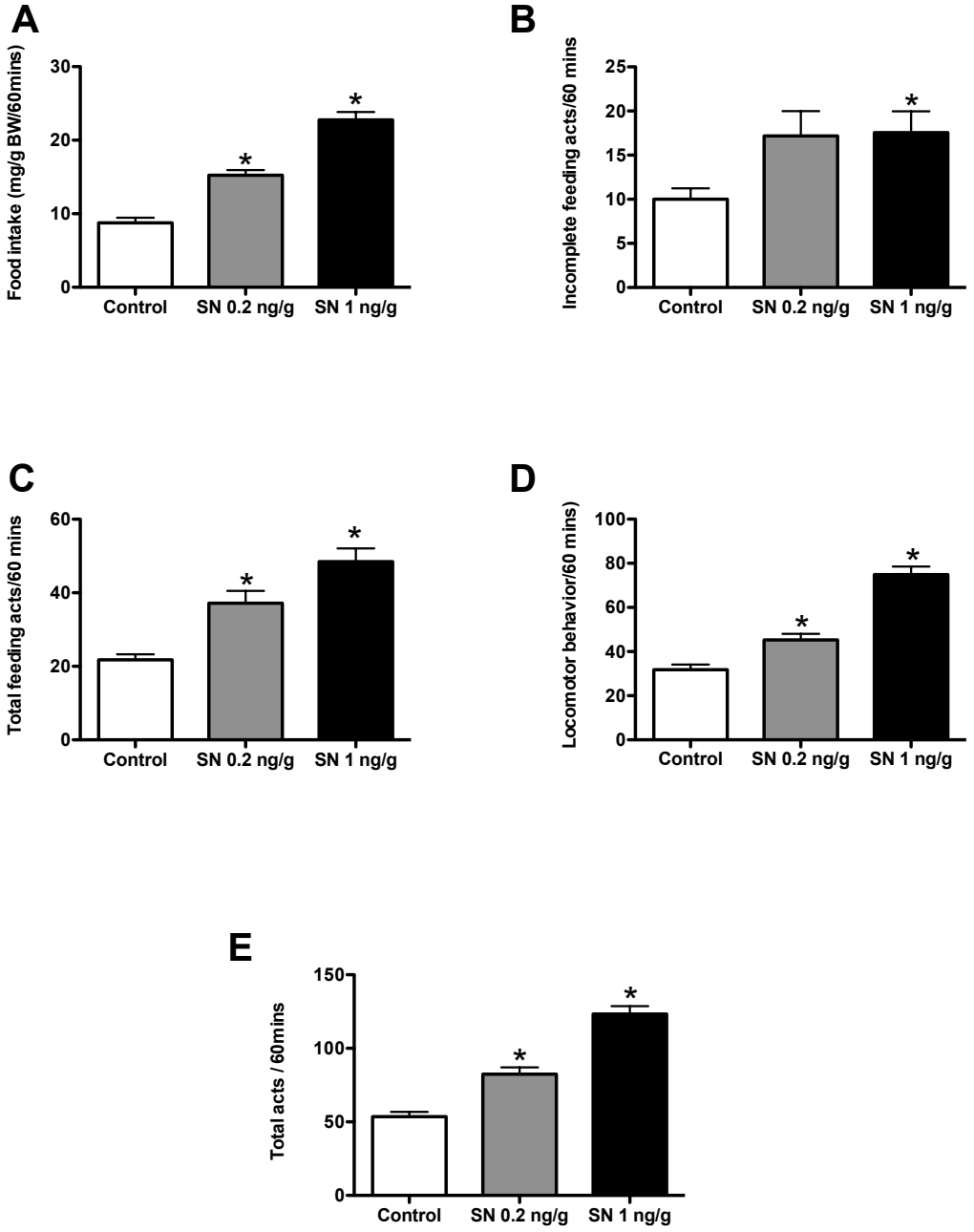

Fig 3.1. The effect of ICV injection of SNa on food intake (A), incomplete feeding acts (B), total feeding acts (C), locomotor behavior (D) and total acts (E). Female goldfish were injected with either saline (n=12 fish per group), SN at 0.2ng/g (n=12) or SNa at 1 ng/g (n=11) then observed for 60 minutes. Food intake was measured by counting the number of pellets eaten by each fish per hour and converted to milligrams of food consumed/wet body weight/time feeding based on the mean pellet weight fed to fish (22 mg). Incomplete feeding acts was monitored by quantifying the number of bumping a pellet with a closed mouth, engulfing of a pellet and then spitting out, or skimming the bottom of the tank. Total feeding acts was assist by quantifying the number of consumed and unconsumed pellets. Locomotor behavior was measured by counting the number of non-feeding acts (bumping into any object in the tank, tank wall or tank mate). Total act was monitored by adding feeding acts to non-feeding acts. Data are presented as mean ± SEM. Stars indicates groups that differ significantly from the control group (ANOVA followed by Dunnet t (2-sided) multiple comparison test.

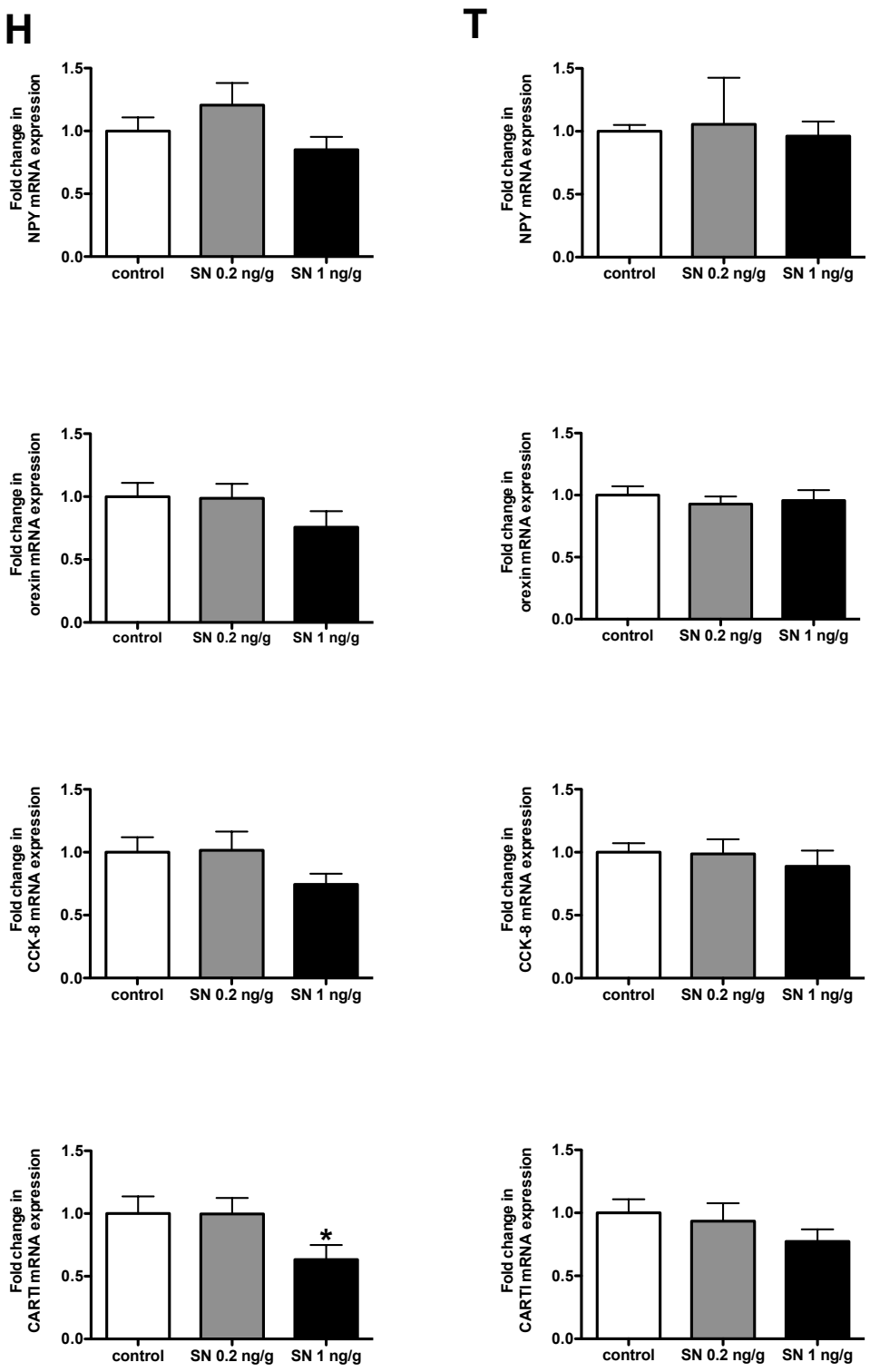

Fig 3.2. The effect of ICV injection of SNa at 0.2ng/g and 1 ng/g on mRNA levels on NPY, pre-pro-orexin (orexin for short), CCK-8 and CARTI in hypothalamus (H) and telencephalon (T) 5 hours after treatment in female goldfish (n= 8-12 fish per group). Stars indicate significant differences between groups by ANOVA followed by Dunnet t (2-sided) multiple comparison test.

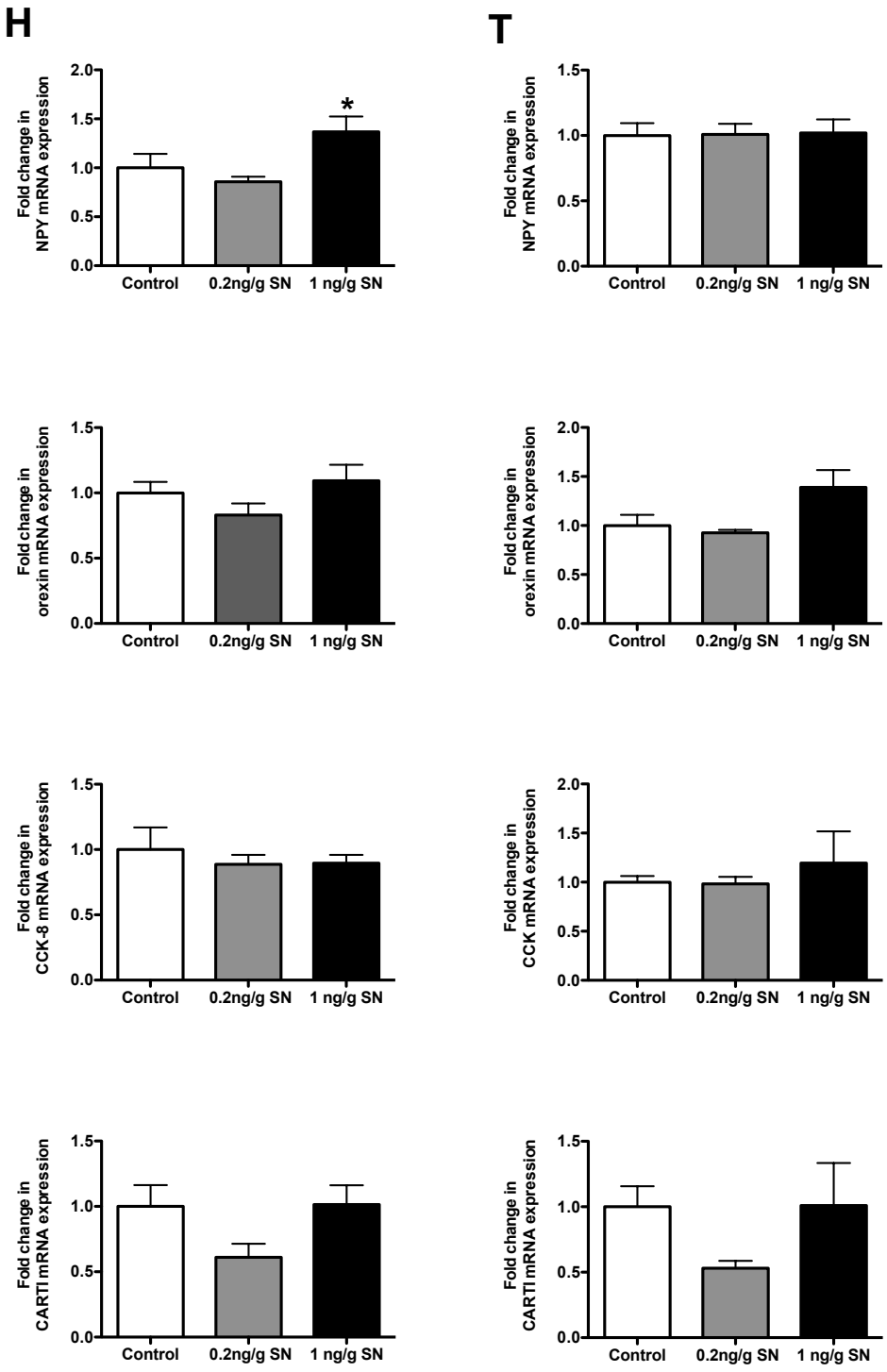

Fig 3.3. The effect of ICV injection of SNa at 0.2 ng/g and 1 ng/g on mRNA levels on NPY, pre-pro-orexin (orexin for short), CCK-8 and CARTI in hypothalamus (H) and telencephalon (T) 2 hours after treatment in female goldfish (n= 7-9 fish per group). Stars indicate significant differences between groups by ANOVA followed by Dunnet t (2-sided) multiple comparison test.

CHAPTER 4

General Discussion

4.1 Thesis results summary

Secretogranin II (SgII) is a secretory protein belongs to the granin family of peptides. Secretogranin II can be proteolytically processed in the secretory granules by prohormone convertases into smaller peptides. The most important peptide released from the SgII precursor is secretoneurin (SN) that plays multiple roles in the endocrine systems in vertebrates. The first chapter in this thesis reviewed details of SgII and its derived peptide SN. Two isoforms of SN named as SNa and SNb maybe generated from SgIIa and SgIIb precursors that likely emerged as a result of early gene duplication in teleosts (Zhao et al., 2010b). All studies to date focus on SgIIa and SNa since nothing is known yet about SgIIb and SNb. The main objective of our research was to examine the potential new role of SNa in regulation of feeding behavior in fish. Establishing that forebrain SgIIa expression responds to fasting and how brain injection of SNa could affect feeding processes might shed some light on this role. In this discussion chapter, the major findings about the SgIIa and SNa peptides in controlling feeding processes will be discussed.

The first study (Chapter 2) examined the distribution of SgIIa mRNA in both central and peripheral tissues related to feeding processes in female goldfish using both RT-PCR for detecting the bands and Q-PCR for measuring mRNA levels. The results showed that SgIIa is presented in all tissues examined. The highest was in the pituitary followed by brain tissues then the peripheral tissues, which confirmed a previous study done on a smaller selection of tissues (Zhao et al., 2006a). Most importantly, SgIIa is expressed in brain tissues (hypothalamus and telencephalon) that have the feeding center and regulate feeding processes directly, and gut that can peripherally control feeding through releasing hormones that act to control feeding behavior. Secondly, we examined the effect of acute (26 h), short (3 days), medium (7 days) and long (14 days) fasting and periprandial changes on SgIIa mRNA and other feeding peptides that are being intensively studied in relation to feeding control. These included NPY, CCK-8 mRNA in hypothalamus, telencephalon, and ghrelin and CCK-8 mRNA in gut. We found SgIIa mRNA is increasing under the effect of acute and short term fasting rather than medium or long term fasting in telencephalon. Even though the increase is statistically significant, it may be considered as a small increase. It is important to compare effect of fasting on

SgIIa with orexigenic peptides such as NPY, OX and ghrelin. For examples, in Brazilian flounder, the effect of fasting was about 2 fold increase in NPY mRNA using Q-PCR method (Campos et al., 2010), in goldfish, Northern blot analysis indicated a 2 and 1.5 fold increase respectively in NPY mRNA levels in hypothalamus and telencephalon (Narnaware & Peter, 2001), a 1.5 fold increase in OX mRNA was observed in fasted goldfish using semi-quantitative RT-PCR analysis method (Nakamachi et al., 2006) and a 2.5 fold increase in hypothalamus ghrelin mRNA in goldfish was detected using slot blot analysis (Unniappan et al., 2004). We observed a 1.25 fold increase in SgIIa mRNA observed 3 h after scheduled feeding time in unfed group (27 h fasting). This finding is somehow in agreement with our hypothesis that fasting would increase the expression of SgIIa in goldfish brain if SgIIa was involved in stimulating food intake.

The second study (Chapter 3) focused on the effect of ICV treatment of SNa on feeding and locomotor behaviors and the expression of some feeding neuropeptides. Two different doses of SNa (0.2 and 1 ng/g) were used for brain injection in the third ventricle. Food intake and locomotor behavior were measured for 60 minutes after ICV injection of SNa. Our results demonstrated that 0.2ng/g SNa increased food consumption and locomotor activity by 150%; and the 1ng/g SNa dose increased food consumption and locomotor activity by 250%. These findings support our original hypothesis that treatment with SNa would increase food intake and fish activity. This result confirmed a previous preliminary study indicating that 1 ng/g SNa induced a significant increase in food intake and fish activity (Trudeau et al., 2012). Also, IP treatment of SNa at 2.5 ng/g increased food intake in goldfish by approximately 150% (Appendix I). Locomotion behavior is somehow linked with feeding behavior. The increase in activity associated with the increase in food intake has also been detected in other orexigenic factors such as OX and ghrelin. For instance, ICV administration of OX A stimulates locomotor activity in goldfish and ornate wrasse (Matsuda et al., 2012a). Moreover, ICV and IP administrations of ghrelin increased goldfish activity (Matsuda et al., 2006). However, ICV treatment with NPY caused a decrease in goldfish swimming activity (Matsuda et al., 2012b). In Chapter 3 we also examined the effect of brain injection of SNa on the expression of some feeding neuropeptides: NPY and OX as feeding stimulators and CCK-8 and CARTI as feeding inhibitors, after short (2 h) and long (5 h) treatment. The results show that SNa caused an increase in NPY mRNA level in fish hypothalamus but not telencephalon at 2 h. However, SNa did not alter OX, CCK-8 or CARTI mRNA in hypothalamus or telencephalon at the 2 h time-point. On the other hand, there were no

observable changes in NPY, OX, CCK-8 mRNA levels in hypothalamus or telencephalon 5 h post-SNa injection. A decrease in CARTI mRNA was observed at 5 h after SNa treatment in hypothalamus but not telencephalon. From the results of gene expression we can conclude that NPY and CARTI are the only neuropeptide mRNAs that were affected by SNa treatment under the conditions of this experiment. Neuroanatomical studies in goldfish brain indicate that SN-IR cell bodies and fibers, and NPY-IR cell bodies are localized in one possible site of interaction in the posterior periventricular nucleus (NPPv) (Canosa et al., 2011; Pontet et al., 1989). Therefore, it is possible that SNa could affect this NPY cells population since the injection was into the third ventricle (V3) of the brain. Other neuroanatomical studies have reported that CART-IR cell bodies are detected in the nucleus of the posterior recess (NRP) in catfish brain (Singru et al., 2007) and SN-IR cell bodies and fibers are also detected in the same area in goldfish brain (Canosa et al., 2011), providing the possibility for SNa and CART interactions. A specific neuroanatomical study has to be performed to determine the relationship between SNa and NPY, and SNa and CART in goldfish brain to confirm these possibilities of interaction.

From the results of Chapter 2 and Chapter 3 we observed that SgIIa mRNA is increasing under the effect of fasting, also food intake is increasing because of SNa treatment. These current findings are the first evidence for SNa to be considered as a feeding peptide, which opens the door to an important question on the physiological role and relative importance of SNa to regulate feeding. To answer this question both the weak and strong evidence for SNa as a feeding regulator will be discussed here. For fasting, a small increase in SgIIa mRNA level in the telencephalon was observed. The vast majority of SN-IR in the telencephalon is in preoptic nucleus. The magnocellular cells co-express SNa and isotocin, and project to the posterior pituitary. There are other areas of the telencephalon containing SN-IR cells, including those that could also be responding to fasting. We hypothesize that only a subset of SN-containing neurons represents the feeding neurons. It may be that the fasting response in SgIIa of specific neurons is larger than that observed in the whole telencephalon examined here by Q-PCR. Based on this data, the SgIIa fasting response is considered as weak but supportive evidence for a role of SgIIa in feeding regulation.

The robust effect of the SNa peptide on food intake is strong evidence for involvement of SNa in feeding regulation. Critically missing is measurements of SgIIa precursor protein, and processed fragments including SNa in response to fasting. This

would be an important connection between SgIIa mRNA responses, and the effects of SNa peptide. The effects of ICV administration of SNa to increase NPY and reduce CARTI are somewhat supportive of the proposed role for SNa as a feeding peptide. Critically missing however is the connection between the fasting response data and the effects of injected SNa on NPY and CARTI systems. The new data obtained as a result of this thesis research does allow the development of a possible model for the role of SNa containing neurons in the regulation of feeding (Fig 4). This model can serve as the foundation for future research that may lead to firm establishment of SNa as a feeding peptide.

4.2 Future directions

Our study has identified a novel role of SNa in controlling food intake in goldfish, but several issues remain unclear and some studies need to be done in the future. It is know that SNa is generated from SgIIa precursor by proteolytic processing at the dibasic cleavage sites by prohormone convertases enzymes (Zhao et al., 2009b). One of the important steps for the next study is measuring the protein levels of SgIIa and SNa under fasting condition to have the full image with the gene expression study. Measuring the SgIIa mRNA in specific cells in brain and gut tissues under the normal and fasting status is important. This could be done by *in situ* hybridization technique to identify which population of SgIIa neurons is responding to fasting. Another important step is examining the co-localization of SNa and NPY, and SNa and CARTI in the fish brain. Exploring the mechanism by which SNa is affecting NPY and CARTI neurons directly or indirectly will also be important. Blocking the effect of SNa by a SNa antagonist will be an important step in defining SN as a key orexigenic peptide. However, this study cannot be done until SN receptors are identified. The SNa receptor is likely to be a G-protein coupled receptor (GPCR), but is still unidentified (Trudeau et al., 2012). Determining the physiological role of the other isoforms, SgIIb and SNb, may reveal other novel roles of the SgII family in the control of food intake. This will require cloning and sequencing of the full cDNA of goldfish SgIIb.

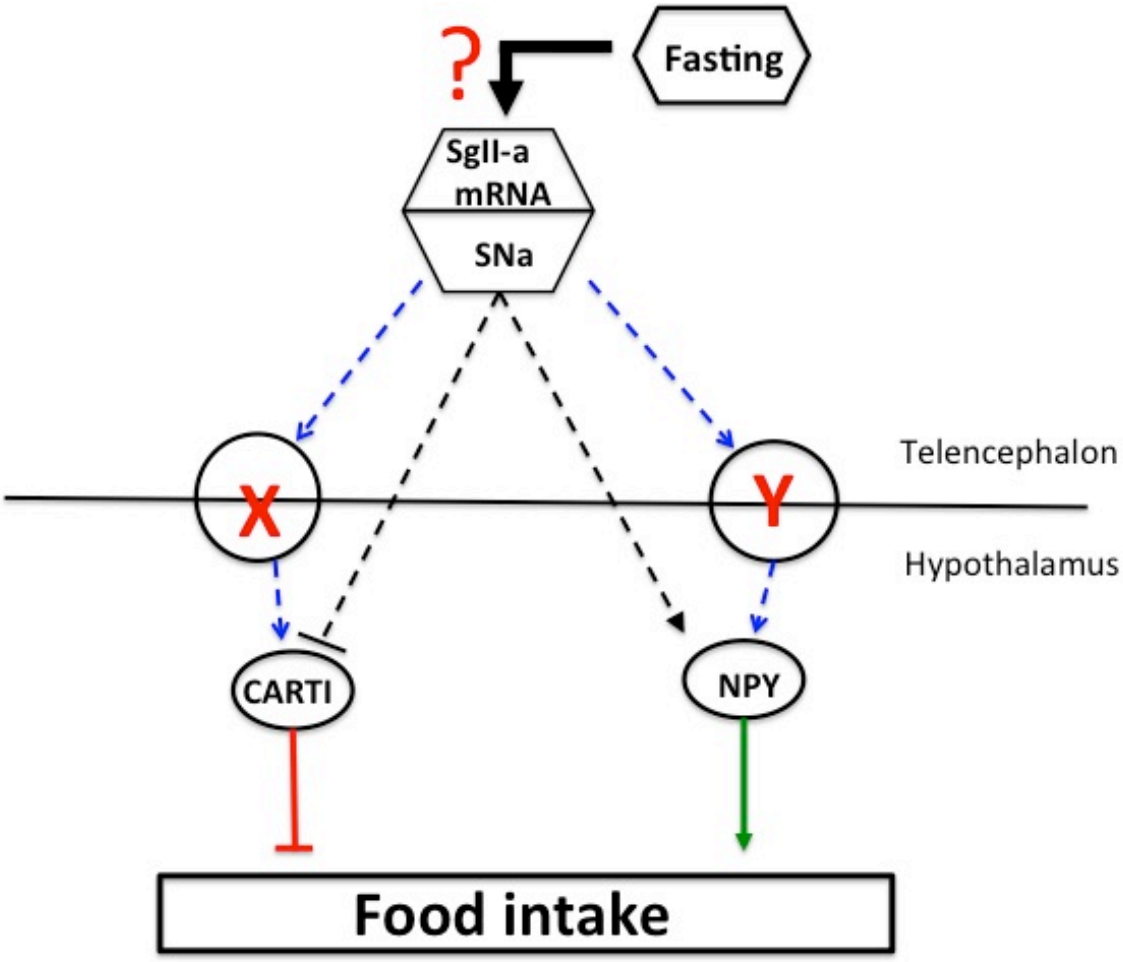

Fig 4. Schematic model for the role of SNa in the regulation of feeding process in goldfish. The X and Y indicate that there are missing data on the effect of SNa on NPY and CART1 that may indicate indirect pathways. The question mark (?) indicated that the SNa cells population that is affected by fasting in telencephalon remains unknown at present.

APPENDIX I

The effect of intrapertonial (IP) injection of SNa on food intake and gene expression

1. Introduction

Intrapertonial (IP) injection was done into tow separate experiments using high and low doses of SNa in female goldfish to see the effect of SNa on food intake and fish activity. In the first experiment three high doses of SNa 50, 250, 500 ng/g were used and we measured only food intake and locomotor activity. In the second experiment lower doses of SNa at 2.5 and 25 ng/g were chosen for the treatment and we assisted food intake, locomotor activity and gene expression of NPY, OX, CCK-8 and CARTI.

2. Materials and Methods

Exactly same method that used for the ICV experiments was done (for details refer to Chapter 3). Female goldfish were injected into the peritoneal cavity, caudal to the pelvic fins, 10 ul/g three different doses of SNa 50, 250, 500 ng/g BW for the first experiment and 2.5, 25 ng/g for the second experiment, saline for vehicle (0.6% NACL2, PH=6.5) using 1CC 26G (3/8) needle attached to a 250 ul Hamilton syringe (80601, 10ul, Model 710 LT SYR). Observation for food intake and locomotor activity was assisted for both experiments after 60 minutes of observation. Gene expression measurement was done only for the second experiment at five hours beyond the treatment for NPY, OX, CCK-8 and CARTI in hypothalamus and telencephalon.

3. Results

IP injection at 50, 250 and 500 ng/g SNa did not alter food intake or locomotor activity in treated fish (Fig 5.1 A, B) which might be due to an overdose effect or receptor desensitization.

Low dose of SNa at 2.5 ng/g elevated an increase in food intake level whereas the high dose of SN at 25 ng/g did not stimulate food intake. However, neither low dose 2.5 ng/g nor high dose of SN 25 ng/g affect fish activity (Fig 5.2 A, B).

For gene expression analysis the high dose of SNa at 25 ng/g elevated a decrease on NPY and CCK-8 mRNA level in hypothalamus but not in telencephalon whereas a low dose at 2.5 ng/g did not affect NPY or CCK-8 mRNA in both brain areas which matches our result that the high dose did not alter food intake which might be due to the

low level of NPY and CCK-8 that known to decrease food intake as an appetite suppressor. Both doses did not affect OX or CARTI mRNA level (Fig 5.3 A, B, C, D).

4. Summary

From the results of these experiments we can infer that SNa shows its effect in affecting appetite and food intake at low doses whereas high doses did not show any change on food intake during the 60 minutes observation. Also the increase in food consumption might be due to the indirect effect of SNa on NPY or CCk-8 neurons.

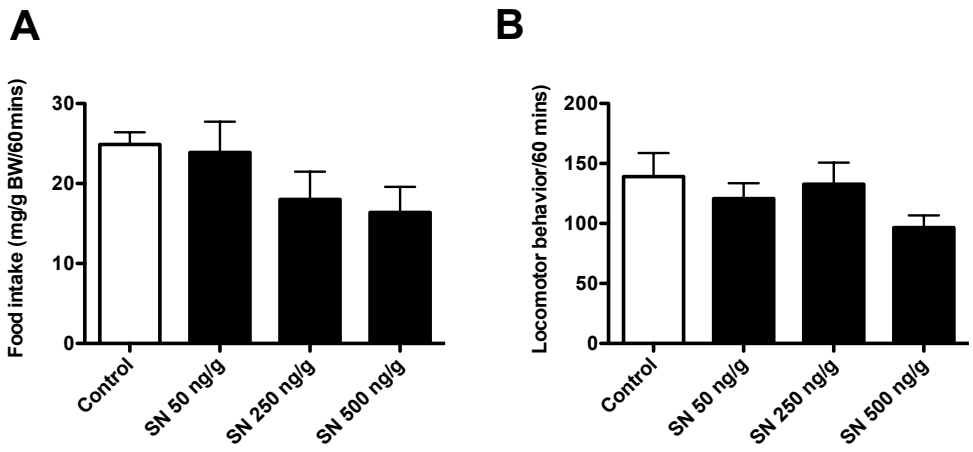

Fig 5.1. The effect of IP injection of SNa on food intake (A) and locomotor activity (B) using three different doses of SNa in female goldfish after 60 minutes of observation. Data are presented as mean ± SEM. Stars indicate groups that differ significantly from the control group (ANOVA followed by Dunnet t (2-sided) multiple comparison test.

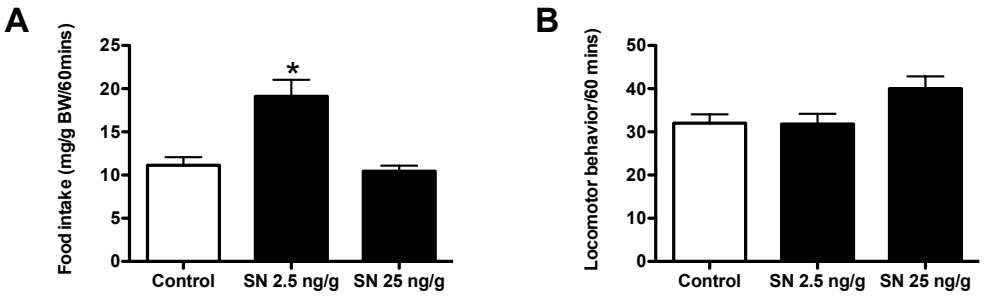

Fig 5.2. The effect of IP injection of SNa on food intake (A) and locomotor activity (B) using two different doses of SNa in female goldfish after 60 minutes of observation. Data are presented as mean ± SEM. Stars indicate groups that differ significantly from the control group (ANOVA followed by Dunnet t (2-sided) multiple comparison test.

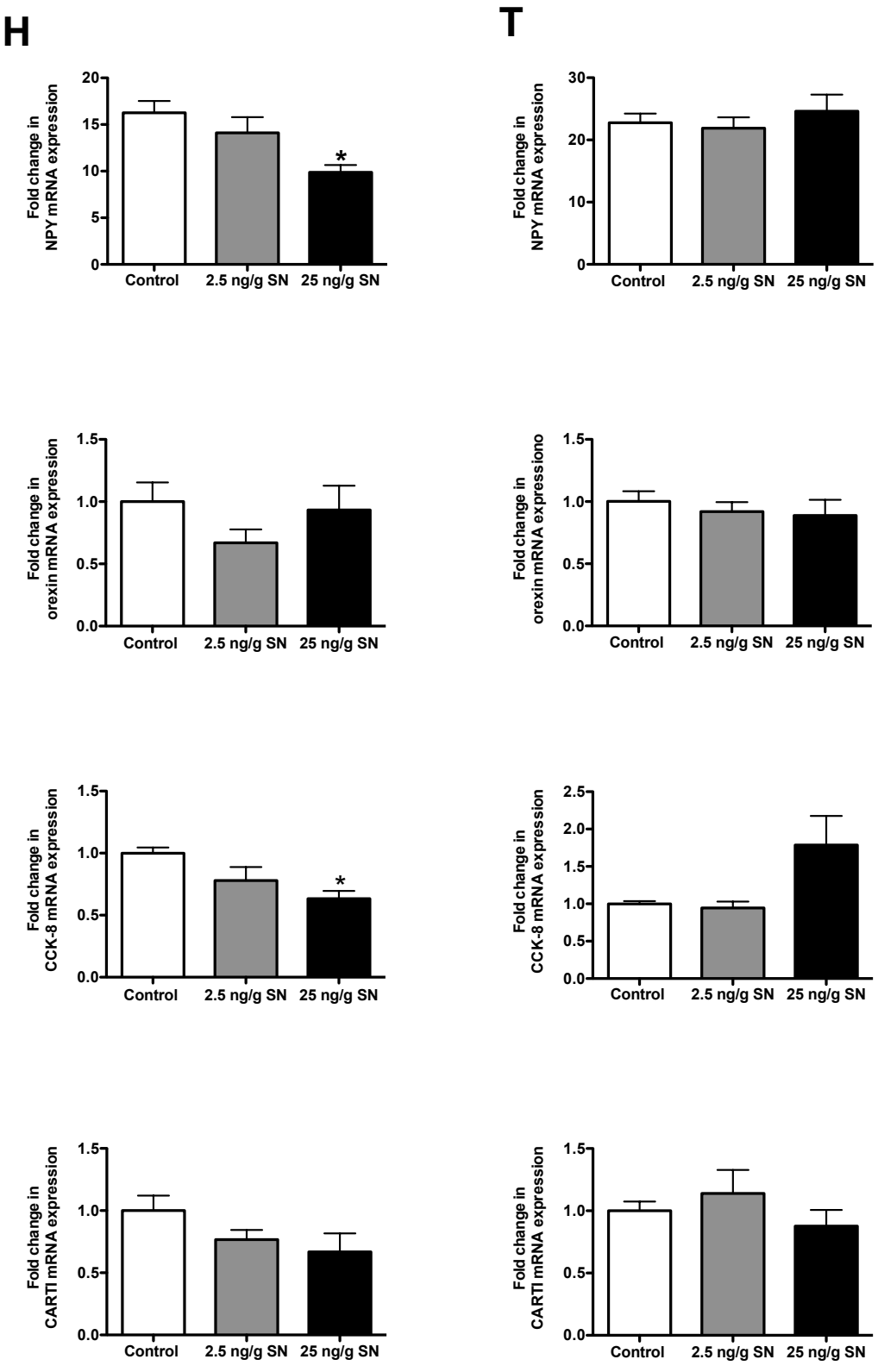

Fig 5.3. The effect of IP injection of SNa at 2.5 and 25 ng/g on mRNA expression of NPY, OX, CCK-8 and CART1 in hypothalamus (H) and telencephalon (T) 5 hours after treatment in female goldfish. Data are presented as mean ± SEM. Stars indicate groups that differ significantly from the control group (ANOVA followed by Dunnet t (2-sided) multiple comparison test.

Appendix II

Table A. Quantitative Q-PCR primers sets and conditions.

Gene	Forward Primer	Reverse Primer	Amplicon Size (bp)	Gene Bank Accession	Primer Concentration (uM)	Annealing Temp °C
SgII	CCTCAGCCAGAGAACTCCAC	ATGCCTCTATCCATCCGAGA	123	AF046002	0.15	63
NPY	CTGGGGATGGGACTCTGTTT	TTCGTCTGCTTGGGAACTCT	206	M87297	0.1	58
CCK-8	AACGCTGGAATCTGTGTGTG	GGGGCTTCATCATCCTCT	193	U70865	0.3	58
β-actin	ACTACTGGTATTGTGATGGACTCC	CGGTCAGGATCTTCATCAGGTAG	142	AB039726	0.1	60
OX	CGAAACGACACGTCCATC	GCGTCTCTCGCCCACTTTAC	99	DQ923590	0.3	60
CARTI	CCATGGAGAGCTCCAAACTC	TCTTGACCCTTTCCTGATGG	280	AF288810	0.15	60
Ghrelin	TGTGCTCTTTCCTTGTGTGTT	ACATCTCTTCTGCCCATCC	236	AF454390	0.4	60
18S	AAACGGCTACCACATCCAAG	CACCAGATTTGCCCTCCA	160	EF90971	0.1	58

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