University of Alberta

Ghrelin Actions and Signalling in GH and LH Release from Goldfish Pituitary Cells

by

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Abstract

Neuroendocrine regulation of vertebrate growth and reproduction is a multi-factorial process and includes growth hormone (GH) and luteinizing hormone (LH) release. In goldfish, the orexigenic peptide ghrelin ($gGRLN_{19}$) stimulates GH and LH release, making it a potential link between energy balance, growth, and reproduction. However, how $gGRLN_{19}$ interacts with other neuroendocrine regulators of GH and LH release and its mechanisms of actions are unknown. To elucidate these aspects, I cloned a partial sequence of the gGRLN receptor (gGHS-R) and demonstrated the presence of gGHS-R mRNA, together with that for gGRLN, in goldfish brain and pituitary tissues using polymerase chain reactions. Preliminary Western blot results supported the presence of gGHS-R1-like proteins in goldfish pituitary extracts. Using goldfish pituitary cell primary cultures in cell column perifusion hormone release and fura-2, AM Ca^{2+}-imaging studies, $gGRLN_{19}$-induced GH and LH release were found to involve increases in intracellular free Ca^{2+} levels via extracellular Ca^{2+} entry through L-type voltage-sensitive Ca^{2+} channels, protein kinase C, and nitric oxide. However, protein kinase A only participated in $gGRLN_{19}$-induced LH release, while its activation potentiated $gGRLN_{19}$-elicited GH release. Utilization of nitric oxide synthase (NOS) isoforms differed, with nNOS and iNOS participating in GH and LH responses, respectively. Pretreatment with pituitary adenylate cyclase-activating polypeptide (PACAP) potentiated $gGRLN_{19}$-induced GH, but not LH, release while pretreatment with $gGRLN_{19}$ followed by PACAP did not enhance hormone secretion. $gGRLN_{19}$ increased GH release in the presence of goldfish GH-releasing hormone (gGHRH) and dopamine (DA), but potentiated GH responses were not observed. Both basal LH release and the LH responses to $gGRLN_{19}$ were inhibited by gGHRH and DA. On the other hand, $gGRLN_{19}$ failed to further elevate GH and LH secretion in the presence of salmon gonadotropin-releasing hormone (sGnRH) and chicken (c)GnRH-II.

Overall, $gGRLN_{19}$ induces both GH and LH release through gGHS-R mediated, cell-selective signalling pathways and exhibits complex interactions with other endogenous regulators of pituitary hormone release in a cell type-, ligand-, and treatment sequence-specific manner. Results support the idea that $gGRLN_{19}$ regulates growth and reproduction, identify gGHRH as a novel LH release inhibitor, and further our understanding of neuroendocrine regulation of these processes.

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List of Abbreviations

$[Ca^{2+}]_i$	intracellular calcium
1400W	N-(3-(aminomethyl)benzylacetamidine
5HT	serotonin
7-Ni	7-nitroindazole
AA	arachidonic acid
AC	adenylate cyclase
AGH	aminoguanidine hemisulfate
ATP	adenosine triphosphate
Bay K8644	1,4-Dihydro-2,6-dimethyl-5-nitro-4-(2-[trifluoromethyl]phenyl)pyridine-3-carboxylic acid methyl ester
Bis-II	bisinololymaleimide II
BLAST	basic local alignment search tool
bp	base pairs
Ca^{2+}	calcium
cAMP	cyclic adenosine monophosphate
cDNA	complementary deoxyribonucleic acid
cGMP	cyclic guanosine monophosphate
cGnRH-II	chicken gonadotropin-releasing hormone-II
cNOS	constitutive nitric oxide synthase
CHO	chinese hamster ovary
CRE	cyclic adenosine monophosphate (cAMP) response element
D1R	dopamine receptor, type 1
D2R	dopamine receptor, type 2
DA	dopamine
DAG	diacylglycerol
DIC	differential-interference contrast
DiC8	1, 2-dioctanoyl-*sn*-glycerol
DMSO	Dimethyl sulfoxide
EF-1α	elongation factor 1 alpha
eNOS	endothelial nitric oxide synthase
ERK	extracellular signal-regulated kinase
FSH	follicle stimulating hormone (a.k.a. GtH-I in fish)
fura-2, AM	5-Oxazolecarboxylic acid, 2-(6-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-(2-(bis(2-((acetyloxy)methoxy)-2-oxoethyl)amino)-5-methylphenoxy)ethoxy)-2-benzofuranyl)-, (acetyloxy)methyl ester
$gGRLN_{12}$	goldfish ghrelin, 12 amino acid form
$gGRLN_{19}$	goldfish ghrelin, 19 amino acid form
GH	growth hormone
GHRH	growth hormone-releasing hormone
gGHRH	goldfish growth hormone-releasing hormone
GHRP-6	growth hormone-releasing peptide 6

GHS-R	growth hormone secretagogue receptor
GnIH	gonadotropin-inhibitory hormone
GnRH	gonadotropin-releasing hormone
Gö 6976	12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole, Go 6976
GOAT	ghrelin o-acyltransferase
GRLN	ghrelin
GtH-I	gonadotropin 1 (a.k.a. FSH)
GtH-II	maturational gonadotropin (a.k.a. LH)
Gα	g-protein, alpha subunit
H-89	N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride
HEK	human embryonic kidney
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIT-T15	clonal hamster beta-cell line
hpf	hours post fertilization
ICV	intracerebroventricular
IGF-I	insulin growth factor I
IL-10	interleukin 10
IL-1β	interleukin 1 beta
iNOS	inducible nitric oxide synthase
IP	interperitoneal
IP3	inositol trisphosphate
kDa	kilodalton
KT 5720	(9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3,2,1-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester
LB	lysogeny broth
LH	luteinizing hormone (a.k.a. GtH-II in fish)
LPS	lipopolysaccharide
LVSCC	L-type voltage-sensitive calcium channels
MAPK	mitogen-activated protein kinase
MEK	mitogen-activated protein kinase kinase
mRNA	messanger ribonucleic acid
NCBI	National Center for Biotechnology Information
NE	norepinephrine
NF-1	nuclear factor I
NF-κB	nuclear factor kappa B
NIF	nifedipine
nNOS	neuronal (a.k.a. brain) nitric oxide synthase
NO	nitric oxide
NOS	nitric oxide synthase
NPY	neuropeptide Y
PAC1	pituitary adenylate cyclase-activating polypeptide type I receptor

PACAP	pituitary adenylate cyclase activating protein
PCR	polymerase chain reaction
PI3K	phosphoinositide-3-kinase
Pit-1	pituitary-specific transcription factor
PKA	protein kinase A
PKC	protein kinase C
PLC	phospholipase C
PTIO	2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide
qPCR	quantitative reverse transcriptase polymerase chain reaction
RACE	rapid amplification of cDNA ends
RNA	ribonucleic acid
RQ	relative quantification ($=2^{-\Delta\Delta Ct}$)
RT-PCR	reverse transcriptase polymerase chain reaction
SERCA	sarco/endoplasmic reticulum calcium ATPase
sGC	soluble guanylate cyclase
sGnRH	salmon gonadotropin releasing hormone
SNP	sodium nitroprusside
SS	somatostatin
Tg	thapsigargin
TMD	transmembrane domain
TNF-α	tumor necrosis factor alpha
USA	united states of America
USF	upstream stimulatory factor
UTR	untranslated region
VERAP	verapamil
VSCC	voltage-sensitive calcium channel

Chapter 1 – Introduction and Background

In vertebrates, body growth, sexual maturation, and many other key physiological functions are regulated by growth hormone (GH) and the gonadotropin luteinizing hormone (LH) [28, 315]. LH stimulates gonadal sex steroid production and plays an important role in enhancing final gamete maturation and the induction of ovulation and sperm release [315]. GH is known to be an important stimulator of somatic growth and it also enhances the gonadal steroidogenic responses to LH [28]. The release of these hormones from the pituitary is controlled by many interacting hypothalamic factors that integrates multiple inputs from various regions of the brain, as well as by factors from other internal organs or tissues [28, 315]. Thus, understanding the signalling mechanisms involved in mediating the effects of these regulators on pituitary GH cells (somatotropes) and LH cells (gonadotropes), as well as how these intracellular mechanisms may interact, is critical to the overall comprehension of growth and reproduction regulation in vertebrates. My thesis focuses on how the neuroendocrine regulator ghrelin (GRLN), a well-known orexigenic peptide acting as the endogenous ligand for the GH secretagogue receptor (GHS-R) [169, 171], exerts its effects on GH and LH (also called maturational gonadotropin or GTH-II in fish; [315]) release from goldfish pituitary cells at the level of receptor-signal transduction and its interactions with several known regulators of GH and LH secretion in this model system. Another pituitary gonadotropin, follicle-stimulating hormone (FSH; also called GTH-I in fish [315]), plays an important role in initiating gametogenesis [315]; however, as a result of limitations within the laboratory, a radioimmunoassay for goldfish FSH is not currently available and thus the effects of GRLN on FSH secretion is not examined in this thesis.

1.1. Ghrelin

1.1.1. Discovery and general structure

The discovery of GRLN was reported by Kojima and colleagues in 1999 [169]. It is a member of a peptide superfamily which includes various forms of GRLN and motilin and has been referred to as the GRLN family of peptides [171, 236]. GRLN has since been shown to be involved in the regulation of many physiological processes, including stimulation of GH secretion and feeding behaviour, as well as regulation of LH secretion and immune function [171, 319]. The amino acid sequence of GRLN varies in different vertebrate groups but the N-terminal sequence is relatively well conserved (Fig. 1.1) [170, 171]. The first 10, 7, and 6 amino acids in the NH_2 terminal are highly conserved, respectively, in mammalian, avian and fish GRLNs [170, 171]. Amphibian forms are not well characterized but appear to exhibit a greater degree of variation [170, 171]. The third residue is almost universally serine (although bullfrogs contain threonine) and contains an acyl modification, generally n-octanoylation [170, 171], which is critical to GRLN's GH-releasing ability. Octanoylation is carried out by the GRLN O-acyltransferase (GOAT) enzyme [98, 358]. Peptide lengths vary from 28 amino acids in mammals to only 12 in some teleost forms [170, 171, 319].

In goldfish, GRLN is predicted to have at least two possible forms based on sequence analysis, a 12 amino acid form ($gGRLN_{12}$) and a 19 amino acid form ($gGRLN_{19}$) (Fig. 1.2; [321]), however only the 19, but not the 12, amino acid form is present among the 11 variants biochemically purified from goldfish intestine [221]. Like other GRLN forms, gGRLN function in the pituitary is dependent on an n-acyl modification on the 3rd residue [321]. Synthesized $gGRLN_{19}$, containing an octanoylation (C8:0) on the 3rd serine residue, has been shown to be active in fish [321]; however, GRLN containing other modifications on the 3rd serine residue, from acetyl (C2:0) up to palmitoyl (C16:0), have also

been shown to be biologically active in CHO-GHS-R_{62} cell lines, as indicated by increases in intracellular free Ca^{2+} concentration ($[Ca^{2+}]_i$) [207].

1.1.2. GRLN gene structure, products, and regulation

The location and expression profile of the GRLN gene locus is unknown in many animal models, however the human GRLN gene has been identified on the short arm of chromosome 3 [279, 280], while the mouse [304] and zebrafish [152, 241] GRLN genes have been localized to chromosome 6. These genes are organized in a similar manner with four coding exons, as well as one upstream exon in the mouse and two in the human loci [279, 280, 304]. The coding exons of the both the murine and human gene translate into a 117 amino acid prepro-GRLN which is cleaved to produce a 94 amino acid pro-GRLN peptide from amino acids 24-117. This pro-GRLN is further processed to form a 28 amino acid GRLN peptide and a 66 amino acid C-terminal peptide called C-GRLN (Fig. 1.3). Detailed post-translational processing information is currently unavailable for the zebrafish pre-proGRLN.

In addition to GRLN, the GRLN gene encodes for other products, including identified hormones, regulatory products, and putative peptides. A recent review [280] describes 7 different gene-derived transcripts from the human GRLN gene and 6 for the mouse (Fig. 1.3 & 1.4.). Peptides, including GRLN, Δ3D peptide, and obestatin, can be translated from various transcripts. GRLN can only be fully translated from the wild-type prepro-GRLN, propro-des-Gln14-GRLN, and Δex2 prepro-GRLN transcripts, all of which are found in both the human and mouse model systems. Truncated forms of GRLN, the function of which remains to be fully understood, can be translated from many human and mouse transcripts, such as Δex2 prepro-GRLN and In2 prepro-GRLN (Fig. 1.4.). Full C-GRLN, which contains the full obestatin sequence, can be obtained from many human forms but only wild-type and prepro-des-Gln14-GRLN in the mouse. Obestatin can be obtained from alternate C-GRLN transcripts in both

mouse and humans, nevertheless, this links the potential production of obestatin directly to production of GRLN from prepro-GRLNs [280, 367]. Although the significance of the various prepro-GRLN gene-derived transcripts, and their products, has not been well-characterized, it has been postulated that some of these products act as regulatory peptides by interacting with either GRLN or its receptor. For example, if alternately spiced transcripts were not further processed, these longer products may bind the GHS-R and decrease the affinity of GHS-R for other molecules and thus increasing the specificity and selectivity for GRLN binding. Alternatively, it has been proposed that smaller products from alternatively spliced prepro-GRLN transcripts may act as GRLN-binding proteins to reduce the turnover rate for GRLN, or they may interact with GOAT, which octanylates the 3rd serine in the GRLN peptide [98, 156, 189], thus modulating GRLN activity.

The gGRLN gene represents the best characterized in regards to teleost GRLN gene structure and processing. The gGRLN gene possesses 4 exons and 3 introns which is transcribed to form a 490 base pair (bp) prepro-GRLN message encoding a 103 amino acid prepro-GRLN peptide (Fig. 1.2) [321]. The 205 bp exon 1 contains the 5' untranslated region (UTR) and the sequence for the first 12 amino acids of mature gGRLN and is followed by a 79 bp exon 2, which translates into the remaining portion of the peptide. Exon 3 is 109 bp in length and encodes part of the C-peptide (C-GRLN) while exon 4 is 95 bp long and contains the remaining C-GRLN sequence and the 3' UTR. Depending on how cleavage occurs, further processing of the 490 bp goldfish prepro-GRLN is predicted, as mentioned earlier, to yield either a 12 or 19 amino acid long mature peptide [321]. Preliminary polymerase chain reaction (PCR) evidence suggests that goldfish tissues express GOAT mRNA (C. Grey, unpublished), which together with the requirement of n-acyl modification on the 3rd residue for the hormone-releasing action of gGRLN [321] suggests that octanylation occurs as part of gGRLN peptide processing.

Although regulation of the GRLN gene has received a lot of attention, there is limited knowledge regarding the mechanisms modulating its expression, aside from work done in human and rat models. A couple of studies in fish models have identified changes in expression related to sexual dimorphism, developmental stages, and seasonality [319]. For example, in tilapia it has been shown that prepro-GRLN mRNA levels are higher in females [248] and in Arctic char prepro-GRLN mRNA levels are higher in the autumn, prior to the sexual maturation season [90]. In the rat and human, molecular cloning and sequencing studies have identified two potential upstream stimulatory factor (USF) binding sites and both distal and proximal transcription initiation sites in the GRLN genes [339]. The core promoter sequence in the human GRLN gene spans bps -667 to -468 and cloned segments of this promoter, found upstream of the proximal transcription initiation site, show the highest level of activity when transfected into stomach and pituitary cell lines [339]. Similarly, the rat core promoter sequence is a 112 bp sequence spanning -581 to -469 upstream of the distal transcription initiation site. Deletion of 5' upstream regions, which includes exon 1 and 2, reduces relative promoter activity in the human GRLN gene but increases relative promoter activity in the rat GRLN gene [339], suggesting the regulation of this gene is species specific.

1.2. The receptor for GRLN, the GHS-R

1.2.1. Discovery and general structure

Prior to the discovery of GRLN, many researchers noted that a handful of synthetic ligands could induce GH release from the pituitary gland of various organisms, and the term GH secretagogues (GHS) was coined to refer to these stimulators [60]. The GHS-R was subsequently discovered in 1996, and was shown to respond to synthetic GHS, including GHRP-6 and hexarelin, as well as pituitary extracts [128]. GHS-R was subsequently supported as the endogenous

receptor for GRLN based on the ability of GRLN to induce increases in $[Ca^{2+}]_i$ in GHS-R transfected CHO cells, as well as GRLN dose-response work with rat primary pituitary cultures [169]. GHS-R has since been characterized as a multiple transmembrane domain (TMD) G-protein coupled receptor and is generally placed within its own superfamily or within the motilin family of receptors, which includes other receptors with gastrointestinal peptides or neuropeptides as endogenous ligands such as GRLN, motilin, neuromedin U, obestatin, and neurotensin [171]. The GHS-R has two identified isoforms: GHS-R1a with seven TMD and GHS-R1b, a truncated form with only five TMD [128, 210]. Only GHS-R1a is considered to be active in mediating GRLN-induced GH release [60, 128]. Recent evidence, however, suggests that the presence of GHS-R1b leads to formation of GHS-R1a/GHS-R1b heterodimers and the translocation of this receptor complex to the nucleus, thus resulting in an inhibitory form of regulation [187].

1.2.2. GHS-R gene structure and regulation

The majority of information on the GHS-R gene comes from mammalian models [254]. In humans, the GHS-R gene is found at chromosomal location 3q26.2 [210, 327] which puts it in close proximity to the GRLN gene, also found on chromosome 3 [279, 280]. The genomic structure of the human GHS-R1a has been characterized as comprising a 5' flanking region, which contains the promoter, followed by two exons, one intron, and the 3' flanking region (Fig. 1.1) [128, 254]. The first exon codes for 796 bp, corresponding to the first 5 TMD, followed by 2152 nucleotides of intron sequence and then 1101 bp, corresponding to TMD 6 and 7 and the C-tail of the GHS-R [128, 254] (Fig. 1.5). Positive regulation promoter regions exist between -951 to -643 bp and between -643 and -460 bp, which are under the control of pituitary-specific transcription factor (Pit-1) [128, 254]. The human GHS-R1a is 366 amino acids long and has a molecular mass of approximately 41 kDa [128, 254]. In contrast, the GHS-R1b is only 289 amino acids long, however the first 265 amino acids are identical to that of GHS-

R1a, suggesting that it is a truncated form of the full-length receptor [60, 128, 254]. Interestingly, recent research suggests distinct subtypes of the GHS-R1a may be present in different tissues. For example, evidence supports that the heart and pituitary cells contain unique GHS-R sequences which show a high degree of similarity with the 41 kDa stomach form, but are 57 and 84 kDa, respectively [24, 242]. The bovine GHS-R gene similarly mapped to BTA1, between BL26 and BMS4004, on chromosome 3 [67]. Not surprisingly, the protein structure of the bovine GHS-R shows a high level of similarity with other mammalian forms, such as ovine (95%) and human GHS-Rs (94%) (Fig. 1.2), and demonstrates a typical 7 TMD structure. Bovine GHS-R1a cDNA has been sequenced as 1101 bp in length, encoding a 366 amino acid receptor while bovine GHS-R1b cDNA is 879 bp in length and encoding for a 292 amino acid receptor [67]. Interestingly, the same study identified many GHS-R polymorphisms using single base extension in conjunction with fluorescent amplified-fragment length polymorphism analysis and allele-specific polymerase chain reaction (PCR), however no functional significance has been identified for these bovine variants.

Molecular cloning and in silico analysis of GHS-R genes from non-mammalian vertebrates also provides a broader understanding of this receptor. Molecular analysis of the chicken GHS-R gene shows that it has a high degree of similarity with its mammalian homologs [103, 305]. However, cloning of the chicken GHS-R cDNA reveals a 48 bp deletion at the 5'end of exon 2 which translates into a lack of TMD 6 in the chicken GHS-R. The significance of this deletion remains unknown but this is likely to have an impact on the function of this receptor (see Section 1.3 below).

Work in fish species has also revealed some interesting findings. Three potential pufferfish GHS-R genes were identified [247]. When the one most similar to the human GHS-R1a (58% similar protein identity) was transfected into HEK-293 cells, they demonstrated similar bioluminescence responses, following GHS treatment, as human GHS-R1a transfected into similar systems [247].

Examination of the transcriptional regulation of the seabream GHS-R gene revealed the presence of many common transcription binding sites, such as activator protein 1 (AP-1), nuclear factor I (NF-1), and USF sites, as well as multiple unique sites including Pit-1, now identified in many other species, and estrogen and glucocorticoid response element sites [359]. These findings suggest that GHS-R gene expression regulation is complex and support the idea that GRLN/GHS-R system plays a role in the regulation of a multitude of physiological processes, such as growth, development, reproduction and immune function ([359] and see Section 1.3 below). In silico analysis followed by immunohistochemistry and intestinal bulb functional characterization identified a zebrafish GHS-R with a high degree of similarity to other fish and mammalian GHS-R forms [241]. Tilapia [154], rainbow trout [153], channel catfish [292], and Atlantic cod [356] GHS-Rs, or GHS-R-like receptors, were all characterized in the last five years. Rainbow trout has two predicted GHS-R1a-like receptor variants, both 387 amino acids long, and 3 predicted GHS-R1b-like orthologs [153]. The rainbow trout GHS-R1a-like receptor and GHS-R1b-like receptor share similar amino acid sequences with other fish GHS-R forms; 67% to pufferfish GHS-R1a, 71% to black seabream GHS-R1a, 67% to zebrafish GHS-R1a and -2a, 60% to chicken GHS-R1a, and 58% to rat GHS-R1a [153]. The tilapia GHS-R-like receptor shows a high level of similarity to the seabream (89%) but is less similar to rat and chicken forms of this receptor, showing only 54% and 60% similarity, respectively [154]. The two channel catfish GHS-Rs also show varying similarity with other identified GHS-Rs, ranging from 76% similar to zebrafish GHS-R1a to only 59% with rainbow trout GHS-R1a-like receptor [292]. Interestingly, tilapia and rainbow trout GHS-R-like receptor expressed in mammalian HEK-293 or CHO cell lines don't show increases in $[Ca^{2+}]_i$ in response to tilapia or rat GRLN [153, 154], suggesting that these may not be true GHS-Rs. Alternatively, these receptors may play a unique role in tilapia and rainbow trout physiological function and/or that these receptors may

require components absent in HEK-293 or CHO cells to be functional, although more research in this area is needed to clarify these possibilities.

While I was in the process of cloning the goldfish (g)GHS-R for this thesis work, researchers in Japan reported four full length gGHS-R sequence variants [151]. These are the gGHS-R1a (1083 bp type-1 and -2), and the gGHS-R-2a, (1104 bp type-1 and 1101 bp type-2), both of which are thought to be active receptors in GRLN activity [151]. Currently no gGHS-R1b forms have been identified in the goldfish and little else is known regarding genomic location, promoter regulation, or other genetic regulatory factors for gGHS-Rs.

1.2.3. GHS-R activity

GHS-R activity is complex. In pituitary cells, GHS mechanisms of action through GHS-R involve activation of phospholipase C (PLC), generation of inositol trisphosphate (IP3), and both mobilization of intracellular Ca^{2+} from stores and Ca^{2+} entry via L-type voltage-sensitive Ca^{2+} channels (LVSCCs) which lead to a biphasic response characterized by a transient increase in $[Ca^{2+}]_i$ followed by secondary sustained rise [25, 60, 121]. However, GHS-R also exhibits a high level of constitutive signalling and heterodimerization with other receptors which leads to switching of G-protein coupling and how ligands may act.

GHS-Rs transfected into COS-7 and HEK-293 display high levels of constitutive activity, as observed through inositol phosphate (IP) turnover and cAMP response element (CRE)-dependent gene transcriptional activity [124]. GRLN or GHS stimulation further increases this activity, while a GHS-R antagonist (inverse agonist) reduces it [124]. The constitutive activity of the GHS-R is approximately 50% of its maximal capacity and this activity is linked to the hydrophobicity of TMDs VI and VII [125], which are the sections absent from its truncated GHS-R1b forms. Unliganded GHS-Rs colocalize with trafficking or recycling compartment proteins, such as Rab-5, Rab-11, and transferrin, and

undergo internalization [123]. This spontaneous GHS-R internalization may be functionally relevant. For example, transfection of seabream GHS-R1a into HEK-293 cells reduces cadmium-induced apoptosis, but GRLN treatment does not modulate the level of apoptosis observed [182].

GHS-R also heterodimerizes with a number of other receptors and this heterodimerization alters its intracellular signalling. GHS-R1a heterodimerizes with dopamine (DA) D1 receptors (D1Rs) and this leads to an increase in DA-induced cAMP accumulation within the cell and a switch in G-protein coupling from the $G\alpha_{11/q}$ to the $G\alpha_{i/o}$ subunits [134]. Formation of GHS-R1a/DA D2 receptor (D2R) heterodimers also modifies classical D2R signalling leading to DA activation of Ca^{2+} mobilization and increases in $[Ca^{2+}]_i$ levels [158]. Moreover, pre-treatment of GHS-R1a and D2R co-expressing cells with GRLN decreases the effects of subsequent DA treatment, demonstrating cross-desensitization [158]. GHS-R1a has also been shown to heterodimerize with the serotonin (5HT) 2C receptor and the melanocortin 3 receptor [277]. It is interesting to note that all of these receptors which heterodimerize with GHS-R1a are also involved in the control of food intake and energy balance, suggesting heterodimerization likely plays a role in regulation of these functions. Thus, it appears that heterodimerization most likely allows for further control and fine-tuning, or even complete alteration, of how GRLN and other neuroendocrine factors exerts their physiological effects.

1.3. GRLN and the GHS-R: Distribution, functions, and signalling

1.3.1. Distribution and functions

GRLN has many functions within the body. To date, known functions of GRLN include regulation of food intake, pituitary hormone (GH, LH, prolactin, thyroid-stimulating hormone) release, pancreatic function, glucose and lipid

metabolism, heart function, gastric motility and acid secretion, sleep duration, memory, learning and behaviour, immune function, cell proliferation, and bone formation (reviewed in [152, 171, 189]). In addition, altered GRLN levels have been associated with several disease states, including anorexia nervosa, Prader-Willi syndrome, obesity, and type-2 diabetes [152, 171, 189]. GRLN's best characterized functions are its orexigenic action and its effects on GH and LH release (the focus of this thesis); these are briefly reviewed for both mammals and fish models below.

1.3.1.1. In mammals

Although GRLN and its receptor, GHS-R, have been identified in multiple vertebrates, including hamster [317], chicken [103, 267, 288, 305], Japanese quail [163, 360], and frog [95, 300], a great deal of our knowledge on the distribution of GRLN and GHS-R, come from human and rat studies. The earliest studies demonstrated the expression of GHS-R in the hypothalamus of pigs, rhesus monkeys and humans, and in rat heart [24, 128]. The current consensus is that in humans, both GHS-R1a and GHS-R1b mRNAs are expressed throughout various tissues, including brain, pituitary, gastrointestinal tract, thyroid, pancreas, spleen, myocardium, adrenal glands, skin, testis and ovary [27, 104]. Human GHS-R1a mRNA expression is highest in the pituitary and, interestingly, significant level of expression is found in somatotropes and the arcuate nucleus, an important hypothalamic area involved in the control of pituitary hormone secretion [27, 104]. In contrast, the highest levels of expression of GHS-R1b mRNA are found in the skin and myocardium, but its expression is only marginally lower in the pituitary [104]. GRLN mRNA is also expressed in most human tissues [27, 104]. Likewise, GHS-R mRNA expression is widespread among tissues in rodent model systems [71, 117, 286, 299, 370]. For example, the expression of GHS-R mRNA has been reported in brain areas, including the hypothalamus and hippocampus, as well as the pituitary, of rats [117] and mice [299, 370], and in testicular tissues of rats [14]. Western blot confirms the expression of GHS-R

protein in the hypothalamus, pituitary, and stomach of rats [286]; the gastrointestinal tract of rats and humans [71]; and the pancreatic islets of Langerhans, including β-cells, of rats [150]. These results support the idea that the GRLN/GHS-R system participates in the regulation of a wide range of physiological functions. Some of these functions will be briefly outlined here and in the next section, to illustrate the importance of GRLN in a larger perspective, however specific details on functions other than GH and LH regulation are beyond the scope of the current thesis. More detail on the following functions can be found in recent reviews [152, 171, 172, 189, 319].

GHS-R1a and GHS-R1b are both highly expressed in human myocardial tissue; however, GRLN mRNA copy numbers are quite low in comparison [104]. The functional significance of this discrepancy is unknown, but when taken together with studies supporting a unique GHS-R isoform in the heart [24], it may suggest that a modified form of GRLN, or a different ligand altogether, might be involved in the regulation of heart function through this receptor.

In support of pancreatic function, as suggested by GHS-R mRNA expression in human pancreatic tissue, GHS-R immunoreactivity has also been found in rat islets, with weak signals in β-cells and both GRLN and GHS-R immunoreactivity in the periphery of the islet of Langerhans. Furthermore, GRLN treatment induces insulin secretion from rat pancreatic islets [72]. These and other results indicate that GRLN is involved in maintenance of glucose levels, glucose and lipid metabolism, promotion of gluconeogenesis, and regulation of glucose uptake and fatty acid oxidization [172, 189, 268, 369].

Interestingly, GHS-R has also been found in human and rat testis [14, 101], and in rat testis this expression has been shown to change throughout development, with the general GHS-R gene expressed continually through postnatal development while the GHS-R1a genes is only detectable from puberty onward [14]. GHS-R, but not GRLN, has also been detected in human ovarian

tissues at different stages of the menstrual cycle [102], suggesting an endocrine target for peripherally released GRLN or that another ligand may utilize this receptor.

GRLN and the GHS-R mRNA have also been identified in many immune cells, including human T-cells, B-cells, and neutrophils [120]. Additional work on human and mouse immune cells has identified an anti-inflammatory role for GRLN. For example, exogenous GRLN treatment decreases the production of pro-inflammatory cytokines interleukin (IL)-1β and tumor necrosis factor (TNF)-α while augmenting the production of the anti-inflammatory cytokine IL-10 in lipopolysaccharide (LPS)-stimulated murine macrophages, a response which can be reduced with treatment of a GHS-R antagonist [337]. Additionally, chronic inflammation has been linked to modulation of GRLN levels in human and rat [244] and GRLN also inhibits pro-inflammatory responses and activation of NF-κB in human endothelial cells [188], further supporting a role for GRLN and its receptor in the immune system.

Although it is generally accepted that GRLN functions as an orexigenic factor, evidence in this regard is not straight forward, based on mammalian work. Multiple studies in mammalian models, including human, rat, and mouse, have reported orexigenic effects for in vivo GRLN treatments [171, 172, 233, 349, 350]; however, work with GRLN gene-knockout mice reveals that GRLN is not critical to regulation of body weight or feeding behaviour. For example, current work links hypothalamic appetite regulation to the involvement of neuropeptide Y (NPY) and AMP kinase (AMPK) signalling [4, 172, 215], and GRLN binding to the GHS-R leads to increases in AMPK and decreases in lipid synthesis [172, 173]; surprisingly, GRLN- or NPY-knockout mice show no abnormalities in body weight or feeding behaviour [80, 260], suggesting that GRLN and NPY are not critical to these functions, despite activating them. On the other hand, knockout of the GHS-R in mice leads to a lack of GRLN-induced GH release and feeding [299], supporting that GRLN does in fact mediate its orexigenic actions through

this receptor. Interestingly, despite GRLNs inability to stimulate GH and feeding behaviour in GHS-R knockout mice, these animals still grow normally and have normal body weights [299]. Early studies attempting to overexpress n-octanoyl-GRLN in mice were unsuccessful, in contrast, overexpressing des-acyl GRLN leads to smaller size, decreased body weight, and reduced serum IGF-1 levels [5, 131]. More recently, the overexpression of active GRLN in mice demonstrated hyperphagia, glucose intolerance, and reduced leptin sensitivity in the animals [18]. Thus it appears that GRLN does play a important role in the regulation of orexigenic behaviour, glucose regulation, metabolism, and body weight regulation, however the fact that removal of GRLN does not lead to significant observable changes in these physiological functions suggests that other factors may compensate for the loss of GRLN or that current models fail to fully identify how GRLN is involved in these processes.

Interestingly, the GRLN receptor GHS-R has been linked to the stimulation of GH release even before the discovery of GRLN. Indeed, once discovered, GRLN was shown to dose-dependently induce GH release via increases in $[Ca^{2+}]_i$ in both humans and rats when administered in vivo, as well as in vitro [169, 171]. Furthermore, co-administration of GHRH and GRLN elicit additive GH release responses in human [119]. However, some controversy regarding a link between GRLN and GH has surfaced. When examining correlations between circulating levels of GRLN and GH, some studies show no relationship [6], while others link them to fasting and time of day [175, 216, 231]. Most early studies on circulating GH and GRLN, however, examined only total GRLN levels and did not differentiate between the GH-releasing form of GRLN, acyl-GRLN, and the non-active form, des-acyl GRLN. A more recent study, focussing on acyl-GRLN, does support a link between GRLN's circulating levels and GH release in fed and fasting men [234, 235].

Although GRLN's ability to induce GH release is well established, and consistent across all organisms tested to date, the direct effects of GRLN on LH

secretion remain somewhat controversial and its mechanisms of action are not fully understood. Many studies, however, have linked GRLN to LH release in multiple vertebrate species [152, 171]. In mammals, GRLN has been reported to stimulate, inhibit, as well as to have no effects on LH release. In human clinical studies, GRLN reduces plasma LH levels and abolishes LH pulsatility in healthy males [167, 181]. On the other hand, GRLN injection does not affect GnRH-induced LH or FSH secretion in normally cycling women, indicating that GRLN may have different effects depending on the sex and reproductive state. In ovariectomized rhesus monkey, intravenous human GRLN treatment also decreases LH plasma levels and pulse frequency, without affecting pulse amplitude [336]. Similarly, an intracerebral 3rd ventricular (ICV) injection of GRLN in ovariectomized rats suppresses LH levels for 1 hr through a reduction in pulse frequency, but not the amplitude, of LH secretory pulses [93]. On the other hand, although GRLN was initially reported not to have direct actions on LH release in rats [169], later studies reveal that GRLN directly stimulates LH release from male and female rat pituitaries in vitro, but this stimulatory effect is affected by the estrus status of the donor female [83, 84]. Furthermore, the same group showed that GRLN-induced LH secretion in vivo is inhibited in prepubertal, as well as gonadectomized male and female rats, and GRLN consistently attenuates gonadotropin-releasing hormone (GnRH)-induced LH release [82, 83]. GnRH neuronal secretion is stimulated by kisspeptin and GRLN inhibits GnRH release and kisspeptin mRNA expression in rats [83, 87]. Taking together these observations, it seems likely that GRLN has multiple effects on the hypothalamic-pituitary axis regulating LH secretion in mammals, with a direct stimulatory effect on basal LH release, as well as inhibitory effects by actions on the kisspeptin-GnRH system and attenuation of GnRH action.

1.3.1.2. In fish

In a few fish species, tissue expression studies of GRLN and the GHS-R have been reported. In rainbow trout, mRNA for the GHS-R1a-like peptide and

GHS-R1b-like peptide are predominantly expressed in brain and pituitary tissues [153]. The mRNA levels of the two identified channel catfish GHS-Rs vary among tissues, with the level of GHS-R1a mRNA expression being highest in the pituitary while GHS-R2a mRNA expression is low in the pituitary but high in other tissues, such as the Brockman bodies, intestine, gall bladder, spleen, and muscles [292]. Similarly, gGHS-R isoforms and subtypes are also differentially expressed in various goldfish tissues [151]. For example, while both of the gGHS-R1a isoforms are expressed in the brain, gonads and gill, the gGHS-R1a-2 isoform appears to have much higher mRNA expression in the liver and testis, as shown via quantitative real-time polymerase chain reaction (qPCR) [151]. On the other hand, the gGHS-R2a-1 isoform shows very low expression level in the gills but the gGHS-R2a-2 isoform appears to have high expression in the gills and lower expression in the ovary, liver and intestine regions of the goldfish [151]. However, protein levels would need to be examined to confirm any differences currently reported. Whether these differences in mRNA expression translate into functional differences remains unknown, but given that GRLN has so many physiological functions (see Section 1.3.2 below), these data suggest that specific functions of GRLN in different fish tissues may be mediated, at least in part, through the use of different receptor isoforms.

In goldfish, ICV and intraperitoneal (IP) injections of the 12 and 19 amino acid gGRLN forms, as well as human GRLN, at doses of 1 and 10 ng/g body weight, increases food intake [320]. On the other hand, serum levels of GRLN decrease within 1 h after feeding while starvation induces a transient increase in serum GRLN levels from days 3-5 [320]. In parallel with these changes in serum GRLN levels, feeding causes a decrease in the postprandial levels of prepro-GRLN mRNA while starvation induces increases in prepro-GRLN mRNA in both the hypothalamus and gut [322]. These results support the idea that GRLN is also orexigenic in fish. Although research in other fish species is limited, GRLNs orexigenic effects have been confirmed in the tilapia, where long-term (21 days)

IP administration of decanoylated GRLN, a major form found in the tilapia, increased both food intake and weight gain [269]. On the other hand, studies in the rainbow trout have produced mixed results. One study demonstrated no effect on feeding at 12 h following IP administration of trout GRLN in 2 year old rainbow trout [149], while another study using juvenile trout and GHSs and rat GRLN found increased food intake at 2 or 5 h post IP injection [282]. Whether this discrepancy is related to the ligand used and/or the age of the trout has yet to be clarified. More work is certainly needed, however, to understand GRLNs involvement in feeding behaviour and weight gain in various fish species.

In vivo and in vitro effects of $gGRLN_{12}$ and $gGRLN_{19}$ on GH release in goldfish have also been explored to some extent. ICV and IP injections increase serum GH levels while static incubation treatments elevate GH secretion from primary cultures of goldfish pituitary cells [322, 323]. GH secretion induced by $gGRLN_{19}$ is abolished by the well-established inhibitory regulator of GH secretion somatostatin-14 (SS-14) in both cell column perifusion and static incubation experiments [322, 323]. Similar findings supporting a GH-inducing role for GRLN can be found among other fish species. For example, in tilapia, IP injections of GHSs lead to increased GH in the circulation [281], whereas rat, eel, and tilapia GRLN all induce increases in pituitary GH and GH mRNA levels [88, 152, 270]. Rat GRLN also increases GH release and GH mRNA expression in orange spotted grouper pituitary fragments [263]. Trout GRLN stimulates GH release from rainbow trout pituitary cells and GRLN IP injection increases GH release and GH mRNA in channel catfish over 3 h [155]. Taken together, these and other findings support the idea that the GH-releasing ability of GRLN is a highly conserved feature of this peptide in fish as in mammals.

Available information also supports a role of GRLN in regulating LH secretion in teleosts, although such data are derived solely from studies on two fish species. In common carp, treatment with human GRLN significant elevates basal and GnRH analogue-induced LH secretion from pituitary cells obtained

from females in 10 h static incubation experiments but has no significant effect on pituitary cells from males. However, in 24 h incubation experiments, human GRLN has no effects on basal LH secretion from pituitary cells derived from either sex, and yet still augments GnRH analogue-induced LH release [293]. Both of the predicted endogenous forms of goldfish hypothalamic/pituitary GRLN ($gGRLN_{12}$ and $gGRLN_{19}$) increase LH release from dispersed goldfish pituitary cells in static incubation and perifusion experiments [322]. Moreover, ICV and IP injections of $gGRLN_{19}$ elevate serum LH levels [322]. $gGRLN_{19}$-induced LH release in vitro is also inhibited by a GHS-R antagonist, d-Lys^3-GHRP-6 [322], indicating that this peptide likely acts via GHS-R at the level of the pituitary.

1.3.2. GRLN signalling

In general, intracellular signalling pathways involved in various GRLN actions include increases in $[Ca^{2+}]_i$, LVSCCs, activation of PLC/IP3-diacylglycerol (DAG)/protein kinase C (PKC), nitric oxide synthase (NOS)/NO, and adenylate cyclase (AC)/cAMP/protein kinase A (PKA) [24, 84, 106, 128, 152, 162, 171, 202, 271, 297]. This is consistent with previous information on the mechanisms of GHS action on pituitary cells (see Section 1.2.3; [25, 60, 121]) since both GRLN and GHS act through GHS-R [60, 128, 169].

In terms of GRLN stimulation of GH release in mammals, Ca^{2+} and PKC signalling are thought to play important roles although most of our knowledge of GRLN-induced GH release and associated signalling is assumed using knowledge from studies on GHSs and the GHS-R, and signalling in this receptor is linked to entry of Ca^{2+} through LVSCCs via activation of the PLC/IP3/DAG/PKC pathway (Fig. 1.6) [27]. This lack of experimental evidence exploring detailed signalling within GRLN target cells does limit conclusions that can be made regarding specific signalling, such as within pituitary somatotropes, thus providing the need for work carried out in this thesis. In a 1999 report, GRLN treatment was shown

to induce increases in $[Ca^{2+}]_i$ in CHO cells transfected with GHS-R [169], supporting the involvement of Ca^{2+}. Previous work with rat somatotropes and the synthetic GHS GHRP-6 demonstrated a dose-response relationship between GHS-R and increases in $[Ca^{2+}]_i$ [25]. Interestingly, the same study also identified that this response was bi-phasic, with an initial large increase in $[Ca^{2+}]_i$ occurring rapidly and followed by smaller sustained oscillations. The removal of extracellular Ca^{2+} or the use of Ca^{2+}-channel blockers resulted in loss of only the sustained portion of the bi-phasic response while pretreatment with thapsigargin (Tg), an inhibitor of the sarcoplasmic/endoplasmicreticulum CaATPase (SERCA) which replenishes Ca^{2+} in intracellular Ca^{2+} stores, resulted in loss of the initial phase [25]. These observations support the presence of differential roles for entry of extracellular Ca^{2+}, likely through LVSCCs, and intracellular Ca^{2+} mobilization in mediating the biphasic GRLN action through the GHS-R in somatotropes. Also, depletion of PKC in these same cells using pretreatment with the PKC-activating phorbol PMA resulted in a loss of the second Ca^{2+} phase [25], supporting a role for PKC in mediating GRLN-induced action through the GHS-R in somatotropes. A similar study with rat somatotropes confirmed GHRP-6 action as both intracellular and extracellular Ca^{2+}-dependent and further supported the involvement of LVSCCs through the use of the dihydropyridine LVSCC blocker nitrendipine or the endogenous inhibitor SS-14 [121], which has been shown to inhibit LVSCCs in multiple systems [41, 133, 198, 334]. Another study using human pituitary somatotrophinomas demonstrated that GHRP-6 treatment dose-dependently elevated PI turnover and increased GH secretion [186], thus linking PLC activation (and by extension Ca^{2+} and PKC) to GHS-R activation and GH release.

Although many studies have not found evidence for the involvement of AC/cAMP/PKA as a key signalling mechanism in GRLN function, there are a few findings to the contrary. The AC inhibitor MDL-12,330A and the PKA inhibitor H-89 blocked GRLN-induced GH release, while GRLN treatment also

induced significant increases in cAMP levels in porcine pituitary cells [202]. Likewise, MDL-12,330A and H-89 inhibited GRLN-induced GH release from primary cultures of female baboon pituitary cells [162]. Interestingly, this study also demonstrated that although GRLN treatment alone had no effect on cAMP levels at 4 h, treatment with both GRLN and growth hormone-releasing hormone (GHRH) together potentiated increases in cAMP levels beyond those induced by GHRH alone [162]. It should also be noted that in HIT-T15 cells, GRLN increases cAMP levels at 5, 10, and 30 min, but not 60 min, post-GRLN treatment [106]. These results suggest that the ability of GRLN to induce increases in cAMP levels is time dependent, and is modulated by the presence or absence of other regulatory factors. Nonetheless, it is thought that GRLN-dependent PKC/Ca^{2+} signalling forms a parallel system to the cAMP/PKA/Ca^{2+}-dependent mechanisms utilized by GHRH, a major stimulatory neuroendocrine factor of GH secretion in mammals [159], to induce GH secretion [171] (also see Section 1.4 below).

GRLN action on GH release in mammals also involves NOS, as with its brain actions on food intake [227], influences on immune functions [100], and protective effects from ischemia in the heart [232, 297]. In dispersed female pig pituitary cells, GRLN-induced GH release is augmented by the addition of L-arginine methyl ester hydrochloride (a substrate for NOS), and GRLN effects are inhibited by haemoglobin (NO scavenger) and N(w)-nitro-L-arginine (NOS inhibitor) [271]. Interestingly, basal GH release is not affect by these inhibitors, suggesting that NOS participates in GRLN-induced GH release, but not the regulation basal GH levels, in this system [271].

Information on the signalling mechanisms mediating direct GRLN actions on LH release in mammals is restricted to one report demonstrating that GRLN-induced LH secretion from rat pituitary cells is NO-dependent [84]. In mammals, three general types of NOS are known, these being the two constitutively expressed NOS isoforms endothelial (e)NOS and brain/neuronal (n)NOS, and the

inducible (i)NOS [335]. Although GRLN enhances iNOS expression in rat myocardiocytes [103], the NOS isoform involved in GRLN stimulation of GH and LH release from mammalian pituitary cells is unknown, although nNOS is a major NOS isoform found in mammalian pituitary cells [262].

In the goldfish, NOS/NO is known to be involved in both GH and LH regulation and various isoforms are expressed (see Sections 1.4 and 1.5, respectively), however there are currently no studies examining NOS/NO involvement in GRLN regulation of pituitary function in any fish models.

1.4. Neuroendocrine regulation of GH

1.4.1. General perspectives

Regulation of GH release has long been studied due to the importance of GH in multiple physiological processes, such as stimulation of muscle formation [69], bone retention [273], energy mobilization [222, 284], appetite [147], osmoregulation [274], and social behaviour [148]. However, some of the traditional effects ascribed to GH, such as tissue growth and differentiation, are actually mediated by insulin-like growth factor 1 (IGF-1) produced either by the liver or locally in response to GH stimulation [22, 73].

GH secretion is thought to be pulsatile in both fish and mammals, and sexual dimorphism has also been shown in mammals. For example, male rats show discrete pulses of GH throughout the day with low inter-peak levels while female rat GH release is characterized as less pulsatile with higher inter-peak levels [28]. In mammals a diurnal rhythm also exist with blood GH levels generally higher in the scotophase relative to the photophase but is also subject to entrainment by the time of feeding [20]. Diurnal variations of GH secretion have also been characterized in a number of other species, such as salmonids [21], goldfish [204], and carp [368]. In fish, we generally see only one to two peaks of

GH a day, with higher overall levels observed at night [81]. In many fish species, seasonal changes have also been observed. In the spring and summer, during sexual maturation, GH levels are higher and generally coincide with vitellogenesis, as seen in salmon [21], carp [192], and rainbow trout [298]. GH has also been shown to stimulate gonadal steroidogenesis [287] and to play a role in gametogenesis [46, 315]. Despite a great deal of support for a role in growth and development, a direct correspondence between fish GH serum levels and growth rates has not yet been established, as blood GH levels often peak months before maximal growth rates are observed [204].

As recently reviewed [195], control of GH regulation in mammalian systems occurs mainly through stimulation by GHRH and inhibition by a 14 amino acid long SS isoform (SS-14). In addition, because GH release also directly stimulates SS-14 release, through action on GHRs located on SS-14 neurons [306], and SS-14 neurons project into the arcuate nucleus to directly inhibit GHRH release [307], GH release is pulsatile in nature as a result of first stimulation by GHRH and then inhibition by SS-14 and the concurrent reduction of GHRH stimulation through GH-induced negative feedback loops. The removal of negative feedback, when GH levels drop, restarts the next cycle of pulsatile release as GH stimulation of SS-14 release is diminished. Thus, "normal" GH release occurs in a pulsatile manner in vivo, as well as in response to stimulators and inhibitors such as IGF-1 and sex steroids [332, 333] with peaks every 3-5 hours [318]. Recent evidence suggest that other neuropeptides also play important roles in GH release at the level of the pituitary cells, such as pituitary adenylate cyclase-activating polypeptide (PACAP) [94] and GRLN (outlined in Sections 1.1 and 1.3).

In comparison to the situation in mammals, stimulatory neuroendocrine regulation in fish is well established as multifactorial and complex in nature, with many GH stimulators characterized. GHRH and PACAP, both members of the glucagon super family of peptides, have both been shown to stimulate GH release

in fish [41, 185]. In fish, GHRH perikarya are found in many regions of the brain, including the pre-optic region, anterior hypothalamus, and the tegmentum, with fibers extending into regions including the telencephalon and pituitary [264]. PACAP distribution is similar to that of GHRH [206]. In goldfish, PACAP- and GHRH-immunoreactive fibers show similar distribution patterns as in other fish, and have been identified in the pars distalis in the vicinity of somatotropes [28, 264, 345]. A purified putative carp GHRH dose-dependently stimulates GH secretion from perifused goldfish pituitary cells, with cells taken from sexually regressed fish showing a greater degree of responsiveness [331]. As expected, PACAP also dose-dependently stimulates GH release from perifused goldfish pituitary cells [345]. Similar effects of GHRH and PACAP have also been reported in salmonids [249] and carp [352]. A number of other neuroendocrine modulators have been characterized as stimulators of GH in fish, such as DA, GnRH, kisspeptin, and neuropeptide Y (NPY) and, as mentioned earlier, GRLN [28, 41]. Adding to the complexity, two GnRH forms are known to be released at the level of the goldfish pituitary – salmon (s)GnRH (also called type III GnRH) and chicken (c)GnRH-II (also called type II GnRH), and both forms enhance GH release [41].

As described earlier, inhibitory regulation of GH is thought to be mainly achieved through SS-14 action in mammals [195]. Although SS-14 is shown to be a highly conserved molecule across vertebrate species and is able to inhibit GH release in all animal models tested [13, 251, 265, 266], inhibitory regulation of GH in fish, however, is much more complex. Multiple SS isoforms exists in fish. In the goldfish there are four distinct isoforms; SS-14, gut SS-28, goldfish brain (gb)SS-28, and [Pro^2]SS-14. Of these, mRNA for SS-14, gbSS-28, and [Pro^2]SS-14 are expressed in the hypothalamus and mRNA for SS-14 and gbSS-28 are also present in pituitary cells and fragments, and all three brain SSs inhibits GH secretion, but each of these SSs have different abilities to attenuate the GH response to stimulatory factors [363, 365]. These observations suggest that

multiple SS isoforms regulate pituitary cell functions in this species and that they have ligand-selective actions. Likewise, multiple SS isoforms have also been reported in the rainbow trout and the orange spotted grouper [225, 353]. In addition to SS, norepinephrine (NE) and 5-HT have been shown to inhibit GH secretion by direct action at the level of goldfish pituitary cells [28, 41]. The ability of NE and 5HT to inhibit GH release not only differs from those of the brain SSs but also from one another. For example, while SS-14, [Pro^2]SS-14 and NE can abolish GnRH-induced GH release, 5HT and gbSS-28 only attenuates these responses. Similarly, while SS-14 and [Pro^2]SS-14 can abolish DA- and PACAP-induced GH secretion, 5HT is only partially effective against these responses and gbSS-28 only attenuates the response to PACAP, but has no effects on DA [41]. Many studies have also linked increases in IGF-I to reductions in GH secretion in fish, as well as in mammals [22, 91, 252, 340], supporting its role in negative feedback on the pituitary.

1.4.2. Intracellular signalling in neuroendocrine regulation of GH release in goldfish

GH release is a Ca^{2+}-dependent exocytotic process [371]. In mammals, GHRH and PACAP elicit stimulatory actions through 7 TMD receptors positively coupled to AC/cAMP/PKA and subsequent elevation of $[Ca^{2+}]_i$ [224, 285, 330]. As described in Section 1.3.2, GRLN stimulation of GH release in mammals is largely mediated by PKC, Ca^{2+}, and NO signalling cascades, although evidence for cAMP involvement has also been indicated. SS reduces GH release via reductions in $[Ca^{2+}]_i$ through intracellular signalling pathways involving AC, PLC, mitogen-activated protein kinase (MAPK), and membrane associated Ca^{2+} and K^+ channels [338]. In addition, SS exerts further control over GH release through action on critical components of the GH exocytotic process, such as Rab3 and SNARE proteins [208].

In fish, the GH release response is also regulated through multiple neuroendocrine regulators acting on various intracellular signal transduction mechanisms, and these are best characterized in goldfish (Fig. 1.7). For example, in the goldfish PACAP and DA stimulation of GH release are known to be mediated by PAC1 and D1R receptors, respectively, and subsequent activation of AC/cAMP/PKA and Ca^{2+} entry through LVSCCs [41, 177, 276]. The resulting increases in $[Ca^{2+}]_i$ is thought to promote fusion and release of vesicles containing GH in a readily releasable pool that is shared between PACAP and DA, since their GH release responses are not additive but both peptides can elevate $[Ca^{2+}]_i$ in the same goldfish somatotrope [41, 363]. On the other hand, DA, but not PACAP, action on GH release involves NOS/NO and arachidonic acid (AA) [41, 56]. The involvement of AC/cAMP/PKA in mediating GHRH stimulation of GH release is assumed based on the ability of synthetic goldfish (g)GHRH to stimulate increases in cAMP in mammalian cell lines transfected with zebrafish GHRH receptors [185] and based on information on mammalian GHRH signalling [209]. Whether this occurs in goldfish somatotropes, however, has not yet been directly examined. PACAP and DA stimulation of GH release also involve mobilization of Ca^{2+} from intracellular stores but the pharmacological properties of these stores differ; in particular, PACAP, but not DA, utilizes stores that are sensitive to caffeine, but not ryanodine [41]. On the other hand, DA, but not PACAP, uses NOS/NO signalling [41, 218].

cGnRH-II and sGnRH, through action on GnRH receptors located on goldfish somatotropes, induce GH release through PKC-dependent pathways, which also lead to entry of extracellular Ca^{2+} through LVSCCs and increases in $[Ca^{2+}]_i$ [41]. Both sGnRH and cGnRH-II also utilize intracellular Ca^{2+} stores; however, while cGnRH-II actions are mediated by store(s) that are sensitive to both caffeine and ryanodine, sGnRH uses IP3- and caffeine-sensitive, but ryanodine-insensitive, stores and only sGnRH action is sensitive to modulation by mitochondrial Ca^{2+} buffering [28, 41]. In addition to PKC and Ca^{2+}, iNOS/NO

and soluble guanylate cyclase (sGC)/cyclic (c)GMP, as well as phosphoinositide-3-kinase (PI3K) pathways, but not AA, also participates in sGnRH and cGnRH-II action on GH secretion [28, 41]. Recently, goldfish kisspeptin is also shown to utilize extracellular Ca^{2+} entry through LVSCC to enhance GH secretion [50].

Interestingly, the two GnRHs can induce $[Ca^{2+}]_i$ increases in the same goldfish somatotrope and GnRH-sensitive cells are also responsive to DA and/or PACAP, suggesting these neuroendocrine stimulators can act on the same goldfish somatotrope [41, 363]. On the other hand, GH release responses to GnRH or synthetic activators of PKC are additive to those to DA, PACAP or synthetic activators of AC/PKA signalling [41, 53, 347]. Taken together, these observations indicate that the readily releasable GH pool in goldfish somatotropes can be separated into largely distinct PKA (DA/PACAP)-sensitive and PKC (GnRH)-sensitive units. How the GH-releasing action of GRLN is manifested in goldfish somatotropes has not been investigated.

In terms of the mechanisms of actions of inhibitory neuroendocrine regulators in goldfish, all three brain SSs can reduce $[Ca^{2+}]_i$ responses. This may be related to their ability to enhance voltage-sensitive K^+ currents, and presumably exert hyperpolarizing influences, but only SS-14 and gbSS-28 attenuates Ca^{2+} currents through LVSCCs in voltage-clamp experiments on isolated currents in goldfish somatotropes [41]. The brain SSs also exerts inhibitory influences at, or downstream of, PKC activation, $[Ca^{2+}]_i$ increases, and NO, but SS-14 and gbSS-28 are generally more effective than $[Pro^2]$SS-14 in inhibiting PKC-mediated responses while gbSS-28 is relatively less effective at sites distal to Ca^{2+} [41]. The three SSs and NE can reduce basal cAMP production. In contrast, SS-14, $[Pro^2]$SS-14 and NE, but not gbSS-28, exerts inhibitory actions distal to cAMP and AA [41]. While both NE and 5HT can also reduce $[Ca^{2+}]_i$ responses to a number of stimulators, NE is generally more effective than 5-HT in this regard and 5-HT also does not exert inhibitory influence downstream of cAMP [41].

The significance of the presence of differences in signal transduction mediating the actions of neuroendocrine stimulators and inhibitors on goldfish GH release is not yet fully understood, but presumably allows for complex fine-tuning of GH release under different conditions.

1.5. Neuroendocrine regulation of LH

1.5.1. General perspectives

In all vertebrate classes, multiple GnRH forms exist with at least 2 forms of GnRH being present in the brain. However, for the longest time, only one brain GnRH form was thought to be hypophysiotropic [46]. In mammals, the main stimulator of pituitary LH release is mammalian (m)GnRH (also called Type I GnRH) which is released from hypothalamic neuronal terminals at the median eminence and then delivered to the pituitary via the hypothalamohypophysial long portal vessel system. Negative regulation of LH release is largely manifested by the negative feedback actions of sex steroid hormone [17, 46, 315]. A majority of the neuroendocrine regulation mechanisms are exerted indirectly via modulation of GnRH neuronal activity [46, 66, 176, 315]. Although this relatively simple model of neuroendocrine regulation by GnRH and steroid feedback is thought to apply to other higher vertebrate species as well, this view is beginning to change. Recently, a gonadotropin release-inhibitory hormone (GnIH), a member of the RFamide family of peptides, has been identified in representative species from all vertebrate classes and it has been shown to exert inhibitory control over LH release from the pituitary in birds and mammals [316]. In addition, another RFamide, kisspeptin, has also been shown to directly affect pituitary LH release in several mammalian model systems [66, 239].

In teleosts, the hypothalamohypophysial long portal vessel system is absent and hypothalamic neurons enter the adenohypohysis and terminate among

the anterior pituitary cells [10, 315]. Current knowledge supports that neuroendocrine control of LH release at the level of the pituitary is more complex in teleosts than in mammals with multiple hypothalamic peptides and neurotransmitters directly exerting inhibitory and stimulatory influence on LH-secreting cells, although the feedback action of gonadal hormones is also important as in mammals. This system of multifactorial control by hypothalamic factors in teleosts is best characterized in the goldfish [46, 315].

As mentioned in Section 1.4.1, two GnRH forms, sGnRH and cGnRH-II are expressed in the brain of goldfish [42]. Both GnRH forms are found in pituitary nerve terminals and both stimulate LH release [253]. In addition, PACAP, kisspeptin, gGRLN, NPY, 5-HT and NE also exert direct stimulatory influences on LH release from pituitary cells and/or fragment preparations, while DA, acting via D2R, inhibits basal as well as stimulated LH secretion [46, 315]. The removal of DA inhibition is an important event which allows the effects of stimulatory neuroendocrine regulators to be manifested to elicit the ovulatory LH surge [46, 315]. Sex steroids, including testosterone and estradiol, can directly enhance the effectiveness of GnRH to stimulate LH release in vitro indicating the presence of a positive feedback influence at the level of pituitary cells [46, 315]. Fish GnIH actions are different from what has been reported for birds and mammals. Goldfish GnIH appears to selectively attenuate cGnRH-II-elicited, but not sGnRH-induced, LH response and its effects on basal LH release from goldfish pituitary cells are mixed with either a stimulatory or no effect being reported, depending on the reproductive status [228, 229]. Likewise, goldfish GnIH stimulates LH release from pituitary cells of sockeye salmon [3].

1.5.2. Intracellular signalling in neuroendocrine regulation of LH release in goldfish

In mammals, mGnRH actions of LH secretion are known to be mediated by PLC/IP3/DAG/PKC, VSCC, Ca^{2+} signalling, lipoxygenase metabolites of AA metabolism, nNOS/NO and cGMP [46, 315].

In goldfish, although both sGnRH and cGnRH-II elicit LH release via GnRH receptors expressed on gonadotropes, there are differences in their intracellular mechanisms of action (Fig. 1.8). Both GnRHs utilize the PLC/DAG/PKC, LVSCC, Ca^{2+}, PI3K, sGC/cGMP, and extracellular signal-regulated kinase (ERK) kinase (MEK)/ERK signalling pathways to stimulate LH release, but cGnRH-II is relatively more dependent on extracellular Ca^{2+} than sGnRH, and only sGnRH utilizes the AA-lipoxygenase pathway [46, 315]. In addition, while cGnRH-II accesses a ryanodine-sensitive intracellular Ca^{2+} store at all times of the seasonal reproductive cycle, this store is only used by sGnRH at sexually regressed state [46, 315]. Furthermore, sGnRH, but not cGnRH-II, action involves IP3- and caffeine-sensitive Ca^{2+} stores, as well as NOS/NO [46, 315].

On the other hand, PACAP stimulation of LH release, via PAC1 receptors, is mediated by cAMP/PKA, MEK/ERK, Ca^{2+}, mobilization of Ca^{2+} from caffeine- and dantrolene-sensitive intracellular stores, and a novel PLC-dependent mechanism that does not involve subsequent IP3 and PKC signalling [46, 315]. LVSCC and NOS/NO signalling also does not participate in PACAP action on LH secretion [46, 315]. Interestingly, PACAP is able to generate increases in $[Ca^{2+}]_i$ in GnRH-responsive identified goldfish gonadotropes and yet the LH responses to PACAP and pharmacological stimulators of AC/cAMP signalling are additive to those elicited by the two GnRHs and synthetic activators of PKC [46]. These results indicate that readily releasable LH within goldfish gonadotropes are largely separated into cAMP/PKA- and PKC-sensitive pools.

Sex steroid positive feedback influence on GnRH-stimulated LH release is in part manifested by enhancing the effectiveness of PKC-mediated hormone release [46]. Goldfish kisspeptin has recently been shown to utilize LVSCC to enhance LH release [50]. The intracellular signalling mechanisms mediating the direct actions of GRLN, NE (α1-like receptors), 5HT (5HT-1 and 5HT-2-like receptors) and NPY (Y2 receptors) on LH release have not yet been identified.

In terms of inhibitory regulation, to date only the mechanisms of DA action have been investigated. DA binding to D2Rs expressed on the surface of goldfish gonadotropes reduces GnRH receptor binding [46]. In addition, DA reduces Ca^{2+} currents through LVSCC and DA inhibitory influences can be manifested at, or downstream of, PLC-mediated IP turnover, and PKC activation, as well as distal to cAMP production; however, DA does not act at steps following NO and AA generation and whether DA affects cAMP or NO production is unknown [46].

1.6. Proposed research, purpose, hypothesis, and experimental design

1.6.1. General overview and purpose

As indicated in Sections 1.1-1.3, GRLN is emerging as an important regulator of pituitary hormone release and especially that of GH and LH. Since GRLN is also involved in the control of food intake, as well as energy metabolism, this peptide likely serves as one of the important links between the regulation of energy status and growth and reproduction. Although there is a fair amount of information regarding the effects of GRLN on GH and LH release, especially in mammals, much is yet to be determined, especially in regards to GRLN intracellular signalling in pituitary cells and its interaction with other endogenous pituitary regulators in many vertebrates, including both mammalian and fish species. Given that GRLN mediates multiple physiological processes,

cell-specific differences such as receptor and/or intracellular signalling pathways and their modulation in response to GRLN and other endogenous factors are sure to be critical to differential regulation of these processes. Understanding the mechanisms of GRLN actions in gonadotropes and somatotropes and whether cell-type specific differences occurs, as well as how GRLN interacts with other endogenous regulators are important aspects in studies on the control of growth and reproduction in vertebrates. In addition, results from such studies may also provide insights into how GRLN may influence so many physiological processes simultaneously in response to changing environmental cues and needs, and by extension to disease states, such as obesity, anorexia nervosa, and diabetes.

With the well-characterized physiological control by multiple neuroendocrine regulators and the understanding of differential intracellular signalling involved (see Sections 1.4 and 1.5), the control of goldfish GH and LH release provides a good study model to examine the effects of GRLN and the mechanisms of action involved in the regulation of GH and LH secretion, as well as the possible cell-type specific actions and interactions with a number of physiological neuroendocrine factors. Goldfish are readily obtained commercially and can be easily maintained in aquaria. The dispersed goldfish pituitary cell primary culture system also allows for the accurate identification of live gonadotropes and somatotropes within a mixed cell population environment, based on morphology observed under differential interference light microscopy alone [329], thus allowing for single cell imaging studies with minimal prior treatments which can often affect cellular responses, such as plague assays, staining, cell sorting, or cell enrichment. Briefly, GH cells are smaller in size, compared to LH cells, have an oval shaped nucleous, a central nucleolus, and cytoplasmic projections while LH cells are larger in size with a kidney shaped nucleus and large spherical shaped globules in the cytoplasmic region (see [329] for further information and figures of cell tyes). In addition, being a representative cyprinid species, results obtained are applicable to other

commercially important carp species in aquaculture, as well as other teleost species in general. With the emerging consensus that neuroendocrine control of GH and LH in mammals is more complex than initially thought, involving the regulation of pituitary somatotropes and gonadotropes by the direct actions of multiple hypothalamic factors and other regulators (see Sections 1.4 and 1.5), results from studies on the neuroendocrine regulation of pituitary hormone release in goldfish also has implications for higher vertebrates.

The focus of my PhD thesis research is to provide further understanding of the regulation of growth and reproduction in vertebrates by examining the effects of GRLN on goldfish GH and LH release from dispersed goldfish pituitary cells in primary culture. Specific aims include 1) elucidating the signal transduction pathways in GRLN-induced GH and LH release, 2) characterizing the GRLN receptor (GHS-R) and its expression in goldfish tissues, and 3) examining the interactions between GRLN and other known endogenous neuroendocrine regulators on GH and LH secretion.

1.6.2. Hypothesis and experimental approach

The present study utilizes synthetic $gGRLN_{19}$, an endogenous form which is known to be effective in stimulating GH and LH release from goldfish pituitary cells [221]. Based on the known mechanisms of GRLN actions in mammals and information on the major intracellular signalling cascades involved in the neuroendocrine regulation of GH and LH release in goldfish, it is hypothesized that in the goldfish pituitary, $gGRLN_{19}$ elicits GH and LH release and changes in $[Ca^{2+}]_i$ via the G-protein coupled gGHS-R1a, as well as the activation of PKC, PKA, and NOS/NO pathways. Furthermore, changes in $[Ca^{2+}]_i$ are hypothesized to involve Ca^{2+} influx through LVSCCs. It is also expected that the gGHS-R1a will be expressed in multiple tissues, including the brain and pituitary of goldfish. Also, given that the same GHS-R antagonist, when applied to static cultures of goldfish pituitaries, inhibits $gGRLN_{19}$-induced LH release but has no effect on

GH release [322], it appears that different gGHS-Rs, intracellular stimulatory pathways, and/or regulatory processes may be utilized in different pituitary cell types to modulate activity of $gGRLN_{19}$.

Investigation of the roles of Ca^{2+}, LVSCCs, PKC, PKA, and NO in $gGRLN_{19}$ signalling will utilize primary cultures of goldfish pituitary cells in established column perfusion assays [37, 200, 203] with appropriate pharmacological inhibitors and stimulators. Hormone release will be quantified through the use of specific GH and LH radioimmunoassays [49, 203]. Cell column perifusion studies enable the easy elucidation of hormone release profiles, as well as the continual removal of media which minimizes potential paracrine and autocrine effects. The role of Ca^{2+} in $gGRLN_{19}$ action will be examined using a combination of hormone release and fura-2, AM Ca^{2+}-imaging studies [145]. Cloning of the gGHS-R cDNA will be performed using molecular techniques, such as rapid amplification of cDNA ends (RACE). Products will be sequenced and compared with known GHS-R sequences using BLAST to confirm their identity and homology. Expression of gGHS-R and gGRLN will be examined using qPCR. Details of these approaches and methods are reported in Chapter 2.

Works on the identification of gGHS-R form(s), their tissue distribution and the dependence of $gGRLN_{19}$ stimulation of LH release on extracellular Ca^{2+} and LVSCCs are presented in Chapter 3. Results on the involvement of LVSCCs and dependence on extracellular Ca^{2+} in $gGRLN_{19}$ actions on GH release are presented in Chapter 4. Chapter 5 focuses on the differential involvement of PKA and PKC in mediating $gGRLN_{19}$-induced GH and LH release while Chapters 6 and 7 examines the possible participation of NOS/NO in mediating the GH and LH responses to $gGRLN_{19}$, respectively. Effects of interactions between $gGRLN_{19}$ and PACAP, as well as $gGRLN_{19}$ and DA, are reported in Chapter 8. Chapter 9 examines the influence of synthetic gGHRH with sGnRH and $gGRLN_{19}$ co-applications. Interactions between $gGRLN_{19}$, sGnRH, and cGnRH-

II are examined in Chapter 10. Chapter 11 provides a summary and discussion on the importance and implications of the thesis findings.

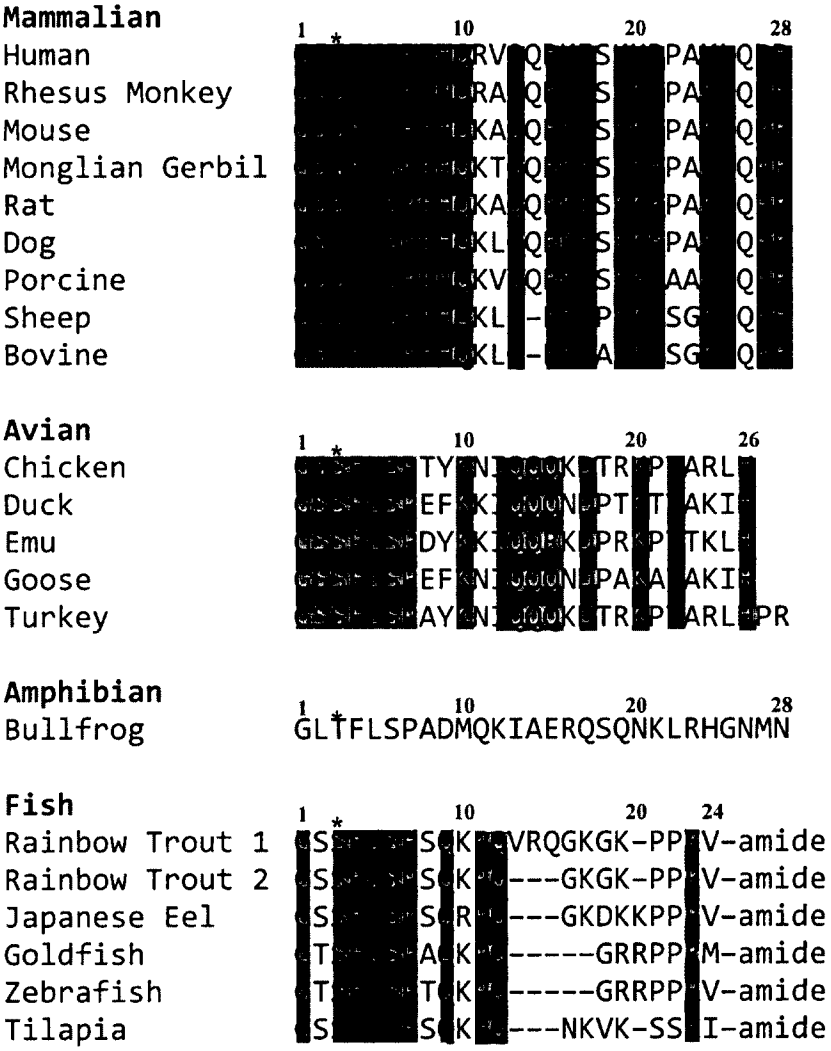

Fig. 1.1. Sequence comparison of various forms of vertebrate GRLN. Identical amino acids in each species are shaded. Asterisks identify acyl-modified third amino acids. (Adapted from [170]).

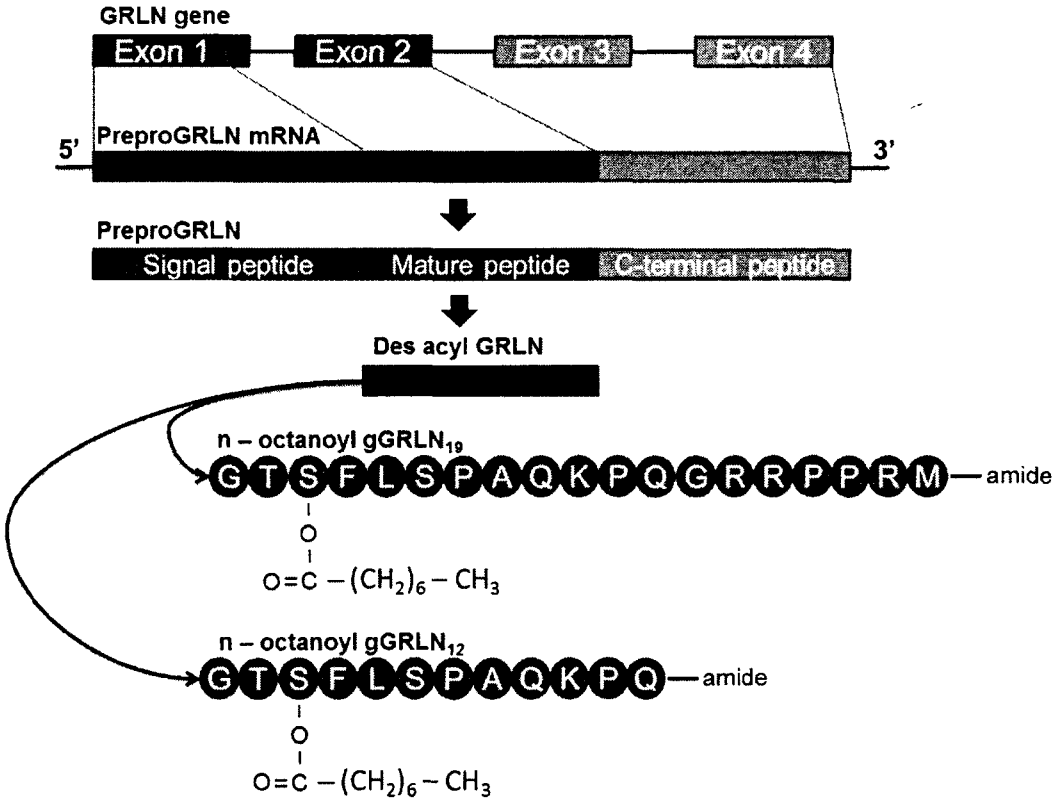

Fig. 1.2. Proposed scheme of the formation of n-octanoylated, amidated, putative GRLN peptides from the GRLN gene in goldfish. In the depiction of the ghrelin gene, boxes represent exons 1, 2, 3 and 4 and lines represent introns 1, 2 and 3. (Adapted from [323]).

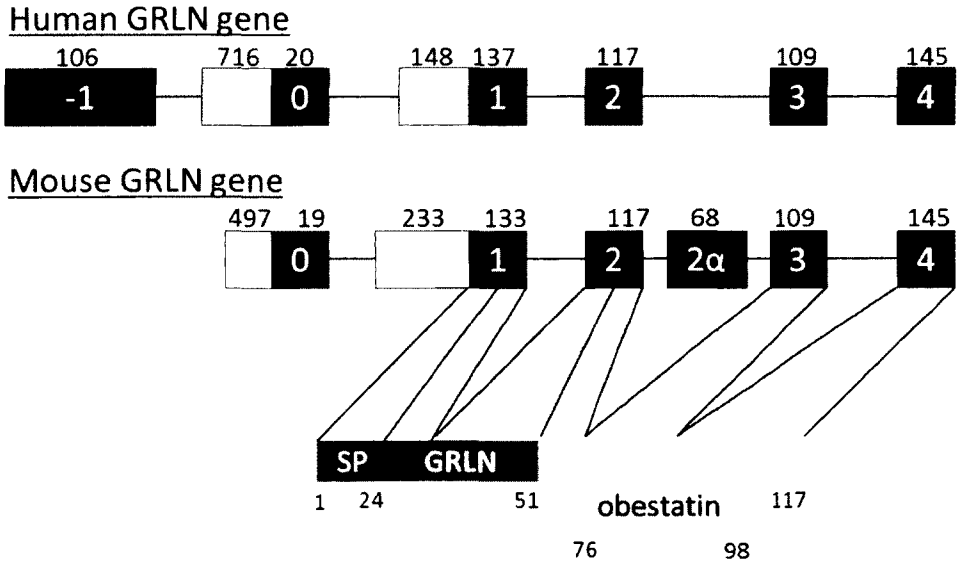

Fig. 1.3. Schematic genomic organization of the human and mouse GRLN genes and the prepro-GRLN coding exons. sizes (in base pairs) are shown above each exon. The preproghrelin is 117 amino acids in length, with different regions depicted by different colors. (Adapted from [280]).

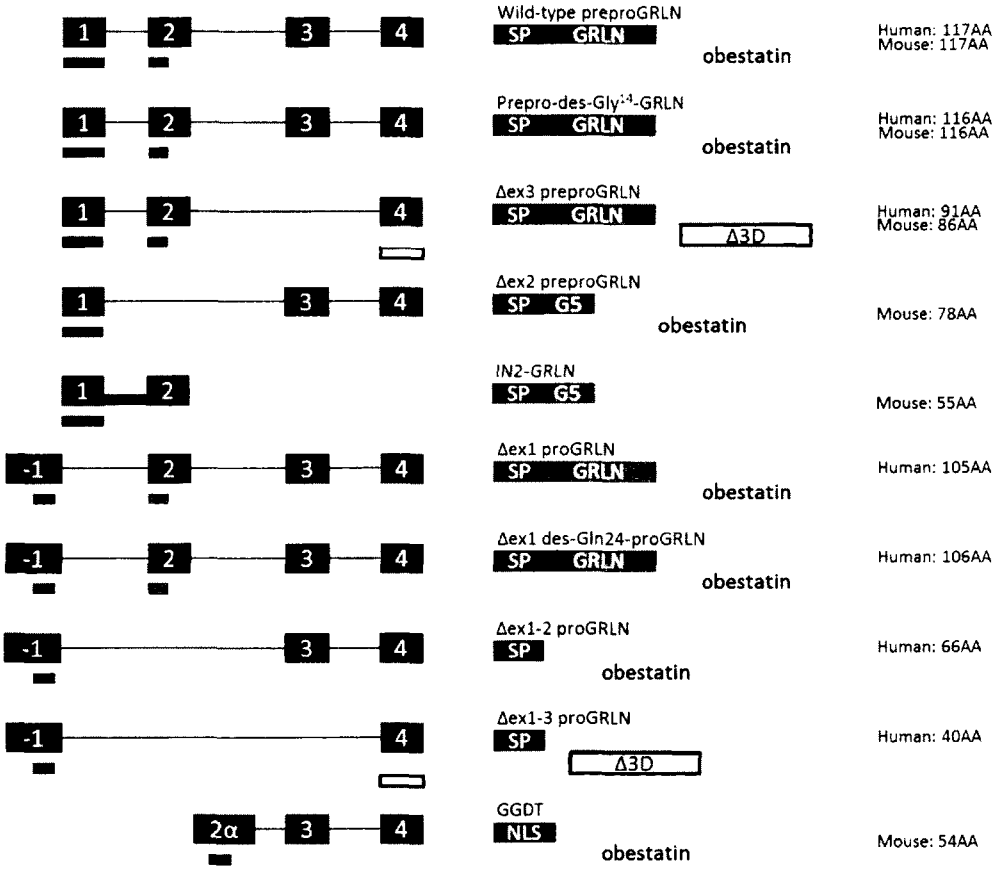

Fig. 1.4. Overview of human and mouse GRLN gene-derived transcripts and putative peptides. Signal peptides are shown in purple, nuclear localization signals in turquoise, ghrelin in red, obestatin in grey, Δ3D peptide in white, In2-GRLN in green, and C-GRLN in orange. G5 denotes putative peptides containing the first 5 amino acids of GRLN (GSSFL). (Adapted from [280]).

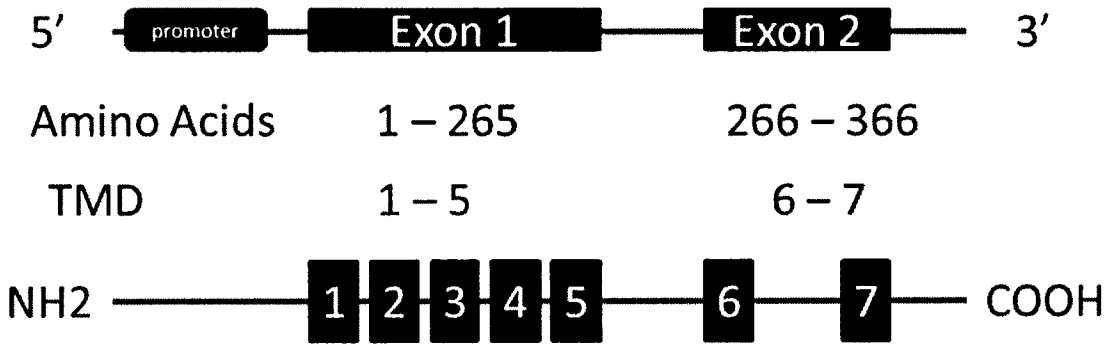

Fig. 1.5. Human GHS-R1a molecular structure. Transmembrane domains (TMD) are numbered in boxes representing corresponding sections of the protein structure. Exon 1 translates into 265 amino acids, including TMD 1-5, while Exon 2 encodes 100 amino acids and TMDs 6 and 7 (adapted from [280]).

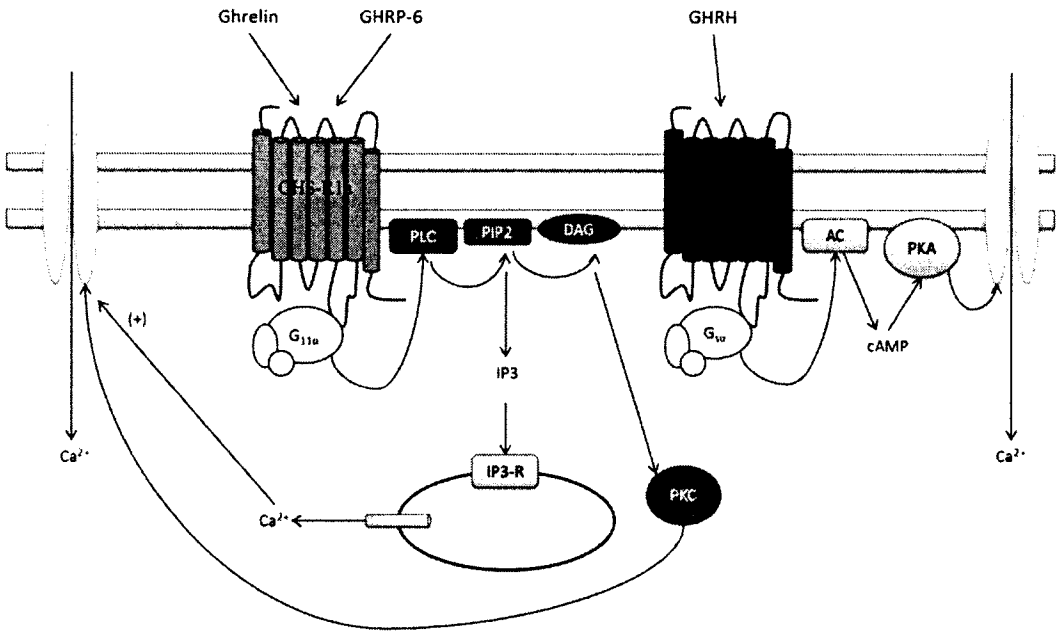

Fig. 1.6. A model for the signal transduction pathway used by GRLN, GHRP-6, and GHRH to trigger intracellular Ca^{2+} mobilization in mammals. The addition of GRLN or GHS, such as GHRP-6, activates PLC through a $G_{11\alpha}$ protein-coupled GHS receptor. PLC hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP2) generating IP3 and DAG. IP3 releases Ca^{2+} from intracellular IP3-sensitive Ca^{2+} pools while DAG activates PKC, both of which activate LVSCCs. On the other hand, GHRH activates AC, through $G_{s\alpha}$ -coupled GHRH receptor, generating cAMP and stimulating PKA and thus activating LVSCCs. (Adapted from [27]).

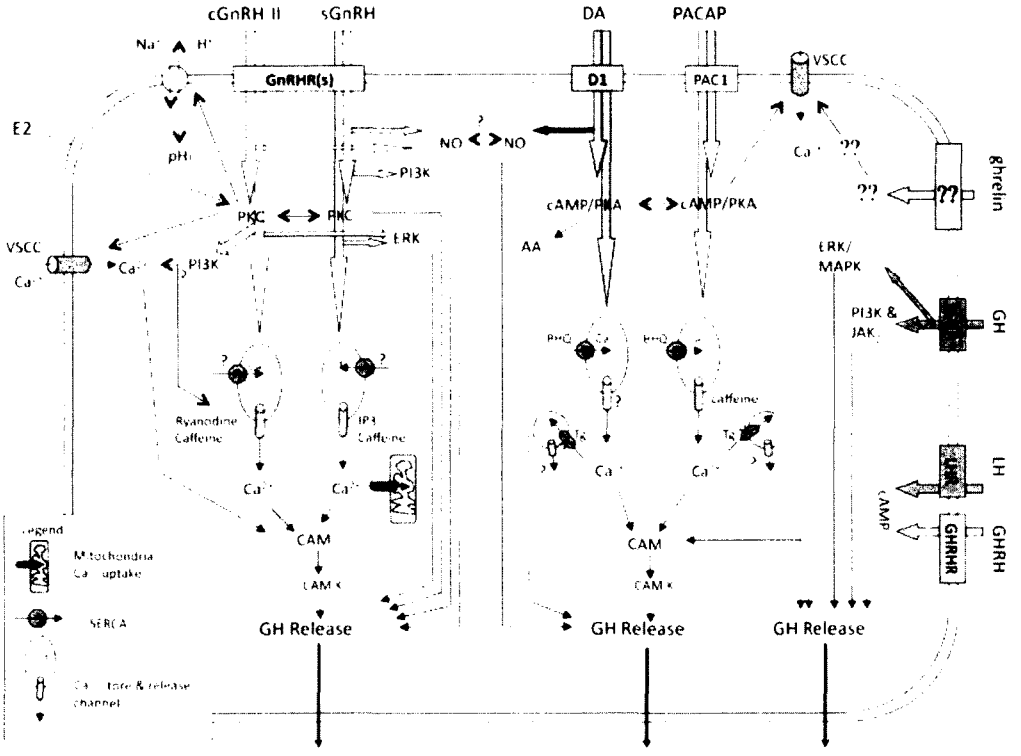

Fig. 1.7. Schematic summary of receptor-signal transduction cascades mediating the stimulatory effects of neuroendocrine factors on GH release from goldfish somatotropes. Briefly, the differential use of second messenger systems, including multiple pharmacologically distinct intracellular Ca^{2+} stores and Ca^{2+} uptake/buffering systems, allows for the ligand-specific interactions controlling induced GH secretion (see Section 1.4.2). The different ligand-selective pharmacologically distinct intracellular Ca^{2+} stores are differentiated by sensitivity to various agents including the Ca^{2+} release channel blocker ryanodine, the SERCA inhibitors Tg and BHQ, and the Ca^{2+} release channel receptor agonists IP3 and caffeine, respectively. In addition, the involvement of calmodulin kinase (CAM K) down stream of Ca^{2+} mobilization, as well as the selective involvement of a Na^{+}/H^{+} antiport system in GnRH action down stream of PKC, are also shown. The potentiating effects of intrapituitary GH and LH on GH release, including the use of Janus kinase (JAK) in GH signalling, as revealed in carp pituitary cells are also included on the right for the sake of completeness. (A version of this figure has been published in [41]).

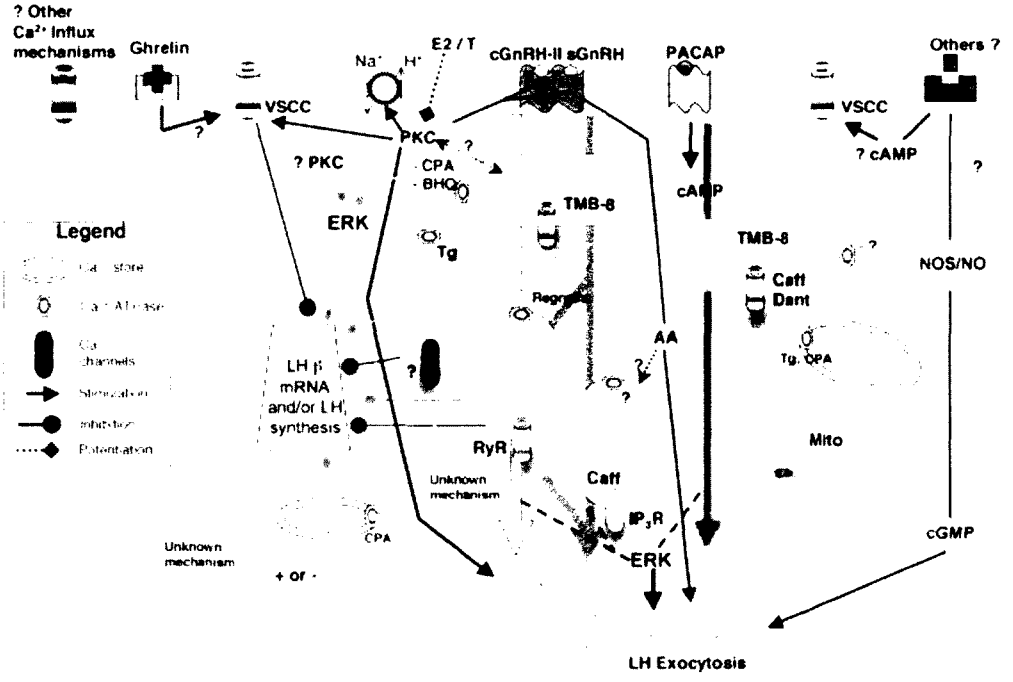

Fig. 1.8. Summary diagram of signal transduction cascades mediating the direct actions of sGnRH, cGnRH-II, PACAP, GRLN and gonadal steroids on goldfish gonadotropes. Differential use of signal transduction cascades allow for differentiation and integration of neuroendocrine regulator effects at the intracellular level (see Section 1.4.3). The ligand-selective intracellular Ca^{2+} stores are differentiated by their sensitivity to various pharmacological agents including the Ca^{2+} release channel blockers TMB-8, dantrolene (Dant), ryanodine (Ry); the SERCA inhibitors Tg, HBQ and CPA; and the Ca^{2+} release channel receptor agonists IP3 and caffeine, respectively. The selective use of a Na^{+}/H^{+} antiport system in GnRH action down-stream of PKC is also shown. In addition, known signalling components affecting LH subunit gene expression, but not discussed in this chapter, are also included for the sake of completeness (A version of this figure has been published in [46]).

Chapter 2 – Materials and methods

2.1. Animals

All animal maintenance and experimental procedures adhere to Canadian Council for Animal Care guidelines and are approved by the University of Alberta Biological Sciences Animal Care Committee. Common goldfish (*Carassius auratus*; 25-30 g BW, post-pubertal) were purchased through Aquatic Imports (Calgary, AB) and maintained in flow-through aquaria (1800 l) at 17-18 °C under a simulated Edmonton, AB, Canada photoperiod (times of graded light on and light off adjusted weekly according to the time of sunrise and sunset). Fish were fed to satiation daily at a scheduled feeding time with commercial fish food. All animals were acclimated to the above conditions for at least 7 days prior to use. As the current work does not focus on sex differences, experiments involved the use of tissues from both male and female fish (mixed sex). In temperate climates, gonadal recrudescence in goldfish starts in late fall/early winter during which time, blood LH and GH levels gradually increase. Serum LH and GH levels continue to increase as gonadal maturation occurs throughout the winter months. Following spawning in early spring, gonadal regression occurs and serum LH levels drops while GH levels remain elevated through the early summer months before decreasing to its lowest levels by fall [56, 315]. In most studies in this thesis, replicate experiments were done within a short time period to minimize the possible effects of gonadal status. However, results from replicate experiments covering more than one gonadal recrudescence state are also generally similar and thus the data were pooled. Nonetheless, to facilitate future comparisons of data, the time of year in which the experiments were performed is presented in the figure legends.

2.2. cDNA synthesis

Fish were anaesthetised in 0.05% tricaine methanesulfonate (Aqualife, Syndel, Canada), a blood sample was collected, followed by perfusion with fish saline (NaCl 100.96 mM, KCl 3.53 mM, $MgSO_4$ 2.41 mM, KH_2PO_4 11.76 mM, $CaCl_2$ 2.52 mM, $NaHCO_3$ 12.5 mM) containing heparin, via cannulation of the heart. Following bulk blood removal, as indicated by whitening of the gills, various tissues were collected (brain, pituitary, spleen, kidney, liver, heart, gill, muscle, ovary, testis, and intestine) and flash frozen in liquid nitrogen. Total RNA was extracted from various tissues using TRIzol™ reagent and cDNA was synthesized with Superscript III and the oligo $(dT)_{12-18}$ primer (Invitrogen, Canada) or using the SMART cDNA amplification kit and Advantage 2 Polymerase (Clontech, USA), for RT-PCR or RACE experiments, respectively.

2.3. Cloning of gGHS-R1a

The list of primers utilized in this study is shown in Chapter 3 (Table 3.1). Primers were designed against conserved regions of the predicted zebrafish GHS-R1a (GeneBank accession NM_001146272; forward primer: base pair (bp) 232 – 256, reverse primer: bp 669 – 692), confirmed in RT-PCR with zebrafish tissue cDNA, and then used to obtain partial sequences of corresponding goldfish cDNA transcripts. The PCR conditions were 94 °C for 2 min and then 32 cycles of 94 °C for 30 sec, 56 °C for 40 sec, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Amplified products were resolved by 1.2% agarose gel electrophoresis and imaged using ethidium bromide. The goldfish β-actin gene was employed as a loading control. Candidate products were excised from the gel, cleaned with a gel extraction kit (Qiagen, USA), subcloned into the PCR 2.1 cloning vector, transformed into competent TOPO TA *E. coli* cells (Invitrogen, USA), plated onto LB-kanamycin plates, and incubated overnight at 37 °C.

Positive clones were identified using vector specific primers (M13 forward and reverse) in colony PCR screens. Positive clones were grown overnight while shaking at 37 °C in 1 ml LB-kanamycin medium and isolated the following day using the QIAquick spin miniprep kit (Qiagen, USA). Isolated plasmids were sequenced using automated DNA sequencing (3730 DNA analyzer, Applied Biosystems, USA). Sequence data from at least 19 clones obtained from 3 separate experiments were used to compile a 500 bp consensus sequence, which was assessed using BLAST analysis against the NCBI database and identified as the GHS-R1a.

Both 3' and 5' RACE were then employed using goldfish cDNA transcripts and goldfish-based, gene-specific, primers (GeneBank accession AB555556; 5' RACE: bp 527 – 504; 3' RACE: bp 299 – 322). Corresponding RACE products were sequenced to yield the remaining portion of our GHS-R1a transcript. PCR conditions were 94 °C for 2 min and then 30 cycles of 94 °C for 30 sec, 59 °C for 45 sec, and 72 °C for 2.5 min, followed by a final extension of 72 °C for 12 min. Amplified products were resolved by 1.2% agarose gel electrophoresis, excised and purified using the QIAquick Gel Extraction Kit (Qiagen, USA), and then subcloned and sequenced as described above for RT-PCR products. Sequences from RACE experiments were appended to the 500 bp sequence and analyzed using BLAST against the NCBI database to confirm the overall identity of the product as highly similar to GHS-R1a.

2.4. Quantitative PCR analysis of gGRLN and gGHS-R1a in goldfish tissues

Total RNA was isolated from the brain, pituitary, spleen, kidney, liver, heart, gill, muscle, ovary, testis, intestine, and whole blood of 4 or 5 individual, healthy, fish using TRIzol™ reagent and Superscript III (Invitrogen, USA), as described above. Primers specific for gGRLN and gGHS-R1a were designed

using Primer Express software (Applied Biosystems, USA) and the expression of these goldfish genes was assessed, relative to the endogenous control gene elongation factor 1 alpha (EF-1α), as established by previous work in goldfish [108, 109], in real time expression analysis (qPCR) using the ΔΔCT analysis method and SYBR®-green reagent (Applied Biosystems, USA). Thermocycling parameters were as follows: 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 sec and 60 °C for 1 min. Analyses of the relative tissue expression data for fish (n = 4 to 5) were carried out using the 7500 Fast software (Applied Biosystems, USA). All primers were tested for specificity using a cDNA dilution series and all protocols included melting point analysis as controls. Resulting RQ values were normalized against the expression seen in the lowest tissue group (liver and muscle, for GHS-R1a and GRLN, respectively).

2.5. RT-PCR expression analysis of zebrafish GHS-R1a in zebrafish tissues and embryos

Total RNA from zebrafish brain, spleen, kidney, liver, heart, gill, muscle, and intestine tissues, as well as from whole fish embryos at 12, 24, 48 and 72 hours post fertilization (hpf), were kindly provided by the laboratory of Dr. James Stafford. The RNA was extracted using TRIzol™ (Invitrogen) and reverse transcribed into cDNA using the Superscript II cDNA synthesis kit (Invitrogen) in accordance to manufacturer's directions. PCR was performed with the following thermocycling parameters: 94 °C for 2 min and then 32 cycles of 94 °C for 30 sec, 56 °C for 40 sec, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. The zebrafish β-actin gene was employed as a loading control, using the same thermocycling parameters.

2.6. RT-PCR expression analysis of gGHS-R1a in goldfish tissues

Total RNA was isolated and reverse transcribed into cDNA using the methods described in Section 2.2 above. The PCR conditions were 94 °C for 2 min and then 32 cycles of 94 °C for 30 sec, 56 °C for 40 sec, and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Amplified products were analyzed by electrophoresis in 1.2% agarose gel and imaged using ethidium bromide. The goldfish β-actin gene was employed as a loading control. All sequences of primers used in this study are provided in Table 3.1.

2.7. In-silico analysis

Comparisons of the putative gGHS-R1a sequence to other GHS-R1a sequences were determined using BLAST against the GenBank database, and amino acid and nucleotide alignments were performed using the Clustal W software (http://www.ebi.ac.uk/Tools/clustalw2/). Nucleotide and amino acid sequences used here were obtained from the GenBank database (accession number): goldfish GHS-R1a, type 1 (AB504275.1); goldfish GHS-R1a, type 2 (AB504276.1); goldfish GHS-R2a, type1 (AB504277.1); goldfish GHS-R2a, type2 (AB504278.1); zebrafish GHS-R1a (NM_001146272.1); rainbow trout GHS-R1a (NM_001124594.1); tilapia GHS-R1a (AB361053.1); black porgy GHS-R1a (AY151040.1); chicken GHS-R1a (NM_204394.1); human GHS-R1a (NM_198407.1); chimpanzee GHS-R1a, variant 1 (XM_001165392.1); rat GHS-R1a (NM_032075.3); mouse GHS-R (NM_177330.4); zebrafish similar to neuromedin U receptor 1 (XM_680313.3); chicken similar to neuromedin receptor 1 (XM_426705.2); human motilin receptor (NM_001507); and mouse neurotensin R2 (BC141019).

2.8. Drugs and reagents

gGRLN$_{19}$ with *n*-octanoyl modification [321] was synthesized in the laboratory of Dr. Jean E. Rivier (Clayton Foundation Laboratories for Peptide Biology, The Salk Institute of Biological Sciences, San Diego, CA). The presence of gGRLN$_{19}$ as one of the native gGRLN forms has been confirmed biochemically in extracts of goldfish intestinal tissues [221]. sGnRH ([Try^7, Leu^8]GnRH) and cGnRH-II, endogenous GnRH forms released at the goldfish pituitary [41, 47], were purchased from Bachem (Torrance, CA). gGHRH with the sequence: HADAIFTNSYRKVLGQISARKFLQTVM and a c-terminal amidation [185] was custom synthesized (Genscript, Piscataway, NJ). PACAP (mammalian PACAP$_{38}$ which has previously been shown to be effective in stimulating GH and LH release from goldfish pituitary cells [346]) and DA were purchased from Sigma-Aldrich (St. Louis, MO). The LVSCC agonist Bay K8644 (+/-), the LVSCC inhibitor nifedipine, and the LVSCC inhibitor verapamil were purchased from Calbiochem via VWR (Mississauga, ON). The PKC activator 1, 2-dioctanoyl-*sn*-glycerol (DiC8), the PKC inhibitor bisinololymaleimide II (Bis-II), the PKC inhibitor 12-(2-Cyanoethyl)-6,7,12,13-tetrahydro-13-methyl-5-oxo-5H-indolo[2,3-a]pyrrolo[3,4-c]carbazole (Gö 6976), the PKA inhibitor N-[2-(p-Bromocinnamylamino)ethyl]-5-isoquinolinesulfonamide dihydrochloride (H-89), the PKA inhibitor (9S,10S,12R)-2,3,9,10,11,12-Hexahydro-10-hydroxy-9-methyl-1-oxo-9,12-epoxy-1H-diindolo[1,2,3-fg:3,2,1-kl]pyrrolo[3,4-i][1,6]benzodiazocine-10-carboxylic acid hexyl ester (KT 5720), the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxyl 3-oxide (PTIO), the NO donor sodium nitroprusside (SNP), the NOS inhibitors aminoguanidine hemisulfate (AGH), N-(3-(aminomethyl)benzylacetamidine (1400W), and 7-nitroindazole (7-Ni) were purchased from EMD (Gibbstown, NJ). Stock solutions of gGRLN$_{19}$, sGnRH, cGnRH-II, gGHRH, PACAP, PTIO, SNP, and AGH were made in distilled de-ionized H_2O. Stock solutions of 1, 2-dioctanoyl-*sn*-glycerol (DiC8), bisinololymaleimide II (Bis-II), Gö 6976, H-89, KT 5720, 1400W, 7-Ni,

and forskolin were made in dimethyl sulfoxide (DMSO). Stock solutions of Bay K8644 (+/-), nifedipine, and verapamil were made in ethanol. Aliquots of concentrated stock solutions were stored at -20 °C until use. Working stock solutions of DA solution was made up fresh, just prior to experiments, in distilled de-ionized H_2O. Final concentrations were achieved by diluting stocks in testing medium. Concentrations of DMSO in final testing solutions did not exceed 0.1% and did not affect basal hormone secretion and $[Ca^{2+}]_i$ (data not shown).

2.9. Goldfish pituitary cell dispersion and preparation

Goldfish were anaesthetised in 0.05% tricaine methanesulfonate (Aqualife, Syndel, Vancouver, Canada) and then decapitated. Pituitaries from both male and female goldfish were removed and pituitary cells were then dispersed by trypsin/DNase treatment as previously described [37]. Cells were cultured overnight on pre-swollen Cytodex-I beads (Sigma, St. Louis, MO; perifusion) or on poly-L-lysine coated cover-slips (Ca^{2+}-imaging) at 28 °C, 5% CO_2, and 100% humidity in plating medium (Medium 199 with Earle's salts (Gibco, Grand Island, NY), 1% horse serum, 25 mM HEPES, 26.2 mM $NaHCO_3$, 100,000 U penicillin/l and 100 mg streptomycin/l, pH adjusted to 7.2 with NaOH) [37, 144].

2.10. Perifusion hormone release experiments

Following overnight incubation at 28 °C, as described above, approximately $1.5x10^6$ cells were loaded into temperature controlled (18 °C) chambers and then perifused with testing medium (Medium 199 with Hanks salts (Sigma, USA), 0.1% bovine serum albumin, 25 mM HEPES, 26.2 mM $NaHCO_3$, 100,000 U penicillin/l and 100 mg streptomycin/l, pH adjusted to 7.2 with NaOH)

for 4 h at a rate of 15 ml/h to stabilize the basal secretion rate of hormones before testing began [37]. After this initial perfusion, 1-min (experiments with nominally Ca^{2+}-free media, testing media prepared without the addition of Ca^{2+} salts) or 5-min fractions (all other experiments with normal Ca^{2+}-containing testing media) were collected and stored at -20 °C until being assayed for GH or LH through the use of well-established radioimmunoassays [49, 203]. Briefly, radioactive GH and LH tracers were made from 5 μg of purified carp GH or LH iodinated with I^{125}, respectively, using lactoperoxidase (Sigma, USA) or chloramine T (Sigma, USA) for LH, followed by column purification with G-50 fine Sephadex beads (Sigma, USA). Samples were pipetted with known amounts of rabbit anti-carp GH or LH antibody (produced at the University of Alberta by Dr. R.E. Peter and used at a dilution of 1:50,000 (Kawauchi carpGH α-seum, rabbit #1) and 1:220,000 (rabbit α-carp GTH378)), respectively, incubated with tracer overnight at 4 °C followed by a subsequent overnight incubation at 4 °C with a commercial secondary goat anti-rabbit gamma globulin antibody (1:40 dilution; Calbiochem via VWR, Mississauga, ON). Samples were spun down to pellet the primary-secondary antibody complexes, decanted to remove unbound radioactivity, and the radioactivity in the pellet quantified in a high throughput gamma counter. Standard curves, employed at the beginning and at the end of each set of samples, were used to calculate ng/ml GH or LH levels. All experiments utilizing Bis-II, Gö 6976, nifedipine, Bay K8644, NO donor and NO inhibitors were performed in the dark to minimize light-induced degradation of reagents. Stimulators were generally applied as a 5-min pulse, unless otherwise indicated. Inhibitors were generally applied at least 20 min prior to stimulator treatment, to allow for the inhibitor to exert its effect. The exact pretreatment periods used are reported in the appropriate data chapters. For experiments investigating interactions between $gGRLN_{19}$ and another stimulator, two types of protocols were used. A "forward" protocol involved application of a 5-min pulse of $gGRLN_{19}$ in the middle of a longer exposure to the other stimulatory agent. A "reverse treatment" paradigm

was also utilized in some experiments in which a prolonged exposure to $gGRLN_{19}$ was used and the other stimulatory agent applied as a 5-min pulse during the exposure to $gGRLN_{19}$. Details of these combinational treatment protocols are presented in the appropriate data chapters. The GH and LH values in the first 5 fractions collected (all obtained prior to drug treatments) were averaged and taken as a measure of the basal rate of secretion (pretreatment) and individual columns were then expressed as a percentage of pretreatment. This normalization allows for the pooling of hormone-response data from different columns without distorting the hormone release profile. Net hormone response was quantified as area under the response curve [58, 341], with base-line subtracted (base-line is defined as the average value of the four fractions prior to the quantification period; net response is the sum of the changes in hormone content in each of the fractions included in the quantification period). Responses in control columns were calculated as area under the response curve using the same response window (quantification period) as used in the treatment columns. All treatments were replicated a minimum of six times in three separate experiments using different cell preparations. Pooled results are presented as mean ± SEM.

2.11. Ca^{2+} imaging

Changes in $[Ca^{2+}]_i$ were measured using the Ca^{2+}-sensitive dye fura-2, AM (Molecular Probes, Eugene, OR) as previously described [144]. Briefly, cells were incubated with 10 μM fura-2, AM dissolved in DMSO with 20% (v/v) Pluronic F-127 (Molecular Probes, Eugene, OR) for 45 min, at 28 °C under 5% CO_2; this concentration and temperature has previously been shown to produce stable and uniform loading of the fura-2, AM dye in goldfish pituitary cell cultures [144]. Following dye incubation, cells were washed three times with imaging media (testing media prepared without phenol red) to terminate the incubation period. Individual somatotropes and gonadotropes were identified

based on distinct morphological characteristics visible under differential-interference contrast (DIC) microscopy, as previously described [329]. Briefly, somatotropes and gonadotropes can be reliably distinguished based on cell size; the location, shape and relative size of their nucleus and nucleolus; and characteristics of cell inclusions. This well-established identification method is specific for GH- or LH-staining cells and has an accuracy of >98% (as shown by repeated, periodic, double-blind verification). Following identification, somatotropes or gonadotropes were excited at 18-21 °C with an Hg-Xe arc lamp (Hamamatsu, Japan) at 340 and 380 nm wavelengths alternately using a computer-controlled filter wheel (Empix Imaging, Mississauga, Canada). Emission fluorescence of 520 nm in response to excitation wavelengths of 340 and 380 nm was recorded into a computer through an oil-immersion objective (1.3 N.A.) mounted on a Zeiss inverted microscope (Carl Zeiss Canada, Don Mills, Canada) with a Paultek digital imaging camera (Grass Valley, CA). Treatment reagents were applied to the cells housed in a closed bath imaging chamber (260 µl chamber volume, series 20; Warner Instruments, Hayden, CT) using a gravity-driven perifusion system at a rate of 1 ml/min, allowing the bath solution to be replaced in approximately 15-16 sec. $gGRLN_{19}$ was applied as a 2-min pulse either alone or 2 min into a longer treatment with an inhibitor or another stimulator. Calcium levels were calculated as: $[Ca^{2+}]_i = k_d \times \frac{F380_{max}}{F380_{min}} \times [\frac{R-R_{min}}{R_{max}-R}]$, where K_d =218nm, $F380_{max}/F380_{min}$ = 1.3523, R_{min}=0.776393, and R_{max}=4.672442, as obtained from a previously performed calibration. Data was quantified as described for perifusion studies. Briefly, data was normalized to the average of the first five recording values (pretreatment level) and then net response was assessed as the area under the response curve, with base-line subtracted. All treatments were replicated a minimum of 9 times in separate experiments using at least 3 different cell preparations. As a control for cell viability and general health throughout the experiment, cells were challenged with

a 2-min depolarizing pulse of KCl (30 mM) following a 10-min washout period with clear testing media alone at the end of all treatment protocols. KCl was able to routinely cause a robust Ca^{2+} signal in >99% of cells and all cells included in the data analysis responded to this control KCl pulse (see Chapters 3 & 4; data not shown in other Chapters).

2.12. Statistical analysis

Statistical comparisons of pooled results were done using one-way analysis of variance (ANOVA). When ANOVA showed that significant differences existed among the experimental groups, a Tukey's comparison test was then used to identify differences between the experimental groups. Where data were not normal, nonparametric comparisons using Kruskal-Wallis test followed by U-tests were employed. Where appropriate, comparisons between only two means were performed using Student's *t*-tests and comparisons against a predicted value were performed using a single sample *t*-test. The level of $P < 0.05$ was considered significant.

Chapter 3 – $gGRLN_{19}$ stimulation of LH release from goldfish pituitary cells: Presence of the gGHS-R1a and involvement of LVSCCs

3.1. Introduction

In vertebrates, LH is one of two pituitary gonadotropins involved in the regulation of gonadal development, sexual maturity and reproduction. Among the numerous functions of GRLN, it has become increasingly evident that GRLN also regulates LH release. However, direct effects of GRLN on LH secretion in mammals remain somewhat controversial and apart from one report of involvement of NO in mediating the stimulatory action on LH secretion in rat pituitary cells [84]; its mechanisms of action are not fully understood (see Chapter 1, Section 1.3; [152, 171]). Whether GRLN directly affects LH release in non-mammalian tetrapods is also unclear. The goldfish represents one of the best studied models in which multifactorial neuroendocrine regulation of LH release, and associated intracellular signaling, have been characterized (Chapter 1, Section 1.5; [46, 315]). $gGRLN_{19}$ has also been shown to stimulate LH release in goldfish in vivo and in vitro [322] but the receptor signalling pathways(s) mediating these effects is unknown.

In this chapter, the aim is to begin to elucidate the signalling components involved in the direct action of $gGRLN_{19}$ on LH release in the goldfish. To this end, I cloned a partial cDNA sequence for a gGHS-R1a, and examined its tissue distribution using molecular techniques. Since Ca^{2+} signalling and LVSCCs are important mediators of sGnRH- and cGnRH-II-stimulated LH release in this species [46, 315], I examined the involvement of extracellular Ca^{2+} and LVSCCs in the LH-releasing action of $gGRLN_{19}$ as the first attempt at elucidating the signal transduction mechanism utilized by gGRLN in goldfish gonadotropes using

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a combination of Ca^{2+} imaging and LH release studies with primary cultures of goldfish dispersed pituitary cells.

3.2. Results

3.2.1. gGHS-R1a molecular sequencing and RT-PCR analysis of tissue expression

As an initial step in cloning the gGHS-R, primers designed against the predicted zebrafish GHS-R1a were used in RT-PCR reactions with zebrafish and goldfish tissues. Predicted, single 500 bp products were observed in adult zebrafish brain, spleen, kidney, heart, and gill, as well as in goldfish brain, pituitary, heart, ovary, testis, and intestine (Fig. 3.1; Fig. 3.2A, lower panel). Positive signals were also obtained from whole zebrafish embryo at hatching (i.e., 48 hpf), and free-swimming (72 hpf) developmental stages, but not at 12 and 24 hpf (Fig. 3.1). 500 bp PCR products from the goldfish brain and pituitary were sequenced and found to have high homology (96%) to the predicted zebrafish GHS-R1a using BLAST analysis against the NCBI database (data not shown). Using the consensus 500 bp sequence obtained, goldfish specific primers were designed and used in subsequent RACE reactions, which generated information allowing for the elucidation of a 720 bp cDNA sequence (data not shown) that encodes a predicted protein of 193 amino acids (Fig. 3.3). In silico analysis predicted that the RACE product encoded a part of the first intracellular loop through the 5^{th} TMD of a 7 TMD, G-protein coupled receptor. This partial receptor sequence showed high amino acid homology to GHS-R1a sequences of tilapia (72%), rainbow trout (73%), black porgy/seabream (74%), chicken (80%), chimpanzee (78%), rat (76%), mouse (76%), and human (78%), as well as 99% homology with the predicted zebrafish GHS-R1a (Fig. 3.3). In addition, the predicted gGHS-R amino acid sequence showed low sequence homology to related receptors, such as zebrafish and chicken neuromedin U receptor 1 (35%

and 34%, respectively), human motilin receptor (54%), or mouse neurotensin receptor (25%), thus suggesting that our partial sequence is the gGHS-R1a. Comparison with the gGHS-R sequences recently made available on NCBI (Fig. 3.3) revealed that our cloned product is identical to the gGHS-R1a, type 2, further confirming that I have characterized a fragment of the gGHS-R1a in our experiments and not a related receptor type. Furthermore, phylogenetic analysis of goldfish GHS-R1a in a recent publication [151] confirmed a high degree of conservation among various species.

3.2.2. Quantitative analysis of gGHS-R1a and gGRLN expression in goldfish tissues.

Using primers designed against the partial gGHS-R1a sequence, expression of this receptor in goldfish tissues was further characterized using real-time, qPCR (Fig. 3.2A, upper panel). Analysis of gGHS-R1a in goldfish tissues revealed the highest expression levels in pituitary (Fig. 3.2A, upper panel), confirming observations obtained through RT-PCR, which also showed expression in the pituitary (Fig. 3.2A, lower panel). The gGHS-R1a expression levels were also relatively greater in spleen, testis and intestine, when compared to other examined tissues (Fig. 3.2A, upper panel), and, with the exception of tissue expression in spleen, these results were also comparable to expression patterns observed in RT-PCR (Fig. 3.2A, lower panel). Lower transcript levels of gGHS-R1a were also observed in brain, kidney, heart, gill, and ovary (Fig. 3.2A, upper panel). It should be noted that the primers used in these latter goldfish tissue surveys are specific to both the type 1 and 2 variants of gGHS-R1a, but not the gGHS-R-2a, type 1 or type 2 forms of this receptor.

To allow a comparison of gGHS-R1a mRNA expression levels to one of its ligands, we also characterized $gGRLN_{19}$ mRNA levels in various goldfish tissues using real-time, qPCR. Using primers designed against the recently

characterized $gGRLN_{19}$ sequence [321], results revealed the highest $gGRLN_{19}$ mRNA expression levels in the spleen, intestine, testis, and pituitary tissues (Fig. 3.2B). Weaker $gGRLN_{19}$ transcript levels were observed in the blood, brain, heart, gill, ovary, liver, and kidney tissues while the lowest expression levels of the hormone were seen in goldfish muscle tissue (Fig. 3.2B). These results are comparable to previous RT-PCR studies using primers against the full length gGRLN cDNA, which revealed strong GRLN mRNA expression in the intestine and spleen and weaker expression in the brain [321]. Furthermore, Southern blot analysis has shown gGRLN expression in the goldfish gill [321]. However, previous work has not shown expression in the pituitary, heart, ovary, testis, nor the blood of goldfish tissues.

3.2.3. Acute LH responses to $gGRLN_{19}$

A previous cell column perifusion study using single dose challenges showed that $gGRLN_{19}$ stimulated LH secretion acutely, but no significant dose-dependency was observed [322]. Using sequential applications of 5-min pulses of various doses of $gGRLN_{19}$ (0.01 to 10 nM; range based on [322]), with challenge pulses applied at 60 min intervals, I evaluated if prior exposure to GRLN affected subsequent LH release responses (Fig. 3.4). A 5-min pulse of a maximal stimulatory dose of sGnRH (100 nM) [37, 38] was applied at the end of the protocol to evaluate whether cell cultures were still responsive following multiple GRLN treatments.

All doses of $gGRLN_{19}$ tested significantly stimulated LH release in a reversible manner (Fig. 3.4). These LH responses were rapid and commenced in the fraction collected immediately following treatment, a delay that can be accounted for by the dead volume in the system. Four repeated 5-min pulses of $gGRLN_{19}$ at 1 nM produced LH responses that were not significantly different from one another (Fig. 3.4C). Interestingly, doses applied in increasing order,

from 0.01 to 10 nM, resulted in decreasing LH release responses (Fig. 3.4A), while decreasing doses, from 10 to 0.01 nM, resulted in LH responses that are not significantly different from one another (Fig. 3.4B), suggesting some form of desensitization in the system.

Treatments of sGnRH (100 nM) consistently produced LH responses in all treatments, suggesting that cells remained healthy and responsive throughout the testing protocols (Fig. 3.4). Due to the lack of a clear dose-response relationship, especially when employing decreasing doses (Fig. 3.4B), a dose of 1 nM was chosen for all following $gGRLN_{19}$ experiments given that it was a physiologically relevant dose [320] which could also produce consistent and repeatable LH responses, in both these and prior experiments [322], and without causing desensitization of subsequent treatments.

3.2.4. $[Ca^{2+}]_i$ in gonadotropes changes in response to $gGRLN_{19}$ and sGnRH

As a first step in evaluating $gGRLN_{19}$-induced LH signalling, whether $gGRLN_{19}$ was able to induce increases in $[Ca^{2+}]_i$ in gonadotropes preloaded with fura-2, AM was examined. Administration of 2-min pulses of $gGRLN_{19}$ increased $[Ca^{2+}]_i$ in identified gonadotropes in mixed pituitary cell populations (Fig. 3.5A). To explore the possible existence of sub-populations of gonadotropes within the pituitary that responded differentially to $gGRLN_{19}$ and sGnRH, I examined if the same cells could respond to both peptides. A comparison of 2-min pulses of sGnRH (100 nM) followed by $gGRLN_{19}$ (1 nM) (Fig. 3.5B, left panel), or $gGRLN_{19}$ followed by sGnRH (Fig. 3B left panel inset), added to the same cell and separated by a washout period, each resulted in significant increases in $[Ca^{2+}]_i$ that were not statistically different from one another in terms of total response magnitude (Fig. 3.5B, right panel). These observations suggest the co-expression of both GnRH and GRLN receptors in single goldfish gonadotropes (Fig. 3.5B). All cells tested responded significantly to a 2-min depolarizing pulse of 30 mM

KCl at the end of the protocol, indicating that they remained healthy and responsive throughout testing (Fig. 3.5).

3.2.5. Effects of limiting the availability of extracellular Ca^{2+}

To examine the possible involvement of extracellular Ca^{2+} in $gGRLN_{19}$ action on LH release, I first tested the effects of limiting available extracellular Ca^{2+}. Transient increases in $[Ca^{2+}]_i$, peaking at about 75-90 sec and returning to around 75% of pretreatment levels by around 3 min, were observed at the onset of nominally Ca^{2+}-free media treatment (Fig. 3.6A inset). Additionally, following reintroduction of extracellular Ca^{2+}, we observed a smaller transient increase in $[Ca^{2+}]_i$, peaking at 30-45 sec and returning to pretreatment levels by 1.5 min. These transient changes in $[Ca^{2+}]_i$ likely reflect changes in cellular Ca^{2+} homeostasis in response to alterations in the electrochemical gradient, and thus application of treatments was delayed until a new equilibrium was reached. Indeed, similar findings have been reported in past studies [110, 139]. Application of a 2-min pulse of $gGRLN_{19}$ 4 min into nominally Ca^{2+}-free treatment, a time when a new electrochemical equilibrium was reached, failed to elevate $[Ca^{2+}]_i$ (Fig. 3.6A), findings that are consistent with the idea that the availability of extracellular Ca^{2+} is required for $gGRLN_{19}$-induced increases in $[Ca^{2+}]_i$ in goldfish gonadotropes. The Ca^{2+} responses to a depolarizing dose of KCl (30 mM) in cells previously exposed to nominally Ca^{2+}-free media alone, $gGRLN_{19}$ alone, and $gGRLN_{19}$ plus nominally Ca^{2+}-free media were not different from one another, indicating that prior exposure to nominally Ca^{2+}-free media did not permanently affect the responsiveness of goldfish gonadotropes.

To further explore the role of extracellular Ca^{2+} in $gGRLN_{19}$-induced signalling in goldfish gonadotropes, I examined the effects of nominally Ca^{2+}-free media on LH release from populations of pituitary cells in perifusion protocols with 1-min, fast fraction collections. Treatment with nominally Ca^{2+}-free media

resulted in a transient rise in LH (Fig. 3.6B). These increases were likely related to the increases in $[Ca^{2+}]_i$ observed in Ca^{2+}-imaging studies. As in previous 5-min fraction collection protocols, treatment of $gGRLN_{19}$ in normal Ca^{2+}-containing media significantly increased LH release in 1-min fraction collection studies. The application of $gGRLN_{19}$ in the presence of nominally Ca^{2+}-free media however, did not result in a significant change in LH release when compared to Ca^{2+}-free media alone (Fig. 3.6B).

Taken together, these LH release and Ca^{2+}-imaging results suggest that extracellular Ca^{2+} entry plays a key role in $gGRLN_{19}$-induced LH release from goldfish pituitary cells. These results are, however, inconclusive due to the high level of variance in LH release following administration of nominally Ca^{2+}-free media and that this treatment also elevated basal LH release.

3.2.6. Effects of the LVSCC agonist, Bay K8644

To further explore the role of extracellular Ca^{2+} in $gGRLN_{19}$ signalling, I examined the effects of the LVSCC agonist Bay K8644. If entry of extracellular Ca^{2+} through L-type VSCCs is critical in $gGRLN_{19}$-induced LH release, treatment of $gGRLN_{19}$ should not result in further increases in $[Ca^{2+}]_i$ and LH secretion when this pathway is already maximally stimulated by Bay K8644. On the other hand, if VSCCs and entry of extracellular Ca^{2+} are not involved in gGRLN-induced signalling in the goldfish gonadotropes, one would expect the responses to treatments of both Bay K8644 and $gGRLN_{19}$ to be additive, as they would be signalling via independent pathways.

Consistent with previous studies showing the ability of Bay K8644 to increase Ca^{2+} flow through LVSCCs on goldfish gonadotropes [53, 137, 328], 10 μM Bay K8644 rapidly increased $[Ca^{2+}]_i$ in identified goldfish gonadotropes. These responses consist of a peak increase at ~1 min following Bay K8644 application followed by sustained elevations at ~150% of pretreatment levels

(Fig. 3.7A). 2-min pulses of $gGRLN_{19}$ alone induced increases in $[Ca^{2+}]_i$ to ~160% of pretreatment levels by 90 sec; however when $gGRLN_{19}$ was applied when gonadotropes were already pre-stimulated with Bay K8644, it was unable to further stimulate $[Ca^{2+}]_i$. These results suggest that $gGRLN_{19}$ is targeting LVSCCs on gonadotropes (Fig. 3.7A).

To further examine the role of LVSSCs in $gGRLN_{19}$-induced LH release, I examined the ability of $gGRLN_{19}$ to stimulate LH secretion in the presence of Bay K8644 in perifusion assays. Consistent with its ability to increase $[Ca^{2+}]_i$ in goldfish gonadotropes, 10 μM Bay K8644 rapidly elevated LH secretion to ~125% pretreatment levels by 10 min, this is followed by sustained increase in basal to ~110% pretreatment throughout the 40 min treatment time (Fig. 3.7B). In contrast to its ability to generate a LH secretion response when applied alone, $gGRLN_{19}$, when applied during Bay K8644 stimulation, did not further elevate LH secretion (Fig. 3.7B). Taken together, these hormone release and Ca^{2+}-imaging results strongly implicate extracellular Ca^{2+} and LVSSCs in $gGRLN_{19}$-induced LH release from the goldfish pituitary.

3.2.7. LVSCC inhibitors abolish $gGRLN_{19}$-induced LH release and $[Ca^{2+}]_i$ signals

To confirm the hypothesis that LVSSCs and extracellular Ca^{2+} entry are important components in $gGRLN_{19}$-induced LH release from goldfish pituitary cells, I tested the effects of LVSCC inhibitors in perifusion and fura-2, AM-based Ca^{2+}-imaging studies. Application of two different LVSCC inhibitors, nifedipine (1 μM) and verapamil (1 μM) [243, 314], each suppressed basal LH release (Fig. 3.8A, B). These two inhibitors have previously been shown to reduce LVSCC currents in goldfish pituitary cells, including gonadotropes [259, 328]. Application of a 5-min pulse of $gGRLN_{19}$ 40 min into nifedipine or verapamil treatment failed to produce a LH response. In, contrast, treatment with $gGRLN_{19}$ alone induced increases in LH release (Fig. 3.8A, B).

The effects of nifedipine on $gGRLN_{19}$-induced $[Ca^{2+}]_i$ in goldfish gonadotropes were also examined as another means of testing the involvement of LVSSCs in $gGRLN_{19}$-induced LH release. To minimize the possible effect of long pretreatment times on the status of intracellular stores with LVSCC blockers, I employed short pretreatment times of only 2 min with the nifedipine in these Ca^{2+}-imaging experiments. Treatment with nifedipine resulted in a transient increase in $[Ca^{2+}]_i$ to ~135% pretreatment level, followed by a return to a slightly elevated, but stable, basal level of ~115% by 2 min (Fig. 3.9). During this time, $gGRLN_{19}$ treatments were unable to stimulate increases in $[Ca^{2+}]_i$ observed in previous Ca^{2+} imaging experiments (Fig. 3.9). On the other hand, 30 mM KCl pulses applied at the end of the treatment protocol were able to stimulate increases in $[Ca^{2+}]_i$ following termination of nifedipine treatment to levels similar to those observed in cells not exposed to the LVSCC inhibitor, indicating that cells were not permanently affected by nifedipine treatment (Fig. 3.9). These observations, when taken together, reinforce the idea that extracellular Ca^{2+} entry through LVSCCs is a critical component in $gGRLN_{19}$ signaling in goldfish gonadotropes.

3.3. Discussion

This thesis chapter aimed at further characterizing the direct effects of GRLN on LH secretion and the role of extracellular Ca^{2+} and LVSCCs in mediating GRLN actions in a representative teleost species, the goldfish, in which neuroendocrine regulation of LH release has been well studied. To this end, I was able to clone a partial sequence of the highly conserved GHS-R1a. Results from RT-PCR and qPCR experiments indicate, for the first time, that gGHS-R1a mRNA is expressed in brain and pituitary tissues in goldfish. gGRLN mRNA has been identified in the brain of goldfish in a previous study [321] and its presence in the pituitary was demonstrated in this study. These GRLN and GHS-R1a

mRNA expression data are consistent with the idea that GRLN is an important neuroendocrine factor in the hypothalamus-pituitary axis of goldfish. Since the primers used do not differentiate between the type 1 & 2 isoforms of gGHS-R1a, whether only one or both forms are expressed in these tissues cannot be ascertained. Nonetheless, the presence of gGHS-R1a mRNA in the pituitary, when taken together with the ability of $gGRLN_{19}$ to elevate $[Ca^{2+}]_i$ in identified gonadotropes and to increase LH secretion from dispersed goldfish pituitary cells in this thesis chapter, as well as the ability of a GHS-R antagonist, D-Lys^3-GHRP-6, to inhibit the LH response to $gGRLN_{19}$ in previous experiments [322], suggest that $gGRLN_{19}$ acts on gGHS-R1a to directly stimulate LH release. Consistent with this hypothesis, evidence supports that GRLN actions in the brain and pituitary of various animals, including rat [220, 313, 370] and baboon [162], are mediated through the GHS-R1a [126, 171, 327]. Future work examining the expression of GHS-R1a mRNA and proteins in different pituitary cell-types would be useful to confirm this hypothesis.

Data in this thesis chapter not only confirmed that $gGRLN_{19}$ consistently induced LH release from dispersed goldfish pituitary cells, with no clearly demonstrable dose-response relationships observed, but importantly, also provides insight into the signal transduction mechanisms involved. My data links $gGRLN_{19}$-induced LH release to Ca^{2+} signalling and involvement of LVSCC in several ways. First, $gGRLN_{19}$ significantly increased $[Ca^{2+}]_i$ in identified gonadotropes. Second, nominally Ca^{2+}-free media inhibited these $[Ca^{2+}]_i$ increases, as well as the LH secretion response, suggesting that extracellular Ca^{2+} availability is a key component in $gGRLN_{19}$-induced intracellular signaling in LH release. Third, when LVSCC functions were already fully activated by Bay K8644, addition of $gGRLN_{19}$ did not further increase $[Ca^{2+}]_i$ in gonadotropes and LH release from dispersed pituitary cells. Fourth, the addition of LVSCC inhibitors abolished the $[Ca^{2+}]_i$ and LH release responses to $gGRLN_{19}$. Taken as a

whole, these results indicate that extracellular Ca^{2+} entry through LVSCC mediates $gGRLN_{19}$ signalling leading to LH release. In support of this idea, the involvement of LVSCC in GRLN action has been reported for GH release in dispersed porcine [202] and rat pituitary cells [357], a rat tumor cell-line [74], and goldfish pituitary cells ([110]; Chapter 4). In addition, the participation of extracellular Ca^{2+} in GRLN action has also been demonstrated in HEK-293 cells expressing transfected fish (porgy/seabream) GHS-R1a [34]. Despite these previous reports indicating the involvement of extracellular Ca^{2+} and LVSCC in GRLN action in pituitary and non-pituitary systems, the current results reveal, for the first time, their involvement in GRLN-induced LH release in any vertebrate model.

The involvement of extracellular Ca^{2+} and LVSCC in GnRH stimulation of LH secretion in goldfish, as well as in other teleosts and mammals, has been clearly demonstrated (Chapter 1, Section 1.5.2; [42, 46]). Since $gGRLN_{19}$-induced goldfish LH release also involves extracellular Ca^{2+} entry through LVSCC, it would be important in further studies to examine the interactions between GnRH and $gGRLN_{19}$ action on goldfish gonadotropes. Indeed, human GRLN has been reported to potentiate the LH response to sGnRH in carp pituitary cells [293] and mGnRH in rat pituitary cells [83]. In the present study, the net $[Ca^{2+}]_i$ response to $gGRLN_{19}$ in cells pre-exposed to sGnRH were not significantly different from responses to treatments with $gGRLN_{19}$ alone or when $gGRLN_{19}$ was applied prior to sGnRH treatment (Fig. 3.5A and B, right panels). When looking at the kinetic profiles, however, there appear to be some differences in the magnitude and duration of both $gGRLN_{19}$- and sGnRH-induced changes in $[Ca^{2+}]_i$ when cells are treated with $gGRLN_{19}$ alone, or with both $gGRLN_{19}$ and sGnRH treatment in either order (Fig. 3.5A and B, left panels). Whether these observations indicate that $gGRLN_{19}$ and sGnRH interact in their

regulation of goldfish gonadotrope functions or that seasonal differences in responsiveness exist requires further study.

Interestingly, sGnRH, cGnRH-II, and PACAP stimulation of LH release in goldfish also involve mobilization of Ca^{2+} from intracellular stores [46]. In addition, mammalian and porgy/seabream GHS-R1a receptors are linked to activation of PLC, which generates the intracellular Ca^{2+}-mobilizing IP3, in expression systems [34]. Given that interference with extracellular Ca^{2+} entry, such as the prolonged exposure to LVSCC antagonists used in LH release testing in the present study, may affect the status of intracellular Ca^{2+} stores, the possible participation of intracellular Ca^{2+} in the Ca^{2+} signaling of $gGRLN_{19}$ leading to LH release remains to be determined and will need to be examined in the future. Complicating the matter, previous studies have demonstrated that global changes in intracellular Ca^{2+} are not linearly related to hormone secretion. Indeed, the SERCA inhibitor Tg causes increases in $[Ca^{2+}]_i$ that are of similar magnitudes as those induced by GnRH and yet the immediate effect of Tg treatment is a decrease in basal LH secretion [142]. The role of other second messenger systems, including the NO pathway, thought to be involved in GRLN stimulation of LH in rats [84], also needs to be considered. Although NO donors stimulate LH release from goldfish pituitary cells, PACAP stimulation of LH release from goldfish pituitary cells does not utilize NO signaling [51].

In several mammalian studies, the ability of GRLN to increase LH secretion appears to be related to the sex and gonadal status of the pituitary donor (Chapter 1, Section 1.5; [82-84]). Similarly, human GRLN is reported to stimulate basal LH release only from pituitary cells obtained from female, but not male, common carps, suggesting that gonadal sex is an important factor [293]. As a result of laboratory limitations and the logistics of separating large numbers of fish based on sex, experiments included in this thesis pooled pituitary cells from both male and female goldfish and thus comparison of the influence of gonadal

sex cannot be made. Such comparisons are also beyond the scope of the current work. Nonetheless, the possible influence of gonadal steroids on $gGRLN_{19}$ effects would be an important element for investigation in future studies. On the other hand, $gGRLN_{19}$, the likely endogenous ligand for the gGHS-R1a, was able to increase LH release from pituitary cells and elevate $[Ca^{2+}]_i$ in gonadotropes prepared from post-pubertal goldfish at all times of the reproductive year in the current work.

In addition to its relatively high level of expression in pituitary and brain, gGHS-R1a mRNA was also detected in the intestine (including stomach), spleen, testis, and heart, while only relatively low levels were observed in other goldfish tissues (Fig. 3.2A). The presence of GHS-R1a mRNA in many of these tissues have also be reported for other teleost species including porgy/seabream [31], orange spotted grouper [62] and the rainbow trout [153]. Recently, is has also been reported that zebrafish intestine stained positively with an antibody against rainbow trout GHS-R1a [241]. Although data in the present thesis chapter failed to show GHS-R1a in the intestine of the zebrafish through RT-PCR, they demonstrated for the first time that GHS-R1a mRNA is expressed in the spleen, liver and brain of adult zebrafish, a commonly used fish model in many physiological and developmental studies because of the relative completeness of its genomic information. It would be interesting to further study the profile of GHS-R1a expression in the zebrafish to clarify the differences observed; however, it is beyond the scope of this thesis. On the other hand, the distribution pattern of GHS-R1a observed in goldfish and other fish species agrees with many of the known physiological functions of GRLN in mammals. For example, the expression of GHS-R1a mRNA in blood and spleen can be correlated with the known ability of GRLN to regulate immune function, and macrophage and T-cell activities [308], and that of GHS-R1a expression in heart with the known effects of GRLN on cardiovascular function and endothelial and cardiomyocyte cell

apoptosis [9, 351]. GHS-R1a expression in the mucosa of gut in humans appears to be related to Crohn disease [127]. The presence of GHS-R1a in gonadal tissues is also consistent with the known direct effects of GRLN on the gonads in mammals and chickens, including estrogen production from ovarian follicles [214, 261, 309]. Interestingly, although GHS-R1a mRNA was expressed in both the ovary and testis of porgy/seabream [31], it appeared that higher levels were present in the testis, as compared to the ovary, of goldfish (Fig. 3.2) and was absent in both testis and ovary of adult orange spotted grouper [62]. Given the lack of statistical difference between the GHS-R1a levels in the testis and ovary (Fig. 3.2A, upper panel) these results may represent normal variance and thus further study is needed to support this finding. Whether these differences were due to species differences and/or the maturational/reproductive status of the animals from which gonadal tissues were harvested would be an interesting study for the future.

GHS-R1a mRNA was not detected in unfertilized and just fertilized eggs of the orange spotted grouper but was present from the neurula stage embryo onwards [62]. In the present thesis chapter, GHS-R1a mRNA expression was not detected in whole zebrafish embryo at 12 and 24 hpf but was present at 48 (hatching) and 72 (free-swimming larvae) hpf. Taken together, these observations indicate that GHS-R1a mRNA expression increases with embryonic development and is not part of the maternal signal in fish embryos.

Results from LH release experiments in which $gGRLN_{19}$ was applied in increasing concentrations suggest that the hormone response to this peptide may undergo desensitization. This was also observed in studies on GH secretion ([110]; Chapter 4). Interestingly, sGnRH in previous studies produced dose-dependent LH secretion and repeated pulse application of a maximally stimulating dose (applied as pulses 1 h apart) showed no desensitization of the LH response [37, 49, 59]. This indicates that, at least for sGnRH, sequential pulse applications

at 1-h intervals do not cause desensitization. Several reports, however, indicate that GHS-R1a can be subject to rapid desensitization and/or receptor down-regulation. Repeated challenges with GRLN or the synthetic GH secretagogue, GHRP-6, desensitized the $[Ca^{2+}]_i$ responses in HEK-293 cells expressing transfected GHS-R1a [26, 27]; in addition, GHS-Rs expression in HEK-293 have been shown to undergo rapid constitutive, as well as ligand-induced, internalization [123]. On the other hand, GHRP-6 increased seabream GHS-R1a promoter activity in HEK-293 cells expressing this promoter [359], suggesting ligand-induced receptor synthesis and potential receptor up-regulation. The partial cloning of the goldfish GHS-R1a provides the basis for future investigation into $gGRLN_{19}$ homologous regulation of GHS-R1a, which will help to clarify some of these differences.

In summary, findings in this thesis chapter strongly support a direct stimulatory role for $gGRLN_{19}$ in goldfish pituitary cells. Furthermore, tissues surveys of the gGHS-R1a, type 1 & 2, support the idea that $gGRLN_{19}$ may act via these receptors at the level of the pituitary. Although the role of intracellular Ca^{2+} has not been examined and functional receptor data has yet to be provided, data in this thesis chapter have clearly demonstrated that both extracellular Ca^{2+} entry and LVSCCs are involved in the LH-releasing process of $gGRLN_{19}$.

Table 3.1. Sequence of primers used in this chapter

Primer	Sequence (5'-3')
[illegible]	[illegible]
Zebrafish GHS-R1a reverse	TGT CTT GTA TAG TCT CAT CGG GCG
[illegible]	[illegible]
Zebrafish β-actin reverse	ATG GGC CAG ACT CAT CGT ACT CCT
[illegible]	[illegible]
Goldfish β-actin reverse	ACT CCT GCT TGC TGA TCC ACA TCT
[illegible]	[illegible]
Goldfish GHS-R1a qPCR reverse	GAG CCC AGA CCT AAT GGC ATA T
[illegible]	[illegible]
Goldfish ghrelin qPCR reverse	CAG TTC GAA CGG AGC ACT CAT
[illegible]	[illegible]
Elongation factor 1-α qPCR reverse	TTG ACA GAC ACG TTC TTC ACG TT
[illegible]	[illegible]
M13 reverse	ACA GCT ATG ACC ATG ATT AC
[illegible]	[illegible]
3' RACE goldfish GHS-R1a	ACC GAC CTT GGA ATT TCG GCA ACG
[illegible]	[illegible]
RACE universal primer (short)	CTA ATA CGA CTC ACT ATA GGG C
[illegible]	[illegible]

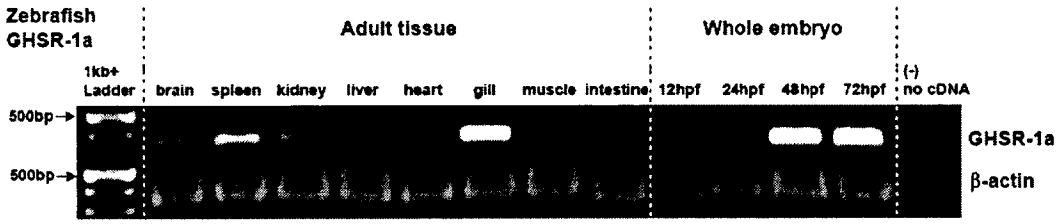

Fig. 3.1. Tissue expression of the GHS-R1a mRNA in zebrafish tissues. Gel view of RT-PCR with GHS-R1a mRNA primers in various zebrafish tissues with β-actin control.

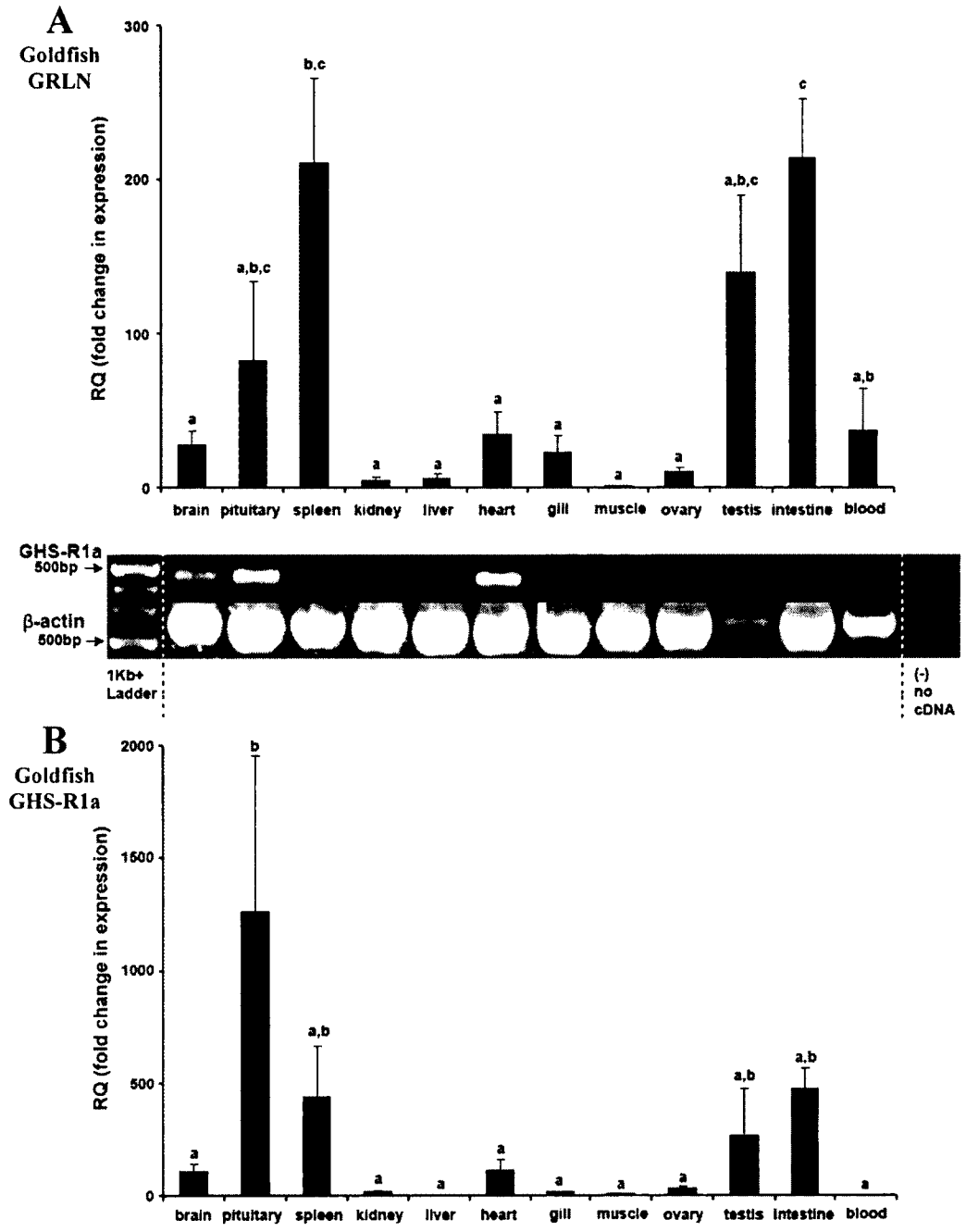

Fig. 3.2. Tissue expression of GRLN and the GHS-R1a mRNA in goldfish tissues. (A, upper) Real-time qPCR analysis of gGHS-R1a mRNA in various goldfish tissues expressed as average fold difference compared to liver tissue ±SEM, n=4-5 fish; (A, lower) corresponding gel view of RT-PCR for GHS-R1a mRNA with β-actin control. (B) Quantitative PCR analysis of gGRLN mRNA in various goldfish tissues expressed as average fold difference compared to muscle tissue ±SEM, n=4-5 fish. qPCR results are normalized to the endogenous control, EF1-α. Tissues with similar relative expression levels share the same letter of the alphabet.

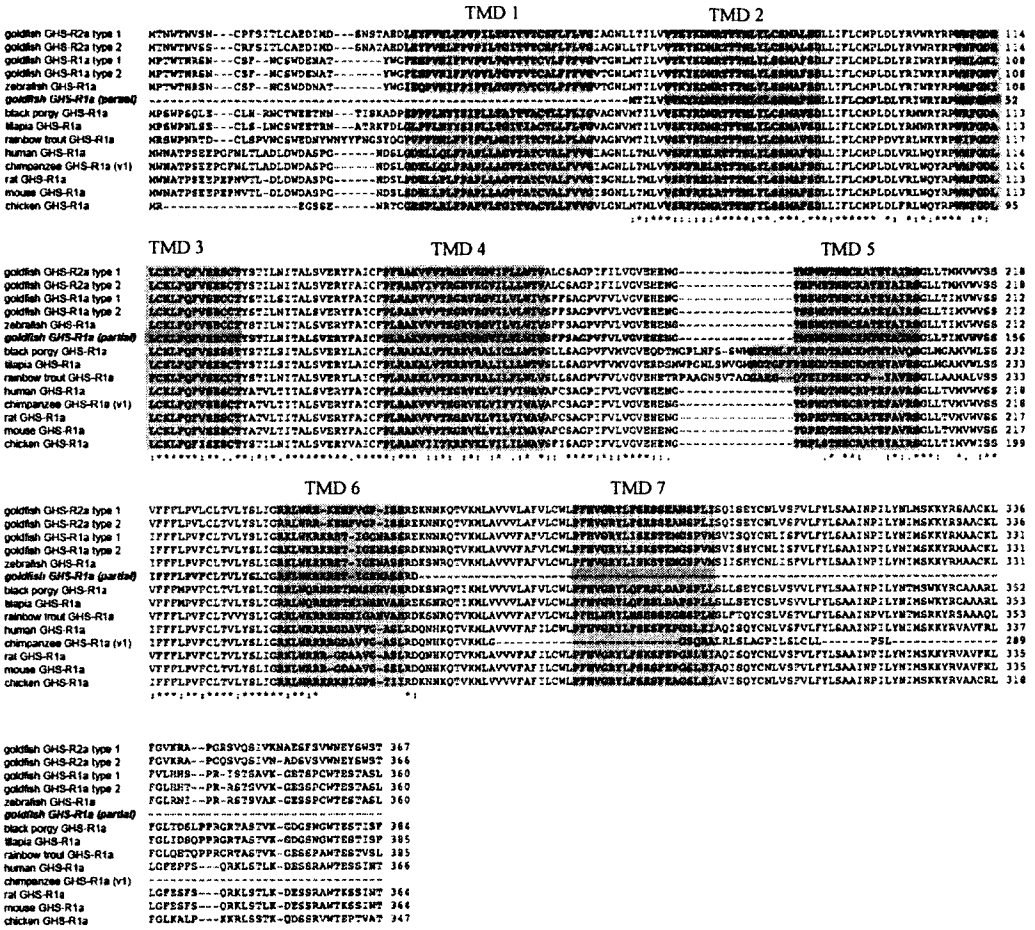

Fig. 3.3. Alignment of the amino acid sequence of the cloned partial gGHS-R1a (highlighted), as compared to GHS-Rs of other species and the recently available full-length gGHS-R sequences. Predicted trans-membrane domains are highlighted in grey. Fully conserved residues are indicated by an asterisk (*), partially conserved and semi-conserved substitutions are represented by ':' and '.', respectively. Amino acid data is deduced from nucleotide sequencing data (not shown).

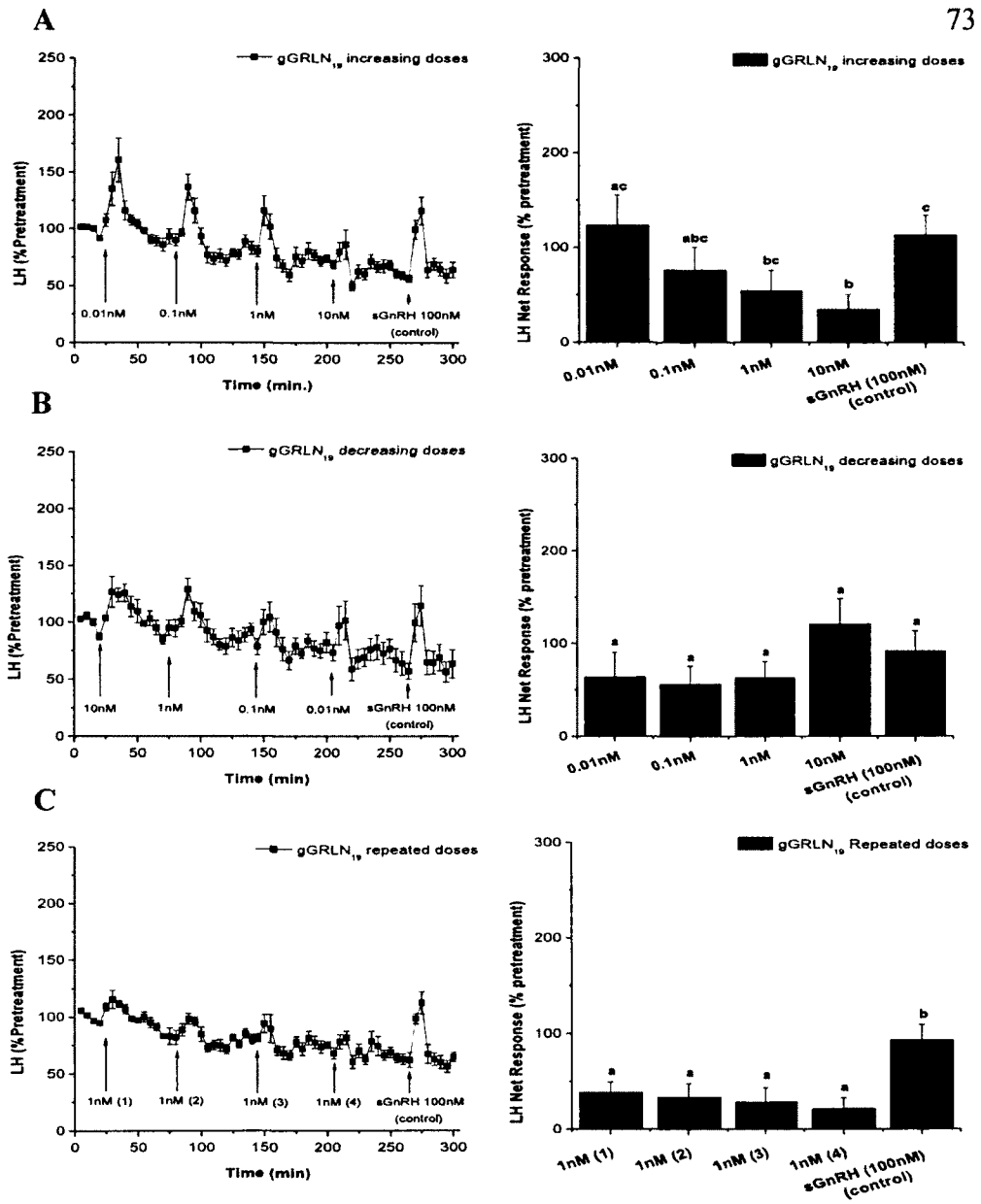

Fig. 3.4. Multiple challenges of $gGRLN_{19}$, applied at intervals of 1 h, induce LH release from dispersed goldfish pituitary cells in perifusion. Arrows indicate applications of 5-min challenges of various doses of $gGRLN_{19}$ applied as increasing (A), decreasing (B), or repeated doses (C) in a column perifusion system. LH values were normalized as % pretreatment (pretreatment value = average values of the first 5 fractions collected; 3.04 ± 0.23 ng/ml). The kinetic profiles of LH release are presented in the left panels and quantified net LH responses are presented on the right. Pooled data are presented as mean ± SEM (n=8 from 4 separate experiments performed between Sept and Dec). Within each treatment series, responses that are not significantly different from one another are denoted by same letters of the alphabet.

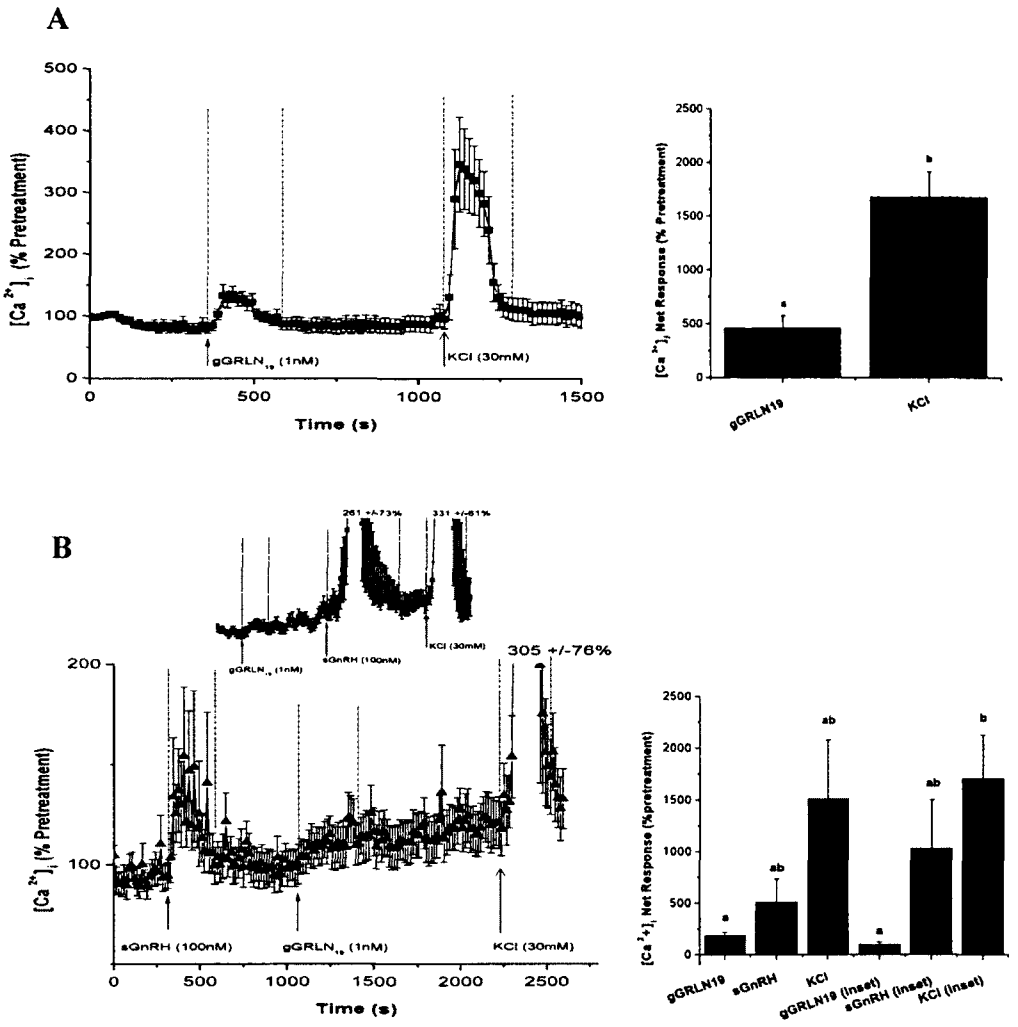

Fig. 3.5. Both $gGRLN_{19}$ and sGnRH elicit changes in $[Ca^{2+}]_i$ in the same individual, identified goldfish gonadotropes within primary cultures of mixed pituitary cells. Cells were treated with 2-min pulses of either 1 nM $gGRLN_{19}$ alone (A) or with 100 nM sGnRH followed by 1 nM $gGRLN_{19}$ (B) or with 1nM $gGRLN_{19}$ followed by sGnRH (B, inset), as indicated by arrows. A 2-min, 30 mM, KCl pulse was applied at the end of the treatment protocol to evaluate if the cells were still healthy and capable of responding to depolarization-induced increase in $[Ca^{2+}]_i$. The kinetic profiles of $[Ca^{2+}]_i$ change are presented in the left panels and quantified net responses are presented on the right. Ca^{2+} levels were normalized to the basal level (% pretreatment; average of the first 5 recorded values; 124.66 ± 32.71 nM) before being pooled. Pooled data are presented as mean ± SEM (n=10 in each case from a total of 8 separate experiments over 3 cell preparations performed in May). Responses that are not significantly different from one another are denoted by same letter of the alphabet.

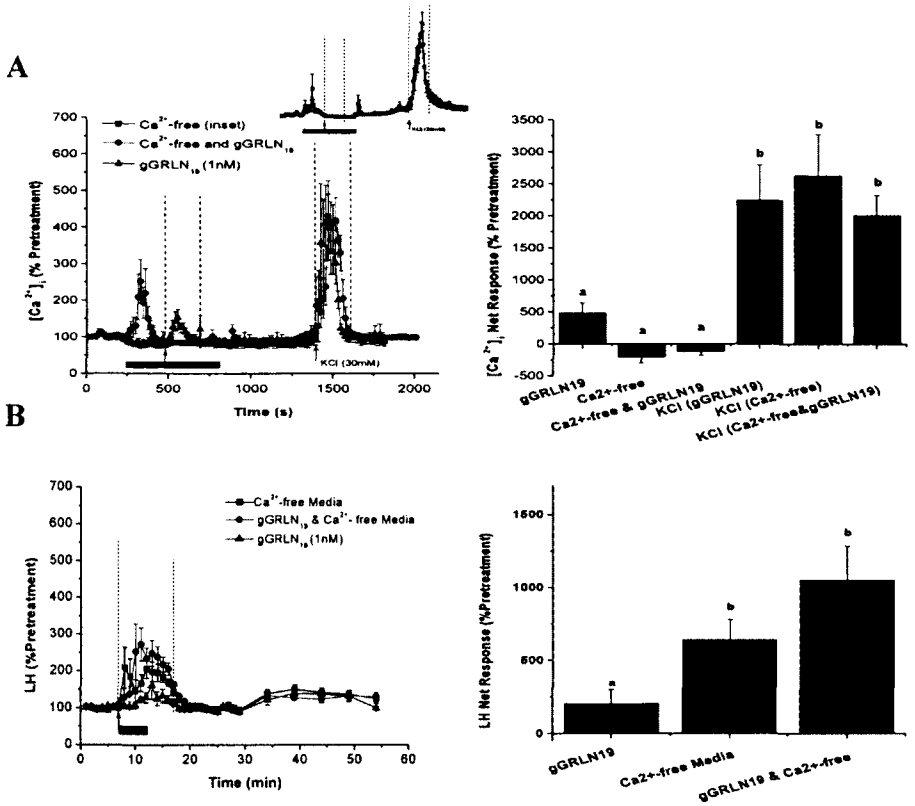

Fig. 3.6. 1 nM $gGRLN_{19}$ did not produce significant changes in $[Ca^{2+}]_i$ (A) and LH release (B) during nominally Ca^{2+}-free media treatment in goldfish pituitary cells. The kinetic response profiles are presented in the left panels and quantified net responses are presented in the right. Vertical dotted lines indicate the duration during which responses to treatments were quantified. Pooled data are presented as mean ± SEM (n=10 from 9 separate experiments over 3 cell preparations performed in July and n=8 from 4 separate experiments performed in May, for $[Ca^{2+}]_i$ and LH release experiments, respectively). Applications of nominally Ca^{2+}-free media are indicated by the solid horizontal bar while $gGRLN_{19}$ applications are indicated by a closed arrow. (A) Cells were pre-treated with nominally Ca^{2+}-free media for 3.25 min prior to a 2-min $gGRLN_{19}$ challenge. Following washout of 10 min, a 2-min pulse of 30 mM KCl (open arrow) was used as a positive control to evaluate if cells remained healthy and responsive to depolarization-induced increases in $[Ca^{2+}]_i$. Ca^{2+} levels were normalized to the basal level (% pretreatment; average of the first 5 recorded values; 118.96 ± 10.33 nM) before being pooled. (B) Cells were pre-treated for 10-min prior to a 5-min $gGRLN_{19}$ challenge. Fractions were collected every minute for the first 30 min, then every 5 min for 25 min. LH values were normalized as % pretreatment (pretreatment value = average values of the first 5 fractions collected; 2.51 ± 0.13 ng/ml). Treatment groups with response values that are not significantly different share the same letter of the alphabet.

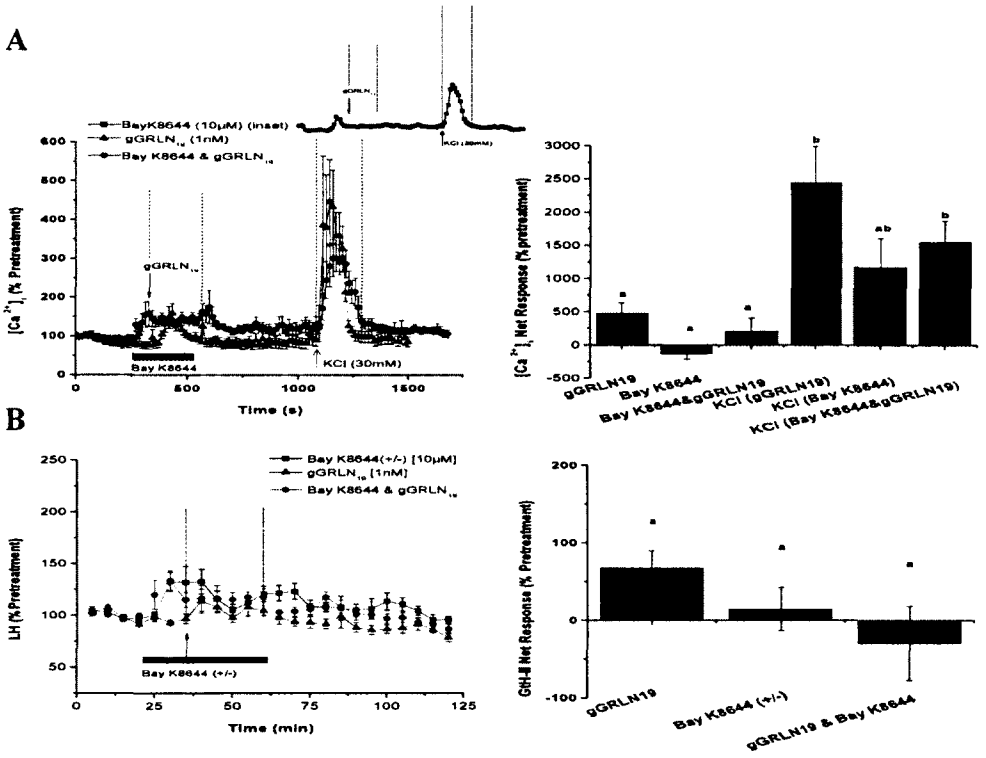

Fig 3.7. 1 nM $gGRLN_{19}$ and 10 μM of the LVSCC agonist Bay K8644 do not produce additive changes in $[Ca^{2+}]_i$ levels (A) or LH release activity (B) in goldfish pituitary cells. The kinetic response profiles are presented in the left panels and quantified net responses are presented in the right panels. The vertical dotted lines indicate the duration during which responses are quantified. Cells were pre-treated with Bay K8644 (solid horizontal bar) prior to $gGRLN_{19}$ challenges (closed arrow). Pooled data are presented as mean ± SEM (n=10 from 8 separate experiments over 3 cell preparations performed in July and n=8 from 4 separate experiments performed in March, for $[Ca^{2+}]_i$ and LH experiments, respectively). (A) Cells were pre-treated with Bay K8644 for 2 min prior to a 2 min pulse of $gGRLN_{19}$. Following a washout of 10 min, a 2-min pulse of 30 mM KCl (open arrow) was used as a positive control to evaluate if cells remained healthy and responsive to depolarization-induced increases in $[Ca^{2+}]_i$. Ca^{2+} levels were normalized to the basal level (% pretreatment; average of the first 5 recorded values; 54.40 ± 5.06 nM) before being pooled. (B) Cells were pre-treated with Bay K8644 for 15 min prior to a 5-min $gGRLN_{19}$ challenge. LH values were normalized as % pretreatment (pretreatment value = average values of the first 5 fractions collected; 2.79 ± 0.10 ng/ml). Treatment groups with response values that are not significantly different share the same letter of the alphabet.

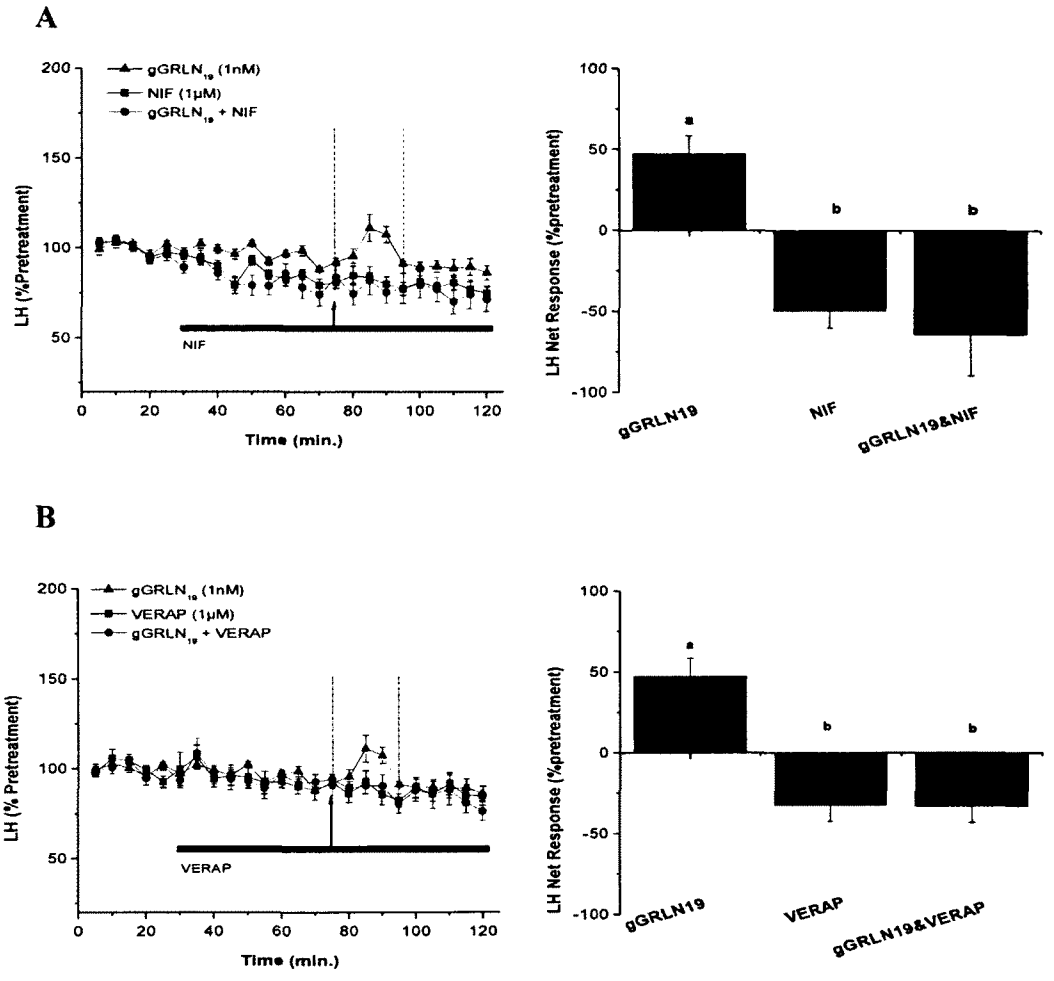

Fig. 3.8. LVSCC inhibitors nifedipine (A) and verapamil (B) eliminate $gGRLN_{19}$-induced LH release from goldfish pituitary cells in perifusion. Cells were pre-treated for 40 min with 1 μM verapamil (VERAP) or nifedipine (NIF) (solid horizontal bars) prior to a 5-min treatment with 1 nM $gGRLN_{19}$ (arrow). The kinetic profiles of the LH responses are shown in the left panels and the quantified net responses are presented in the right panels. The duration of net response quantification is indicated by the two dotted vertical lines. LH values were normalized as % pretreatment (pretreatment value = average values of the first 5 fractions collected; 7.92 ± 0.55 ng/ml). Pooled data are presented as mean ± SEM (n=16 ($gGRLN_{19}$) or n=8 (other treatments), from 5 separate experiments performed between Dec and April). Treatment groups with response values that are not significantly different share the same letter of the alphabet.

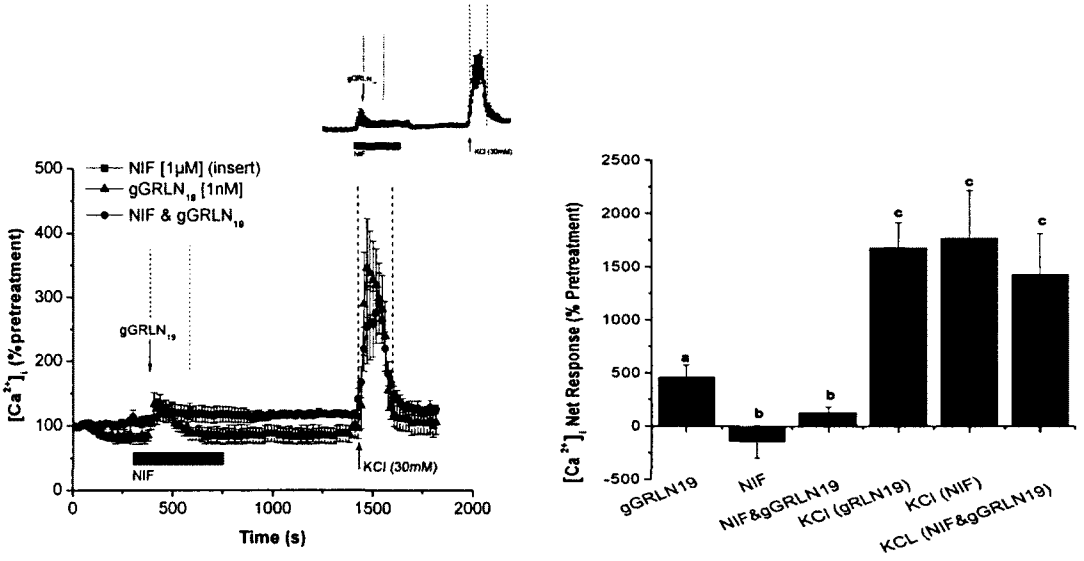

Fig. 3.9. The LVSCC inhibitor nifedipine (NIF) inhibits $gGRLN_{19}$-induced changes in $[Ca^{2+}]_i$ in individual, identified goldfish gonadotropes in primary cultures of mixed pituitary cell. Cells were pre-treated with 1 μM nifedipine (solid horizontal bar) for 2 min prior to a 2-min, 1 nM, $gGRLN_{19}$ challenge (closed arrow). Following washout of 10 min, a 2-min, 30 mM, KCl treatment (open arrow) was used as a positive control to evaluate if cells remained healthy and responsive to depolarization-induced increases in $[Ca^{2+}]_i$. The kinetic profiles of $[Ca^{2+}]_i$ change are presented in the left panel and quantified net responses are presented on the right. Vertical dotted lines indicate the duration of response quantification. Ca^{2+} levels were normalized to the basal level (% pretreatment; average of the first 5 recorded values; 34.10 ± 4.04 nM) before being pooled. Pooled data are presented as mean ± SEM (n=10 from 9 separate experiments over 3 cell preparations performed in August). Treatment groups with response values that are not significantly different share the same letter of the alphabet.

Chapter 4 – $gGRLN_{19}$-induced GH release from goldfish pituitary cells involves LVSCCs

4.1. Introduction

The presence of gGRLN mRNA in the goldfish brain and hypothalamus [321] suggests a role for gGRLN in the regulation of pituitary hormone secretion in this model system and $gGRLN_{19}$ elevates GH release in vivo and in vitro (Chapter 1, Section 1.3.1; [322]). Little knowledge, however, is available regarding intracellular signalling cascades that lead to gGRLN-induced GH release in the goldfish pituitary. On the other hand, stimulation of black seabream GHS-R1a expressed in HEK-293 cells with GHSs demonstrates that stimulation of PLC, increases in $[Ca^{2+}]_i$, and activation of LVSCCs are possible mechanisms linked to GRLN action in fish [31, 32]. Given the importance of increases in $[Ca^{2+}]_i$ in GRLN-elicited GH release in mammals (Chapter 1, Section 1.4.2; [162, 202]) and the relative importance of Ca^{2+} signalling and LVSCCs in mediating the GH release response to many neuroendocrine regulators of GH release in goldfish (Chapter 1, Section 1.4.2; [48]), the hypothesis that Ca^{2+} entry through LVSCCs mediates GRLN action on GH release in goldfish pituitary cells is tested here.

To this end, I examined GH release from primary cultures of dispersed goldfish pituitary cells in a column based perifusion system and changes in $[Ca^{2+}]_i$ using fura-2, AM-based Ca^{2+}-imaging experiments using morphologically identified, individual goldfish somatotropes in mixed pituitary cell culture. The involvement of Ca^{2+} entry through LVSCCs was investigated using commercially available inhibitors or stimulators of LVSCCs and nominally Ca^{2+}-free media. $gGRLN_{19}$ was employed for all studies, as it is an endogenous form found in the

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goldfish [221], and it effectively stimulate GH release responses in the goldfish both in vivo and in vitro in previous experiments [322].

4. 2. Results

3.2.1. Acute GH responses to $gGRLN_{19}$

I employed a perifusion system to examine the acute GH secretion response from goldfish pituitary cells to 5-min challenges of various doses of $gGRLN_{19}$ (0.01 to 10 nM, range is based on previous work using goldfish cells and $gGRLN_{19}$ in static incubation experiments [322]). A sequential application protocol with challenge pulses applied at 60 min intervals was used, allowing washout of treatment and recovery of cells, to evaluate if prior exposure to $gGRLN_{19}$ affects the GH release response to subsequent challenges (Fig. 4.1). At the end of the testing protocol, a 5-min pulse of a maximal stimulatory dose of sGnRH (100 nM) [39] was employed as a positive control to ensure the cell cultures were still viable and responsive following GRLN treatments.

All doses of $gGRLN_{19}$ tested stimulated GH release. Repeated applications of 4 pulses of 1 nM $gGRLN_{19}$ produced GH responses that were not significantly different from one another (Fig. 4.1). Increases in GH release were seen in the 5-min fraction collected immediately after the application of $gGRLN_{19}$. This delay can be totally accounted for by the dead volume of the system, suggesting that the onset of GH response is rapid. When pulses of 0.01, 0.1, 1 and 10 nM $gGRLN_{19}$ were applied in a descending concentration series, the GH responses followed a dose-dependent relationship. In contrast, when the different $gGRLN_{19}$ concentrations were applied in a series of pulses with increasing concentration, the GH response to 10 nM $gGRLN_{19}$ was the smallest

and significantly different from that elicited by 0.01 nM $gGRLN_{19}$, which was the largest (Fig. 4.1A).

In all experiments, cells responded well and consistently to the 100 nM sGnRH treatment applied at the end of each of the test sequences, indicating that the cells remained healthy and capable of responding with increased GH secretion even at the end of the testing protocol. Thus, the lack of dose-response relationships, especially when $gGRLN_{19}$ was applied in increasing concentrations, suggest that prior exposure to high concentrations of $gGRLN_{19}$ has adverse effects on subsequent responses to the same peptide. To avoid such possible complications, single $gGRLN_{19}$ exposures were used in all subsequent experiments. Given that 1 nM $gGRLN_{19}$ produced consistent and repeatable responses, this dose was used in all other experiments.

4.2.2. $[Ca^{2+}]_i$ changes in response to $gGRLN_{19}$ and sGnRH

As a first step in evaluating the possible participation of Ca^{2+} in the intracellular signalling cascade leading to induction of GH release from goldfish pituitary cells by $gGRLN_{19}$, I examined whether $gGRLN_{19}$ increased $[Ca^{2+}]_i$ in identified somatotropes preloaded with the fura-2, AM Ca^{2+}-imaging dye. Application of a 2-min pulse of 1 nM $gGRLN_{19}$ caused a significant elevation in $[Ca^{2+}]_i$ (Fig. 4.2A), consistent with the hypothesis that $gGRLN_{19}$ utilizes Ca^{2+} signalling. The onset of the increase in $[Ca^{2+}]_i$ is relatively rapid, occurring often within ~30 sec from the time of $gGRLN_{19}$ application. Considering that the theoretical exchange time for the imaging chamber is at least 15-16 sec, this "latency" to onset of response is minimal.

Since sGnRH was used as a positive control in experiments in Figure 4.1 to argue that cells previously responding to $gGRLN_{19}$ were healthy and capable of releasing GH when challenged with another ligand, I needed to examine the possibility that the ability of sGnRH to induce GH release in pituitary cell

populations following treatment with GRLN was entirely due to the existence of subpopulations of somatotropes each responding separately to sGnRH and $gGRLN_{19}$. To ascertain if the same cells can respond to both sGnRH and $gGRLN_{19}$, the $[Ca^{2+}]_i$ response in individual somatotropes to sequential 2-min applications of sGnRH (100 nM) and $gGRLN_{19}$ (1 nM) was examined (Fig. 4.2B). Treatments with sGnRH and $gGRLN_{19}$ each produced significant changes in $[Ca^{2+}]_i$. The $[Ca^{2+}]_i$ response to sGnRH appeared larger than that of $gGRLN_{19}$, however there is no significant differences between them. All cells tested in both protocols showed an acute response to a depolarizing 2-min 30 mM KCl treatment, indicating they were healthy and responsive following the exposure to sGnRH and/or $gGRLN_{19}$. The results support the co-expression of receptors for both sGnRH and $gGRLN_{19}$ in at least some goldfish somatotropes.

4.2.3. Effects of limiting the availability of extracellular Ca^{2+}

To explore the source of $gGRLN_{19}$-induced Ca^{2+} signals, I first tested the effects of incubation with nominally Ca^{2+}-free media on $gGRLN_{19}$-induced changes in $[Ca^{2+}]_i$. Application of nominally Ca^{2+}-free media resulted in a transient elevation in $[Ca^{2+}]_i$, which peaked at approximately 30-45 sec, and a sustained lowering of $[Ca^{2+}]_i$ to about 75% of pretreatment levels by about 2 min (Fig. 4.3). The re-introduction of Ca^{2+}-containing media also lead to a transient rise in $[Ca^{2+}]_i$ which peaked at approximately 30 sec followed by a return to basal $[Ca^{2+}]_i$ levels by 2 min. Application of a 2-min pulse of 1 nM $gGRLN_{19}$ generated a significant increase in $[Ca^{2+}]_i$ but a significant response was not observed when $gGRLN_{19}$ was applied at 3.25 min into nominally Ca^{2+}-free treatment (Fig. 4.3). Given that cell viability and responsiveness through much longer protocols had already been well established (Fig. 4.1 & 4.2), sGnRH controls were no longer used in these and subsequent experiments.

Next, I compared the $gGRLN_{19}$-induced GH release responses in the presence of normal Ca^{2+}-containing media or nominally Ca^{2+}-free media using a perifusion protocol with fast fraction collections (Fig. 4.4). Treatment with nominally Ca^{2+}-free media produced a rapid increase in basal GH secretion. As in previous experiments with 5-min fraction collection, treatment with a 1 nM $gGRLN_{19}$ pulse increased GH release in the presence of normal Ca^{2+}-containing media in a 1-min fraction collection protocol. In contrast, $gGRLN_{19}$ applied together with nominally Ca^{2+}-free media did not produce a GH response greater than that observed with the nominally Ca^{2+}-free media alone. Although these results are suggestive of a role of extracellular Ca^{2+} entry in $gGRLN_{19}$-induced GH release from goldfish pituitary cells, they are inconclusive because administration of the nominally Ca^{2+}-free media resulted in a large amount of variance, as well as an increase in basal GH release.

4.2.4. Effects of the LVSCC agonist Bay K8644

To further explore the role of extracellular Ca^{2+} in $gGRLN_{19}$-induced GH release from goldfish pituitary cells, the LVSCC agonist Bay K8644 was used [366]. I hypothesized that if $gGRLN_{19}$ utilized entry of extracellular Ca^{2+} through LVSCCs in its signal transduction cascade, then when these channels were already activated by a high dose of a LVSCC agonist, an additional $gGRLN_{19}$ treatment would not result in a further response, as $gGRLN_{19}$ would be triggering the same channels. However, if LVSCCs were not important in $gGRLN_{19}$ signalling, the responses to both chemicals should be additive. I began by examining the effects of Bay K8644 on $[Ca^{2+}]_i$ in identified somatotropes and by comparing the $[Ca^{2+}]_i$ signals induced by $gGRLN_{19}$ alone or in conjunction with Bay K8644 treatment (Fig. 4.5). Consistent with the presence of LVSCCs on goldfish somatotropes as revealed by previous patch-clamp studies [53] application of 10 μM Bay K8644 rapidly increased $[Ca^{2+}]_i$ in identified goldfish somatotropes. This response is characterized by a peak $[Ca^{2+}]_i$ response within

approximately 1 min followed by a sustained elevation of lower magnitude by approximately 2 min. Application of a 2-min pulse of 1 nM $gGRLN_{19}$ increased $[Ca^{2+}]_i$ when applied alone. However, when $gGRLN_{19}$ was applied 2.5 min into the Bay K8644 treatment, this combination treatment failed to produce a $[Ca^{2+}]_i$ signal beyond what was observed with Bay K8644 alone. Negative responses, as depicted in the right panel of Fig 4.6, are expected in this case given that base-line is subtracted to produce the cumulative net reponse values and base-line values here reflect the peak response magnitude of the pre-exposure to a stimulator. The lack of additive changes in $[Ca^{2+}]_i$ observed in these experiments supports the hypothesis that $gGRLN_{19}$ utilizes extracellular Ca^{2+} entry through LVSCCs as part of its signalling cascade leading to changes in $[Ca^{2+}]_i$.

To further explore this hypothesis, I also examined the GH release response to both $gGRLN_{19}$ and Bay K8644 challenges in perifusion experiments utilizing goldfish mixed pituitary cell populations (Fig. 4.6). Treatment with a 40-min pulse of 10 μM Bay K8644 elicited a GH response consisting of a rapid increase to a maximal elevation lasting for about 20 min and a prolonged sustained response of lower magnitude afterwards. 1 nM $gGRLN_{19}$ increased GH release when applied alone as a 5-min pulse. In contrast, when $gGRLN_{19}$ was applied 15-min into the Bay K8644 treatment, the response obtained with this combination treatment was not significantly different from that observed with Bay K8644 alone over the same time period. These results further support the involvement of extracellular Ca^{2+} and LVSCCs in $gGRLN_{19}$-induced GH release.

4.2.5. LVSCC inhibitors abolish $gGRLN_{19}$-induced GH release and $[Ca^{2+}]_i$ signals

Another approach to examine the role of LVSCCs in $gGRLN_{19}$-induced GH release was to employ the LVSCC inhibitors nifedipine and verapamil [243, 314] in perifusion experiments with goldfish mixed pituitary cell cultures.

Pretreatment of 40 min with inhibitors was employed to allow the cells to re-establish a baseline level of GH secretion following blockage of LVSCCs prior to challenge with $gGRLN_{19}$. Treatment with 1 μM nifedipine reduced basal GH secretion with maximal sustained suppression reached by 15 to 20 min of treatment (Fig. 4.7A). Verapamil applied at 1 μM concentration likewise produced a gradual reduction in basal GH release (Fig. 4.7B). Application of 5-min pulses of 1 nM $gGRLN_{19}$ resulted in significant increases in GH release when applied alone; however, in the presence of nifedipine (Fig. 4.7A) or verapamil (Fig. 4.7B), $gGRLN_{19}$ treatment failed to elicit a GH release response. These results further implicate LVSCCs as a key signalling component in $gGRLN_{19}$-induced GH release.

Finally, the role of LVSCCs in $gGRLN_{19}$-induced changes in $[Ca^{2+}]_i$ were examined in Ca^{2+}-imaging studies. In these experiments, I employed a shorter pretreatment time of only 2 min with 1 μM nifedipine to reduce the possible effects that a long pretreatment with LVSCC inhibitor might have on the status of intracellular Ca^{2+} stores. Nifedipine did not significantly affect basal $[Ca^{2+}]_i$ levels (Fig. 4.8). In the presence of nifedipine, a 2-min pulse of 1 nM $gGRLN_{19}$ failed to elicit a significant change in $[Ca^{2+}]_i$, suggesting that LVSCCs plays a role in the $gGRLN_{19}$ signalling pathway in goldfish somatotropes (Fig. 4.8) in contrast to the effects of $gGRLN_{19}$ alone in previous experiments in the current thesis chapter (e.g., Fig. 4.2, 4.3 & 4.5). I also used a 2-min pulse of 30 mM KCl to establish that cells were able to respond to depolarization with an increase in $[Ca^{2+}]_i$ following washout of the LVSCC inhibitor. In these experiments, the magnitude of the KCl-induced $[Ca^{2+}]_i$ response was similar to those in earlier experiments following stimulation by $gGRLN_{19}$ and/or sGnRH (Fig. 4.1).

4.3. Discussion

Previous studies on goldfish have identified gGRLN mRNA in the brain and hypothalamus [321], suggesting that in addition to the peripheral source of gGRLN, gGRLN from hypothalamic origin may also directly regulate pituitary hormone secretion in goldfish. Previous in vitro and in vivo work has established a role for gGRLN as a GH secretion stimulator in this species [322]. The present GH-release and Ca^{2+}-imaging results not only confirm that $gGRLN_{19}$ is an effective stimulator of GH release, but also suggest that $gGRLN_{19}$ exerts direct action on goldfish somatotropes. These observations, when taken together with the in vitro ability of GRLN to increase GH release from tilapia pituitary cells [88], also suggests that GRLN is an important effector of GH release in fish species in general. More importantly, the results from this thesis chapter provide the first insights into the mechanisms of action of GRLN on GH release in fish, as well as information on other functional characteristics of the receptor(s) for GRLN on somatotropes in goldfish.

Several lines of evidence from my results in the present thesis chapter point to the involvement of extracellular Ca^{2+} entry through LVSCC in mediating $gGRLN_{19}$ stimulation of GH release. First, $gGRLN_{19}$ elicited an increase in $[Ca^{2+}]_i$ in goldfish somatotropes and this action was not seen when nominally Ca^{2+}-free media was used as the testing media, suggesting that $gGRLN_{19}$ action requires an adequate supply of extracellular Ca^{2+}. Second, when GH secretion was already elevated by a 10 μM concentration of the dihydropyridine LVSCC agonist Bay K8644, which also effectively increased $[Ca^{2+}]_i$ in single identified goldfish somatotropes, application of $gGRLN_{19}$ failed to further increase GH release, as well as $[Ca^{2+}]_i$. Bay K8644 has been shown to increase the mean open times of LVSCCs in the 10 μM range, the dosage used in the present study [15, 366]. Third, two LVSCC inhibitors, nifedipine and verapamil, abolished the ability of $gGRLN_{19}$ to increase GH secretion. These two antagonists have been

shown to inhibit LVSCC by different mechanisms in the 1 μM range [243, 314]. Furthermore, $gGRLN_{19}$ failed to increase $[Ca^{2+}]_i$ in the presence of nifedipine and both verapamil and nifedipine reduced Ca^{2+} currents through LVSCCs in goldfish pituitary cells in previous studies [259]. The idea that extracellular Ca^{2+} availability and LVSCC participate in $gGRLN_{19}$ action in goldfish somatotropes is supported by findings from other pituitary and non-pituitary test models. Nifedipine treatment leads to a significant decrease in GRLN-induced GH release in dispersed rat pituitary cells [357] and porcine pituitary cells [202]. GRLN also up-regulates high-voltage activated LVSCCs in GH-secreting cells derived from a rat pituitary tumor cell-line, the GC somatotropes [74]. Furthermore, the importance of availability of extracellular Ca^{2+} in GRLN action has been demonstrated in rat arcuate nucleus [168] and in HEK-293 cells transfected with GHS-R [32].

Although the GRLN-induced increase in $[Ca^{2+}]_i$ appears to be entirely dependent on extracellular Ca^{2+} entry in porcine pituitary cells [202], GRLN action in rat somatotropes additionally involves mobilization of Ca^{2+} from Tg-sensitive intracellular stores [357]. Despite the fact that a 2-min pre-treatment with nifedipine was adequate to abolish the entire $[Ca^{2+}]_i$ response to $gGRLN_{19}$ and that $gGRLN_{19}$ failed to further increase $[Ca^{2+}]_i$ in cells pre-treated with Bay K8644, I cannot entirely rule out the possibility that mobilization of Ca^{2+} from intracellular stores also participate in $gGRLN_{19}$ action on goldfish somatotropes. First, the present imaging system gives an overall measurement of $[Ca^{2+}]_i$ across the cell and small regional increases may not be adequate to produce a detectable change in signal. Second, the filling status of intracellular Ca^{2+} stores is tightly tied to entry of Ca^{2+} through membrane Ca^{2+} channels in goldfish pituitary cells [138]. Like the present study, acute exposure to nominally Ca^{2+}-free media elevates both GH [348] and LH [146] secretion from goldfish pituitary cells. Such a paradoxical increase in hormone release has been interpreted as the result

of release of Ca^{2+} from intracellular stores in response to the disturbance of Ca^{2+} homeostasis caused by the reduction of the availability of extracellular Ca^{2+} for entry through LVSCC and other plasma Ca^{2+} channels. This is consistent with the existence of a transient elevation in $[Ca^{2+}]_i$ observed at the onset of the application of nominally Ca^{2+}-free media. The importance and involvement of Tg-sensitive and -insensitive intracellular Ca^{2+} stores in mediating the GH responses to GnRH, DA and PACAP have been demonstrated for goldfish (Chapter 1, Fig 1.7; [28, 58, 143, 146]). Activation of mammalian and fish GRLN receptor systems is also linked to PLC with the generation of IP3, which mobilizes Ca^{2+} from intracellular stores (see Chapter 1, section 1.4.2). The possible involvement of intracellular Ca^{2+} stores in mediating $gGRLN_{19}$ action on GH release has yet to be examined in goldfish. Further studies utilizing SERCA inhibitors, such as Tg, and IP3 receptor inhibitors, such as xestospongin C, will be essential in understanding the role of intracellular stores in GRLN-induced GH release from goldfish pituitary cells.

$gGRLN_{19}$ produced significant GH release responses from mixed goldfish pituitary cultures in the present thesis chapter as in a previous report using static incubation and single dose perifusion protocols [322]. Interestingly, the response sizes to $gGRLN_{19}$ were related more to the positioning of a given treatment rather than the concentration of a given dose. The first test pulses in a protocol often produced larger responses irrespective of their concentration. These results suggest that receptor desensitization, trafficking, and/or other form of down-regulation in the subsequent intracellular pathway rapidly occurs following GHSR activation in goldfish. As mentioned previously (Chapter 3), repeated treatment of GRLN or synthetic GHSs such as GHRP-6 resulted in attenuated $[Ca^{2+}]_i$ signals in HEK-293 cells transfected GHSR-1a [26, 27] while transiently transfected GHS-Rs within HEK-293 cells have been shown to undergo a high level of constitutive and ligand-induced internalization [123], resulting in very

low number of receptors being available following a ligand challenge. Further characterization of the cloned goldfish GHS-Rs (see Chapter 3) will be needed before evaluation of the different means of receptor and post-receptor desensitization can proceed. Demonstration of GHS-R expression on goldfish somatotrope will also help to confirm that $gGRLN_{19}$ acts directly on goldfish somatotropes although the ability of this hormone to rapidly elevate $[Ca^{2+}]_i$ in identified goldfish somatotropes and GH secretion in mixed pituitary cell population is supportive of such a possibility.

Recently, it has been established that GnRH-induced LH secretion in rat pituitaries is potentiated by GRLN co-treatment [84]. Results in this thesis chapter provides evidence that identified somatotropes within goldfish mixed pituitary cultures are reactive to both sGnRH and $gGRLN_{19}$, supporting the co-expression of both types of receptors on single somatotropes. Interestingly, the $[Ca^{2+}]_i$ increase induced by $gGRLN_{19}$ following prior exposure to sGnRH is, on average, 277% smaller than that induced by $gGRLN_{19}$ alone, suggesting that interactions between the Ca^{2+} signalling components of two ligands exist. sGnRH has long been known to stimulate GH release from goldfish pituitaries via the PLC/IP3/PKC pathway and with the involvement of both extra- and intra-cellular Ca^{2+} stores [138, 143, 145]. With the demonstration of the involvement of extracellular Ca^{2+} entry through LVSCC in $gGRLN_{19}$ action in the present study and the potential activation of the PLC/IP3/PKC in GRLN signalling in other systems [170], investigation of the interactions of sGnRH and $gGRLN_{19}$ in the control of GH release in goldfish will be needed in future studies. These studies should also include evaluation of the mechanism(s) whereby $gGRLN_{19}$ activates LVSCC. Given that LVSCC can be regulated by G-protein subunits, PKA, PKC, and phosphatidylinositol 3,4,5-trisphosphate [1, 311], these should be possible targets in such investigations.

The regulation of GH release from the goldfish pituitary is under a complex, multi-factorial control network in which gGRLN plays a role. Results reported in this thesis chapter have provided clear evidence that $gGRLN_{19}$ can elicit significant GH-releasing activity in a repeatable and reversible manner from goldfish pituitary cells, and for the first time that the $gGRLN_{19}$ signalling pathway in somatotropes may be subject to rapid desensitization. Moreover, the ability of $gGRLN_{19}$ to induced GH release from goldfish pituitary cells is highly dependent on the availability of extracellular Ca^{2+} and the activity of LVSCCs. These results when taken together provide the basis for future studies into the signal transduction mechanisms of $gGRLN_{19}$ in a physiological system of regulation of GH secretion.

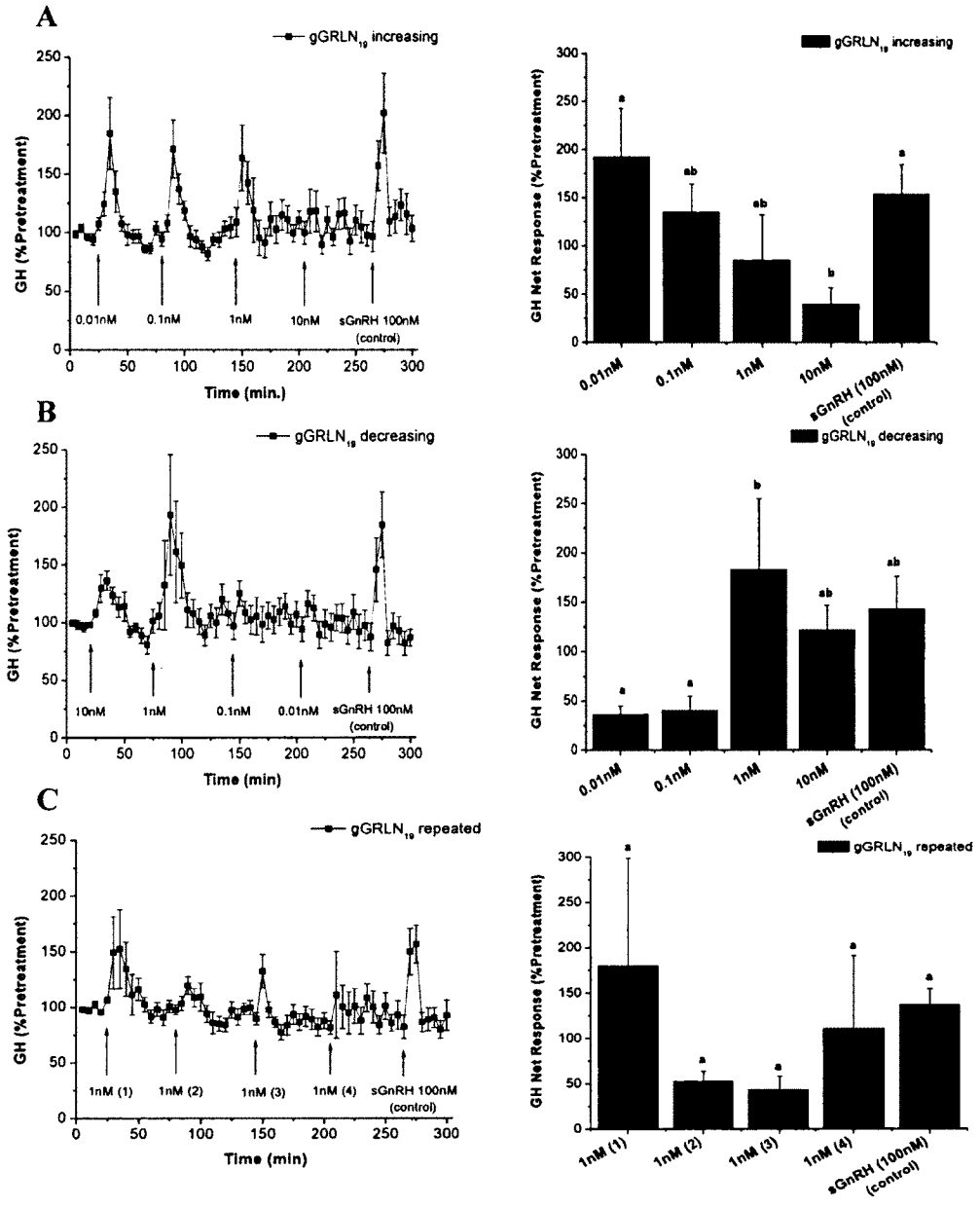

Fig 4.1. Multiple challenges of $gGRLN_{19}$, applied at intervals of 1 h, induce GH release from dispersed goldfish pituitary cells in perifusion. Arrows indicate applications of 5-min challenges of various doses of $gGRLN_{19}$ applied as increasing (A), decreasing (B), or repeated doses (C) in a column perifusion system. GH values were normalized as % pretreatment (pretreatment value = average values of the first 5 fractions collected; 32.97 ± 1.37 ng/ml). The kinetic profiles of GH release are presented in the left panels and quantified net GH responses are presented on the right. Pooled data are presented as mean ± SEM (n=8 from 4 separate experiments performed between Sept and Dec). Within each treatment series, responses that are not significantly different from one another are denoted by same letters of the alphabet.

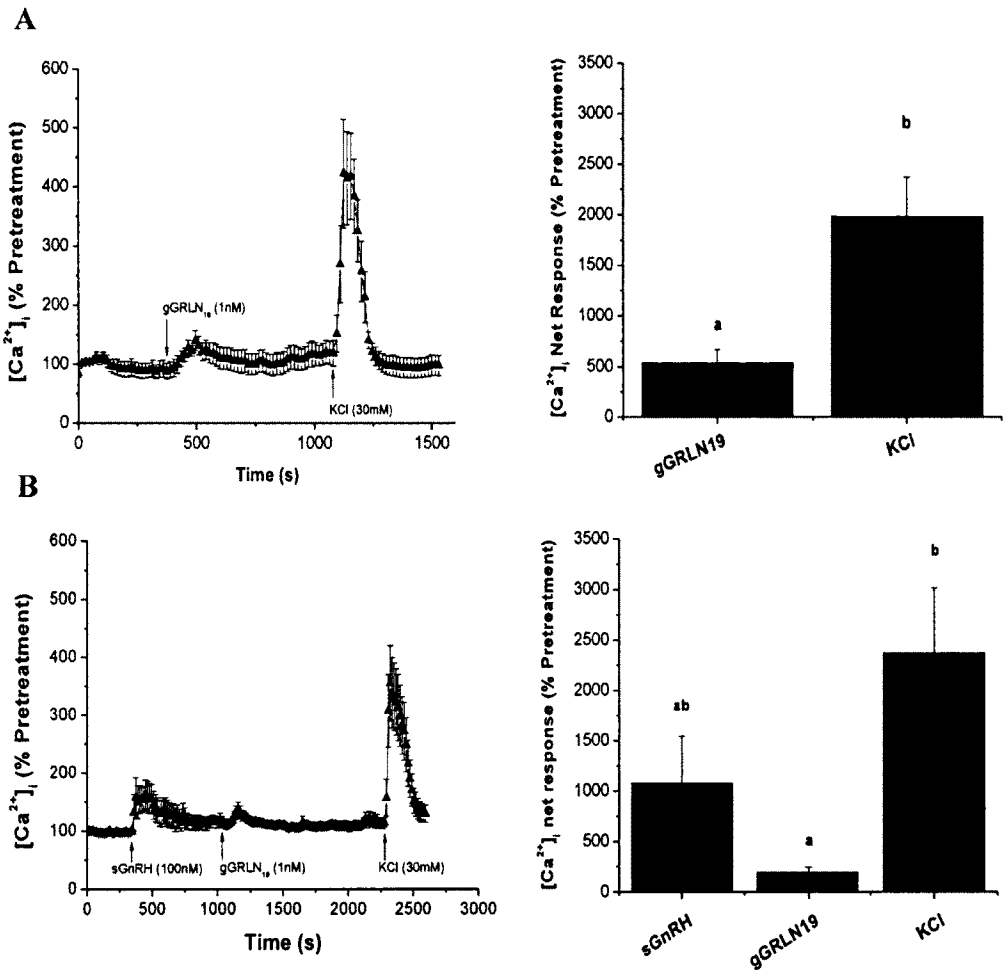

Fig 4.2. Both $gGRLN_{19}$ and sGnRH elicit changes in $[Ca^{2+}]_i$ in the same individual, identified goldfish somatotropes within primary cultures of mixed pituitary cells. Cells were treated with 2-min pulses of either 1 nM $gGRLN_{19}$ alone (A) or with 100 nM sGnRH followed by 1 nM $gGRLN_{19}$ (B), as indicated by arrows. A 2-min, 30 mM, KCl pulse was applied at the end of the treatment protocol to evaluate if the cells were still healthy and capable of responding to depolarization-induced increase in $[Ca^{2+}]_i$. The kinetic profiles of $[Ca^{2+}]_i$ change are presented in the left panels and quantified net responses are presented on the right. Calcium levels were normalized to the basal level (% pretreatment; average of the first 5 recorded values; 129.32 ± 4.84 nM) before being pooled. Pooled data is presented as mean ± SEM (n=10 in each case from a total of 8 separate experiments over 3 cell preparations performed in May). Responses that are not significantly different from one another are denoted by same letter of the alphabet.

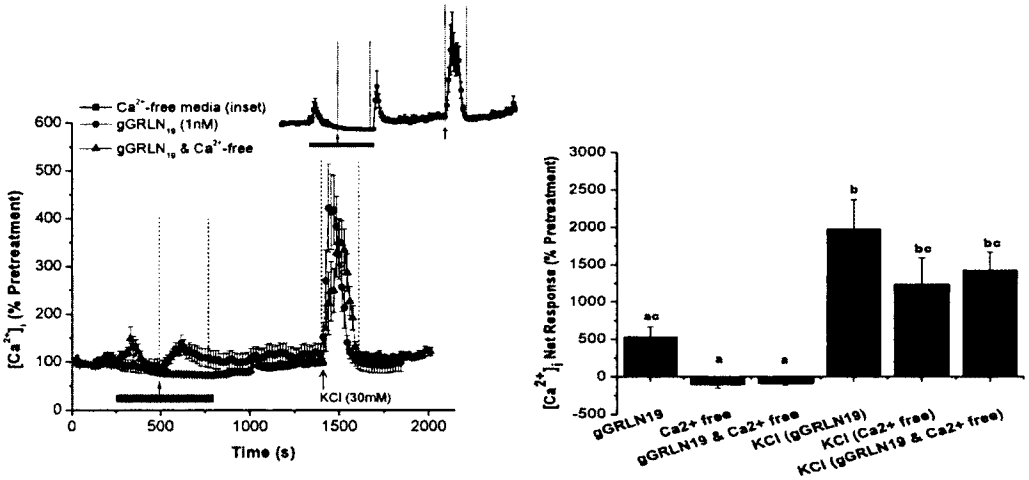

Fig 4.3. 1 nM $gGRLN_{19}$ did not produce changes in $[Ca^{2+}]_i$ levels during nominally Ca^{2+}-free media treatment in individual identified goldfish somatotropes in primary cultures of mixed pituitary cells. Cells were pre-treated with nominally Ca^{2+}-free media (solid horizontal bar) for 3.25 min prior to a 2-min $gGRLN_{19}$ challenge (solid arrow). Following washout of 10 min, a 2-min pulse of 30 mM KCl (open arrow) was used as a positive control to evaluate if cells remained healthy and responsive to depolarization-induced increases in $[Ca^{2+}]_i$. The kinetic profiles of $[Ca^{2+}]_i$ change are presented in the left panel and quantified net responses are presented on the right. Vertical dotted lines indicate the duration during which responses to $gGRLN_{19}$, Ca^{2+}-free, and $gGRLN_{19}$ & Ca^{2+}-free (left) or KCl (right) treatments are quantified. Calcium levels were normalized to the basal level (% pretreatment; average of the first 5 recorded values; 128.06 ± 10.65 nM) before being pooled. Pooled data is presented as mean ± SEM (n=10 from 9 separate experiments over 3 cell preparations performed in July).

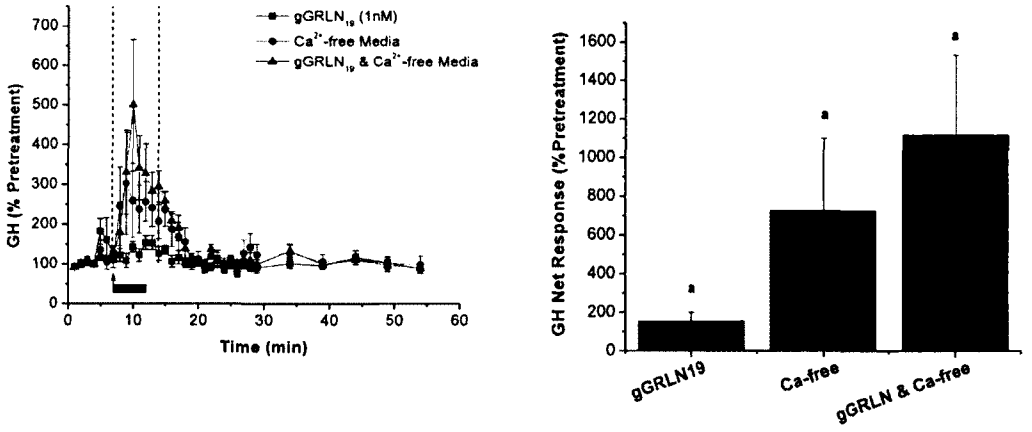

Fig 4.4. Effects of nominally Ca^{2+}-free media on $gGRLN_{19}$-induced GH release form goldfish pituitary cells in perifusion. Fractions were collected every minute for the first 30 min, then every 5 min for 25 min. Exposure to nominally Ca^{2+}-free media is indicated by the solid horizontal bar whereas the 5-min challenge of 1 nM $gGRLN_{19}$ is denoted by the arrow. The kinetic profiles of GH release are presented in the left panel and the quantified net responses are presented on the right. The duration of net response quantification is indicated by the two dotted vertical lines. GH values were normalized as % pretreatment (pretreatment value = average values of the first 5 fractions collected; 17.90 ± 3.01 ng/ml). Pooled data is presented as mean ± SEM (n=8 from 4 separate experiments performed in May).

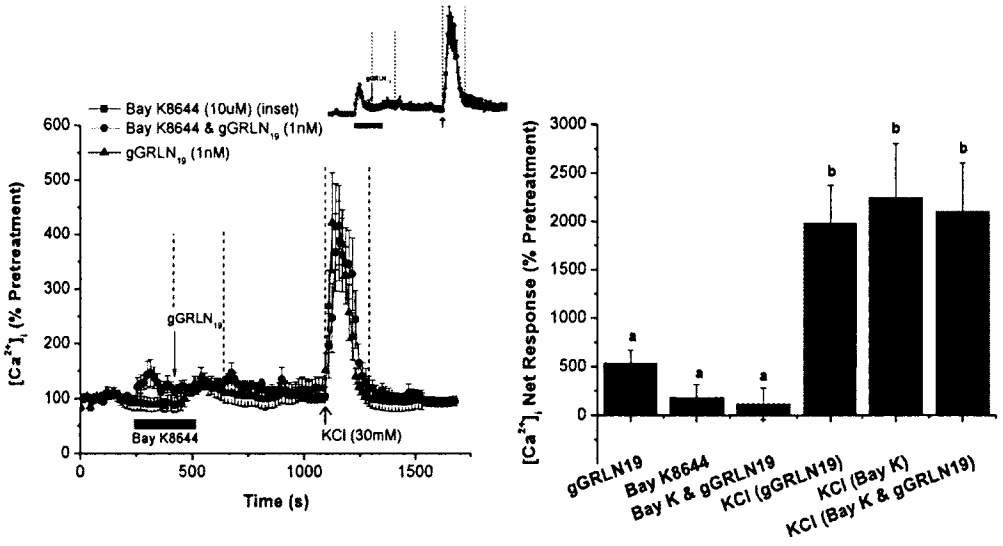

Fig 4.5. 1 nM $gGRLN_{19}$ and 10 μM of the LVSCC agonist Bay K8644 do not produce additive changes in $[Ca^{2+}]_i$ levels in individual identified goldfish somatotropes in primary cultures of mixed pituitary cells. Cells were pre-treated with Bay K8644 (solid horizontal bar) for 2.5 min prior to a 2-min $gGRLN_{19}$ challenge (solid arrow). Following washout of 10 min, a 2-min pulse of 30 mM KCl (open arrow) was used as a positive control to evaluate if cells remained healthy and responsive to depolarization-induced increases in $[Ca^{2+}]_i$. The kinetic profiles of $[Ca^{2+}]_i$ change are presented in the left panel and quantified net responses are presented on the right. The vertical dotted lines indicate the duration during which responses to $gGRLN_{19}$, Bay K8644, and Bay K8644 & $gGRLN_{19}$ (left) or KCl (right) treatments are quantified. Calcium levels were normalized to the basal level (% pretreatment; average of the first 5 recorded values; 174.73 ± 25.42 nM) before being pooled. Pooled data is presented as mean ± SEM (n=10 from 8 separate experiments over 3 cell preparations performed in July).

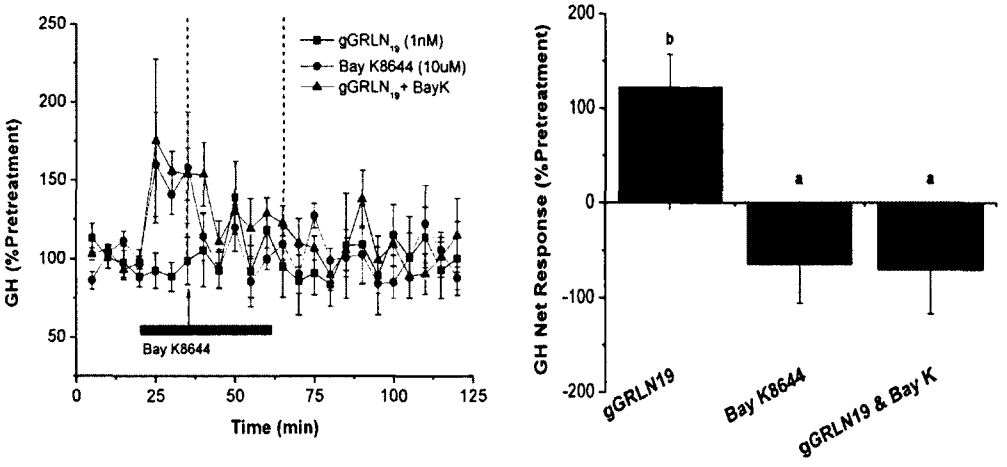

Fig 4.6. Effects of the LVSCC agonist Bay K8644 on $gGRLN_{19}$-induced GH release. Cells were pretreated with 10 μM Bay K8644 (solid horizontal bar) for 15 min prior to a 5-min, 1 nM, $gGRLN_{19}$ challenge (arrow). The kinetic profiles of GH release are presented in the left panel and the quantified net GH responses are presented on the right. The two vertical dotted lines indicate the duration through which responses to $gGRLN_{19}$, Bay K8644, and $gGRLN_{19}$ & Bay K8644 treatments (left) were quantified. GH values were normalized as % pretreatment (pretreatment value = average values of the first 5 fractions collected; 14.13 ± 3.07 ng/ml). Pooled data is presented as mean ± SEM (n=8 from 4 separate experiments performed in March).

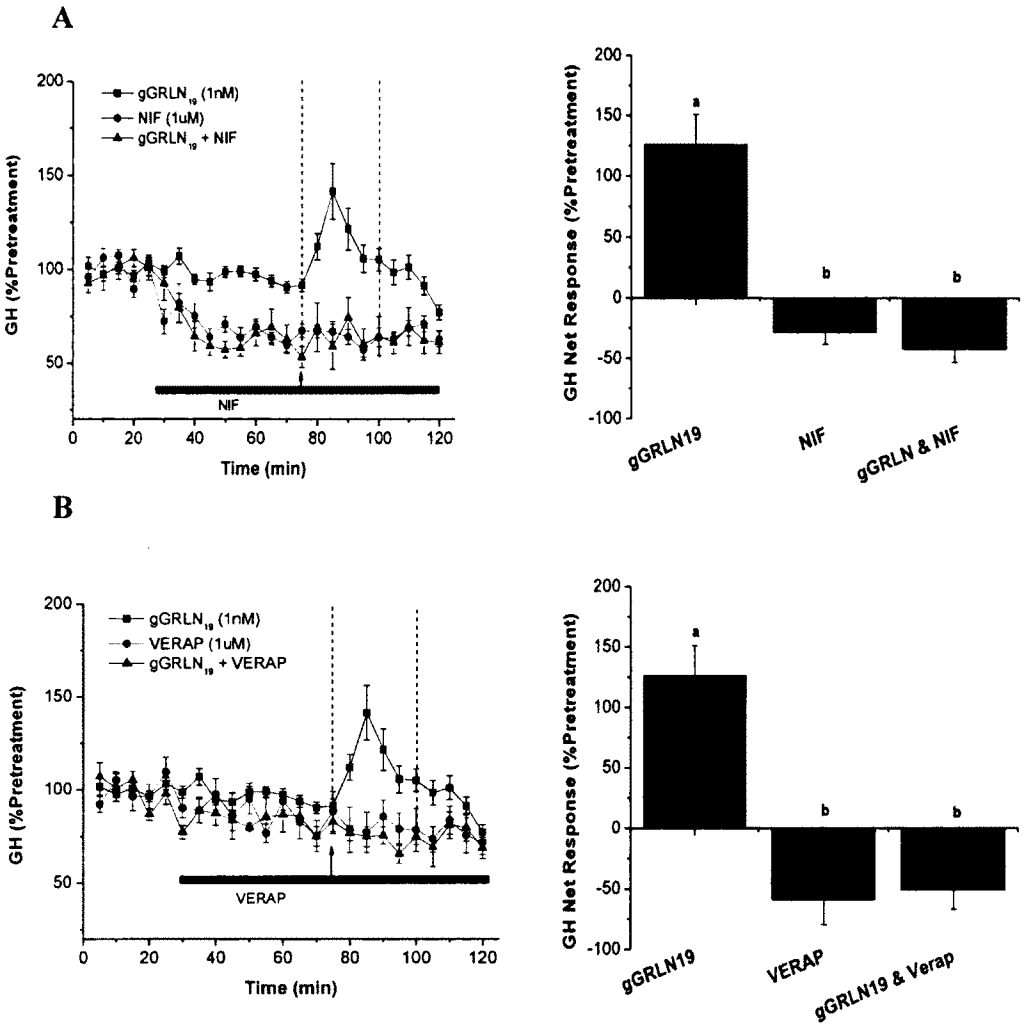

Fig 4.7. LVSCC inhibitors nifedipine (NIF; A) and verapamil (VERAP; B) eliminate $gGRLN_{19}$-induced GH release from goldfish pituitary cells in perifusion. Cells were pre-treated for 40 min with 1 μM VERAP or NIF (solid horizontal bars) prior to a 5-min treatment with 1 nM $gGRLN_{19}$ (arrow). The kinetic profiles of the GH responses are shown in the left panels and the quantified net responses are presented in the right panels. The duration of net response quantification is indicated by the two dotted vertical lines. GH values were normalized as % pretreatment (pretreatment value = average values of the first 5 fractions collected; 26.61 ± 1.86 ng/ml). Pooled data presented as mean ± SEM (n=16 ($gGRLN_{19}$) or n=8 (other treatments), from 5 separate experiments performed between Dec and April).

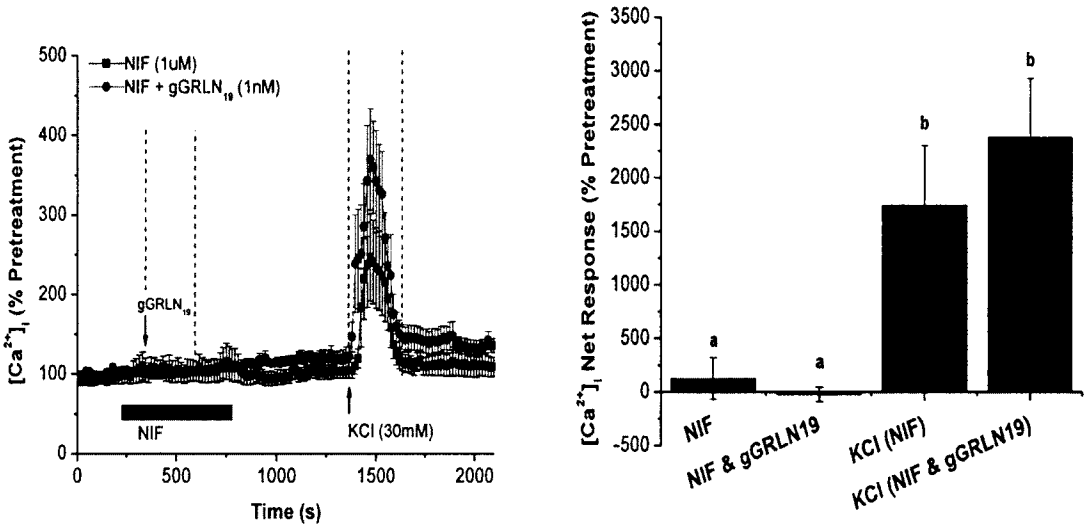

Fig 4.8. The LVSCC inhibitor nifedipine (NIF) inhibits $gGRLN_{19}$-induced changes in $[Ca^{2+}]_i$ in individual identified goldfish somatotropes in primary cultures of mixed pituitary cell. Cells were pre-treated with 1 μM nifedipine (solid horizontal bar) for 2 min prior to a 2-min, 1 nM, $gGRLN_{19}$ challenge (arrow). Following washout of 10 min, a 2-min, 30 mM, KCl treatment (open arrow) was used as a positive control to evaluate if cells remained healthy and responsive to depolarization-induced increases in $[Ca^{2+}]_i$. The kinetic profiles of $[Ca^{2+}]_i$ change are presented in the left panel and quantified net responses are presented on the right. Vertical dotted lines indicate the duration of response quantification. Calcium levels were normalized to the basal level (% pretreatment; average of the first 5 recorded values; 125.29 ± 16.92 nM) before being pooled. Pooled data is presented as mean ± SEM (n=10 from 9 separate experiments over 3 cell preparations performed in August).

Chapter 5 – Differential involvement of PKC and PKA in $gGRLN_{19}$-induced GH and LH release from goldfish pituitary cells

5.1. Introduction

As indicated in Chapter 1, GRLN regulates growth and reproduction by its effects on pituitary GH and LH release. In mammals, the consensus is that GRLN actions on somatotropes involve PLC/PKC/Ca^{2+}, and although controversial, the participation of AC/cAMP/PKA signalling has also been shown (Chapter 1, Section 1.3.2; [169, 271]). GRLN's ability to directly induce LH release is also contentious in mammals and not much is known regarding GRLN's signal transduction mechanisms in mammalian gonadotropes (Chapter 1, Sections 1.3.1.1 & 1.3.2; [171, 172, 230]).

In teleosts, GRLN action in GH and LH secretion is not well understood but $gGRLN_{19}$-induces GH and LH release from goldfish in vivo, as well as from primary cultures of goldfish pituitary cells in vitro (Chapter 1, Section 1.3.1.2; [110, 115, 322]). In Chapters 3 & 4, I have shown that these $gGRLN_{19}$ actions are dependent on extracellular Ca^{2+} entry through LVSCCs [110, 115], however, whether PKC and/or PKA signalling are also involved is unknown. Despite the shared involvement of LVSCCs, the GHS-R antagonist D-Lys^3-GHRP-6 inhibits $gGRLN_{19}$-elicited LH, but not GH, release [322], and the profiles for $gGRLN_{19}$-induced Ca^{2+} signals are dissimilar in goldfish somatotropes and gonadotropes, with increases in $[Ca^{2+}]_i$ in response to $gGRLN_{19}$ treatment occurring more rapidly in gonadotropes than in somatotropes (Chapters 3 & 4; [110, 115]). Whether these observations represent significant differences between cell types in is currently unknown, but these results suggest that $gGRLN_{19}$ utilizes non-

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identical intracellular signalling complements in enhancing hormone release from goldfish gonadotropes and somatotropes.

In goldfish pituitary cells, PKA and PKC are two key mechanisms in the GH and LH release responses to various stimulators and inhibitors, and the differential use of these two signalling pathways is an important element in the selective regulation of LH and GH secretion by neuroendocrine regulators in goldfish (Chapter 1, Sections 1.4.2 & 1.5.2). In this thesis chapter, I tested the hypothesis that PKA and PKC differentially participate in the signalling of $gGRLN_{19}$ on hormone release from goldfish gonadotropes and somatotropes by examining the effects of stimulators and inhibitors of PKC and PKA on GH and LH release responses from goldfish pituitary cells to $gGRLN_{19}$ in column perifusion experiments. To gain insight into the relationships between PKC and PKA activation and the Ca^{2+} signal evoked by $gGRLN_{19}$ leading to hormone release, I monitored $[Ca^{2+}]_i$ in identified somatotropes and gonadotropes with fura-2, AM Ca^{2+}-imaging in parallel experiments.

5.2. Results

5.2.1. Effects of PKC inhibitors, Bis-II and Gö 6976 on $gGRLN_{19}$-induced hormone release and changes in $[Ca^{2+}]_i$

Previous immunochemical studies have shown that conventional, novel and atypical PKC isoforms are expressed in the goldfish pituitary and that conventional PKCs are present in goldfish somatotropes [135, 165]. Thus, to investigate the role of PKC in $gGRLN_{19}$-induced GH and LH release we first examined the effects of two PKC inhibitors, the PKCα and PKCβ isoforms-selective Gö 6976 [205] and the general PKC inhibitor Bis-II [312] in column perifusion assays. At a dose of 100 nM, both of these inhibitors have been shown

to abolish the $[Ca^{2+}]_i$ responses to the PKC agonist DiC8 in goldfish pituitary cells [362]. Application of a 5-min pulse of 1 nM $gGRLN_{19}$, increased GH and LH secretion in the present study to levels comparable with previously reported results (Fig. 5.1; Chapters 3 & 4; [110, 115]). Treatment with either 100 nM Gö 6976 or Bis-II alone did not significantly alter basal secretion of GH and LH (Fig. 5.1A and C) and in the presence of these inhibitors, $gGRLN_{19}$ did not produce hormone responses that were different from those to PKC inhibitors alone (Fig. 5.1). These results implicated a critical role for PKC in $gGRLN_{19}$-induced GH and LH release.

To gain an insight into the relationship between PKC and the $gGRLN_{19}$-induced Ca^{2+} signal important for stimulated GH and LH release [110, 115], I examined the effects of the two PKC inhibitors (100 nM) on $[Ca^{2+}]_i$ responses to 2-min applications of 1 nM $gGRLN_{19}$. For clarity, only $[Ca^{2+}]_i$ traces for Gö 6976 and corresponding treatments are shown in the main panels while Bis-II traces are shown in the inset (Fig. 5.2A and C). In addition, the 30 mM depolarizing pulse of KCl (used as a positive control as in previous protocols in Chapters 3 & 4) is also not depicted in the interest of clarity. In both gonadotropes and somatotropes, a 2-min pulse of $gGRLN_{19}$ was able to significantly induce a change in $[Ca^{2+}]_i$ levels. Gö 6976 treatment alone, but not Bis-II alone, lead to a transient rise in $[Ca^{2+}]_i$ levels in both GH and LH cell types but $[Ca^{2+}]_i$ returned to near pretreatment levels before $gGRLN_{19}$ was administered (Fig. 2A and C). In the presence of either Gö 6976 or Bis-II, the $[Ca^{2+}]_i$ responses to $gGRLN_{19}$ were reduced relative to those elicited by $gGRLN_{19}$ alone (Fig. 5.2 B and D). These observations suggest that PKC is involved in $gGRLN_{19}$ Ca^{2+} signalling in both GH and LH cell types in the goldfish pituitary.

5.2.2. $gGRLN_{19}$ and the PKC agonist DiC8 do not induce additive responses of GH and LH release and $[Ca^{2+}]_i$ increases.

As a further evaluation of the possible involvement of PKC in $gGRLN_{19}$ actions on goldfish somatotropes and gonadotropes, I examined the effects of combined treatments of $gGRLN_{19}$ and a synthetic PKC activator, DiC8. The rationale is that if PKC is not a central element in $gGRLN_{19}$ signalling leading to increased hormone release and changes in $[Ca^{2+}]_i$ in goldfish somatotropes and gonadotropes, $gGRLN_{19}$ should still be able to induced unattenuated hormone release responses even when the PKC pathway is fully activated by a maximally stimulatory dose of a synthetic PKC agonist.

Working under the assumption that administration of a maximally stimulating dose of 100 μM DiC8 [44, 137] will fully saturate the PKC pathway, I compared the hormone responses to a 35-min treatment with 100 μM DiC8 alone, a 5-min pulse of 1 nM $gGRLN_{19}$ alone and the combined treatment of 35 min 100 μM DiC8 applied 10 min prior to a 5-min pulse of $gGRLN_{19}$ (Fig. 5.3). As in previous experiments, $gGRLN_{19}$ was able to induce significant GH and LH release from the goldfish pituitary cells by itself, and the responses to combined treatment of $gGRLN_{19}$ and DiC8 were not significantly different from those to DiC8 alone (Fig. 5.3). Similarly, although application of a 2-min pulse of 1 nM $gGRLN_{19}$ alone significantly elevated $[Ca^{2+}]_i$ in goldfish somatotropes and gonadotropes, the responses to $gGRLN_{19}$ applied 2.5 min into a 10-min continuous treatment with 100 μM DiC8 were not significantly different from those to DiC8 alone in both cell types (Fig. 5.4). These results are consistent with the hypothesis that cellular responses to $gGRLN_{19}$ in both GH and LH cells in the goldfish pituitary are PKC dependent.

5.2.3. PKA inhibitors H-89 and KT 5720 inhibit gGRLN$_{19}$-induced responses in LH, but not GH, cells.

As a first step in examining the role of PKA in gGRLN$_{19}$-induced hormone release from the goldfish pituitary, dispersed pituitary cells in primary culture were exposed to gGRLN$_{19}$ alone, or gGRLN$_{19}$ during treatment with a PKA inhibitor, H-89 or KT 5720. 10 μM H89 has been shown previously to be maximally effective against cAMP-induced GH and LH release from goldfish pituitary cells but does not interfere with PKC agonist-stimulated hormone secretion [137, 347] and KT 5720 is reported to be specific for PKA in the nM range and does not affect PKC even at μM doses [157]. 10 μM H-89, but not 100 nM KT 5720, induced a transient rise in GH and LH release from goldfish pituitary cells (Fig. 5.5A and C). This rise, however, quickly subsided and a new equilibrium was reached within 25 min for GH and 35 min for LH (Fig. 5.5). As in previous experiments, 1 nM gGRLN$_{19}$ alone induced significant increases in both GH and LH release (Fig. 5.5). On the other hand, when administered 45 min following commencement of treatment with either PKA inhibitor, the LH responses to gGRLN$_{19}$ were significantly attenuated to levels not different from those of the PKA inhibitors alone; however, the GH responses to gGRLN$_{19}$ were not significantly different from those to gGRLN$_{19}$ alone (Fig. 5.5B and D).

Similar to work with PKC inhibitors, I further explored gGRLN$_{19}$-induced changes in $[Ca^{2+}]_i$ in GH and LH cells with PKA inhibitors in fura-2, AM imaging studies. Treatment with 1 nM gGRLN$_{19}$ alone elevated $[Ca^{2+}]_i$ in both GH and LH cell types (Fig. 5.6). Treatments with either one of the two PKA inhibitors alone did not lead to large changes in basal $[Ca^{2+}]_i$ in either GH or LH cell types. In somatotropes, the gGRLN$_{19}$-induced $[Ca^{2+}]_i$ was slightly delayed in terms of the time of onset in the presence of PKA inhibitors; however, the quantified total responses in the presence of inhibitors were not significantly different from values in the absence of the inhibitors but these were significantly greater than

corresponding values of inhibitors alone (Fig. 5.6A and B). In gonadotropes, the $[Ca^{2+}]_i$ responses to $gGRLN_{19}$ in the presence of PKA inhibitors were significantly lower than those to $gGRLN_{19}$ alone but not different from levels in the PKA inhibitors alone groups (Fig. 5.6C and D). These results support the hypothesis that PKA may have differential roles in $gGRLN_{19}$ signalling in goldfish gonadotropes and somatotropes.

5.2.4. An AC activator, forskolin, affects $gGRLN_{19}$-induced hormone release and $[Ca^{2+}]_i$ responses in a cell-type specific manner

To further explore the differential role of PKA in $gGRLN_{19}$-induced GH and LH cell responses, the interactions between the AC activator forskolin and $gGRLN_{19}$ were examined in hormone release studies. Forskolin was used at a concentration of 10 μM, a concentration that has previously been shown to maximally stimulate PKA-dependent hormone release in goldfish pituitary cells, as well as to increase cAMP production [55, 347]. As expected, application of either 1 nM $gGRLN_{19}$ alone or 10 μM forskolin alone induced significant increases in both GH and LH levels (Fig. 5.7). The net GH responses to $gGRLN_{19}$ in the presence of forskolin (applied commencing 10 min prior to $gGRLN_{19}$) were significantly greater than those to $gGRLN_{19}$ alone and to forskolin alone quantified over the same duration (Fig. 5.7B). The quantified GH response to the combined $gGRLN_{19}$ and forskolin treatment was approximately double that of the estimated sum of the responses to either stimulator alone. In contrast, combination treatments of $gGRLN_{19}$ and forskolin did not elicit LH responses that were significantly different in magnitude from those induced by either $gGRLN_{19}$ or forskolin alone (Fig. 5.7D), indicating that LH responses to $gGRLN_{19}$ and forskolin were not additive (Fig. 5.7B and D).

In an attempt to further understand the role of PKA in $gGRLN_{19}$ signalling in goldfish gonadotropes and somatotropes, I examined $gGRLN_{19}$'s ability to

further augment changes in $[Ca^{2+}]_i$ levels when treated in combination with forskolin. Treatments of either $gGRLN_{19}$ or forskolin alone resulted in significant increases in $[Ca^{2+}]_i$ in both somatotropes and gonadotropes when compared to basal $[Ca^{2+}]_i$ levels, and forskolin elicited a slower but longer-lasting increase in $[Ca^{2+}]_i$ relative to $gGRLN_{19}$ in both cell types (Fig. 5.8). Interestingly, the somatotrope and gonadotrope $[Ca^{2+}]_i$ responses to $gGRLN_{19}$ in the presence of forskolin were not significantly different from those that can be accounted for by forskolin alone or $gGRLN_{19}$ alone over the same time period (Fig. 5.8B and D). These results are not at variance with the hypothesis that PKA plays different roles in gonadotrophs and somatotrophs in terms of $gGRLN_{19}$ action.

5.3. Discussion

This thesis chapter sets out to explore the role of PKC and PKA in $gGRLN_{19}$-induced GH and LH release and their corresponding intracellular Ca^{2+} signals, as induced by $gGRLN_{19}$ in goldfish pituitary somatotropes and gonadotropes.

5.3.1. *PKC involvement in GH and LH cells*

Two lines of evidence reveal that $gGRLN_{19}$-induced GH and LH release and increases in $[Ca^{2+}]_i$ in somatotropes are PKC dependent. First, in the presence of either a general PKC inhibitor, Bis-II, or a PKCα and PKCβ isoform-selective inhibitor, Gö 6976, $gGRLN_{19}$ was neither able to significantly stimulate GH and LH release nor increase $[Ca^{2+}]_i$ in somatotropes and gonadotropes. Second, $gGRLN_{19}$ failed to further stimulate GH and LH release and elevate $[Ca^{2+}]_i$ in both cell-types when the PKC pathway was already maximally stimulated by DiC8. Consistent with the proposed involvement of PKC in $gGRLN_{19}$ actions on goldfish somatotropes and gonadotropes, teleost GHS-Rs

expressed in mammalian cell lines have been shown to be linked to PLC/PKC/Ca^{2+} signalling [33] and the involvement of PKC in mediating GRLN action on GH secretion in mammals is well demonstrated [60, 162, 170, 202]. Nonetheless, this is the first time that PKC involvement in GRLN stimulation of GH release has been shown for any teleost species, as well as in LH release in vertebrates.

Results in Chapters 3 and 4 have shown that extracellular Ca^{2+} entry through LVSCCs is a major component in $gGRLN_{19}$ stimulation of GH and LH release and the accompanying elevations in $[Ca^{2+}]_i$ in goldfish pituitary cells. Whether PKC affects LVSCC currents in goldfish somatotropes and gonadotropes has not been directly investigated, but PKC is known to enhance LVSCC functions in mixed pituitary cell cultures [53, 135, 137, 347]. Furthermore in goldfish, LVSCC inhibitors decrease PKC agonists-induced GH and LH release [137, 347] and $[Ca^{2+}]_i$ changes in somatotropes [362]. When viewed together with these previous findings, results in this thesis chapter indicate that PKC plays a role in the $gGRLN_{19}$-elicited increases in $[Ca^{2+}]_i$ in goldfish somatotropes and gonadotropes via actions on LVSCC.

The exact PKC isoform involved in $gGRLN_{19}$ actions on GH and LH release is not known but is likely to include member(s) of the conventional PKC forms. The PKCα- and/or PKCβ-selective inhibitor Gö 6976 inhibits $gGRLN_{19}$'s hormone-releasing action and corresponding changes in $[Ca^{2+}]_i$ at the level of the goldfish pituitary cells. In addition, immunoreactivity for conventional PKCα has been detected in goldfish pituitary cells [165], and down regulation of conventional PKCs is associated with an attenuation of the LH response to GnRH [136].

5.3.2. PKA involvement in GH and LH cells

Results indicate that PKA is also a critical signalling component in $gGRLN_{19}$-induced LH release and changes in $[Ca^{2+}]_i$ in gonadotropes. This is supported by the observed inability of $gGRLN_{19}$ to induce significant increases in LH release and elevations in $[Ca^{2+}]_i$ in gonadotropes in the presence of either H-89 or KT 5720, as well as the failure of $gGRLN_{19}$ to further induce LH release and $[Ca^{2+}]_i$ increase when the PKA pathway was already stimulated by the AC activator, forskolin. These results also place PKA upstream of the increases in $[Ca^{2+}]_i$ induced by $gGRLN_{19}$ actions in gonadotropes which is known to involve LVSCC (Chapter 4; [110, 115]). Although results from the present thesis chapter do not directly examine whether $gGRLN_{19}$/PKA-induced $[Ca^{2+}]_i$ in gonadotropes is caused by increased Ca^{2+} entry through LVSCC, this hypothesis is likely since cAMP increases the magnitude of LVSCC currents in goldfish gonadotropes in previous studies [47]. The involvement of cAMP/PKA in $gGRLN_{19}$ signalling leading to LH release is interesting as this pathway is not well-established in GRLN actions in mammalian pituitary cells [60, 170]. The current work thus supports a role for PKA in GRLN action, as shown in previous findings in baboon [162], and porcine [202], primary pituitary cell cultures; my results, however, only implicate PKA as a potentiator of $gGRLN_{19}$ action on GH release while presenting novel evidence that PKA is critical to $gGRLN_{19}$-induced LH release. In this regard, it would be of interest to evaluate the ability of $gGRLN_{19}$ to increase cAMP production in goldfish pituitary gonadotropes in future studies.

In contrast to the situation with LH release, neither the GH nor the $[Ca^{2+}]_i$ responses to $gGRLN_{19}$ in goldfish somatotropes were significantly reduced by the PKA inhibitors H-89 and KT 5720, suggesting the non-involvement of PKA in $gGRLN_{19}$ actions on GH release in goldfish. This idea that PKA is not a necessary component in $gGRLN_{19}$-induced GH release is also supported by the finding that $gGRLN_{19}$ can further increase GH release in the presence of the AC

activator forskolin. Previously results have demonstrated that PKA- and PKC-stimulated GH release are at least additive in 2-h static incubation studies [347] and this is not at variance with the current findings that $gGRLN_{19}$ stimulation of GH release requires PKC but not PKA. Interestingly, the GH response to combined forskolin and $gGRLN_{19}$ treatment is at least twice that of the sum of the responses to either stimulator alone, suggesting that the cAMP/PKA signalling pathway may interact with, and potentiate, $gGRLN_{19}$ (PKC) actions in terms of GH release. However, this enhanced GH release to the combination treatment with forskolin and $gGRLN_{19}$ is only accompanied by a $[Ca^{2+}]_i$ change that is of the same magnitude as that to either forskolin or $gGRLN_{19}$ alone. Why the $gGRLN_{19}$-induced $[Ca^{2+}]_i$ increases are not additive to that elicited by forskolin is not known but the paradoxical observations with the combined treatment with forskolin and $gGRLN_{19}$ indicate that the potentiating effects of forskolin/PKA are occurring independently and/or downstream of $[Ca^{2+}]_i$ increases. Synergistic interactions between PKA and PKC signalling in hormone release are not without precedence. GRLN (PKC/Ca^{2+}) similarly potentiates GHRH (PKA/Ca^{2+})-induced GH release in cows [310] and humans [119]. In HeLa-T4 cells expressing GHS-Rs and GHRH-Rs, stimulation of these two receptors also produced synergistic effects [70]. Likewise, exposure to PKC agonists DiC8 and tetradecanoyl 14β-phorbol acetate can enhance the LH and GH release response to forskolin, respectively, in goldfish pituitary cells [53, 137]. Downstream targets for the PKA pathway to potentiate $gGRLN_{19}$-induced GH release are likely related to increases in the number of primed GH-containing vesicles making up the readily releasable or immediately releasable pool [2] and/or an increase in the efficiency of the vesicle trafficking and docking via regulation of the various proteins involved in these processes [29, 130]. Although vesicle trafficking in neuroendocrine cells is not fully understood, possible targets for the PKA pathway, as induced by $gGRLN_{19}$, might include Rab-, ARF-, or Rho-GTPases, SNARE proteins, or other proteins critical to GH release from vesicles in somatotropes [100, 130]. Indeed,

it would be interesting to examine changes in trafficking proteins in response to $gGRLN_{19}$ treatment in future studies.

5.3.3. Differential signalling in $gGRLN_{19}$ actions on GH and LH cells and multiplicity of signalling cascades

The above findings clearly indicate that $gGRLN_{19}$ utilizes multiple signalling pathways in stimulating hormone release in goldfish pituitary cells in a cell-type specific manner, namely PKC/Ca^{2+} in both LH and GH cells, and PKA/Ca^{2+} in LH cells. Comparing the time course of $[Ca^{2+}]_i$ increases induced by $gGRLN_{19}$ between the two cell-types also revealed that the $gGRLN_{19}$-elicited Ca^{2+} signal in gonadotropes increased more rapidly and peaked approximately 1 min earlier than that in somatotropes (Fig 5.2 A&C). How these differences are manifested is not clear but differential activation of receptor types and or receptor isoforms may be involved.

mRNAs encoding for four gGHS-R isoforms are present in the goldfish pituitary, and mRNA for gGHS-R1a, type 1 and gGHS-R1a, type 2 predominates in this tissue [151]. However the identity of gGHS-R type(s) present in goldfish somatotropes and gonadotropes remains to be determined. Nevertheless, given that 1) gGHS-R1a mRNAs are predominant in goldfish pituitary [115, 151]; 2) proteins immunoreactive to antibody against mammalian GHS-R1a are detected in goldfish pituitary extracts in preliminary Western blotting studies (CL Grey and JP Chang, unpublished; Appendix 1); 3) both gGHS-R1a, type 1 and gGHS-R1a, type 2 receptor isoforms are linked to increases in $[Ca^{2+}]_i$ in transfection-expression studies in HEK cells [151]; 4) $gGRLN_{19}$ stimulation of LH and GH release involves Ca^{2+} entry through LVSCC (Chapters 3 & 4; [110, 115]); and 5) Ca^{2+} signals are rapidly elicited by $gGRLN_{19}$, and essentially instantaneously in both somatotropes and gonadotropes, in this thesis chapter and in Chapters 3 and 4 ([110, 115]), $gGRLN_{19}$ likely activates gGHS-Rs of the 1a lineage on LH and

GH cells to exert its effect in goldfish. Further experiments, however, would be required to thoroughly test this hypothesis. Whether gGHS-Rs of the 1a lineage are linked to different intracellular signalling cascades and/or are differentially expressed on goldfish somatotropes and gonadotropes also needs clarification. In addition, work on HIT-T15 β-cells showed that both PKC and PKA pathways were involved in GRLN-induced cytoprotective effects, however these cells expressed GRLN, but not the GHS-R [106], suggesting that GRLN may target receptor types other than GHS-R. Results from the literature also suggest that the GHS-R may heterodimerize with other receptors, such as the DA receptor or GHRH-R (Chapter 1, Section 1.2.3; [134, 162]), providing another possibility for differential signalling in GH and LH cell types.

That $gGRLN_{19}$ stimulation of pituitary hormone release in goldfish, and even the release of a single hormone type (LH), involves not just one, but at least two, signalling cascade pathways may be unexpected but not without precedent in neuroendocrine factor actions. Such examples include 1) GHRH and GHRP-2 actions on membrane ion currents in ovine and human somatotropes [60], 2) GnRH receptor signal transduction coupling to multiple signalling cascades in rat and mouse cell lines [63], and 3) PACAP stimulation of somatolactin from cultured goldfish pituitary cells [7]. As described in Chapter 1, Section 1.3.2, GRLN also utilizes NO signalling to stimulate GH release in mammals. Whether NO signalling similarly participates in $gGRLN_{19}$ effects on goldfish LH and GH release remains to be examined; however, the involvement of NO in neuroendocrine stimulation of GH release in the goldfish pituitary cell model has been demonstrated [217, 324, 325].

Results from this thesis chapter also add support for the multiplicity of signalling cascades mediating the ligand- and cell-type-specific control of LH and GH release in goldfish by direct actions of neuroendocrine regulators, as has previously been shown for the two endogenous goldfish GnRH forms (sGnRH

and cGnRH-II), PACAP and DA (Chapter 1, Sections 1.4.2 & 1.5.2; [45]). My work now adds $gGRLN_{19}$ as an activator of the PKC, PKA and LVSCC/Ca^{2+} signalling cascades in LH, and PKC and LVSCC/Ca^{2+} in GH, cell-types. Given the ability of forskolin (PKA) to enhance $gGRLN_{19}$ (PKC) effects on GH release, such multiplicity of second messenger systems not only forms the basis for the ligand-specific control of pituitary hormone release by neuroendocrine regulators, but also allows for the possible synergistic interactions of hormone-releasing actions of select stimulatory neuroendocrine factors. It would be of interest to investigate the interactions of $gGRLN_{19}$ with different known neuroendocrine regulators of GH and LH release in the goldfish pituitary cell system in future studies.

5.3.4. Summary

Taken as a whole, results from this thesis chapter add to the overall understanding of GRLN and its activity at the cellular level by elucidating the signalling pathways utilized in $gGRLN_{19}$-induced GH and LH release from goldfish pituitary cells. Findings from this chapter suggest that $gGRLN_{19}$-induced GH and LH release in goldfish pituitary cells are PKC-dependent, while $gGRLN_{19}$-induced LH release is also PKA dependent. Furthermore, current results suggest that the PKA pathway acts in goldfish somatotropes to potentiate $gGRLN_{19}$-induced GH release downstream and/or independent of increases in $[Ca^{2+}]_i$. To further understand the complex role GRLN plays in animal physiology in general, and in pituitary cell signalling in particular, it would be important to look at the role of other signalling cascades and the GHS-R isoforms involved in GRLN actions in different pituitary cell types, as well as GRLN's interactions with other known neuroendocrine regulators in future studies.

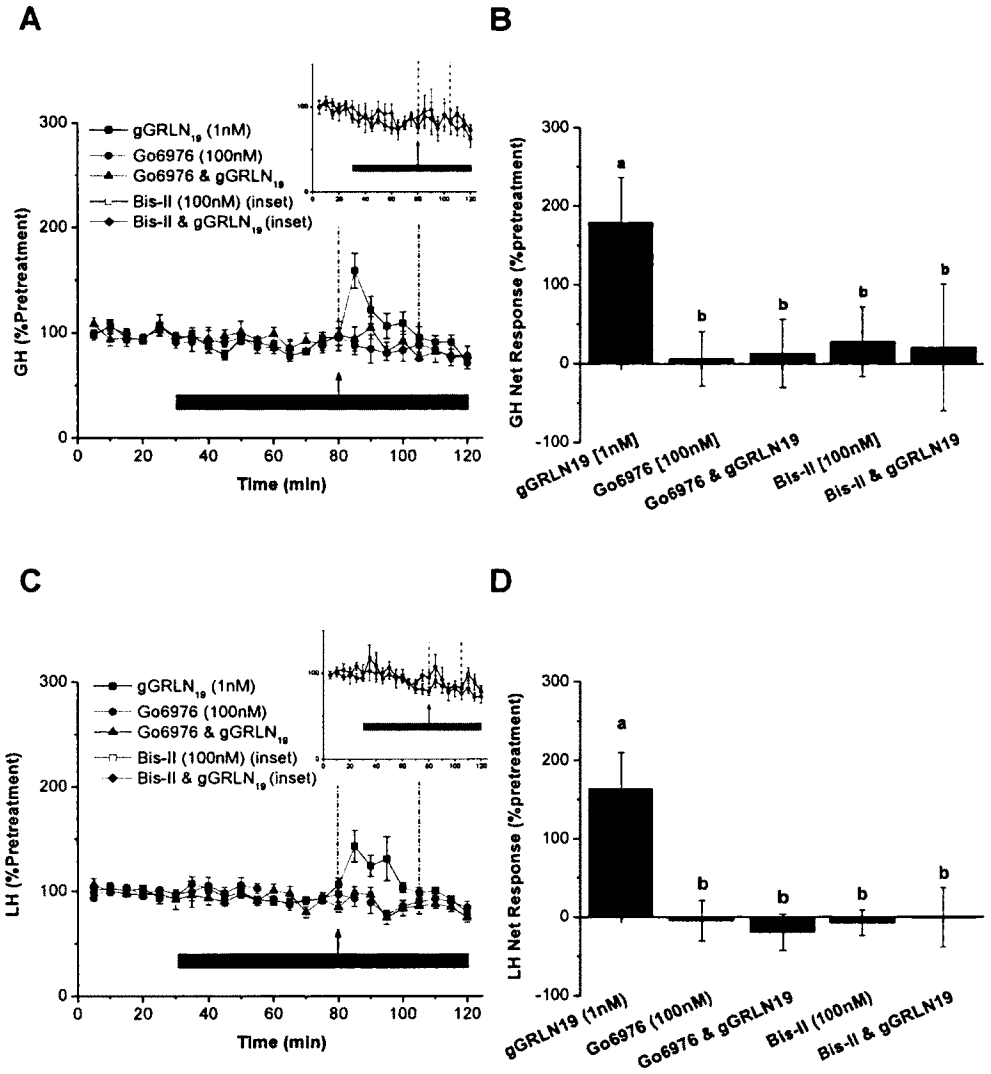

Fig. 5.1. Two PKC inhibitors, 100 nM Bis-II and 100 nM Gö 6976, inhibit 1 nM $gGRLN_{19}$-induced hormone secretion from dispersed goldfish pituitary cells in column perifusion. Following a 45 min pretreatment with the PKC inhibitors (black bar), cells were challenged with a 5-min pulse of $gGRLN_{19}$ (arrow), followed by a 35 min washout with inhibitor alone. GH (A) and LH (C) values in hormone release traces were normalized to pretreatment averages (average of first 5 fractions; 57.0 ± 3.8 ng/ml and 3.9 ± 0.2 ng/ml, for GH and LH, respectively). Quantified net responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, inhibitor alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (n=8 from 4 separate experiments performed between May and June). Treatment values statistically different from each other have different letters of the alphabet.

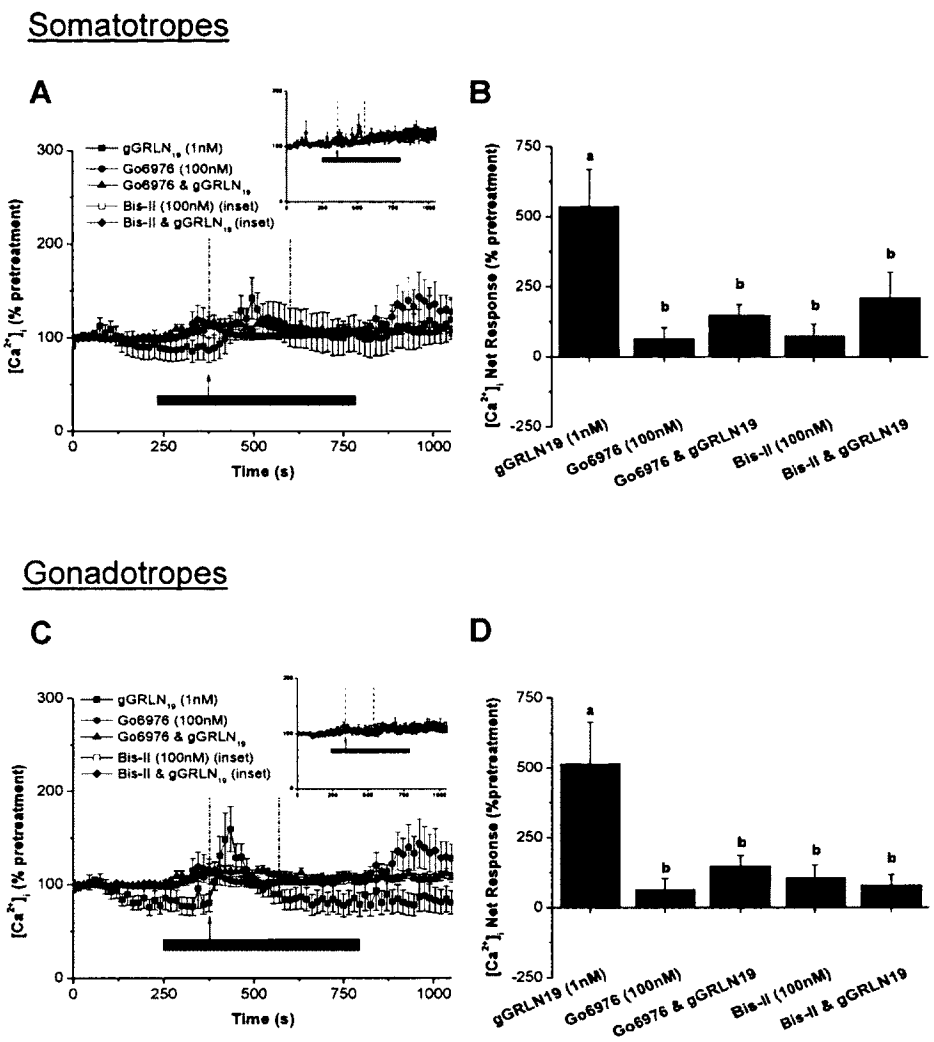

Fig. 5.2. Two PKC inhibitors, 100 nM Bis-II and 100 nM Gö 6976, inhibits 1 nM $gGRLN_{19}$-induced increases in $[Ca^{2+}]_i$ in individually identified goldfish somatotropes and gonadotropes loaded with fura-2, AM. Following a 2.5 min pretreatment with the PKC inhibitors (black bar), cells were challenged with a 2-min pulse of $gGRLN_{19}$ (arrow), followed by a 5.5 min washout with inhibitor alone. Ca^{2+} response traces for somatotropes (A) and gonadotropes (C) were normalized to pretreatment averages (average of first 5 fractions: 151.3 ± 13.4 nM and 132.0 ± 18.1 nM for somatotropes and gonadotropes, respectively). Quantified net $[Ca^{2+}]_i$ responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, inhibitor alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (Bis-II, n=9 from 5 separate experiments performed in January; Gö 6976, n=10 from 6 separate experiments performed in August). Treatment values statistically different from each other have different letters of the alphabet.

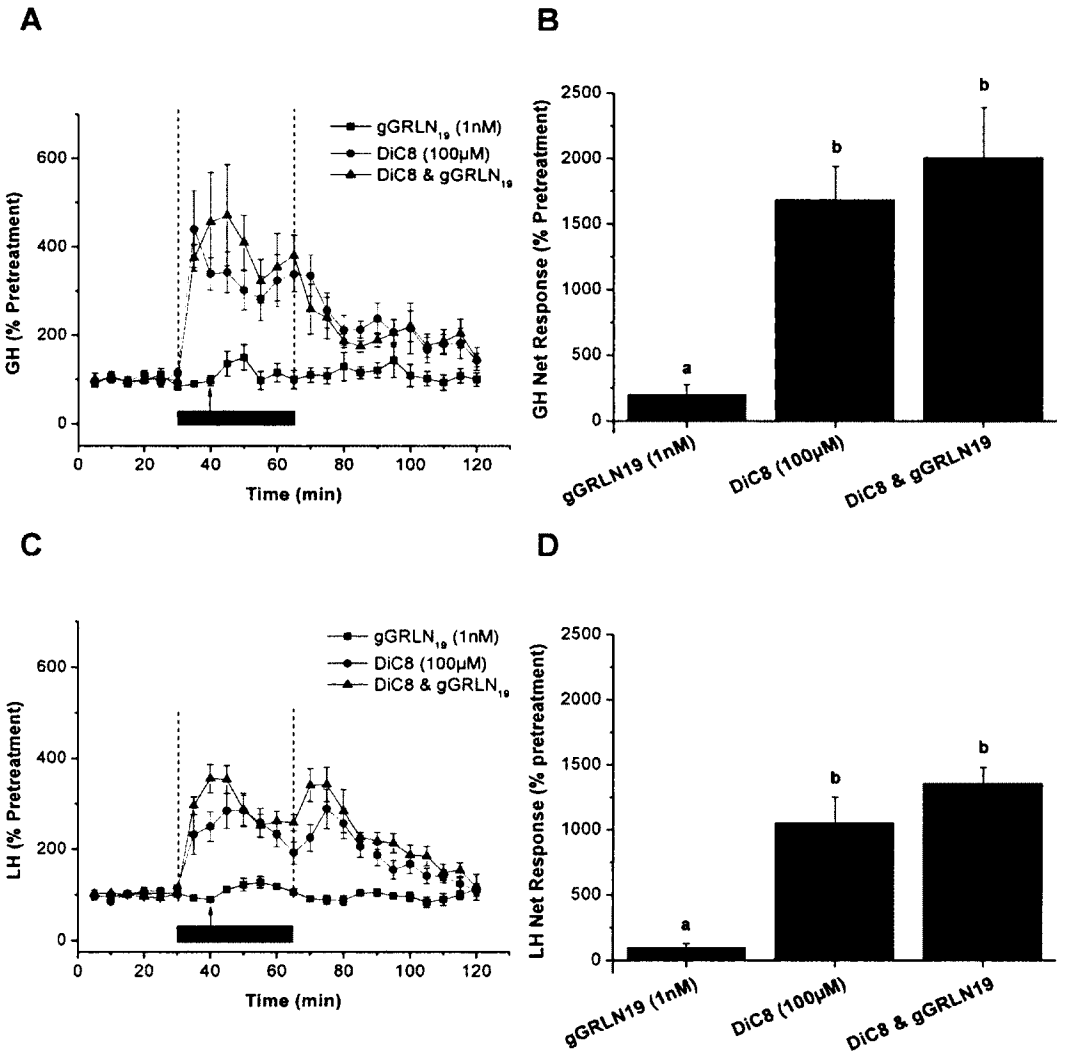

Fig. 5.3. 1 nM $gGRLN_{19}$ fails to further stimulate hormone release when pituitary cells are pre-treated with the PKC agonist DiC8 (100 μM). Following a 10 min pretreatment with the PKC agonist DiC8 (black bar), cells in column perifusion were challenged with a 5-min pulse of $gGRLN_{19}$ (arrow), followed by a 20 min washout with DiC8. GH (A) and LH (C) hormone release traces were normalized to pretreatment average (average of first 5 fractions: 8.3 ± 0.7 ng/ml and 2.8 ± 0.2 ng/ml, for GH and LH, respectively). Quantified net responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, DiC8 alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (n=8 from 4 separate experiments performed between April and May). Treatment values statistically different from each other have different letters of the alphabet.

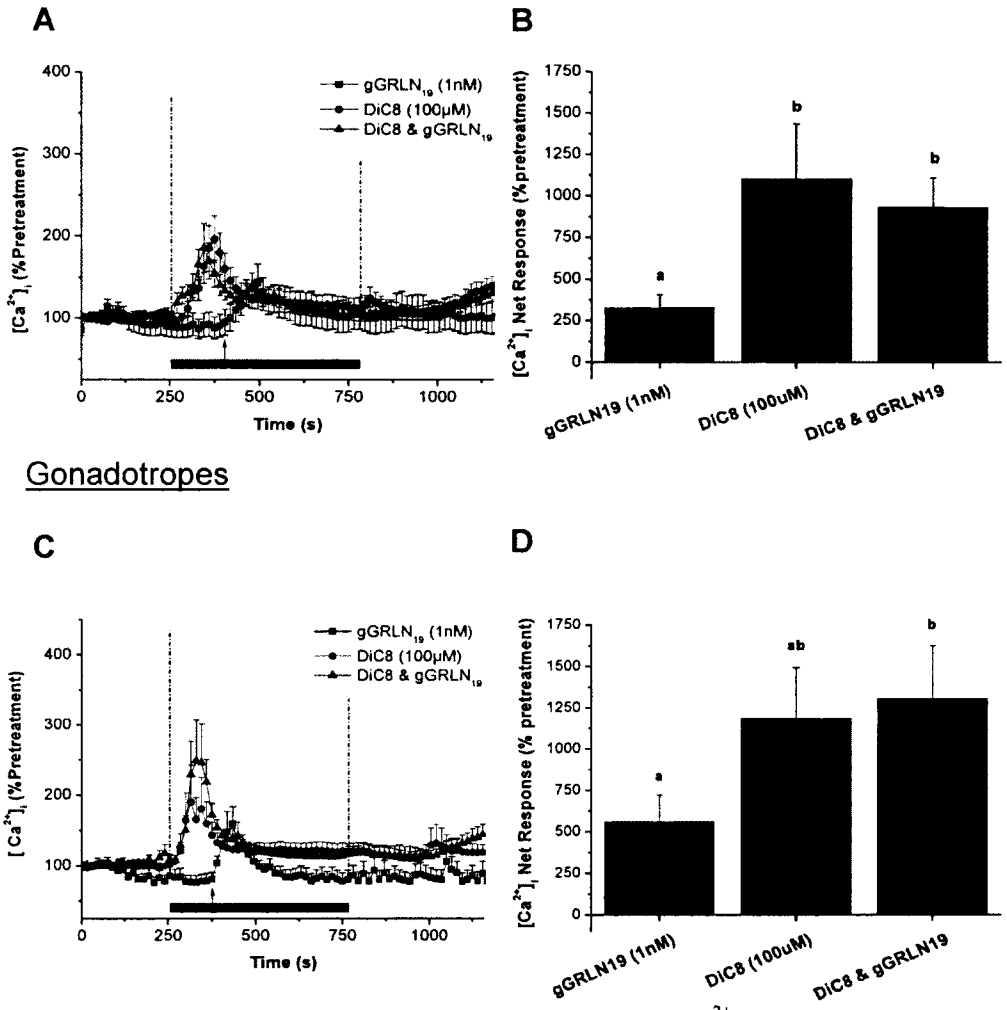

Fig. 5.4. 1 nM $gGRLN_{19}$ fails to further stimulate changes in $[Ca^{2+}]_i$ when pituitary cells are pre-treated with the PKC agonist DiC8 (100 μM). Following a 2.5 min pretreatment with the PKC agonist DiC8 (black bar), cells loaded with fura-2, AM on poly-l-lysine coverslips were challenged with a 2-min pulse of $gGRLN_{19}$ (arrow), followed by a 5.5 min washout with DiC8. Ca^{2+} response traces in somatotropes (A) and gonadotropes (C) were normalized to pretreatment averages (average of first 5 fractions: 124.0 ± 9.1 nM and 168.7 ± 23.2 nM, for somatotropes and gonadotropes, respectively). Quantified net responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, DiC8 alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (n=10 for each treatment performed between January and February using 3 separate cell preparations). Treatment values statistically different from each other have different letters of the alphabet.

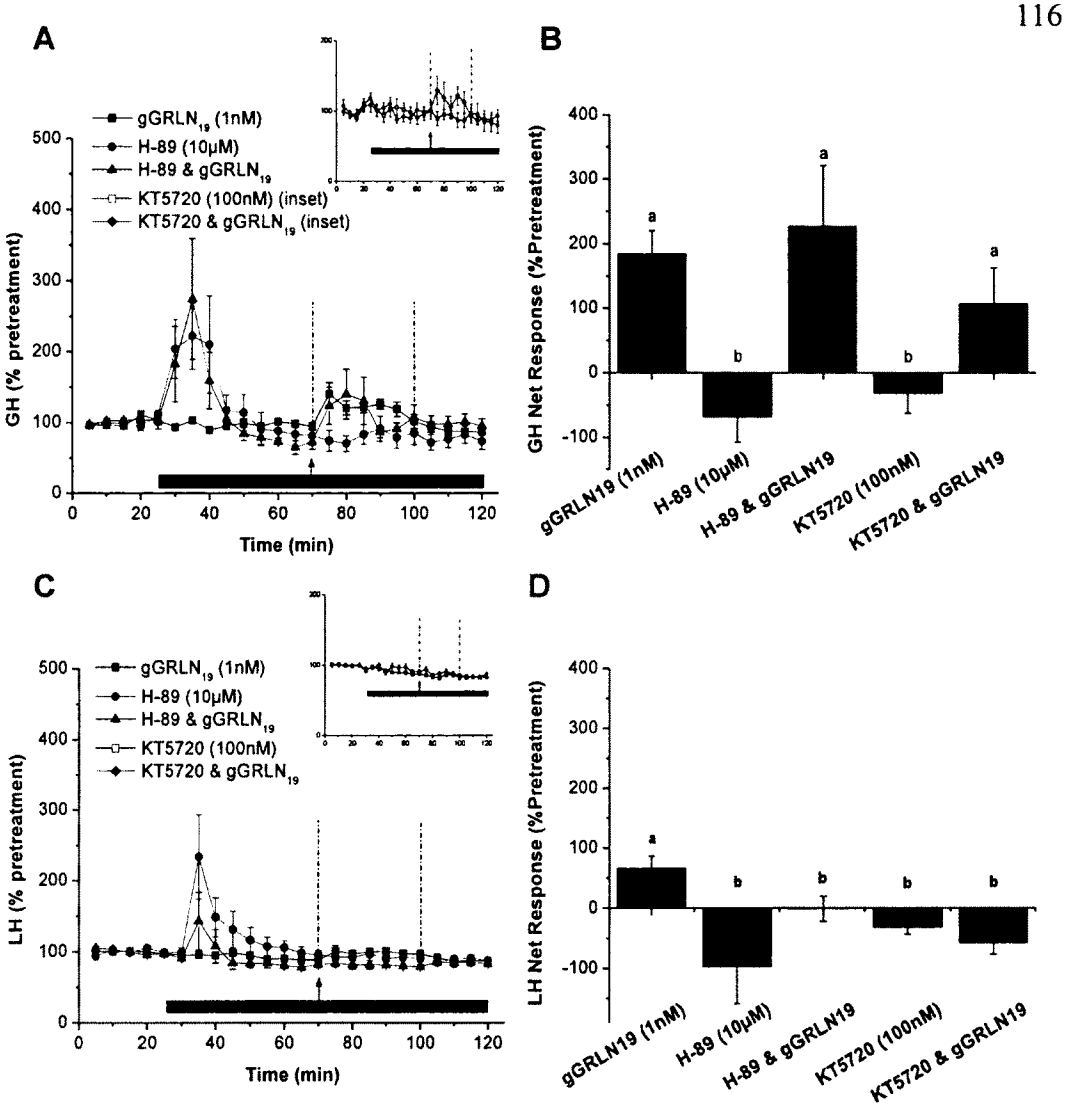

Fig. 5.5. Two PKA inhibitors, 10 μM H-89 and 100 nM KT 5720, abolish 1 nM $gGRLN_{19}$-induced LH, but not GH, secretion from dispersed goldfish pituitary cells in column perifusion. Following a 45 min pretreatment with the PKA inhibitors (black bar), cells were challenged with a 5-min pulse of $gGRLN_{19}$ (arrow), followed by a 45 min washout with inhibitor alone. GH (A) and LH (C) release traces were normalized to pretreatment averages (average of first 5 fractions: 15.9 ± 1.5 ng/ml and 4.0 ± 0.1 ng/ml for GH and LH, respectively). Quantified net responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, inhibitor alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (n=8 from 4 separate experiments performed between January and February). Treatment values statistically different from each other have different letters of the alphabet.

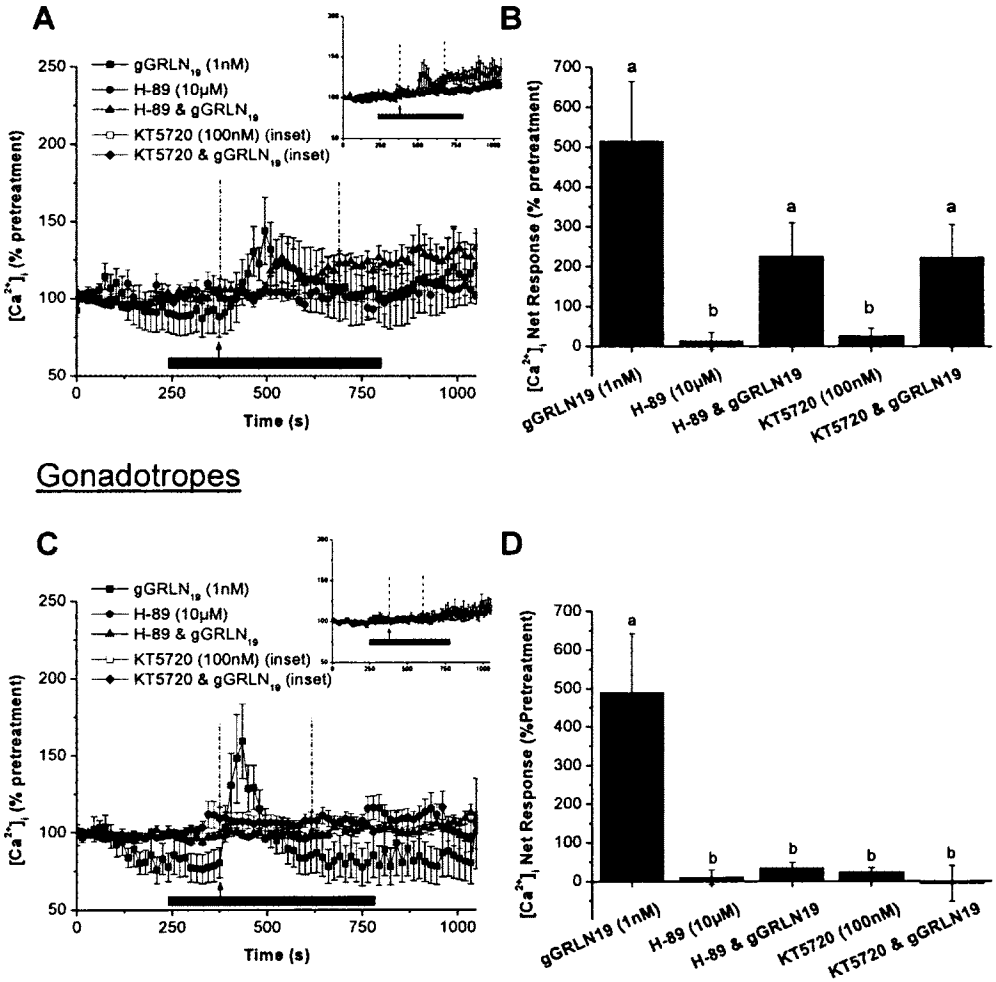

Fig. 5.6. Two PKA inhibitors, 10 μM H-89 and 100 nM KT 5726, abolish 1 nM $gGRLN_{19}$-induced increases in $[Ca^{2+}]_i$ in individually identified goldfish pituitary cells loaded with fura-2, AM. Following a 2.5 min pretreatment with the PKA inhibitors (black bar), cells were challenged with a 2-min pulse of $gGRLN_{19}$ (arrow), followed by a 5.5 min washout with inhibitor alone. Ca^{2+} response traces for somatotropes (A) and gonadotropes (C) were normalized to pretreatment averages (average of first 5 fractions: 157.6 ± 9.1 nM and 162.6 ± 12.2 nM for somatotropes and gonadotropes, respectively). Quantified net $[Ca^{2+}]_i$ responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, inhibitor alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (H-89, n=10 from 3 separate experiments performed between June and July; KT 5720, n=10 from 4 separate experiments run in July). Treatment values statistically different from each other have different letters of the alphabet.

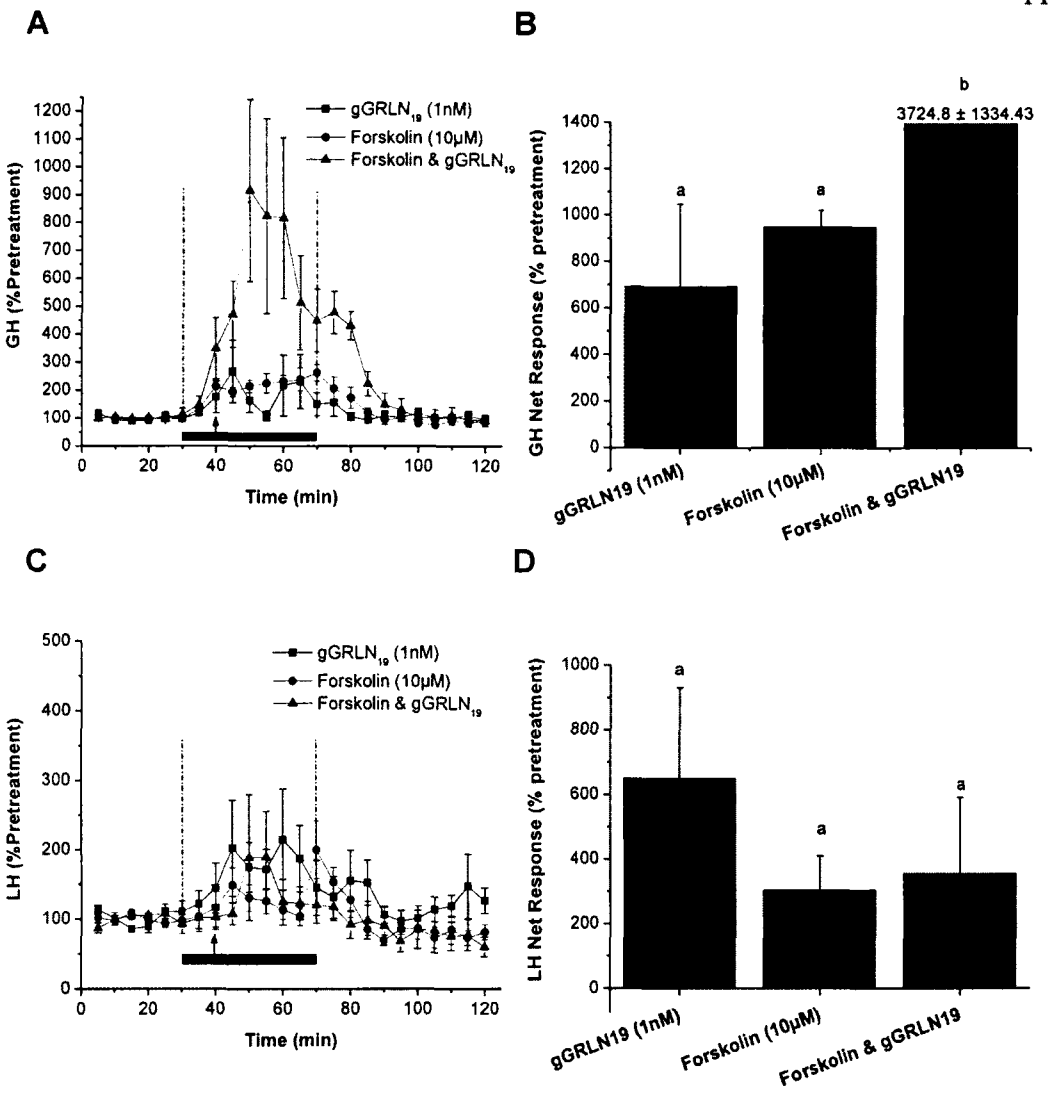

Fig. 5.7. An adenylate cyclase activator, forskolin (10 μM), potentiates the GH, but not LH, release response to 1 nM $gGRLN_{19}$ from dispersed goldfish pituitary cells in column perifusion. Following a 10 min pretreatment with forskolin alone, cells were challenged with a 5-min pulse of $gGRLN_{19}$, followed by a washout of 25 min with forskolin alone. GH (A) and LH (C) response traces were normalized to pretreatment averages (average of first 5 fractions: 11.7 ± 1.0 ng/ml and 12.3 ± 1.9 ng/ml for GH and LH, respectively). Quantified net responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, forskolin alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in April). Treatment values statistically different from each other have different letters of the alphabet.

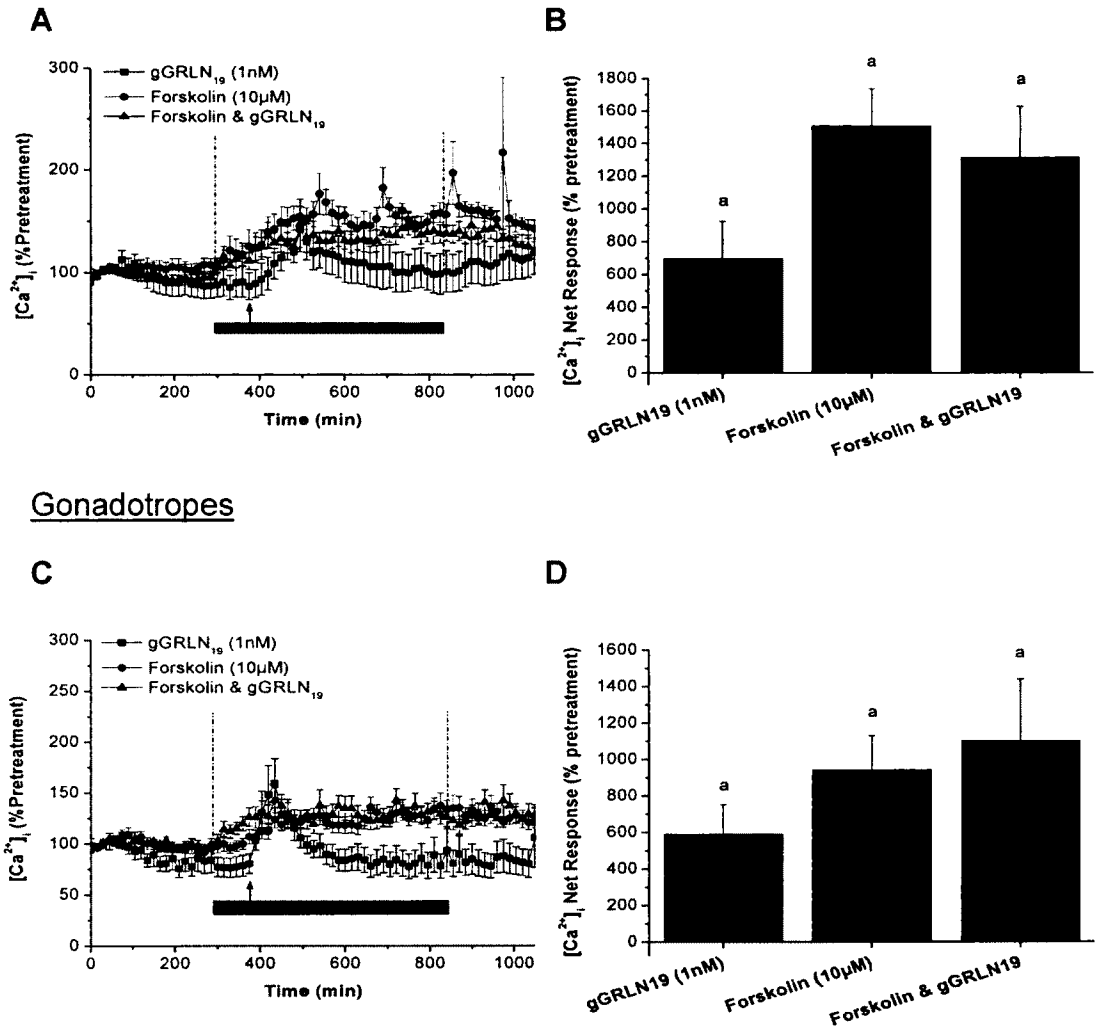

Fig. 5.8. 1 nM $gGRLN_{19}$ did not further increase $[Ca^{2+}]_i$ in dispersed goldfish pituitary cells loaded with fura-2, AM in cells pretreated with an adenylate cyclase activator, forskolin (10 μM). Following a 2.5 min pretreatment with forskolin alone, cells were challenged with a 2-min pulse of $gGRLN_{19}$, followed by a washout of 5.5 min with forskolin alone. Ca^{2+} response traces in somatotropes (A) and gonadotropes (C) were normalized to pretreatment averages (average of first 5 fractions: 155.9 ± 11.9 nM and 187.6 ± 19.9 nM for somatotropes and gonadotropes, respectively) Quantified net $[Ca^{2+}]_i$ responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, forskolin alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (n=10 from 3 separate experiments run in August). Treatment values statistically different from each other have different letters of the alphabet.

Chapter 6 – $gGRLN_{19}$-induced GH release from goldfish pituitary cells is NO dependent

6.1. Introduction

Results from Chapters 4 and 5 demonstrate that $gGRLN_{19}$ stimulation of GH release from goldfish pituitary cells involves extracellular Ca^{2+} entry through LVSCC and activation of PKC. In addition to Ca^{2+} and PKC, NO signalling participates in GRLN actions at different targets in multiple organisms (Chapter 1, Section 1.3.2). For example, in dispersed female pig pituitary cells, GRLN-induced GH release is augmented by the addition of L-arginine methyl ester hydrochloride (a substrate for NOS), and GRLN effects are inhibited by haemoglobin (NO scavenger) and N(w)-nitro-L-arginine (NOS inhibitor) [271]. GRLN increases NO production and inducible iNOS protein levels in rat heart cells [297], suggesting that NO is important to the cardiac effects of GRLN, such as protection from ischemia and improvement in cardiac contractility [232]. ICV administration of GRLN to NOS-knockout mice also fails to induce food intake, indicating that NO mediates brain actions of GRLN [227].

As reviewed in Chapter 1, Section 1.4.2, NOS/NO signalling also plays a role in the neuroendocrine regulation of GH release in goldfish. In particular, immunoreactivity for nNOS and iNOS have been identified in dispersed goldfish pituitary cells [324] and extracts [325], and sGnRH-, cGnRH-II-, and DA-induced GH release in goldfish involve NO signalling [28, 40]. However, whether $gGRLN_{19}$ stimulation of GH release from goldfish pituitary cells involves NOS/NO signalling is unknown.

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Given that NOS/NO signalling participates in GRLN-induced GH secretion from porcine pituitary cells and the known involvement of this signalling pathway in the neuroendocrine regulation of GH release in goldfish, I tested the hypothesis that NOS/NO signalling mediates $gGRLN_{19}$-induced GH release from goldfish pituitary cells. To accomplish this, the effects of an NO scavenger (PTIO), an NO donor (SNP) and three NOS inhibitors (1400W, AGH, 7-Ni) on the GH release response to $gGRLN_{19}$ from primary cultures of dispersed goldfish pituitary cells were examined in a cell column perifusion system.

6.2. Results

6.2.1. The NO scavenger PTIO abolishes $gGRLN_{19}$-induced GH release

I first examined whether limiting NO availability, using an NO scavenger, affected the ability of a 1 nM dose of $gGRLN_{19}$ to induce GH release, a dose previously shown to be maximally effective [110]. Treatment with 10 μM PTIO has previously been shown to abolish NO donor-elicited GH release [325]. Treatment with $gGRLN_{19}$ increased GH release (Fig. 6.1A & B), a result consistent with those demonstrated in Chapters 4 and 5. In contrast, when $gGRLN_{19}$ was administered 15 min into a 50 min PTIO treatment, GH release was reduced to a level that was not significantly different from PTIO treatment alone (Fig. 6.1A & B).

6.2.2. The NO donor SNP and $gGRLN_{19}$ do not produce additive release of GH

Given that an NO scavenger attenuated the ability of $gGRLN_{19}$ to induce GH release, I was interested in whether an NO donor would be able to further increase GH release in the presence of $gGRLN_{19}$ stimulation. The reasoning was that if $gGRLN_{19}$-induced GH release is independent of NO, additive responses should be seen with co-administration with SNP, as seen in previous studies with

PACAP and SNP where PACAP further stimulates GH release in the presence of SNP, indicating that while the current dose of SNP maximally stimulates the NO pathway, it does not exhaust GH-releasing potential [219]. However, if $gGRLN_{19}$ utilizes the NO pathway the GH responses to maximally stimulating doses of SNP (100 μM) [219] and $GRLN_{19}$ (1 nM) would not be additive, since they would share similar NO-sensitive GH release signalling components. To ensure $gGRLN_{19}$ effects could be visualized, the 5-min SNP treatment was administered at a time corresponding to that of 20 min into the $gGRLN_{19}$ treatment. Both $gGRLN_{19}$ and SNP treatments alone resulted in significant GH release (Fig. 6.2A & B). However, the response to the combined SNP and $gGRLN_{19}$ treatment was not significantly different than that of SNP alone (Fig. 6.2A & B), a result consistent with the hypothesis that NO is a signalling component of $gGRLN_{19}$-induced GH release in goldfish pituitary cells.

6.2.3. NOS isoform-selective inhibitors differentially blocks $gGRLN_{19}$-induced GH release

My next step in elucidating the role of the NOS/NO signalling pathway in $gGRLN_{19}$-induced GH release in the goldfish pituitary was to look into the role of NOS. The first NOS inhibitor employed, 1400W, has been previously shown to inhibit the 3 NOS isoforms in the micro-molar range (IC_{50} ~0.2, 7.3 and 1000 μM for the i-, n-, and e-NOS isoforms, respectively) [250]. Treatment with 1 μM 1400W, a concentration that should not affect eNOS, did not result in significant changes to basal GH release when applied alone, while $gGRLN_{19}$ alone induced a significant elevation in GH release (Fig. 6.3A & B). However, when $gGRLN_{19}$ was administered in the presence of 1400W, it failed to significantly increase GH release (Fig. 6.3A & B).

The next NOS inhibitor employed, 7-Ni, has been shown to selectively inhibit both eNOS and nNOS at the low micro-molar range (IC_{50} ~0.7, 0.8 and 30

μM for n-, e- and iNOS, respectively) [164]. Application of 1 μM 7-Ni did not affect basal GH secretion but abolished the GH response to 1 nM $gGRLN_{19}$ treatment (Fig. 6.4A & B).

Finally, I examined the effects of the NOS inhibitor AGH, which has been shown to inhibit both iNOS and eNOS with IC_{50} values of ~250 and 530 μM, respectively [116]. Application of 1 mM AGH did not significantly alter basal GH release or the GH response to 1 nM treatment with $gGRLN_{19}$ (Fig. 6.5A & B).

6.3. Discussion

I set out to examine the role of NO in the intracellular signalling of $gGRLN_{19}$-induced GH release. Three lines of evidence support the conclusion that the NOS/NO pathway is a critical component of $gGRLN_{19}$-induced GH release from goldfish pituitary cells. First, the NO donor SNP has been shown to effectively increase NO in goldfish pituitary cell cultures [219, 325] but co-treatment with SNP and $gGRLN_{19}$ did not lead to additive changes in GH release, a response that previous findings have shown is possible if co-treated stimulators utilize different pathways [219]. Second, the NO scavenger PTIO, which effectively reduces NO donor-elicited GH release from goldfish pituitary cells in a previous study [325], significantly reduced $gGRLN_{19}$-induced GH release. The fact that PTIO does not suppress PACAP-induced GH release in a previous study [219] indicates that PTIO does not inhibit GH release nonspecifically in the goldfish pituitary cell study system. Third, two NOS inhibitors (7-Ni and 1400W) inhibited the ability of $gGRLN_{19}$ to stimulate GH secretion. Although whether $gGRLN_{19}$ can induce NO production still remains to be elucidated, these findings support the hypothesis that NOS/NO signalling is an integral component of $gGRLN_{19}$ stimulation of GH release in goldfish. That NOS/NO signalling participates in the neuroendocrine regulation of GH release from goldfish

pituitary cells is not novel since it has been demonstrated for GnRH and DA [219, 325]. Nonetheless, results from the present thesis chapter are the first to implicate NOS/NO signalling in direct GRLN action at the level of the pituitary cells in any teleost species, as well as for GRLN action in other tissues in teleost in general.

The participation of NOS/NO in direct GRLN stimulation of pituitary GH release is controversial in mammals. Although NO is reported not to mediate direct pituitary action of a 3-h treatment of GRLN on GH release in adult rats [256], long-term (10 days) in vivo administration of GRLN induces increases in GH release in prepubertal rats, a result which is not seen with the administration of NO inhibitors [255]. On the other hand, the participation of NO in the direct action of GRLN on GH is clearly demonstrated in porcine systems [271] and, as discussed in the Sections 1.3.2 and 6.1 of this thesis, the involvement of NOS/NO in GRLN actions in many other physiological functions including appetite control [227], immune functions [291], and cardiac performance [232, 297] is well-established. Taken together with results from the present thesis chapter, it appears that utilization of NOS/NO signalling is one of the evolutionary conserved intracellular signalling mechanisms central to the physiological functions of GRLN in vertebrates.

Results from this thesis chapter also provides insight into the possible identity of the NOS isoform involved in mediating $gGRLN_{19}$ effects on goldfish GH release. Three types of NOS (i-, n- and e-NOS) are found in tetrapods [335]. Goldfish pituitary cells express immunoreactivity for iNOS and nNOS [324, 325] and partial cDNA fragments of i- and n-NOS have been cloned from goldfish [174, 179]. Recently, complete cDNA sequences for two goldfish iNOS isoforms (A, accession: AY904362.1; B, accession: AY904363.1) have also been reported. Interestingly, eNOS has not been identified in the goldfish. My results show that while 7-Ni (should inhibit eNOS and nNOS at the dose used) and 1400W (should not inhibit eNOS at the dose used) treatments eliminated $gGRLN_{19}$-induced GH

release, the NOS inhibitor AGH (should inhibit eNOS and iNOS isoforms at the dose employed) was unable to inhibit $gGRLN_{19}$-induced GH release. Assuming that the specificity of these inhibitors for mammalian NOS isoforms are similar for goldfish NOSs, these observations suggest that nNOS is the primary NOS isoform involved in $gGRLN_{19}$ intracellular signalling in goldfish somatotropes. Although studies support the involvement of NOS/NO in mammalian GH release responses in several model systems [23, 326], these studies have not looked at NOS isoform specific roles in mammalian GH release, as induced by GRLN. Thus the novel finding that nNOS may be the preferred NOS isoform mediating $gGRLN_{19}$-induced GH release in goldfish pituitary cells may have implications in future studies on the neuroendocrine control of GH release in mammalian and non-mammalian model systems.

Although $gGRLN_{19}$, GnRH, and DA all utilize NOS/NO signalling in stimulating GH release from goldfish pituitary cells, the spectrum of sensitivity to the different NOS inhibitors differs between these neuroendocrine factors. $gGRLN_{19}$-elicited GH secretion is attenuated by 7-Ni and 1400W (Fig. 6.3 & 6.4) while GnRH-induced GH release is sensitive to AGH and 1400W [324, 325] and DA-stimulated GH release is only sensitive to 7-Ni [219]. Despite the fact that the sensitivity of goldfish NOS isoforms to these inhibitors has yet to be directly determined, these differences indicate that linkages to and the use of different NOS isoform(s) plays an important role in the ability of goldfish somatotropes to respond to multiple neuroendocrine stimulators.

In mammalian cardiomyocytes, nNOS and eNOS are constitutively expressed and their activation require increases in $[Ca^{2+}]_i$ [85, 191, 237, 278, 354]. Activation of seabream and goldfish GHS-Rs in expression systems has also been linked to increases in PLC activity and subsequent elevations in $[Ca^{2+}]_i$ [35, 151]. Likewise, $gGRLN_{19}$ increases $[Ca^{2+}]_i$ in goldfish somatotropes and the GH release response is Ca^{2+}-dependent (Chapter 3; [110]). How $gGRLN_{19}$

activates NOS remains to be studied but it is likely that the presumed activation of nNOS in $gGRLN_{19}$ action on goldfish somatotropes is mediated by $[Ca^{2+}]_i$ increases.

The soluble sGC/cGMP system is a common downstream target of NO signalling [166, 258]. Likewise, cGMP has been linked to GRLN-induced GH release in other animal models, including mammals. Recent work on female pig pituitary cells demonstrated that an inhibitor of sGC abolished the NO-dependent GRLN-induced GH release [271]. Similarly, sGC/cGMP has been implicated in the NOS/NO-sensitive GnRH action on GH release from goldfish pituitary cells [325]. On the other hand, sGC/cGMP is not always the downstream target of the NO pathway. For example, in female pig pituitary cell cultures, GHRH and low doses of SS have been shown to stimulate GH in a NOS/NO dependent manner, yet inhibition of sGC only blocks GHRH-induced, not low dose SS-induced, hormone release [199]. NO can also signal independently of sGC and cGMP via S-nitrosylation, an attachment of NO to proteins which can cause conformational and functional changes in protein targets [191, 295]. This type of protein modification by NO is thought to be required for NO activation of ryanodine receptors in cardiomyocytes [296, 355]. Interestingly, ryanodine receptors have been previously implicated in goldfish pituitary GH release [140, 143], thus presenting the activation of these receptors, and subsequent release of Ca^{2+}, as a potential target for NO, as stimulated by $gGRLN_{19}$. To further understand the $gGRLN_{19}$ signal transduction cascade in goldfish somatotropes, it would be important to examine whether sGC, cGMP, and ryanodine receptors are also involved in the ability of $gGRLN_{19}$ to increase GH secretion in future studies. Since it has been shown that SNP-induced $[Ca^{2+}]_i$ signals in goldfish GH cells are linked to the availability of extracellular Ca^{2+} and VSCC activity [363], how NO may interact with these and other signalling pathways in mediating $gGRLN_{19}$-induced GH release would also be useful.

Interestingly, the net GH response to a 5-min application of $gGRLN_{19}$ in 7-Ni experiments (Fig. 6.4) is of greater magnitude, by about 3 fold, relative to those in the other experiments. Whether this reflects a change in the GH responsiveness to $gGRLN_{19}$ along the seasonal reproductive stages remains to be ascertained but seasonal reproductive variations in the GH-releasing ability of GnRH, DA and PACAP have been demonstrated in goldfish [28, 40].

In conclusion, the current study clearly demonstrates that $gGRLN_{19}$ utilizes NOS, likely nNOS, and the NO pathway to induce GH release from the goldfish pituitary. This adds to the overall understanding of the complex, multifactorial signalling of hormone release from the goldfish pituitary and the functioning of GRLN in various physiological target systems.

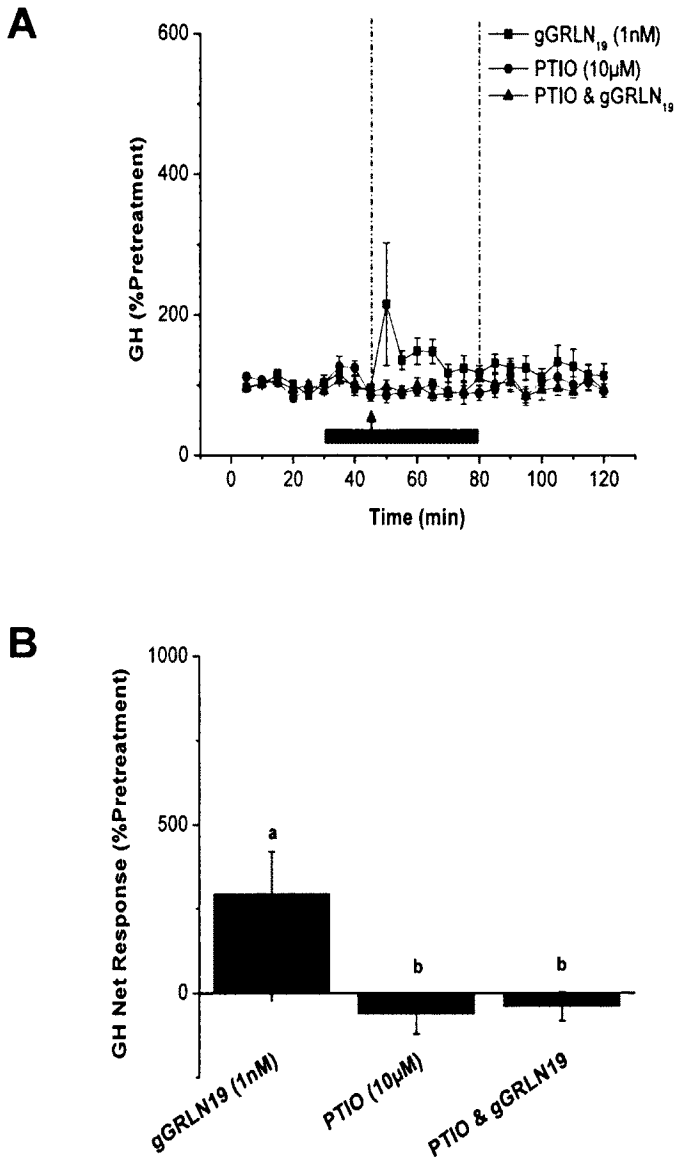

Fig. 6.1. $gGRLN_{19}$ fails to stimulate GH release in the presence of the NO scavenger PTIO. PTIO (black bar; 10 μM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net GH responses were quantified (area between vertical dotted lines) for PTIO alone, $gGRLN_{19}$ alone, and PTIO with $gGRLN_{19}$ for statistical analysis. Pretreatment average (first 5 fractions) was 0.55 ± 0.03 ng/ml. Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in October). Treatment values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

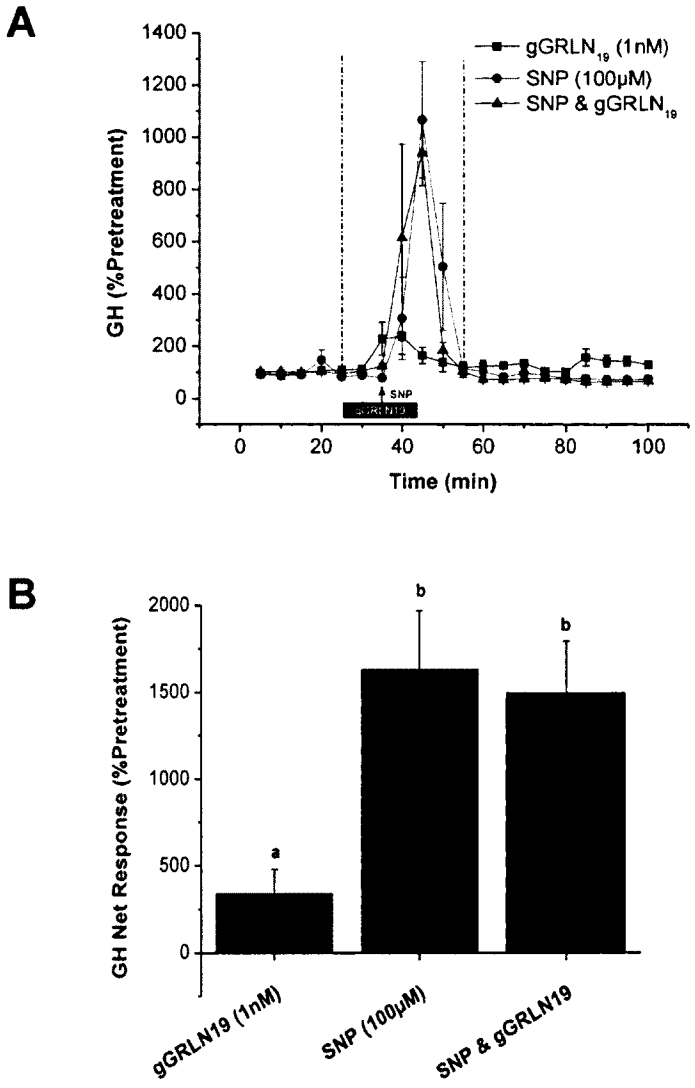

Fig. 6.2. $gGRLN_{19}$ and the NO donor SNP do not induce an additive GH release response. $gGRLN_{19}$ (black bar; 1 nM) was administered 10 min prior to a 5-min pulse of SNP (arrow; 100 μM). Net GH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, SNP alone, and $gGRLN_{19}$ with SNP for statistical analysis. Pretreatment average (first 5 fractions) was 10.13 ± 1.11 ng/ml. Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in January and February). Treatment values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

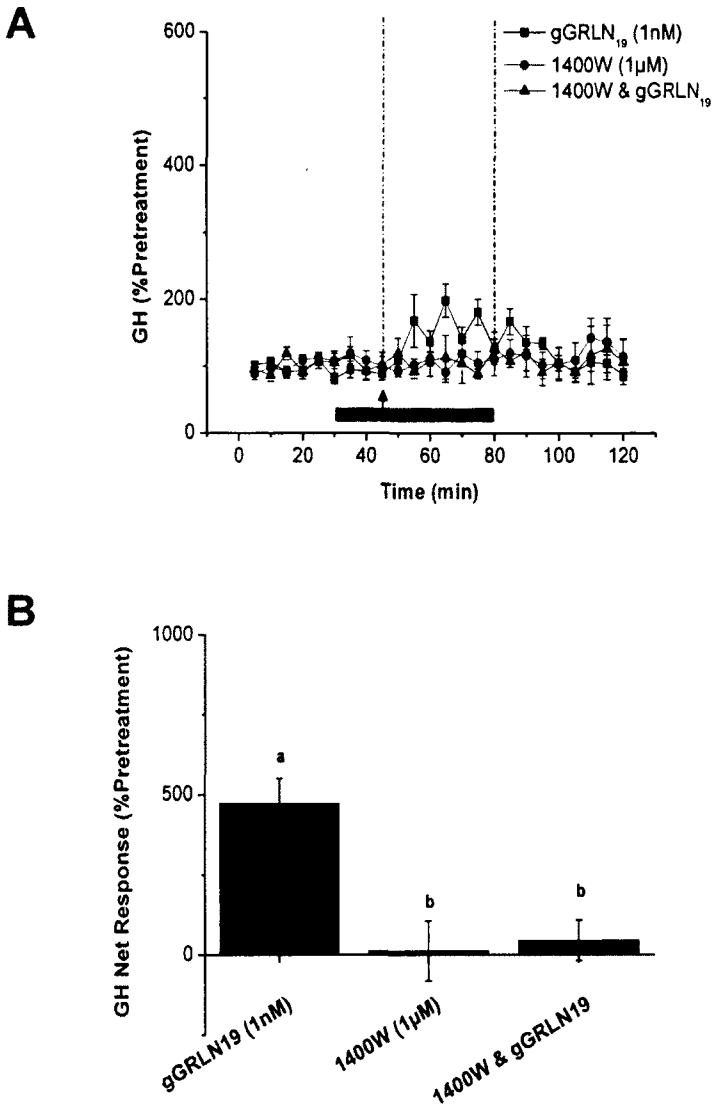

Fig. 6.3. $gGRLN_{19}$ fails to induce significant GH release in the presence of the NO inhibitor 1400W. 1400W (black bar; 1 μM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net GH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, 1400W alone, and $gGRLN_{19}$ with 1400W for statistical analysis. Pretreatment average (first 5 fractions) was 3.93 ± 0.48 ng/ml. Data are presented as mean ± SEM (n=6 from 3 separate experiments performed in May). Treatment values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

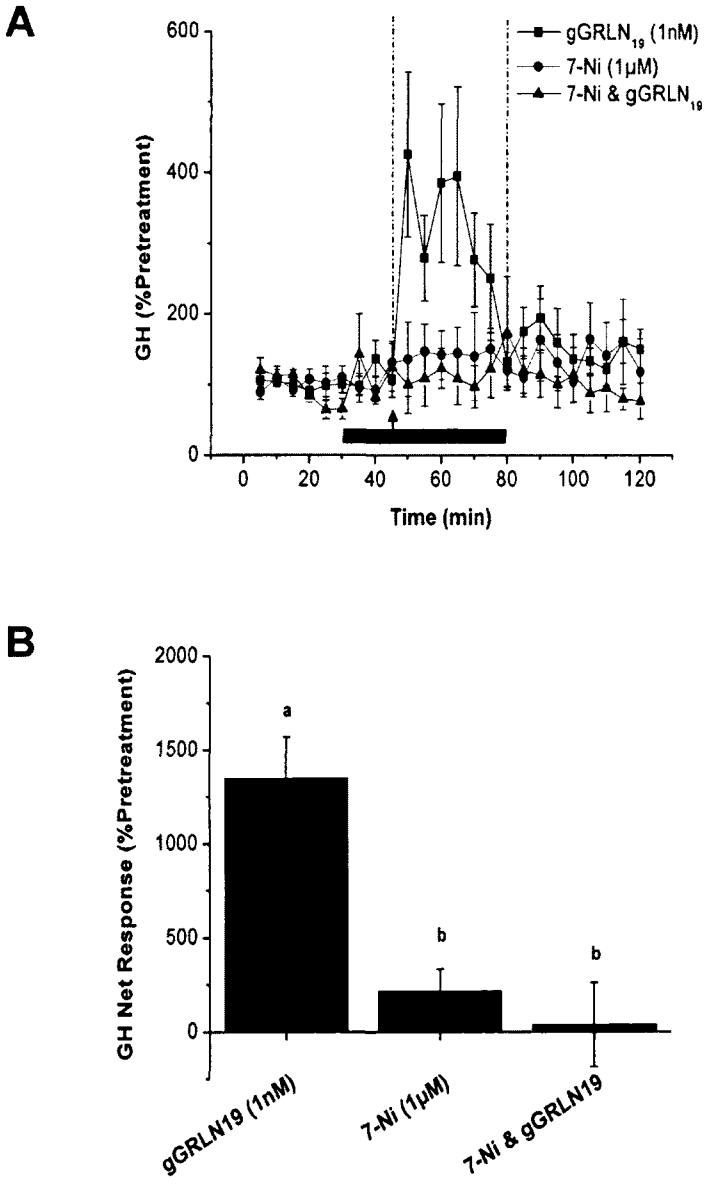

Fig. 6.4. $gGRLN_{19}$ fails to induce significant GH release in the presence of the NO inhibitor 7-Ni. 7-Ni (black bar; 1 μM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net GH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, 7-Ni alone, and $gGRLN_{19}$ with 7-Ni for statistical analysis. Pretreatment average (first 5 fractions) was 5.17 ± 1.18 ng/ml. Data are presented as mean ± SEM (n=6 from 3 separate experiments performed in June). Treatment values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

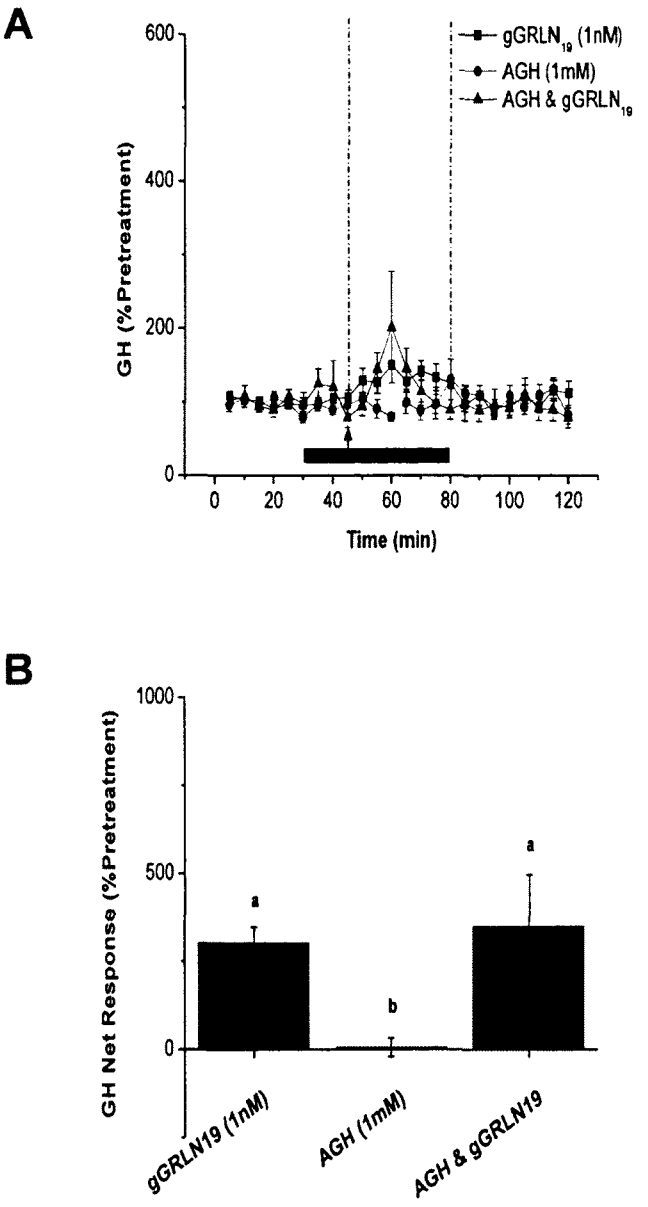

Fig. 6.5. $gGRLN_{19}$ induces a significant GH release in the presence of the NO inhibitor AGH. AGH (black bar; 1 mM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net GH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, AGH alone, and $gGRLN_{19}$ with AGH for statistical analysis. Pretreatment average (first 5 fractions) was 3.80 ± 0.40 ng/ml. Data are presented as mean ± SEM (n=6 from 3 separate experiments performed in April and May). Treatment values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

Chapter 7 – NO signalling in $gGRLN_{19}$-induced LH release from goldfish pituitary cells

7.1. Introduction

Despite some controversial findings, GRLN is recognized as a LH release regulator with actions at the levels of the hypothalamus, pituitary and gonads (Chapter 1, Section 1.3.1; [196, 230]). Direct GRLN stimulation of LH release from rat pituitary cells is at least in part mediated by NOS/NO [84]. Although the NOS isoform involved in this GRLN effect is not known, GRLN up-regulates NO production, iNOS protein expression, and iNOS gene expression in rat hearts [297]. Likewise, GnRH-induced LH release in mammals has also been reported to utilize NO signalling but this likely involves nNOS [8, 16, 61, 99, 197].

In the goldfish, immunoreactivity for both iNOS and nNOS has been identified in dispersed pituitary cells, including gonadotropes [324]. The involvement of NOS/NO signalling in goldfish pituitary hormone release is well established, with evidence supporting NO involvement in sGnRH-, cGnRH-II-, and DA-elicited GH release, as well as in sGnRH-induced LH release (Chapter 1, Section 1.5.2; [212]). In Chapter 6, I have also shown that $gGRLN_{19}$ stimulation of goldfish GH release has an NOS/NO component. However, signalling mediating $gGRLN_{19}$-stimulated GH & LH secretion is known to be non-identical (Chapter 5) and the involvement of NO in $gGRLN_{19}$-induced LH release has yet to be examined.

In this chapter, I tested the hypothesis that the NOS/NO pathway is a critical intracellular signalling component of $gGRLN_{19}$ stimulation of LH release from goldfish pituitary cells. To this end, I analyzed the samples collected in

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experiments reported in Chapter 6 for LH release responses in order to examine the role of NO, and the involvement of previously identified NOS isoforms, in $gGRLN_{19}$-stimulated LH release from goldfish pituitary cells in primary culture.

7.2. Results

7.2.1. The NO scavenger PTIO reduces $gGRLN_{19}$-induced LH release

To explore the overall involvement of NO in $gGRLN_{19}$-induced LH release from goldfish pituitary cells I used the NO scavenger PTIO in column perifusion, thus examining if $gGRLN_{19}$ could still induce significant increases in LH without the availability of NO. Application of $gGRLN_{19}$ at 1 nM, a maximally stimulatory and physiologically relevant dose previously established to induce repeatable and reversible increases in LH [115, 320] lead to an approximately net 150% increase in LH levels when compared to basal levels (Fig. 7.1A & B). Treatment with 10 μM PTIO alone [122], a dose that effectively abolished NO donor-induced LH release in a previous study [212], did not have a significant effect on basal LH levels (Fig. 7.1A & B). However, when $gGRLN_{19}$ was applied 15 min into a PTIO treatment, $gGRLN_{19}$-induced LH secretion was reduced to levels not significantly different from PTIO alone (Fig. 7.1A & B).

7.2.2. The NO donor SNP and $gGRLN_{19}$ do not stimulate an additive release of LH

To further explore the role of NO in $gGRLN_{19}$-induced LH release, I next utilized the NO donor SNP. If NO is not a critical component to $gGRLN_{19}$-induced LH release, co-treatment with SNP and $gGRLN_{19}$ should lead to additive release of LH from goldfish pituitary cells in column perifusion, a finding that has been previously observed under similar conditions where PACAP was able to further stimulate LH release from goldfish pituitary cells in perifusion in the

presence of SNP, when compared to SNP alone [52]. Given that SNP alone induces a large change in LH release, we chose to employ a longer, 20-min treatment of 1 nM $gGRLN_{19}$ with a 5-min 100 μM SNP pulse administered 10 min after the onset of $gGRLN_{19}$ treatment to facilitate the detection of additive LH release, if present (Fig. 7.2A). The 20 min $gGRLN_{19}$ treatment alone induced a significant net increase in LH release of approximately 275% (Fig. 7.2B). Treatment with a maximally stimulating dose of SNP alone (100 μM), which has also been shown to effectively enhance NO production in goldfish pituitary cells [52, 212, 240], induced a large, approximately 850% net increase in LH release while co-treatment with $gGRLN_{19}$ and SNP resulted in a net response (~750%) not significantly different than SNP alone (Fig. 7.2A & B).

7.2.3. NOS isoform-selective inhibitors differentially block $gGRLN_{19}$-induced LH release

Next, I turned upstream of NO and examined the involvement of NOS by utilizing three NOS isoform-selective inhibitors.

First, I examined the effects of 1 μM 1400W, a dose that would likely inhibit iNOS selectively [250]. A 5-min pulse of 1 nM $gGRLN_{19}$ produced a significant net increase in LH release but this was not seen in the presence of 1400W (Fig. 7.3A & B). Treatment with 1400W did not significantly affect basal LH secretion levels over the same quantification time period (Fig. 7.3A & B). These results implicated the involvement of iNOS in $gGRLN_{19}$-induced LH release in the goldfish pituitary.

Next I examined the effects of 7-Ni at 1 μM, a dose that would likely inhibit nNOS and eNOS, but not iNOS [164]. A 5-min treatment with 1 nM $gGRLN_{19}$ produced significant net increases in LH release when applied either alone or in the presence of 7-Ni; the magnitudes of these two responses were also not significantly different from one another (Fig. 7.4A & B). 7-Ni treatment

alone did not result in a significant change in net LH release over the same quantification period (Fig. 7.4A & B). These results suggested that nNOS and eNOS were unlikely to be involved in $gGRLN_{19}$-induced LH release from goldfish gonadotropes.

Finally, I examined the effects of 1 mM AGH, which should inhibit both iNOS and nNOS, but not eNOS [116], in order to allow me to further examine if iNOS is indeed the NOS isoform involved in $gGRLN_{19}$-induced LH release. A 5 min (1 nM) $gGRLN_{19}$ treatment produced a significant net increase in LH release when applied alone but not in the presence of AGH (Fig. 7.5A & B). The net changes in LH release elicited by AGH alone and the combination of $gGRLN_{19}$ and AGH were also not significantly different from one another (Fig. 7.5B). These observations were consistent with the involvement of iNOS in $gGRLN_{19}$ actions on LH release.

7.3. Discussion

Several lines of evidence from this thesis chapter support the hypothesis that NOS/NO is a critical component in $gGRLN_{19}$-induced LH release and identify iNOS as the most likely NOS isoform involved. First of all, in the presence of the NO scavenger PTIO, $gGRLN_{19}$ failed to induce a significant LH release response while co-treatment with the NO donor SNP did not lead to additive responses, both of which support the involvement of NO in the $gGRLN_{19}$ intracellular signalling cascade in goldfish gonadotropes. In addition, the NOS inhibitors 1400W and AGH abolished $gGRLN_{19}$-induced LH release, adding further support for the involvement of NO in $gGRLN_{19}$-induced gonadotrope signalling. Furthermore, assuming that the NOS isoform selectivity of the NOS inhibitors used in this study is similar in goldfish as in mammals, the sensitivity of the $gGRLN_{19}$-induced LH response to 1400W (iNOS selective) and AGH (iNOS

and nNOS selective), but not 7-Ni (nNOS and eNOS selective) indicates that iNOS is likely the NOS isoform mediating $gGRLN_{19}$ stimulation of LH release in this system. The presence of iNOS-immunoreactivity in goldfish gonadotropes is also consistent with the involvement of an iNOS-like molecule in signalling transduction events leading to LH release [324, 325]. The conclusion that NOS/NO mediates $gGRLN_{19}$-induced LH release in goldfish, however, is also supported by the known involvement of this signalling mechanism in GRLN stimulation of LH release in rats [84].

How $gGRLN_{19}$ activates iNOS in goldfish gonadotropes has not been examined. In mammals, iNOS is generally not a constitutively expressed enzyme, but it is active at basal levels $[Ca^{2+}]_i$ once its expression is induced, whereas nNOS and eNOS are constitutively expressed but require increases in $[Ca^{2+}]_i$ to be active [16]. On the other hand, iNOS-like immunoreactivity has been detected in pituitary cells and extracts from normal goldfish suggesting that it may be constitutively present in this tissue [324, 325]; however, its sensitivity to $[Ca^{2+}]_i$ has not been examined. It has been previously demonstrated that $gGRLN_{19}$ increases $[Ca^{2+}]_i$ in identified goldfish gonadotropes and that the LH response to $gGRLN_{19}$ is sensitive to Ca^{2+} entry through LVSCC [115]. It would be important to examine in future studies whether $gGRLN_{19}$ activation of iNOS in goldfish gonadotropes is downstream of increases in $[Ca^{2+}]_i$ and/or other $gGRLN_{19}$ signalling pathways (e.g., PKC; Chapter 5, [111]), as well as whether the expression of iNOS in the goldfish pituitary can be induced by $gGRLN_{19}$ and/or varies according to seasonal reproductive stages. Interestingly, the NO donor SNP has been shown to increase $[Ca^{2+}]_i$ in a LVSCC-dependent manner in goldfish somatotropes [363]. Thus, it is also possible that NO production is proximal to increases in $[Ca^{2+}]_i$ during $gGRLN_{19}$ stimulation of LH release. Furthermore, NO is known to activate sGC/cGMP/PKG in many systems [166, 258] but activation of sGC/cGMP/PKG signalling can also occur independently of

NO in goldfish gonadotropes during cGnRH-II stimulation [212]. Whether GC/cGMP/PKG also participates in $gGRLN_{19}$-induced LH release in goldfish and its relationship to NO is another topic that needs to be addressed. Future work focusing on these proven and postulated components of $gGRLN_{19}$-induced signalling and cross-talk between pathways would not only be interesting but also important.

Recently, sGnRH-induced, but not cGnRH-II- and PACAP-elicited, LH release was shown to involve NOS/NO signalling [212]. The present results add $gGRLN_{19}$ as one of the neuroendocrine factors that utilize NOS/NO signalling in stimulating LH release in goldfish. Interestingly, whereas only 1400W and AGH affected $gGRLN_{19}$ stimulation of LH release, all three of the NOS inhibitors used in this study (1400W, 7-Ni and AGH) effectively reduced the LH response to sGnRH [212]. These observations suggest that the NOS isoform (or complement of isoforms) involved in sGnRH and $gGRLN_{19}$ actions on goldfish gonadotropes are different. Furthermore, although results from this thesis chapter indicate that 1400W- and AGH-sensitive NOS is the isoform involved in mediating $gGRLN_{19}$ stimulation of LH release, the NOS isoform(s) mediating the GH response to $gGRLN_{19}$ is not identical, being sensitive to 7-Ni and 1400W, but not AGH (Chapter 6, [112]). Likewise, the LH and GH responses to sGnRH are also affected by different complements of NOS inhibitors (1400W, 7Ni and AGH for LH; 1400W and 7Ni for GH) [212, 324, 325]. Thus, the NOS isoform (or sets of isoforms) used by a particular neuroendocrine regulator also differ between goldfish somatotropes and gonadotropes. These results have an important implication: that the selective use of NOS/NO signalling, as well as the involvement of different NOS isoform(s), in intracellular signalling is part of the complexity within signal transduction events that allows for ligand- and cell-type-specific neuroendocrine control of pituitary LH and GH release in goldfish.

Given that $gGRLN_{19}$ stimulation of GH release also involves NOS/NO (Chapter 6) and NO is a diffusible molecule [112], a question that must be asked is whether the NOS-sensitive LH response to $gGRLN_{19}$ is the result of paracrine stimulation by NO released from neighboring cells such as somatotropes, and vice versa. It has been shown that identified goldfish gonadotropes and somatotropes both express NOS immunoreactivity [324, 325], indicating that goldfish gonadotropes and somatotropes likely produce NO via NOS. Although we have yet to demonstrate directly that $gGRLN_{19}$ can stimulate NO production in individual goldfish gonadotropes and somatotropes, the differing spectrum of sensitivity to NOS inhibitors in the $gGRLN_{19}$-elicited LH and GH responses described above strongly suggests that activation of NOS present in gonadotropes must contribute to the NOS/NO-dependence of $gGRLN_{19}$ action on LH release, while the NOS in somatotropes must also contribute directly to the corresponding GH response. Regardless of the differences in NOS isoform involved in the generation of $gGRLN_{19}$-induced NO signal in goldfish gonadotropes and somatotropes, these results indicate that NOS/NO signalling is one of the conserved mechanisms of action for $gGRLN_{19}$. Interestingly, NO generally has an inhibitory effect on ovarian steroidogenesis and follicular growth, survival and maturation in mammalian model systems [75-77] and $gGRLN_{19}$ inhibits final oocyte maturation in zebrafish [283]. Whether NOS/NO signalling participates in the gonadal actions of $gGRLN_{19}$ would be an important aspect of future investigation into the integrated neuroendocrine role of $gGRLN_{19}$ in reproduction.

In conclusion, in addition to revealing, for the first time, that NOS/NO participates in the direct action of $gGRLN_{19}$ on LH release in goldfish, and in any teleost species for that matter, results from this chapter also add to our understanding on the complexity of NOS/NO involvement in the multifactorial neuroendocrine control of LH and GH release in this model system. In particular, in addition to differences observed in the involvement of PKC and PKA

signalling in $gGRLN_{19}$-induced secretion from GH and LH cell types (Chapter 5, [111]), results from this thesis chapter, when taken with those of Chapter 6 on NO involvement in $gGRLN_{19}$-induced GH, indicate that there are also differences in the NOS/NO signalling pathways between somatotropes and gonadotropes in response to this stimulatory hormone. These results also support NO as a conserved component for GRLN signalling among vertebrates in various physiological functions, including cardiac, immune and hormone release [16], thus adding to the overall understanding of GRLN actions both at the pituitary level and at other target tissues in vertebrates.

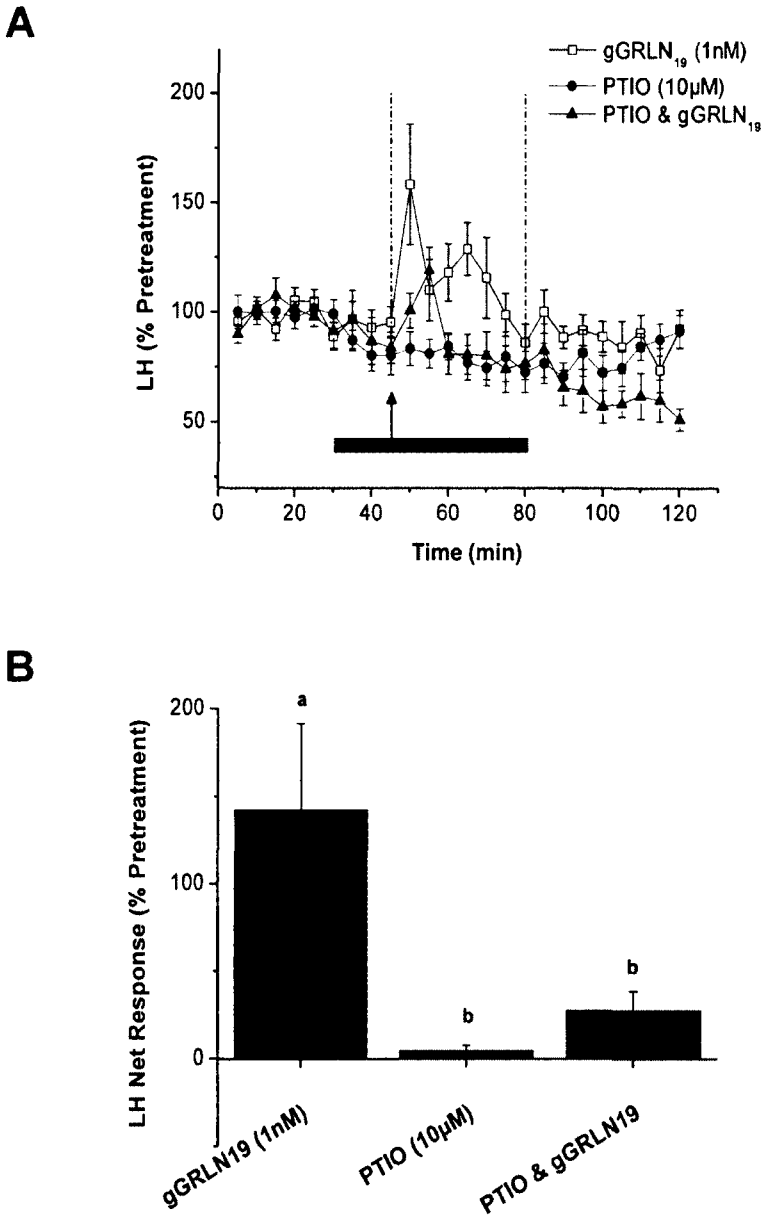

Fig. 7.1. $gGRLN_{19}$-stimulated LH release is significantly reduced in the presence of the NO scavenger PTIO. PTIO (black bar; 10 μM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net LH responses were quantified (area between vertical dotted lines) for PTIO alone, $gGRLN_{19}$ alone, and PTIO with $gGRLN_{19}$ for statistical analysis. Pretreatment average (first 5 fractions) was 4.12 ± 0.45 ng/ml. Data are presented as mean ± SEM (4 separate experiments were performed in October, n=8). LH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

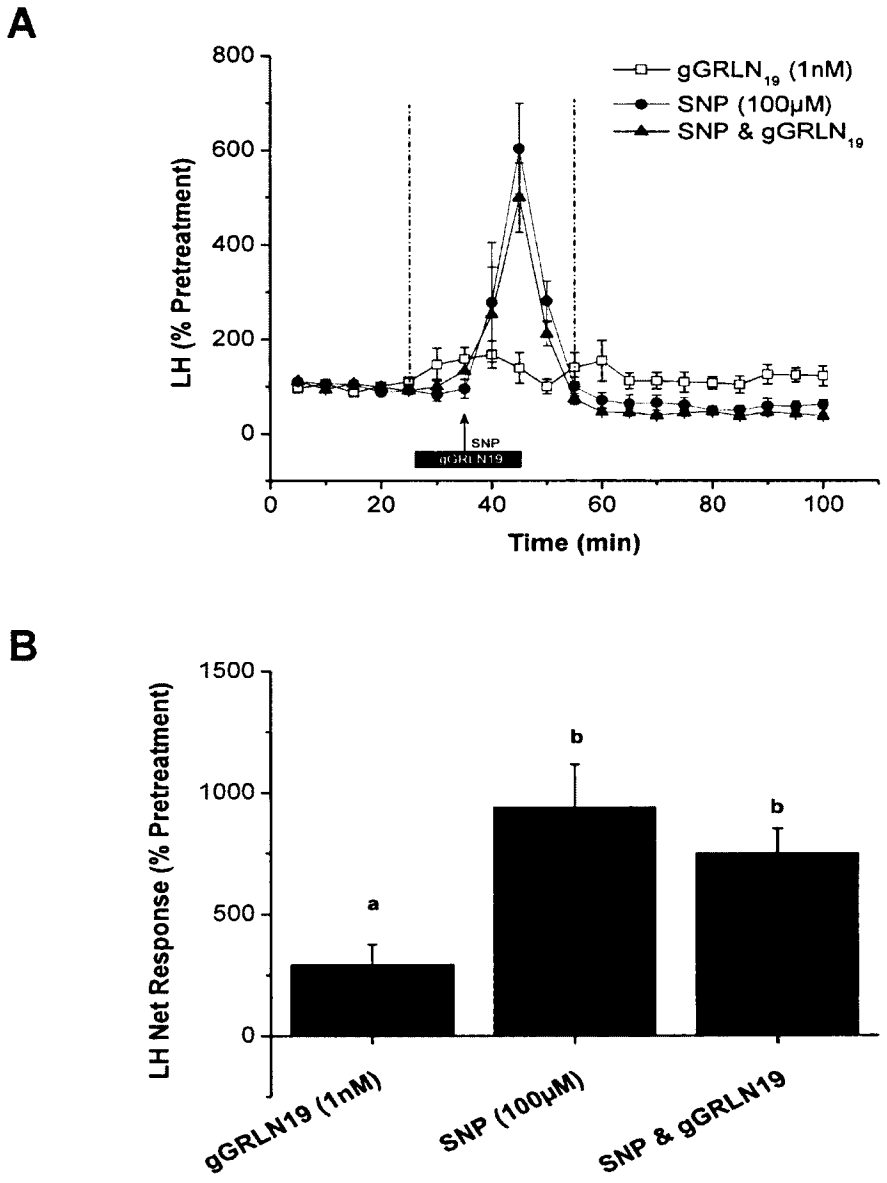

Fig. 7.2. $gGRLN_{19}$ and the NO donor SNP do not induce an additive LH release response. $gGRLN_{19}$ (black bar; 1 nM) was administered 10 min prior to a 5-min pulse of SNP (arrow; 100 μM). Net LH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, SNP alone, and $gGRLN_{19}$ with SNP for statistical analysis. Pretreatment average (first 5 fractions) was 12.86 ± 1.45 ng/ml. Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in January and February). LH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

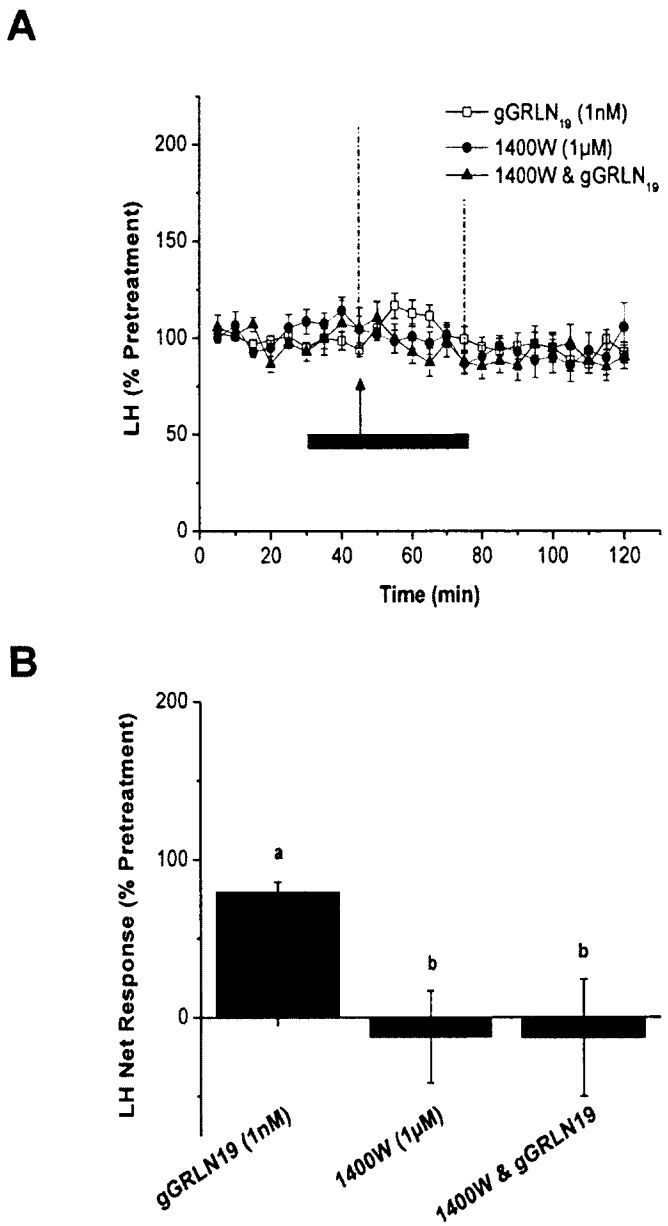

Fig 7.3. $gGRLN_{19}$ fails to induce significant LH release in the presence of the NO inhibitor 1400W. 1400W (black bar; 1 μM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net LH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, 1400W alone, and $gGRLN_{19}$ with 1400W for statistical analysis. Pretreatment average (first 5 fractions) was 2.31 ± 0.34 ng/ml. Data are presented as mean ± SEM (n=6 from 3 separate experiments performed in May). LH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

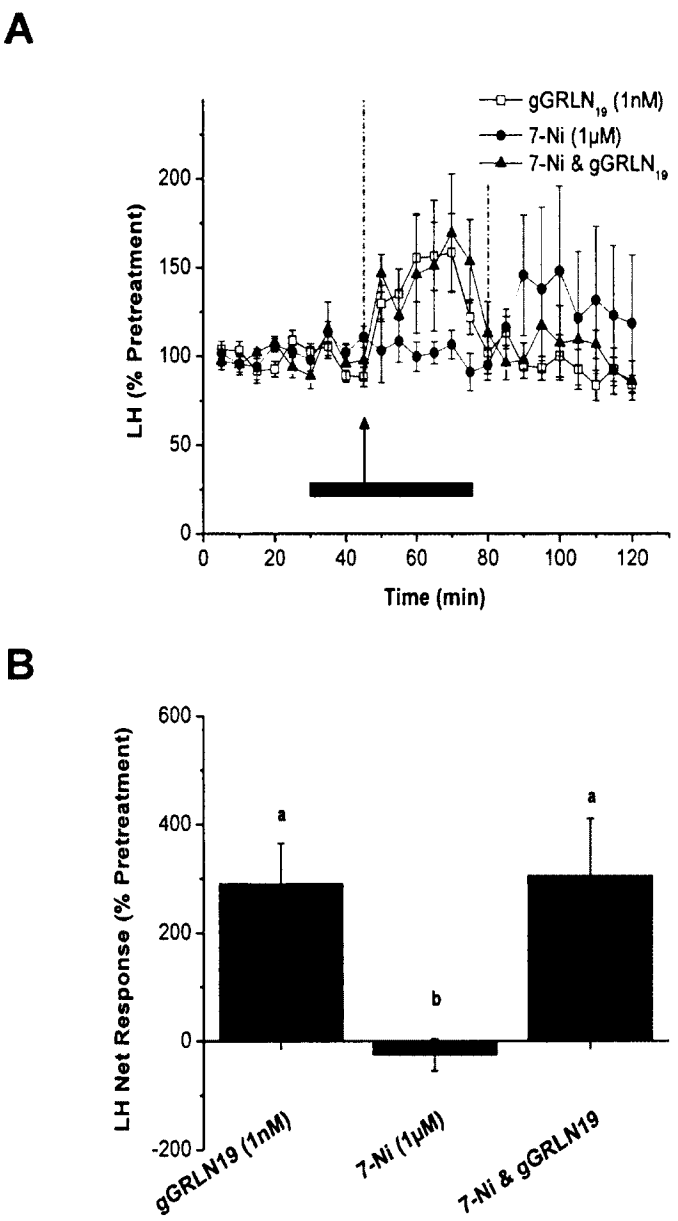

Fig. 7.4. $gGRLN_{19}$ induces a significant LH release response despite the presence of the NO inhibitor 7-Ni. 7-Ni (black bar; 1 μM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net LH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, 7-Ni alone, and $gGRLN_{19}$ with 7-Ni for statistical analysis. Pretreatment average (first 5 fractions) was 1.92 ± 0.16 ng/ml. Data are presented as mean ± SEM (n=6 from 3 separate experiments performed in June). LH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

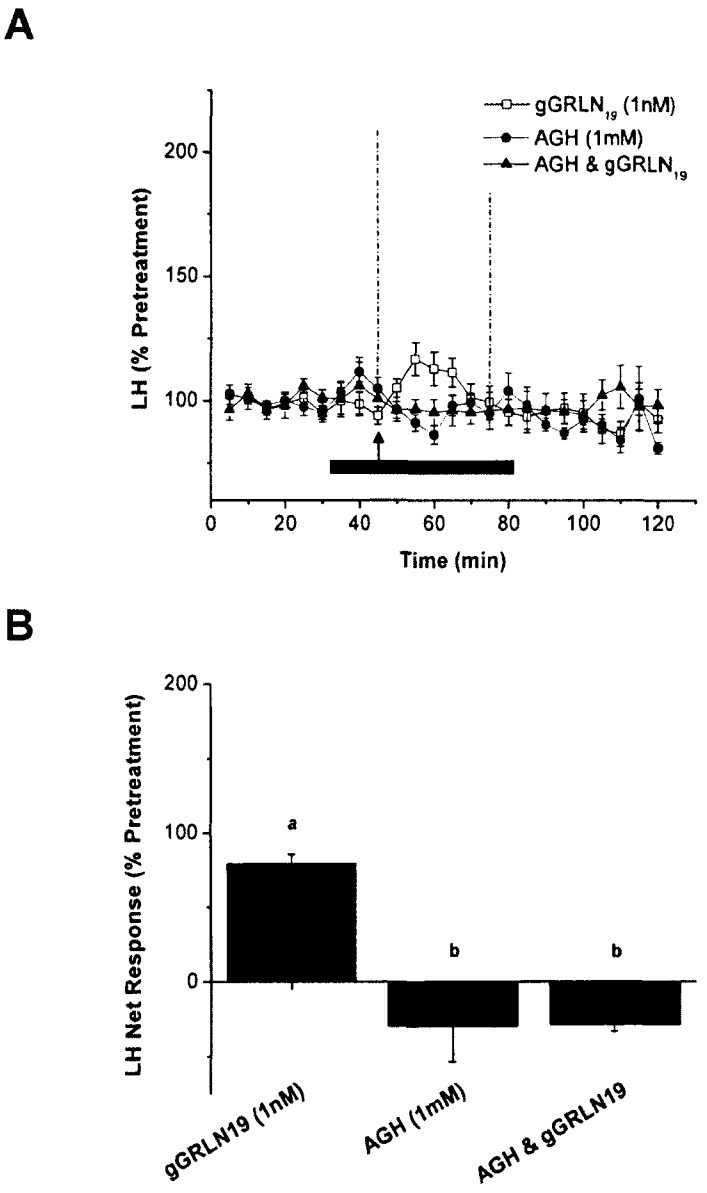

Fig 7.5. $gGRLN_{19}$ fails to induce significant LH release in the presence of the NO inhibitor AGH. AGH (black bar; 1 mM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net LH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, AGH alone, and $gGRLN_{19}$ with AGH for statistical analysis. Pretreatment average (first 5 fractions) was 2.23 ± 0.27 ng/ml. Data are presented as mean ± SEM (n=6 from 3 separate experiments performed in April and May). LH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test)

Chapter 8 – Differential modulation of $gGRLN_{19}$-induced GH and LH release by PACAP and DA in goldfish pituitary cells

8.1. Introduction

In goldfish, neuroendocrine regulation of pituitary GH and LH release are both multifactorial in nature and involves common, as well as distinct neuroendocrine factors (reviewed in Chapter 1, Sections 1.4.2 & 1.5.2). Among these neuroendocrine factors, PACAP and DA have been shown to affect both LH and GH secretion [41, 46]. PACAP directly stimulates both LH and GH secretion via PAC1 receptor-mediated cAMP/PKA signalling [346]. DA D1R-mediated increase in GH secretion similarly involves cAMP/PKA but has an additional NOS/NO component [218]. On the other hand, DA inhibits LH release via D2Rs and actions at or downstream of PKC and PKA activation [328]. Results from Chapters 3-7 of this thesis have also revealed that $gGRLN_{19}$ stimulates GH and LH release via common, as well as target-selective, signalling pathways. Specifically, while $gGRLN_{19}$ stimulation of GH and LH release both involve Ca^{2+}, LVSCC, NO and PKC, only $gGRLN_{19}$-induced LH release has an additional PKA component. Furthermore, the NOS isoform utilized is specific to each cell-type.

Interestingly, in mammals, both D1R and D2R have been shown to modulate GRLN effects by heterodimerization with GHS-R1a (Chapter 1, Section 1.2.3; [134, 158, 277]), and cAMP/PKA-dependent GHRH actions may potentiate the PKC-dependent GRLN-induced GH release (Chapter 1, Section 1.3.2; [119, 171]. In goldfish GH and LH release, PKC and PKA also form two major distinct intracellular signalling pathways (Chapter 1, Sections 1.4.2 & 1.5.2; [41, 46]), and

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the AC activator forskolin enhances $gGRLN_{19}$-elicited GH release in Chapter 5. These results suggest that DA and the PKA-utilizing PACAP may exert important modulatory effects on $gGRLN_{19}$ regulation of GH and LH release from goldfish pituitary cells; however, how DA and PACAP interacts with $gGRLN_{19}$-induced GH and LH secretion have not been investigated in this model system.

To further understand the multifactorial neuroendocrine control of GH and LH release in goldfish, as well as $gGRLN_{19}$ actions, this thesis chapter investigates if the abilities of $gGRLN_{19}$ to elicit GH and LH secretion are affected by DA and PACAP using primary cultures of dispersed goldfish pituitary cells. Results indicate that $gGRLN_{19}$ interacts with PACAP and DA on GH and LH release in a cell-type- and ligand-specific manner.

8.2. Results

8.2.1. Treatment with $gGRLN_{19}$ does not alter DA action on GH or LH release

The interactions between treatments with $gGRLN_{19}$ and DA, an established stimulator of GH release and inhibitor of LH secretion [41, 46], on hormone release from primary cultures of goldfish pituitary cells was first examined. Goldfish used were at times of gonadal regression. As expected, a 50-min application of DA at 1 μM, a maximally stimulatory dose [29], induced a large net GH response of approximately 8000% pretreatment levels (Fig. 8.1A & B). Likewise, a 5-min pulse of $gGRLN_{19}$ at 1 nM, a maximally stimulating and physiologically relevant dose [110, 115, 322], produced a net GH response of approximately 500% pretreatment. However, the addition of $gGRLN_{19}$ in the middle of the DA treatment was not able to further elevate the total net GH response above levels of that observed with DA alone (Fig. 8.1A & B). DA treatment alone reduced basal LH release as expected and inhibited $gGRLN_{19}$-

induced LH release (Fig. 8.1C & D). Application of $gGRLN_{19}$ alone elevated LH secretion with a net response of approximately 380% pretreatment; when applied in the presence of DA however, the net LH response to the combination treatment was not significantly different from that to DA alone (approximately -225 and -290 % pretreatment, respectively; Fig. 8.1C & D).

8.2.2. PACAP potentiates $gGRLN_{19}$-induced GH, but not LH, release

The interactions of $gGRLN_{19}$ and the known cAMP/PKA signalling peptide PACAP was examined next. Goldfish used were at times of late gonadal recrudescence. Treatment with a maximally stimulating dose of PACAP (10 nM) [54, 345] for 55 min resulted in a net GH response of approximately 600% pretreatment, a response magnitude that was similar to that induced by a 5-min application of 1 nM $gGRLN_{19}$ (Fig. 8.2A & B). However, when the $gGRLN_{19}$ pulse was applied 15 min into the PACAP treatment, this combination resulted in an enhanced total net GH release response (~2500% pretreatment) that was significantly greater than the responses to either $gGRLN_{19}$ or PACAP alone, as well as doubled the approximated sum of these two individual responses (Fig. 8.2A & B). This increase in total net GH release with the combined PACAP and $gGRLN_{19}$ treatment was largely due to the elevated GH levels in fractions collected following $gGRLN_{19}$ application (Fig. 8.2A). On the other hand, prior treatment with PACAP had no significant effect on $gGRLN_{19}$-induced LH release (Fig. 8.2 C&D). Here the PACAP-induced net increase in LH release (~150% pretreatment) was approximately half that of $gGRLN_{19}$ while $gGRLN_{19}$ alone and the combination treatment of both stimulators each resulted in a net LH release response of approximately 310% pretreatment (Fig. 8.2C & D).

8.2.3. Prior treatment with $gGRLN_{19}$ has no effect on PACAP-induced GH or LH release

GRLN levels in goldfish change in response to many physiological functions including energy levels and hunger status [319, 322] and thus these changes in GRLN release may occur at different times, relative to the release of other neuroendocrine factors including PACAP. Given the large potentiating effect of the combined PACAP and $gGRLN_{19}$ treatment on induced GH release observed in the previous protocol, I was interested in whether the treatment order was important in eliciting this enhanced GH release response. To this end, I pretreated with $gGRLN_{19}$ prior to PACAP challenge and measured both the GH and LH release responses. Pituitary cells used in these experiments were obtained from goldfish with gonads at mid-recrudescence. Given that a prolonged 1 nM treatment with $gGRLN_{19}$ induces GH and LH release in a bi-phasic manner and with the maximal peak release generally observed within 10 min of the treatment onset [110, 115], we chose a shorter 10-min pretreatment with $gGRLN_{19}$, compared to the previous experimental protocol with PACAP, to maximize the chances of observing additivity or potentiation with PACAP application, if present. As expected, treatment with either 1 nM $gGRLN_{19}$ alone for 35 min or a 10 nM PACAP alone pulse of 5 min induced significant release of both GH (net GH response to $gGRLN_{19}$ and PACAP ~220 and 830% pretreatment, respectively; Fig. 8.3A & B) and LH (net LH response to $gGRLN_{19}$ and PACAP ~100 and 160% pretreatment, respectively; Fig. 8.3C & D). The combination of a 35-min exposure to $gGRLN_{19}$ together with a 5-min treatment with PACAP, applied 10 min into the $gGRLN_{19}$ treatment, produced net GH and LH responses (approximately 900 and 190% pretreatment, respectively) that were of similar magnitudes to those of PACAP application alone (Fig. 8.3), suggesting that this combination treatment protocol did not result in a potentiation of either the GH or the LH release response.

8.2.4. Treatment with PACAP prior to $gGRLN_{19}$ potentiates Ca^{2+} signals in somatotropes, but not gonadotropes

Previous work has shown that hormone release response to $gGRLN_{19}$ and PACAP in goldfish somatotropes and gonadotropes involve elevations in $[Ca^{2+}]_i$ [110, 115, 276]. As an initial step to understand the intracellular basis for the potentiating effect of PACAP pretreatment on the GH response to $gGRLN_{19}$ observed in Section 8.2.2, I examined whether the $gGRLN_{19}$-elicited changes in $[Ca^{2+}]_i$ signals in goldfish somatotropes and gonadotropes were affected by prior exposure to PACAP. Pituitary cells used were obtained from goldfish at late gonadal recrudescence and pre-spawning stages. As in previous studies [46, 110, 115, 276, 364], we observed significant elevations in $[Ca^{2+}]_i$ within somatotropes and gonadotropes when either a 9.5 min, 1 nM, PACAP alone or a 2 min, 1 nM, $gGRLN_{19}$ alone treatment was applied (Fig. 8.4). Co-treatment with PACAP and $gGRLN_{19}$, with the $gGRLN_{19}$ plus PACAP application commencing 2 min into the PACAP treatment, resulted in enhanced $[Ca^{2+}]_i$ signals in somatotropes observed at time points shortly after the onset of the combined $gGRLN_{19}$ plus PACAP exposure (Fig. 8.4 A & B). Averaged peak $[Ca^{2+}]_i$ magnitudes observed within the 2 min following the onset of $gGRLN_{19}$ exposure in the presence of PACAP were about 3 times those observed in the absence of PACAP (Fig. 8.4A). Just as importantly, the cumulative net $[Ca^{2+}]_i$ response to combined $gGRLN_{19}$ and PACAP treatment was significantly greater those produced by either of these peptides alone and nearly doubled the expected sum of the responses to PACAP and $gGRLN_{19}$ alone (cumulative net increase induced by PACAP alone, $gGRLN_{19}$ alone and the combination treatment were ~1400, 800 and 4200% pretreatment, respectively; Fig. 8.4B). In contrast, no significant difference between the total net response with the combined $gGRLN_{19}$ and PACAP treatment and PACAP alone was observed in gonadotropes, although both were significantly greater than that to $gGRLN_{19}$ alone (Fig. 8.4C & D). These results demonstrate that pre-

exposure to PACAP selectively increased the $gGRLN_{19}$-elicited $[Ca^{2+}]_i$ signal within somatotropes, but not gonadotropes, in parallel with its effects on hormone secretion.

8.3. Discussion

The cAMP/PKA intracellular signalling mechanism has been implicated as a modulator of GRLN action in mammalian models [41, 46]. In an attempt to further understand the complex regulation of growth and development through modulation of pituitary hormone release in goldfish, as well as the mechanisms of GRLN actions in general, we explored interactions between $gGRLN_{19}$ and two other endogenous regulators, DA and PACAP, both of which have been shown to utilize the cAMP/PKA pathway in stimulating goldfish hormone release from pituitary cells. Novel findings in this thesis chapter include: 1) demonstration of a cell-type-specific potentiated hormone release response, and associated $[Ca^{2+}]_i$ signals in somatotropes, but not gonadotropes, in response to PACAP and $gGRLN_{19}$ co-treatment; 2) differences in the effects of the two PKA activating ligands, PACAP and DA, on $gGRLN_{19}$ actions on GH secretion; and 3) the ability of DA to inhibit $gGRLN_{19}$-induced LH release.

8.3.1. DA and $gGRLN_{19}$ action on GH and LH release

In many teleosts, DA is an inhibitor of basal and stimulated LH release by actions directly at the level of pituitary cells and the removal of this inhibitory influence is an integral part of the neuroendocrine events leading to the ovulation-inducing LH surge [78, 372]. Results in this thesis chapter extend this inhibitory action to GRLN-induced LH release for the first time in any teleost species. Results from Chapter 5 have implicated both PKC and PKA pathways in mediating $gGRLN_{19}$ stimulation of LH secretion from goldfish pituitary cells

[111], whereas DA inhibits stimulated LH release by actions on both PKC and PKA signalling via D2Rs in goldfish gonadotropes [46, 328]. How DA exerts its inhibitory effects on $gGRLN_{19}$-elicited LH secretion has not been directly examined but it seems likely that interference at both the PKC and PKA pathways may be involved.

On the other hand, DA is known to stimulate GH secretion via D1Rs and PKA activation in goldfish somatotropes while $gGRLN_{19}$ elicits GH release through PKC signalling (Chapter 5 and references [41, 53, 111, 347]). Previous studies have shown that PKC- and PKA-dependent GH release are at least additive in this system [41]. For example, further enhancement of GH release to maximally stimulating doses of DA, or pharmacological activators of PKA, can be achieved with co-treatment of maximally stimulating doses of pharmacological activators of PKC, or the PKC-dependent stimulator GnRH, with DA [41, 53, 347, 363]. Thus, the observation that co-treatment with DA and $gGRLN_{19}$ failed to produce an additive or a potentiated GH response is unexpected, especially given that the AC activator forskolin, as well as PACAP, pretreatment enhance $gGRLN_{19}$-elicited GH secretion ([111] and see Section 8.4.2 below). Furthermore, in a previous study using prolonged treatment with caffeine which also generated an increase in GH release reaching peak magnitudes of ~1000% pretreatment or greater, as with DA in this study, further stimulation of GH secretion above that seen with DA alone can be attained by the pulse application of another secretagogue [276]. These observations indicate that the releasable GH pool in goldfish somatotropes are not depleted by maximal stimulation DA and further elevation in GH secretion is possible if a separate major pathway is activated. The reason(s) for this surprising result with DA and $gGRNL_{19}$ co-treatment on GH release are not known but literature information on DA receptor and GHS-R1a interactions may provide a clue.

Goldfish somatotropes express D1R [342] and are also believed to express GHS-R1a [111, 322]. In HEK cells co-expressing mammalian D1R and GHS-R1a, these two receptors are thought to heterodimerize and co-treatment with GRLN enhances DA-induced cAMP response as a result of a switch in GRLN-GHS-R1a signalling from $G\alpha_{11/q}$, known to be linked to the PKC pathway, to a pertussis toxin-sensitive $G\alpha_{i/o}$-dependent pathway [134]. Furthermore, D1R-GHS-R1a heterodimers also co-internalize with DA and GRLN treatment, and their presence attenuates GRLN-induced increases in $[Ca^{2+}]_i$ [277]. If such heterodimerization of receptors and switch in GHS-R1a G-protein coupling occurs in goldfish somatotropes, it could explain the lack of additive GH response with the co-application of DA and $gGRLN_{19}$, since $gGRLN_{19}$ may no longer be signalling via $G\alpha_{11/q}$-PKC and/or its ability to elevate $[Ca^{2+}]_i$ may be impaired in the presence of DA stimulation. However, whether these events occur in goldfish somatotropes remains to be determined and further study would be required to elucidate this. Interestingly in mammals, GHS-R1a heterodimerization with D2R is also important in the neuroendocrine regulation of GH release and GRLN regulation of food intake. For example, D2R knockout in mice reduces GRLN-elicited and GHRH-induced GH release in mice [97]. Moreover, subsets of neurons in the hypothalamus expressing GHS-R1a/D2R heterodimers have been identified with florescence resonance energy transfer (FRET) and time resolved FRET, and treatment of wild type and $GRLN^{-/-}$ mice with a D2R agonist induces anorexia [158]. Although, D2Rs are not expressed on goldfish somatotropes, they are present on goldfish gonadotropes [328]. Whether D2R and GHS-R1a heterodimerization also plays a role in the inhibition of $gGRLN_{19}$-induced LH release by DA is unknown but it seems unlikely given that GHS-R1a/D2R heterodimer acts via activation of PLC in mammalian expression systems [158] and DA application inhibits $gGRLN_{19}$-stimulated LH release in this thesis chapter (Fig, 8.1). Nonetheless, the possibility of DA receptor heterodimerization with

GHS-R1a is an important area to consider in future studies on the neuroendocrine regulation of goldfish LH and GH release.

8.3.2. PACAP and $gGRLN_{19}$ action on GH and LH release and $[Ca^{2+}]_i$ signals in somatotropes

PACAP is a major modulator of hormone release from the pituitary gland, including GH and LH release in teleosts [94, 224, 249, 272]; however, the present thesis chapter is the first known study to look at co-treatment of PACAP and GRLN on GH and LH release responses in any vertebrate pituitary cell system. Since GRLN release changes in response to many inputs, such as hunger and starvation, the examination of the order in which $gGRLN_{19}$ is applied, relative to PACAP, is also important to consider as the relative order that goldfish pituitary cells may be exposed to different neuroendocrine factors may vary significantly depending on the physiological conditions. Indeed within the goldfish, preproGRLN mRNA levels in the hypothalamus and serum GRLN levels have both been shown to change in response to starvation and following feeding [152, 320], however longer term changes have not been examined to date. In the goldfish, PACAP induces GH and LH release from pituitary cells via a cAMP/PKA dependent pathway, but not PKC and NOS. Although PACAP-induced GH and LH release both involve increases in $[Ca^{2+}]_i$ through intracellular Ca^{2+} mobilization, only its GH-releasing actions involve Ca^{2+} entry through LVSCCs [54, 345, 346]. On the other hand, $gGRLN_{19}$-induced GH release involves PKC, LVSCCs and nNOS while the LH response is mediated by PKA, PKC, LVSCCs and iNOS (Chapters 3-7; [110, 112, 113, 115]). Consistent with these data is the present results on LH release, which demonstrates no additivity for PACAP and $gGRLN_{19}$ co-treatment in either the PACAP followed by $gGRLN_{19}$ or the reverse paradigm (Fig. 8.2 & 8.3), supporting that PACAP and $gGRLN_{19}$ both share the major, converging, PKA-dependent pathway to elicit LH release in goldfish gonadotropes. On the other hand, PACAP treatment prior to

$gGRLN_{19}$ results in a potentiation of GH release and an elevated $gGRLN_{19}$-induced $[Ca^{2+}]_i$ signal (Fig. 8.2 & 8.4). These observations fit in with results shown in Chapter 5 with forskolin pre-treatment, which also potentiates $gGRLN_{19}$-elicited GH release while having no effect on the LH response. These results suggest that the PKC-dependent, but PKA-independent, $gGRLN_{19}$-induced GH release is positively modulated by PKA-activating ligands in goldfish somatotropes. Given that the enhancement of GH release is only seen when PACAP treatment commences prior to $gGRLN_{19}$ application, but not in the reverse application sequence, it is likely that the activation of the PKA pathway in goldfish somatotropes "primes" the hormone-release response to PKC-mediated stimulation. PKA activation may recruit more GH-containing vesicles, increase the readily releasable pool, or increases the turnover of vesicles at the membrane, thus allowing for higher levels of hormone release in response to the $gGRLN_{19}$ treatment. In support of these possibilities are studies linking cAMP to synaptic vesicle activity. In primary cultures of rat hippocampal cells, activation of PKA, and associated increased phosphorylation of the vesicle phosphoprotein synapsin I, produces increased levels of vesicle exocytosis when cells are subsequently stimulated [213]. Also, studies in *Drosophila* link cAMP levels to enhancement of synaptic vesicle release and demonstrate that mutation-induced defects in the cAMP/PKA system result in decreased synaptic vesicle recruitment [160].

It is interesting to note that pretreatment with PACAP followed by $gGRLN_{19}$ also resulted in an increase in the $[Ca^{2+}]_i$ response in the present study but a similar elevation in $[Ca^{2+}]_i$ response was not observed with forskolin pre-exposure in experiments shown in Chapter 5. This difference is likely due to the fact that PACAP effects are receptor-mediated events while forskolin directly stimulates AC. Application of PACAP not only results in activation of AC and PKA, it is also linked to other signalling pathways [41, 218, 276] (also see above); one or more of which may allow the $gGRLN_{19}$-elicited $[Ca^{2+}]_i$ signal to be

amplified. The exact intracellular mechanism(s) involved in this enhancement of the $[Ca^{2+}]_i$ by PACAP remains to be determined. Likewise, whether the increased $[Ca^{2+}]_i$ response is a result of enhanced Ca^{2+} entry through LVSCCs and/or intracellular Ca^{2+} mobilization, as well as whether $gGRLN_{19}$ actions on goldfish somatotropes has an intracellular Ca^{2+} store-dependent component is unknown. While we cannot rule out the possibility that activation of PKA also allows for a more effective $[Ca^{2+}]_i$-exocytosis coupling, especially when we have only examined the interactions between PACAP or forskolin with $gGRLN_{19}$ at maximal effective doses, it appears that the elevated GH release response can occur independent of further increase in $[Ca^{2+}]_i$ beyond that induced by $gGRLN_{19}$ alone.

The effects of co-treatment with PACAP and $gGRLN_{19}$ on GH release observed in the present thesis chapter resemble those of GHRH and GRLN co-application in mammals, where GHRH is thought to be the major and PKA-activating stimulator of GH release and positive interactions between GHRH and GRLN on GH release have been reported [159, 171]. Interestingly, co-application of synthetic goldfish (g)GHRH and $gGRLN_{19}$ in goldfish pituitary cells did not produce an additive or a potentiated GH release response (see Chapter 9). However, how gGHRH exerts its GH-releasing effects in goldfish pituitary cells has not been directly investigated, although it is thought to also utilize PKA signalling based on receptor expression studies in cell-lines [185]. Interestingly, evidence in the literature supports the idea that GHRH is the major stimulator of GH release in birds and mammals, while PACAP and GHRH appear to work together to stimulate GH secretion in fish and amphibians [94]. These differences between how gGHRH (and PACAP) interacts with $gGRLN_{19}$ in goldfish as compared to mammals are not at odds with this proposed change in the relative importance of PACAP and GHRH peptides in the neuroendocrine regulation of GH secretion across vertebrate species.

8.3.3. Other implications and summary

The ability of PACAP to enhance the GH, but not the LH, response to $gGRLN_{19}$, as well as the differential actions of DA on $gGRLN_{19}$ effects on GH and LH release, makes sense from the perspective of overall regulation of physiological functions by pituitary hormones. Given that GRLN appears to be a link between energy status, growth, and reproduction, its action at the level of the pituitary on GH and LH release must be selectively modulated under various circumstances. For example, during periods of body growth, stimulation of orexigenic behavior and release of large amounts of GH would be required but LH release would not be expected to be elevated, or may even be reduced if energy stores are being expended in somatic growth. The major period of somatic growth in goldfish commences in the post-spawning season of late spring and early summer and the serum GH level is also much elevated while serum LH is low in vivo [56, 315]. At this time of the year, the ability of PACAP to stimulate GH release is greatest in vitro [56] and PACAP's ability to selectively enhance $gGRLN_{19}$ action of GH release may be part of the neuroendocrine mechanisms in maintaining a relatively high serum GH level. On the other hand, during gonadal recrudescence in the winter months, energy supply for gonadal growth, as well as appropriate gonadal steroidogenesis is required. It is known that GH enhances the ability of LH to stimulate sex steroid production [183, 287] and is a positive modulator of spermatogenesis induction [194]. DA inhibitory action on LH release-responsiveness to neuroendocrine stimulators, including $gGRLN_{19}$ and others, allows for an adequate LH release while preventing the premature elicitation of the ovulation inducing LH surge. At the same time, DA's own GH-releasing actions, together with its non-potentiating interactions with $gGRLN_{19}$, as well as the effects of other neuroendocrine factors on GH release, maintains a level of GH secretion adequate to maintain energy balance and to enhance the steriodogenic response to LH necessary for gonadal development. It would be

very interesting to look at how PACAP release and actions differs under different stages of somatic growth, as well as gonadal maturation, to further understand the role(s) of PACAP and GRLN interaction at the level of the pituitary. Likewise, whether the influence of DA on $gGRLN_{19}$-elicited GH and LH release varies seasonally is also an important area of future research in the differential neuroendocrine regulation of the release these two pituitary hormones.

Overall, results from this thesis chapter provide novel evidence of the differential modulation of $gGRLN_{19}$'s hormone-releasing activity by two known neuroendocrine regulator of LH and GH release, DA and PACAP, in goldfish pituitary cells. Specifically, results demonstrate that PACAP is able to potentiate $gGRLN_{19}$-induced GH, but not LH, release while DA inhibits $gGRLN_{19}$ effects on gonadotropes and does not enhance $gGRLN_{19}$ actions in somatotropes. Furthermore, these results fit with the current model that $gGRLN_{19}$-induced GH release is dependent on PKC, but not PKA, and is potentiated by stimulators of PKA signalling, while $gGRLN_{19}$-induced LH release relies on both PKC and PKA-dependent signalling mechanisms. The differences in the effects of pretreatment with DA or PACAP on $gGRLN_{19}$-elicited GH release indicate that potentiation of $gGRLN_{19}$ stimulation of GH release may not be a universal feature of PKA-activating ligands, but may be hormone-receptor-system specific. In addition, complex interactions, such as receptor heterodimerization, which has been shown to affect GRLN receptor actions in other systems, may be important and profitable avenues for further research into $gGRLN_{19}$ neuroendocrine actions. Furthermore, given that pretreatment with PACAP prior to $gGRLN_{19}$ application, but not the reverse treatment sequence, leads to an enhanced GH release response, the order of exposure to hypothalamic factors is probably an important aspect in the multifactorial neuroendocrine control of the release of GH and other pituitary hormones that will require attention in future investigations.

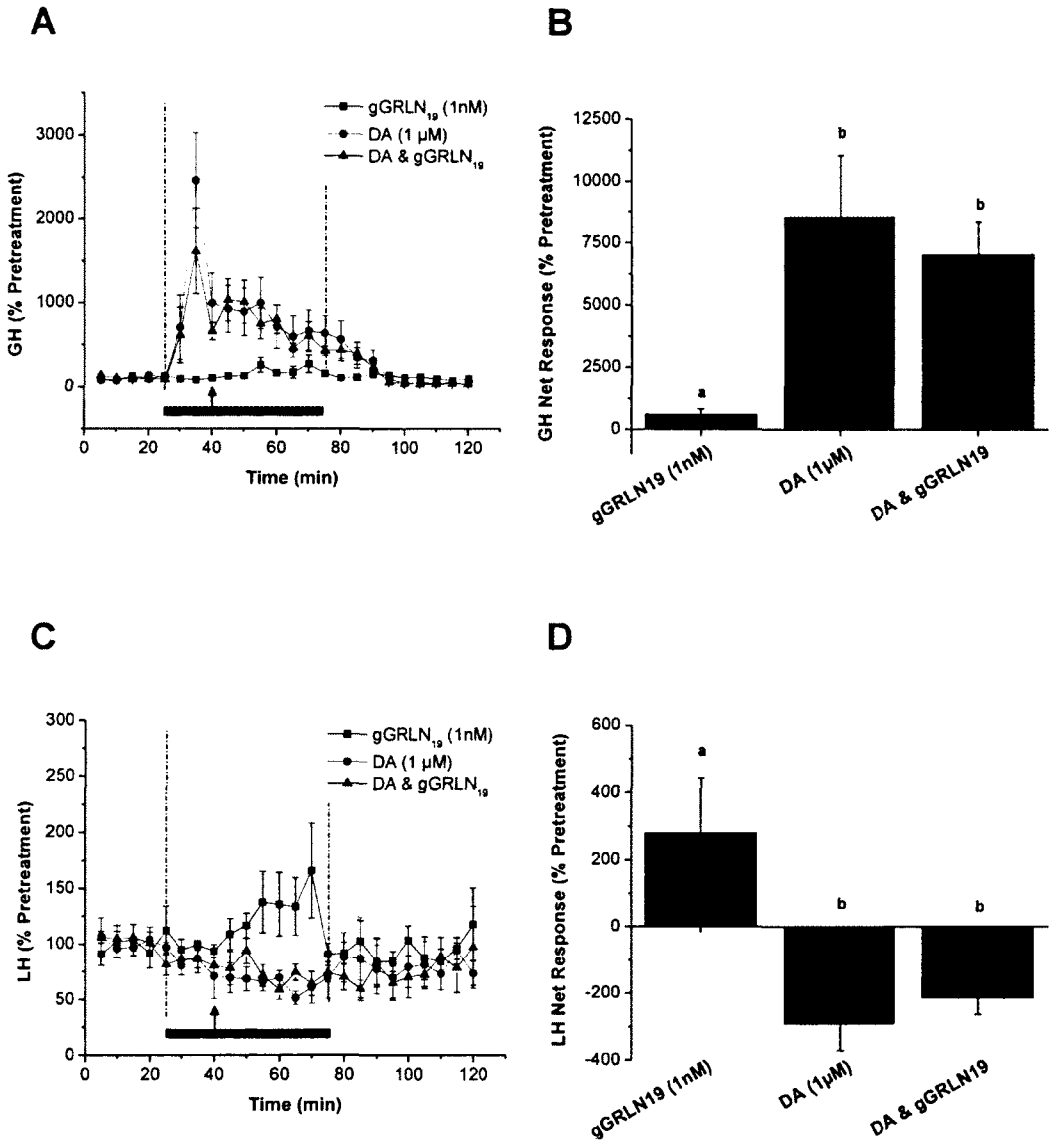

Fig. 8.1. $gGRLN_{19}$ does not modulate DA action on either GH or LH release. DA (black bar; 1 μM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net GH and LH responses were quantified (area between vertical dotted lines) for DA alone, $gGRLN_{19}$ alone, and DA with $gGRLN_{19}$ for statistical analysis. Pretreatment average (first 5 fractions) was 3.97 ± 0.66 ng/ml for GH and 1.01 ± 0.16 ng/ml for LH. Data are presented as mean ± SEM (4 separate experiments were performed in June and July, n=8). GH release profiles are shown in panel A (GH) and C (LH) while the quantified net responses are shown in panel B (GH) and D (LH). Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

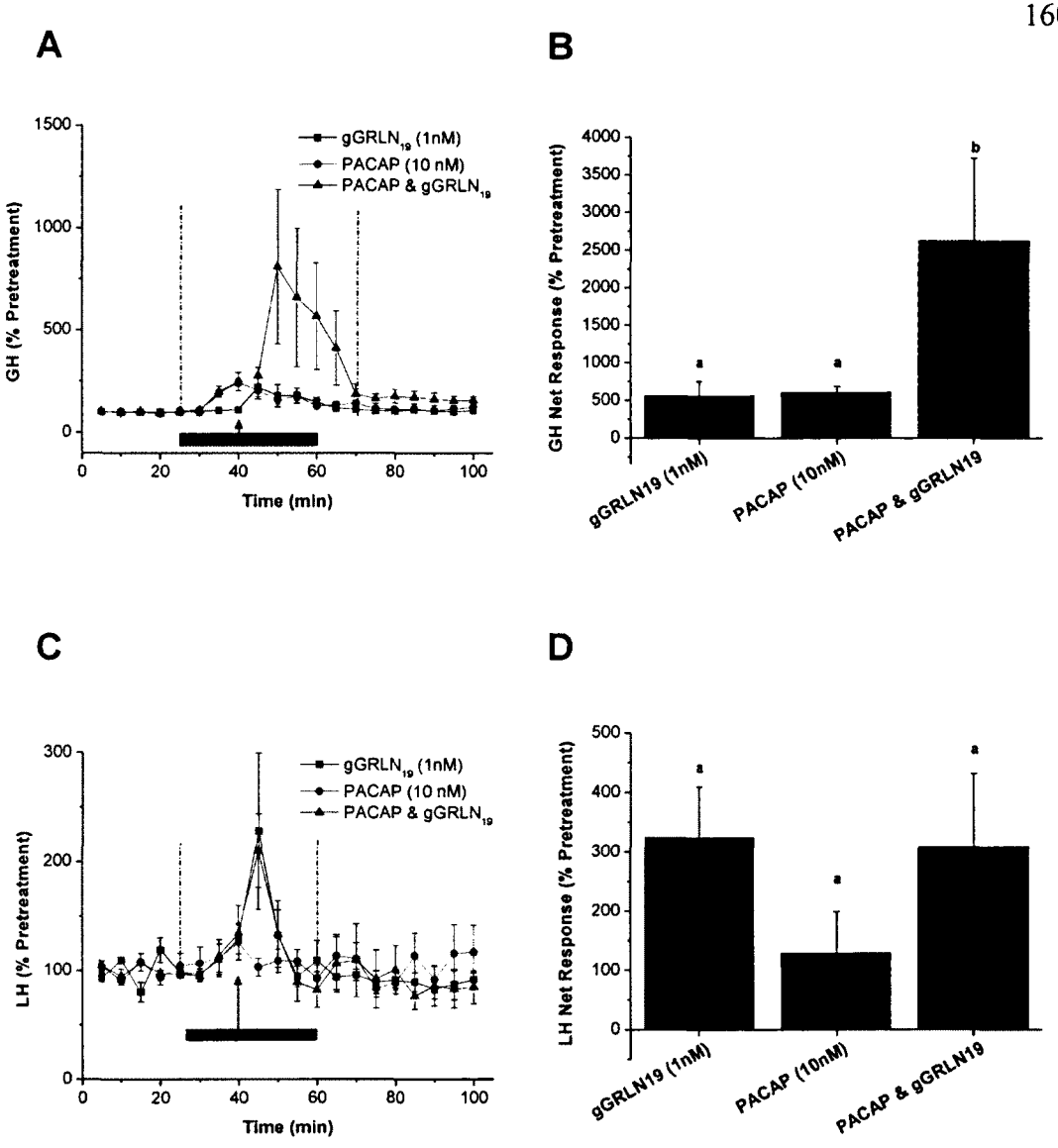

Fig. 8.2. PACAP treatment potentiates $gGRLN_{19}$-induced GH, but not LH, release. PACAP (black bar; 10 nM) was administered 15 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). Net GH and LH responses were quantified (area between vertical dotted lines) for $gGRLN_{19}$ alone, PACAP alone, and $gGRLN_{19}$ with PACAP for statistical analysis. Pretreatment average (first 5 fractions) was 10.58 ± 0.64 ng/ml (GH) and 6.21 ± 0.93 (LH). Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in March and April). Hormone release profiles are shown in panel A (GH) and C (LH) while the quantified net responses are shown in panel B (GH) and D (LH). Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

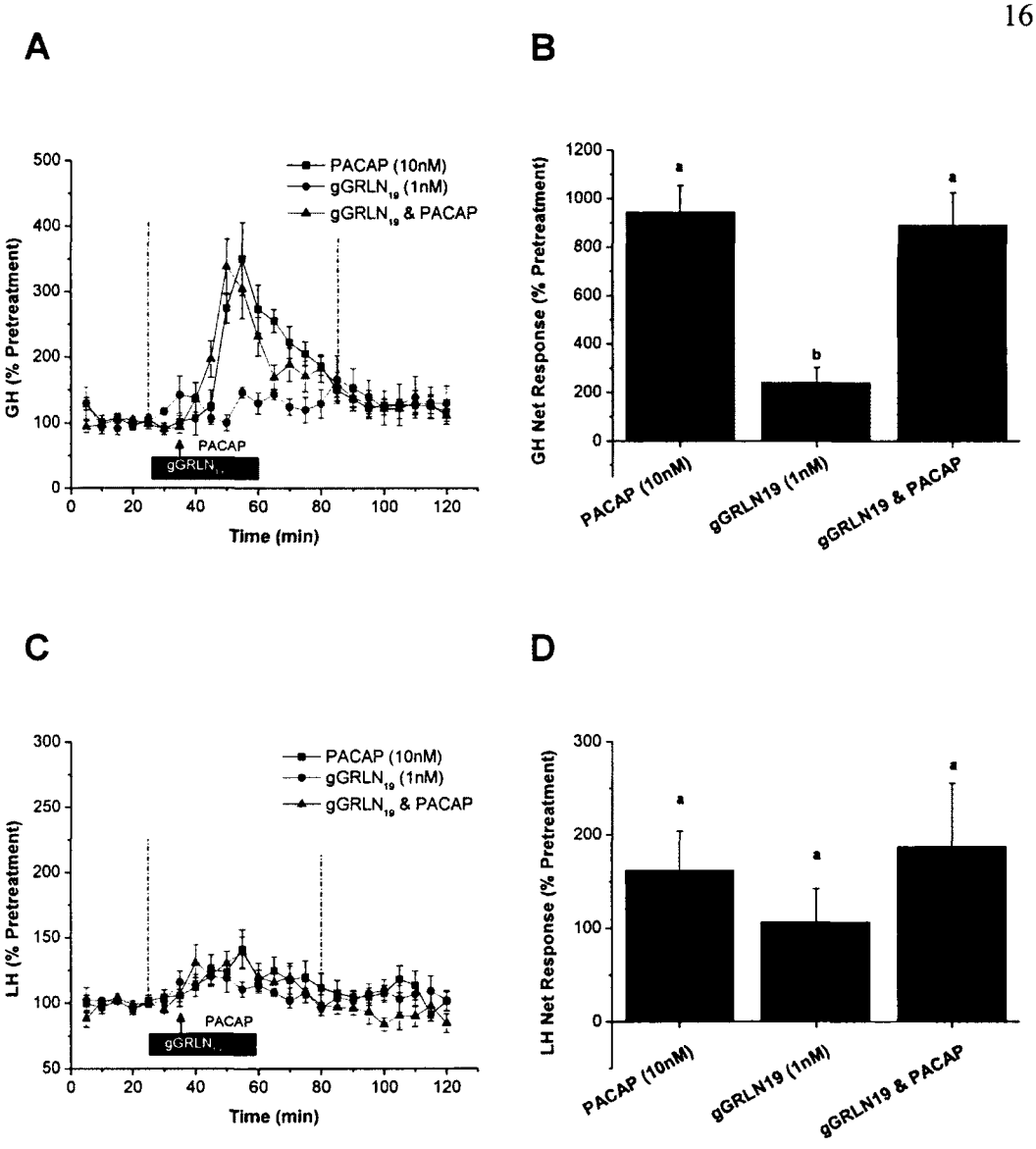

Fig. 8.3. $gGRLN_{19}$ treatment fails to potentiate PACAP-induced hormone release. $gGRLN_{19}$ (black bar; 1 μM) was administered 10-min prior to a 5-min pulse of PACAP (arrow; 10 nM). Net hormone responses were quantified (area between vertical dotted lines) for PACAP alone, $gGRLN_{19}$ alone, and $gGRLN_{19}$ with PACAP for statistical analysis. Pretreatment average (first 5 fractions) was 5.41 ± 0.41 ng/ml (GH) and 3.82 ± 0.21 ng/ml (LH). Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in January and February). Hormone release profiles are shown in panel A (GH) and C (LH) while the quantified net responses are shown in panel B (GH) and D (LH). Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test).

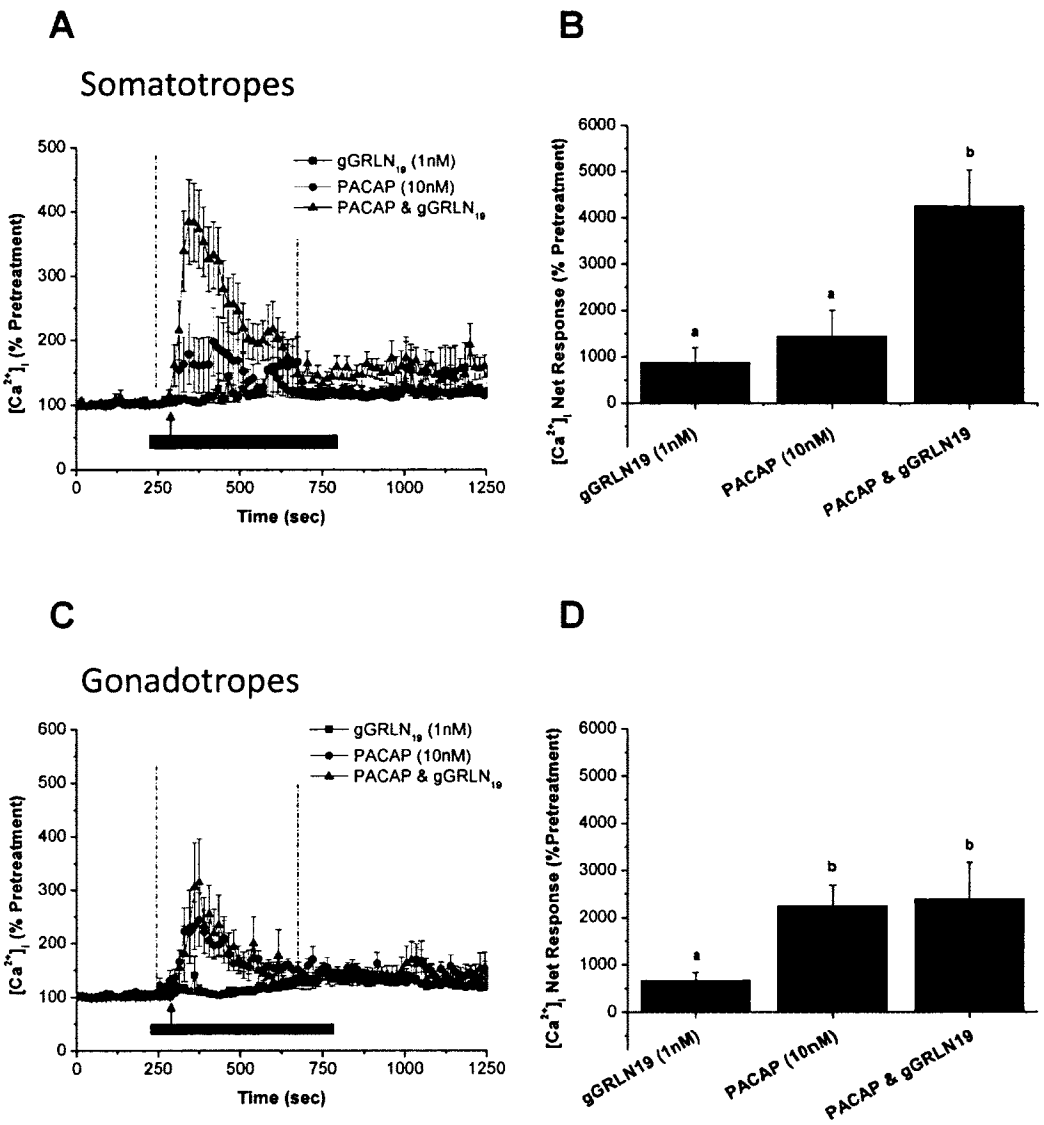

Fig. 8.4. 1 nM $gGRLN_{19}$ potentiates changes in $[Ca^{2+}]_i$ when somatotropes, but not gonadotropes, are pre-treated with PACAP (10 nM). Following a 2 min pretreatment with PACAP (black bar), cells loaded with fura-2, AM on poly-l-lysine coverslips were challenged with a 2-min pulse of $gGRLN_{19}$ (arrow), followed by a washout of 5.5 min with PACAP. Ca^{2+} response traces in somatotropes (A) and gonadotropes (C) were normalized to pretreatment averages (average of first 5 fractions: 227.21 ± 11.99 nM and 242.39 ± 14.12 nM for somatotropes and gonadotropes, respectively). Quantified net responses (area between vertical dotted lines) for $gGRLN_{19}$ alone, PACAP alone, or combination treatments were summarised for statistical comparison (B and D). Data are presented as mean ± SEM (n=12 for each treatment performed between March and May from 4 separate cell preparations). Treatment values statistically different from each other have different letters of the alphabet.

Chapter 9 – gGHRH stimulates GH release but inhibits $gGRLN_{19}$- and sGnRH-induced LH release from goldfish pituitary cells

9.1. Introduction

As reviewed in Chapter 1, GHRH and GnRH are the main stimulatory factors for GH and LH release, respectively, in mammals (Chapter 1, Sections 1.4 & 1.5). In addition GRLN also participates in the neuroendocrine regulation of GH and LH release (Section 1.3.1). GRLN directly stimulates pituitary GH secretion via receptor-signalling mechanisms that are largely dissimilar to those activated by GHRH (mainly PKC and PKA, respectively), and GRLN and GHRH can synergistically enhance each other's GH responses [152, 170, 171]. This positive interaction between GRLN and GHRH is likely to be important when rapid increases in GH release are required and is thus thought to be necessary for maximal functioning of GH release responses [119, 171].

In addition to its major role in GH release, GHRH has also been reported to have other functions in mammals [12, 159], including actions on gonadotropes. A subset of rat gonadotropes express GHRH binding sites [65], and long-term treatment with GHRH (2 weeks) reduces the number of LH-β mRNA positive cells [246]. On the other hand, bovine-based studies demonstrate a short-term (5-30 min) increase in LH serum levels in response to GHRH injection [223], while studies on dogs show that GHRH treatment has no significant effect on LH release [19]. Interestingly, a combination treatment of GnRH, corticotropin-releasing hormone, GHRH, and thyrotropin-releasing hormone in dogs lead to a significantly lower LH release response when compared to GnRH treatment alone

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[211], suggesting that GHRH may act in conjunction with other endogenous pituitary regulatory hormones to modulate the LH response to GnRH.

In goldfish, GnRH is an important neuroendocrine stimulator of both LH and GH release (Chapter 1, Sections 1.4.2 & 1.5.2; [41, 46]). Similarly, $gGRLN_{19}$ increases LH and GH secretion via direct actions on gonadotropes and somatotropes (Chapter 1, Section 1.3; [110, 115]). Like other vertebrate systems, synthetic gGHRH increases GH release from goldfish pituitary cells and gGHRH stimulation of zebrafish GHRH receptors expressed in cell expression systems elevates cAMP production [185]. In addition, mRNAs for gGHRH and gGHRH receptor are expressed in goldfish pituitary extracts [185], suggesting that gGHRH is also a physiological regulator of GH release in this system.

Reminiscent of the interactions between GHRH and GRLN in mammals [152, 170, 171], treatment with either the PKA-dependent PACAP (Chapter 8) or the AC activator forskolin (Chapter 5) enhances the GH response to the PKC-dependent $gGRLN_{19}$ in goldfish pituitary cells. However, how gGHRH interacts with other neuroendocrine stimulators in GH secretion, including $gGRLN_{19}$, and whether gGHRH affects LH secretion is unknown in the goldfish. To begin to address these gaps in knowledge in the neuroendocrine control of GH and LH release in goldfish, I examined the effect of gGHRH on both GH and LH release from primary goldfish pituitary cell cultures, alone and in conjunction with $gGRLN_{19}$ and sGnRH. More specifically, experiments in this chapter look at whether treatment with gGHRH alters the ability of $gGRLN_{19}$ and sGnRH to induce GH and LH secretion, using a cell column perifusion protocol which allows for easy visualization of hormone release profiles. $gGRLN_{19}$ and sGnRH were used at maximally effective doses of 1 and 100 nM, respectively [110, 115]. gGHRH was applied at a dose of 100 nM which elicited a maximum or near maximum increase in GH secretion from goldfish pituitary cells in 4-h static incubation experiments [185].

9.2. Results

9.2.1. $gGRLN_{19}$ induced GH release in the presence of gGHRH

Whether gGHRH affected the ability of $gGRLN_{19}$ to elicit GH release was examined first. A 5 min (1 nM) $gGRLN_{19}$ pulse was administered 20 min into the 55 min (100 nM) gGHRH treatment (Fig. 9.1A). Treatment with $gGRLN_{19}$ alone produced a net increase in GH release to approximately 250% pretreatment levels while gGHRH alone produced a net GH response of approximately 160% pretreatment (Fig. 9.1A & B). The combination of $gGRLN_{19}$ and gGHRH induced a net GH response of approximately 430% when quantified over the total 55 min duration of gGHRH presence, and this total net response was statistically different from that to gGHRH treatment alone but not $gGRLN_{19}$ alone (Fig. 9.1A & B). When only the net responses to $gGRLN_{19}$ applied in the absence and in the presence of gGHRH were compared (i.e., quantified over the 35 min following $gGRLN_{19}$ application), no statistical difference between these values was demonstrated (Fig. 9.1B, inset). These results demonstrated that $gGRLN_{19}$ was able to induce significant increases in GH levels in the presence of gGHRH. These experiments were performed using pituitary cells from fish at early stages of gonadal recrudescence (December and January).

9.2.2. sGnRH induced GH release in the presence of gGHRH

The influence of gGHRH on the ability of sGnRH to induce GH release was investigated next using a similar protocol as with the experiments with $gGRLN_{19}$ above. Treatment with a 5-min pulse of 100 nM sGnRH alone produced a net GH release response of approximately 300% pretreatment (Fig. 9.2A & B). Treatment of 100 nM sGnRH alone resulted in a net increase in GH secretion of approximately 425% pretreatment (Fig. 9.2A & B). When quantified over the entire duration of gGHRH application, co-treatment with both gGHRH and sGnRH did not lead to a response greater than either treatment alone (Fig.

9.2A & B). Importantly, when only the GH release responses following sGnRH applications were quantified (i.e., over the 35 min following sGnRH application), no significant difference was observed between those elicited by sGnRH alone and sGnRH applied in the presence of gGHRH (Fig. 9.2B inset). These results indicate that sGnRH was able to elicit a GH response in the presence of gGHRH. These experiments were performed using pituitary cells from fish at stages of gonadal regression (May and June).

9.2.3. $gGRLN_{19}$ and sGnRH failed to induce LH release in the presence of GHRH

To examine the potential effects of GHRH on LH release, I also analyzed the samples collected in the above experiments for LH. As expected based on results in Chapter 3, 1 nM $gGRLN_{19}$ induced a net LH release response of approximately 170% pretreatment (Fig. 9.3). Likewise 100 nM sGnRH elicited a net LH response of approximately 100% pretreatment (Fig. 9.4). On the other hand, gGHRH treatment alone produced a cumulated net reduction in LH values (net change = -74 ± 20% pretreatment levels, $p=0.002$ vs. no change or a value of zero, pooled results from Fig. 3 & 4, $n=16$, single sample t-test). Interestingly, neither $gGRLN_{19}$ nor sGnRH treatment caused significant increases in LH release when applied in the presence of GHRH. The quantified responses to the combination treatment of either $gGRLN_{19}$ or sGnRH with gGHRH were similar to those of gGHRH alone and all were significantly lower than responses to $gGRLN_{19}$ or sGnRH alone, regardless of whether the responses were quantified over the equivalent time of the entire gGHRH application or just following the application of either $gGRLN_{19}$ or sGnRH (Fig. 9.3B & 9.4B, and insets). These results demonstrated an inhibitory effect for gGHRH on stimulated and basal LH release.

9.3. Discussion

In this chapter, I have set out to elucidate gGHRH effects on goldfish pituitary cells, specifically examining if it could induce changes in GH and LH release from primary pituitary cell cultures and how it may interact with two other endogenous stimulators of GH and LH secretion, $gGRLN_{19}$ and sGnRH. Results not only further our understanding of gGHRH action but also have important implications on the neuroendocrine regulation of GH and LH release.

Early GH release studies with biochemically purified "carp GHRH" and "salmon GHRH" suggest that fish GHRH are weak stimulators of GH release in teleosts [30, 36, 264]. However, current molecular evidence indicates that these molecules are not genuine fish GHRHs but are related peptides in the PACAP/GHRH family, and should better be termed PACAP-related peptides [302]. Results from 4-h static incubation experiments, using authentic gGHRH synthesized based on genomic information, indicate that gGHRH is an effective stimulator of GH release from goldfish pituitary cells [185]. Results from this thesis chapter not only supports the idea that gGHRH is a stimulator of GH release from goldfish pituitary cells but also provides information on the kinetics of the GH response to this peptide. The response to gGHRH is biphasic and consists of an immediate increase in GH secretion to a peak magnitude within 5 min (the time resolution limit of the experimental protocol) and this is followed by a decrease in GH secretion upon continual exposure to gGHRH (Fig. 9.1A & 9.2A). When quantified, the net GH response to 100 nM gGHRH in experiments performed using pituitary cells from fish with regressed or regressing gonads was twice that of experiments performed using cells with gonads at early recrudescence (Fig. 9.1A & 9.2A; regressed: 306 ± 43% pretreatment; early recrudescence: 161 ± 26% pretreatment; $p = 0.012$, regressed vs. early recrudescence, unpaired *t*-test). These results indicate a potential seasonal reproductive difference in the effectiveness of gGHRH in this respect. The

neuroendocrine basis for these seasonal changes to gGHRH effectiveness is unknown but the effects of gonadal steroids may play a role. For example, in mice removal of the gonads impairs the maturation of GHRH neurons, specifically altering the developmental changes of action potential frequency and voltage-dependent potassium current [105]. In addition, castration of mice reduces the percentage of GHRH responsive cells in pituitaries, while ovariectomy leads to an increase in the percentage of GHRH-responsive cells; effects which can be reversed with treatment of either testosterone or estradiol, respectively [275] and support a steroid dependency and/or modulation of cells specifically responding to GHRH in the pituitary. In the goldfish there are also many seasonal differences in hormone responsiveness at the level of the pituitary related to seasonal changes in reproduction and growth [56, 315]. For example, DA is more effective in stimulating GH release from goldfish pituitaries collected from sexually regressed than in sexually matured fish [343]; whereas castration enhances the GH-release response to DA D1 stimulation [344]. On the other hand, castration reduces the GH response to sGnRH [344] while direct applications of estradiol enhances the ability of sGnRH to increase GH release from dispersed goldfish pituitary cells [28]. These observations, when taken together, are consistent with the possibility that gonadal steroids play a role in the modulation of the seasonal variations in GH-release responsiveness to GHRH treatment in the goldfish.

Our findings also demonstrate that $gGRLN_{19}$ and sGnRH are able to further stimulate GH release in the presence of gGHRH. These results are consistent with the known mechanisms of GHRH and GRLN actions on GH release in general, as well as the known signalling transduction coupling of sGnRH in goldfish somatotropes. GHRH has been shown to signal through PKA in mammals [126]. Likewise, various non-mammalian GHRHs, including gGHRH, increase intracellular cAMP levels in zebrafish GRHR receptor

expression systems [185], thus PKA likely mediates gGHRH-stimulated GH release in goldfish pituitary cells, although this has not been directly examined. On the other hand $gGRLN_{19}$ and sGnRH are known to signal GH release via the PKC pathway, independently of PKA (Chapter 5; [111]). Because of the use of separate signalling pathways, the ability of $gGRLN_{19}$ and sGnRH to increase GH release in the presence of gGHRH is not surprising.

However, results with gGHRH and $gGRLN_{19}$ are at odds with findings in mammalian models demonstrating synergistic or potentiating interactions between GHRH and GRLN on GH release [126, 301]. Work with goldfish pituitary cells also showed that the adenylate cyclase activator forskolin (i.e., PKA activation and presumably mimicking gGHRH signalling) potentiates $gGRLN_{19}$ stimulation of GH release (Chapter 5). Thus the inability of gGHRH to potentiate $gGRLN_{19}$ stimulation of GH release is surprising and suggests the existence of species differences in how GHRH and GRLN may interact in terms of GH secretion. Studies directly examining the mechanisms of gGHRH actions, as well as further understanding of $gGRLN_{19}$ signalling in goldfish pituitary somatotropes will be needed to understand why such differences in GHRH effects exist.

In addition to the effects on GH release, the current work demonstrates for the first time that gGHRH has an inhibitory effect on both $gGRLN_{19}$- and sGnRH-induced LH release, as well as basal LH release levels. The magnitude of the net gGHRH-induced changes in LH release also did not vary between data obtained in early recrudescence (-88 ± 37 %; Fig. 3) and gonadal regression (-55 ± 18 %; Fig. 4; $p = 0.439$ vs. early recrudescence, unpaired *t*-test). These observations suggest that gGHRH is a negative neuroendocrine modulator of stimulated and basal LH secretion. Since this is the first study to examine the direct in vitro effects of GHRH on stimulated LH response in any species, we cannot rule out the possibility that these inhibitory actions may be specific to gGHRH action in

the goldfish pituitary. However, while injection of GHRH alone has no effects on basal LH levels in dogs, co-injection of CRH, GHRH, TRH, and GnRH leads to a decrease in LH release response when compared to GnRH-induced LH release alone [79]. In addition, long-term exposure to GHRH reduces the number of LH-β mRNA positive cells in rat pituitary cultures [246]. Whether gGHRH affects LH-β mRNA expression in goldfish gonadotropes is unknown but since the present protocol only utilized a relatively short (20 min) gGHRH pretreatment, reduction of gene expression is unlikely to be the reason behind its inhibitory actions on sGnRH- and $gGRLN_{19}$-stimulated LH release. Nevertheless, the results in mammals are consistent with the idea that GHRH may be inhibitory to gonadotrope functions in a number of vertebrate species. Whether gGHRH acts directly on goldfish gonadotropes and how its inhibitory influence on LH secretion is manifested requires further studies, but the presence of GHRH receptors on a subset of rat gonadotropes supports the idea that GHRH directly affect gonadotrope functions [64, 65]. Since both $gGRLN_{19}$ and sGnRH use PKC and NO signalling to elicit LH secretion in goldfish (Chapters 5 and 7; [46, 111, 114]), whether gGHRH's inhibitory effect is restricted to neuroendocrine stimulators that utilize these transduction mechanisms is also another important question to be addressed.

This study represents the first time that the direct pituitary effects of GHRH on both GH and LH release and the interactions of GHRH with other known stimulatory neuroendocrine regulators have been investigated in any teleost species. Results support a role for gGHRH in both GH and LH regulation in the goldfish pituitary. Possible seasonal differences in the effectiveness of gGHRH in stimulating GH secretion and the ability of two stimulators of GH release ($gGRLN_{19}$ and sGnRH) to elicit increases in GH secretion in it presence are revealed. Importantly, gGHRH is shown to be an inhibitor of basal and stimulated LH release. This resembles the actions of another known

hypothalamic neuroendocrine factor that directly inhibits LH release, that of DA D2 mechanism which also affects both basal and stimulated LH secretion [46]. Taken together with the fact that gGHRH and gGHRH-receptor mRNAs are expressed in goldfish pituitary tissues [185], gGHRH is likely to be a physiological neuroendocrine factor for the regulation of GH and LH release in this system. Although elucidating the exact physiological role of gGHRH in the multifactorial neuroendocrine regulation of GH and LH release requires more thorough examinations in the future, some postulations can be made. Goldfish in the temperate climate is a seasonal breeder with spawning occurring in spring and the major period of somatic growth in the summer. With its increased effectiveness in eliciting GH secretion during the spring/summer transition and the ability to inhibit stimulated LH response, and especially of sGnRH, gGHRH may play an important role in the co-ordination of growth and reproduction. From an energy expenditure perspective, it would be beneficial for an organism to inhibit the stimulation of reproduction during times of rapid somatic growth and physical development. Regardless of whether this hypothesized role of gGHRH is true, it would be important in future studies to examine the intracellular signalling pathways involved in both gGHRH-induced GH release and it's inhibition of basal and stimulated LH release responses. Studies into possible seasonal differences in gGHRH interactions with $gGRLN_{19}$, sGnRH and other neuroendocrine regulators on LH and GH will also be important to fully understand the effects of gGHRH in growth and reproduction in this species.

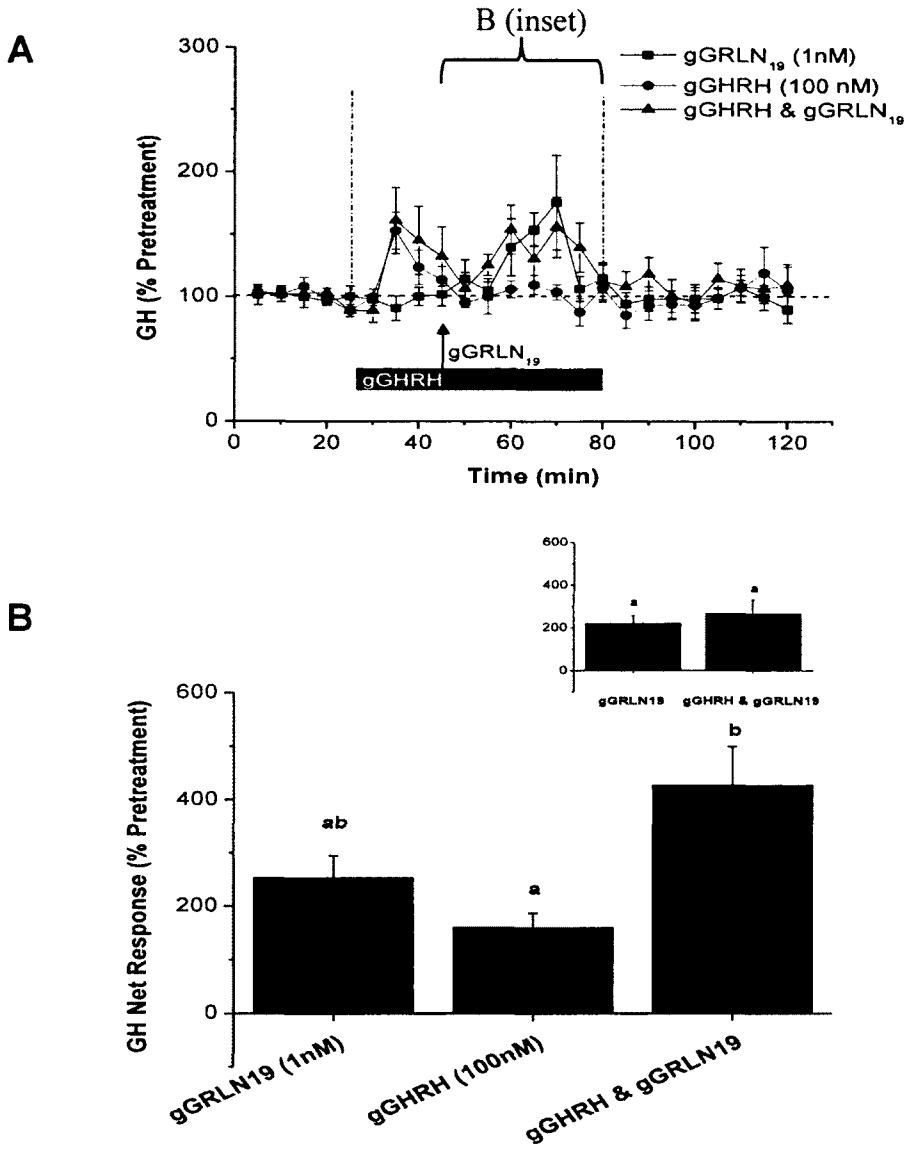

Fig. 9.1. $gGRLN_{19}$ stimulates GH release in the presence of gGHRH. gGHRH (black bar; 100 nM) was administered 20 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). GH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net GH responses were quantified for gGHRH alone, $gGRLN_{19}$ alone, and gGHRH with $gGRLN_{19}$ over the equivalent duration of gGHRH exposure (area between vertical dotted lines), as well as for $gGRLN_{19}$ in the absence or presence of gGHRH over the 35 min following $gGRLN_{19}$ exposure (indicated by the horizontal bracket). Pretreatment average (first 5 fractions) was 1.34 ± 0.11 ng/ml. Data are presented as mean ± SEM (4 separate experiments performed in December and January, n=8). Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test, main panels; unpaired *t*-test, inset).

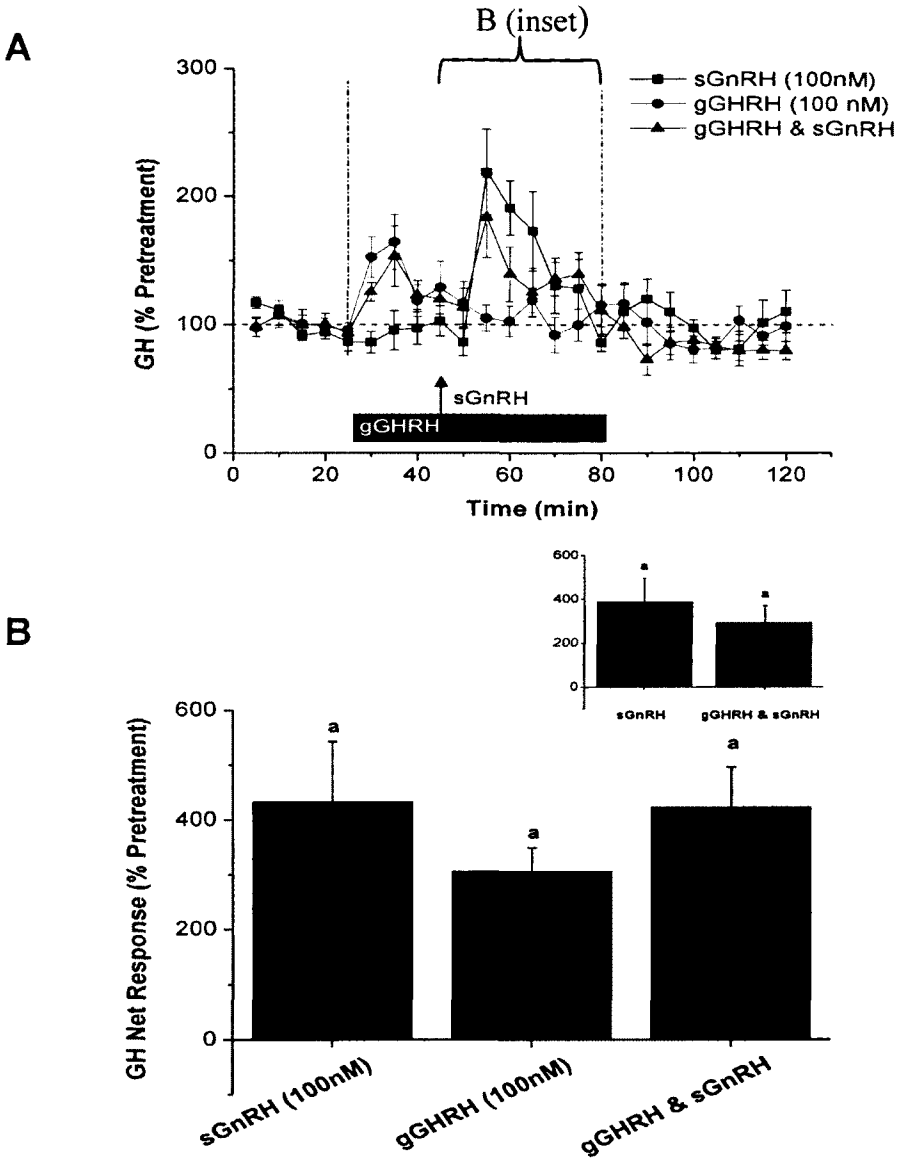

Fig. 9.2. sGnRH does not further stimulate GH release in the presence of gGHRH. gGHRH (black bar; 100 nM) was administered 20 min prior to a 5-min pulse of sGnRH (arrow; 100 nM). GH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net GH responses were quantified for sGnRH alone, gGHRH alone, and sGnRH with gGHRH over the equivalent duration of gGHRH exposure (area between vertical dotted lines), as well as for sGnRH in the absence or presence of gGHRH over the 35 min following sGnRH exposure (indicated by the horizontal bracket). Pretreatment average (first 5 fractions) was 7.64 ± 0.65 ng/ml. Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in May and June). Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test, main panels; unpaired *t*-test, inset).

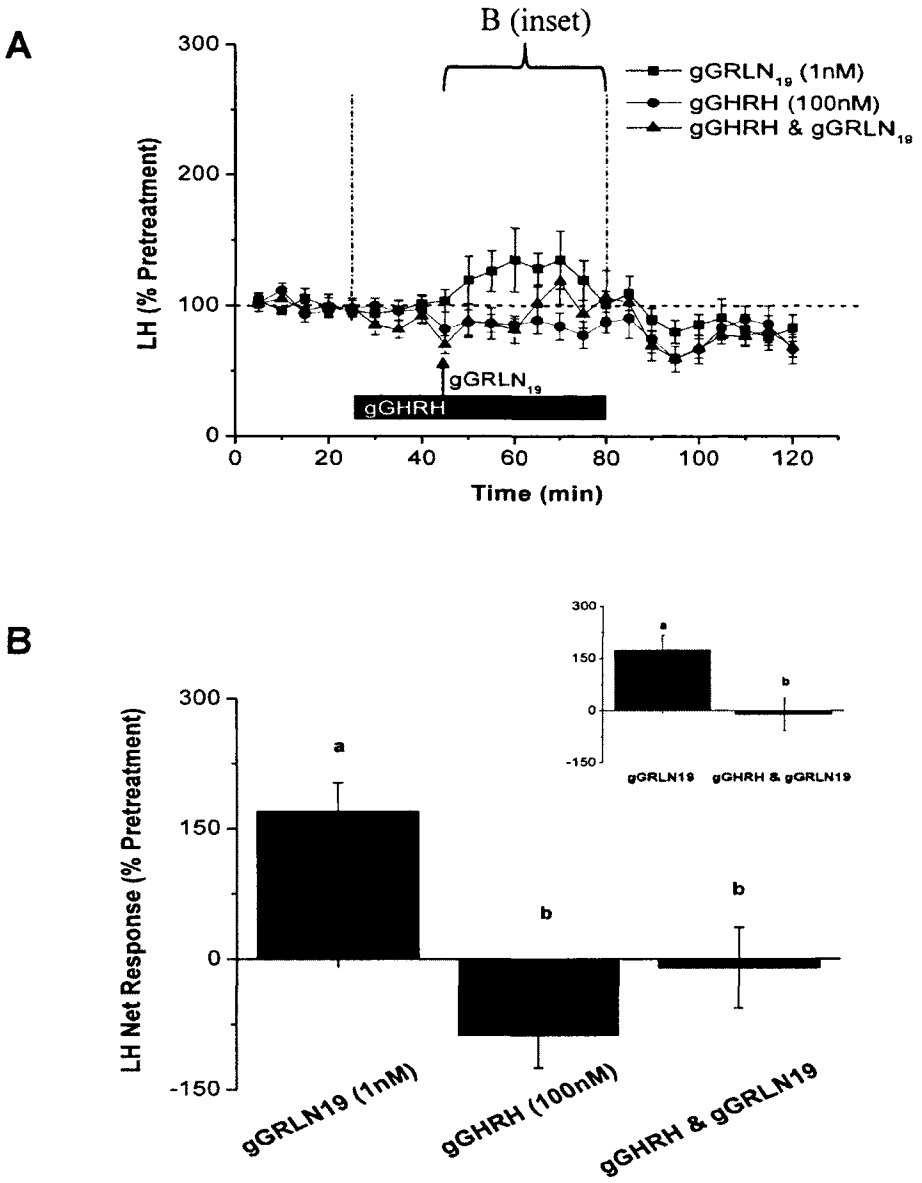

Fig. 9.3. $gGRLN_{19}$ fails to induce significant LH release in the presence of gGHRH. gGHRH (black bar; 100 nM) was administered 20 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM). LH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net LH responses were quantified for $gGRLN_{19}$ alone, gGHRH alone, and $gGRLN_{19}$ with gGHRH over the equivalent duration of gGHRH exposure (area between vertical dotted lines), as well as for $gGRLN_{19}$ in the absence or presence of gGHRH over the 35 min following $gGRLN_{19}$ exposure (indicated by the horizontal bracket). Pretreatment average (first 5 fractions) was 5.79 ± 0.52 ng/ml. Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in December and January). Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test, main panels; unpaired *t*-test, inset).

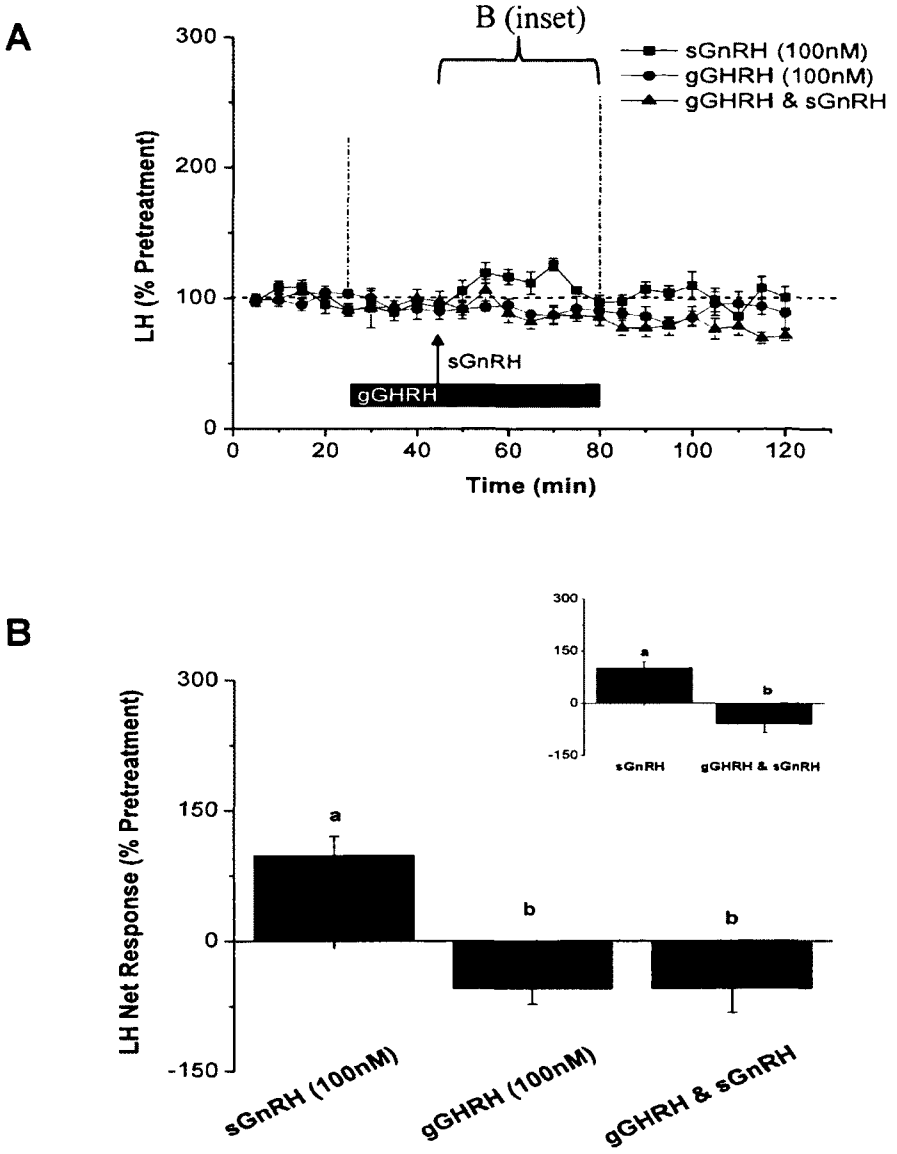

Fig. 9.4. sGnRH fails to induce a significant LH release response in the presence of gGHRH. gGHRH (black bar; 100 nM) was administered 20 min prior to a 5-min pulse of sGnRH (arrow; 100 nM). LH release profiles are shown in panel A while the quantified net responses are shown in panel B. Net LH responses were quantified for sGnRH alone, gGHRH alone, and gGHRH with sGnRH over the equivalent duration of gGHRH exposure (area between vertical dotted lines), as well as for sGnRH in the absence or presence of gGHRH over the 35 min following sGnRH exposure (indicated by the horizontal bracket). for statistical analysis. Pretreatment average (first 5 fractions) was 1.58 ± 0.10 ng/ml. Data are presented as mean ± SEM (n=8 from 4 separate experiments performed in May and June). Net response values statistically different from each other have different letters of the alphabet ($P<0.05$; ANOVA followed by Tukey test, main panels; unpaired *t*-test, inset).

Chapter 10 – Interactions between $gGRLN_{19}$, sGnRH, and cGnRH-II in the regulation of GH and LH release in goldfish pituitary cells

10.1. Introduction

As reviewed in Chapter 1, Sections 1.4.2 and 1.5.2, and references [41, 46], neuroendocrine regulation of GH and LH release in goldfish is multifactorial. Among the known neuroendocrine regulators, several stimulate both GH and LH secretion. These include sGnRH, cGnRH-II, PACAP and $gGRLN_{19}$, and individual goldfish somatotropes and gonadotropes can respond to all of these ligands in terms of increases in $[Ca^{2+}]_i$. Thus, knowing how each of these regulators exerts its effects at the cellular and intracellular level, as well as how they interact to affect GH and LH secretion at the level of pituitary cells, are essential for understanding the multifactorial neuroendocrine regulation of pituitary hormone secretion.

It has been established that sGnRH and cGnRH-II stimulatory actions on GH and LH secretion are mediated by overlapping, as well as ligand- and cell-type-specific, signalling cascades (reviewed in Chapter 1, Sections 1.4.2 & 1.5.2; [41, 46]). Briefly, sGnRH and cGnRH-II both increase GH and LH release from goldfish pituitary cells via LVSCC- and PKC-dependent pathways, but not PKA. However, differences in properties of the intracellular Ca^{2+} stores utilized by these two regulators exist in GH and LH cell types [41, 46]. In addition, only sGnRH-induced LH release involves AA [41, 46]. Furthermore, while iNOS/NO signalling is involved in both sGnRH and cGnRH-II actions on GH release [324, 325], NO only participates in sGnRH-induced, but not cGnRH-II-stimulated, LH release [212].

Results from Chapters 3 to 7 have also revealed that $gGRLN_{19}$ stimulates GH and LH secretion by direct actions on goldfish pituitary cells via common, as well as cell-type-specific, intracellular signalling pathways. While $gGRLN_{19}$-induced GH and LH release both involve increased Ca^{2+} entry through LVSCCs and the activation of PKC, $gGRLN_{19}$-elicited LH secretion has an additional PKA component (Chapters 3 to 5). Furthermore, although NOS/NO pathway is utilized by $gGRLN_{19}$ in both GH and LH release, the NOS isoform involved differs, with nNOS being critical in $gGRLN_{19}$-induced GH release (Chapter 6) and iNOS in $gGRLN_{19}$-induced LH release (Chapter 7).

It is evident that although signal transduction pathways mediating sGnRH, cGnRH-II and $gGRLN_{19}$ pituitary actions in goldfish share common elements, such as PKC and LVSCC, differences also exist. In goldfish pituitary cells, PKC and PKA form two largely distinct pathways leading to additive GH, as well as LH, release [53, 54, 57, 347]. For example, PACAP and GnRH produce additive GH and LH release responses due to their differential use of PKA and PKC signalling, respectively [41, 46, 53, 54, 347]. Likewise, results from Chapter 8 demonstrate that PACAP enhances $gGRLN_{19}$-induced GH release, which may also reflect their differential use of intracellular signalling. When these observations are viewed together, the presence of non-overlapping intracellular signalling components suggests that $gGRLN_{19}$ may interact with GnRH in the control of goldfish pituitary hormone secretion.

To further understand $gGRLN_{19}$ action in the multifactorial neuroendocrine control of GH and LH release in goldfish, I examined the combined actions of $gGRLN_{19}$ and either sGnRH or cGnRH-II on GH and LH release from primary cultures of dispersed goldfish pituitary cells in column perifusion experiments. Two treatment protocols, i.e., a 5-min $gGRLN_{19}$ pulse applied in the middle of a longer GnRH treatment and the reverse application paradigm, were used to more thoroughly investigate their possible interactions.

10.2. Results

10.2.1. Effects of co-treatment of $gGRLN_{19}$ and cGnRH-II on GH and LH release

I began by examining the interactions between $gGRLN_{19}$ and cGnRH-II using maximally stimulating doses of 100 nM for cGnRH-II [37] and 1 nM for $gGRLN_{19}$ [110, 115, 320]. The first experiment employed a 5-min pulse of $gGRLN_{19}$ administered 20 min into a 50 min cGnRH-II treatment ("forward paradigm"; Fig. 10.1 A&B). In contrast to application of $gGRLN_{19}$ alone where a rapid elevation in GH release ("peak response") can be seen within the 5-10 min following $gGRLN_{19}$ treatment (closed squares Fig. 10.1A), no such increase was seen when $gGRLN_{19}$ was applied together with cGnRH-II (closed triangles Fig. 10.1A). The net GH responses to $gGRLN_{19}$ alone, cGnRH-II alone, and the combined $gGRLN_{19}$ and cGnRH-II treatment were all of similar magnitude (~300 - 400% pretreatment) and not significantly different from one another when quantified over the equivalent time duration of the 50 min cGnRH-II application. To examine the possible influence of treatment order, a "reverse paradigm" was also employed in which a 5-min pulse of cGnRH-II was applied 20 min into a 50 min $gGRLN_{19}$ treatment (Fig. 10.1 C&D). Interestingly, the magnitude of net increase in the "peak response" to cGnRH-II observed within the 5-10 min following cGnRH-II exposure was almost halved when cGnRH-II was applied in the presence of $gGRLN_{19}$ (~increase of 150% pretreatment vs. 80% pretreatment; closed squares vs closed triangles, Fig. 10.1C). The net GH release response to the combined cGnRH-II and $gGRLN_{19}$ treatment (~700% pretreatment) was somewhat larger than those in response to either cGnRH-II or $gGRLN_{19}$ alone (~400-500 % pretreatment); but no significant difference was observed between these values when quantified over the equivalent time duration of the 50 min $gGRLN_{19}$ application.

Samples from these experiments were also analyzed for LH to examine the interactions between $gGRLN_{19}$ and cGnRH-II and on LH release from goldfish pituitary cells. As expected, $gGRLN_{19}$ and cGnRH-II each increased LH secretion (Fig. 10.2; [46]). However, the magnitude of the increase in "peak response" to $gGRLN_{19}$ and cGnRH-II were greatly attenuated (or abolished) when each of these neuroendocrine regulators were applied in the presence of the other (Fig. 10.2). Furthermore, in both experimental paradigms, the combined neuropeptide applications did not produce net LH release responses that were significantly different to those of their respective $gGRLN_{19}$ alone and cGnRH-II alone controls when quantified over the equivalent duration of either cGnRH-II in the "forward" and $gGRLN_{19}$ in the "reverse paradigm", respectively (Fig. 10.2; net GH responses ~40-130% and ~60-130% pretreatment, forward and reverse paradigm, respectively). Net LH responses to cGnRH-II alone and $gGRLN_{19}$ treatments were also not significantly different from one another in both experimental treatment paradigms (Fig. 10.2).

10.2.2. Effects of co-treatments of $gGRLN_{19}$ and sGnRH on GH and LH release

I next looked at $gGRLN_{19}$ and sGnRH co-treatment. Maximally stimulating doses of 1 nM for $gGRLN_{19}$ [110, 115, 320] and 100 nM for sGnRH [37] were used. We employed a 10-min pretreatment protocol in these experiments, where a 5-min $gGRLN_{19}$ pulse was applied 10 min into a 20 min sGnRH treatment ("forward paradigm") or a 5-min sGnRH pulse was applied 10 min into a 35 min $gGRLN_{19}$ treatment ("reverse paradigm"). Past experiments indicated that both a 20-min and 10-min pretreatment time would allow for the observation of additive hormone release responses to $gGRLN_{19}$, cGnRH-II, or sGnRH in perifusion [110, 115, 212].

As expected, $gGRLN_{19}$ and sGnRH were both able to stimulate GH (Fig. 10.3; [41, 110]) and LH release (Fig. 10.4; [46, 115]) when applied alone.

Distinct "peak" GH and LH release responses to $gGRLN_{19}$ were not easily discernible when $gGRLN_{19}$ was applied in the presence of sGnRH (Fig. 10.3A & 10.4A); likewise, a distinct "peak" GH response to sGnRH was not apparent when it was applied in the presence of $gGRLN_{19}$ (Fig. 10.3C). In addition, the LH values in fractions collected 15-35 min following sGnRH application in the presence of $gGRLN_{19}$ were lower than those in the absence of $gGRLN_{19}$ (Fig. 10.4C). More importantly, net GH release responses to combined treatment of $gGRLN_{19}$ and sGnRH were not significantly different from responses to the corresponding $gGRLN_{19}$ alone and sGnRH alone treatments, in either the "forward" or "reverse" paradigm (Fig. 10.3 B & D; net GH responses: ~300-550% pretreatment, "forward paradigm" experiments; ~450-500% pretreatment, "reverse paradigm" experiments). Likewise, the net LH responses to combined treatment of $gGRLN_{19}$ and sGnRH were not significantly different from those to either of the peptides alone in both experimental paradigms (Fig. 10.4 B & D; net LH responses: ~325-450% pretreatment, "forward paradigm" experiments; ~50-110% pretreatment, "reverse paradigm" experiments).

10.3. Discussion

Interactions of neuroendocrine actions at the level of the pituitary play an important role in the modulation of GRLN action. For example, GHRH potentiates GRLN action on GH release in mammals [119, 169]. However, this type of enhancement of GH release was not seen in studies with goldfish using gGHRH and $gGRLN_{19}$ (Chapter 9). On the other hand, pretreatment with PACAP enhanced $gGRLN_{19}$-induced GH release from goldfish pituitary cells in perifusion (Chapter 8). These observations suggest that differences between mammalian models and other species exist in terms of how GRLN actions may be modulated by neuroendocrine factors. Results from the present thesis chapter add to our

understanding of whether two other major neuroendocrine factors known to regulate GH and LH release in goldfish, sGnRH and cGnRH-II, may interact with $gGRLN_{19}$ in the control of goldfish GH and LH secretion.

sGnRH and cGnRH-II signalling transduction pathways in goldfish gonadotropes differ from those of $gGRLN_{19}$. These differences include the involvement of cAMP/PKA in $gGRLN_{19}$, but not sGnRH and cGnRH-II, action on LH release and the lack of NOS/NO involvement in cGnRH-II-induced LH secretion relative to that in response to $gGRLN_{19}$ [112, 114, 212, 325]. In addition, the pharmacology properties of the NOS involved in sGnRH and cGnRH-II action on GH release also differ from that mediating $gGRLN_{19}$ stimulation of GH secretion, suggesting the use of an unidentical hormone-specific suite of NOS isoforms in somatotropes [112, 114, 212, 325]. PKA activators (e.g., cAMP analogues) and PKA-utilizing ligands (e.g., PACAP) produce LH responses that are addition to those to sGnRH and cGnRH-II in previous studies [46, 54, 57], and one would expect a similar interaction with $gGRLN_{19}$ and GnRH co-treatment. Furthermore, human GRLN has been shown to enhance the in vitro pituitary cell LH release responses to a sGnRH superactive analogue in common carp and to mGnRH in rats [83, 293]. Surprising, LH responses to the combined treatment of $gGRLN_{19}$ and either sGnRH or cGnRH-II were not significantly different from either $gGRLN_{19}$ alone or GnRH alone treatments regardless of the sequence of neuropeptide application in our perifusion studies. It should be noted that the experiments with carp pituitary cells [18] were performed over a 10 and 24 h static incubation and thus results may not be directly comparable. On the other hand, the two endogeneous goldfish GnRHs and $gGRLN_{19}$ also share major intracellular signalling components in the form of LVSCC and PKC in goldfish gonadotropes [46, 111]. Taken together, these results suggest that PKC/Ca^{2+} intracellular signalling likely play a more important role in $gGRLN_{19}$ stimulation of LH release relative to

PKA, and perhaps NOS/NO, in the goldfish. Similarly, the inability of $gGRLN_{19}$ and either of the two GnRHs to produce additive GH release responses are consistent with the idea that the hormone-releasing actions of these neuropeptides converge at major sites involving and/or downstream of PKC, LVSCC (Ca^{2+}) and NOS/NO, known common signalling pathways involved in GH release induced by these neuroendocrine factors [111, 112].

Goldfish pituitary tissue expresses gGHSR-1a mRNA (Chapter 3; [115]) and these receptors are thought to mediate $gGRLN_{19}$ actions on somatotropes and gonadotropes (Chapters 3 & 4). Although the presence of gGHSR-1a on goldfish somatotropes and gonadotropes have not been directly determined, these two pituitary cell-types expresses GnRH-Rs on their cell surfaces [41, 46, 68]. Whether gGHSR-1a and GnRH-R mRNA and/or protein are co-expressed on goldfish somatotropes and gonadotropes has not been directly examined; however, results from Chapters 3 & 4 demonstrating that Ca^{2+} signals to $gGRLN_{19}$ and sGnRH can be generated in the same identified somatotrope and identified gonadotrope support the hypothesis that gGHSR-1a and GnRH-Rs are present on the same goldfish somatotropes and gonadotropes. In addition, results from the present thesis chapter are consistent with the idea that $gGRLN_{19}$ and GnRH share common readily releasable GH and LH pools and by extension, the presence of both gGHSR-1a and GnRH receptors on the same goldfish somatotropes and gonadotropes.

Mobilization of Ca^{2+} from intracellular and extracellular sources is an important element in signal transduction secretion coupling in many systems including goldfish somatotropes and gonadotropes [41, 46]. Although $gGRLN_{19}$ stimulation of GH and LH release are accompanied by increases in $[Ca^{2+}]_i$ levels in goldfish somatotropes and gonadotropes in a LVSCC- and PKC-dependent fashion [110, 111, 115], whether mobilization of Ca^{2+} from intracellular Ca^{2+} stores also participates in $gGRLN_{19}$ actions on GH and LH secretion is not known

and needs to be determined. Regardless of the non-additive nature of the acute $gGRLN_{19}$-elicited and GnRH-induced GH and LH release, it would also be interesting and important in the future to investigate whether the Ca^{2+} signals induced by $gGRLN_{19}$ in goldfish somatotropes and gonadotropes are affected by GnRH treatment (and vice versa). Results from such experiments may reveal similarities and/or differences in the use of Ca^{2+} pools, which can form the basis for further studies examining how these pools may be involved in the differential regulation of GH and LH relese by $gGRLN_{19}$. In addition, studies on the longer-term interactions between GnRH and $gGRLN_{19}$ on other aspects of gonadotropes and somatotropes functions, such as hormone mRNA expression and protein synthesis may also be important.

In summary, this is the first study on the interactions between GRLN and GnRH action on pituitary GH secretion at the level of pituitary cells in any vertebrate species and one of a few studies investigating these direct pituitary cell level interactions on LH release. Given that $gGRLN_{19}$ likely signals through gGHS-R1a [115], and that sGnRH and cGnRH-II utilize GnRH-Rs on goldfish pituitary cells to induce hormone release [47, 68, 361], it is unlikely that these endogenous regulators compete for ligand binding sites on similar receptors. Results are consistent with current knowledge that LVSCC and PKC are critical and central intracellular signalling elements in both $gGRLN_{19}$-induced GH and LH release from goldfish pituitary cells, and that these pathways converges with similar components in sGnRH and cGnRH-II action on GH and LH secretion. These findings add to the overall understanding of $gGRLN_{19}$ action and the neuroendocrine regulation of growth and reproduction at the level of the pituitary and provide a basis for further work on the complex interaction between $gGRLN_{19}$, the two native GnRH forms, as well as other neuroendocrine factors in the control of pituitary cell functions.

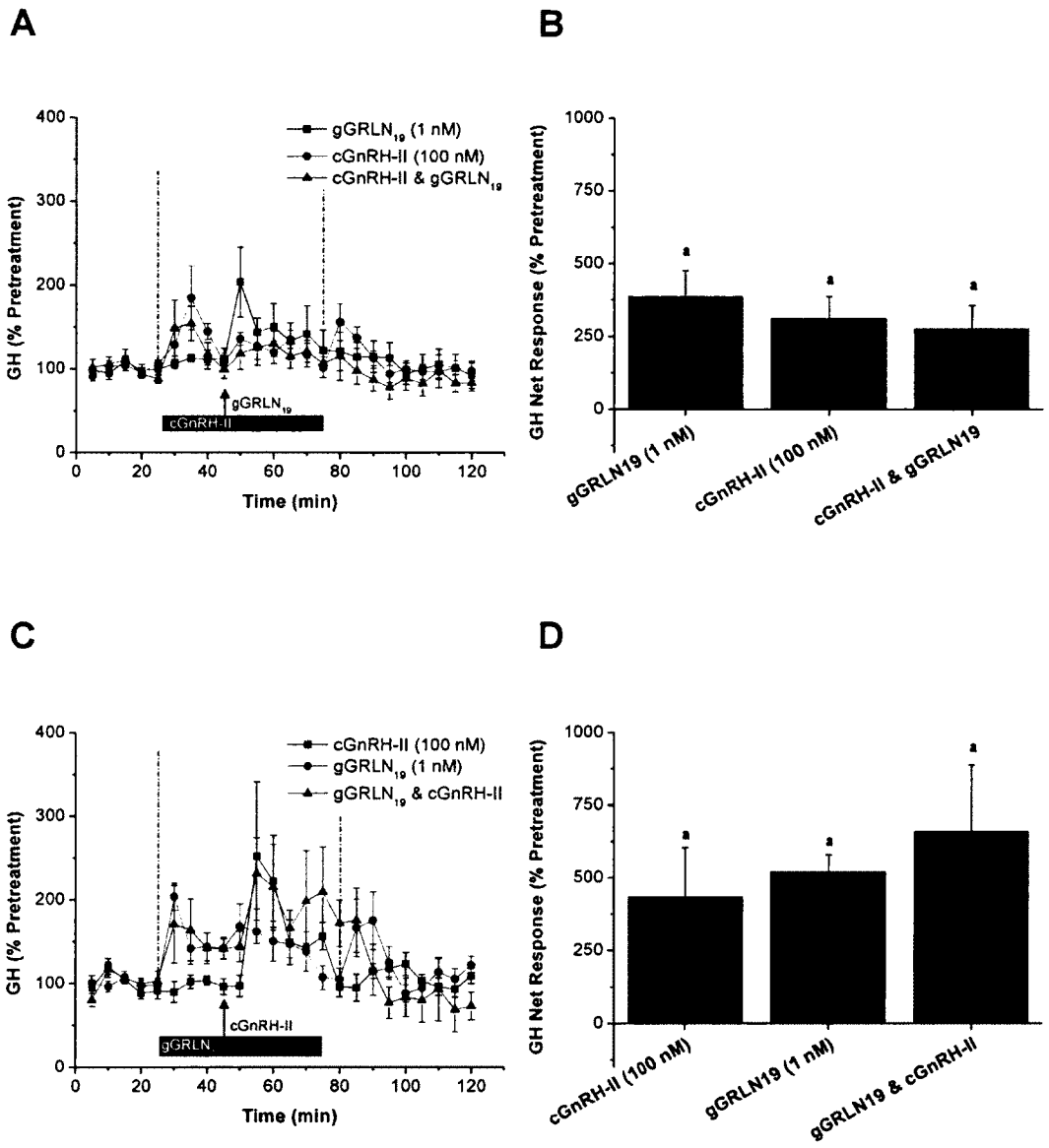

Fig. 10.1. cGnRH-II and $gGRLN_{19}$ do not induce additive GH release responses from goldfish pituitary cells in either "forward" (A & B, a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM) applied 20 min into a 50 min cGnRH-II (black bar; 100 nM) treatment) or "reverse" (C & D, a 5-min pulse of cGnRH-II (arrow; 100 nM) applied 20 min into a 50 min $gGRLN_{19}$ (black bar; 1 nM) treatment) paradigms. (B&D) Net GH responses were quantified (area between vertical dotted lines) for cGnRH-II alone, $gGRLN_{19}$ alone, and cGnRH-II with $gGRLN_{19}$ for statistical analysis. Pretreatment average (first 5 fractions) was 0.55 ± 0.03 ng/ml and 10.3 ± 0.75 ng/ml for "forward" and "reverse" paradigms, respectively. Data are presented as mean ± SEM (4 separate experiments were performed in November and between May-June, for "forward" and "reverse" paradigms respectively, n=8). Net response values that are not statistically different from each other share the same letter of the alphabet ($P<0.05$; ANOVA).

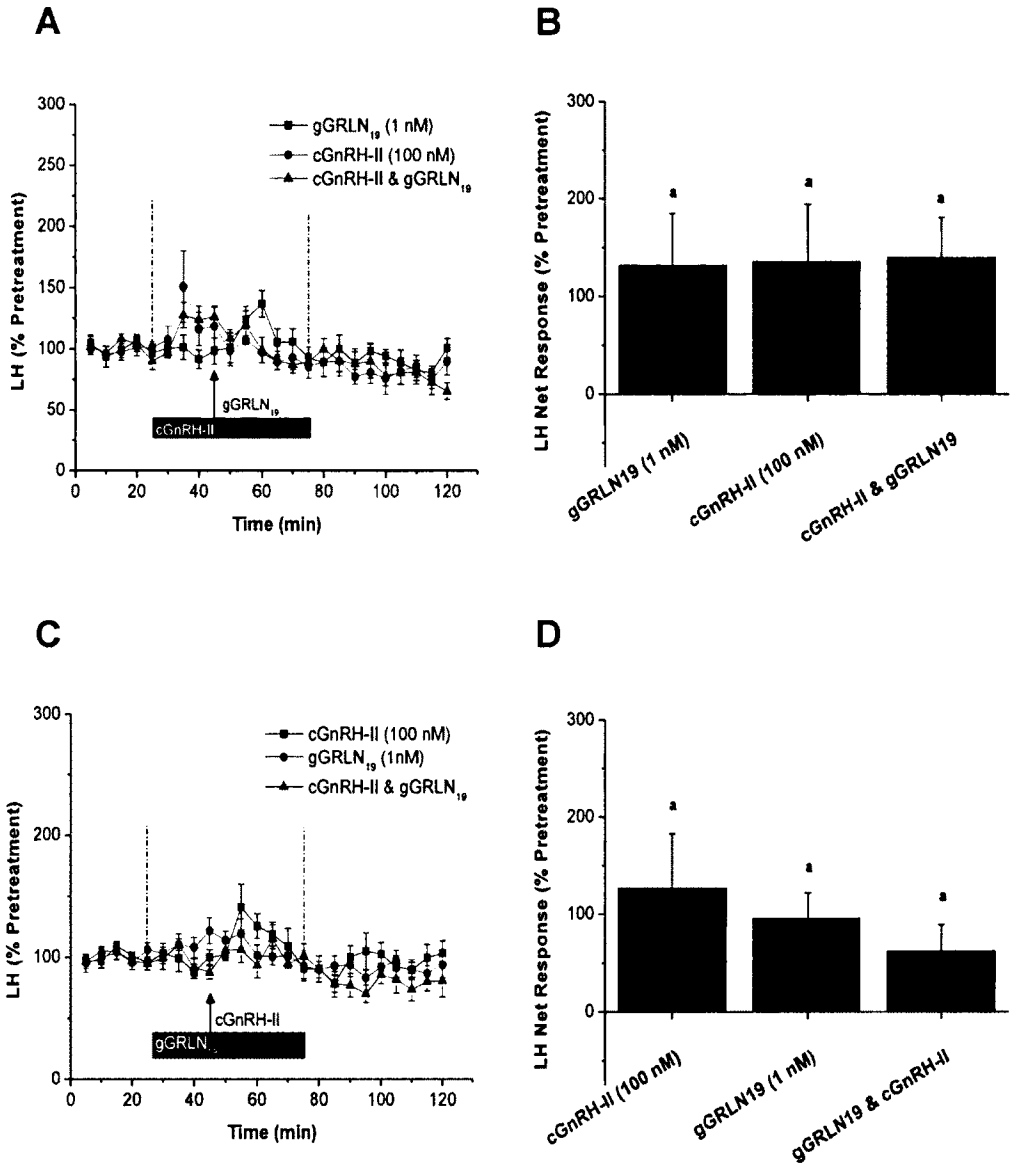

Fig. 10.2. cGnRH-II and $gGRLN_{19}$ do not induce additive LH release responses from goldfish pituitary cells in either "forward" (A & B, a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM) applied 20 min into a 50 min cGnRH-II (black bar; 100 nM) treatment) or "reverse" (C & D, a 5-min pulse of cGnRH-II (arrow; 100 nM) applied 20 min into a 50 min $gGRLN_{19}$ (black bar; 1 nM) treatment) paradigms. (B&D) Net LH responses were quantified (area between vertical dotted lines) for cGnRH-II alone, $gGRLN_{19}$ alone, and cGnRH-II with $gGRLN_{19}$ for statistical analysis. Pretreatment average (first 5 fractions) was 5.63 ± 0.50 ng/ml and 1.92 ± 0.06 ng/ml for "forward" and "reverse" paradigms, respectively. Data are presented as mean ± SEM (4 separate experiments were performed in November and between May-June, for "forward" and "reverse" paradigms respectively, n=8). Net response values that are not statistically different from each other share the same letter of the alphabet ($P<0.05$; ANOVA).

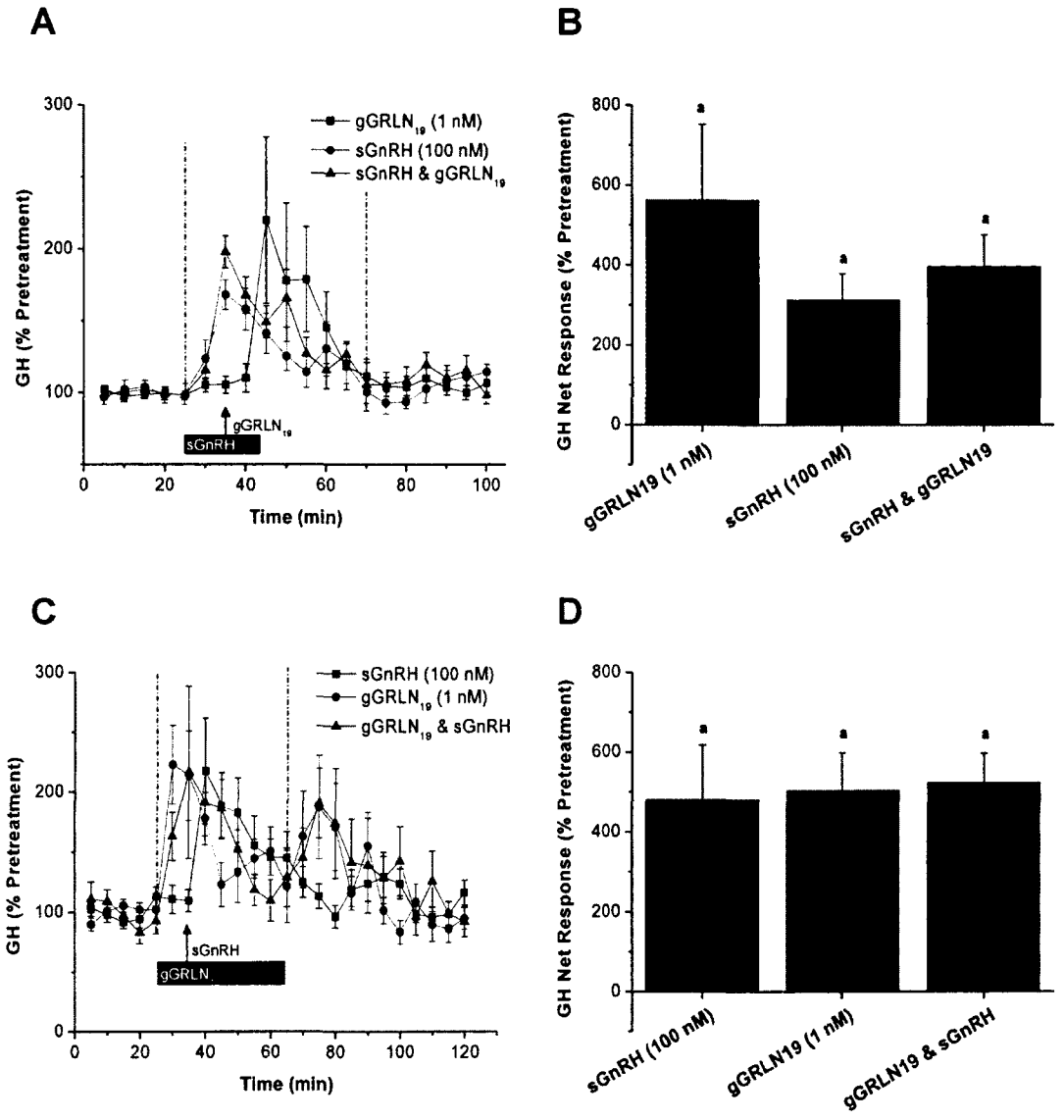

Fig. 10.3. sGnRH and $gGRLN_{19}$ do not induce additive GH release responses from goldfish pituitary cells in either "forward" (A & B, a 20 min sGnRH treatment (black bar; 100 nM) administered 10 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM)) or "reverse" (C & D, a 40 min $gGRLN_{19}$ treatment (black bar; 1 nM) administered 10 min prior to a 5-min pulse of sGnRH (arrow; 100 nM)) paradigms. (B&D) Net GH responses were quantified (area between vertical dotted lines) for sGnRH alone, $gGRLN_{19}$ alone, and sGnRH with $gGRLN_{19}$ for statistical analysis. Pretreatment average (first 5 fractions) was 10.59 ± 0.80 ng/ml and 3.63 ± 0.29 ng/ml for "forward" and "reverse" paradigms, respectively. Data are presented as mean ± SEM (4 separate experiments were performed in between Feb-Mar and Jan-Feb, for "forward" and "reverse" paradigms respectively, n=8). Net response values that are not statistically different from each other share the same letter of the alphabet ($P<0.05$; ANOVA).

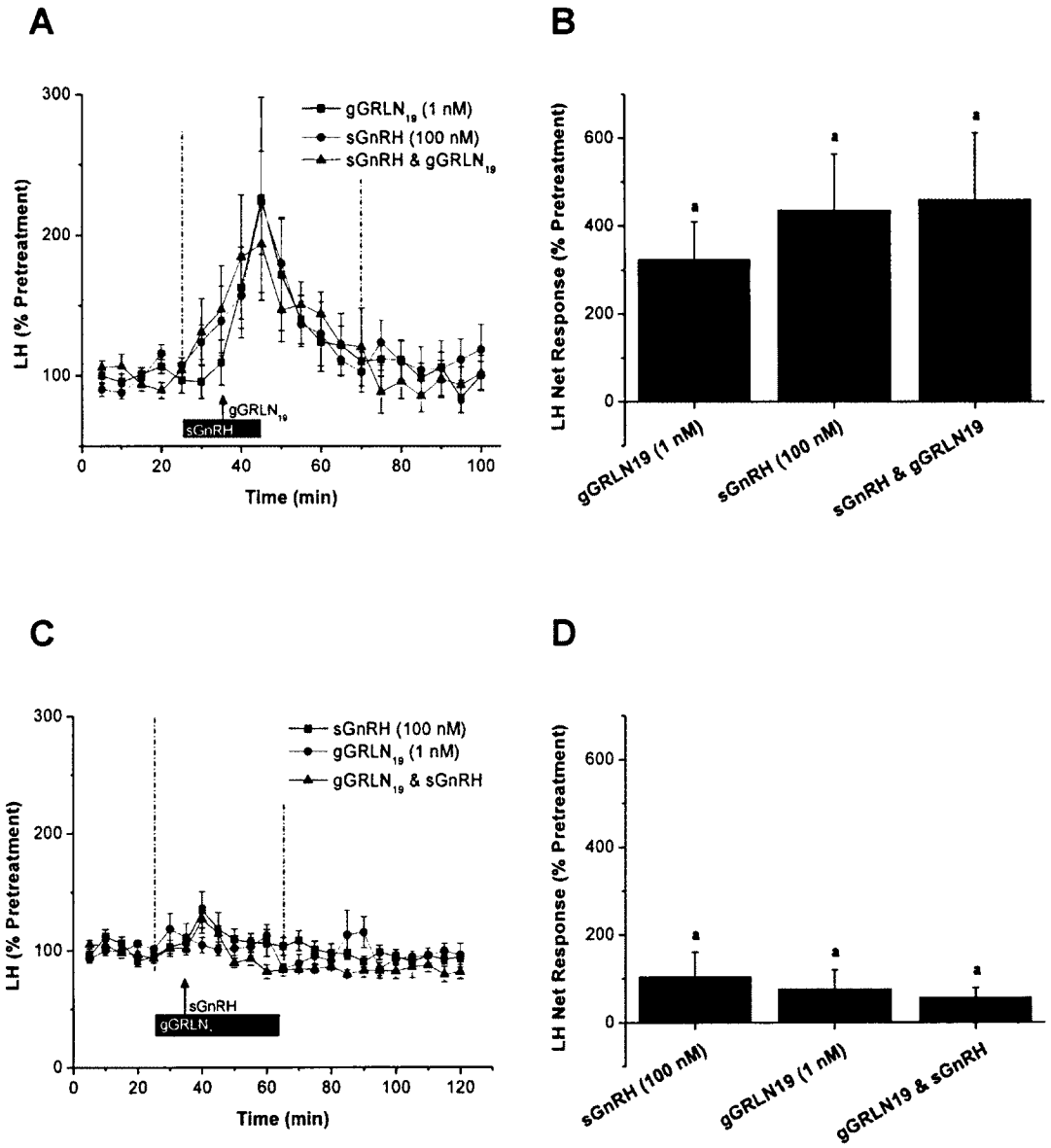

Fig. 10.4. sGnRH and $gGRLN_{19}$ do not induce additive LH release responses from goldfish pituitary cells in either "forward" (A & B, a 20 min sGnRH treatment (black bar; 100 nM) administered 10 min prior to a 5-min pulse of $gGRLN_{19}$ (arrow; 1 nM)) or "reverse" (C & D, a 40 min $gGRLN_{19}$ treatment (black bar; 1 nM) administered 10 min prior to a 5-min pulse of sGnRH (arrow; 100 nM)) paradigms. (B&D) Net LH responses were quantified (area between vertical dotted lines) for sGnRH alone, $gGRLN_{19}$ alone, and sGnRH with $gGRLN_{19}$ for statistical analysis. Pretreatment average (first 5 fractions) was 3.41 ± 0.22 ng/ml and 2.92 ± 0.17 ng/ml for "forward" and "reverse" paradigms, respectively. Data are presented as mean ± SEM (4 separate experiments were performed in between Feb-Mar and Jan-Feb, for "forward" and "reverse" paradigms respectively, n=8). Net response values that are not statistically different from each other share the same letter of the alphabet ($P<0.05$; ANOVA).

Chapter 11 – General Discussion

Co-ordinated regulation of pituitary GH and LH release is critical to many physiological processes in vertebrate species, including growth and development. The discovery of the orexigenic peptide GRLN as an endogenous ligand for the GHS-R, and as a potent inducer of GH release and regulator of LH release, has spurred many studies into these and other functional roles of GRLN in the overall physiology of animals; however, its effect on LH secretion is controversial in mammals (Chapter 1). As outlined in Chapter 1, regulation of GH and LH release in vertebrates involves the coordination of multiple stimulatory and inhibitory signals and thus requires complex feedback and regulatory signalling mechanisms which must be coordinated for different hormones and under different environmental and physiological circumstances. Using goldfish pituitary cells, this thesis sets out to expand upon our understanding of GRLN's ability to regulate GH and LH release activity, its intracellular signalling processes, and its interactions with several known neuroendocrine regulators of GH and LH release. Results not only support the hypothesis that LVSCCs, increases in $[Ca^{2+}]_i$, PKC, PKA, and NO are important pathways in $gGRLN_{19}$'s direct action on pituitary hormone release but also reveal gonadotrope- and somatotrope-specific differences in the involvement of PKA and NOS isoforms (Chapters 3-7). These results represent the first time that the intracellular signalling pathways involved in GRLN action on GH and LH release have been examined and elucidated in any teleost species. Observations from combination treatments with other neuroendocrine regulators suggest that interactions between $gGRLN_{19}$ and GnRH, DA and PACAP on GH and LH release are complex and reveal novel inhibitory effects of GHRH on pituitary basal and stimulated LH secretion, as well as DA on $gGRLN_{19}$ stimulation of LH release (Chapters 8-10). These findings, when taken together with results from gGHS-R1 mRNA expression studies (Chapter 3), add

to our overall comprehension of the neuroendocrine regulation of GH and LH release, and by extension growth and development in fish and other vertebrates, and in particular the possible role of GRLN in these processes.

11.1. Expression of gGRLN and the gGHS-R

As outlined in Chapter 1, GRLN has been shown to act directly via the GHS-R to signal the release of GH from pituitary cells in mammals. The findings in Chapters 3 and 4, when taken together with previous work with GRLN in the goldfish [322], support the idea that $gGRLN_{19}$ directly signals via the gGHS-Rs on goldfish somatotropes and gonadotropes to induce GH and LH release, respectively. Complementing the results on pituitary gGHS-R1 mRNA expression (Fig. 3.2), responses to $gGRLN_{19}$ in Ca^{2+}-imaging studies, as seen in Chapters 3 and 4, are very rapid and virtually instantaneous, taking into account the dead-volume of the system. Furthermore, given that this Ca^{2+}-imaging protocol involves the use of continually replaced bath medium, paracrine effects have also been minimized, further supporting direct action in both somatotropes and gonadotropes within the goldfish pituitary. In addition, preliminary Western blot studies with a mammalian antibody against rat GHS-R1 indicate that gGHS-R1 proteins are expressed in goldfish pituitary cells (Appendix Fig. A.1). Although the cellular localization of gGHS-R1 protein still remains to be confirmed, these results fit in with the concept of direct $gGRLN_{19}$ pituitary cell actions via gGHS-R1 in goldfish, as in other animal models (reviewed in [172, 230]).

It is interesting to note, however, that a previous study in fish with a GHS-R antagonist inhibited $gGRLN_{19}$-induced LH, but not GH, release in static incubation experiments, thus supporting direct action on the GHS-R in gonadotropes but not in somatotropes [322]. Although there are a few possible

explanations for this discrepancy, the most likely is protocol choice. In the current, and in comparable mammalian studies, protocols chosen allow for real-time examination of changes in cellular responses over short periods of time (5 min for perifusion studies and 15 sec for Ca^{2+}-imaging studies in the current work). On the other hand, static incubations utilized in previous goldfish experiments used exposure times of 2 h, looking at cumulative hormone levels [322]. The current work also demonstrated that multiple challenges of GRLN resulted in rapid desensitization of both LH and GH release responses (Chapters 3 & 4, Figures 3.4 and 4.1). Thus, comparisons between the two protocols are quite difficult given the differences in how data were collected and examined. Likely, differences in the rates of desensitization and recovery from desensitization, as well as both paracrine and autocrine effects might have played a role in these differences. Indeed, the presence of both GRLN and its receptor has been demonstrated in multiple mammalian tissues (Chapter 1), including human myometrium [238] and cancer [92, 201] tissue. In addition, both gGRLN and gGHS-R mRNAs have been shown to be expressed in the goldfish pituitary (Chapter 3), adding support to the possible involvement of paracrine and autocrine effects of $gGRLN_{19}$ in long-term incubation studies.

The expression patterns of both gGRLN and the gGHS-R observed in this thesis (Chapter 3) are generally consistent with those of other species, with high levels of both the receptor and GRLN found in brain and intestine regions (see Chapter 1). This expression pattern makes sense with regards to GRLN's functions as an orexigenic peptide and as a potent stimulator of GH release. Expression of both the receptor and GRLN in heart tissue also fits with current evidence supporting GRLN as a modulator of cardiac function (see Chapter 1), while expression in the gonads fits with recent work demonstrating that GRLN has wide ranging effects on the reproductive axis, from alteration of LH and FSH secretion to direct action within ovaries [230]. It is interesting that GRLN mRNA

is up-regulated in zebrafish embryo between 24 hpf and 48 hpf. Current studies in humans, focussed on changes in GRLN during development, demonstrate that the highest levels of GRLN are during early post-natal life, coinciding with the onset of growth hormone effects on somatic growth [294]. In the zebrafish, 24-48 hpf coincides with significant somatic growth and neuromuscular development as there is continued rapid lengthening of the body, development of the heartbeat, early touch reflex, early motility, rudimentary pectoral fins, and pigmentation of the retina, body stripes, and tail [161]. Thus, the up regulation of GRLN message at this time is likely related to the development of these processes and, given its established role in cardiac function and GH release [171, 172], it is highly likely that GRLN is critically involved in the rapid body lengthening and development of the heart.

That GRLN mRNA is present in the pituitary supports its action on pituitary cells and, given that both GRLN and its receptor are found in this tissue, it suggests that autocrine or paracrine effects may also be present, although this would need to be confirmed through future work (also see discussion above). GRLN, when modified by the GOAT enzyme, has also been shown to cross the blood brain barrier (BBB) [11], and in addition to its presence in the hypothalamus and pituitary, it is also produced in the fundus of the stomach [171], suggesting that there are multiple sources of this hormone acting at the level of the pituitary. How these different sources of GRLN affect GH and LH release is currently unknown, however a recent study examining the transport of human GRLN across the BBB in mice has found that the amount of GRLN crossing the BBB changes in response to energy-related factors [11]. For example, obesity in older mice leads to decreases in GRLN transport across the BBB while fasting and increased triglyceride serum levels promote its transport [11], suggesting that the energy status changes lead to changes in the amount of

GRLN available in the brain and, as a result, changes in hormone release at the level of the pituitary may be directly related to changes in energy status.

11.2. $gGRLN_{19}$-induced GH and LH release

In agreement with mammalian findings, outlined in Section 1.3, work in this thesis provides strong support for gGRLN as a reliable stimulator of GH release in the goldfish pituitary, and this effect can be demonstrated in all experiments performed regardless of the time of the year in which the cell preparations were made (Fig. 11.1A). Given that GRLN is able to induce GH release in all vertebrates tested to date [152, 171], it appears that GRLN's GH release ability is well conserved across all vertebrate species. Comparison of the response to net GH responses to 5-min applications of 1 nM $gGRLN_{19}$ across all the experiments performed in this thesis revealed that the magnitude of the GH response with pituitary cells obtained from fish in July and August was about double those observed with cells prepared from fish in the fall to winter months (September to February) although no significant differences can be demonstrated. Interestingly, although maximal body growth in goldfish in the temperate zone occurs in the summer, serum GH levels begin to wane by July and August from their summer maximum and the GH-inducing abilities of PACAP, DA, GnRH, TRH, and NPY are low [56]. In contrast, increased IGF-1 levels are observed in this time of year [56]. Perhaps the ability of $gGRLN_{19}$ to enhance GH release at this time of the year, coupled with its ability to affect feeding and metabolism, plays a role in maintaining serum GH at levels still adequate for the stimulation of IGF release and continual growth in the late summer period. This idea is supported by recent work in Arctic charr demonstrating that prepro-GRLN mRNA levels rise during July, peak in Aug, and that maximal growth rates occur during this time of year [90]. On the other hand, since acylation of GRLN is

critical for its GH-inducing activity (see Chapter 1), it is important to follow these studies up with the examination of serum acylated GRLN levels throughout the year (see future directions, Section 11.5.3).

GRLN as a regulator of LH release has also been established in mammalian models, however whether it acts as a stimulator or inhibitor remains controversial (Section 1.5). The results in this thesis work support GRLN as a direct stimulator of LH release by action at the level of the pituitary cells (also see Section 11.1 above). In male rats, however, GRLN has been shown to reduce pulse frequency of GnRH [184]. GRLN treatment of hypothalamus fragments taken from female rats also demonstrate reduced GnRH secretion [83] while decreases in LH pulse frequency following GRLN treatment have also been demonstrated in rats, sheep, monkeys, and humans [230]. Furthermore, GRLN treatment has been linked to reduction in FSH secretion and hypothalamic Kiss1 mRNA and a delay of puberty onset [230]. On the other hand, and in agreement with the findings in this thesis, GRLN has also been shown to stimulate LH release in many animal models, and under different reproductive conditions (Section 1.3.1), and is linked to stimulation of gonadal activity in birds [319]. Taken together with current work, it appears that species specific, as well as gender and gonadal reproductive state-specific, differences in GRLNs regulation of the reproductive system exist. Although not a specific aim of the current work, analysis of the net LH release data in response to 5-min 1 nM $gGRLN_{19}$ applications collected in this thesis revealed that although $gGRLN_{19}$ effectively stimulate LH release throughout different times of the seasonal reproductive year, significant differences exist depending on the time of year the experiments were performed (Fig. 11.1). The average magnitude of the LH response to 1 nM $gGRLN_{19}$ in experiments performed in July and August is significantly lower than those observed at the other months of the year. On the other hand, the greatest magnitude of the LH responses to $gGRLN_{19}$ tended to occur in experiments

performed in the spring (April and May; corresponding to the time of spawning [315]). Likewise, the abilities of sGnRH, cGnRH-II and PACAP to each induce LH release in vitro are also at a maximal at times of late gonadal recrudescence and the spawning seasons [193, 276]. Thus it is likely that, in addition to GnRH and PACAP, $gGRLN_{19}$ also plays an important role in the stimulating gonadal maturation and reproduction in the goldfish.

The observed seasonal changes in LH release-responsiveness to $gGRLN_{19}$ is quite interesting, as the time of lowest LH response to $gGRLN_{19}$ coincides with a time in which $gGRLN_{19}$-induced GH release is apparently at its largest (Fig. 11.1). These patterns of changes in GH and LH responsiveness to $gGRLN_{19}$ throughout the seasonal reproductive/body growth year are consistent with the known timing of changes in reproductive readiness and the requirement for increasing LH levels to stimulate gonadal recrudescence during winter and final oocyte maturation and spawning in spring [56, 315], as well as the role of GH in potentiating steroidogenic responses of the gonads to LH during gonadal recrudescence [315] and the assumed importance of GH in somatic growth in the summer months [56]. Although these observations are suggestive of the presence of distinct seasonal effects of $gGRLN_{19}$ on pituitary GH and LH release, more work is needed to examine these effects fully and to elucidate the mechanisms underlying these potential differences.

11.3. Intracellular signalling

Results from this thesis indicate that $gGRLN_{19}$ utilizes multiple common, as well as cell-type specific, intracellular signalling mechanisms to elicit GH and LH release (Fig. 11.2 & 11.3).

11.3.1. Ca^{2+} and LVSCCS in $gGRLN_{19}$-induced GH and LH release

It is not surprising that both LVSCCs and entry of extracellular Ca^{2+} are involved in $gGRLN_{19}$-induced LH and GH release (Chapters 3 & 4). As mentioned in Chapter 1, Ca^{2+} is a critical component to vesicle fusion within pituitary cells, and although both intracellular and extracellular sources may contribute to this process, the entry of Ca^{2+} via LVSCCs is common in many stimulator pathways, including sGnRH, cGnRH-II, PACAP, and DA actions in the goldfish pituitary cell system (Fig. 1.7 & 1.8), as well as in other hormone secretion systems [96, 314]. Hormone secretion is also increased with Ca^{2+} ionophore treatment and application of a depolarizing dose of KCl, which promotes Ca^{2+} entry [41, 46, 141] and, as seen in this thesis, by treatment with the LVSCC agonist Bay K8644 (Fig. 3.7 & 4.6). Likewise, treatment with LVSCC inhibitors nifedipine and verapamil decreases basal hormone release (Fig. 3.8 & 4.7), further supporting the role of Ca^{2+} in basal hormone release. These observations all indicates a role for Ca^{2+} entry through LVSCC in the control of basal as well as $gGRLN_{19}$-stimulated GH and LH release. How $gGRLN_{19}$ enhances LVCSS activity is not known but previous work supports a possible involvement of channel phosphorylation and activation through PKC or PKA action [41, 46]. Given that PKC and/or PKA are critical in $gGRLN_{19}$-induced hormone release (Chapter 5) and that use of LVSCC inhibitors (Chapters 3 & 4) and inhibitors of PKC and PKA (Chapter 5) lead to decreased hormone release and abolishment of increases in $[Ca^{2+}]_i$, it is highly likely that $gGRLN_{19}$-induced LVSCC activity is related to activation of these Ca^{2+} channels via the PKC and/or PKA pathway (Fig. 11.2 & 11.3).

Although not examined in the current thesis, intracellular Ca^{2+} stores are also involved in neuroendocrine regulation of GH and LH release from goldfish pituitary cells. Multiple, pharmacologically distinct, intracellular Ca^{2+} stores are

present in goldfish pituitary cells and they differentially contribute to the release of GH or LH in response to different neuroendocrine regulators, as outlined in Chapter 1 [41, 46, 142, 143, 146]. Evidence from experiments with the GHS GHRP-6 supports that GRLN activity may also involve mobilization of Ca^{2+} from intracellular stores. The $[Ca^{2+}]_i$ responses to GHRP-6 in Ca^{2+}-imaging studies using rat somatotropes in primary culture are biphasic (see Sections 1.2.3 and 1.3.2; [25, 121]). It is interesting to note, however, that only doses of GHRP-6 at 10 nM or above produced the first phase response, considered to be intracellular Ca^{2+} dependent, and only in 12.5 to 36% of cells imaged while second phase occurred in 20 to 75% of cells with the same doses [25], suggesting that the ability to increase Ca^{2+} release from intracellular stores is dose-dependent and may occur in a sub-set of somatotropes in response to GHS-R activation. Whether this intracellular Ca^{2+} mobilization phase is present with $gGRLN_{19}$ treatment in somatotropes and/or gonadotropes, and is related to the induction of hormone release remain be determined. However, results in this thesis do not rule out the presence of an intracellular Ca^{2+} mobilization phase in $gGRLN_{19}$ actions on goldfish (Fig. 11.2 & 11.3).

The first phase of GHRP-6-induced increase in $[Ca^{2+}]_i$, regarded as the intracellular Ca^{2+}-dependent component, occurs within approximately 10 sec of the exposure to GHRP-6 [25]. In comparison, the methods utilized in this thesis have a time resolution of only 15 sec in Ca^{2+}-imaging and 5 min in perifusion assays. Although the current work did not demonstrate any significant changes in hormone release or $[Ca^{2+}]_i$ when $gGRLN_{19}$ was applied in the presence of LVSCC inhibitors or Ca^{2+}-free media, $gGRLN_{19}$-induced GH and LH release were slightly larger than the response with Ca^{2+}-free media alone (Fig. 3.6 & 4.4). Although not significantly different, the quantified $[Ca^{2+}]_i$ signal in gonadotropes was generally greater following nifedipine plus $gGRLN_{19}$ treatment compared to that observed with nifedipine alone (Fig. 3.9). Perhaps further experiments with finer

time resolution may help to reveal an intracellular Ca^{2+}-dependent element in the early part of the $[Ca^{2+}]_i$ response.

Very few studies have followed up on the involvement of intracellular Ca^{2+} stores in GRLN and GHS-R activity, but one study, using rat pancreatic AR42J cells, confirms a biphasic Ca^{2+} response for GRLN treatment, with an initial transient rise followed by a sustained increase in $[Ca^{2+}]_i$ [178]. This study also finds that removal of extracellular Ca^{2+} abolished the GRLN-induced sustained increase in Ca^{2+} while maintaining the transient rise [178], supporting a model in which initial GRLN treatment signals entry of Ca^{2+} via VSCCs and release of Ca^{2+} from intracellular stores, followed by further release of intracellular Ca^{2+} in response to this initial rise. Whether this model applies to $gGRLN_{19}$ actions on goldfish GH and LH is not known but the idea that Ca^{2+} entry can induce release of Ca^{2+} from intracellular stores is not at odds with current knowledge of goldfish pituitary cells and intracellular Ca^{2+} stores. Further work would be need to examine this possibility as well as the downstream actions of increases in $[Ca^{2+}]_i$ in general, such as the involvement of CAM kinases, leading to hormone release (see future directions, Section 11.5.5).

11.3.2. PKC and PKA

As indicated by results from Chapter 5, PKC and PKA are differentially utilized in GRLN-induced GH and LH release, with PKC signalling being involved in both the GH and LH response while PKA only participates in the LH response (Fig. 11.2 & 11.3). As discussed in Chapter 5, PKC is accepted as the major pathway for GRLN-induced GH release, although the involvement of PKA has also been demonstrated. Uniquely, the involvement of PKA in $gGRLN_{19}$ action is related to its effects on gonadotropes, but not somatotropes, in goldfish. In the goldfish GH and LH release model, PKC and PKA form two major and largely independent pathways (Chapter 1). In this system, while sGnRH and

cGnRH-II utilize PKC, but not PKA, PACAP uses PKA, but not PKC, in both LH and GH release (Fig 1.7 & 1.8; [41, 46]), results from this thesis represent the first time that a neuroendocrine regulator has been shown to utilize both PKA and PKC in one, but not another, cell type in teleost pituitary cell study models.

11.3.3. NOS/NO pathway

The current work also demonstrates that the NOS/NO pathway is important in $gGRLN_{19}$-induced GH and LH release (Fig. 11.2 & 11.3), while the different pharmacological profile of effective NOS inhibitors reveals a cell-type specific utilization of NOS isoforms (Chapters 6 & 7). Thus, these results also reinforce the idea that $gGRLN_{19}$ utilizes divergent intracellular signalling pathways in goldfish gonadotropes as compared to somatotropes. The exact identity of the NOS isoforms involved in these $gGRLN_{19}$ actions remains to be confirmed but the participation of nNOS and iNOS, respectively, in the GH and LH responses to $gGRLN_{19}$ is suggested (Chapters 6 & 7). Interestingly, the constitutive (c)NOS, nNOS, is activated by increases in $[Ca^{2+}]_i$ in mammalian systems. Whether activation of NOS during $gGRLN_{19}$ stimulation of pituitary hormone release occurs as a result of the $[Ca^{2+}]_i$ increases and/or downstream of PKC and PKA, which may drive increased LVSCC activities (Sections 11.3.1), as well as the implication of differential PKC and PKA involvement in gonadotropes and somatotropes, will be interesting avenues for future investigations.

As already discussed in Chapters 6 and 7, results from this thesis supports the idea the NOS/NO signalling is a common feature of GRLN actions in general. Interestingly, GRLN modulation of cardiac functions also involves increases in iNOS gene expression and protein production [297]. On the other hand in *H. pylori*-induced gastritis, which causes damage via lipopolysaccharide-induced impairment of cNOS, GRLN treatment counters impairment of cNOS activity and thus counters the effects of this infection [291]. The regulation of feeding by

GRLN, as well as by NPY and CCK, are also linked to nNOS activity. A recent study using nNOS knockout mice and ICV administration of GRLN, NPY, and CCK demonstrates that without nNOS, GRLN and NPY fail to induce food intake while CCK fails to inhibit food intake [129, 227]. Thus, it appears that the differential use of NOS isoforms in a target-specific fashion may be one of the features of how GRLN affects a myriad of physiological functions.

How the differential use of NOS isoforms, and for that matter PKA, by $gGRLN_{19}$ in goldfish pituitary hormone release is unknown but given the presence of gGHS-R isoforms mRNA in pituitary tissues (Sections 1.1.3.2 & 4.3), the presence of cell-type unique receptor isoform(s) may play a role. In addition, GHS-R heterodimerization may also be involved. As discussed in Section 1.2.3, previous work has demonstrated that the GHS-R1a undergoes heterodimerization with various other receptors, including DA, 5-HT 2C, and melanocortin 3 receptors [277]. Heterodimerization of GHS-R1a with D1Rs is also associated with changes in G-protein coupling from $G\alpha_{11/q}$ to $G\alpha_{i/o}$ [134] and heterodimerization with D2Rs is also associated with changes in intracellular signalling [158]. Since D1Rs and D2Rs participate in the regulation of GH and LH release, respectively, from goldfish pituitary cells (Sections 1.4 & 1.5; [41, 46]), heterodimerization with different DA receptor types between the two cell-types may lead to cell-specific $gGRLN_{19}$ signalling in goldfish somatotropes relative to gonadotropes. This hypothesis, however, needs to be tested in more detail.

11.4. Implications of results examining $gGRLN_{19}$'s interactions with other endogenous regulators

Results from Chapters 8 to 10 are largely consistent with the idea that $gGRLN_{19}$ utilizes PKC in stimulating GH and LH release and that PKA and PKC forms largely distinct signalling mechanisms leading to GH and LH release in goldfish (Fig. 11.2 & 11.3). For example, $gGRLN_{19}$ (PKC-dependent) can still elicit GH and LH release responses in the presence of a PKA-activating neuroendocrine factor (PACAP) but not a PKC-activating neuroendocrine factor (sGnRH or cGnRH-II). In addition, the ability of PACAP exposure to enhance the GH-releasing ability of $gGRLN_{19}$ in an application-order specific manner not only illustrates the potential importance of PKA signalling ligands in facilitating $gGRLN_{19}$ (PKC)-dependent GH release, but also that the sequence of exposure to these factors, and by implication of the sequence of activation of intracellular signalling pathways, may also be part of the complexity in neuroendocrine regulation by multiple regulators (see Discussion section in Chapter 8). However, the lack of potentiating effects of gGnRH (presumed to be PKA-dependent) on $gGRLN_{19}$-stimulated GH release, as opposed to that expected based on mammalian studies (discussed in Chapter 9), not only reveal species differences but also suggest that the presence of ligand-specific interactions of receptor signal transduction pathways.

Surprisingly, two sets of observations are not completely consistent with the idea that PKC and PKA target pathway-specific releasable hormone pools in goldfish somatotropes and gonadotropes, namely DA effects on $gGRLN_{19}$ on GH release (Chapter 8) and the influence of the two GnRHs on $gGRLN_{19}$-induced LH release (Chapter 10). While the inability of $gGRLN_{19}$ to further induce LH release in the presence of GnRH is not at variance that both share a common PKC-dependent mechanism, and supports the idea that PKC-dependent mechanisms are central to the ability of $gGRLN_{19}$ to stimulate both GH and LH

release (Chapter 10), one would have expected at least a partial ability of $gGRLN_{19}$ to further elicit LH release in the presence of GnRH. On the other hand, GHS-Rs are known to heterodimerize with other receptors, including DA receptors, leading to changes in G-protein coupling [277], and this is postulated to explain the inability of $gGRLN_{19}$ to elicit a GH-release response in the presence of DA (discussed in Chapter 8). Whether a similar receptor heterodimerization and switch in receptor coupling occur during combined treatment with $gGRLN_{19}$ and GnRH occur that leads to the loss of a significant PKA-dependent component in $gGRLN_{19}$-induced LH release will also be a possibility to consider. Interestingly, GRLN also stimulates DA release from rat brain [132], while pretreatment with a D1R antagonist reduces GRLN-induced food intake [245]. Taken together, it appears that there are complex, intercommunicating signals between DA, GRLN, pituitary hormone release, and food intake. Once again, these differences, although only preliminary in nature at the moment, are likely to form the basis for GRLN's ability to differentially regulate a multitude of physiological process simultaneously, while also enhancing the notion that GRLN forms a link between energy status, growth, and reproduction.

In addition to revealing a novel direct inhibitory role of gGHRH on basal and stimulated LH release (Fig. 11.3), results from Chapter 9 has other implications. Previous experiments with a presumptive biochemically purified carp GHRH indicate that it is only effective in stimulating GH release in goldfish with regressed gonads or gonads at very early stages of recrudescence (Aug to Dec). On the other hand, the synthetic gGHRH stimulated GH release in experiments in Dec to Jan (early to mid-recrudescence) and in May to June (post-spawning and beginning of gonadal recrudescence) (Chapter 9). These data support the idea that the biochemically purified carp GHRH is likely a PACAP-related peptide, rather than a genuine GHRH. Given that PACAP-related peptides have been identified in a number of vertebrates [303], the role of gGHRH and the

goldfish PACAP-related peptide in the neuroendocrine regulation of LH and GH release requires further examination in the future.

11.5. Future Directions

11.5.1. Identification of the gGHS-R on different pituitary cell-types

The current work has presented strong support for the direct action of $gGRLN_{19}$ on both somatotropes and gonadotropes and that this action occurs via the gGHS-R1. It has not, however, confirmed the identity of the receptors on the individual pituitary cell types with any direct method and this is an important aspect that needs to be examined in the future.

Although antibodies against mammalian GHS-Rs exist, and at least one of which appears to cross-react with gGHS-R1a, antibodies against mammalian GHS-Rs may not adequately differentiate the different gGHS-R isoforms. Thus, I would develop and use antibodies against the gGHS-R1a and gGHS-R2a, preferentially with the ability to differentiate between the truncated and full form of the receptors. These antibodies could then be used in conjunction with fluorescent stains to identify the nucleus and cell surface proteins (e.g. gangliosides), allowing localization and identification of the cellular expression of gGHS-Rs through the use of immunocytochemistry techniques. Since a protocol already exists for single cell identification, as utilized in all fura-2, AM studies in the current work (Chapter 2; [329]), this cell-identification system can be used to reveal the presence of the gGHS-R and its colocalization within the plasma membrane of individual goldfish gonadotropes and somatotropes, as well as to track the internalization of receptors following treatments with neuroendocrine factors. Furthermore, use of enriched GH- and LH-cell fractions, which could be obtained via established percoll gradient separation [43, 329] or other cell sorting

techniques, followed by co-immunoprecipitation assays with pull-down of the gGHS-R followed by probing for D1R, D2R, PAC1R, and/or other receptor types with appropriate antibodies, could be used to support heterodimerization and identify any cell-type specific differences within gonadotropes and somatotropes. In this regard, teleost D1R, D2R and PAC1R cDNAs have been cloned (GenBank EF377327.1; [89, 180, 226], EF382625.1; [257], and AF048820.1; [177], for goldfish D1R, D2R, and PAC1R, respectively) and it is possible that fish specific antibodies against these molecules can be raised if antibodies against mammalian receptors are not adequate. This information would greatly add to the current work by further supporting the presence of direct $gGRLN_{19}$ action on GH and LH cell types within the goldfish pituitary, as well as evaluate cell-type specific heterodimerizations between gGHS-Rs and other receptors, as hypothesized in this thesis (Chapter 8). In addition, this will help to clarify the basis of differential signal transduction coupling in $gGRLN_{19}$ actions between gonadotropes and somatotropes revealed here (Chapters 5 to 8) and in a previous work with a GHS-R antagonist in goldfish pituitary cells [322]. Since the morphology-based single cell identification technique can also accurately identify lactotropes, and an enriched lactotropes cell fraction can also be obtained with the established percoll gradient separation [43, 329], one can also extend similar studies to these prolactin-containing cells, which would be of interest given that many studies have established GRLN-induced changes in PRL secretion (Section 1.3; [230, 327]).

11.5.2. Expression of GHS-R: responses to treatments

Results in Chapters 3, 4, and 8, demonstrating rapid desensitization and the presence of differences in the $gGRLN_{19}$-induced hormone release responses in the presence of DA or PACAP, clearly indicate that further work examining the changes in GHS-R protein expression levels in response to treatments with $gGRLN_{19}$, as well as other endogenous pituitary regulators, should be done.

Preliminary data suggest that the levels of gGHS-R1a-like protein in goldfish pituitary cells are differentially modulated by 4-h static incubation treatments with various neuroendocrine factors (Appendix Fig. A.1). It appears that 4-h exposures to $gGRLN_{19}$, cGnRH-II, PACAP and DA increase total gGHS-R1a-like protein levels in goldfish pituitary cells, whereas gGHRH reduces it. However, these results do not differentiate between gGHS-R1a-like protein levels in different cell-types, nor do they provide information on the amount of receptors on the cell surface. This information could be greatly expanded upon with other experiments. First, further work on the mRNA and protein expression levels of both $gGRLN_{19}$ and the GHS-R1a would be invaluable to define the starting points for subsequent work. This can be achieved through the use of qPCR and Western blotting, preferably done throughout the year to be useful in seasonality experiments (see 11.5.3 below), and with goldfish-based, and not rat-based, antibodies which are not currently available. This should be followed up with an examination of treatment-induced changes, focusing on protein and mRNA changes in response to $gGRLN_{19}$, DA, PACAP, sGnRH, cGnRH-II, and others, both alone and in combination and at various time points. Comparison of the gGHS-R1a protein levels in plasma membrane preparations relative to total protein extracts, coupled with imaging (see Section 11.5.1, above) would provide additional information on membrane receptor protein expression and trafficking. It would also be interesting to follow this work up with examination of gGHS-R1a protein and mRNA expression in tissues extracted from animals under different energy states, which could be established with changes in feeding prior to tissue extraction.

11.5.3. Seasonality of hormone release responses and expression of gGRLN

Given that GRLN is involved in growth, reproduction, and feeding behavior, and thus is a potential link between these processes, it is important to examine possible seasonal changes in its expression and activity, particularly in

an organism such as the goldfish which progresses through yearly cycles of gonadal maturation, spawning and regression, as well as seasonal body growth. To this end, there are many experiments which could be performed. Although data from this thesis hinted at the presence of seasonal differences in responsiveness in LH, and perhaps GH, secretion (Fig. 11.1), this needs to be more thoroughly investigated since this was not the focus of the current work and thus data were not extensively taken evenly throughout the year. This work would be best followed up by seasonal examination of acylated and unacylated gGRLN as well as gGHS-Rs, both at the mRNA and protein levels at baseline and in response to treatments with neuroendocrine factors and changes in energy status or feeding. This data can be obtained via qPCR and Western blot. Particular focus on brain, pituitary, gonadal, and intestinal tissues would be prudent, as these tissues represent the major production and targets for GRLN in relation to hormone release, reproduction, and feeding behavior [152, 171, 172].

11.5.4. Involvement of intracellular Ca^{2+} stores

Perhaps one of the foremost ideas on my mind throughout my work was the possibility of differential use of intracellular Ca^{2+} stores in pituitary somatotropes and gonadotropes, in response to $gGRLN_{19}$ treatment. As outlined in Section 11.3.1, activation of GHS-R produces biphasic $[Ca^{2+}]_i$ responses in mammals, and these phases have been identified as intracellular and extracellular Ca^{2+} specific [25]. Previous work in goldfish pituitary cells also clearly demonstrates the existence of pharmacologically distinct intracellular Ca^{2+} stores which are differentially utilized by various pituitary regulators (Chapter 1; [41, 46]). These are known to be accessed selectively in a ligand- and cell-type specific manner during sGnRH, cGnRH-II, PACAP, and DA actions [41, 46]. Given that the current work demonstrates that $gGRLN_{19}$ differentially utilizes intracellular signalling molecules, such as PKA (Chapter 5) and NO (Chapters 6 & 7), it is quite likely that differences in usage of intracellular Ca^{2+} stores exist as

well. To start, it would be interesting to examine $[Ca^{2+}]_i$ responses in GH and LH cell types under shorter time periods, such as 5-s data collection, in the fura-2, AM Ca^{2+}-imaging studies, to identify if biphasic responses are present in goldfish pituitary cells, as seen in mammals. In conjunction with these experiments, the use of pharmacological manipulation of the various pharmacologically distinct intracellular stores through the use of a broad spectrum Ca^{2+} release channel blocker, TMB-8, and intracellular Ca^{2+}-store specific drugs BHQ, CPA, caffeine, ryanodine, dantrolene, and xestospongin C [41, 46] in both perifusion hormone-releasing and Ca^{2+}-imaging studies could be used to examine which intracellular stores are utilized by $gGRLN_{19}$ in gonadotropes and somatotropes, and if any cell-specific differences exist. Following these studies, the relationship(s) between extracellular Ca^{2+} entry and the refilling and release of Ca^{2+} from stores, as well as their relationships with the other known signalling transduction mechanisms involved in $gGRLN_{19}$ actions can be addressed. These results will further the understanding of $gGRLN_{19}$ signalling in goldfish pituitary cells, as well as form the basis for future examinations on how $gGRLN_{19}$ may interact with other neuroendocrine regulators. In addition, such knowledge may also enhance our understanding how GRLN action in target tissues other than pituitary cells may be manifested.

11.5.5. Others

Many other experiments come to mind which could provide useful information regarding the use of GRLN in physiological processes. For example, both perifusion and Ca^{2+}-imaging work with goldfish pituitary cells, focussing on other intracellular signalling components currently known to be involved in GRLN signalling, such as 1) AMPK, described as critical to GRLN signalling in multiple tissues, including rat hypothalamus, liver, and adipose tissue [190]; 2) MAPK, shown to increase with GRLN treatment in cultured porcine ovarian granulosa cells [289]; and 3) PI3K, involved in transient (2 h), insulin-induced,

repression of prepro-GRLN gene expression in hypothalamic cell line neurons, mHypoE-38 [86], would all be appropriate. Interestingly, MAPK (especially ERK1/2) and PI3K have also been shown to play a role in the neuroendocrine regulation of goldfish pituitary LH and GH release and/or gene expression [41, 46].

As with GRLN, obestatin is also reported to be a regulator of appetite and GH secretion. Although the effects of obestatin are controversial at the moment, it is generally thought to have opposite effects to GRLN, acting to inhibit GH release and to reduce feeding behavior [118]. Despite evidence that obestatin binds its own receptor, the GPR39, and that its ability to inhibit GH release from the pituitary occurs indirectly, at the level of the hypothalamus, it has also been shown to directly compete with GRLN for binding to the GHS-R in pancreatic cell lines [107, 118]. Given that support is present for GRLN directly acting through the GHS-R in pituitary cells, both in fish (Chapter 3) and in mammals (Chapter 1.3; [169, 171], it would also be very interesting to examine the interactions between $gGRLN_{19}$ and obestatin, in similar protocols to those used in this thesis, examining any changes to both GH and LH release, and associated changes in Ca^{2+} signals, using goldfish pituitary cells.

Following up on GRLN's role in reproduction, as shown through the stimulation of goldfish LH release demonstrated in this thesis and via activity in the gonads, placenta, and in parturition in mammals [230, 319], further work on gender specific effects of $gGRLN_{19}$ would be interesting. In particular, changes in estradiol and testosterone levels of goldfish, in response to $gGRLN_{19}$ treatment, would be a strong initial experiment into the gonadal effects of this peptide. Exploration into gender specific differences in $gGRLN_{19}$-induced GH and LH release, particularly in a seasonal manner, would also prove interesting, forming a complement to those proposed in Sections 11.5.2 and 11.5.3, and together will

provide a better understanding on $gGRLN_{19}$'s overall role in the regulation of reproduction in goldfish.

11.6. Conclusion

Overall, novel results as presented and discussed in Chapters 3-10 in this thesis work has significantly added to our understanding of the complex regulation of not only GH and LH release, but also how signalling of $gGRLN_{19}$, a hormone with far reaching physiological functions and effects, occurs within specific cell-types. The results support $gGRLN_{19}$ as a potent stimulator of GH and LH release, signalling through GHS-Rs, which induces Ca^{2+} entry via LVSCC and PKC in both gonadotropes and somatotropes while utilizing cell-type specific NOS isoforms and the PKA pathway to achieve differential modulation of activity (Fig. 11.2 & 11.3). Furthermore, current work identifies PACAP as an endogenous potentiator of $gGRLN_{19}$-induced GH, but not LH, release in a treatment specific manner while demonstrating that sGnRH and cGnRH-II do not modulate $gGRLN_{19}$'s stimulatory effects on pituitary hormone release. DA has been shown to significantly reduce $gGRLN_{19}$-induced LH release, while not inducing significant increases in $gGRLN_{19}$-induced GH release, identifying a manner in which $gGRLN_{19}$ action on gonadotropes can be modulated without changing its GH-releasing effects. Finally gGHRH has been revealed as a novel inhibitor of basal and $gGRLN_{19}$-induced LH release, while not significantly altering $gGRLN_{19}$-induced GH release. Together, a model has been identified in which $gGRLN_{19}$ is able to differentially modulate GH and LH release in multiple ways and, given GRLN's orexigenic activity, these differences are almost certainly related to energy status and the various inputs received for different physiological reasons in the pituitary, which require different responses in GH and LH release (Fig 11.2 - 11.4). The findings in this thesis contribute to an

overall understanding of pituitary hormone release, control of growth and reproduction, and differential, cell-specific, signalling within endocrine cells while providing a foundation for a great deal of future work, not only on GRLN action but also differential signalling and the modulation of pituitary hormone release and hormonal regulation of physiological functions, in general.

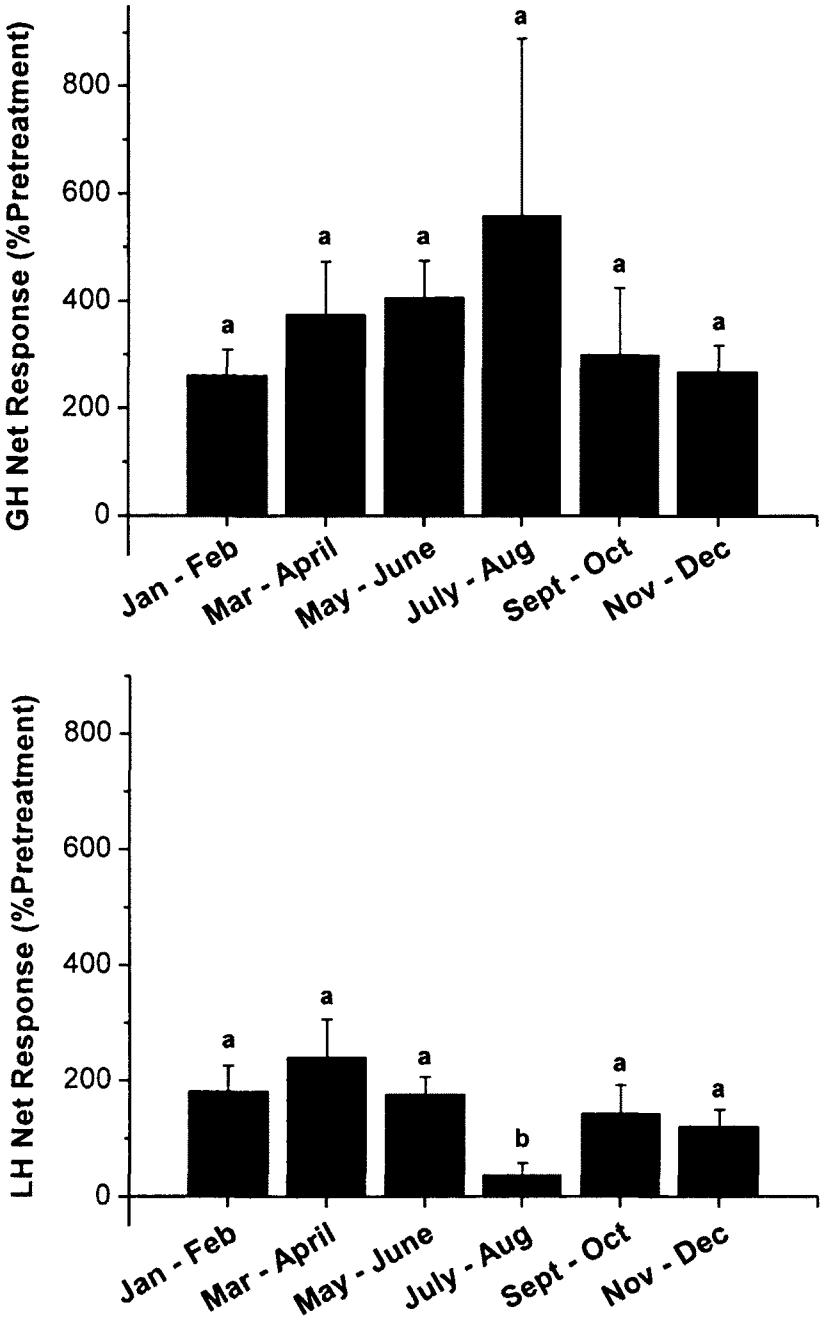

Fig 11.1. Seasonal averages of $gGRLN_{19}$-induced hormone release. Averages (mean ± SEM) of 1 nM, 5-min application, $gGRLN_{19}$-induced hormone release responses from experiments presented in Chapters 3-10 were grouped into two-month periods and analyzed. Different letters of the alphabet identify groups that are significantly different ($P < 0.05$; Kruskal-Wallice and ANOVA tests for GH and LH, respectively).

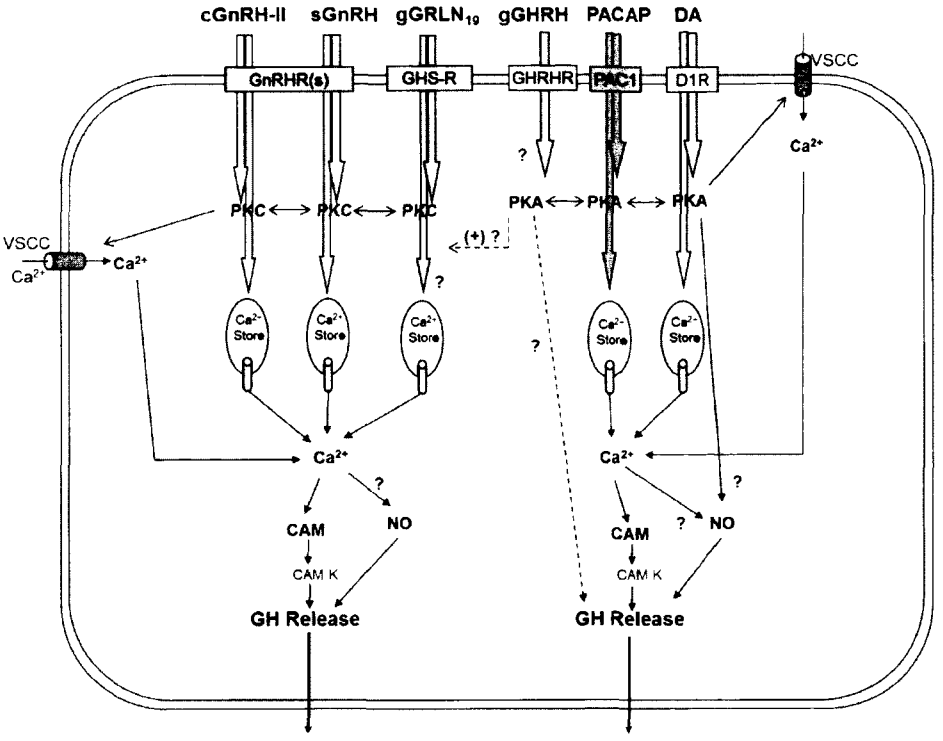

Fig 11.2. A proposed model of $gGRLN_{19}$ signalling within goldfish somatotropes. $gGRLN_{19}$, likely activates the GHS-R to induce GH release via enhanced Ca^{2+} entry through LVSCCs and the stimulation of PKC- and nNOS/NO-dependent pathways. $gGRLN_{19}$ and the two GnRHs share a similar PKC-dependent releasable GH pool. Potentiation of $gGRLN_{19}$-induced GH release can be effected by pre-exposure to PACAP, signalling via PKA and likely occurring through the PAC1 receptor. Potential heterodimerization with the D1R has not been shown, but may play a significant role in GH regulation.

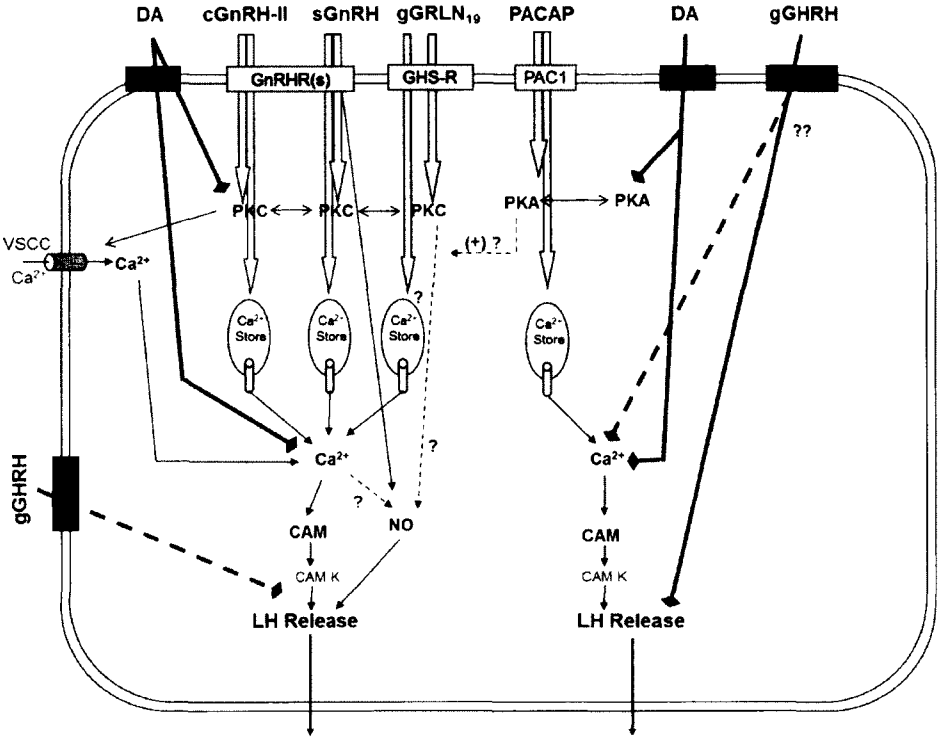

Fig 11.3. A proposed model of $gGRLN_{19}$ signalling within goldfish gonadotropes. $gGRLN_{19}$, likely signals through the GHS-R to induce LH release via activation of Ca^{2-} entry through LVSCCs, as well as PKC-, PKA-, and iNOS/NO-dependent pathways. $gGRLN_{19}$ and the two GnRHs share a common PKC-dependent releasable LH pool. Activation of PKA signalling, such as that elicited by PACAP, may lead to enhancement of PKC-dependent LH release as has been demonstrated previously with GnRH. The novel gGHRH inhibitory effects on LH secretion, as well as that of DA-D2R activation, are also depicted (diamond-ended lines). Potential heterodimerization with the D2R has not been shown here, but may play a significant role in LH release regulation.

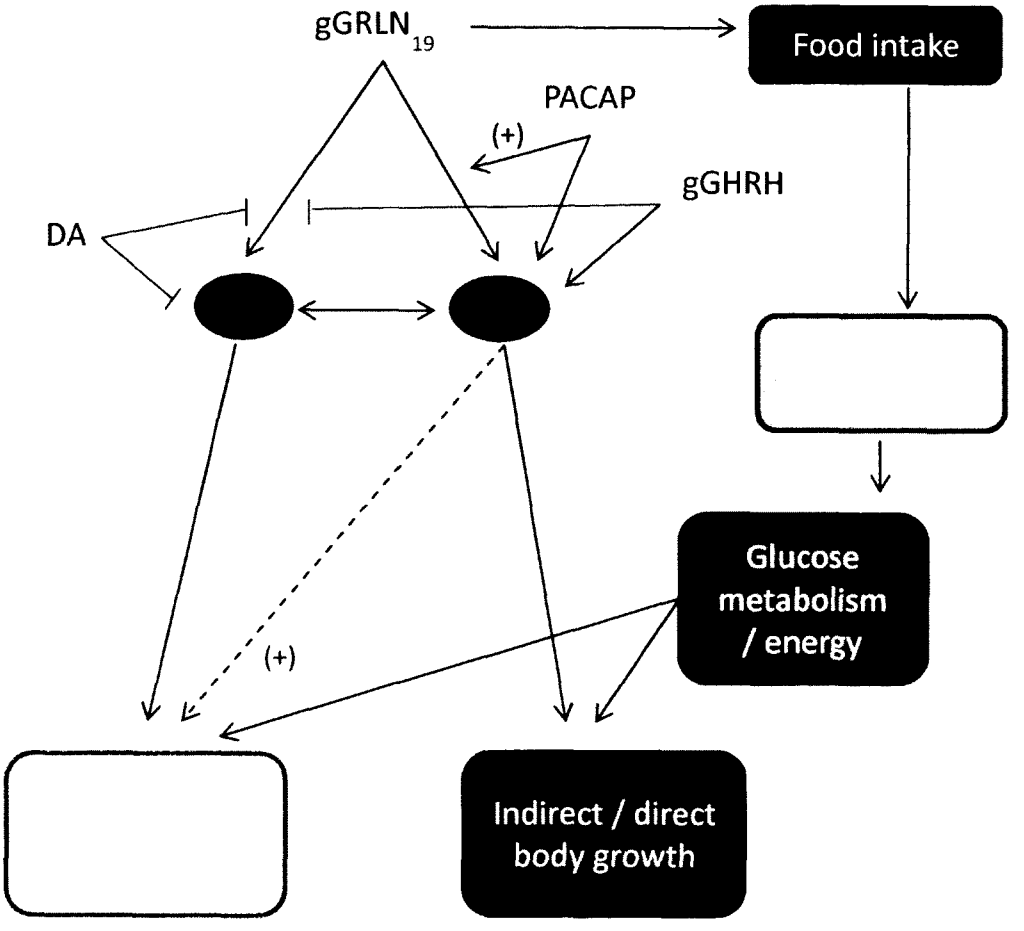

Fig 11.4. A working model demonstrating links between $gGRLN_{19}$, growth, reproduction, and energy levels in goldfish and modulation of gGRLN19 action by gGHRH, DA, and PACAP. Inhibitory influences of DA and gGHRH on LH responses are indicated by the T-ended lines.

Chapter 12 – References

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Appendix 1 – GHS-R1a protein expression

A.1. Introduction

Results from Chapters 3, 4, and 5 indicate that gGHS-R1a mRNA is present in goldfish pituitary and suggest that $gGRLN_{19}$ is acting directly through the gGHS-R1a, as shown in mammals [6, 7]. However, whether gGHS-R1a proteins are present is not known. Here I preformed preliminary Western blot analysis of the GHS-R-like proteins in the goldfish pituitary under basal conditions and following exposure to neuroendocrine factors. Given that 1) $gGRLN_{19}$ induces GH and LH release from goldfish pituitary cells (Chapters 3-10), 2) GRLN may have autocrine/paracrine effects at target tissues [2, 8, 9], and 3) $gGRLN_{19}$ treatment has been suggested to induce rapid desensitization in GH and LH release responses (Chapters 3 & 4), I chose to use treatments of other endogenous regulators of GH and LH release in addition to $gGRLN_{19}$. Specifically, the current work looks at changes in immunoreactive gGHS-R-like proteins, compared to basal levels, in response to DA, PACAP, sGnRH, cGnRH-II, gGHRH, and $gGRLN_{19}$.

A.2. Materials and Methods

A.2.1. Animals, tissue collection and drugs treatments

Animals were maintained as described in Section 2.1. Hormone stock solutions were prepared as in Section 2.8. Following anaesthetization by immersion in 0.05% tricaine methanesulfonate (Aqualife, Syndel, Vancouver, Canada) and subsequent decapitated, pituitaries (12 per treatment, placed in individual tubes and kept separate throughout the protocol) from both male and

female goldfish were removed, washed 3 times with dispersion media, cut into fragments, and placed in 5 ml of testing media in six-well plates (Primaria culture plates, Falcon). Following a 4-h treatment with maximally stimulatory concentrations of neuroendocrine regulators, samples were transferred to tubes, washed 3 times with PBS, and media was removed by centrifugation prior to cell lysis. Protein was extracted using cell lysis buffer (50 mM Tris, 150 mM NaCl, 1% triton X-100) containing phosphatase and protease inhibitor cocktails (Roche, Mannheim, Germany).

A.2.2. *Western Blot analysis*

Protein concentrations were determined by the DC Protein Assay (Bio-Rad, Hercules, CA). For Western detection of gGHS-R-like, protein samples (2.5 μg/lane) were subjected to electrophoresis on a 12% gel by SDS-PAGE. Separated proteins were blotted onto a 0.2 μm supported nitrocellulose membrane (Bio-Rad, Hercules, CA). Membranes were washed in Tris-buffered saline (TBS; 20 mM Tris hydroxymethyl aminomethane, 144 mM NaCl, pH 7.6 with HCl; Bioshop, Burlington, ON) containing 0.8% (v/v) Tween-20 (Polyoxyethylene 20-Sorbitan Monolaurate; Fisher, Fairlawn, NJ) for 5 min followed by a 1-h blocking treatment at room temperature with 5% skim milk (BD, Sparks, MD). Following blocking, membranes were then incubated with the rabbit anti-rat GHS-R1a IgG (Alpha Diagnostic, San Antonio, TX) at a 1:800 dilution, overnight at 4 °C. Specificity for GHS-R1a was tested by preadsorption of the primary antibody with its homologous antigen. After 3, 10-min, washes in TBS with 0.8% Tween and 3 10-min washes in TBS alone, membranes were incubated with horse radish peroxidase-labelled anti-rabbit IgG (Sigma, USA) at a 1:5000 dilution for 1 h at room temperature. After thorough washing in TBS with Tween and TBS alone, the immunocomplexes were visualized with the Peirce Western Blotting Substrate (Thermo scientific, Rockford, IL) followed by autoradiography on X-ray films (Kodak, Rochester, NY). For β-actin detection, membranes were first stripped in

stripping buffer (70 mM sodium dodecyl sulfate, 0.5 M Tris hydroxymethyl aminomethane, pH 6.8 with HCl, 0.8% v/v β-mercaptoethanol), followed by overnight incubation at 4 °C with rabbit anti-actin antibody (Sigma, USA) at a 1:800 dilution. Following 3, 10-min, washes in TBS with 8% Tween and 3, 10-min, washes in TBS alone, membranes were incubated with horse radish peroxidase-labelled anti-rabbit IgG (Sigma, USA) at a 1:5000 dilution for 1 h at room temperature and visualized with Peirce Western Blotting Substrate (Thermo scientific, Rockford, IL) followed by autoradiography on X-ray films (Kodak, Rochester, NY). Bands were quantified using Image J software. Intensity of the gGHS-R-like band was normalized to that observed of the corresponding β-actin band. Responses to treatment were expressed as a percentage of untreated controls.

A.3. Results

Both GHS-R and β-actin immunoreactive bands were detected in all treatment samples (Fig. A.1A). Exposure to 1 nM $gGRLN_{19}$, 10 nM PACAP, 100 nM cGnRH-II, 100nM sGnRH, and 1 μM DA elevated gGHS-R-like protein levels by roughly 75, 80, 120, 125, and 100% over controls, respectively, while 100 nM gGHRH treatment was accompanied by a reduction in gGHS-R-like protein levels by approximately 85%, relative to untreated controls (Fig. A.1B). Staining was not observed when using primary antibody against rat GHS-R1a that had been preadsorbed with antigen (results not shown).

A.4. Discussion

Although preliminary in nature, and without proper replicates for statistical analysis, the current results suggest that gGHS-R-like proteins are

present in goldfish pituitaries and that gGHS-R-like protein expression is differentially affected by 4-h treatments with various endogenous neuroendocrine regulators. The gGHS-R-like protein band has an approximate molecular size of around 41 kDa, a size similar to that reported for mammalian GHS-R1a [3], and the predicted size of the gGHS-R, as based on its cDNA sequence (Chapter 3; [4]).

The exact pituitary cell-types expressing gGHS-R-like proteins are not known. Likewise, whether the neuroendocrine factor-induced changes represent changes in all, or only in selective cell-types expressing these gGHS-R-like proteins, remains to be determined. Nonetheless, these changes likely lead to modulation of GH and/or LH release responses induced by $gGRLN_{19}$ treatment, given that $gGRLN_{19}$ has been shown to work directly through this receptor [6, 7], and thus may represent a manner in which regulation of $gGRLN_{19}$ action at the level of the goldfish pituitary occurs.

The ability of $gGRLN_{19}$ treatment to elevate gGHS-R-like protein levels is unexpected, given that the LH and GH responses to $gGRLN_{19}$ appear to rapidly desensitize (Chapters 3 & 4). On the other hand, these effects on gGHS-R-like protein expression may represent a long-term regulatory response related to recovery from desensitization, given that perifusion protocols utilize 5-min pulses and here we used 4-h treatments. Interestingly, $gGRLN_{19}$ increases prior to, and decreases following, meals [5] while GHRH cycles throughout the day in response to changes in GH levels (Chapter 1; [11, 12]) and GHRH release increases in times of somatic growth [1]. Thus the changes in GHS-R-like protein levels following $gGRLN_{19}$ and gGHRH treatments are likely linked to the physiological regulation of growth and metabolism. In support of this idea are recent findings which demonstrate a significant increases in avian hypothalamic GHS-R1a and GRLN mRNA levels in response to food restriction [10].

Notably, PACAP treatment leads to an increase in gGHS-R-like protein expression here, and PACAP exposure prior to $gGRLN_{19}$ treatment leads to a potentiation of the GH-release response (Chapter 8), suggesting that this increase in receptors for $gGRLN_{19}$ may contribute to PACAPs ability to potentiate $gGRLN_{19}$-induced GH release. In contrast, DA treatment similarly increased gGHS-R-like protein expression but DA pre-exposure did not enhance $gGRLN_{19}$-induced GH release (Chapter 8). Although the time-course of changes in gGHS-R-like protein expression following DA application is not known, these results with DA are not at odds with the ideas proposed in Chapter 8 that other mechanisms, such as receptor heterodimerization, may play a role when $gGRLN_{19}$ and DA are co-applied.

Preliminary results from this appendix chapter show that both endogenous GnRHs are capable of increasing pituitary GHS-R-like protein expression (Fig, A.1). Despite elevating GHS-R-like protein expression, treatment with GnRH does not magnify the LH and GH release responses to $gGRLN_{19}$, nor are the hormone-release responses to $gGRLN_{19}$ and GnRH additive (Chapter 10). Thus these findings on GnRH-induced GHS-R protein level changes reinforce the idea that the sharing of common post-receptor signalling pathway (i.e., PKC) is very likely the reason that sGnRH and cGnRH-II did not have any significant effect on $gGRLN_{19}$-induced GH or LH release. How GnRH-induced elevation in pituitary GHS-R-like protein expression is unknown and requires further study but the two GnRHs are known to utilize similar, as well as non-identical, signal transduction cascades in both goldfish somatotropes and gonadotropes (reviewed in Chapter 1).

The faint lower molecular size band that can sometimes be seen may represent the presence of an alternate gGHS-R isoform. Given that there are two isoforms of each subtype in the cDNA analysis (Chapter 3), this is not too surprising.

Further work would need to be done to confirm that changes observed here are statistically significant and to examine membrane-bound GHS-R protein levels compared to total amounts. Although changes in GRLN and GHS-R1a mRNA in response to obestatin and GRLN treatment have been recently examined [10], this work represents the first time that the effects of other neuroendocrine factors (sGnRH, cGnRH-II, PACAP, DA and $gGRLN_{19}$) and changes in GHS-R protein levels have been examined. Thus these preliminary results add to our understanding of how gGRLN action at the level of the goldfish pituitary can be modulated, as well as the overall complexity of neuroendocrine regulation of pituitary hormone release, in general.

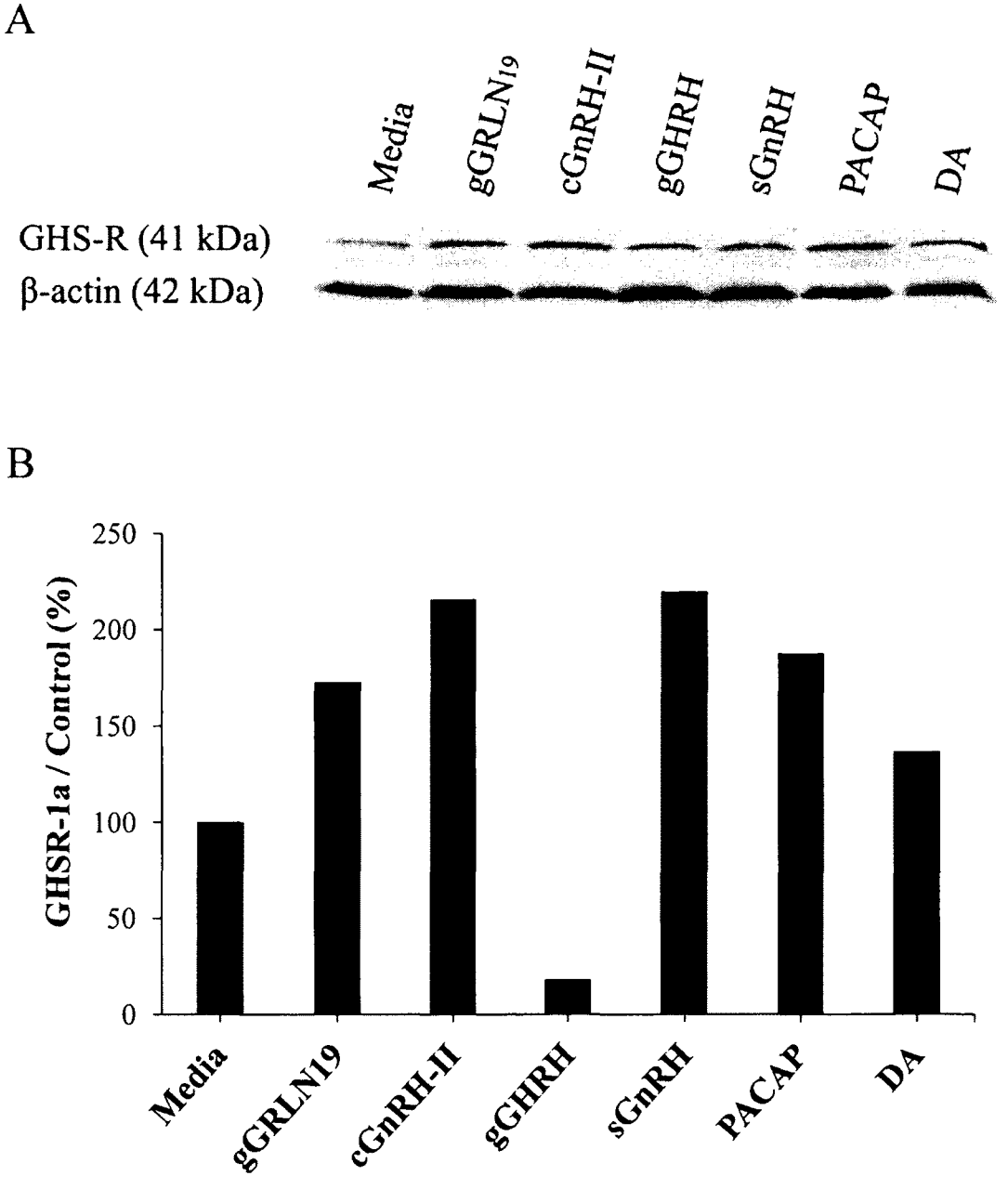

Fig. A.1. Expression of GHS-R-like protein in goldfish pituitary fragments following 4-h treatment with various neuroendocrine regulators. Following SDS-PAGE electrophoresis, GHS-R-like protein was detected using anti-rat GHS-R1a antibody, followed by ECL conjugated secondary antibody and visualization with autoradiography (A). Bands were quantified using Image J software, normalized to β-actin levels, and then represented as a ratio of media only controls for comparison (B).

A.5. **References**

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