Guanine-Nucleotide Binding Proteins, Tyrosine Kinases, and Associated Regulators in the Integrated Control of Basal and GnRH-Dependent Goldfish Pituitary Hormone Secretion

by

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Abstract

In vertebrates, the pituitary gland is a major regulator of physiological processes through the production and release of chemical signal messengers called hormones. In turn, central control of pituitary cell functions is exerted through neurohormones secreted from hypothalamic neurons. The fidelity of hormonal communication is achieved via recognition molecules, termed receptors, which elicit intracellular responses when specifically bound and activated by cognate hormone ligands. Among these receptors is the family of guanine-nucleotide (G) protein-coupled receptors (GPCRs), which are the predominant class of cell-surface transmembrane recognition systems for a variety of hormones and neuropeptides.

Among pituitary hormones, luteinizing hormone (LH) controls processes of sexual maturation and reproduction, whereas growth hormone (GH) influences somatic growth, metabolism, and immune function. In the goldfish (*Carassius auratus*), the two natural variants of hypothalamic gonadotropin-releasing hormone, GnRH2 and GnRH3, both stimulate LH and GH release by activating a shared population of plasma membrane G protein-coupled GnRH receptors (GnRHRs) on pituitary gonadotrophs and somatotrophs (LH- and GH- secreting cells, respectively). Prior research in our lab has characterized multiple post-receptor signal transduction mechanisms mediating the goldfish pituitary hormone release responses to GnRH. In addition to the shared use of several conserved intracellular signalling modules, GnRH-isoform-, cell-type-, and function-selective engagement of signalling effectors also exists in this system. Although ligand-selective recruitment of effectors following GnRHR activation is established, the functions of proximal receptor elements in driving such “biased signalling” remains unresolved.

In GPCR systems across taxa, the classical immediate effectors including heterotrimeric guanine nucleotide-binding (G) proteins, β-arrestins, and G protein-coupled receptor kinases (GRKs) play agonist-specific roles in facilitating both GPCR signal transduction and signal termination. Additionally, monomeric "small GTPases" are another relevant class of intracellular effectors in secretory cell types, due to their highly conserved cellular functions in modulating vesicle exocytosis via dynamic control of the actin cytoskeleton. However, the roles of these effectors in neuroendocrine systems and GPCR networks, especially in basal vertebrate models, have been under studied. Likewise, GPCR engagement of intracellular protein tyrosine kinases and other transmembrane receptor systems through transactivation may represent an important non-canonical route of directing selective cell functions. Altogether, these effectors represent important nodes of signal transduction that may be engaged by GnRHRs in ligand-selective fashions to control hormone secretion from goldfish pituitary cells.

The aims of my thesis were to characterize the involvement of classical GPCR effectors, small GTPases, and non-canonical tyrosine kinase elements in the control of goldfish pituitary cell secretion in basal (unstimulated) states and in response to GnRH isoforms. To achieve these aims, I utilized the established dispersed pituitary cell column perifusion system to monitor LH and GH secretion responses following transient pharmacological manipulation of the effectors of interest, in basal and GnRH-stimulated conditions. Results show that several classical GPCR-interacting proteins play important roles in mediating secretion responses to GnRH. In particular, multiple G protein alpha subtypes control GnRH-dependent effects, while β-arrestins and GRKs are identified for the first time as novel effectors involved in both mediating and terminating hormone release responses to GnRH, in a ligand- and pituitary-cell-type-dependent manner. In addition, studies of small GTPase proteins reveal how these cellular effectors can be

differentially utilized in basal and agonist-stimulated cell states to control hormone exocytosis. Furthermore, intracellular protein tyrosine kinases, receptor tyrosine kinases, and additional non-canonical cell-surface elements are identified for the first time in mediating selective GnRH-dependent and basal effects in this system.

Overall, results from my thesis expand the known repertoire of GnRH signalling networks across taxa and highlight the diversity in transduction machineries utilized to evoke cellular responses in response to extracellular signals. Findings also reveal, and lay the foundation for understanding, how the selective usage of intracellular effectors can be leveraged during the coordinated control of reproductive and growth processes by hypothalamic neuropeptide-GPCR systems. Just as importantly, this thesis represents one of only a handful of detailed investigations into natural ligand bias in physiological study systems, especially from any basal vertebrate model.

Preface

This thesis is an original work by Enezi Khalid. The research project, of which this thesis is a part, received research ethics approval from the University of Alberta Animal Care and Use Committee, Biological Sciences, Project Title "Neuroendocrine Control and Transduction Mechanisms in Fish Pituitary Hormone Secretion"; Study ID: AUP00000080).

Chapter 3 of this thesis has been published in part as Khalid, E., and Chang, J.P., 2020, "β-Arrestin-dependent signaling in GnRH control of hormone secretion from goldfish gonadotrophs and somatotrophs" in General and Comparative Endocrinology, vol 287:113340. Parts of Chapter 4 have been published as Khalid, E., and Chang, J.P., 2022, "Receptor-proximal effectors mediating GnRH actions in the goldfish pituitary: Involvement of G protein subunits and GRKs" in General and Comparative Endocrinology, vol 319: 113991. Parts of Chapter 5 have been accepted for publication as Khalid, E., and Chang, J.P., 2023, "Small GTPase control of pituitary hormone secretion: Evidence from studies in the goldfish (*Carassius auratus*) neuroendocrine model" in General and Comparative Endocrinology, 339:114287. This paper also contains the validation of modified LH and GH radioimmunoassays described in Chapter 2.

Perifusion experiments using the matrix metalloprotease inhibitor GM6001 (Chapter 6) were performed in conjunction with Joshua Pemberton (former PhD student in the Chang Lab), for which I later ran the hormone assays and data analysis. I was responsible for all other experiments, data collection, and analysis in this thesis document, as well as the literature review in Chapter 1 and concluding analysis in Chapter 7. Dr. John P. Chang (supervisor) provided conceptual and technical guidance, and manuscript composition editing.

Parts of the illustrations in this thesis (Chapter 1, and Chapters 3-7) were drawn using pictures from Servier Medical Art, licensed under a Creative Commons Attribution 3.0 Unported License (https://creativecommons.org/licenses/by/3.0/).

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List of Abbreviations

AA	**arachidonic acid**
AC	**adenylyl cyclase**
ADAM	**a disintegrin and metalloproteinase**
Akt	**Ak strain transforming**
ANOVA	**analysis of variance**
AP2	**assembly polypeptide 2**
Arf	**ADP-ribosylation factor**
ArhGAP	**Rho-GTPase activating protein**
ARNO	**Arf nucleotide-binding-site-opener**
AT1R	**angiotensin II type 1 receptor**
ATPase	**adenosine trisphosphatase**
β1AR	**β1 adrenergic receptor**
β2AR	**β2 adrenergic receptor**
BSA	**bovine serum albumin**
Btk	**Bruton's tyrosine kinase**
CaM	**calmodulin**
CaMK	**Ca^{2+}/calmodulin-dependent kinase**
cAMP	**cyclic adenosine monophosphate**
CCP	**clathrin-coated pits**
CCS	**clathrin-coated structures**
cdc42	**cell division control protein 42 homolog**
cGMP	**cyclic guanosine monophosphate**

CHO	**Chinese hamster ovary**
CSK	**c-terminal Src kinase**
CT	**calcitonin**
DAG	**diacylglycerol**
DMSO	**dimethyl sulfoxide**
ECL	**extracellular loop (of GPCR)**
ECM	**extracellular matrix**
EGF	**epidermal growth factor**
EGFR	**epidermal growth factor receptor**
ERK	**extracellular signal-regulated kinase**
FAK	**focal adhesion kinase**
FEME	**fast endophilin-mediated endocytosis**
FRET	**fluorescence resonance energy transfer**
FSH	**follicle-stimulating hormone**
Gα	**G protein alpha subunit**
Gαβγ	**heterotrimeric G protein complex**
$G\alpha_{i/o}$	**G protein alpha subunit type i/o**
$G\alpha_{s}$	**G protein alpha subunit type s**
$G\alpha_{q/11}$	**G protein alpha subunit type q/11**
$G\alpha_{s}$	**G protein alpha subunit type s**
Gβ	**G protein beta subunit**
Gβγ	**G protein beta/gamma subunit dimeric complex**
Gγ	**G protein gamma subunit**

GAP	**GTPase-activating protein**
GDI	**GDP-dissociation inhibitor**
GDP	**guanosine diphosphate**
GEF	**guanine nucleotide exchange factor**
GfA	**goldfish gonadotropin-releasing hormone receptor type A**
GfB	**goldfish gonadotropin-releasing hormone receptor type B**
GH	**growth hormone**
GHRH	**growth hormone-releasing hormone**
GnRH	**gonadotrophin-releasing hormone**
GnRH2	**[His^{5}Trp^{7}Tyr^8]GnRH; chicken-GnRH-II**
GnRH3	**[Trp^{7}Tyr^8]GnRH; salmon GnRH**
GnRHR	**gonadotropin-releasing hormone receptor**
GPCR	**G-protein coupled receptor**
GRK	**G-protein coupled receptor kinase**
GTP	**guanosine triphosphate**
GTPase	**guanosine triphosphate hydrolase**
Hb-EGF	**heparin-bound extracellular growth factor**
ICL	**intracellular loop (of GPCR)**
IP_3	**inositol 1,4,5-trisphosphate**
IP_3R	**inositol 1,4,5-trisphosphate receptor**
LH	**luteinizing hormone**
LHRH	**luteinizing hormone-releasing hormone**
MAPK	**mitogen-activated protein kinase**

MEK	**extracellular signal-regulated protein kinase kinase**
MOR	**μ-opioid receptor**
MMP	**matrix metalloproteinase**
mTOR	**mammalian target of rapamycin**
NCAM	**neural transmembrane cell adhesion molecule**
NO	**nitric oxide**
NOS	**nitroc oxide synthase**
p140Ras-GRF	**guanine-nucleotide releasing factor for p21 Ras**
p-ERK	**phosphorylated ERK1/2 (Tyr^{202}/Thr^{204})**
p-Src	**phosphorylated Src (Tyr^{416})**
PACAP	**pituitary adenylyl cyclase-activating polypeptide**
PBR	**polybasic region**
PDE	**phosphodiesterase**
PDK1	**3-phosphoinositide-dependent protein kinase 1**
PH	**pleckstrin homology**
PI3K	**phosphoinositide 3-kinase**
PI(4)P5K	**phosphatidylinositol-4-phosphate 5-kinase**
PIP_2	**phosphatidylinositol 4,5-bisphosphate**
PIP_3	**phosphatidylinositol 3,4,5-trisphosphate**
PIT-1	**antagonist of PIP_3-PH domain interactions**
PKA	**protein kinase A**
PKC	**protein kinase C**
PKG	**protein kinase G**

PLA_2	**phospholipase A2**
PLC	**phospholipase C**
PLD	**phospholipase D**
POA	**preoptic area of the hypothalamus**
PP2A	**protein phosphatase 2A**
PRL	**prolactin**
PTEN	**phosphatase and tensin homolog**
PTX	**pertussis toxin**
Pyk2	**protein tyrosine kinase 2 beta**
Rab	**Ras-associated binding**
Rac	**Ras-related C3 botulinum toxin substrate**
Raf	**rapidly accelerated fibrosarcoma**
Ran	**Ras-related nuclear protein**
Ras	**rat sarcoma virus**
Ras-GRP	**Ras guanyl nucleotide-releasing protein**
RGS	**regulator of G-protein signalling**
RH	**regulator of G-protein signalling homology (domain)**
RhoA	**Ras homolog family member A**
RIA	**radioimmunoassay**
ROCK	**Rho-associated protein kinase**
RTK	**receptor tyrosine kinase**
RyR	**ryanodine receptor**
SERCA	**sarco/endoplasmic reticulum Ca^{2+} ATPase**

SH2	**Src homology 2 (domain)**
SH3	**Src homology 3 (domain)**
SFK	**Src family kinases**
SOS	**Son of Sevenless**
Src	**sarcoma (from Rous sarcoma virus)**
TGF	**transforming growth factor**
TM	**transmembrane (domain of GPCR)**
TPA	**tetradecanoylphorbol acetate (PKC activator)**
TRPC	**transient receptor potential canonical channel**
VEGF	**vascular endothelial growth factor**
VGCC	**voltage-gated Ca^{2+} channel**

Chapter One

Introduction

1.1 The pituitary gland and endocrine axis

The endocrine system is a systemic network of cells, tissues, and soluble factors that play essential roles in coordinating physiological processes. The soluble factors are chemical messengers, known as hormones, which can facilitate cell-cell communication in the organism to bring about desired cellular responses. These hormones, in turn, are synthesized by endocrine cells and tissues. Among these tissues is the pituitary gland, classically referred to as the "master" endocrine gland, and its primary function is the production and secretion of a variety of hormones that are essential to the regulation of a multitude of physiological functions, including, but not limited to, metabolism, body growth, reproduction, and immune competence. Pituitary cells, in turn, can be regulated by the products of upstream neurons originating in the brain, i.e., neurosecretory substances/hormones, local intra-pituitary mechanisms, as well as hormonal feedback from distant peripheral sites in the body. Together, this integrated control is essential to the timed regulation of whole-organism physiology and proper responses to stimuli (Figure 1.1). The success of such integration at each level is reliant on, and enabled by, structure-specific interactions between the chemical messengers (ligands) and their recognition proteins (receptors), including plasma-membrane proteins. Upon activation, receptors initiate diverse ligand-specific responses, in a process known as signal transduction, which ultimately dictates cellular responses to a particular messenger. These fundamental principles of information transfer are generally common to the various levels of organization (i.e., cells, tissues, organ systems) across all domains of life.

In particular, I am interested in understanding how the control of the secretory activities of growth hormone (GH)-synthesizing somatotrophs and luteinizing hormone (LH)-producing gonadotrophs are regulated at the level of signal transduction upon stimulation by the

neuropeptide gonadotrophin-releasing hormone (GnRH) in the goldfish. In goldfish as in other vertebrates, LH regulates gonadal steroidogenesis and reproduction while GH regulates metabolism and body growth; in addition, GH can also potentiate the gonadal steroidogenic response to LH (Van Der Kraak et al., 1990). Although it is commonly believed that LH and GH secretion are selectively stimulated by GnRH and GH-releasing hormone (GHRH), respectively, based on studies in higher vertebrates, GnRH is also a major stimulator of GH secretion in addition to GHRH and several other factors in the goldfish and other teleost species (Holloway and Leatherland, 1997; Melamed et al., 1995; Wong et al., 1998). That GnRH can also stimulate GH secretion is not unique to teleosts. GnRH receptors can be expressed in normal human somatotrophs (La Rosa et al., 2000) and GnRH stimulates GH release from primary rat somatotrophs (Badger et al., 1987). In addition, GnRH can stimulate GH release in humans in certain cases, such as in patients with acromegaly (Watanobe et al., 1993; Watanobe and Tamura, 1995), pituitary adenomas (Lania et al., 2004), Klinefelter's syndrome (Dickerman et al., 1981), and other clinical conditions such as schizophrenia and depression (Amsterdam et al., 1982; Brambilla, 1978; Cantalamessa et al., 1985). Interestingly, two hypophysiotropic GnRH isoforms also exist in goldfish and GnRH stimulation of LH and GH in the goldfish pituitary cell model system also involves overlapping and yet distinct complements of signal transduction elements in both a cell-type- and GnRH-isoform-dependent manner (Chang and Pemberton, 2018); the latter is a form of ligand-biased signalling (Shonberg et al., 2014; Urban et al., 2007). Thus, elucidation of GnRH signal transduction mechanisms in goldfish pituitary gonadotrophs and somatotrophs is a fundamental part of understanding the coordinated neuroendocrine control of growth and reproduction, as well as how ligand-biased and cell-type-dependent signal

transduction events leading to secretory functions may be manifested in pituitary cells; the latter may also have implications for understanding cell physiology and its regulation.

In the following sections within this Introduction chapter, I will briefly summarize the current literature pertaining to the actions of GnRH in the control of pituitary cell function using information from several vertebrate model systems. To understand GnRH actions, information pertaining to its natural variants and receptor subtypes, as well as common signal transduction elements from broad receptor families, will also be reviewed, prior to the description of specific aims of this thesis.

1.2 GnRH functions, isoforms, and distribution

GnRH is a hypothalamic neuropeptide classically associated with the stimulation of pituitary gonadotrophin release, thus playing a pivotal role in regulating the hypothalamic-pituitary-gonadal axis across vertebrates. Secreted from a dispersed population of hypothalamic neurons, it is delivered to the anterior pituitary where it binds and activates GnRH receptors (GnRHRs) expressed on target cells, the gonadotrophs. Through release of the gonadotrophins, LH and FSH (follicle-stimulating hormone), GnRH ultimately exerts central control over gametogenesis, puberty onset, ovulation, and reproductive competence, and has long been considered a master regulator of reproduction (Gore, 2002). In mammals, changes in GnRH release pulsatility also regulate gonadotrophin release, and this is manifested through changes in transcription, translation, and secretion of GnRH (McArdle et al., 2021). At least twenty isoforms of GnRH have been described and have been sorted into three groupings, based on genomic structure, function, and syntenic associations; these groups are GnRH1, which includes the prototypical mammalian form (i.e., luteinizing hormone-releasing hormone, LHRH);

GnRH2, which is conserved in jawed vertebrates and with [His^5, Trp^7, Tyr^8]GnRH or chicken (c)-GnRH-II being the prototype; and GnRH3, which is primarily specific to teleosts and includes the prototype [Trp^7, Tyr^8]GnRH or salmon GnRH (Ogawa et al., 2021; Roch et al., 2014). Three GnRH paralogs are also present in the extant agnathans (such as lamprey), although the relationship to vertebrate classes of GnRH remains to be clarified (Ogawa et al., 2021), and GnRH3 is postulated to be secondarily lost in tetrapods (Decatur et al., 2013; Kim et al., 2011; Tostivint, 2011). Within teleosts, considerable variation exists in the presence of multiple GnRH isoforms, and in the resulting GnRH neuronal distribution patterns in the brain. Salmonids and cyprinids possess two GnRH isoforms, GnRH2 and GnRH3, whereas eel and catfish possess GnRH1 and GnRH2, and still other teleost species, such as medaka, sea bass, and fugu, have all three. Regardless, it is established that at least two GnRH types are expressed in nearly all vertebrate brains (with the exception of some rodents; Desaulniers et al., 2017): the conserved GnRH2 form (c-GnRH-II), and one form of GnRH1 or GnRH3 (Ogawa et al., 2021). Where present, the GnRH1 neurons originating in the preoptic area of the hypothalamus (POA) are typically the hypophysiotropic form, capable of stimulating pituitary hormone release. In their absence (such as in goldfish and zebrafish), this role is recapitulated by POA GnRH3 neurons instead. In fish with two GnRH isoforms, GnRH2 neurons projecting from the midbrain tegmentum region also innervate the pituitary and can regulate pituitary cell functions (Muñoz-Cueto et al., 2020). Besides this hypophysiotropic role, these midbrain GnRH2 neurons also project to other areas of the brain and have variable species-specific roles in regulating behaviour such as food intake (Kauffman and Rissman, 2004; Marvel et al., 2019; Matsuda et al., 2008; Xia et al., 2014).

1.3 The goldfish neuroendocrine model and GnRH

The experiments presented in this thesis employ the well-characterized goldfish (*Carassius auratus)* model system, which has been used for neuroendocrine studies at the University of Alberta since the 1970s (Peter, 1972; Peter and McKeown, 1975). Goldfish express two forms of GnRH: GnRH2 (c-GnRH-II) and GnRH3 (salmon GnRH), both of which are released from goldfish hypothalamic slices and pituitary fragments (Yu et al., 1991). Similar to other teleosts, goldfish do not possess the median eminence vasculature found in mammals, which serves as a conduit between the hypothalamus and the anterior pituitary (adenohypophysis). Instead, regions of the adenohypophysis are directly innervated by hypothalamic neurons delivering modulatory factors (Ball, 1981; Peter et al., 1990). Over twenty such hypothalamic factors have been characterized in teleosts for their capacity in regulating pituitary gonadotrophs (Trudeau, 2018; Zohar et al., 2010), whereas at least ten factors are known to control somatotroph cell functions in goldfish (Chang et al., 2012). Unlike in mammals where co-expression of LH and FSH in a single gonadotroph is common (Naor and Childs, 1986), LH and FSH are largely synthesized in distinct gonadotrophs in teleosts (Nozaki et al., 1990; Schmitz et al., 2005; Weltzien et al., 2014). Although GnRH has been shown to stimulate both FSH and LH release in teleosts such as tilapia (Aizen et al., 2007), coho salmon (Dickey and Swanson, 2000), and rainbow trout (Vacher et al., 2001), whether GnRH also enhances FSH release in goldfish has not been elucidated due to the lack of a FSH assay. Due to the inability to monitor goldfish FSH, all subsequent mention of goldfish gonadotrophin and gonadotrophs in this thesis pertains to the LH equivalent (formerly known as maturational gonadotrophin or gonadotrophin II in the literature; Van Der Kraak et al., 1992) unless otherwise specified.

1.3.1 Pituitary organization

In goldfish, GnRH3 neurons that project to the pituitary originate in the preoptic-anterior hypothalamic region of the brain, while the major source of GnRH2 delivered to the pituitary is from midbrain neurons, although GnRH2-secreting neurons are also found overlapping with the preoptic-anterior hypothalamus GnRH3 population (Figure 1.2 schematic; Kim et al., 1995; Yu et al., 1991). Interestingly, despite there being no clear difference in the number of GnRH2- and GnRH3-immunoreactive fibers detected in the pituitary gland (Kim et al., 1995), other studies have reported higher contents of GnRH3 in the pituitary relative to GnRH2 as detected by HPLC and radioimmunoassay (Yu et al., 1988). Regardless, both GnRH forms can be detected in goldfish serum, and are similarly capable of stimulating pituitary hormone release *in vitro* and *in vivo* (Chang et al., 1990; Peter et al., 1990). Interestingly, in addition to regulating LH secretion, both GnRH2 and GnRH3 also act on pituitary somatotrophs to control the production and release of GH, a finding similarly demonstrated in some other teleosts including species of carp, tilapia, grouper, and salmon (Kumar Bhandari et al., 2003; Li et al., 2002; Melamed et al., 1995).

1.3.2 Seasonal variations in pituitary hormones

Goldfish are seasonal breeders; consequently, the levels of serum LH and GH fluctuate over the breeding cycle. Being a spring-spawning species, circulating LH levels in goldfish in the North American temperate zone are highest during the pre-spawning (early spring) and spawning stages (April-June), following which they decrease with gonadal regression until late fall when gonadal recrudescence commences and levels start to rise again through the winter months (Peter, 1981; Peter and Crim, 1979). Levels of GH display a nadir during the late fall, followed by a gradual rise through winter and a peak during the spring months, remaining elevated

through the summer when the highest somatic growth rates occurs (Marchant and Peter, 1986). Despite the seasonality in blood LH and GH levels, both GnRHs are still able to stimulate the release of these two pituitary hormones from goldfish pituitary cells *in vitro*, although the magnitude of the responses also vary seasonally (Johnson and Chang, 2002).

1.4 Guanine nucleotide-binding (G)-protein coupled receptors

GnRHRs belong to the Class A group within the superfamily of G-protein coupled receptors (GPCRs; Millar et al., 2004). In general, GPCRs constitute the largest class of plasma membrane receptors for a diverse array of intercellular signalling ligands, including neurotransmitters, cytokines, ions, lipids, steroids, peptides, and protein hormones (Pierce et al., 2002). Besides these chemical molecules, GPCRs can also serve as receptors for light (rhodopsin; Stenkamp et al., 2005) and stretch-mediated stimuli (i.e., mechanotransduction; Erdogmus et al., 2019), but this section will focus primarily on the aforementioned GPCRs that bind chemical ligands as a first messenger. In this respect, since the >800 known GPCRs represent the dominant class of receptors for an overwhelming number of physiological chemical messengers, they also represent drug targets for approximately 35% of the approved pharmaceuticals on the market as of 2017, signifying their importance to overall health and disease physiology (Sriram and Insel, 2018).

1.4.1 General elements of Class A GPCR structure and function

GPCRs, of which GnRHRs are members, are plasma membrane receptors which typically possess an extracellular N-terminus, followed by seven membrane-spanning transmembrane (TM) domains, and an intracellular C-terminus “tail”. The ability of GPCRs to propagate signals

initiated by ligand binding is dependent on them being able to change their shape through structural rearrangements of the TM domains and the extracellular and intracellular loops (ICLs). These structural changes then allow for binding of transducers, such as G proteins, to the exposed intracellular face of the receptor transmembrane core, which in turn allows transducers to transmit signalling through downstream signalling pathways. Thus, GPCR activation is a process initiated by binding of the cognate ligand to the ligand-binding (orthosteric) pocket, causing reorganization of TM bundles, and culminating in binding and activation of downstream transducers, which is also facilitated by a number of conserved structural motifs. While the extracellular domains and orthosteric sites exhibit considerable variation allowing for fine-tuned ligand specificity, the movements of hydrophobic transmembrane alpha-helices and the ensuing rearrangements facilitating access of downstream transducers to exposed intracellular motifs are better conserved features of this family of GPCRs at large (Zhou et al., 2019); these highly conserved structure-function relationships allow for inferences about function across taxa. Beyond the information gathered from genome sequencing and annotation projects, these insights have been gleaned from a growing number of GPCR crystal structures in active and inactive states, as well as novel methodologies utilizing biosensors such as conformation-specific nanobodies or small molecule FRET (Fluorescence Resonance Energy Transfer; Zhou et al., 2021). From these studies, consensus mechanisms of GPCR activation have been determined. In general, Class A GPCRs are activated following agonist binding to the orthosteric site which triggers the “toggle switch” located in TM3/6, leading to changes in a conserved motif in TM6. This causes an outward movement of TM6 on the intracellular side of the receptor, creating an opening where G proteins can insert and bind, leading to initiation of G protein signalling.

1.4.2 GnRHRs

The human GnRHR, designated as type I GnRHR, is an interesting case study among Class A GPCRs, due to its unique lack of a typical intracellular C-terminus tail, as well as the preceding amphipathic helix region (helix 8). Helix 8 typically contains cysteine residues which serve as sites that may be modified by palmitoylation, a mechanism allowing GPCRs to be mobilized into membrane lipid rafts (Goddard and Watts, 2012). C-terminus tails of GPCRs contain Ser/Thr residues which, when present, are potential sites of phosphorylation by GPCR kinases (GRKs) as well as other downstream protein kinases such as protein kinase A (PKA) and protein kinase C (PKC). Classically, phosphorylation of the C-terminus is considered an essential prerequisite for arrestin binding and subsequent receptor internalization (Krupnick and Benovic, 1998). Indeed, early studies on mammalian GnRH receptors established that they did not undergo desensitization, at least not the rapid desensitization event associated with arrestin actions, but instead displayed more prolonged constitutive internalization patterns (Millar et al., 2004). This adaptation is postulated to support elevated and sustained LH release during the pre-ovulatory LH surge in mammals which is driven by high-frequency, high-amplitude pulsatile GnRH release (McArdle, 2012; Millar et al., 2004)

On the other hand, non-mammalian GnRH receptors (including those in amphibians, birds, and fish) retain the intracellular C-terminus tail, and are thereby designated type II GnRHRs (Flanagan and Manilall, 2017; Roch et al., 2014). As such, chimeras of mammalian GnRHRs fused with a GnRHR C-terminus tail from African catfish (*Clarias garepinus*) display rapid agonist-induced desensitization (Lin et al., 2014). Conversely, mice (which normally express type I tail-less GnRHRs) engineered with a chimera carrying the chicken GnRHR C-terminus tail display impaired gonadotrophin release responses and lower serum gonadotrophin

levels, as well as various reduced fertility indices in both male and female animals (Toufaily et al., 2021). In addition, post-receptor cellular responses to GnRH (such as G protein activation and Ca^{2+} mobilization) also differed depending on the identity of the C-tail fused with the murine GnRHR, with differences observed using catfish *vs.* lizard *vs.* frog GnRHR C-tails, implying that the specific C-tail sequence (and not merely the presence or absence of one) also has a role to play, depending on the specific endpoint measured.

Two GnRHRs have been cloned from goldfish, named GfA and GfB, and both belong to the type II GnRHR group (Illing et al., 1999). Primary sequences of GfA and GfB show retention of both typical helix 8 and C-tail residues containing putative palmitoylation and phosphorylation sites, respectively (Figure 1.3). Similar to the catfish GnRHR, both GfA and GfB receptors have known phosphorylation sites in the C-tail region [SXXS] (Blomenröhr et al., 1999; Illing et al., 1999). Other serine residues in both Helix 8 and C-tail regions are also known targets of phosphorylation by intracellular protein kinases, such as PKC (Caunt et al., 2004). Aside from the presence of the C-tail relative to the mammalian tail-less GnRHRs, other structural features and motifs are well conserved, not only between fish and mammalian GnRHRs, but also extending to common structures of mammalian Class A GPCRs in general. These conserved regions are typically present in the TM domains towards the central and cytosolic interior, and are involved in maintaining conformation-independent interhelical contacts which stabilizes overall receptor structure (Venkatakrishnan et al., 2013). Examples of such elements include the TM7 motif (D/N)PXXY, which plays an essential role in the transition to activated GPCR states; CWxPY in the TM6 region, which directly controls activation of the ”transmission switch” leading to opening of the G-protein binding pocket; and PxxxxxxCY in TM5, which also surrounds the G protein binding site. Natural mutations of GnRHR in the

CWxPY domain result in disrupted folding during biosynthesis, reduced membrane expression, and severely abrogated responses to GnRH, and are associated with the pathological state of congenital hypogonadotrophic hypogonadism in humans (Flanagan and Manilall, 2017). Another essential conserved sequence in Class A GPCRs is the DRY amino acid triplet located at the junction between TM3 and ICL2, which is an important part of the "ionic lock" which stabilizes the inactive conformation of the GPCR, and once broken, forms new contacts with TM5 as part of the receptor activation mechanism, then allowing for G protein binding (Rosenbaum et al., 2009). Interestingly, the third residue in this highly conserved DRY motif is changed in all known GnRHRs. Type I GnRHRs have DRS and type II tailed GnRHRs possess DRH/DRQ (Kosugi and Sower, 2010). Goldfish GfA and GfB sub-types both have DRH (Illing et al., 1999). The basal vertebrate lamprey also carries the DRH motif in its GnRHR, and point mutations in this third position reveal that the His residue enables proper cell-surface expression of the receptor as well as downstream inositol phosphate responses (Kosugi and Sower, 2010).

Interestingly, some early vertebrates such as the skate and coelocanth also possess tail-less (type I) GnRHRs that are similar to the mammalian homologs, suggesting that these variants existed early on in evolutionary time and that the C-tail may not have undergone a secondary loss in mammals (Roch et al., 2014). On the other hand, lampreys express type II tailed GnRHRs, and truncation analysis reveals that the intracellular tail in these receptors is essential for rapid ligand-dependent internalization (Silver and Sower, 2006).

The first crystal structure for any GnRHR was recently reported using the human type I GnRHR stabilized in complex with an antagonist drug, which revealed an unusual orthosteric site where the receptor N-terminus can occupy the binding pocket alongside the antagonist (Yan et al., 2020). Besides this unique finding, the study also confirms the functional importance of

select conserved motifs involved in ligand recognition and signal transmission through the TM bundle (i.e., "microswitches"), especially in TM3, TM6, and TM7. Interestingly, a number of putative and confirmed GnRH binding sites are identical between mammalian and goldfish GnRHR primary sequences (Flanagan and Manilall, 2017; Illing et al., 1999). Although premature, it would be tempting to suggest that the receptor N-terminus is similarly involved in the orthosteric binding pocket of type II GnRHRs such as those of the goldfish. Regardless, this is an exciting development for reproductive endocrinology in general, and bolsters possibilities of future structural characterization of a type II tailed GnRHR and subsequent comparisons. While antagonist-stabilized receptor structures have reveal important information regarding the landscape of transitional receptor states, future structural analyses incorporating natural ligands and/or in complex with downstream transducers would be especially informative towards understanding basic GnRHR function.

Overall, these highly conserved regions (DRY, CWxPY, D/NPxxY, and others) in Class A GPCRs are demonstrably involved in a consensus receptor activation "pathway" through the TM bundle and intrahelical networks, as recently shown in an analysis of 234 available Class A GPCR structures (Zhou et al., 2019). As such, while evolutionary and functional divergence has played a part in dictating responses to diverse ligands through variable orthosteric sites, mechanisms of receptor activation and subsequent intracellular transducer binding follow predictable patterns. Thus, even in the absence of a solved tailed-GnRHR crystal structure, it is possible to integrate findings from studies on goldfish GnRHRs with information from the family of related Class A GPCRs with known structures.

1.5 GPCR-proximal signal transducers

A common and important theme of cell-surface receptor systems is signal amplification, whereby increasing numbers and diversity of molecules are engaged at further points downstream from initial receptor activation, and GPCRs are no exception to this phenomenon. Following activation of the GPCR by a ligand, the initial transfer of information to the interior of the cell is handled by G proteins, which then further recruit and direct overlapping and divergent signalling pathways through several conserved intracellular modules and circuits that enables fine-tuned coordination of various cellular responses.

1.5.1 G protein subunits

G proteins, which have long been recognized as the main transducers of activated GPCR signals, are generally assembled in heterotrimeric complexes consisting of alpha, beta, and gamma subunits. These subunits are part of the G protein superfamily of regulatory GTP hydrolases, of which the small monomeric G proteins are also a part (see Section 1.6 below). All GPCR-interacting G proteins have a conserved region for recognition of guanine nucleotides on the Gα subunit. Binding or hydrolysis of GTP trigger conformational shifts in the Gα subunit protein through movement of the "switch" region, and these conformations are major determinants of effector recognition and how it interacts with binding partners. The 21 identified vertebrate Gα subunits belong to four families: $G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$ (Downes and Gautam, 1999; Simon et al., 1991). Additionally, there are six identified Gβ and twelve Gγ subunits, leading to hundreds of potential heterotrimeric complexes that may be assembled, specific combinations of which regulate downstream signal specificity in ligand- and receptor-specific fashions (Sivertsen et al., 2013). Gβ and Gγ subunits form constitutive Gβγ heterodimers which bind Gα in a nucleotide-dependent fashion. In the inactive state, Gαβγ subunits exist as a

heterotrimer, wherein Gα is associated with GDP, and it is in this state that G proteins initially interact with GPCRs. Following receptor activation by ligand, GPCR-catalyzed exchange of GDP for GTP on Gα occurs, and the Gβγ subunits dissociate. Active Gα-GTP complexes, once free of the receptor, may then engage downstream effectors, initiating distinct signal transduction pathways. Each Gα family is also generally linked to specific interaction partners and downstream signal transduction; thus, signal specificity of GPCRs is believed to be largely driven by Gα identity (Figure 1.5). Following hydrolysis of GTP through auto-hydrolytic activity on the Gα subunit and/or externally catalyzed by a GTPase-activating protein (GAP), the Gα subunit associates once again with GDP and then reassembles into a heterotrimer with Gβγ subunits, thus completing the cycle of G protein subunit dissociation and GDP/GTP exchange.

Classically, GnRHRs are primarily considered to couple to the $G\alpha_{q/11}$ subtype due to the well-described links to phospholipase C (PLC)-β and Ca^{2+}-dependent signalling (Grosse et al., 2000; Hsieh and Martin, 1992; Naor, 2009; White et al., 2008), but evidence also exists for coupling to $G\alpha_s$ and $G\alpha_{i/o}$ proteins (Hawes, 1993; Liu et al., 2002b; Naor, 2009; Stanislaus et al., 1998). Interestingly, recent evidence from studies in mouse models suggests that dual coupling to $G\alpha_{q/11}$ and $G\alpha_s$ is physiologically relevant, and that GnRHRs may transduce signals through different $G\alpha$ types to influence discrete gonadotroph functions *in vivo* (Stamatiades et al., 2022). In the case of goldfish GnRHRs, $G\alpha_{q/11}$ signalling can be inferred from the elevation of inositol 1,4,5-trisphosphate (IP_3) production following GnRH application (Chang et al., 1995; see also Section 1.8), although the involvement of $G\alpha_{q/11}$ has never been directly tested.

Being a classical receptor-interacting effector, G proteins are well poised to be major players in controlling diverse outcomes downstream of GPCRs such as GnRHRs. Specifically, the sensitivity of G protein complexes to disruption by GTP binding is shown to be ligand-

dependent; this provides a molecular basis for biased agonism one step removed from alterations in GPCR structural states (Furness et al., 2016). There are several broad routes for possible diverging responses at the level of G protein heterotrimers – distinct active conformations of $G\alpha_{q/11}$ and/or GTP occupancy time dictating varied responses, or involvement of non-$G\alpha_q$ subunits (other families of Gα and/or Gβγ which are important signal transduction mediators in their own right; Khan et al., 2013), or some combination of the above. Perhaps unsurprisingly, nature reveals to us examples of all of these permutations. For example, using the calcitonin (CT) receptor system and two ligands, human CT and salmon CT, one group has shown that differences in conformation of G protein subunits are induced in a ligand-dependent fashion, which was independent of the amount of subunits recruited (Furness et al., 2016). Notably, stimulation of the receptor with one ligand promoted a Gα subunit conformation which enabled increased rates of both initial GTP binding and turnover per unit time. Alongside this, receptor-residency time and second messenger generation similarly varied. Together, these observations provide evidence that the stabilized conformational intermediate of the GPCR reliably translates into conformations of the G proteins in the ternary complex with a direct functional consequence (i.e., rates of nucleotide exchange). Additionally, in the cannabinoid receptor system, several endogenous and synthetic ligands of CB1 and CB2 cannabinoid receptors provoke differential usage of G protein subunits, both across families (i.e., $G_{i/o}$ *vs.* $G_{q/11}$ utilization) and within the same family (i.e., differential usage of G_{i1}, G_{i2}, G_{i3}); these differences often lead to distinct downstream effects (Diez-Alarcia et al., 2016; Manning et al., 2021; Priestley et al., 2017). Likewise, differential engagement of Gα subunits has been shown for other Class A GPCRs including β1 adrenergic receptors (β1ARs; Ippolito and Benovic, 2021; Martin et al., 2004), M2 muscarinic receptors (Michal et al., 2007; Randáková et al., 2020), and dopamine D2 receptors

(Von Moo et al., 2022). While these data are largely generated in expression systems, such examples of promiscuous GPCR coupling to G proteins has also been documented in native cellular contexts, such as for the β2AR in cardiac myocytes (G_s/$G_{i/o}$ coupling; Xiao, 2001), the thyrotrophin receptor in human thyroid cell membranes (G_s, $G_{q/11}$, $G_{i/o}$, $G_{12/13}$; Laugwitz et al., 1996), and recently for the GnRHR in mouse gonadotrophs ($G\alpha_{q/11}$ and $G\alpha_s$; Stamatiades et al., 2022). It is probable that GPCR-G protein coupling in native cellular environments is more complex than can be adequately described in reconstituted systems (Masuho et al., 2015). Since distinct Gβγ complexes also have varying selectivity for different Gα subunits and distinct Gβγ dimers made up of different isoforms of β and γ subunits exhibit differences in GPCR-induced responses, the coupling to different heterotrimeric G proteins would also lead to the selective involvement of Gβγ subunits and their downstream elements (Masuho et al., 2021; Tennakoon et al., 2021).

1.5.2 G-protein Coupled Receptor Kinases (GRKs) and β-Arrestins

Following ligand-binding and initial engagement of second-messenger systems, GPCRs are known to undergo a process of desensitization to regulate signal transduction. Receptor desensitization typically requires the coordinated actions of GRKs and β-arrestins (Gurevich and Gurevich, 2019a). The GRK family of enzymes belongs to the AGC kinase family group (Arencibia et al., 2013). Of the seven members of the GRKs family, four (GRK2, GRK3, GRK5, and GRK6) are ubiquitously expressed, with GRK2 and GRK3 being the best characterized for their roles in GPCR desensitization. In particular, GRK2 and GRK3 contain pleckstrin homology (PH) domains, which enable interaction with phospholipids and Gβγ subunits, and have identified contact sites that allow them to directly interact with Gα subunits (Gurevich et al.,

2012). GRKs enzymatically modify activated GPCRs through phosphorylation of specific sites on the ICLs and C-terminus tails of activated GPCRs. This results in the generation of a specific phosphorylation pattern or "barcode" upon these regions of the receptor that promotes β-arrestin recruitment and binding of β-arrestin to the receptor, which ultimately leads to receptor desensitization (Figure 1.5; Chen and Tesmer, 2022; Gurevich and Gurevich, 2019a).

Along with G proteins and GRKs, β-arrestins are among the earliest proteins to engage GPCRs in response to ligand binding and receptor activation (Gurevich and Gurevich, 2019b). Across the family of vertebrate GPCRs, recruitment of β-arrestin to the receptor following GRK-induced phosphorylation is a common event in receptor desensitization. Of the four members of the arrestin family, arrestin-1 and arrestin-4 are limited to the visual system, whereas arrestin-2 and arrestin-3 (later renamed as β-arrestin-1 and β-arrestin-2) are ubiquitously expressed (Craft et al., 1994; Wilden et al., 1986). Arrestins play an immediate role in receptor desensitization by competing with G proteins for receptor binding at intracellular cavities which open up following outward movement of GPCR TM5 and TM6 helices, an universal feature of agonist binding throughout the GPCR superfamily (Hilger et al., 2018; Hauser et al., 2021). Besides this role at the activated receptor, β-arrestins can additionally mediate receptor internalization into clathrin-coated structures/pits (CCS/CCP; Figure 1.5), which is carried out through specific interactions with clathrin and the associated adaptor protein AP2 (Laporte et al., 2000). Specifically, interactions with active GPCRs releases the C-terminus tail of β-arrestin (Zhuo et al., 2014), which exposes the binding sites for clathrin and AP2 contained in this region (Kim and Benovic, 2002).

Interestingly, receptor fates can vary following incorporation into CCS. Internalized GPCRs can stimulate further waves of "endosomal" signalling, or are targeted for degradation

through ubiquitin-dependent endo-lysosomal pathways, or may be recycled back to the plasma membrane (Han et al., 2013; Thomsen et al., 2018). In some cases, dynamin-dependent scission of the CCP leading to matured endosomes may also be required for internalization and subsequent effects, although there is considerable variation along this theme depending on the specific GPCR and/or cellular context (Claing et al., 2000; Eichel et al., 2016).

Beyond the initial identified roles in desensitization, a growing body of evidence has also shown β-arrestins to be part of normal GPCR signal transduction linking to downstream intracellular effectors, including extracellular regulated protein kinases (ERKs) of the mitogen-activated protein kinase (MAPK) family and phosphoinositide 3-kinase (PI3K) of the family of membrane lipid kinases (Peterson and Luttrell, 2017; Figure 1.5; also see Section 1.5.3 below). As such, a number of studies have examined the roles of arrestin and dynamin proteins in GnRH-GnRHR actions in several cell model systems (Table 1.2), and while the involvement of these proteins are mixed, some conclusions can be drawn. In general, type I mammalian tail-less GnRHRs internalize in a dynamin-dependent, but arrestin-independent manner, and utilize dynamin-dependent mechanisms to engage signalling leading to ERK activation (Bonfil et al., 2004; Heding et al., 2000; Hislop et al., 2001). On the other hand, type II tailed GnRHRs (such as those from frogs and African catfish, as well as from marmoset monkeys) can utilize both arrestin and dynamin-dependent mechanisms leading to receptor internalization, and further utilize arrestins in ERK signalling, where tested (Caunt et al., 2006; Heding et al., 2000; Hislop et al., 2005, 2001). Notable exceptions are the chicken type II GnRHR and the bullfrog Bf3-GnRHR, which internalize in a dynamin-dependent, but arrestin-independent, fashion (Acharjee et al., 2002; Pawson et al., 2003). Interestingly, two other isoforms of the bullfrog GnRHR, Bf1 and Bf2, do utilize arrestin and dynamin, and internalize in a rapid fashion (Acharjee et al.,

2002). Other than ERK activation, one study utilizing the LβT2 and αT3-1 cell lines, as well as primary pituitary cells from sheep, has also shown utilization of dynamin-dependent signalling downstream of GnRHRs as a requirement for GnRH-induced extracellular Ca^{2+} influx through voltage-gated Ca^{2+} channels (VGCCs), induction of FSHβ gene expression, and reorganization of the actin cytoskeleton (Edwards et al., 2016). Importantly, propagation of signalling downstream of dynamin may be uncoupled from the roles of this protein in internalization, as seen for the human GnRHR, where dynamin is required for GnRH-induced ERK signalling, but not receptor internalization (Table 1.2). This may be attributed to dynamin's scaffolding functions through its SH3-binding proline-rich and PH domains (Achiriloaie et al., 1999; Hinshaw, 2000). However, whether either of these mechanisms play roles in GnRH-dependent hormone release has not been addressed; furthermore, the majority of these studies are in expression systems and many studies use GnRH analogues rather than native hormones which may result in different conformations of the activated GnRHRs.

Similar to findings discussed for G proteins, ligand-dependent conformations of intracellular proteins, has also been described in the case of "activated" β-arrestins, leading to differential signalling downstream of the same GPCR (Latorraca et al., 2020; Shukla et al., 2008). This is manifested through particular phosphorylation patterns on GPCR residues variably exposing binding determinants on the arrestin molecule itself, which in turn dictates which downstream effector can interact with arrestin, as well as altering stability of the receptor-arrestin complex. For example, angiotensin II analogs were shown to promote distinct conformational rearrangements in β-arrestin, which variably affected the half-life of the angiotensin II type 1 receptor (AT1R)-arrestin complex and also correlated with the degree of ERK1/2 activation (Lee et al., 2016; Zimmerman et al., 2012). Another study using a panel of β2AR agonists also

showed ligand-specific conformational changes at two distinct sites on arrestin, which correlated with levels of either receptor internalization or ERK activation (Liu et al., 2020). Thus, ligand-bound GPCR transitional states, in conjunction with variable GRK-dependent phosphorylation patterns ("barcodes"), stabilize one of multiple possible active arrestin conformations that have different propensities for signalling and/or receptor internalization.

Therefore, like Gα and Gβγ subunits, β-arrestins are part of the small suite of important receptor-proximal elements in GPCR signal transduction serving as the molecular basis for receptor- and ligand-specific recruitment of a number of downstream transduction pathways, signal amplification, and regulation of cellular functions (Peterson and Luttrell, 2017; Smith and Rajagopal, 2016). Indeed β-arrestin has been shown to be involved in signalling bias downstream of several hormone-GPCRs (Reiter et al., 2017). Despite the importance of β-arrestins in GPCR functions, as of 2022, only five GPCR-arrestin complexes have been successfully crystallized, in contrast to the nearly 300 known structures of GPCR-G protein complexes (Chen and Tesmer, 2022).

In addition to being recruited to the intracellular C-terminus tails of GPCR following receptor phosphorylation by GRKs, β-arrestins may also bind ICLs of GPCRs (i.e., the receptor "core"), and also N/DPXXY motifs in TM7; thus a lack of C-tail does not necessarily preclude arrestin interactions (Chen and Tesmer, 2022; Leo et al., 2022; Madziva et al., 2015). It is therefore not surprising that type I (tail-less) as well as type II (tailed) GnRHRs such as catfish and xenopus GnRHRs, can be internalized in dynamin-dependent or dynamin-independent fashions (Acharjee et al., 2002; Blomenröhr et al., 2002, 1999; Hislop et al., 2005; Millar et al., 2004; Perrett and McArdle, 2013). Accordingly, the potential exists that type I and II GnRHRs can similarly employ β-arrestins and/or dynamin, following GRK-mediated phosphorylation of

the receptors in signal amplification and linkages to a diversity of select signal transduction cascades. However, information on the involvement of GRKs in GnRHR systems is lacking, understandably due to the prevalence of mammalian laboratory models lacking GnRHR C-tails, but some evidence exists, showing that type II mammalian receptors from marmoset monkeys (expressed in COS-1 monkey cells) require GRK actions (Madziva et al., 2015; Ronacher et al., 2004a). Thus GRKs may also be an important element in GnRHR signalling, including that of the goldfish GnRHR system which has also been shown to undergo receptor desensitization following prolonged exposure to the two native goldfish GnRHs (Habibi, 1991a, 1991b).

1.5.3 G protein *vs.* GRK/arrestin bias

As mentioned above, recruitment of intracellular signal transduction cascades following GPCR activation can be achieved via G protein subunits as well as by β-arrestin. When G protein subunits and β-arrestin are linked to divergent signal transduction elements, distinct and diverse cellular responses can be expected, such as activation of Src family of non-receptor tyrosine kinases (SFKs), which is typically attributed to direct actions of arrestin rather than G protein subunits (Peterson and Luttrell, 2017). However, in some instances, the same downstream cascade(s) can be activated by both G protein- and β-arrestin-dependent mechanisms. As mentioned in Section 1.5.2 above, β-arrestins can be a mechanism through which ERK activation in GPCR signalling is manifested. β-arrestins recruit, and act as scaffold for, members of the three-tiered p44/42 MAPK cascade in endosomal compartments, and direct associations with β-arrestin have been described for Raf (MAPKKK), MEK1/2 (mitogen-activated protein kinase kinase; MAPKK) and ERK1/2 (MAPK) (Song et al., 2009; Jean-Charles et al., 2017). Activation of the ERK cascade in GPCR signalling can also occur via β-arrestin-independent (i.e., solely G-

protein-dependent) mechanisms depending on the specific GPCR and cellular context (Grundmann et al., 2018; O'Hayre et al., 2017; Shenoy et al., 2006). Interestingly, the same effectors can bring about distinct cellular outcomes depending on whether MAPKs are engaged as a result of G-protein *vs.* arrestin-dependent actions, (i.e., the result of G protein- *vs.* arrestin-biased signalling). There is evidence for G-protein dependent ERK activation being early, transient, and transcription promoting, whereas arrestin-dependent ERK activation may be delayed yet sustained over a longer period of time while localizing ERK to the cytosol; this has been shown for both AT1Rs (Ahn et al., 2004) and β2ARs in transfected HEK293 cells (Shenoy et al., 2006).

Other examples in which G protein *vs.* arrestin signalling bias plays a role in determining the outcome of ligand-receptor activation can be found in receptor pharmacology. Whereas "G protein-biased" agonists of the μ-opioid receptor (MOR) promoted analgesia with fewer side effects, other arrestin-utilizing agonists elicited larger undesirable side effects such as respiratory depression and sedation (DeWire et al., 2013). Following such initial observations, a lead compound which is largely analgesic has now gained FDA approval for use in adults (Lambert and Calo, 2020). In the same vein, angiotensin II can act through AT1Rs to increase blood pressure through the $G\alpha_q$ pathway, whereas selectively engaging the arrestin pathway leads to reduced blood pressure and increased cardiac performance (Ikeda et al., 2015; Rajagopal et al., 2006; Turu et al., 2019). Such G protein *vs.* arrestin bias has also been exploited in the development of therapeutics targeting GPCRs for cannabinoids (Leo and Abood, 2021), dopamine (Park et al., 2016), serotonin (Sniecikowska et al., 2020), opioids (Kappa subtype; Mores et al., 2019), catecholamines (Ippolito and Benovic, 2021), and other hormones and

neuropeptides (Bond et al., 2019), underscoring the relevance of this particular facet of signalling bias to overall health and physiology.

Since β-arrestins recruitment to GPCR requires GRKs, it follows that GRKs are also important receptor proximal molecules determining the relative use of β-arrestin-dependent *vs.* β-arrestin-independent (i.e., G-protein-dependent) intracellular transduction modules. Indeed, for the β2AR, mutation of a ICL3 residue that disrupts GRK binding leads to a "G-protein-biased" signalling phenotype (Choi et al., 2018).

1.6 Small G protein functions across secretory cell types and their integration in GPCR networks

In addition to the already outlined functions of heterotrimeric G proteins, another important group of G proteins is the monomeric small G proteins (17-30 kDa), which are also referred to as small GTPases. In general, small GTPases alternate between active GTP-bound and inactive GDP-bound conformations, the dynamics of which are tightly controlled by type-specific regulatory molecules in order to ensure spatial and temporal specificity. The rate-limiting exchange of GDP for GTP (activation step) is facilitated by guanine nucleotide exchange factors (GEFs), whereas GTPases activating proteins (GAPs) increase the intrinsic GTPase function leading to hydrolysis of GTP to GDP and deactivation. In addition, GDP-dissociation inhibitors (GDIs) inhibit small G protein activation by preventing GDP release (Cherfils and Zeghouf, 2013). Comprising over 100 members in eukaryotes, this small GTPase superfamily consists of at least five subgroups: the Arf (ADP-ribosylation factor GTPases), Ras (rat sarcoma), Rab (Ras-associated binding protein), Rho (Ras homologous), and Ran (Ras-related nuclear protein) families (Wennerberg et al., 2005). Together, they participate in

mediating important cellular functions, including cytoskeletal reorganization, vesicle trafficking, gene expression, cell migration and proliferation, as well as key events associated with cell cycle transitions (Takai et al., 2001). Some of these GTPases are known to affect phospholipid-dependent signalling, such as that of the PI3K pathway (Yang et al., 2012); this is in addition to their well-characterized roles in the recruitment and regulation of MAPK, as well as PLC-γ, signalling in growth factors receptors and other receptor tyrosine kinase (RTK) systems (Bar-Sagi and Hall, 2000; Frost, 1997; Lyon and Tesmer, 2013). Similarly, some members of the Arf, Rho, and Ras families participate in GPCR signalling cascades, and are also important players in the exocytotic pathway of secretory cell types, including endocrine, neural, and immune cells (Figure 1.8; Collins, 2003; Gasman et al., 2003; Watson, 1999). However, the role of these molecules in neuroendocrine systems and pituitary hormone secretion is not as well described, especially in studies utilizing basal vertebrates such as teleosts.

1.6.1 Arf GTPases

Arfs are classified into type I (Arf1-3), type II (Arf4, Arf5) and type III (Arf6) families and play important functions in cargo-sorting, vesicle formation, and recruitment of lipid-modifying enzymes at the Golgi, and these roles are conserved from yeast to mammals (Takai et al., 2001). Recent research has additionally elaborated on their involvement throughout the secretory and endocytic pathways (Adarska et al., 2021), as well as remodelling of plasma membrane important for exocytosis (Arous and Halban, 2015; Porat-Shliom et al., 2013) in basal and agonist-dependent hormone release across various endocrine cell types. For example, Arf1 stimulates budding of nascent secretory vesicles containing GH and prolactin (PRL) from the trans-Golgi network in the GH3 rat pituitary cell line, through indirect actions on phosphatidic

acid synthesis (Austin and Shields, 1996; Chen and Shields, 1996; Siddhanta et al., 2000). Likewise, Arf1 plays a role in the generation of competent corticotrophin secretory granules in the mouse AtT-20 pituitary cell line (Moore et al., 2002). Arf6, through its actions on PI(4)P-5-kinase and modulation of the cellular phosphoinositide pool is required for sustained insulin secretion (Lawrence and Birnbaum, 2003; Laychock, 1983; Prentki and Matschinsky, 1987) and participates in Ca^{2+}-dependent exocytosis in rat neuroendocrine PC12 cells engineered to secrete human GH (Aikawa and Martin, 2003).

In addition, Arf6-induced actin cytoskeletal rearrangement and its co-ordination with Rac1 and RhoA (both members of the Rho subfamily; Hall, 2012) are important downstream elements of stimulation of endogenous bombesin receptors in CHO cells (Boshans et al., 2000). Interestingly, at least 8 different GEFs for Arf6 have been identified in mammals, likely indicative of a high degree of spatiotemporal control in the widespread functions of this GTPase (Casanova, 2007).

1.6.2 Rho family GTPases: Rac1 and RhoA

Rac1 is ubiquitously expressed and is a common central transducer for RTKs, GPCRs, adhesion receptors, and cytokine receptors (Bosco et al., 2009). Rac1 plays important roles in neurons, endocrine cells and immune cell types, where membrane remodelling is required for granule exocytosis (Ibanga et al., 2022; Kowluru, 2010; Li et al., 2003; Sheshachalam et al., 2017). Rac1 is also implicated in GnRH stimulation of MAPK cascades in mammalian systems (Harris et al., 2002; Naor, 2009), possibly enabling GnRH-dependent engagement of the actin cytoskeleton through Rac1's actions on mammalian target of rapamycin complex 2 (mTORC2; Edwards et al., 2017). RhoA is also a key regulator of actin polymerization, and is implicated in

Ca^{2+}-dependent exocytotic events in multiple secretory cell types (Komuro et al., 1996; Norman et al., 1996), through downstream effectors such as the Ser/Thr kinase ROCK (Rho-associated protein kinase; Amano et al., 2010). Rho and Rac family GTPases are also important in the long-term control of motility and cell membrane neurite-like outgrowths in LβT2 gonadotrophs (Godoy et al., 2011), and may play a role in chemotactic migration in response to GnRH, which has been observed in both αT3-1 cell lines and mouse gonadotrophs in live pituitary slices (Edwards et al., 2017; Navratil et al., 2007).

1.6.3 Ras

The Ras family is perhaps the best studied grouping of small GTPases, due to early interest stemming from the finding that mutations in Ras proteins, which are promoters of mitogenic signalling, were implicated in human cancers. The Ras family has three known members (K-, N- and H-Ras) and all three can directly bind and activate Raf protein kinases (i.e., MEKK) (Andersen et al., 1981). Through Raf, as well as their effects on PI3Ks, Ras GTPases are central drivers of growth and mitogenic pathways via the ERK and Akt (Ak strain transforming; protein kinase B) cascades (Castellano and Santos, 2011). In addition to activation via tissue-specific GEFs such as Ras-GRP and p140Ras-GRF, Ras activation can also be achieved through interactions with the ubiquitous protein SOS (son of sevenless), an adaptor which links Ras to activated cell-surface receptors through phosphotyrosine-dependent mechanisms (Figure 1.9; Hennig et al., 2015). In LβT2 mouse gonadotrophs, GnRH analogue (buserelin) stimulation of gonadotrophin subunit expression through the ERK pathway can be inhibited by a dominant-negative mutant of Ras (Bonfil et al., 2004). Alternatively, in αT3-1 rat gonadotrophs, GnRH activation of ERK is only partially Ras-dependent, and direct PKC

activation of Raf plays a greater role in the input to ERK (Levi et al., 1998; Reiss et al., 1997). In addition, the calcium-binding protein calmodulin (CaM), and the associated Ca^{2+}/CaM-dependent kinase (CaMK) may also influence MAPK cascades through actions on Raf kinases (Agell et al., 2002; Illario et al., 2003; Moretó et al., 2008), possibly in concert with the proline-rich tyrosine kinases (Pyk2 tyrosine kinases; also see Section 1.7.2). Together, these results indicate that modulation by multiple signalling factors acting at multiple sites along the entire canonical Ras-Raf-MEK-ERK cascades is of importance in GnRH signalling.

1.7 Protein Tyrosine Kinases and integration with GPCR signalling

Beyond the engagement of classical G protein cascades leading to intracellular signalling, many GPCRs are capable of additionally recruiting tyrosine kinase-containing receptors to influence a diversity of cellular actions in a process often called receptor transactivation. RTKs are a large family of membrane receptors which serve to transduce signals downstream of numerous growth factors, cytokines, and other ligands. In turn, RTKs propagate downstream signalling to modulate a variety of functions and maintain cellular homeostasis, ranging from cell metabolism and survival to proliferation and differentiation (Wheeler and Yarden, 2015).

To achieve such transactivation, activated GPCRs relay through intracellular molecule(s)/module(s) (including tyrosine kinases such as c-Src and Pyk2, and other routes involving Ca^{2+}/PKC) to stimulate catalytic activity of membrane matrix metalloproteinase (MMP) enzymes, which in turn cleave and activate cell-surface pro-ligand molecules (Figure 1.8). Such a pathway is especially well described for the family of epidermal growth factors (EGFs) and transforming growth factors (TGFs and heparin-bound EGF (Hb-EGF). Once the membrane-anchored pro-ligand is cleaved, the released soluble ligand can then act on its cell-

surface tyrosine kinase growth factor receptor. Collectively, this phenomenon is termed "triple-membrane-bypass" signalling (Schafer and Blaxall, 2017). As such, GnRHRs were shown to transactivate EGF receptors (EGFRs) in LβT2 gonadotrophs, which was required for GnRH-induced ERK activation and induction of the immediate early genes c-Fos and c-Jun (Roelle et al., 2003). In GT1-7 hypothalamic neurons, GnRH activation of EGFRs through MMP actions is dependent on PKC (Shah et al., 2004). Other known examples of growth factor receptors shown to be activated via GPCR-elicited transactivation are the receptors for insulin-like growth factor (IGF), TGFβ, and platelet-derived growth factor (PDGF), although these may involve intracellular relays rather than MMP-dependent release of a surface-anchored ligand (Cattaneo et al., 2014). Whether transactivation, either through MMPs or intracellular relays, occurs in the case of stimulated GnRHRs in goldfish pituitary cells leading to hormone release is undetermined. Interestingly, RTKs can also function upstream of GPCRs and GPCR-associated transducers to carry out cellular actions (Waters et al., 2004); thus, in general, such bi-directional receptor cross-talk may represent important aspects of normal cell physiology.

1.7.1 Src family kinases

The Src family kinases (SFKs) are a group of non-receptor protein tyrosine kinases (PTKs) that couple to various receptor systems, and control cellular functions such as cell growth, metabolism, and survival pathways. In humans, this family comprises eleven members and are commonly divided into two major groupings: group I (Src, Fyn, Fgr, Yes) and group II (Blk, Hck, Lck, Lyn), and group III (Frk, Srm, Brk) which is distantly related to the first two (Roskoski, 2015). They are characterized by the SH1 catalytic kinase core domain, the presence of SH2 and SH3 Src-homology domains which mediate protein-protein interactions, and a C-

terminus tail which typically contains sites for phosphorylation by upstream kinases such as the C-terminal Src kinase Csk (Roskoski, 2015). While SFK activation downstream of RTKs is well defined, involving known receptor-associated adaptors and phosphotyrosine-based interactions, the mechanisms underlying their recruitment and engagement by GPCRs is still unclear (Berndt and Liebscher, 2021). Although several GPCRs are known to activate SFKs, a common pathway of activation remains to be elucidated. However, three major routes have been documented: direct GPCR interactions with SFKs (via ICLs and C-terminus tails of the GPCRs), G protein-dependent recruitment, and β-arrestin-dependent activation. Interactions of Src have been shown with $G\alpha_s$ and $G\alpha_{i/o}$ subunits *in vitro*, and association with Gβγ subunits can also occur (Luttrell et al., 1997; Ma et al., 2000). Src activity also appears to be sensitive to $G\alpha_{i/o}$ inhibition in whole cells, but whether other signalling intermediates may be involved is unclear (Luttrell et al., 1996; Parra-Mercado et al., 2019). On the other hand, arrestin-based interactions have a clearer structural basis, due to both β-arrestin1 and β-arrestin2 containing multiple polyproline (PxxP) motifs which are able to bind SH3-domain-containing proteins; indeed, this interaction has been shown to contribute to activation of SFKs downstream of GPCR activation (Luttrell et al., 1999; F. Yang et al., 2018), although the two β-arrestin isoforms may also oppositely regulate Src activity in some cases (Kuo et al., 2006). Finally, some GPCRs contain these same SH3-binding PxxP motifs in their ICLs and C-terminus tails, and interactions with Src proteins has been shown in some cases, although it is not clear whether other adaptors are required and how Gα subunits or β-arrestins may also contribute to this interaction. Interestingly, the GfA receptor contains one such PxxP motif in its C-terminus tail (Figure 1.3), and mammalian GnRHRs may also use receptor tyrosine kinase transactivation to activate Src in transfected COS-7 cells (Kraus et al., 2020), while others have shown that GnRHR engagement of RTK/Src is cell type-

dependent (Shah et al., 2003a). In transfected HEK293 cells, GnRH stimulation of ERK1/2 occurred in a Src- and focal adhesion kinase (FAK)-dependent fashion, downstream of $G_{q/11}$-PLC-β activation (Davidson et al., 2004; see also Section 1.9.2). Further, GnRH activation of Src kinases and FAK led to cytoskeletal remodelling, and the authors suggest that Src/FAK-dependent scaffolds are necessary for ERK induction in this model system. Src also functions as a platform to activate other MAPK cascades (such as the Jun N-terminal Kinase JNK) in the αT3-1 mouse gonadotroph cell line (Levi et al., 1998), and in these same cells, Src-dependent mechanisms can drive promoter activity of the glycoprotein alpha subunit (common to pituitary gonadotrophin hormones) following stimulation by the GnRH analog [D-Trp^6]-GnRH (Harris et al., 2003).

1.7.2 Pyk2/FAK

The proline-rich tyrosine kinase 2 (Pyk2) is another intracellular protein tyrosine kinase that transduces signals from various cell-surface receptor systems, in turn influencing cellular processes such as cell migration, proliferation, and survival (Schaller, 2010). Pyk2, and its closely related paralog FAK, are the only members of the FAK family of non-receptor tyrosine kinases (Parsons, 2003), and were initially characterized for their roles in cellular adhesion and motility. Importantly, Pyk2 is linked to signalling downstream of several PLC-coupled receptors, and is a known Ca^{2+}-dependent effector due to the presence of a calmodulin-binding element (Kohno et al., 2008; Lev et al., 1995; Momin et al., 2022). In agreement with this general function, GnRHR activation of Pyk2 is downstream of Ca^{2+}-dependent mechanisms in both GT1-7 cells and αT3-1 gonadotrophs, and both Pyk2 and CaMKII are involved in GnRH-induced shedding of the pro-ligand proHB-EGF from GT1-7 cells (Okitsu-Sakurayama et al.,

2021, 2019). Other studies in the mouse αT3-1 gonadotrophs have also demonstrated that Pyk2 is phosphorylated following GnRHa (buserelin) treatment, and that Pyk2 catalytic domains directly associate with calmodulin in a Ca^{2+}-dependent manner, which are both upstream of ERK activation (Xie et al., 2008). Given that calmodulin responds to Ca^{2+} influx through L-type VGCCs in αT3-1cells (Mulvaney et al., 1999; Xie et al., 2008), and both VGCCs and CaMKII are also involved GnRH pituitary cell actions in primary cultures of rat pituitary cells (Durán-Pastén and Fiordelisio, 2013; Haisenleder et al., 2003), Pyk2 kinases may also be an important element in GnRHR signalling in untransformed pituitary cells. As discussed above (Section 1.6.3), Pyk2 can also act as a GPCR "relay" molecule for MMP-dependent RTK activation in several model systems (George et al., 2013; Yu et al., 2014; Figure 1.8), which makes it a potentially important node in understanding post-receptor mechanisms for GnRH in general.

1.8 Signal transduction utilized by GnRH in hormone release from goldfish pituitary

Since the initial findings showing that both goldfish GnRH isoforms stimulate LH and GH release, characterization of the intracellular mechanisms utilized by GnRH has been an active area of research using a number of approaches and experimental conditions. These include monitoring LH and GH release responses from primary cultures of dispersed goldfish pituitary cells to GnRH in the absence or presence of inhibitors of signalling cascades under static incubation or in cell column perifusion. While static incubation enables the study on longer-term actions on hormone release, as well as facilitates examinations on hormone synthesis and availability when combined with measurements of cellular hormone contents and mRNA expression, cell column perifusion provides the opportunity to examine acute effects and in an environment that minimizes paracrine and autocrine influences. In conjunction with experiments

monitoring changes in the level of signalling molecules in pituitary cell populations or identified gonadotrophs or somatotrophs, results from these hormonal studies results reveal that a number of signal transduction mechanisms are engaged following goldfish GnRHR activation (Figure 1.6). In many cases, utilization of these pathways also varies in GnRH isoform-, cell-type, time- and context-dependent fashions. (i.e., acute *vs.* prolonged secretion, release *vs.* synthesis/availability, and basal secretion *vs.* agonist stimulated conditions) (Chang and Pemberton, 2018).

1.8.1 PKC/Ca^{2+}-dependent signalling

The major event of signal transduction following GnRHR activation is engagement of the membrane enzyme PLC (presumably PLC-β), which cleaves the membrane phospholipid PtdIns(4,5)P_2, generating two active signalling molecules, the membrane-bound diacylglycerol, and the soluble messenger IP_3 (Morgan et al., 1987). Subsequently, diacylglycerol leads to activation of PKC isoforms, whereas IP_3 evokes release of Ca^{2+} from intracellular stores (Streb et al., 1983). Consistent with these findings in mammals, GnRH2 and GnRH3 stimulation of goldfish pituitary cells also generate IP_3 and/or IP_2 and IP, indicative of the participation of PLC (Chang et al., 1995). The role of PKC, as well as intracellular Ca^{2+} stores, in GnRH stimulation of goldfish LH and GH secretion has subsequently been confirmed; however, the pharmacological properties of the Ca^{2+} stores involved differ between GnRH isoforms and between gonadotrophs and somatotrophs indicating the selective use of distinct Ca^{2+} stores and/or Ca^{2+} release channels (Chang et al., 2012; Johnson and Chang, 2000). In addition, L-type VGCCs play a role in both the LH and GH responses to the two GnRHs and the activation of these channels in part involves PKC (Chang et al., 2000; Wong et al., 1994). The involvement of

CaMK has also been demonstrated in both LH and GH release (Chang et al., 2000; Jobin et al., 1996b). PKC also appears to play a role in mediating the universal involvement of Na^+/H^+ exchangers (and by implication cellular pH regulation) in GnRH actions on LH and GH secretion in the goldfish dispersed pituitary cells system (Li et al., 2010; Van Goor et al., 1997, 1996; Table 1.1). Classically, the linkage between GPCR and PLC-β signalling is via $G\alpha_{q/11}$ subunits. On the other hand, although results from goldfish pituitary cells point to the involvement of PLC in GnRH signalling, the involvement of $G\alpha_{q/11}$ has not been directly tested in the goldfish system.

Another common Ca^{2+}-sensitive signalling effector is the nitric oxide synthase (NOS) system. NOS generates the diffusible gas NO from L-arginine and NO stimulates soluble guanylate cyclase to produce cGMP leading to the activation of protein kinase G (PKG) (Denninger and Marletta, 1999). Interestingly, although cGMP/PKG is involved in the LH and GH responses to both GnRHs, GnRH3-stumulated LH and GH release, as well as GnRH3-elicited LH secretion, but not GnRH2 stimulation of LH secretion, involve NOS (Meints et al., 2012; Uretsky et al., 2003; Uretsky and Chang, 2000). How the divergence between the use of NOS and cGMP/PKG occur in GnRH2 action on goldfish gonadotrophs is at present unknown.

Although $G\alpha_s$-cAMP dependent signalling is involved in GnRH action in tilapia and in some mammalian study models (Melamed et al., 1996; Naor and Huhtaniemi, 2013), its participation in GnRH2 and GnRH3 actions in goldfish LH and GH secretion has been ruled out based on the inability of GnRH to increase cAMP production in dispersed goldfish pituitary cells and the inability of inhibitors of cAMP-dependent PKA to reduce hormone secretion responses to the two GnRHs (Chang et al., 1993; Wong et al., 1994). On the other hand, $G\alpha_s$ and $G\alpha_{i/o}$

appear to participate in the longer-term control of basal (unstimulated) hormone secretion (Chang et al., 1993).

1.8.2 MEK-ERK

Another important signalling axis engaged downstream of GnRHR activation is the MAPK module. This module is generally organized in three tiers of serine/threonine kinases, MAPKKK, MAPKK and MAPK, which subsequently phosphorylate and activate the next kinase. Of the three families of MAPKs, the best-characterized in GPCR networks is the ERK, for which the upstream MAPKK in the axis is MEK (MAPK/ERK Kinase), and the corresponding MAPKKK is Raf (Lewis et al., 1998). This cascade, and ERK activation in particular, is a component of virtually all vertebrate GnRH systems studied thus far. The importance of ERK in GnRH biology is underscored by findings that gonadotroph- or pituitary-specific knockouts of ERK can render mice infertile, in part due to abrogated responsiveness to GnRH (Bliss et al., 2009; Brown et al., 2018). In the goldfish pituitary model, except for GnRH2-elicited acute GH secretion, ERK proteins play a role in regulating acute hormone secretion as well as LH and GH biosynthesis at the transcriptional and translational levels (Klausen et al., 2008; Pemberton et al., 2013; Table 1.1). Although PKC can activate ERK in goldfish pituitary cells, PKC does not mediate the ability of GnRH to enhance LH and GH mRNA levels indicating that goldfish GnRHRs recruit MEK/ERK signalling via other mechanism(s) in addition to PKC (Klausen et al., 2008; Pemberton et al., 2013). The exact mechanism(s) by which goldfish GnRHR activation leads to the use of this MEK/ERK module is not known but arrestin involvement is a candidate to consider since goldfish GnRHRs can undergo receptor desensitization (Habibi, 1991a, 1991b). In addition, a receptor transactivation

mechanism via MMPs to subsequently activate growth factor receptors (in particular EGFR) which has been shown to mediate GnRH action in LβT2 gonadotrophs, is also a possibility (Roelle et al., 2003). Transactivation via cytosolic relay factors, as well as direct receptor interaction using Src as described in Section 1.7.1; and/or through the Ca^{2+}-CaM Pyk2 relay (Xie et al., 2008; Section 1.7.2) are avenues still to be explored.

Interestingly, in mammalian GnRH systems, the MEK-ERK module is also known to integrate information pertaining to pulses of GnRH (Kanasaki et al., 2012; Perrett et al., 2014), which may also contribute to differential control of LH and FSH release from the same gonadotroph. In teleost fish, however, evidence for pulsatile GnRH secretion is lacking, although *in vitro* LH release responses are known to desensitize during prolonged stimulation, which can be overcome by using pulsatile administration of GnRH instead (Habibi, 1991a), and pacemaker activities of GnRH neurons in medaka and dwarf gourami have also been found (Abe and Oka, 2002; Kanda et al., 2010). Furthermore, the secretion of GH in grass carp is reported to be episodic in nature, an observation not at variance with the presence of pulsatile hypothalamic inputs to the pituitary (Zhang et al., 1994).

1.8.3 PI3K and other lipid-mediated actions

PI3K, a membrane phospholipid-dependent signalling system, is also utilized by goldfish GnRHRs. Classically in this signalling module, Class I PI3K action converts $PI(4,5)P_2$ to $PI(3,4,5)P_3$ (PIP_3). PIP_3 recruits 3-phophoinositide-dependent protein kinase 1 (PDK1) and Akt to the plasma membrane and facilitates Akt activation by PDK1-mediated phosphorylation. PI3K-dependent downstream elements modulate various cellular functions and Akt in particular is a central node integrating inputs from several receptor systems (Manning and Cantley, 2007).

Of the four Class I PI3K isoforms, p110α only participates in the modulation of basal hormone secretion whereas p110β, p110γ and p110β additionally participate in GnRH2- and GnRH3-induced LH and GH secretion in a ligand- and cell-type-selective manner (Table 1.1; Pemberton et al., 2015). PI3Ks are also differentially involved in the regulation of cellular LH and GH availability (Pemberton, 2015). How PI3Ks are coupled to the activation of goldfish GnRHRs has not been examined directly but Gβγ subunits are likely involved since two of these PI3K isoforms (p110β and p110γ) are known to be activated by Gβγ in other systems and Gβγ is involved in goldfish GnRH stimulation of hormone release (Pemberton et al., 2015; Pemberton and Chang, 2016). Likewise, arrestin is also a candidate to consider, since arrestins can directly interact with PI3K catalytic and regulatory subunits (Jean-Charles et al., 2017; Peterson and Luttrell, 2017; Song et al., 2017). Interestingly, despite the general involvement of PI3Ks in GnRH actions on LH and GH release, only GnRH3-induced GH release is sensitive to modulation of Akt, suggesting that downstream PI3K-dependent element(s) other than Akt are also differentially recruited by GnRHR activation in goldfish gonadotrophs and somatotrophs in a ligand specific manner (Table 1.1; Pemberton and Chang, 2016). Interestingly the involvement of the PtdIns(3,4,5)P_3-dependent signalling effector Bruton's tyrosine kinase (Btk) in GnRH-induced hormone release from goldfish pituitary has been demonstrated (Pemberton and Chang, 2016), and Btk requires phosphorylation by SFKs (particularly c-Src) for its full activation (Afar et al., 1996). Additionally, the observed uncoupling of the PI3K-Akt axis in goldfish pituitary cells may reflect activation of Akt through protein tyrosine-kinase dependent mechanisms (Pemberton, 2015), and SFKs are good candidates for direct regulation of Akt (Chen et al., 2001; Jiang and Qiu, 2003).

PLA_2 (phospholipase A2)-mediated liberation of arachidonic acid (AA) and subsequent metabolism of AA via lipoxygenase enzyme also plays a role in mediating the LH release response to GnRH3. However, this intracellular signalling module is not involved in GnRH2 stimulation of LH release nor GnRH2- and GnRH3-induced GH secretion (Chang et al., 1996, 1991; Table 1.1)

1.9 Functional selectivity in GnRH receptor signalling

In addition to the aforementioned differential use of signalling components (Table 1.1), GnRH2-induced LH secretion has been shown to be more sensitive to changes in extracellular-Ca^{2+} availability and to PKC inhibition relative to GnRH3 (Chang et al., 1990b). Thus the degree of involvement of (or sensitivity to) certain signalling components is a part of the selective post-receptor signalling pathways for GnRH2 and GnRH3. How this selective use of signalling components is achieved and whether the differential use of the two goldfish GnRHRs on gonadotrophs and somatotrophs is involved are interesting questions.

Regarding the two goldfish GnRHR isoforms GfA and GfB, although they show selectivity to GnRH analogs with GfB unable to recognize His or Trp substitutions at position 8 of the peptide, both receptors have greater sensitivity to GnRH2 over GnRH3 in total inositol phosphate production assays in a COS-1 transient expression system (Illing et al., 1999). The preference for GnRH2 is consistent with findings from binding studies in goldfish pituitary membrane preparations (Habibi et al., 1992). In *in situ* hybridization experiments, expression of GfA and GfB mRNA can be found in LH- and in GH-staining cells using adjacent pituitary sections although the expression of GfA overlaps more with LH-staining which might tempt one to postulate that GfA is more a LH-releasing receptor (Illing et al., 1999). However, hormone

release from perifused fragments in response to GnRH analogs with position 8 substitutions would suggest that GfA is most similar to the GH-releasing GnRHRs instead (Habibi et al., 1992). In addition, the existence of GfA and GfB by itself is not sufficient to explain the diversity of responses in response to the two GnRH ligands, due to several lines of evidence. Goldfish GnRH2 and GnRH3 compete for the same class of high-affinity, low-capacity sites in pituitary membrane preparations (Habibi et al., 1989, 1987). Both isoforms also similarly displace an avidin gold-labelled sGnRH analog from immunohistochemically identified gonadotrophs (Cook et al., 1991). Additionally, hormone release responses to GnRH2 and GnRH3 are not additive, and pulse application of GnRH2 during prolonged treatment with GnRH3 (and *vice versa*) do not further stimulate release (Chang et al., 1993). Taken together, these findings support the notion that GnRH ligands act through shared populations of goldfish GnRH receptors; thus, any differences in intracellular effector usage likely arise as a consequence of distinct post-receptor signalling dynamics, rather than being solely attributed to signalling through unique receptor isoforms (i.e., GfA *vs.* GfB).

It is also important to recognize that the studies of GfA and GFB receptor affinity and properties reported in Illing et al. (1999) have limitations. They were carried out in heterologous expression systems (transiently transfected COS-1 monkey cells) and may not necessarily reflect the behaviour of these receptor isoforms in native cellular environments. Indeed, in recent years, the importance of different GPCR-transducer/effector complements as well as lipid environments even between cell types within the same species on receptor functions, properties and elicited cellular responses have been emphasized (Atwood et al., 2011; Mystek et al., 2016; Polit et al., 2021). Furthermore, while the rarer IP_3 is relevant for Ca^{2+} signalling leading to exocytosis (Tse et al., 1993, 1997), total inositol phosphate production (which does not necessarily correlate with

functional hormone release responses) is the only signalling endpoint downstream of GnRHR activation monitored in the study by Illing and colleagues. Thus, there is insufficient evidence to definitively assign a single receptor subtype to one pituitary cell type, and GfA and GfB are likely both part of the mechanisms utilized by both goldfish GnRH isoforms in stimulating pituitary LH and GH release.

Regardless of the situation concerning the possible role(s) of GfA and GfB, the observations of divergent post-receptor signal transduction in the goldfish GnRHR mediated responses are in agreement with well-established models of GPCR function. GPCRs are dynamic proteins that undergo rapid changes in a range of inactive and active conformational states. Agonist binding directs a change in the proportion of time the receptor spends in one or more possible active states. In the past two decades, a wealth of biochemical and structural evidence has been revealed which describes the phenomenon of distinct ligand-stabilized receptor states for a multitude of GPCRs, as had been predicted by molecular dynamic modelling (Kenakin, 2011). Thus, in contrast to initial models of GPCRs as two-state, binary, "on-off" switches, an updated view suggests that they are closer to allosteric microprocessors, selectively transducing extracellular signals in a ligand-specific fashion (Smith et al., 2018). It is now appreciated that GPCRs dynamically exist in an equilibrium of several distinct conformations. Interestingly, while agonist binding to receptors shifts this equilibrium, multiple possible conformations can still exist even in the presence of ligand since biophysical evidence reveals that not only are unbound and ligand-bound receptor states clearly different, but similar ligands for the same receptor can also lead to stabilization of disparate active conformations. In turn, this diversity of GPCR conformational equilibria has been shown to alter coupling and activation dynamics of the first line of receptor-interacting effectors, including the G protein hetero-trimer and arrestin

proteins. Thus, it has been suggested that agonist-specific structural changes in GPCRs translates into intracellular signalling specificity through ligand-selective allosteric coupling (Gurevich and Gurevich, 2020; Wingler and Lefkowitz, 2020; Wisler et al., 2014). While many of the foundational studies in this area have been performed using the adrenergic and angiotensin II receptor systems, functional studies have now demonstrated biased agonism for many other ligand-receptor systems reflecting both a wide range of neuropeptides/hormones and multiple GPCR families; these include receptors for serotonin, dopamine, opioid, parathyroid hormone, oxytocin, thyroid-stimulating hormone, ghrelin, somatostatin, and calcitonin (Reiter et al., 2017). Just as importantly, many of these observations are mirrored in natural systems, utilizing native ligands in primary cells and tissue and the goldfish GnRH2/3-GnRHR system in LH and GH release fits into this current understanding of GPCR receptor activation.

1.10 Central hypothesis and experimental objectives

The broad objective of my doctoral thesis research is to characterize important regulatory steps in the physiological control of hormone secretion from goldfish pituitary cells. In doing this, I sought to address some gaps in knowledge and emergent questions in the goldfish neuroendocrine model of GnRH regulation of LH and GH secretion (as reviewed in Sections 1.3 and 1.8 above). In particular, the role of GnRHR-interacting proteins, as well as their putative links to known downstream signal transduction elements, are understudied in this system. In addition, the possible integration of protein tyrosine kinase effectors, as well as growth factor receptor involvement, are open questions with regard to GnRH actions on pituitary hormone release, and in the control of signalling cascades leading to MAPK/ERK activation. Just as importantly, the roles of various putative regulators that exert dynamic control over general

vesicle trafficking and hormone exocytosis in goldfish pituitary cells have been largely overlooked. These regulators likely represent target nodes for several of the identified neuromodulators in the goldfish model system in addition to GnRH although GnRH signalling is the best characterized, and knowledge on their involvement will contribute to the understanding of how multiple signalling modules are engaged and coordinated following receptor activation by a single ligand, as well as how the multifactorial regulatory control of LH and GH secretion by neuroendocrine factors are integrated at the level of intracellular signal transduction.

To address some of these deficiencies highlighted immediately above, my research is divided into five inter-related major focus areas. These are (1) to establish the involvement of β-arrestins and associated effectors in mediating GnRH-induced LH and GH secretion, (2) to examine the involvement of the complement of G protein subunits in GnRH-evoked hormone release, (3) to address the roles of small monomeric GTPase proteins in basal and agonist-stimulated hormone release, (4) to determine whether pituitary GnRH signalling networks also utilize protein tyrosine kinases and/or RTK transactivation, and (5) to pharmacologically link activation of the central kinase MAPK/ERK to these major routes of signalling. Since pituitary pars distalis of teleost are directly innervated and function nerve terminals remain in pituitary fragments, experiments will be performed with primary cultures of goldfish pituitary cells devoid of such terminals (Chang et al., 1990). Overall, the central hypothesis of my thesis is that classical GPCR effectors are involved in goldfish GnRHR actions and mediate the biased responses to GnRH2 and GnRH3, while additionally engaging protein tyrosine kinase-based signalling networks and small GTPase effectors in order to elicit hormone secretion responses.

1.11 Thesis overview

(1) Chapter 2 contains details pertaining to experimental methodologies used to conduct my thesis research, including primary culture of goldfish pituitary cells, immunoblotting procedures, cell column perifusions and associated radioimmunoassays, and bioinformatics approaches.

(2) Studies in Chapter 3 tested the involvement of β-arrestins and dynamin in acute GnRH-induced LH and GH release and basal hormone secretion, as well as the contribution of β-arrestin to GnRH-dependent ERK activity.

(3) Chapter 4 contains investigations of the complement of Gα subunits and GRKs in GnRHR signal transduction to ERK and in GnRH-dependent hormone release.

(4) Experiments in Chapter 5 tested the involvement of a selected complement of small monomeric G proteins for their roles in basal and GnRH-dependent pituitary hormone release.

(5) In Chapter 6, I examined the contributions of protein tyrosine kinases and RTKs to GnRH actions, specifically in the control of GnRH-dependent ERK activity and LH and GH release.

(6) Chapter 7, the Discussion Chapter, provides an overview of the findings in this thesis and integrates them with the current understanding of vertebrate GnRH actions in the control of hormone release.

Table 1.1. Overview of signal transduction mechanisms in GnRH control of LH and GH release from goldfish pituitary. Select examples of signal transduction mechanisms utilized in GnRH2- and GnRH3-dependent stimulation are presented. These results are obtained from perifusion studies using dispersed goldfish pituitary cells in primary culture. While several mechanisms are commonly used between the two pituitary cell types and downstream of both GnRH isoforms, some elements are selectively utilized in a cell-type- or ligand-dependent fashion (reviewed in Chang et al., 2009, 2012; Pemberton et al., 2015, 2016).

	Gonadotrophs (LH)		**Somatotrophs (GH)**	
Target	**GnRH2-stimulated**	**GnRH3-stimulated**	**GnRH2-stimulated**	**GnRH3-stimulated**
PLC	Yes[a]	Yes	Yes	Yes
PKC	Yes	Yes	Yes	Yes
Cyclic GMP	Yes	Yes	Yes	Yes
Na^+/H^+ antiport	Yes	Yes	Yes	Yes
NOS	No	Yes	Yes	Yes
Arachidonic acid	No	Yes	No	No
PI3K isoforms	p110β, p110δ[b]	p110δ, p110γ	p110β, p110γ	p110β, p110δ, p110γ
Akt	No	No	No	Yes
MAPK (MEK/ERK)	Yes	Yes	No	Yes
Voltage-gated Ca^{2+} channels	Yes	Yes	Yes	Yes
Intracellular Ca^{2+} stores and channels	RyR[c]	IP_3, Caffeine, RyR	RyR, Caffeine	Caffeine, IP_3

[a] Yes, involved; No, not involved.
[b] Involvement of individual PI3K isoforms
[c] Involvement of Ryanodine (RyR)-, IP_3- and Caffeine-sensitive calcium stores

Table 1.2. **Overview of arrestin and dynamin involvement in GnRHR functions.** An intracellular C-terminus tail, a general determinant of arrestin interactions, is present in type II (e.g., xenopus, catfish, bullfrog, chicken, and monkey) but not Type I GnRHRs (human, mouse, rats). Arrestin and dynamin are generally involved in receptor internalization, but can also mediate GnRHR-dependent signalling. Some type II receptors such as bullfrog Bf3 and chicken GnRHR can also internalize in an arrestin-independent fashion. Information in table sourced from Acharjee et al., 2002; Blomenröhr et al., 1999; Bonfil et al., 2004; Caunt et al., 2006; Edwards et al., 2016; Heding et al., 2000; Hislop et al., 2005, 2001; Madziva et al., 2015; Pawson et al., 2003; Ronacher et al., 2004; Vrecl et al., 1998.

GnRHR	**Cell**	**Arrestin Involvement**		**Dynamin Involvement**	
		Internalization	***Signalling***	***Internalization***	***Signalling***
Human (type I)	HeLa[a]	No[b]	No (ERK[c])	No	Yes (ERK)
Xenopus (type II)		Yes	Yes (ERK)	Yes	Yes (ERK)
Rat (type I)	COS-7, HEK-293	No	-	Yes	-
Catfish (type II)		Yes	-	Yes	-
Rat + Catfish (C-tail) chimera		Yes	-	Yes	-
Mouse (type I)	αT3-1	-	-	-	Yes (ERK, Ca^{2+} influx)
Rat (type I)	LβT2	-	No (ERK)	-	Yes (ERK, Ca^{2+} influx, *FSHβ* expression)
	HEK-293	No			
Monkey (type II)	COS-1	Yes*	Yes (ERK)	Yes	-
Bullfrog (bf) (type II; 3 subtypes)	HEK-293	Bf1: Yes	-	Bf1: Yes	-
		Bf2: Yes		Bf2: Yes	
		Bf3: No		Bf3: Yes	
Chicken (type II)	COS-7, HEK-293	No	-	Yes	-

[a] HeLa, COS-7 and HEK-293 cells are expression systems, whereas αT3-1 and LβT2 gonadotrophs have endogenous GnRHR expression.
[b] Yes, involved; No, not involved; (-), not tested.
[c] Signal transduction response monitored as the target of arrestin or dynamin involvement.
* Expression-level dependent – internalization occurs only when arrestin is overexpressed.

Figure 1.1. Overview of the hypothalamic-pituitary-target axis. Across vertebrates, whole-body control of homeostasis by the endocrine system generally follows the outlined paradigms of control. In general, higher centres in the brain integrate information pertaining to various internal and external stimuli, and hypothalamic neuronal populations generally control anterior pituitary (adenohypophysis) cell functions through the release of stimulatory and inhibitory neuromodulators such as gonadotrophin-releasing hormone, gonadotrophin inhibitory hormone, growth hormone-releasing hormone, somatostatin, dopamine, and others, whereas some hypothalamic neurons project and form the posterior pituitary (neurohypophysis, not shown). In terms of the control of the adenohypophysis, the mode of delivery to the pituitary varies across taxa. In mammals and higher vertebrates, hypothalamic neuronal projections terminate in the median eminence vascular network, from where they are carried through a system of portal vessels to the adenohypophysis. In teleosts, however, the median eminence is absent, and hypothalamic neurons directly project to the adenohypophysis, and depending on the particular species, can terminate at or near individual populations of pituitary cell types (such as gonadotrophs and somatotrophs). Regardless of the method of delivery, hypothalamic neuromodulators modulate anterior pituitary cell functions in order to influence both synthesis and release processes of major pituitary hormone-secreting cell types, including the gonadotrophins (luteinizing hormone and follicle-stimulating hormone), growth hormone, prolactin, thyrotrophin, corticotrophin, and melanotrophin. Once released from the gland, pituitary hormones enter systemic circulation and act at target tissues to carry out their primary function(s). For example, luteinizing hormone is responsible for stimulating gonadal development and steroid production, whereas growth hormone influences metabolism and growth processes through actions on the liver, skeletal muscle, and adipose tissue. Hormonal products of target tissues (such as sex steroids) can in turn regulate upstream locations in the axis, by acting at the level of the hypothalamus, pituitary, and other brain centres (long-loop feedback). Similarly, pituitary hormones can also regulate upstream brain and hypothalamic centres (short-loop feedback). Shown in the example schematic is the neuroendocrine-endocrine axis for luteinizing hormone and growth hormone. Adapted from Hadley and Levine, 2006.

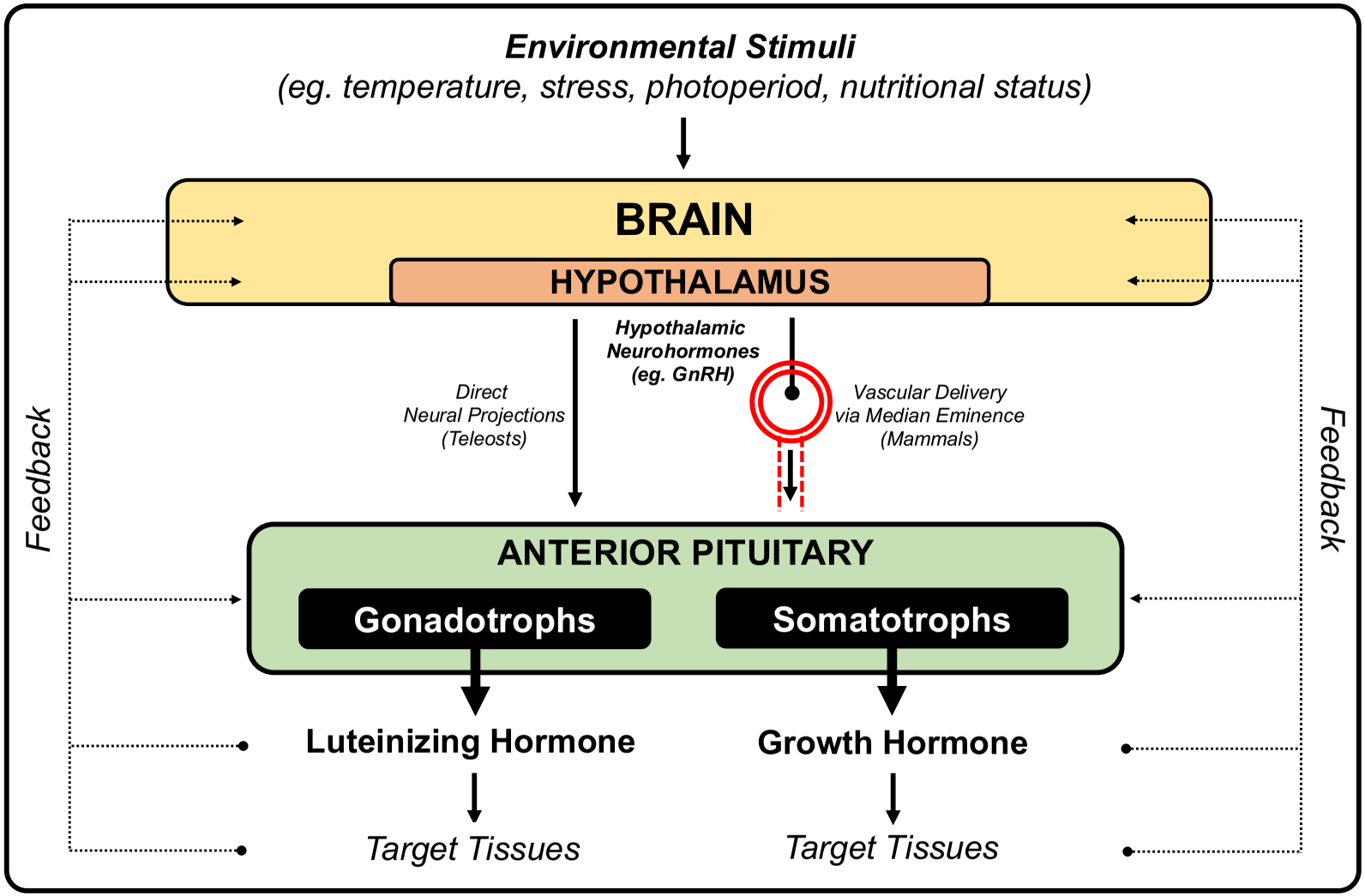
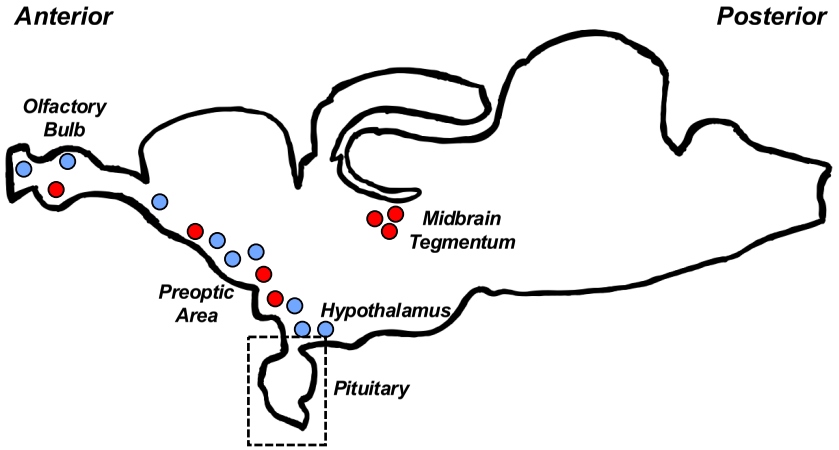

Environmental Stimuli
(eg. temperature, stress, photoperiod, nutritional status)
BRAIN
HYPOTHALAMUS
Hypothalamic Neurohormones (eg. GnRH)
Direct Neural Projections (Teleosts)
Vascular Delivery via Median Eminence (Mammals)
Feedback
Feedback
ANTERIOR PITUITARY
Gonadotrophs
Somatotrophs
Luteinizing Hormone
Growth Hormone
Target Tissues
Target Tissues

Figure 1.2. GnRH neuron distribution and pituitary innervation in goldfish. A. In goldfish (*Carassius auratus*), GnRH2-expressing neuronal cell bodies are primarily concentrated in the midbrain tegmentum region, while the GnRH3-expressing cell bodies are found distributed in the olfactory bulb and along the ventral diencephalic and telencephalic regions, including the ventral preoptic area and basal hypothalamus. GnRH2 and GnRH3 can both also be detected in the pituitary gland itself. B. While GnRH neurons project to several brain regions, the major origins of GnRH neurons projecting to the pituitary gland (boxed area from (A) is expanded) are the preoptic population (GnRH3) and midbrain population (GnRH2). GnRH fibers terminate either in the proximal pars distalis region of the adenohypophysis, where gonadotroph and somatotroph cell populations are located, or at a basal membrane separating the adenohypophysis from neurohypophysis. Adapted from Lethimonier et al., 2004; Muñoz-Cueto et al., 2020; Peter et al., 1990; Zohar et al., 2010.

A. Distribution of GnRH2 and GnRH3 neuron cell bodies in goldfish (Carassius auratus) brain.



B. GnRH neuron fibers terminate in the pituitary gland.

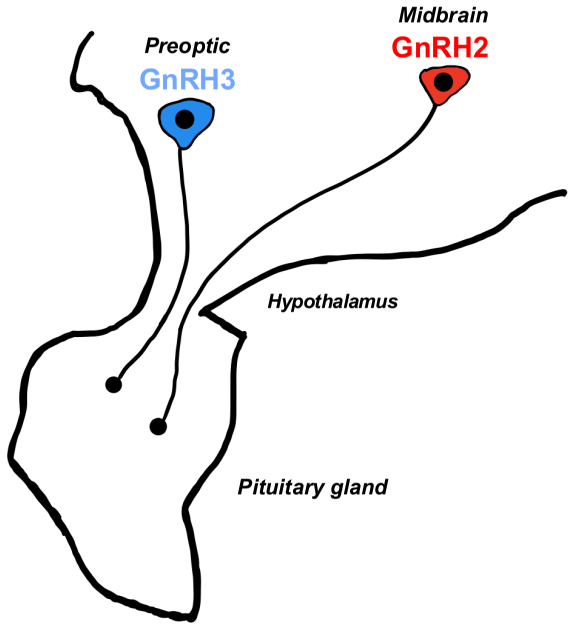


Figure 1.3. GnRHR receptor primary amino acid sequence comparison. The GnRH receptor belongs to the Class A superfamily of G-protein coupled receptors (GPCRs). Goldfish type A (GfA) and type B (GfB) receptors are compared to the human GnRHR (mammalian type I). GPCRs structures span the plasma membrane 7 times (TM: transmembrane domains indicated in red text), with extracellular N-terminus extensions, intracellular (ICL) and extracellular (ECL) loops, and typically a COOH-terminus intracellular tail (all indicated in blue text). Structural motifs/domains highlighted in grey are generally conserved in Class A GPCRs and play roles in receptor activation and transducer binding. Importantly, goldfish GnRHRs retain the Helix8 amphipathic region and intracellular C-terminus tails which are lost in human GnRHRs, which has consequences for intracellular kinase phosphorylation, arrestin-binding, and receptor internalization (see main text; Section 1.4.2). Underneath the sequence alignments, an asterisk (*) indicates positions which have a single, fully conserved residue, whereas a colon (:) indicates conservation of residues with *strongly* similar properties (> 0.5 in the Gonnet PAM 250 matrix). Lastly, a period (.) indicates conservation between groups with *weakly* similar properties (between 0 and 0.5 in the Gonnet PAM 250 matrix). Adapted from Illing et al., 1999 and Flanagan et al., 2017. Receptor domain information was sourced from the GPCR database (https://gpcrdb.org; Pándy-Szekeres et al., 2018).

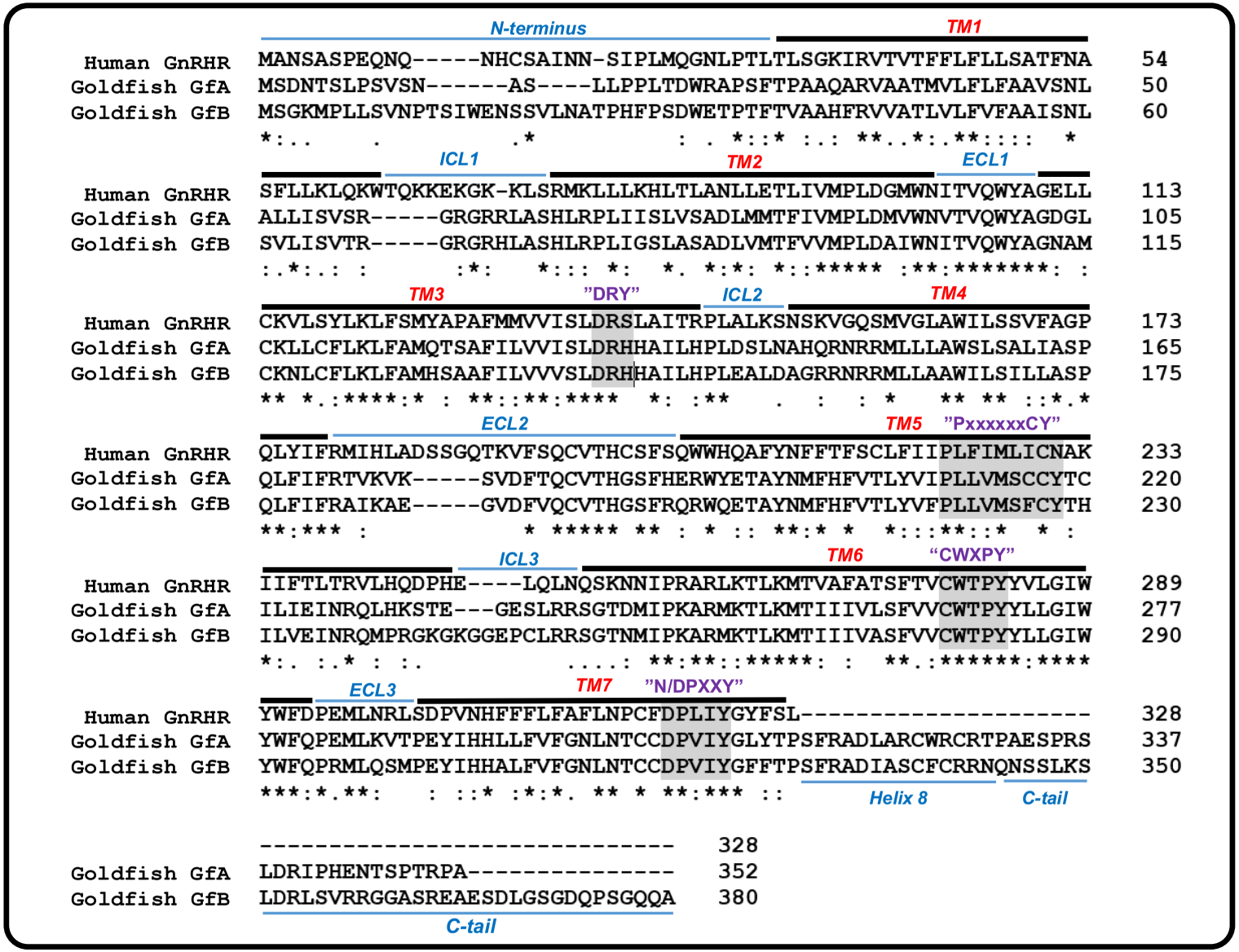

N-terminus
TM1
Human GnRHR MANSASPEQNQ-----NHCSAINN-SIPLMQGNLPTLTLSGKIRVTVTFFLFLLSATFNA 54
Goldfish GfA MSDNTSLPSVSN------AS----LLPPLTDWRAPSFTPAAQARVAATMVLFLFAAVSNL 50
Goldfish GfB MSGKMPLLSVNPTSIWENSSVLNATPHFPSDWETPTFTVAAHFRVVATLVLFVFAAISNL 60
ICL1
TM2
ECL1
Human GnRHR SFLLKLQKWTQKKEKGK-KLSRMKLLLKHLTLANLLETLIVMPLDGMWNITVQWYAGELL 113
Goldfish GfA ALLISVSR-----GRGRRLASHLRPLIISLVSADLMMTFIVMPLDMVWNVTVQWYAGDGL 105
Goldfish GfB SVLISVTR-----GRGRHLASHLRPLIGSLASADLVMTFVVMPLDAIWNITVQWYAGNAM 115
TM3
"DRY"
ICL2
TM4
Human GnRHR CKVLSYLKLFSMYAPAFMMVVISLDRSLAITRPLALKSNSKVGQSMVGLAWILSSVFAGP 173
Goldfish GfA CKLLCFLKLFAMQTSAFILVVISLDRHHAILHPLDSLNAHQRNRRMLLLAWSLSALIASP 165
Goldfish GfB CKNLCFLKLFAMHSAAFILVVVSLDRHHAILHPLEALDAGRRNRRMLLAAWILSILLASP 175
ECL2
TM5
"PxxxxxxCY"
Human GnRHR QLYIFRMIHLADSSGQTKVFSQCVTHCSFSQWWHQAFYNFFTFSCLFIIPLFIMLICNAK 233
Goldfish GfA QLFIFRTVKVK-----SVDFTQCVTHGSFHERWYETAYNMFHFVTLYVIPLLVMSCCYTC 220
Goldfish GfB QLFIFRAIKAE-----GVDFVQCVTHGSFRQRWQETAYNMFHFVTLYVFPLLVMSFCYTH 230
ICL3
TM6
"CWXPY"
Human GnRHR IIFTLTRVLHQDPHE----LQLNQSKNNIPRARLKTLKMTVAFATSFTVCWTPYYVLGIW 289
Goldfish GfA ILIEINRQLHKSTE---GESLRRSGTDMIPKARMKTLKMTIIIVLSFVVCWTPYYLLGIW 277
Goldfish GfB ILVEINRQMPRGKGKGGEPCLRRSGTNMIPKARMKTLKMTIIIVASFVVCWTPYYLLGIW 290
ECL3
TM7
"N/DPXXY"
Human GnRHR YWFDPEMLNRLSDPVNHFFFLFAFLNPCFDPLIYGYFSL---------- 328
Goldfish GfA YWFQPEMLKVTPEYIHHLLFVFGNLNTCCDPVIYGLYTPSFRADLARCWRCRTPAESPRS 337
Goldfish GfB YWFQPRMLQSMPEYIHHALFVFGNLNTCCDPVIYGFFTPSFRADIASCFCRRNQNSSLKS 350
Helix 8
C-tail
---------- 328
Goldfish GfA LDRIPHENTSPTRPA---------- 352
Goldfish GfB LDRLSVRRGGASREAESDLGSGDQPSGQQA 380
C-tail

Figure 1.4. Major, canonical pathways of G protein subunit-specific transduction downstream of activated GPCRs. Following agonist-binding-induced structural rearrangements in GPCRs, binding of G proteins is facilitated through opening of intracellular cavities in the receptor. In inactive states, G protein subunits exist in heterotrimeric complexes (Gαβγ), during which a GDP molecule is bound to Gα. In this conformation, Gαβγ complexes can interact with activated receptors, whereupon the receptor catalyzes GDP exchange for GTP, leading to active, and dissociated, α-GTP and βγ heterodimers, which both influence downstream signalling pathways and cellular functions. Depending on the identity of Gα subunit engaged, specific downstream pathways (colour-coded) can then be stimulated downstream. $G\alpha_s$ subunits typically stimulate, and $G\alpha_i$ subunits inhibit, activity of the adenylyl cyclase (AC) enzyme, respectively, whereas the $G\alpha_{q/11}$ family couples to phospholipase C (PLC) activity, and the $G\alpha_{12/13}$ group is linked to activation of Rho-GEFs. $G\alpha_{q/11}$ are also linked to Rho activation through p63 Rho-GEF, whereas $G\alpha_{i/o}$ subunits can engage Src-family kinases (SFKs). Phospholipase A2 (PLA_2) can be activated downstream of $G\alpha_{q/11}$-PKC actions and is also linked to $G\alpha_{i/o}$-dependent pathways but a direct interaction with $G\alpha_{i/o}$ has not been verified. Known direct binding effectors of Gα are shown associated to the subunits, whereas downstream/indirect targets are linked by a vertical line. Similarly, although the influence of individual isoforms of Gβγ is relatively unknown, these G protein subunit dimers, once dissociated from Gα, influence activity of PI3K (phosphoinositide 3-kinase) p110β and p110γ subunits, G-protein-gated inwardly rectifying potassium (GIRK) channels, as well as GRKs (GPCR kinases), all through direct protein-protein interactions. Importantly, although presented as discrete groupings, considerable heterogeneity exists in GPCR-G protein coupling, and recent studies show that only half of all GPCRs selectively activate one Gα family, while the other half activate isoforms from two or more of the four families (Hauser et al., 2022; Inoue et al., 2019). Figure adapted from Pemberton (2015).

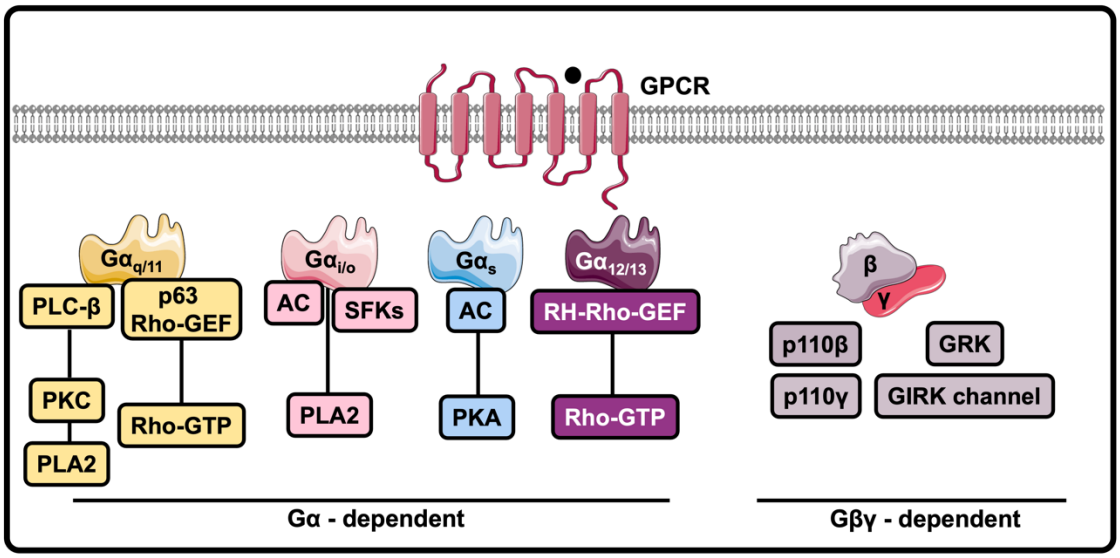

GPCR
Gαq/11
PLC-β
p63 Rho-GEF
PKC
Rho-GTP
PLA2
Gαi/o
AC
SFKs
PLA2
Gαs
AC
PKA
Gα12/13
RH-Rho-GEF
Rho-GTP
Gα - dependent
β
γ
p110β
GRK
p110γ
GIRK channel
Gβγ - dependent

Figure 1.5. Multifaceted roles of β-arrestins in GPCR desensitization and signalling. β-arrestin recruitment to activated GPCRs is mediated by phosphorylation of GPCR intracellular loops and/or C-terminus tails, which then facilitates arrestin binding. Arrestins then compete with Gα for the same receptor-binding sites, which can lead to receptor desensitization (inhibiting further Gα-dependent signalling). In addition, arrestins can target GPCRs to clathrin (Cla)-coated structures/pits (CCS/CCP), through direct interactions with clathrin and the adaptor protein AP2. From here, GPCRs may continue to actively signal through arrestin-dependent scaffolding of effectors (inset; signalling module groups colour coded) in a process called endosomal signalling, be recycled to the plasma membrane, or be targeted to endo-lysosomal degradation pathways. Among the arrestin interactome is the classical MAPK cascade proteins (Raf, MEK, ERK), phospholipid-dependent effectors (PI3K, Akt, and PTEN, a phosphatase for PtdIns(3,4,5)P_3), Src family kinases (SFKs) such as c-Src, phosphodiesterase (PDE) for cAMP, and E3 ubiquitin ligases such as Mdm2 and parkin. This latter interaction is implicated in both ubiquitination of arrestin, which is necessary for assembly of receptor signalling complexes, or alternatively leads to ubiquitination of the arrestin-bound GPCR, leading to proteasomal degradation. Information from Peterson et al., 2017 and Gurevich and Gurevich, 2019b.

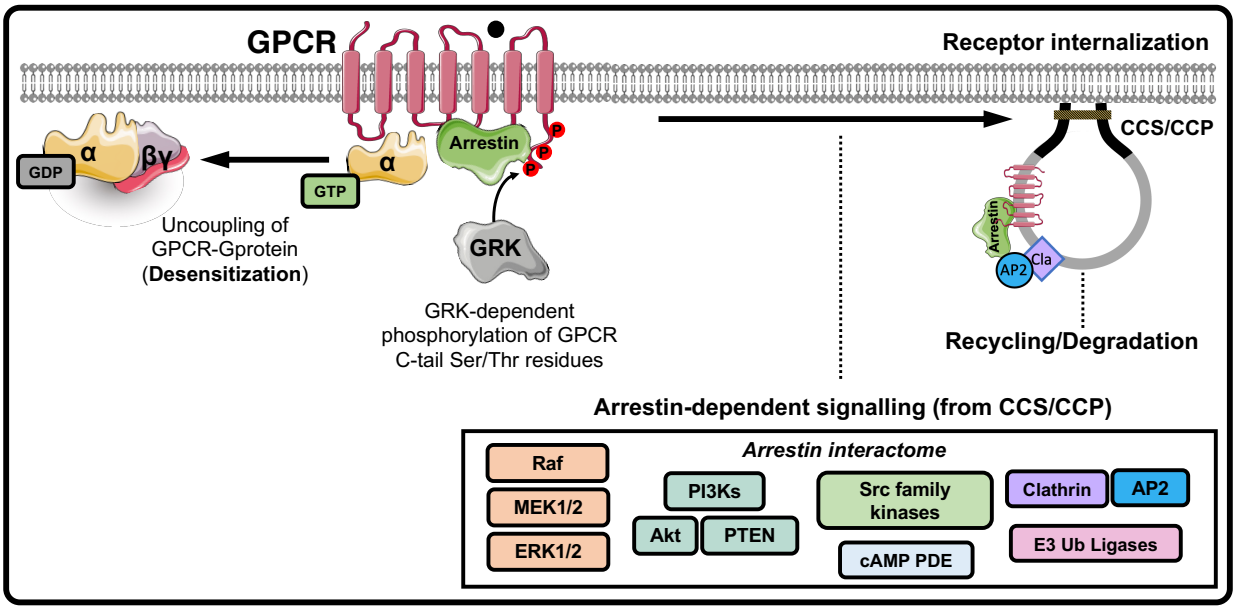

GPCR
Receptor internalization
α
βγ
GDP
GTP
α
Arrestin
P
P
P
GRK
Uncoupling of
GPCR-Gprotein
(Desensitization)
GRK-dependent
phosphorylation of GPCR
C-tail Ser/Thr residues
CCS/CCP
Arrestin
AP2
Cla
Recycling/Degradation
Arrestin-dependent signalling (from CCS/CCP)
Arrestin interactome
Raf
MEK1/2
ERK1/2
PI3Ks
Akt
PTEN
Src family
kinases
cAMP PDE
Clathrin
AP2
E3 Ub Ligases

Figure 1.6. Common signal transduction mechanisms utilized by goldfish GnRHRs. GnRH2 (red) and GnRH3 (blue; primary amino acid sequences shown in top left corner) bind and activate cell-surface receptors (GnRHRs) from the Class A GPCR superfamily. Following receptor-binding and activation, a number of pathways are activated within goldfish pituitary cells. For example, the canonical $G\alpha_{q/11}$-linked recruitment of phospholipase C-β (PLC-β) and the subsequent production of protein kinase C (PKC) are generally involved in both pituitary gonadotrophs and somatotrophs, and engaged by both GnRH isoforms, but cell-type and ligand-specific dependent differences in the usage of other effectors exist (see Table 1 for examples). Similarly, while Ca^{2+}-dependent mechanisms are generally important to the control of hormone release, variation exists in the specific intracellular stores and release channels involved. Additionally, the regulation of acute hormone release responses can also be uncoupled from hormone synthesis and longer term hormone release and synthesis responses can also further vary in a time-dependent fashion (Chang et al., 2009, 2012; Pemberton et al., 2015). Overall, while considerable information exists regarding major intracellular effectors and their roles in pituitary hormone secretion, the possible upstream receptor-associated transducers (unlabelled units in shaded box below GnRHR) are relatively understudied in this system. Figure adapted from Pemberton (2015).

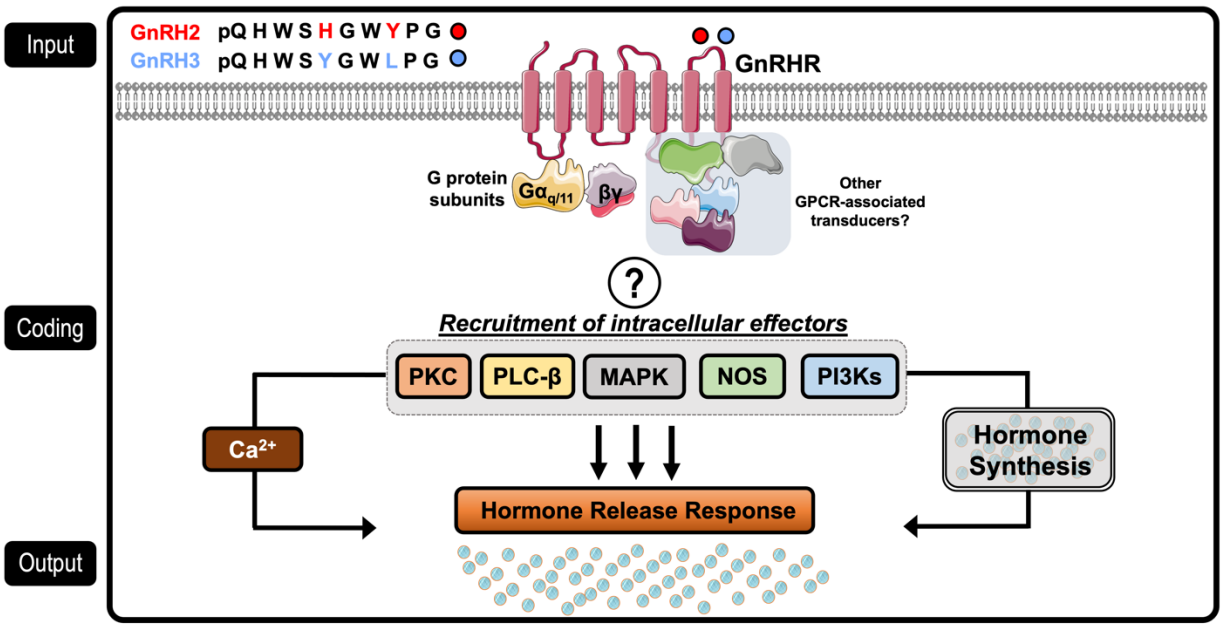

Input
Coding
Output
GnRH2 pQ H W S H G W Y P G
GnRH3 pQ H W S Y G W L P G
GnRHR
G protein subunits
Gαq/11
βγ
Other GPCR-associated transducers?
?
Recruitment of intracellular effectors
PKC
PLC-β
MAPK
NOS
PI3Ks
Ca2+
Hormone Synthesis
Hormone Release Response

Figure 1.7. Roles of small monomeric G proteins (GTPases) in secretory cells. Select functions of the small GTPase proteins Arf1, Arf6, Rac, Rho, and Ras are presented, with an emphasis on those functions relevant to exocytosis/recycling pathways in secretory cell types, as well as known links to GPCR-dependent signal transduction. In particular, Rho GTPases have well-established links to $G\alpha_{q/11}$ and $G\alpha_{12/13}$ subunits, whereas Arf and Rac GTPases are typically activated downstream of PI3K (phosphoinositide 3-kinase) actions and the subsequent generation of the rare membrane phospholipid PtdIns(3,4,5)P_3 (PIP_3). Arf and Rac GTPases can also be sequentially activated by each other, and Rac can sequentially activate Rho GTPases as well. Regardless, all three are known to play major roles in the control of F-actin dynamics, and Rho and Rac also influence secretory vesicle transport along microtubules, whereas Arf1 is known to regulate secretory vesicle budding from the trans-Golgi network. On the other hand, Ras GTPase leads to activation of the MAPK module, and can be activated through multiple different mechanisms downstream of GPCRs (refer to Figure 1.9). *Inset:* Activation mechanisms for the superfamily of small G proteins are generally the same (Rac is shown as an example). Upstream guanine-nucleotide exchange factors (GEFs) catalyze GDP/GTP exchange, leading to activation of GTPases, whereas GTPase-activating proteins (GAP) accelerate the GTP hydrolysis reaction, promoting the transition to the inactive GDP-bound state.

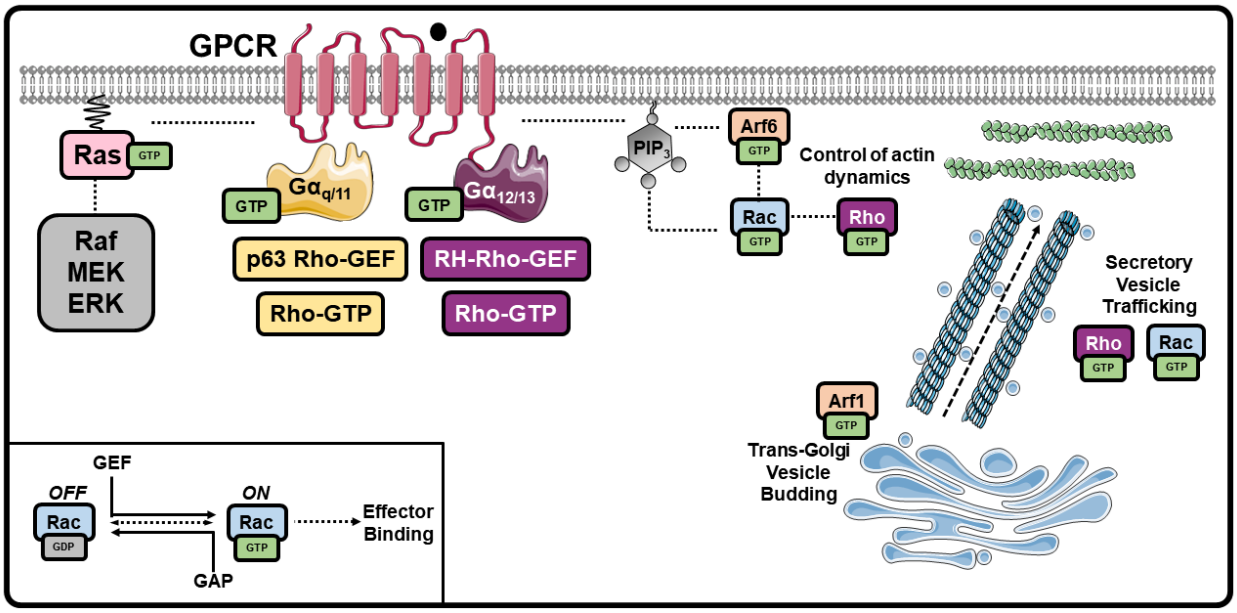

GPCR
Ras
GTP
Raf
MEK
ERK
GTP
$G\alpha_{q/11}$
GTP
$G\alpha_{12/13}$
p63 Rho-GEF
Rho-GTP
RH-Rho-GEF
Rho-GTP
PIP_3
Arf6
GTP
Control of actin dynamics
Rac
GTP
Rho
GTP
Secretory Vesicle Trafficking
Rho
GTP
Rac
GTP
Arf1
GTP
Trans-Golgi Vesicle Budding
GEF
OFF
Rac
GDP
ON
Rac
GTP
Effector Binding
GAP

Figure 1.8. Mechanisms of receptor tyrosine kinase (RTK) transactivation by GPCRs. Following receptor activation and initiation of G-protein dependent signalling, there are two major routes leading to subsequent activation of receptor tyrosine kinases. One or more engaged intracellular "relay" molecules (orange box) can lead to activation of membrane-expressed protease enzymes (MMP: matrix metalloproteinase; ADAM: A Disintegrin and Metalloproteinase), which can cleave inactive growth factor ligands tethered to the membrane (i.e., pro-ligand), thus allowing the now released growth factor for growth factor receptor activation often on the same cell, leading to engagement of typical growth factor mitogenic cascades (e.g., MAPK: the Raf/MEK/ERK module indicated here; and/or the PI3K-Akt module). Alternatively, intracellular protein kinases (PKC, Src, Pyk2) within the subset of relay molecules can directly phosphorylate intracellular domains on RTKs, although this mechanism is not as prevalent or well characterized. Adapted from George et al., 2013.

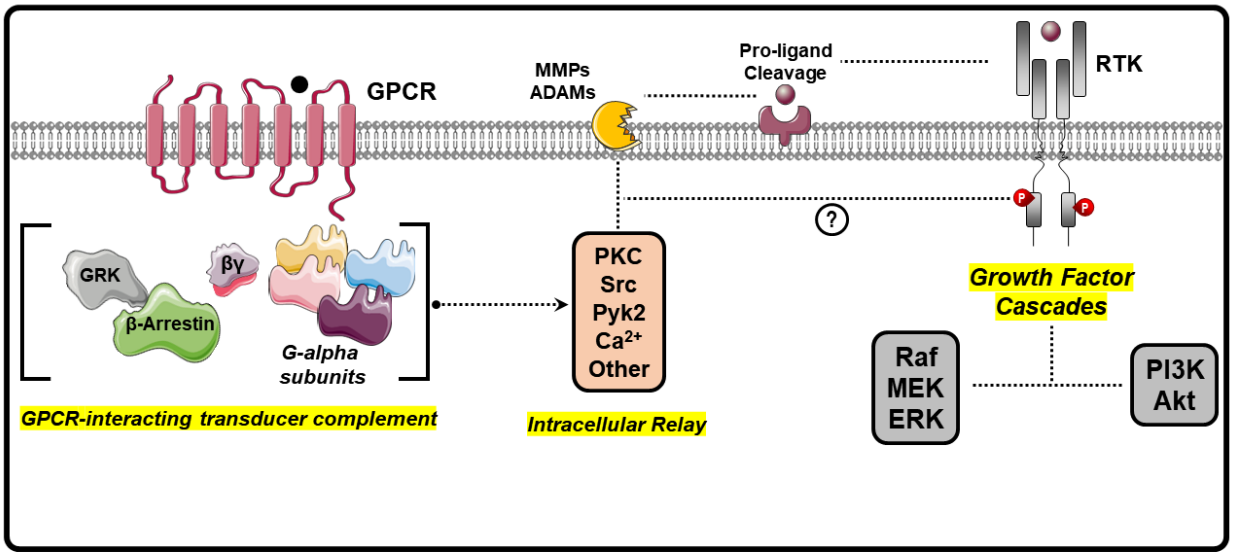
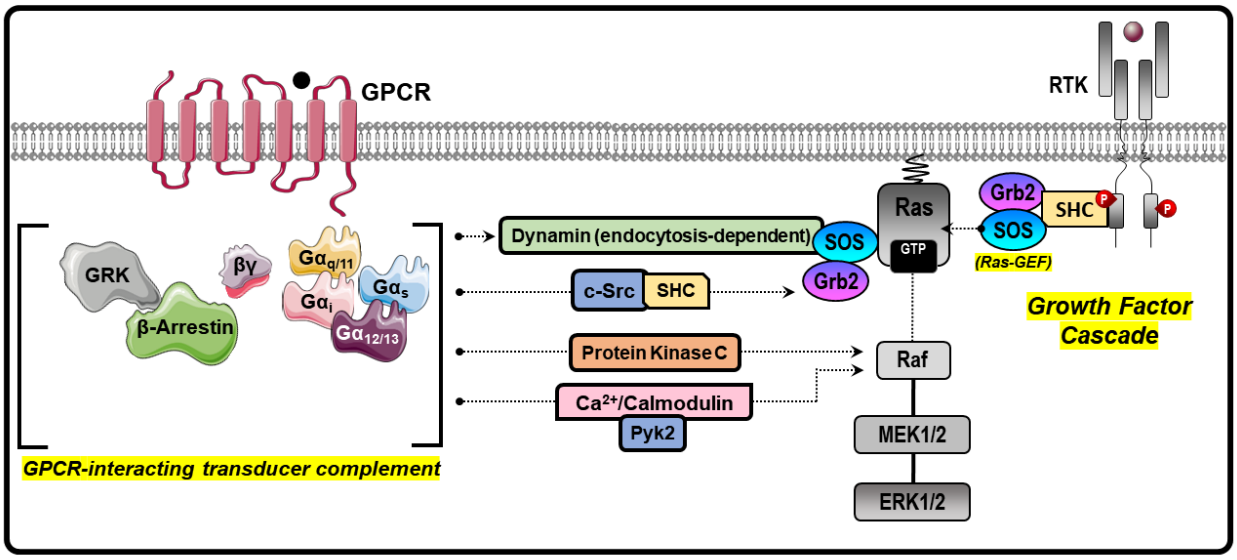

GPCR
MMPs
ADAMs
Pro-ligand
Cleavage
RTK
GRK
βγ
β-Arrestin
G-alpha
subunits
GPCR-interacting transducer complement
PKC
Src
Pyk2
Ca2+
Other
Intracellular Relay
?
Growth Factor
Cascades
Raf
MEK
ERK
PI3K
Akt

Figure 1.9. Receptor-mediated pathways leading to mitogen-activated protein kinase (MAPK) cascade activation. The canonical MAPK cascade is a major effector of GnRH-dependent actions across vertebrate model systems. Shown here is the cascade for one of the three major MAPK families, that of extracellular regulated protein kinase (ERK). This module integrates several membrane-receptor inputs and is organized in a tiered fashion, where activation of the upstream kinase leads to activation of the subsequent kinase, i.e., Raf (MAPKKK) phosphorylates MEK (MAPKK) which then phosphorylate ERK (MAPK). Depending on the specific isoform, the lipid-anchored small GTPase Ras is tethered to the plasma membrane through the use of either S-palmitoyl cysteine residues or polybasic motifs, and activated Ras leads to recruitment of cytosolic Raf kinase and subsequently plays a role in its activation. A number of possible mechanisms exist leading to initiation of this module from receptor-dependent pathways. Typically, the ubiquitous Ras-GEF SOS (son-of-sevenless) and Grb2 adaptor are required to initiate the cascade by conversion of Ras-GDP to Ras-GTP. Growth factor receptors (often receptor tyrosine kinases, RTKs) can interact with SOS/Grb2 through phosphotyrosine-based SHC adaptors, whereas GPCR-dependent pathways can utilize the tyrosine kinase c-Src, or arrestin/dynamin mechanisms (coded in green) (and possibly also in concert with Src) to engage with Grb2 and SOS. Recruitment of arrestin classically involves GPCR kinase (GRK). Alternatively, other GPCR-initiated pathways such as PKC-, Ca^{2+}/calmodulin- and Pyk2-dependent mechanisms can bypass Ras, and directly act at the MAPKKK level (Raf kinase), which ultimately also leads to MEK-ERK activity and subsequent functions. In the goldfish pituitary, GnRH is known to activate PKC, which is also upstream of MEK-ERK, but other possible pathways, including the potential contribution of RTK transactivation (see Figure 1.8) have not been evaluated.


GPCR
RTK
GRK
βγ
β-Arrestin
Gαq/11
Gαi
Gαs
Gα12/13
GPCR-interacting transducer complement
Dynamin (endocytosis-dependent)
SOS
Grb2
c-Src
SHC
Protein Kinase C
Ca2+/Calmodulin
Pyk2
Ras
GTP
Raf
MEK1/2
ERK1/2
Grb2
SOS
SHC
(Ras-GEF)
Growth Factor Cascade

Chapter Two

Materials and Methods

2.1 Animal care and maintenance

Animal use protocols were approved by the University of Alberta Animal Care and Use Committee (AUP #000080) and were carried out in accordance with Canadian Council for Animal Care (CCAC) guidelines. Post-pubertal, 4-5 inch male and female goldfish (*Carassius auratus*) were purchased from Aquatic Imports (Calgary, Canada), which sources their fish from the USA. Upon arrival, fish were maintained in flow-through aquaria at 18 °C, under a simulated natural Edmonton, AB, Canada photoperiod. Within each aquarium, fish of both sexes were held together. Fish were fed to satiation daily (Mazuri® Koi Platinum Bits; PMI Nutrition International) by staff of the Faculty of Science Animal Support Services (SASS). Fish were allowed to acclimate to these conditions for a minimum of 1 week prior to being used as pituitary donors and were typically used within six weeks of arrival.

2.2 Dispersed cell preparations from goldfish pituitary glands

On the day prior to experiments, goldfish of both sexes were anesthetized using 0.05% tricane methane-sulfonate (Syndel Laboratories, Vancouver, BC) buffered with 0.1% sodium bicarbonate, and cervically transected. Pituitary glands were removed and placed in ice-cold dispersion medium (Medium 199 with Hank's salts, Gibco; supplemented with 26 mM NaHCO3, 25 mM HEPES, 100 mg/L streptomycin, 100,000 units/L penicillin and 0.3% bovine serum album (BSA); pH 7.2). Pituitaries were then washed three times with room temperature dispersion medium and diced into fragments of approximately 1 mm^3 using a #10 surgical scalpel blade, and subsequently processed using an established trypsin/DNAse cell enzymatic dispersion protocol (Chang et al., 1990a). At the end of the dispersion, cells were re-suspended in calcium-free medium (Medium 199 with Hank's salts, without calcium chloride anhydrous,

Gibco; supplemented with 25 mM HEPES, 51.2 mM NaCl, 100 mg/L streptomycin, 100,000 units/L penicillin and 0.3% BSA; pH 7.2) to minimize cell clumping, and a 10 μL aliquot of the suspension was used to assess cell yield and viability (Trypan Blue exclusion; viability was routinely >98%). 1.5 x 10^6 dispersed cells were then allowed to attach onto preswollen Cytodex beads (Cytodex 1, 131-220 μm; MilliporeSigma) in 6 mL of plating medium (Medium M199 with Earle's salts, Gibco, containing 26 mM $NaHCO_3$, 25 mM HEPES, 100 mg/L streptomycin, 100,000 U/L penicillin, 1% (vol/vol) horse serum, pH 7.2), and incubated overnight (28 °C, 5% CO_2, saturated humidity) in 60 x 15 mm petri dishes prior to use in perifusion studies the following day. Alternatively, for immunoblotting experiments, 2 x 10^6 dispersed goldfish pituitary cells were cultured overnight in flat-bottomed tubes (TPP 10, Techno Plastic Products AG, Switzerland) in 15 mL plating medium. For perifusion experiments, pituitaries from approximately 25 fish were typically collected for a single experiment, whereas approximately 30 fish pituitaries were pooled in preparations used for immunoblotting studies.

2.3 Mammalian cell line culture

AD293 (a derivative of HEK293 cells; Stratagene) cells were provided by Dr. James Stafford (Dept. of Biological Sciences, University of Alberta) and used as representative mammalian controls for expression of intracellular proteins in Western blot analysis (Section 2.7). Cells were grown at 37 °C and 5% CO_2 in complete Dulbecco's modified Eagle's medium (Gibco) containing 10% (vol/vol) heat-inactivated fetal bovine serum (FBS; Sigma #F1051), 100,000 U/L penicillin and 100 mg/L streptomycin (Gibco) in 6-well BioLite microwell cell culture plates (Thermo Scientific).

2.4 Drugs and Reagents

2.4.1 GnRH peptides

cGnRH-II ([His^5, Trp^7, Tyr^8]GnRH; GnRH2) and salmon GnRH ([Trp^7, Leu^8]GnRH; GnRH3) were purchased from Bachem (Torrance, CA) and 100 μM stock solutions were made up in ddH2O, and stored at -20 °C. Stock solutions of GnRH were diluted down (1:1000 vol/vol) to working experimental concentrations prior to use in testing medium (Medium 199 with Hank's salts, Gibco; 26 mM $NaHCO_3$, 25 mM HEPES, 100 mg/L streptomycin, 100,000 U/L penicillin, 0.1% BSA; pH 7.2).

2.4.2 Pharmacological inhibitors

All compounds utilized in this thesis were obtained from suppliers in powder form, and stock solutions were prepared in dimethyl sulfoxide (DMSO) for storage at -20 °C. Inhibitors were then diluted down to working concentrations (1:1000 vol/vol) in testing medium. Final concentrations of DMSO solvent did not exceed 0.1% (vol/vol), a concentration at which there is no measurable effect on LH and GH release, as well as goldfish pituitary cell membrane electrophysiological properties (Johnson & Chang, 2000; Johnson et al., 2002; Wong et al., 1992).

The β-arrestin inhibitor, Barbadin (3-Azanyl-5-[4-(phenylmethyl)phenyl]thieno[2,3-d]pyrimidin-4-one), the Arf1/6 inhibitor NAV-2729 [3-(4-Chlorophenyl)-5-(4-nitrophenyl)-2-(phenylmethyl)pyrazolo[1,5-*a*]pyrimidin-7(4*H*)-one], the Rac1 inhibitor EHT 1864 [5-(5-(7-(Trifluoromethyl)quinolin-4-ylthio)pentyloxy)-2-(morpholinomethyl)-4*H*-pyran-4-one dihydrochloride], the RhoA inhibitor Rhosin [D-Tryptophan (2*E*)-2-(6-quinoxalinylmethylene)hydrazide hydrochloride], the SOS-Ras interaction inhibitor BAY-293

[(*R*)-6,7-Dimethoxy-2-methyl-*N*-[1-[4-[2-[(methylamino)methyl]phenyl]thiophene-2-yl]ethyl]quinazolin-4-amine], the Src inhibitor DGY-06-116 [4-((2-acrylamidophenyl)amino)-N-(2-chloro-6-methylphenyl)-2-((4-(4-methylpiperazin-1-yl)phenyl)amino)pyrimidine-5-carboxamide] and the Pyk2/FAK inhibitor PF-562271 [N-[3-[[[2-[(2,3-Dihydro-2-oxo-1H-indol-5-yl)amino]-5-(trifluoromethyl)-4-pyrimidinyl]amino]methyl]-2-pyridinyl]-N-methyl-methanesulfonamide] were purchased from Cedarlane (Burlington, ON). The dynamin inhibitor, Dyngo4a (3-Hydroxy-*N*′-[(2,4,5-trihydroxyphenyl)methylidene]naphthalene-2-carbohydrazide) was purchased from Abcam (Cambridge, MA). The $G\alpha_{q/11}$ inhibitor YM-254890 ((*R*)-1-((3*S*,6*S*,9*S*,12*S*,18*R*,21*S*,22*R*)-21-acetamido-18-benzyl-3-((*R*)-1-methoxyethyl)-4,9,10,12,16,22-hexamethyl-15-methylene-2,5,8,11,14,17,20-heptaoxo-1,19-dioxa-4,7,10,13,16-pentaaza-cyclodocosan-6-yl)-2-methylpropyl (2*S*,3*R*)-2-acetamido-3-hydroxy-4-methylpentanoate) was purchased from Focus Biomolecules (Plymouth Meeting, PA). The pan-Gα inhibitor BIM-46187 [(S,2R,2′R)-3,3′-Disulfanediylbis(2-amino-1-((S)-8-(cyclohexylmethyl)-2-phenyl-5,6-dihydroimidazo[1,2-a]pyrazin-7(8H)-yl)propan-1-one) Tetrahydrochloride], the $G\alpha_{i/o}$ inhibitor pertussis toxin, the matrix metalloproteinase (MMP) inhibitor GM6001 [N-[(2R)-2-(hydroxamidocarbonylmethyl)-4-methylpentanoyl]-L-tryptophan methylamide] and the EGFR inhibitor BIBW-2992 (Afatinib; (2E)-N-[4-[(3-Chloro-4-fluorophenyl)amino]-7-[[(3S)-tetrahydro-3-furanyl]oxy]-6-quinazolinyl]-4-(dimethylamino)-2-butenamide, (S,E)-N-(4-(3-Chloro-4-fluorophenylamino)-7-(tetrahydrofuran-3-yloxy)quinazolin-6-yl)-4-(dimethylamino)but-2-enamide) were purchased from Sigma-Aldrich (St. Louis, MO)**.** The GRK2/3 inhibitor CMPD101 **(**3-[[[4-Methyl-5-(4-pyridinyl)-4*H*-1,2,4-triazol-3-yl]methyl]amino]-*N*-[[2-(trifluoromethyl)phenyl]methyl] benzamide) was purchased from Tocris (Bristol, United Kingdom)

2.4.3 Antibodies

Antibodies against phosphorylated (p)-ERK$^{Tyr202/Thr204}$ (D13.14.4E Rabbit mAb #4370), total ERK (137F5 Rabbit mAb #4695S), phosphorylated (p)-Src^{Tyr416} (D49G4 Rabbit mAB #6943), endogenous Src (36D10 Rabbit mAb #2109), RhoA (clone 67B9), Rac (#2465 polyclonal), Ras (clone 27H5), and β-actin (#4697 Rabbit polyclonal) were all purchased from Cell Signaling Technologies (Whitby, ON). Arf1 antibody (NBP2-29906, polyclonal) was purchased from Novus Biologicals (Centennial, CO, USA). Secondary antibody for immunoblot visualization, goat anti-rabbit IgG (H+L)-HRP conjugate, was purchased from BioRad (Mississauga, ON).

2.5 Cell column perifusion hormone release studies

Following overnight culture as described in Section 2.2, cytodex beads with dispersed goldfish pituitary cells attached were transferred from culture dishes to 50 mL centrifuge tubes and washed (200 x g; 10 min) in testing medium, before being loaded into temperature-controlled columns (1.5 x 10^6 cells/column, 18 °C, 500 µL chamber volume) to be perifused with testing medium (15 mL/h flow rate). Following a 4-h perifusion to stabilize secretion levels, the experiment was started, with 5-min fractions collected for the duration of the 2-h experiment. For the columns receiving pharmacological treatments, inhibitors were administered continuously between 25 and 90 min, and a 5-min pulse of GnRH was delivered between 45-50 min (in the presence or absence of inhibitor as appropriate). Release responses to the GnRH pulse commence at 5-6 min following application based on the flow rate and dead volume of the perifusion system (Wong et al., 1992). For experiments utilizing pertussis toxin in Chapter 4,

inhibitor was included during overnight culture and during the 4-h acclimation period for those treatment groups receiving inhibitor. All perifusion experiments were carried out in a dark room to minimize light-induced damage to reagents. Perifusate fractions were collected and stored at -20 °C for future analysis using radioimmunoassay. Perifusion treatments were done in duplicate within each experimental run and each experiment was repeated a minimum of four times using four independent cell preparations.

2.5.1 Analysis of acute GnRH-induced hormone release

LH and GH release levels from individual perifusion columns were expressed as a percentage of the corresponding pretreatment value (%pretreatment), which refers to the average of the first 5 fractions collected, during which cells are exposed to media alone. This enables pooling of results from multiple cell preparations and experimental columns without distorting the shape of hormone release kinetics. Secretion responses to the pulse application of GnRH were quantified as net response of LH or GH levels, calculated as the area under the response curve with baseline subtraction (baseline defined as the average of hormone values in the three pre-pulse fractions collected prior to the time of GnRH application) for fractions with values higher than mean + 1 SEM of the average baseline value (Wong et al., 1992). Accordingly, the net responses for treatment columns with inhibitor alone or inhibitor + GnRH were quantified over the same period (Figure 2.1).

2.5.2 Analysis of basal hormone release

In some cases, the effects of pharmacological inhibitors on basal (unstimulated) LH and GH release were also evaluated by comparing the average of %pretreatment values over the

duration of inhibitor treatment (30-95 min) to the average of values from 0 to 20 min (during which cells are exposed to media alone) within the same matched columns.

2.6 Radioimmunonassays for measurement of LH and GH levels

2.6.1 Barbital buffer-based radioimmunoassay for GH

Measurement of growth hormone (GH) levels in collected perifusion samples was performed using an established radioimmunoassay (Marchant et al., 1987). Radioactive GH tracer was made using 5 μg of recombinant carp GH protein (rcGH; GenWay Biotech Inc., CA) iodinated with 1 μCi ^{125}I using lactoperoxidase (Sigma; Oakville, ON). ^{125}I-labeled GH was column purified with G-50 fine Sephadex beads (Sigma; Oakville, ON) and eluted using diluent (0.08 M barbital buffer (5g/L Na barbital, 3.25g/L Na acetate.3H_2O, 0.1g/L thimerosal; pH 8.6 with HCl) with 0.5% BSA added).

Tracer was also prepared in diluent, resulting in known amounts of radioactivity (13,000 to 15,000 cpm per 100 μL tracer). Perifusion samples (adjusted to 25 μL with diluent as needed to bring sample values into range; normally 15 μL sample + 10 μL diluent; samples measured in duplicate) were pipetted on ice with 100 μL of rabbit anti-carp GH antibody (Marchant et al., 1987; antibody subsequently registered as AB_2631195) at a 1:50,000 dilution, in diluent containing 2% normal rabbit serum, followed by incubation at 4 °C in for 20 h. Volume-matched GH standard curves (rcGH; 0.32 to 200 ng/mL) including zero hormone tubes (antibody in diluent alone), and tubes used to assess non-specific binding (2% normal rabbit serum in diluent) were also pipetted in parallel at the beginning and end of each set of samples. Following a 20 h incubation, 100 μL of ^{125}I-GH tracer was then added to all tubes, including a set of total count tubes (tracer alone) followed by a further incubation at 4 °C for 20 h. Then, 200 μL of goat-anti

rabbit gamma globulin secondary antibody (1:14 initial dilution in diluent; Antibody Incorporated #51-155) was added to induce precipitation of antibody-bound hormone over a 20 h incubation period at 4 °C. Finally, tubes were centrifuged at 1,000 x g for 20 min and 4 °C to pellet the bound primary-secondary antibody complex, decanted to remove the unbound fraction, and the radioactivity within was measured using a gamma counter (Packard Cobra II).

2.6.2 Barbital buffer-based radioimmunoassay for LH

LH tracer was prepared using 5 μg of purified carp LH (gifted by the late Dr. R.E. Peter, Department of Biological Sciences, University of Alberta; formerly called maturational gonadotrophin 2 or GTH-II) and 1 μCi ^{125}I, using a modified chloramine-T method (Peter et al., 1984). ^{125}I-labeled LH was column purified with G-50 fine Sephadex beads, eluted using 0.08 M barbital buffer, and tracer prepared in diluent, resulting in known amounts of radioactivity (15,000 to 17,000 cpm per 200 μL tracer). Perifusion samples, adjusted to 50 μL volume as needed to bring values in samples into assay range (normally 25 μL sample + 25 μL diluent; samples measured in duplicate), were pipetted on ice with 200 μL of LH antibody (rabbit anti-carp cGTH-378; produced at University of Alberta by the late Dr. R.E. Peter, subsequently registered as AB_2631196) at a 1:220,000 dilution, in diluent containing 1% normal rabbit serum. Standard curves comprising volume-matched LH standards (purified carp pituitary LH; 0.16 to 100 ng/mL), zero hormone tubes (antibody in diluent alone), and tubes used to assess non-specific binding (1% normal rabbit serum in diluent) were pipetted in parallel at the beginning and end of each set of samples. On the same day, 200 μL of ^{125}I-LH tracer was added to all tubes containing samples/standards mixed with primary antibody, and incubated for 48 h at 4 °C. Next, 200 μL of goat-anti rabbit gamma globulin secondary antibody (1:14 initial dilution

in diluent; Antibody Incorporated #51-155) was added. Following a 20-h incubation at 4 °C, tubes were centrifuged and pellet radioactivity measured as above (2.6.1).

2.6.3 Modifications to Barbital-based RIA

Due to a recent shortages in sodium barbital availability and resulting difficulty in obtaining this chemical (Cooney and Titcombe, 2022), which necessitated development of alternative buffer systems for LH and GH RIAs, we tested a Na 150 mM / Tris 50 mM (hereafter denoted as Na/Tris) buffer, and a Tris 25 mM (hereafter denoted as Tris) buffer, both at pH 8.6, with 0.1g/L thimerosal and 5% BSA as in the reference barbital buffer systems. Na/Tris and Tris are common buffers for many protein purification and analysis systems, as well as replacements for barbital buffer in some applications (DeCaprio and Kohl, 2017; Laemmli, 1970; Ambler and Rodgers, 1980; Monthony et al., 1978; Petzold and Shaw, 2007). Both Na/Tris and Tris buffers were evaluated in each assay (LH and GH) against the reference barbital buffer system across a range of reference hormone standards (recombinant carp (rc)GH for the GH assay, Marchant et al., 1987; purified carp maturational gonadotropin 2 (GTH-II, LH) for the LH assay, Peter et al., 1984), serial dilutions of pituitary homogenate, and perifusion samples. All other conditions were kept the same as per the previously validated RIAs (GH: Marchant et al., 1987; LH: Peter et al., 1984), including the iodination procedures, sephadex column purification of iodinated hormones, primary and secondary antibodies, concentrations and incubation times, as well as radioactive tracer amounts. Cross-reactivity was tested against several biochemically purified teleost pituitary hormone preparations gifted by the late Dr. R.E. Peter (Department of Biological Science, University of Alberta): carp pituitary glycoproteins (GP1, predicted to be FSH; GP2, i.e., LH or maturational GTH-II used in the LH radioimmunoassays; and GP3, presumable

thyrotropin; Van Der Kraak et al., 1992), carp PRL (Miyajima et al., 1988) chum salmon GH (Kawauchi et al., 1986), and salmon gonadotropin (SG-G100; Donaldson et al., 1972).

2.6.4 Modified GH RIA: Tris 25 mM buffer system

For the GH assay, Tris buffer was deemed a suitable replacement, based on the observed parallelism on log-logit plots for full standard curves (rcGH, 0.32 ng/mL to 200 ng/mL) as well as serial dilutions of goldfish pituitary homogenate (Figure 2.2). The Tris buffer was also a better match with regard to binding properties of the assay (% of tracer bound at zero hormone - Barbital: 34.0%, Tris: 34.6%, Na/Tris: 19.5%). Intra- and inter-assay variability in the GH assay utilizing Tris buffer were 4.7% and 4.7%, respectively, when tested using perifusion samples and rcGH standards. Overall, samples measured in the Tris system have values of 112.3 ± 5.9% (mean ± SEM) of those measured with the reference barbital buffer system across a range of 0.48 ng/mL to 76.5 ng/mL over 36 samples. No displacement of the GH tracer was observed with any of the fish hormones listed above in full curves (0.64 to 200 ng/mL) with the Tris buffer system.

2.6.5 Modified LH RIA: Na 150 mM / Tris 50 mM buffer system

For the LH assay, the Na/Tris system was deemed an appropriate substitute for the barbital buffer-based RIA. Despite both Tris and Na/Tris buffers showing parallelism in terms of displacement in the standard curves in the LH assay, the binding properties of the Na/Tris buffer was a closer match to the reference system (Barbital: 37.6%, Na/Tris: 34.4%, Tris: 51.3%). Thus, the Na/Tris buffer system was chosen for further validation. Samples assayed in the Na/Tris buffer (ranging from 0.33 ng/mL to 52 ng/mL LH concentration over 24 samples) measured at 100.4 ± 5.0% (mean ± SEM) of the reference values assayed in barbital buffer. As with the

standard curves, pituitary dilutions in the Na/Tris and barbital buffers displayed parallelism on log-logit plots (Figure 2.3). Inter- and intra-assay variation in the LH assay utilizing the Na/Tris buffer were 3.7% and 4.7%, respectively. In testing for cross-reactivity, GP1 and SG-G100 showed some displacement of the LH tracer; however, parallelism was not observed. Cross-reactivity for GP1 was 0.1 relative to maturational GTH-II (LH) based on ED_{50} values, and was comparable to prior immunological characterization of these two hormones in the barbital buffer-based system (Van Der Kraak et al., 1992). SG-G100 displaced the LH tracer only at very high concentrations, and cross-reactivity was 0.0064 based on ED_{80} values.

Based on the tested parameters, these Tris and Na/Tris substitute buffers were thereafter used to assay perifusion samples for GH and LH concentrations, respectively, for all experiments in Chapter 5 (small GTPases), and the experiments employing BIBW-2992 (EGFR inhibitor) and PF-562271 (Pyk2/FAK inhibitor) in Chapter 6, where samples for all other experiments were analyzed using the barbital buffer-based assays.

2.7 Immunoblotting experiments and western blot analysis

Following overnight culture in flat-bottomed culture tubes (Section 2.2), dispersed pituitary cells were collected, spun down (200 x g, 10 min) and resuspended in 10 mL of testing medium. Following exposure to inhibitor pre-treatment (or equivalent DMSO vehicle) for 30 min, GnRH2 or GnRH3 was added (final concentration of 100 nM; 5 min exposure) for treatment groups receiving agonist stimulation. Treatments were terminated by placing the tubes on ice and the addition of ice-cold PBS (phosphate-buffered saline). Cells were then washed twice with 10 mL PBS (200 x g, 10 min per spin), and the cell pellet resuspended in ice-cold lysis buffer (50 mM Tris-Cl, 150 mM NaCl, 0.1% nonylphenoxypolyethoxy-ethanol) containing

complete EDTA-free protease inhibitor cocktail and phosSTOP phosphatase inhibitor cocktail (Roche; Mississauga, ON). Cells were then sonicated (VirSonic 100 Ultrasonic Cell Disrupter; VirTis) in lysis buffer, and the lysates agitated for 30 min at 4 °C, before being centrifuged at 16,000 x g for 20 min. Lysate supernatants were diluted 1:1 with 2x reducing buffer (Laemmli sample buffer with 2β-mercaptoethanol; Bio-Rad) and boiled for 10 min at 95 °C. Lysates from AD-293 cells, where applicable, were processed in the same manner. Proteins were separated on 10% polyacrylamide SDS-PAGE gels and transferred to 0.45 μm Trans-Blot Pure nitrocellulose membranes (Bio-Rad), followed by PonceauS staining to verify transfer efficiency. Membranes were then blocked for 60 min at 21 °C in blocking buffer (1x Tris-buffered saline and 0.1% Tween 20 (TBS-T) with 5% BSA), followed by overnight incubation at 4 °C with primary antibodies against p-ERK (1:2500), total-ERK (1:5000), p-Src (1:1000), or total-Src (1:5000), made up in blocking buffer. Following overnight incubation and washes in TBS-T and TBS (3x, 10 min each), membranes were incubated in secondary goat anti-rabbit antibody (1:5000 in TBS-T and 5% skim milk; Gibco) for 2 h at 21 °C, after which washes were repeated and membranes imaged using Pierce ECL Western Blotting Substrates (Thermo Fisher Scientific) on a ChemiDoc system (BioRad). For each of the replicate experiments using independent cell preparations, lysates were ran in duplicate gels and individually probed using phospho- and endogenous-target antibodies for the phosphoprotein of interest (ERK or Src). Membranes were then stripped of antibody using mild stripping buffer (15 g/L glycine, 1 g/L SDS, 1% Tween-2; pH 2.2) prior to re-blocking and probing with anti-β-actin antibody (1:5000) to normalize for total protein within each lane. Densitometry was performed using ImageLab software (BioRad). For both ERK and Src, ratios of phospho-target/actin and endogenous-target/actin were

determined to adjust for loading, and a final ratio of phospho/endogenous index was calculated, followed by normalization to vehicle controls within the experiment.

2.8 Bioinformatics

Where possible, *in silico* analysis was conducted to verify that residues and three-dimensional pockets targeted by compounds designed against mammalian signalling protein homologs are conserved in basal vertebrate species. Protein sequences retrieved from Universal Protein Resource Knowledgebase (UniProtKB; https://www.uniprot.org) or the available goldfish genome, following organism-specific BLAST (*Carassius auratus*, NCBI Taxonomy ID 7956), were aligned using the Clustal Omega (https://www.ebi.ac.uk/Tools/msa/clustalo/) Web Portal. In some cases, confirmed protein sequences from the closely related cyprinid species zebrafish (*Danio rerio*) were also used for comparison. Representative species for homology modelling were chosen based on sequence completeness in the protein regions of interest, as well as available three-dimensional structural data retrieved from the Research Collaboratory for Structural Bioinformatics (RCSB) Protein Data Bank (https://www.rcsb.org/). Structural homology models were generated by submitting query sequences to the Protein Homology/AnalogY Recognition Engine, version 2.0 (Phyre2; http://www.sbg.bio.ic.ac.uk/~phyre2/html/page.cgi?id=index). Three dimensional molecular model graphical images were generated using UCSF Chimera version 1.15, developed by the Resources for Biocomputing, Visualization, and Informatics at the University of California, San Francisco, with NIH P41-GM103311 (https://www.cgl.ucsf.edu/chimera/).

2.9 Statistics

Pooled results from phospho-immunoblotting experiments and hormone release responses are presented as mean ± SEM. Relevant information regarding sample size is presented in all figure legends. Levene's test was used to assess homoscedasticity of data. Parametric data resulting from multiple treatment groups were compared using ANOVA followed by Fisher's Least Significant Difference post-hoc tests, whereas non-parametric data were compared using Kruskal-Wallis followed by Dunn's multiple comparisons post-hoc tests, where applicable. Additionally, Student's paired *t* tests were employed to compare the overall effects of pharmacological inhibitors on unstimulated release to basal release prior to inhibitor application within the same columns. Single-sample *t*-tests were used to compare a treatment value against zero (i.e., no change) and two-sample *t*-tests were used to compare the effects of two treatments only. The level of significance for all comparisons was set at $P < 0.05$.

Figure 2.1. Schematics of column perifusion studies and data quantification. A: Following overnight culture, aggregates of dispersed pituitary cells and Cytodex beads are loaded into temperature-controlled columns. After an initial acclimation period (4 h) where cells are perifused with M199 medium alone to stabilize secretion levels, perifusate fractions from six columns (three treatment groups, in duplicate) are collected every 5 min for 2 h. B: Inhibitor treatment is initiated at 25 min for those columns receiving inhibitor treatment and lasts until 90 min (horizontal grey bar), followed by a washout period where inhibitor is replaced with M199 medium. A 5-min pulse of GnRH2 or GnRH3 (shown: GnRH3) is administered between 45-50 minutes for groups receiving GnRH stimulation. The flow rate and dead-space volume of the system is such that release responses commence at 5-6 min following application. Hormone release (LH or GH; LH is shown as an example) is represented as a percentage of the corresponding pre-treatment values within an individual column, where 100% is the average hormone content released in the first five fractions (green box). C: Secretion responses to the pulse application of GnRH were quantified as net response of LH or GH levels, calculated as the area under the response curve with baseline subtraction (baseline defined as the average of hormone values in the three pre-pulse fractions collected prior to the time of GnRH application; magenta box). Accordingly, the net responses for treatment columns with inhibitor alone or inhibitor + GnRH were quantified over the same time period (duration indicated by vertical dotted lines in B).

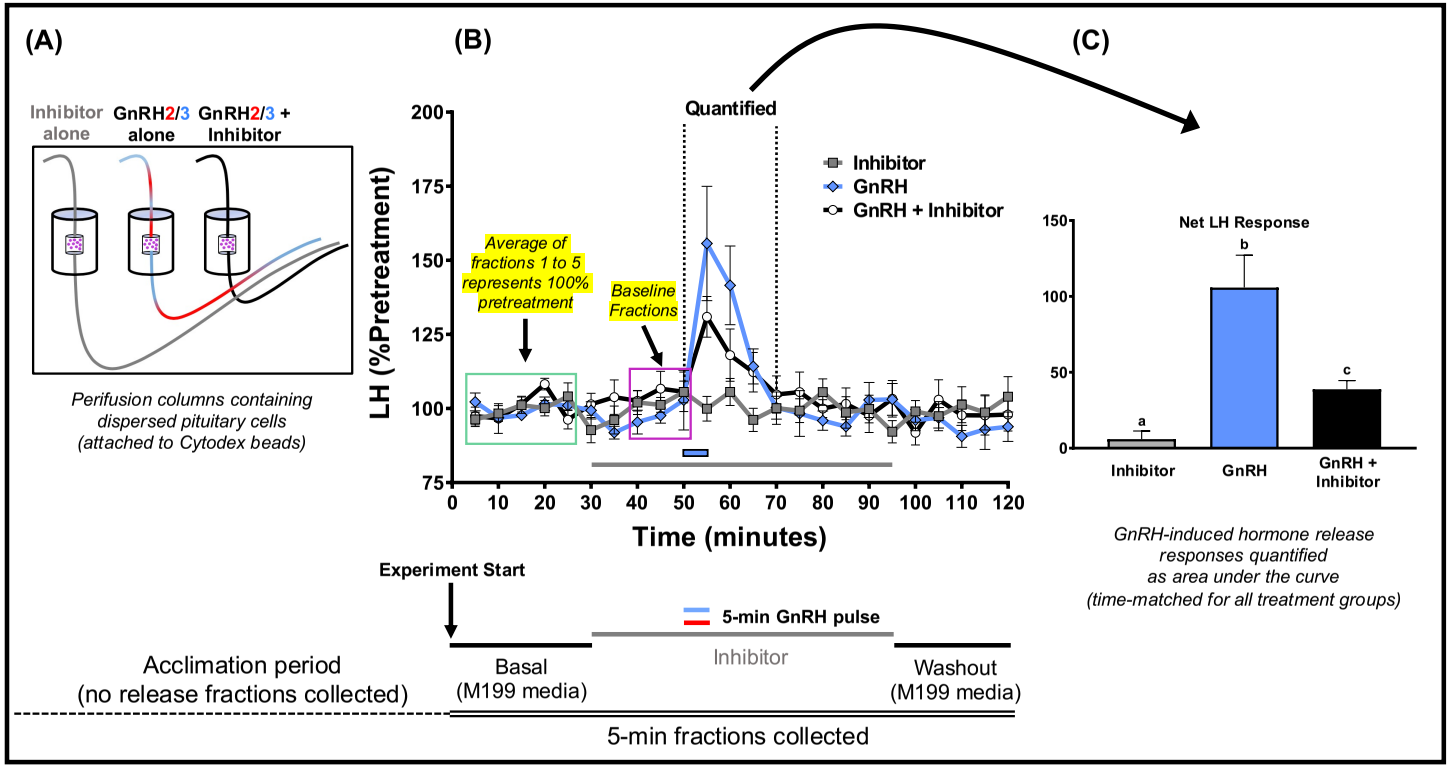
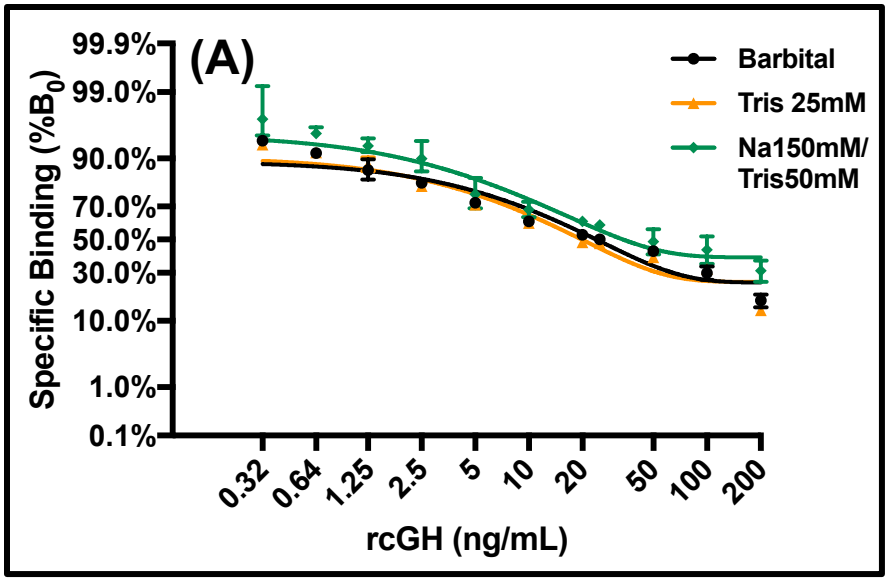

(A)
Inhibitor alone
GnRH2/3 alone
GnRH2/3 + Inhibitor
Perifusion columns containing dispersed pituitary cells (attached to Cytodex beads)
(B)
Quantified
Inhibitor
GnRH
GnRH + Inhibitor
Average of fractions 1 to 5 represents 100% pretreatment
Baseline Fractions
LH (%Pretreatment)
Time (minutes)
Experiment Start
5-min GnRH pulse
Inhibitor
Acclimation period (no release fractions collected)
Basal (M199 media)
Washout (M199 media)
5-min fractions collected
(C)
Net LH Response
Inhibitor
GnRH
GnRH + Inhibitor
GnRH-induced hormone release responses quantified as area under the curve (time-matched for all treatment groups)

Figure 2.2. Validation of Tris 25 mM buffer for GH RIA. A: Displacement of ^{125}I-rcGH (recombinant carp growth hormone) tracer by increasing concentrations of rcGH standard, compared between Barbital, Na 150 mM/Tris 50 mM, and Tris 25 mM buffer GH RIA systems, using rabbit anti-carp GH antisera (AB_2631195). Binding data are presented as mean ± SEM (Barbital and Tris 25, n = 3 runs with each hormone standard in duplicate; Na150/Tris50, n=2 runs with each hormone standard in duplicate) on a log-logit scale. B: Dilutions of pituitary homogenate extract compared between Barbital (solid black: pituitary #1, dotted black: pituitary #2) and Tris 25 mM (solid orange: pituitary #1, dotted orange: pituitary #2) buffer systems. A.U.: arbitrary unit.


(A)
Barbital
Tris 25mM
Na150mM/
Tris50mM
Specific Binding (%B0)
99.9%
99.0%
90.0%
70.0%
50.0%
30.0%
10.0%
1.0%
0.1%
0.32
0.64
1.25
2.5
5
10
20
50
100
200
rcGH (ng/mL)

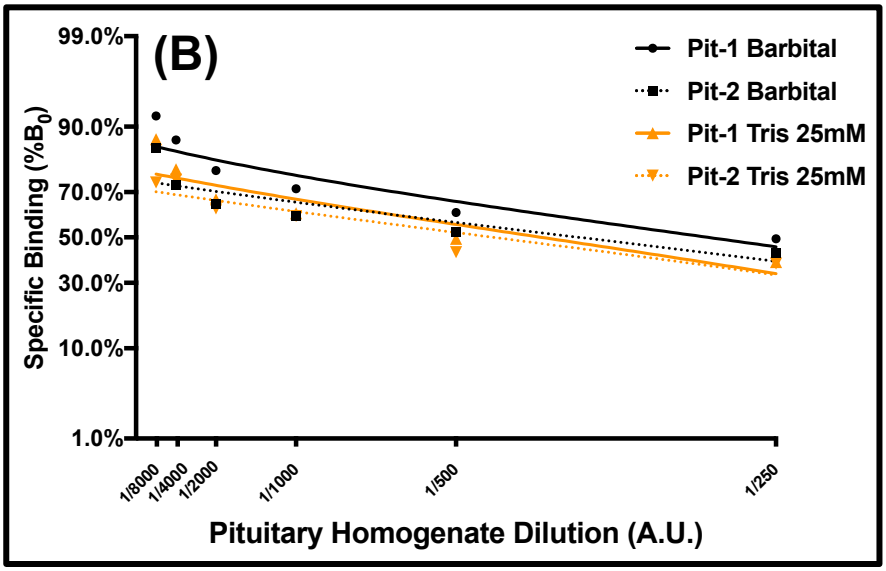
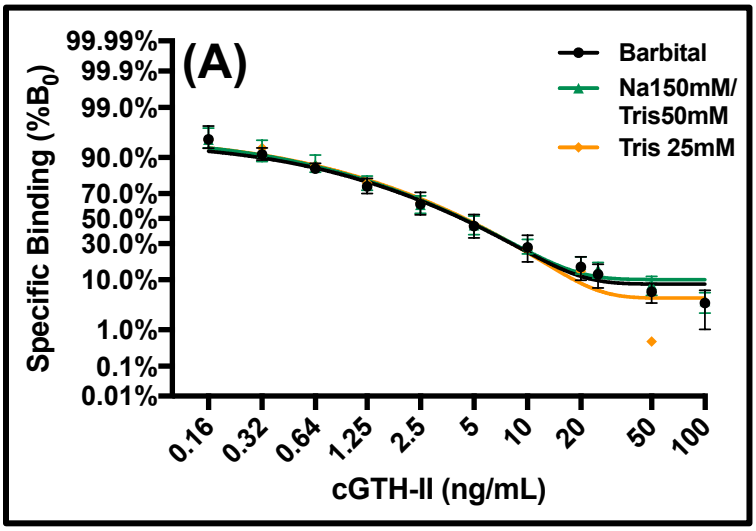
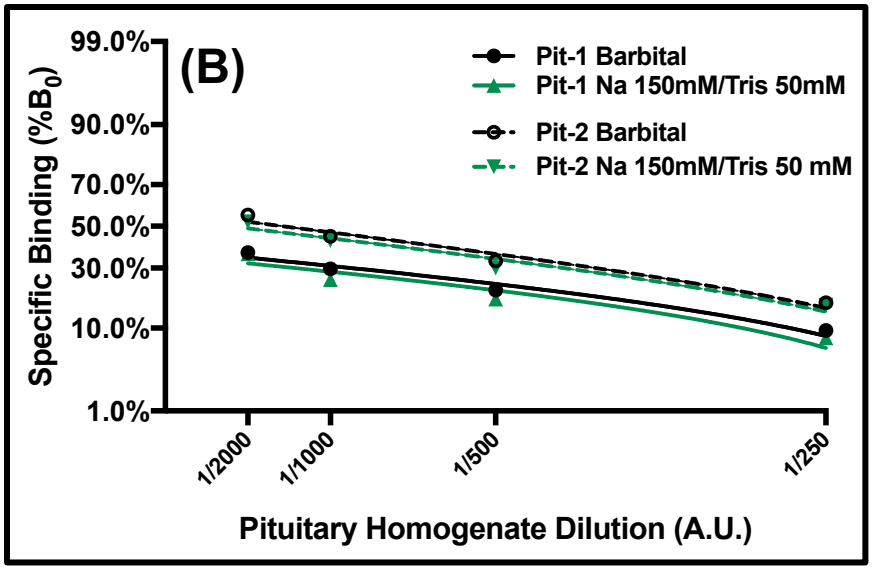
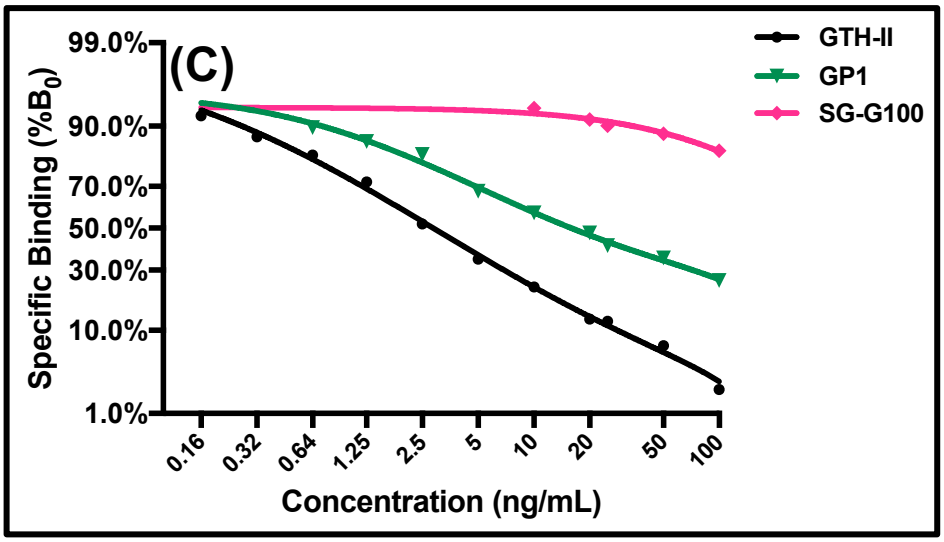

(B)
Pit-1 Barbital
Pit-2 Barbital
Pit-1 Tris 25mM
Pit-2 Tris 25mM
Specific Binding (%B0)
99.0%
90.0%
70.0%
50.0%
30.0%
10.0%
1.0%
1/8000
1/4000
1/2000
1/1000
1/500
1/250
Pituitary Homogenate Dilution (A.U.)

Figure 2.3. Validation of Na / Tris buffer for LH RIA. A: Displacement of ^{125}I-cGTH-II (purified carp gonadotrophin II; i.e. LH) tracer by increasing concentrations of GTH-II standard, compared between Barbital, Na 150 mM/Tris 50 mM, and Tris 25 mM buffer LH RIA systems, using rabbit anti-carp GTH (RAC) antisera (AB_2631196). Binding data are presented as mean ± SEM for Barbital and Na 150 mM/Tris 50 mM (n = 4 runs with each hormone standard in duplicate), whereas data for Tris 25 mM represent the arithmetic mean from 1 run with each hormone standard in duplicate. B: Dilutions of pituitary homogenate extract compared between Barbital (solid black: pituitary #1, dotted black: pituitary #2) and Na 150 mM / Tris 50 mM (solid green: pituitary #1, dashed green: pituitary #2) buffer systems. C: Displacement of ^{125}I-GTH II tracer by cGTH-II (LH), GP1 (carp glycoprotein 1, presumably FSH), and salmon gonadotrophin (SG-G100). A.U.: arbitrary unit.


(A)
Barbital
Na150mM/
Tris50mM
Tris 25mM
Specific Binding (%B0)
99.99%
99.9%
99.0%
90.0%
70.0%
50.0%
30.0%
10.0%
1.0%
0.1%
0.01%
0.16
0.32
0.64
1.25
2.5
5
10
20
50
100
cGTH-II (ng/mL)


(B)
Pit-1 Barbital
Pit-1 Na 150mM/Tris 50mM
Pit-2 Barbital
Pit-2 Na 150mM/Tris 50 mM
Specific Binding (%B0)
99.0%
90.0%
70.0%
50.0%
30.0%
10.0%
1.0%
1/2000
1/1000
1/500
1/250
Pituitary Homogenate Dilution (A.U.)


(C)
GTH-II
GP1
SG-G100
Specific Binding (%B0)
99.0%
90.0%
70.0%
50.0%
30.0%
10.0%
1.0%
0.16
0.32
0.64
1.25
2.5
5
10
20
50
100
Concentration (ng/mL)

Chapter Three

Arrestin- and dynamin-dependent mechanisms in basal and GnRH-evoked hormone release from pituitary gonadotrophs and somatotrophs

3.1 Introduction

GPCR receptor complexes are known to internalize into endosomal compartments following their activation and this phenomenon has also been shown for various type II tailed-GnRHRs in expression systems (McArdle et al., 2002). A central protein implicated in this process is β-arrestin. In addition to their role in terminating GPCR signaling, β-arrestins also participate in coupling GPCRs to second messenger systems known to be important for hormone secretion, including ERK, Class I PI3Ks, AA, and Ca^{2+}-dependent pathways (Peterson and Luttrell, 2017; Shenoy et al., 2006; Song et al., 2009), in part through endosomal signaling pathways (Jean-Charles et al., 2017). Additionally, following GPCR incorporation into CCS via β-arrestin-dependent mechanisms, commitment to vesicle maturation and subsequent internalization requires the actions of the GTPase dynamin in vesicle scission from the plasma membrane (Cocucci et al., 2014; Morlot and Roux, 2013; schematic in Figure 3.1); furthermore, dynamin-dependent internalization has also been shown to mediate cellular responses downstream of several GPCRs, including the mammalian GnRHR. As such, arrestins and dynamin are potentially engaged relatively early in GPCR signal transduction and are also good candidates for mediating the observed ligand bias in the goldfish pituitary to the two native isoforms of goldfish GnRH, as reviewed in Chapter 1.

However, while some measurements of signalling endpoints have been described (such as ERK activation), the studies on arrestin and dynamin involvement in GnRHR actions available in the literature have nearly all been performed in expression systems or in the clonal cell gonadotroph models (LβT2 and αT3-1 cells), and only one study has examined physiological endpoints (GnRH-induced actin remodelling; Edwards et al., 2016). Furthermore, all studies of non-mammalian GnRHRs (catfish and frog) have been performed by heterologous expression in

mammalian cell types (also see Table 1.2). Additionally, due to difficulties in selectively studying arrestin-dependent mechanisms in primary untransformed cells, few studies to date have directly addressed the roles of β-arrestins in hormone secretion in vertebrate models, and none to our knowledge in any primary pituitary cell systems (as indexed by NCBI PubMed).

Recently, a novel small molecule, β-arrestin/β2-adaptin interaction inhibitor (Barbadin), has been characterized, which allows for the examination of a subset of arrestin-dependent mechanisms downstream of GPCR activation (Beautrait et al., 2017). Barbadin selectively perturbs binding of β-arrestin and the β2-adaptin subunit of the clathrin adaptor protein complex AP2, which, along with β-arrestin-clathrin interactions, is a necessary interaction for arrestin-driven incorporation of GPCRs into CCS and/or early endosomes (Kim and Benovic, 2002; Laporte et al., 2000). Importantly, Barbadin does not affect GPCR-β-arrestin interactions, nor does it alter β2-adaptin-clathrin association, and is highly specific for the β-arrestin-AP2 binding hotspot (Beautrait et al., 2017; Sundqvist et al., 2020).

In this data chapter, I examined whether β-arrestin and dynamin activities are required for GnRH2/GnRH3-dependent stimulation of hormone release in physiological contexts using the goldfish pituitary cell model system in conjunction with the inhibitor of β-arrestin-AP2 interaction Barbadin and the dynamin inhibitor Dyngo4a.

3.2 Results

3.2.1 Barbadin affects ERK phosphorylation in dispersed goldfish pituitary cells

Barbadin targets the highly conserved β-arrestin binding pocket on AP2 (Laporte et al., 2000), and it inhibits binding of both β-arrestin1/2 with comparable efficacy and in a reversible fashion (Beautrait et al., 2017). This interaction hotspot (Phe388, Phe391, and Arg395 residues

on the C-terminal of β-Arrestin1/2, interacting with Glu849, Tyr888, and Glu902 residues on β2-adaptin) is 100% conserved at the protein level between zebrafish and humans, as well as in predicted goldfish protein sequences based on the completed goldfish genome project (NCBI Assembly ASM336829v1; Figure 3.2A,B). This high degree of conservation is also predicted by protein modelling (Figure 3.2C). These observations suggest that the 25 μM dose of Barbadin used in the present study should be effective (IC_{50} of 7.9 μM for inhibition of receptor-induced signalling, in the original HEK293T cell expression system). Barbadin was also tested on one primary cell type (rat aortic smooth muscle cells) and a 20 μM dose was deemed effective in inhibiting arrestin actions in the original characterization of this molecule (Beautrait et al., 2017), and it has also been used at a 10 μM doses in subsequent publications in multiple *in vitro* cell systems (He et al., 2021; Sundqvist et al., 2020).

ERK is a well-known target of GnRH post-receptor signalling in multiple systems, and changes in phospho-$ERK^{T202/Y204}$ levels have been used as an index of ERK activation (Liu et al., 2002a; Naor et al., 2000). Consistent with previous findings by Klausen et al. (2008), stimulation with maximally stimulatory concentrations (100 nM) of either GnRH2 or GnRH3 for 5 min significantly elevated phospho-ERK levels in extracts of primary cultures of goldfish pituitary cells above unstimulated controls by approximately 0.7 to 0.8 fold (Figure 3.3). Barbadin application alone also increased phospho-ERK levels by about 0.4 fold, although these changes were not significant relative to controls. Combination GnRH + Barbadin treatment resulted in elevations of phospho-ERK levels of approximately 1.3 to 1.4 fold; these changes were significantly greater than those observed with either GnRH or Barbadin alone. These further increases in GnRH-induced phospho-ERK levels in the presence of Barbadin could, to a large

extent, be accounted for by the effects of Barbadin on basal phospho-ERK alone, although a greater than additive effect could also not be definitely ruled out.

3.2.2 β-arrestins participate in GnRH2/3-induced LH secretion

The involvement of β-arrestins in GnRH control of LH secretion from gonadotrophs was investigated in column perifusion experiments. Application of 5-min pulses of maximally stimulatory concentrations of GnRH2 and GnRH3 (100 nM; Chang et al., 1990) significantly stimulated LH release (Figure 3.4). Treatment with Barbadin alone transiently elevated LH release, this returned to pre-exposure levels within 20 min; more importantly, Barbadin significantly suppressed the LH response to both GnRH isoforms (Figure 3.4). Upon termination of Barbadin application, a further increase in LH release was observed in the GnRH2 + Barbadin group, but not in other treatment groups (washout response quantified in Figure 3.4C). The divergent release response kinetics following inhibitor washout in the combination treatments may indicate differences in GnRH isoform-selective actions on arrestin-dependent mechanisms on distal exocytotic machinery in gonadotrophs.

3.2.3 β-arrestins have differential effects on GnRH2/3-induced GH secretion

Treatment with 5-min pulses of 100 nM GnRH2 or GnRH3 stimulated GH secretion as in previous studies (Chang et al., 1990). Application of Barbadin elicited a transient increase in GH release. In the presence of Barbadin, the GH response to GnRH2 was attenuated while that to GnRH3 was further increased (Figure 3.5). Transient elevations in GH release also occurred upon termination of inhibitor treatment, which were most prominent in the GnRH2 + Barbadin combination and Barbadin alone groups (Figure 3.5C).

3.2.4 β-arrestins modulate basal pituitary hormone secretion

The effects of Barbadin treatment alone on basal hormone release were also analyzed by comparing the average hormone concentrations over the treatment duration (30-95 min) to the average pre-treatment values from the first 20 min when cells were exposed to media alone, from the same columns. Despite transient spikes in both the kinetics of LH and GH secretion (grey traces; Figures 3.4 & 3.5), significant elevations in averaged hormone release over the entire Barbadin treatment period was only detected for GH, but not LH (Figure 3.6).

3.2.5 Selective effects of dynamin inhibition on GnRH-induced hormone release profiles

Dyngo4a is a widely-used inhibitor of dynamin actions especially in the context of receptor internalization pathways. A 30 μM dose was selected based on several studies in multiple primary and clonal cell systems (Basagiannis et al., 2021; Heldin et al., 2019; Kockx et al., 2014), including a study examining GnRH actions in the αT3-1 mouse gonadotroph cell line (Edwards et al., 2016). In this thesis chapter using dispersed goldfish pituitary cells, the quantified acute stimulation of LH and GH releases following GnRH application were not altered by pre-treatment with Dyngo4a (Figures 3.7 and 3.8). However in the presence of Dyngo4a, GnRH2-induced LH release selectively exhibited a prolonged release pattern with LH secretion remaining elevated over the inhibitor-alone baseline for the remainder of the perifusion period (Figure 3.7A). Average LH values in the GnRH2 + Dyngo4a group over the next 6 fractions following the termination of the acute response quantification (65 to 90 min of the perifusion experiment) were significantly higher than the corresponding average values in the

Dyngo4a alone group (GnRH + Dyngo4a : 118.18 ± 7.79 % pretreatment; Dyngo4a : 100.22 ± 5.73 % pretreatment; $P < 0.05$, Student's t-test).

3.2.6 Dynamin inhibition leads to elevation of unstimulated hormone release

The overall effects of Dyngo4a on basal (unstimulated) LH and GH release were assessed by comparing hormone release levels before and after application of this inhibitor. Application of Dyngo4a resulted in a transient elevation in LH secretion which returned to baseline pre-treatment levels during the inhibitor treatment, followed by a further reduction during the inhibitor washout period (grey traces in Figure 3.7). On the other hand, elevations in GH release upon Dyngo4a administration were sustained throughout the duration of inhibitor treatment and trended towards a return towards baseline levels during the inhibitor washout period (grey traces in Figure 3.8). Quantification of hormone release levels over the duration of inhibitor treatment showed that unstimulated GH release, but not LH secretion, were significantly different from pre-treatment levels (Figure 3.9).

3.3 Discussion

Results from the present experiments indicate that β-arrestins affect basal LH and GH release and ERK activity in dispersed pituitary cells, as well as reveal the presence of differential effects of β-arrestins in GnRH-isoform-specific-induced LH and GH release. In addition, observations from pharmacological manipulation of dynamin GTPase activity reveal non-identical roles for this protein in the control of basal LH *vs.* GH exocytosis, as well as its selective roles in modulating GnRH2-stimulated LH secretion.

3.3.1 β-Arrestin-dependent component of ERK activation

GnRHR stimulation leads to a rapid and robust stimulation of ERK1/2 in various systems, including the goldfish pituitary (this study and Klausen et al., 2008), and ERK1/2 are archetypal interaction partners of β-arrestin-dependent signalling (Eishingdrelo et al., 2015). Barbadin was shown to inhibit agonist-induced clustering of the vasopressin receptor V2R into CCP, which was correlated with ERK activation, at 2.5 and 5 min (Beautrait et al., 2017). Accordingly, the effects of Barbadin on GnRH-induced post-receptor signalling were first assessed using the phosphorylation status of ERK1/2 as a readout at the 5 min timepoint, time-matched with GnRH stimulation in perifusion experiments. Paradoxically, Barbadin elevated the GnRH-induced increases in phospho-ERK levels in goldfish pituitary dispersed cell extracts. These results would suggest that β-arrestins do not mediate GnRH-stimulated ERK signalling and may even have an inhibitory role in the regulation of ERK signalling in these cells. Such unexpected observations are not without precedence. While many investigations support a facilitating role for arrestin-dependent engagement of ERK, other studies show no contribution of arrestins towards modulating ERK signalling, or even the opposite effect. For example, knockdown of β-arrestin1/2 elevated agonist-induced ERK activation downstream of M3 muscarinic receptors within 5 min (Luo et al., 2008), the same timeframe tested in the present results. Likewise, an arrestin-binding-deficient mutant of the free fatty acid GPCR, FFA4 (also known as GPR120), upon agonist stimulation, demonstrated significantly enhanced activation of ERK phosphorylation while also extending the kinetics of Ca^{2+} elevation (Alvarez-Curto et al., 2016). The authors proposed that the impaired receptor internalization due to poor association with arrestins led to sustained G-protein-dependent Ca^{2+} signalling and ERK activation. Whether the enhancement of GnRH-induced phospho-ERK levels following inhibition of β-arrestins is

similarly due to potentiated agonist-induced Ca^{2+} mobilization responses and subsequent facilitation of ERK via PLC-β/PKC signalling remains to be explored, but PKC-dependent ERK activation in goldfish gonadotrophs and somatotrophs has been reported (Klausen et al., 2008; Pemberton et al., 2013). Interestingly, Barbadin also has a positive influence on basal phospho-ERK levels (although this was not statistically different from vehicle controls). This is in line with prior work describing constitutive actions of β-arrestins in the absence of activated receptors (Song et al., 2006; Zhan et al., 2011) and the reported ability of β-arrestins to localize pools of ERK to microtubules, sequestering them away from membrane-generated signals, thereby dampening basal ERK activity (Coffa et al., 2011; Hanson et al., 2007). A limitation of the current findings on ERK activation is that phospho-ERK levels were measured from mixed populations of dispersed goldfish pituitary cells; consequently, specific changes in ERK activation in gonadotrophs and somatotrophs might have been masked or diluted by the phosphorylation state of ERK in the other pituitary cell-types.

Interestingly, recent work using genome-edited HEK293 cells has shown that G proteins are necessary to initiate ERK signalling (Grundmann et al., 2018; O'Hayre et al., 2017). Although arrestins may not be sufficient to initiate ERK activation via upstream MAP3Ks in the absence of active G proteins, it is thought that they still play significant roles in GPCR signal propagation through scaffolding of the three-tiered active MAPK cascade (Gurevich and Gurevich, 2018; and see Section 1.5.3) and fine-tune active spatiotemporal dynamics of ERK signalling (Gutkind and Kostenis, 2018). Furthermore, there is considerable variation in the interplay between β-arrestin-mediated GPCR internalization and activation of ERK, with some GPCRs requiring internalization (Banerjee and Mahale, 2018; May et al., 2014; Perez-Aso et al., 2013; Pierce et al., 2000; Smith et al., 2016), whereas others propagate signals to ERK without

receptor internalization (Kramer and Simon, 2000; O'Hayre et al., 2017; Schramm and Limbird, 1999); this may further vary in ligand- and receptor-isoform-specific fashions (Halls et al., 2016; Perez-Aso et al., 2013). Thus, based on the present data, β-arrestin- and AP2-dependent targeting of GnRHRs to clathrin structures are not major components of acute GnRH-induced ERK activation (and suggests that G protein-dependent actions are sufficient), but whether this plays a role in longer-term/endosomal signalling following GnRHR internalization in goldfish pituitary cells and how this might vary for GnRH2 and GnRH3 is not currently known.

3.3.2 β-arrestin-dependent mechanisms play permissive roles in GnRH-induced LH secretion from goldfish gonadotrophs

Treatment with Barbadin suppressed both GnRH2- and GnRH3-induced acute LH secretion responses from gonadotrophs. These results indicate that β-arrestins may have rapid stimulatory effects downstream of GnRHR activation, engaging downstream signalling components leading to hormone secretion (Figure 3.10). Since the actions of Barbadin are to prevent GPCR-associated-β-arrestins from engaging AP2/clathrin-dependent internalization machinery, it is likely that under normal conditions, the internalized endosomal complexes participate in GnRH signalling through recruitment and/or sequestration of active kinase effectors at sites distal to the plasma membrane (DeWire et al., 2007). The downstream signalling partner(s) recruited by arrestin-dependent mechanisms following GnRHR activation in goldfish gonadotrophs is unknown at present but might include elements of the MAPK cascade (including Raf, MEK, and ERK), Class I PI3Ks, as well as other protein tyrosine kinases and Ca^{2+}-dependent pathways (Gurevich and Gurevich, 2019b; Peterson and Luttrell, 2017). Many of these signalling mechanisms are known to mediate GnRH2 and GnRH3 actions on goldfish LH

release (Chang et al., 2009; Pemberton et al., 2015). However, given that Barbadin did not attenuate GnRH-induced ERK activation, arrestin-dependent recruitment of ERK may not be a major component of GnRH action in goldfish gonadotrophs. On the other hand, these results provide important support to the idea that GnRHR-stimulated physiological endpoints, such as hormone secretion, involve and/or continue following receptor internalization, adding to growing evidence of endosomal signalling across GPCRs (Kim et al., 2021; Thomsen et al., 2018; Vilardaga et al., 2014).

3.3.3 β-arrestin-dependent mechanisms play differential roles in GnRH2/3-stimulated GH secretion from goldfish somatotrophs

Similar to the observations in gonadotrophs, treatment with Barbadin suppressed GnRH2-induced GH secretion from somatotrophs, suggesting that GnRHR-arrestin-containing endosomes are similarly involved in GnRH2-dependent recruitment of downstream signalling effectors to stimulate GH release. Notably, GnRH2-induced acute GH release is independent of MEK-ERK in somatotrophs (Pemberton et al., 2013); therefore, the effector(s) recruited to these endosomal scaffolds are likely to be one of the other signalling candidates, some of which are discussed in Section 1.5.3. In particular, arrestins are known to post-translationally activate iNOS, and NO mediates the GH response to GnRH2 (Meints et al., 2012; Uretsky et al., 2003). Similarly, arrestins directly bind and control localization of CaM, and CaMK is part of the mechanisms utilized by both GnRHs in somatotrophs (Chang et al., 2012). Additionally, arrestins interact with phospholipid-dependent signalling through context-specific actions on PI3K subunits as well as the lipid phosphatase PTEN (phosphatase and tensin homolog), which antagonizes PI3K functions (Jean-Charles et al., 2017; Lima-Fernandes et al., 2011), and some

Class I PI3K isoforms facilitate GnRH2-induced GH responses (Pemberton et al., 2015); whether arrestins link to PI3K actions in the goldfish pituitary remains to be examined.

In contrast to GnRH2, GnRH3-elicited GH was enhanced in the presence of Barbadin, suggesting that β-arrestins may be playing entirely different roles for the same population of GnRHRs within the one cell-type in a ligand-dependent fashion. Considering the mechanisms of action of Barbadin as an inhibitor of arrestin-AP2-mediated receptor internalization while preserving receptor-arrestin interactions (Beautrait et al., 2017), these differences suggest that arrestin-GnRHR complexes are directed to distinct fates in a ligand-dependent (i.e., GnRH2 *vs.* GnRH3) fashion within somatotrophs (Figure 3.10). In the case of GnRH3-stimulated receptors, CCS may be directed towards ubiquitin-dependent endo-lysosomal degradation pathways and attenuation of the stimulatory actions on GH secretion (Han et al., 2013), rather than promoting scaffolding of signalling effectors leading to GH release as in the case of GnRH2-engaged receptors. Similar agonist-specific effects have been observed for other Class A GPCRs such as the MORs, where there are notable differences in downstream signalling, desensitization, and endosomal trafficking, when comparing the agonists morphine and etorphine (Molinari et al., 2010; Whistler and von Zastrow, 1998; Zheng et al., 2008). These opposing ligand-selective functions of β-arrestins are reconciled by observations that their active conformations are numerous and vary in both receptor- and ligand-specific fashions, suggesting that this information is encoded within the activated arrestin conformation (Lee et al., 2016; Nuber et al., 2016; Shukla et al., 2008).

Another possibility which cannot be ruled out in GnRH3-dependent GH secretion is the recruitment of negative regulators of hormone secretion to arrestin-GnRHR complexes. As such, activated β-arrestins have been shown to form signalosome complexes with Akt and the protein

phosphatase PP2A. Within this complex, β-arrestin locally inhibits PP2A functions, leading to activation of Akt and its downstream target glycogen synthase kinase 3β (Kendall et al., 2011). Catalytic inhibition of Akt in goldfish somatotrophs similarly enhances GnRH3-dependent GH release (Pemberton and Chang, 2016), which is consistent with an overall stimulatory role for β-arrestins upstream of Akt. This may reflect functions of β-arrestins in concert with receptor internalization for GnRH3-stabilized GnRHRs. The present findings are not only in agreement with the previously reported cell-type specific effects between gonadotrophs and somatotrophs in terms of GnRH3-stimulated GnRHRs, as well as between actions of the two GnRHs within somatotrophs (Chang et al., 2012), but they also further extend these cell-specific mechanisms to include differential involvement of β-arrestins-dependent regulatory components, such as possible endosomal sorting and trafficking of these receptors.

3.3.4 Barbadin effects on basal hormone secretion and the observed "washout rebound increase in hormone release"

Despite having a transient effect on basal LH and GH release upon application, Barbadin generally had more persistent influences in elevating basal GH secretion relative to LH release over the treatment period. Previously, a MEK inhibitor U0126 was shown to elevate basal GH release in static cultures of dispersed goldfish pituitary cells (Pemberton et al., 2013), suggesting that the MEKs (and likely the ERKs) generally exert negative control over GH secretion. These findings, together with the observation that Barbadin positively influenced basal phospho-ERK levels in goldfish pituitary cells (Figure 3.3), suggest the presence of arrestin-dependent negative regulation of ERK pools in basal scenarios at least in somatotrophs in the long-term, disruption of which accounts for the overall elevated basal GH release in the presence of Barbadin.

Likewise, a "washout rebound increase" in basal hormone release upon termination of Bardadin treatment alone was only seen for GH but not LH. Whether these differences are also related to the postulated distinct roles of β-arrestins in basal ERK activation in somatotrophs *vs.* gonadotrophs is unknown, but these observations are consistent with the hypothesis that β-arrestins play different roles in the regulation of basal hormone release between the two cell-types. In general, information regarding non-GPCR-linked functions of arrestins is sparse, and control of basal hormone secretion in the absence of receptor-inputs represents an unexplored aspect of the role of arrestins in cell biology and warrants further investigation.

Interestingly, prominent transient increases in hormone release were also seen in the GnRH2 + Barbadin group in both LH and GH secretion upon termination of Barbadin application, but not significantly so with the GnRH3 + Barbadin group (quantified "washout" response for the GnRH3 + Barbadin group not significantly different from zero, one-sample t-test, $P>0.05$, for both LH and GH; corresponding LH and GH responses for GnRH2 + Barbadin group, $P<0.05$ *vs*. zero). Such rapid washout rebound increases in hormone release have been observed in pituitary cell types following removal of tonic dopamine-mediated inhibition of PRL release, and changes in Ca^{2+} dynamics (especially involving L-type VGCCs) are thought to play a role in this phenomenon (del Mar Hernández et al., 1999; Gregerson et al., 1994; Mau, 1997). Similar rebound effects are observed in GH release from goldfish pituitary cells following removal of the inhibitory influences of gonadotrophin-inhibitory hormone (GnIH; Moussavi et al., 2014) and of norepinephrine (Lee et al., 2001), although the underlying mechanisms are not known. Also, in bovine chromaffin cells, while an inhibitor of the sarco/endoplasmic reticulum Ca^{2+} ATPase (SERCA) predictably decreases catecholamine exocytosis in response to a neurotransmitter, removal of the inhibitor potentiates subsequent responses (Martínez-Ramírez et

al., 2021). Thus, whether and how arrestin-dependent mechanisms in goldfish pituitary cells lead to alterations in Ca^{2+} handling, possibly in concert with distal exocytotic machinery, needs to be addressed. Regardless of how these washout rebound increases are manifested, these observations lend further support to the idea that GnRH isoform-selective mechanisms are present in both goldfish somatotrophs and gonadotrophs and for the involvement of β-arrestins in mediating such biased GnRH signalling mechanisms as previously postulated (Pemberton and Chang, 2016).

3.3.5 Dynamin functions in GnRH-stimulated LH and GH release

Although a number of studies have evaluated the functions of dynamin GTPase in GnRHR actions across taxa, this is the first to examine its involvement in GnRH-dependent hormone release. Despite the known roles of dynamin in GnRHR internalization and signalling within 5-10 min of agonist stimulation in some studies (Acharjee et al., 2002; Edwards et al., 2016; Hislop et al., 2001), perturbation of dynamin activity in goldfish pituitary cells did not abrogate acute GnRH-induced LH and GH release. However, there was a selective and prolonged enhancement of LH release during dynamin inhibition, specific to responses evoked by GnRH2, but not GnRH3. This suggests that dynamin-dependent mechanisms are involved in desensitization of GnRH2-induced LH release, and that GnRH2-stabilized receptors in gonadotrophs are selectively targeted to pathways including receptor internalization, and possibly in concert with β-arrestins (Figure 3.9). On the other hand, receptors activated by GnRH3 in the same cells, while similarly engaging arrestin-AP2 dependent mechanisms and presumably localizing to CCS, may not fully internalize into mature endosomes, or do so utilizing dynamin-independent pathways. Finally, it has been noted previously that

desensitization of hormone secretion responses to both native GnRHs vary depending on the duration and type (pulsatile *vs.* continuous administration) of GnRH stimulation (Habibi, 1991a, 1991b). As such, whether dynamin GTPases also participate in long-term actions of GnRH in LH and GH responses should be evaluated in future studies.

3.3.6 Dynamin's roles in basal LH and GH release

Similar to inhibition of arrestin-AP2 interactions, inhibition of dynamin GTPase activity resulted in elevation of unstimulated hormone release, which was more pronounced and sustained in somatotrophs as compared to gonadotrophs. These observed effects on hormone release in the absence of agonist-stimulation likely involve functions of this GTPase in multiple steps along the secretory pathway (González-Jamett et al., 2013b), and especially the influence of dynamin in the pore dynamics following fusion of secretory vesicles to the plasma membrane.

It has been shown using neuroendocrine cell types such as PC12 and bovine adrenal chromaffin cells that disruption of dynamin function leads to increases in hormone (catecholamine) release, through alterations in both quantal size and duration of exocytotic events (González-Jamett et al., 2013a). Similarly, inhibiting dynamin actions either through overexpression of the SH3 domain of amphiphysin (a dynamin-binding partner), or through nonhydrolyzable GTP analogues, resulted in elevated duration (specifically increased half-width and fall-time) of detected amperometric spikes, which indicate release of hormone from single granules (Graham et al., 2002). These observations are consistent with the proposed role of dynamin GTPase in closure of secretory vesicles following expansion of the fusion pore which controls the type and amount of molecules released in exocytotic events, as shown in PC12 cells and the insulin/glucagon/somatostatin/ghrelin-secreting murine MIN6 β-cells (Holroyd et al.,

2002; Tsuboi et al., 2004). Disrupting dynamin expression also enhances vesicular cargo release in the rat insulinoma INS-1 cell line and MIN6 β-cells (Trexler et al., 2016; Tsuboi et al., 2004). Recently, these predicted exocytotic fusion pore dynamics have been visualized in live bovine chromaffin neuroendocrine cells, and pharmacological and genetic manipulations of dynamin confirm that normal dynamin-dependent pore constriction limits the rates and amounts of vesicular cargo release (Shin et al., 2018). Consistent with this role, gain-of-function dynamin mutations impair exocytosis and disrupt fusion pore dynamics in immortalized human myoblasts engineered with an exocytotic reporter protein (Bayonés et al., 2022). Similarly, in neuronal cell types, acute pharmacological inhibition of dynamin induces increased spontaneous release of neurotransmitters, with mixed effects on stimulated exocytosis (Arranz et al., 2014; Douthitt et al., 2011; Hofmann and Andresen, 2017). It is tempting to postulate that these dynamin-dependent effects on the secretory fusion pore dynamics likewise participate selectively in the enhancement of GnRH2-induced LH secretion response by dynamin, as well as dynamin's action on basal GH secretion.

On the other hand, multiple studies have shown a facilitating role for dynamin in regulating exocytosis in various cell types, the opposite of results observed in the present experiments (Fan et al., 2021, 2015; Jackson et al., 2015; Kockx et al., 2014; Min et al., 2007). The discrepancies in these facilitating *vs.* inhibitory roles of dynamin GTPase in secretion across studies may, in part, be attributed to the methods used to assess dynamin function (such as expression of mutants, pharmacological inhibition), as well as the specific cell systems, secretory vesicle characteristics (e.g., synaptic vesicles *vs.* dense core vesicles), and varied secretory pathways for unique releasable pools. As such, differences in basal hormone release in

somatotrophs and gonadotrophs from the present study may also indicate different dynamin GTPase-dependent control of secretory vesicle dynamics in these two cell types.

3.4 Summary

Results from this chapter provide the first direct evidence of a role for β-arrestins in propagating signals downstream of goldfish GnRHRs, as well as novel roles for β-arrestin-mediated endosomal actions in the control of hormone secretion from untransformed pituitary gonadotrophs and somatotrophs in any vertebrate species. Furthermore, β-arrestin-dependent events likely participate in conjunction with heterotrimeric G proteins in differential, isoform-selective GnRH signalling in goldfish gonadotrophs and somatotrophs (Pemberton and Chang, 2016). How G-protein- and β-arrestin-dependent events selectively contribute to the known functional selectivity in response to the two native goldfish GnRHs remains to be further elucidated, but the present results emphasize β-arrestins as an important platform for divergent actions of type II tailed GnRHRs in the control of hormone secretion.

As discussed in Chapter 1, despite the widespread roles of arrestins described for a number of GPCRs (Reiter et al., 2017), the mammalian type I GnRH receptor is an absentee from this list, due to its unique absence of a C-terminus tail (Stojilkovic et al., 1994; Tsutsumi et al., 1992). Since arrestins are recruited to GPCRs via phosphorylation of C-terminus Ser/Thr residues by GRKs (Sente et al., 2018; Yang et al., 2017), the absence of the tail likely explains the lack of arrestin engagement by mammalian type I GnRHRs. However, β-arrestin association has been described for catfish (Blomenröhr et al., 1999; Pawson et al., 2008) and bullfrog (Acharjee et al., 2002) type II tailed GnRHRs, and goldfish GnRHRs similarly contain Ser/Thr residues in the C-terminus tail (Illing et al., 1999), thus enabling arrestin recruitment.

One limitation of the results in this chapter is that potential differential effects of β-arrestins 1 and 2 cannot be ruled out, which may have select non-redundant roles in both GPCR desensitization and arrestin-dependent signalling (reviewed in Srivastava et al., 2015). In the goldfish pituitary, this difference may be manifested through agonist-selective GnRHR coupling to one β-arrestin isoform over another, thus affecting downstream outcomes (e.g., in GnRH2 *vs.* GnRH3 stimulation of GH secretion). Future availability of isoform-selective inhibitors will allow for a clearer understanding of the roles of β-arrestin1/2 as they pertain to hormone secretion. Additionally, the use of the novel Barbadin molecule represents an early line of investigation into the role of β-arrestins in goldfish pituitary, and follow-up studies targeting G-proteins are necessary to highlight potential non-overlapping roles of these signalling arms. Importantly, employing transient pharmacological inhibition to study this phenomenon in untransformed cells provides valuable information to complement data from arrestin-knockout models.

Although the effects of Dyngo4a on ERK phosphorylation have not been examined as in the case with Barbadin, results from experiments utilizing Dyngo4a reveal that dynamin-dependent mechanisms are not a major component of acute goldfish GnRHR actions, except in the case of GnRH2-stabilized receptors in gonadotrophs, where a desensitization-type response is normally mediated by dynamin. This adds to the body of work involving GnRHRs across taxa (Table 1.2) and contributes to our understanding of the physiological role(s) of dynamin actions in GnRH-dependent hormone release. Just as importantly, pituitary cell type-specific functions of this GTPase in the control of basal hormone secretion are also uncovered.

Overall, the findings in this chapter add to the knowledge of GnRHR transduction networks in goldfish gonadotrophs and somatotrophs, and additionally provide insights regarding

β-arrestins in the control of hormone release responses and neuropeptide receptor signalling from a primary cell model, as well as their role in GPCR biology at large. Furthermore, the involvement of β-arrestins and dynamin provide a rational basis for the investigation of other receptor-interacting/proximal mechanisms in goldfish GnRHR actions. In addition, differences in the effects of Barbadin and Dyngo4a on basal and GnRH-elicited release reveal that the canonical arrestin and dynamin interactions leading to internalization and receptor downregulation is undoubtedly an over-simplification of the importance of these two molecules.

Figure 3.1. Overview of the major pathways of agonist-induced GPCR internalization and signalling. Activated GPCRs can be targeted to multiple pathways leading to internalization and numerous functional outcomes, with varying involvement of arrestin and dynamin proteins. Although arrestins are variably involved in agonist-induced internalization, dynamins are typically always required, with few exceptions. One major pathway utilizes arrestin-dependent targeting to clathrin-coated structures (CCS), facilitated by direct interactions between arrestins and the clathrin adaptor protein complex AP2. This is followed by dynamin-dependent scission of the vesicle leading to complete internalization (A). From here, GPCR-arrestin endosomal complexes may continue to signal through scaffolding of effectors (right side green endosome) or be targeted to lysosomal pathways for degradation (left side red endosome). Receptors may also be recycled to the plasma membrane. Alternatively, arrestin-receptor complexes may recruit effectors and signal from CCS, without the need for internalization (B). Arrestin-independent mechanisms of internalization also exist (C). In this paradigm, AP2 and other clathrin-adaptors may interact directly with GPCR residues and mediate dynamin-dependent internalization; similar to panel A, endosomal fates can vary by context, and internalization-independent signalling requiring dynamin has also been reported. (Note that the pathways presented in this schematic are generalized and many additional variations exist. For example, some GPCRs recruit and transiently activate arrestins to signal but are trafficked to CCS in an arrestin-independent manner; thereafter, receptor internalization may or may not occur. Similarly, G protein subunits may remain associated with arrestin-bound receptors, as novel arrestin-GPCR-Gα endosomal signalling complexes have also been reported. In addition, other dynamin-dependent internalization pathways may not involve clathrin or its adaptors but these events are not as well characterized. Furthermore, internalization can also be dependent on cholesterol-containing lipid rafts, caveolae, and endophilins). Figure adapted from Von Moo et al., 2021 and Eichel et al., 2016.

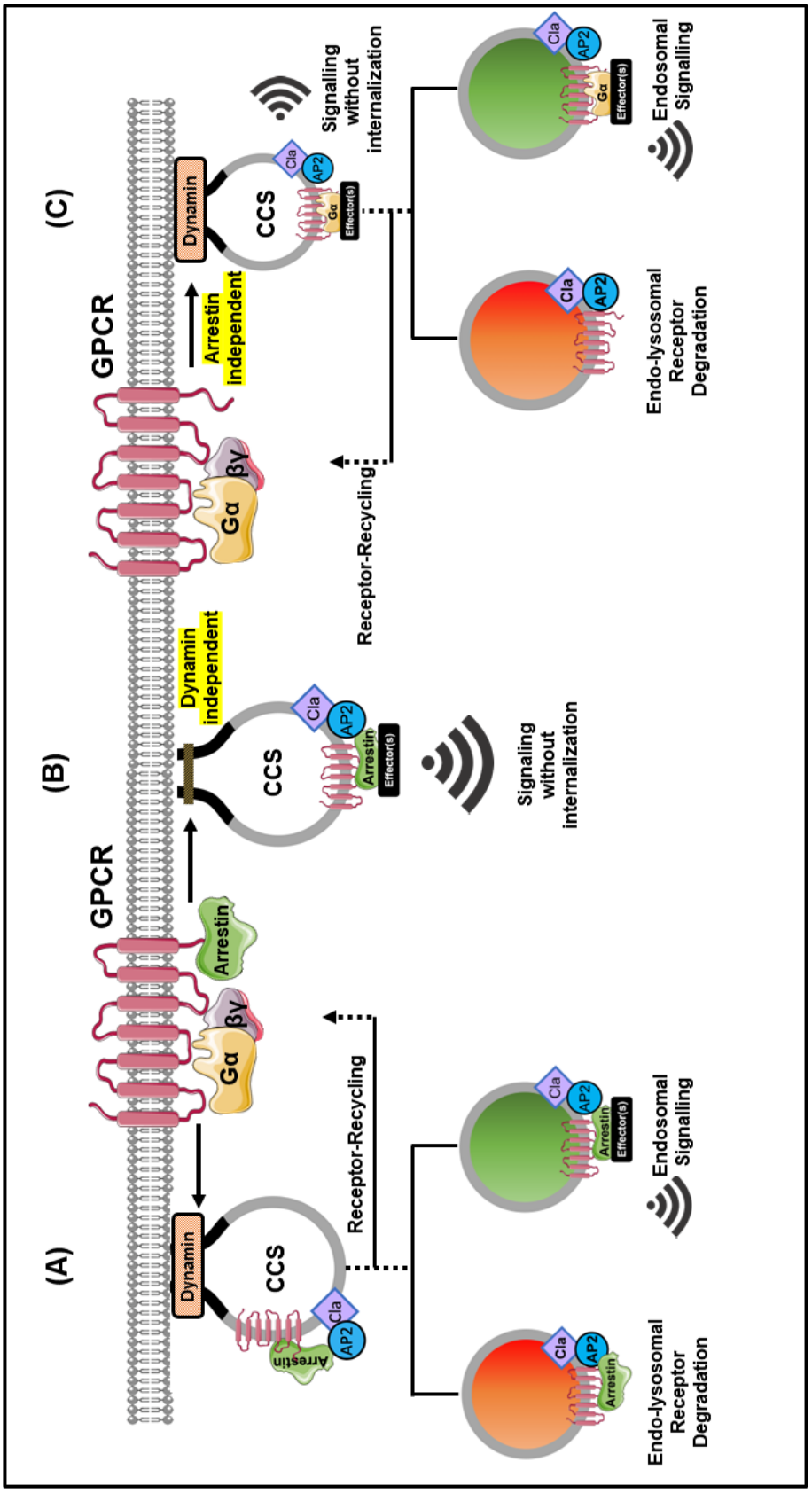

(A)
GPCR
Gα
βγ
Arrestin
Dynamin
CCS
Cla
AP2
Arrestin
Receptor-Recycling
Endosomal Signalling
Endo-lysosomal Receptor Degradation
(B)
GPCR
Gα
βγ
Arrestin
Dynamin independent
CCS
Cla
AP2
Arrestin
Effector(s)
Signaling without internalization
Receptor-Recycling
Endosomal Signalling
Endo-lysosomal Receptor Degradation
(C)
GPCR
Gα
βγ
Arrestin independent
Dynamin
CCS
Cla
AP2
Gα
Effector(s)
Signalling without internalization
Receptor-Recycling
Endosomal Signalling
Endo-lysosomal Receptor Degradation

Figure 3.2. Primary amino acid sequence alignments of (A) the beta subunit of the AP2 adaptor complex and (B) β-arrestins 1 and 2. Alignment demonstrates a high degree of conservation between humans and teleosts based on confirmed sequences (zebrafish) and predicted sequences from genome assembly (goldfish). Of note, the key residues critical for interaction of AP2 with β-arrestins are 100% conserved - as indicated by arrows: Glu849, Tyr888, Glu902 on AP2 interact with Phe388, Phe391, Arg395 on β-arrestin (Laporte et al., 2000; Beautrait et al., 2017). Underneath the alignments, an asterisk (*) indicates positions which have a single, fully conserved residue, whereas a colon (:) indicates conservation of residues with *strongly* similar properties (> 0.5 in the Gonnet PAM 250 matrix). Lastly, a period (.) indicates conservation between groups with *weakly* similar properties (between 0 and 0.5 in the Gonnet PAM 250 matrix). (C) The structures of the β-arrestin-binding pocket of human and zebrafish AP2β1s (left; residues 845-931 shown), as well as the AP2 interaction hot spot on human and zebrafish β-arrestins (shown on the right), are also highly conserved in predicted 3-D models. The small molecule inhibitor Barbadin, designed against human AP2, occupies the β-arrestin-binding-site on AP2 and prevents binding of both β-arrestin isoforms with similar efficacy and in a reversible fashion (Beautrait et al., 2017). See section 2.8 for details regarding bioinformatics approaches.

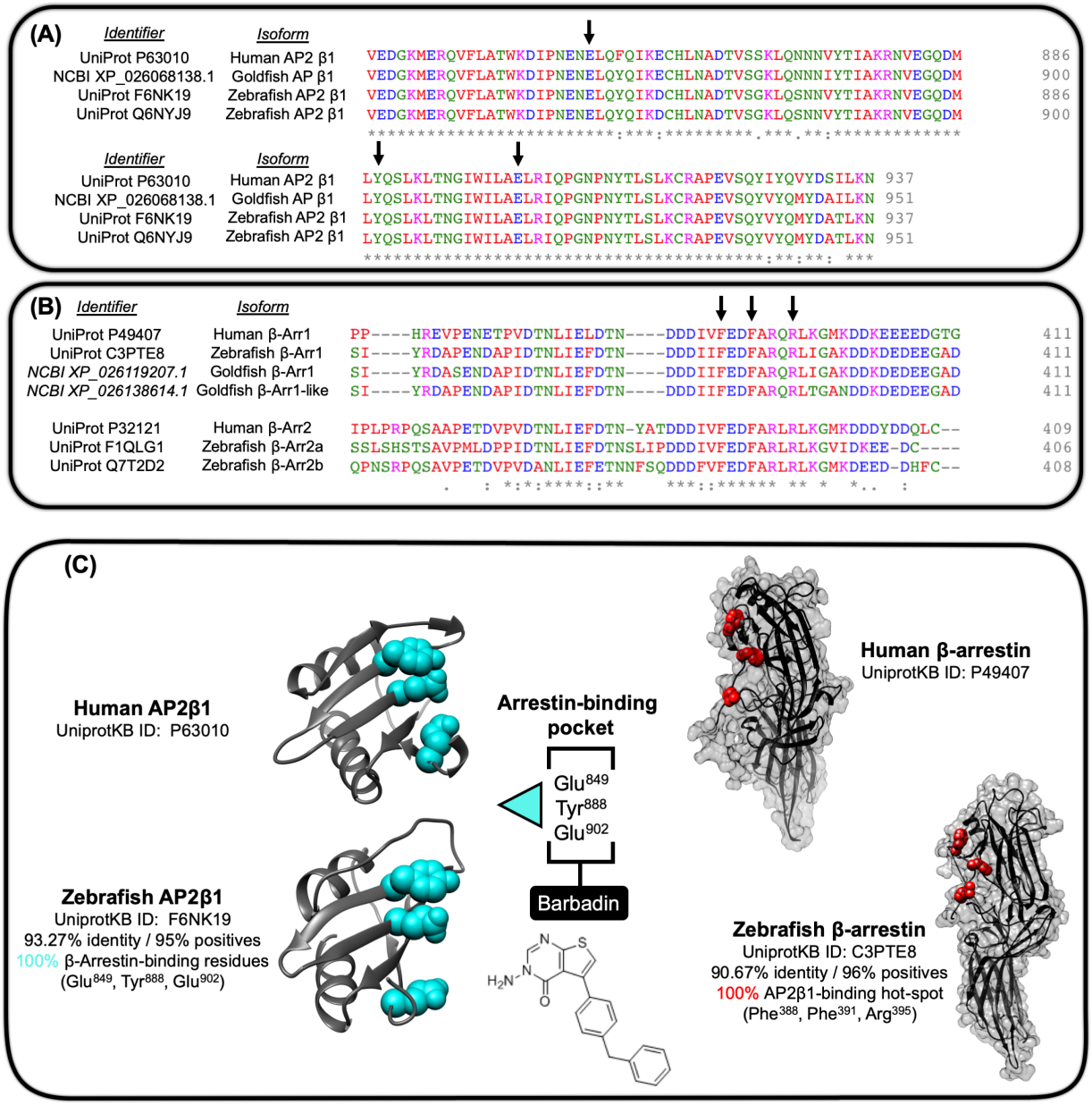

(A)
Identifier
Isoform
UniProt P63010
Human AP2 β1
VEDGKMERQVFLATWKDIPNENELQFQIKECHLNADTVSSKLQNNNVYTIAKRNVEGQDM 886
NCBI XP_026068138.1
Goldfish AP β1
VEDGKMERQVFLATWKDIPNENELQYQIKECHLNADTVSGKLQNNNIYTIAKRNVEGQDM 900
UniProt F6NK19
Zebrafish AP2 β1
VEDGKMERQVFLATWKDIPNENELQYQIKDCHLNADTVSGKLQSNNVYTIAKRNVEGQDM 886
UniProt Q6NYJ9
Zebrafish AP2 β1
VEDGKMERQVFLATWKDIPNENELQYQIKDCHLNADTVSGKLQSNNVYTIAKRNVEGQDM 900
Identifier
Isoform
UniProt P63010
Human AP2 β1
LYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYIYQVYDSILKN 937
NCBI XP_026068138.1
Goldfish AP β1
LYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYVYQMYDAILKN 951
UniProt F6NK19
Zebrafish AP2 β1
LYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYVYQMYDATLKN 937
UniProt Q6NYJ9
Zebrafish AP2 β1
LYQSLKLTNGIWILAELRIQPGNPNYTLSLKCRAPEVSQYVYQMYDATLKN 951
(B)
Identifier
Isoform
UniProt P49407
Human β-Arr1
PP----HREVPENETPVDTNLIELDTN----DDDIVFEDFARQRLKGMKDDKEEEEDGTG 411
UniProt C3PTE8
Zebrafish β-Arr1
SI----YRDAPENDAPIDTNLIEFDTN----DDDIIFEDFARQRLIGAKDDKDEDEEGAD 411
NCBI XP_026119207.1
Goldfish β-Arr1
SI----YRDASENDAPIDTNLIEFDTN----DDDIIFEDFARQRLIGAKDDKDEDEEGAD 411
NCBI XP_026138614.1
Goldfish β-Arr1-like
SI----YRDAPENDAPIDTNLIEFDTN----DDDIIFEDFARQRLTGANDDKDEDEEGAD 411
UniProt P32121
Human β-Arr2
IPLPRPQSAAPETDVPVDTNLIEFDTN-YATDDDIVFEDFARLRLKGMKDDDYDDQLC-- 409
UniProt F1QLG1
Zebrafish β-Arr2a
SSLSHSTSAVPMLDPPIDTNLIEFDTNSLIPDDDIVFEDFARLRLKGVIDKEE-DC---- 406
UniProt Q7T2D2
Zebrafish β-Arr2b
QPNSRPQSAVPETDVPVDANLIEFETNNFSQDDDFVFEDFARLRLKGMKDEED-DHFC-- 408
(C)
Human AP2β1
UniprotKB ID: P63010
Zebrafish AP2β1
UniprotKB ID: F6NK19
93.27% identity / 95% positives
100% β-Arrestin-binding residues
(Glu849, Tyr888, Glu902)
Arrestin-binding pocket
Glu849
Tyr888
Glu902
Barbadin
Human β-arrestin
UniprotKB ID: P49407
Zebrafish β-arrestin
UniprotKB ID: C3PTE8
90.67% identity / 96% positives
100% AP2β1-binding hot-spot
(Phe388, Phe391, Arg395)

Figure 3.3. Effects of Barbadin on ERK phosphorylation in dispersed pituitary cells. Following overnight culture, dispersed pituitary cells were pre-treated with Barbadin (25 μM) or DMSO vehicle for 30 min, followed by addition of GnRH2 or GnRH3 (100 nM) for 5 min in the presence of DMSO or Barbadin. Cells were then harvested and lysed, and protein extracts probed for phospho-ERK (Thr202/Tyr204), total ERK, and β-actin. Example blots are presented in panel A. Densitometry readings normalized to the unstimulated vehicle control are presented in panel B. Results (mean ± SEM) are pooled from n = 5 individual cell preparations from goldfish at early gonadal recrudescence (October-November). Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$).

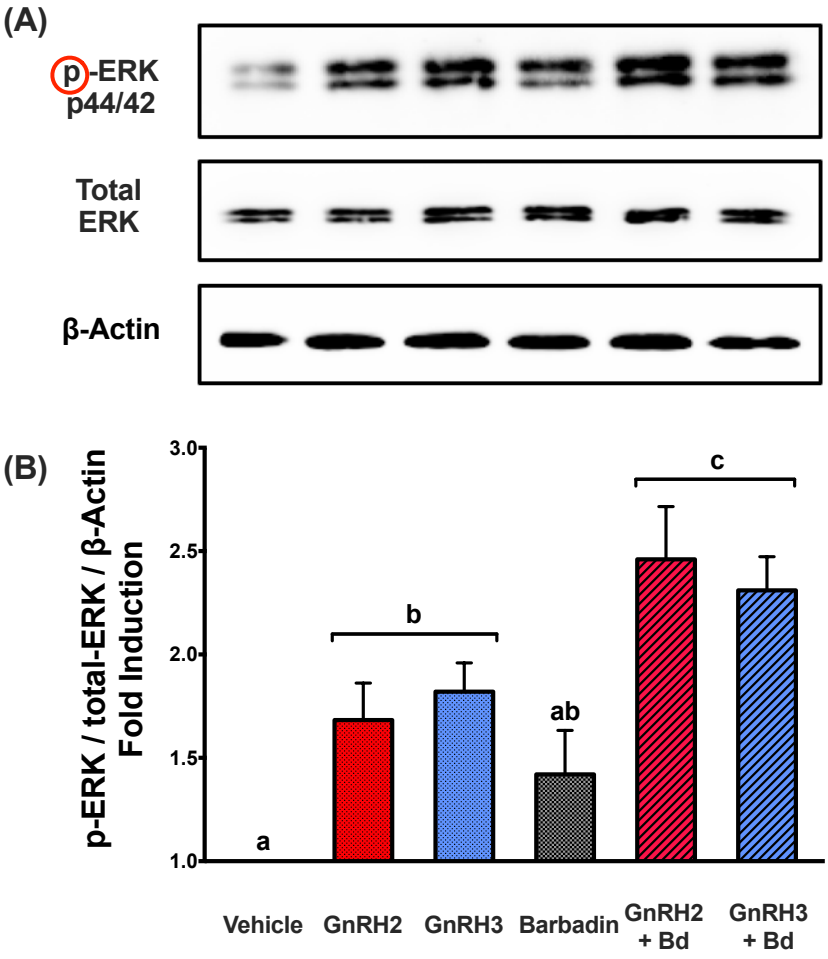
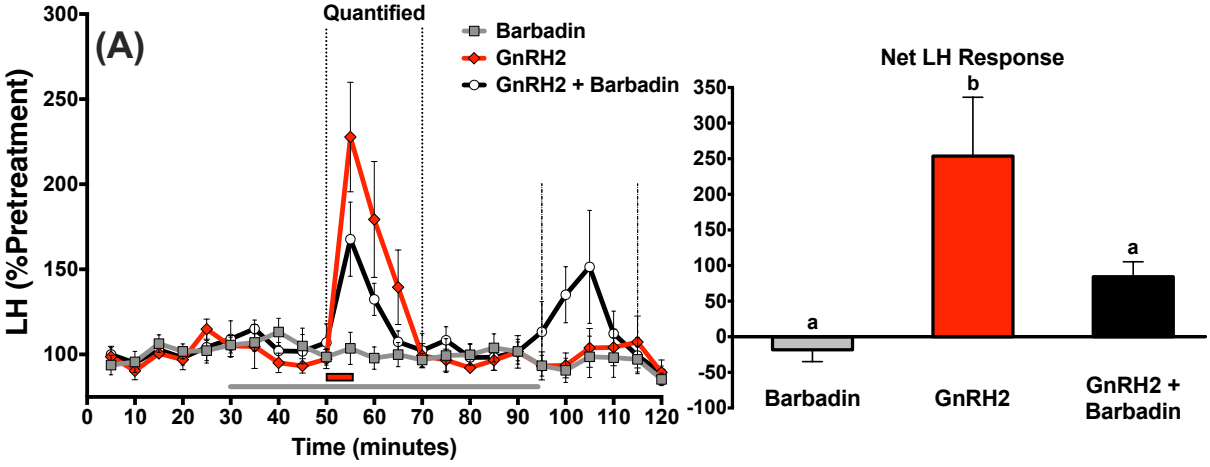
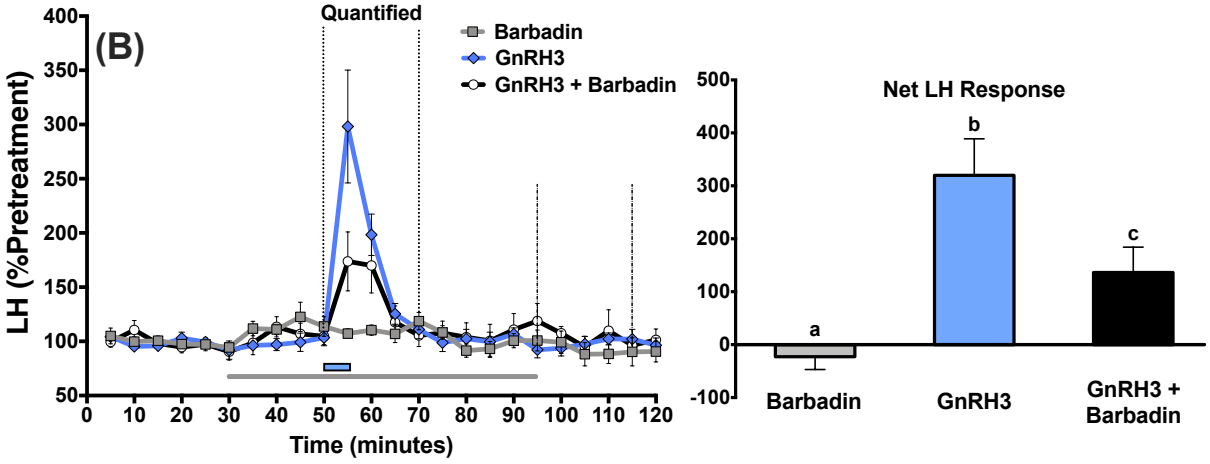

(A)
p-ERK
p44/42
Total
ERK
β-Actin
(B)
p-ERK / total-ERK / β-Actin
Fold Induction
3.0
2.5
2.0
1.5
1.0
a
b
ab
c
Vehicle
GnRH2
GnRH3
Barbadin
GnRH2
+ Bd
GnRH3
+ Bd

Figure 3.4. Effects of Barbadin (25 µM) treatment on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). LH release kinetics are displayed on the left (grey solid square, inhibitor alone; coloured solid diamond, GnRH alone; open white circle, GnRH + Barbadin) and the corresponding quantified net responses to agonist stimulation (between vertical dotted lines) are presented on the right. The grey horizontal line underneath the kinetics indicates duration of Barbadin treatment, and the coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. The quantified net responses upon removal of inhibitor (95 to 115 min) are presented in panel C. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 2.37 ± 0.04 ng/mL, n = 48, from eight independent cell preparations from goldfish at early gonadal recrudescence, December to January). Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).


(A)
Quantified
Barbadin
GnRH2
GnRH2 + Barbadin
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
a
Barbadin
GnRH2
GnRH2 + Barbadin


(B)
Quantified
Barbadin
GnRH3
GnRH3 + Barbadin
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
Barbadin
GnRH3
GnRH3 + Barbadin

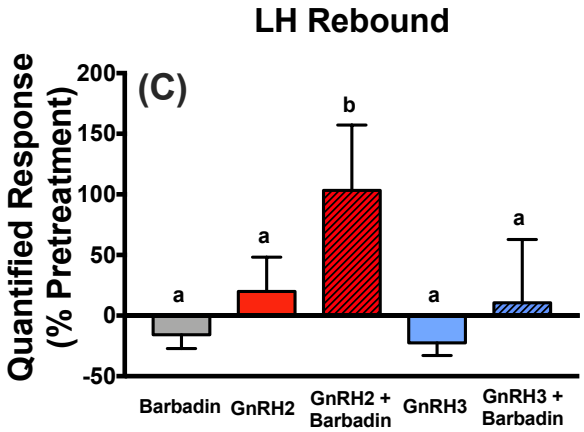
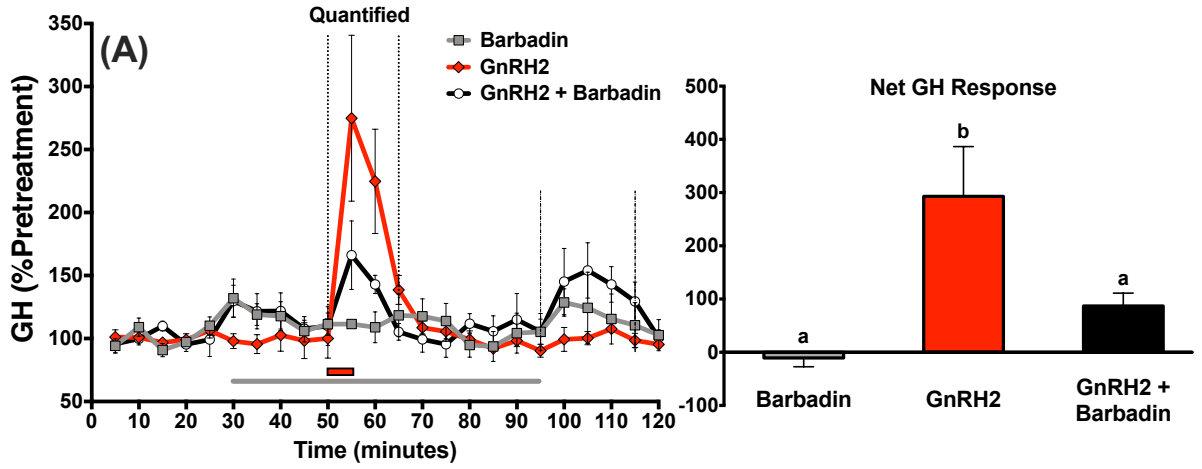
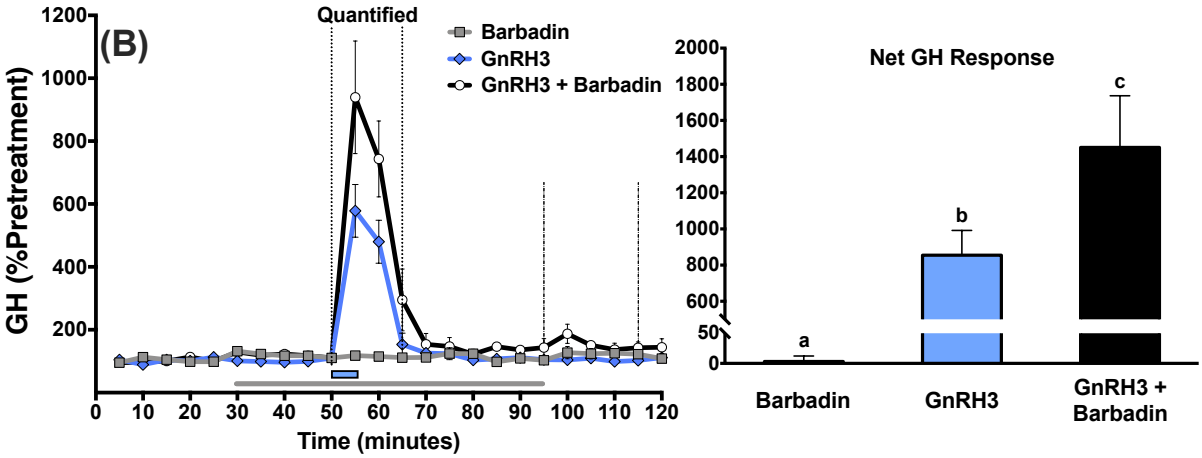

LH Rebound
(C)
Quantified Response
(% Pretreatment)
a
a
b
a
a
Barbadin
GnRH2
GnRH2 + Barbadin
GnRH3
GnRH3 + Barbadin

Figure 3.5. Effects of Barbadin (25 µM) treatment on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). GH release kinetics are displayed on the left (grey solid square, inhibitor alone; coloured solid diamond, GnRH alone; open white circle, GnRH + Barbadin) and the corresponding quantified net responses to agonist stimulation (between vertical dotted lines) are presented on the right. The grey horizontal line underneath the kinetics indicates duration of Barbadin treatment, and the coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. The quantified net responses to removal of inhibitor (95 to 115 min) are presented in panel C. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 6.12 ± 0.25 ng/mL, n = 48, from eight independent cell preparations from goldfish at early gonadal recrudescence, December to January). Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).


(A)
Quantified
Barbadin
GnRH2
GnRH2 + Barbadin
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
a
Barbadin
GnRH2
GnRH2 + Barbadin


(B)
Quantified
Barbadin
GnRH3
GnRH3 + Barbadin
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
c
Barbadin
GnRH3
GnRH3 + Barbadin

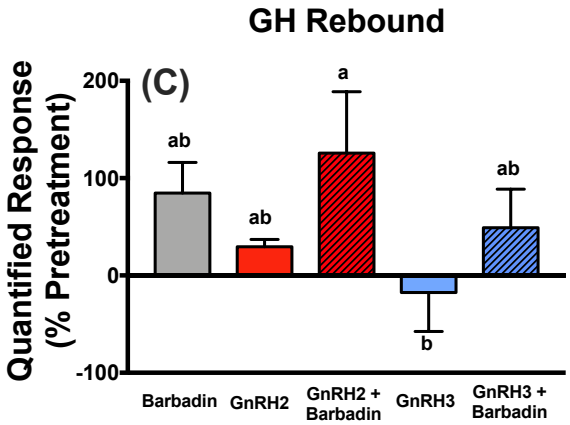

GH Rebound
(C)
Quantified Response (% Pretreatment)
ab
ab
a
b
ab
Barbadin
GnRH2
GnRH2 + Barbadin
GnRH3
GnRH3 + Barbadin

Figure 3.6. Quantified effects of 65 min perifusion with Barbadin (25 µM) on basal LH (panel A) and GH (panel B) release. Results (mean ± SEM) from all Barbadin treatment alone columns in LH and GH release perifusion studies were pooled (n = 16 from 8 individual cell preparations). Basal release prior to inhibitor treatment was calculated as the average of the first four fractions (expressed as "%pretreatment value") when cells were perifused with media alone prior to any pharmacological manipulation. Hormone release during inhibitor-alone treatment was calculated as the average of the percentage pretreatment values over the duration of Barbadin treatment (30 to 95 min; grey horizontal bars from kinetics graphs in Fig. 3.4 & 3.5). An asterisk (*) denotes the presence of significant differences from the pretreatment values prior to inhibitor application (paired Student *t* test; $P < 0.05$).

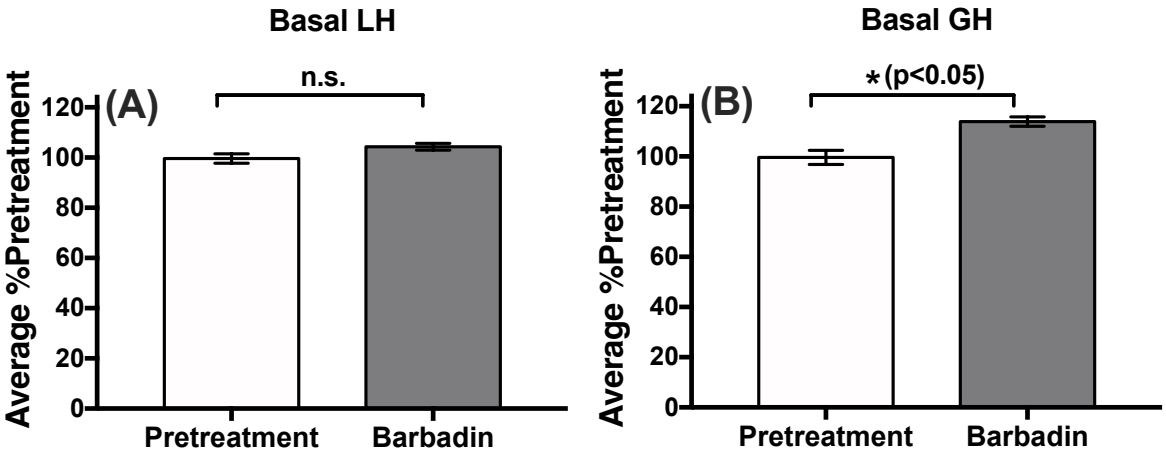

Basal LH
n.s.
(A)
Average %Pretreatment
120
100
80
60
40
20
0
Pretreatment
Barbadin
Basal GH
∗(p<0.05)
(B)
Average %Pretreatment
120
100
80
60
40
20
0
Pretreatment
Barbadin

Figure 3.7. Effects of Dyngo4a (30 µM) treatment on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). LH release kinetics are displayed on the left (grey solid square, inhibitor alone; coloured solid diamond, GnRH alone; open white circle, GnRH + Dyngo4a) and the corresponding quantified net responses to agonist stimulation (between vertical dotted lines) are presented on the right. The grey horizontal line underneath the kinetics indicates duration of Dyngo4a treatment, and the coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 1.83 ± 0.16 ng/mL, n = 48, from eight independent cell preparations from goldfish with regressed gonads or gonads at early recrudescence (September to October). Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

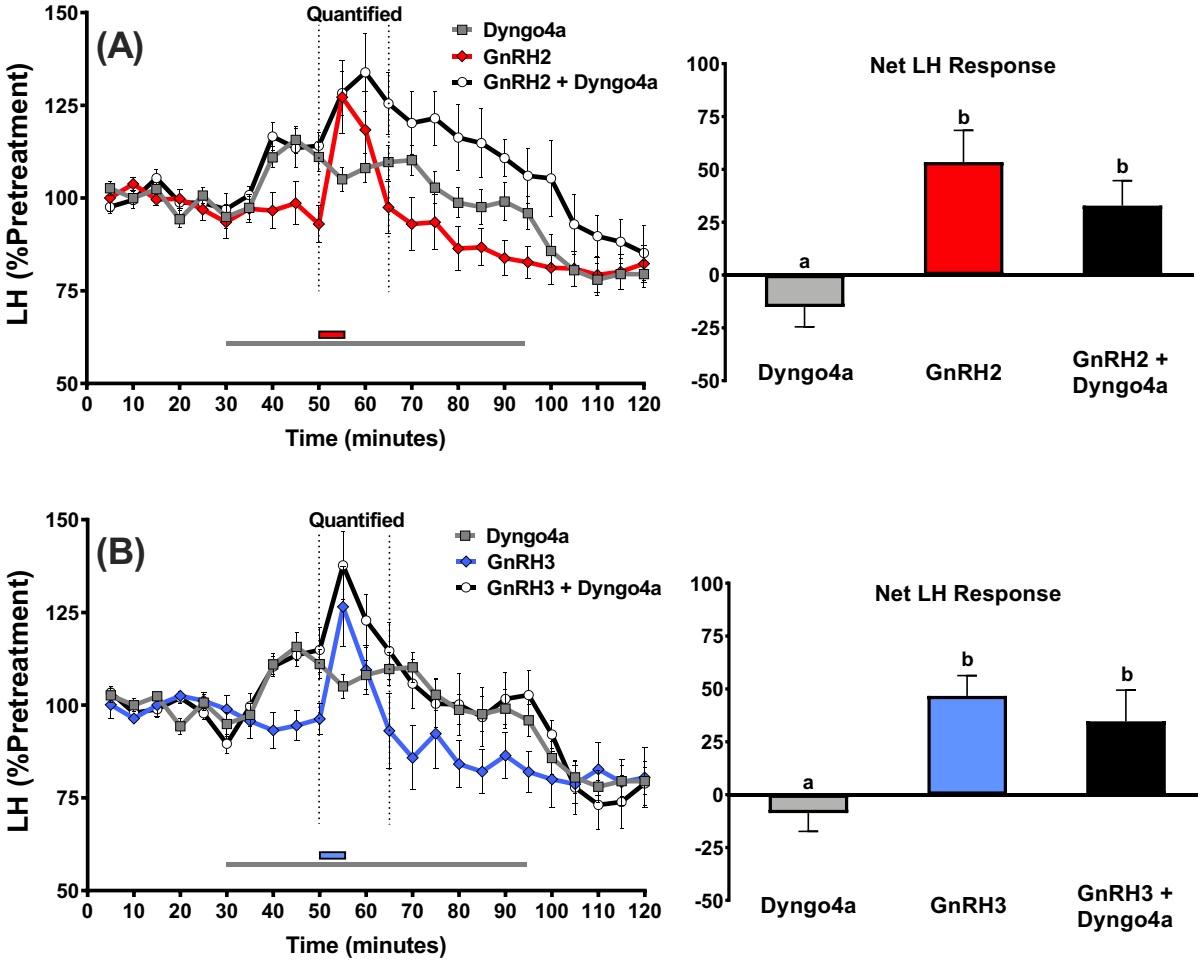

(A)
Quantified
Dyngo4a
GnRH2
GnRH2 + Dyngo4a
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
Dyngo4a
GnRH2
GnRH2 + Dyngo4a
(B)
Quantified
Dyngo4a
GnRH3
GnRH3 + Dyngo4a
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
Dyngo4a
GnRH3
GnRH3 + Dyngo4a

Figure 3.8. Effects of Dyngo4a (30 µM) treatment on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). GH Release kinetics are displayed on the left (grey solid square, inhibitor alone; coloured solid diamond, GnRH alone; open white circle, GnRH + Dyngo4a) and the corresponding quantified net responses to agonist stimulation (between vertical dotted lines) are presented on the right. The grey horizontal line underneath the kinetics indicates duration of Dyngo4a treatment, and the coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 18.99 ± 1.42 ng/mL, n = 48, from eight independent cell preparations from goldfish with regressed gonads or gonads at early recrudescence (September to October). Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

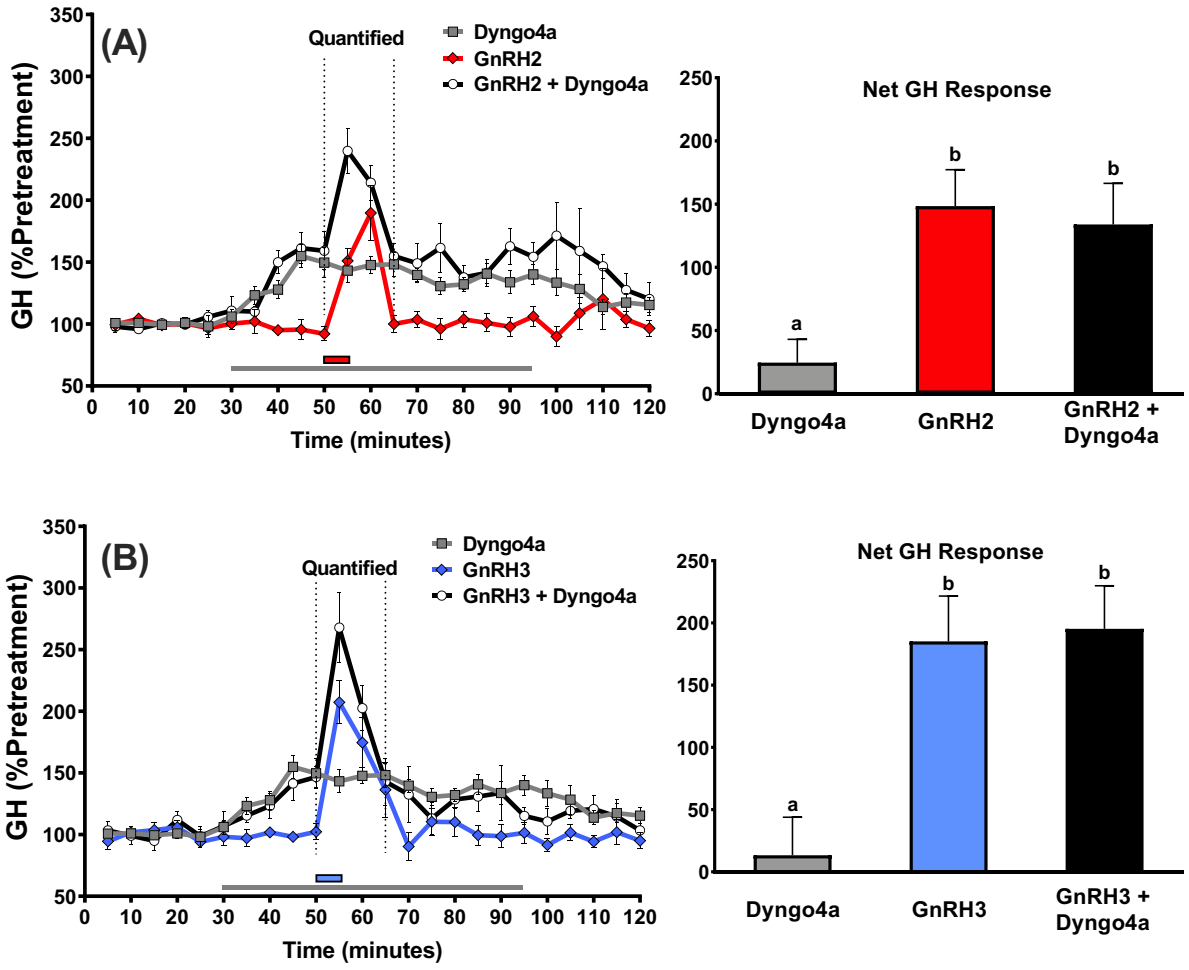

(A)
Quantified
Dyngo4a
GnRH2
GnRH2 + Dyngo4a
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
Dyngo4a
GnRH2
GnRH2 + Dyngo4a
(B)
Quantified
Dyngo4a
GnRH3
GnRH3 + Dyngo4a
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
Dyngo4a
GnRH3
GnRH3 + Dyngo4a

Figure 3.9. Quantified effects of 65 min perifusion with Dyngo4a (30 µM) on basal LH (A) and GH (B) release. Results (mean ± SEM) from all Dyngo4a treatment alone columns in LH and GH release perifusion studies were pooled (n = 16 from 8 individual cell preparations). Basal release prior to inhibitor treatment was calculated as the average of the first four fractions (expressed as "%pretreatment value") when cells were perifused with media alone prior to any pharmacological manipulation. Hormone release during inhibitor-alone treatment was calculated as the average of the percentage pretreatment values over the duration of Dyngo4a treatment (30 to 95 min; grey horizontal bars from kinetics graphs in Fig. 3.7 and 3.8). An asterisk (*) denotes the presence of significant differences from the pretreatment values prior to inhibitor application (paired Student *t* test; $P < 0.05$).

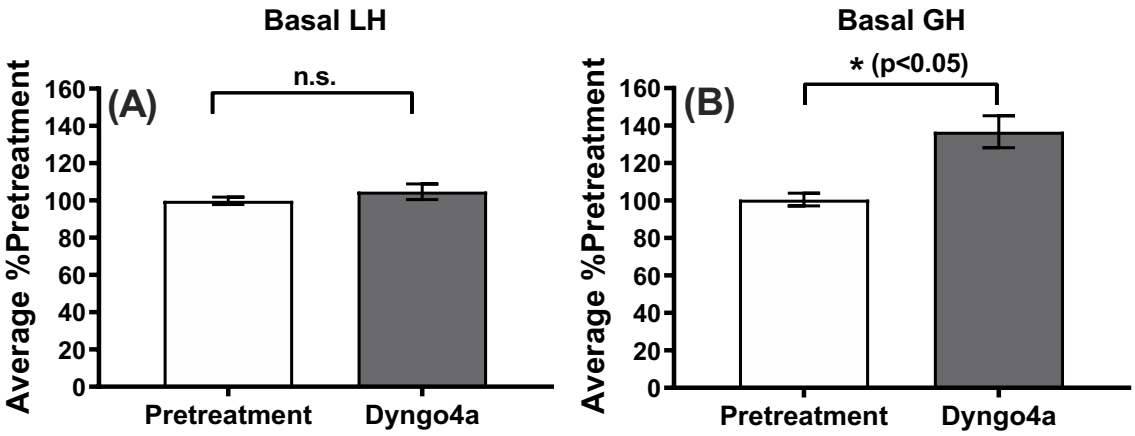
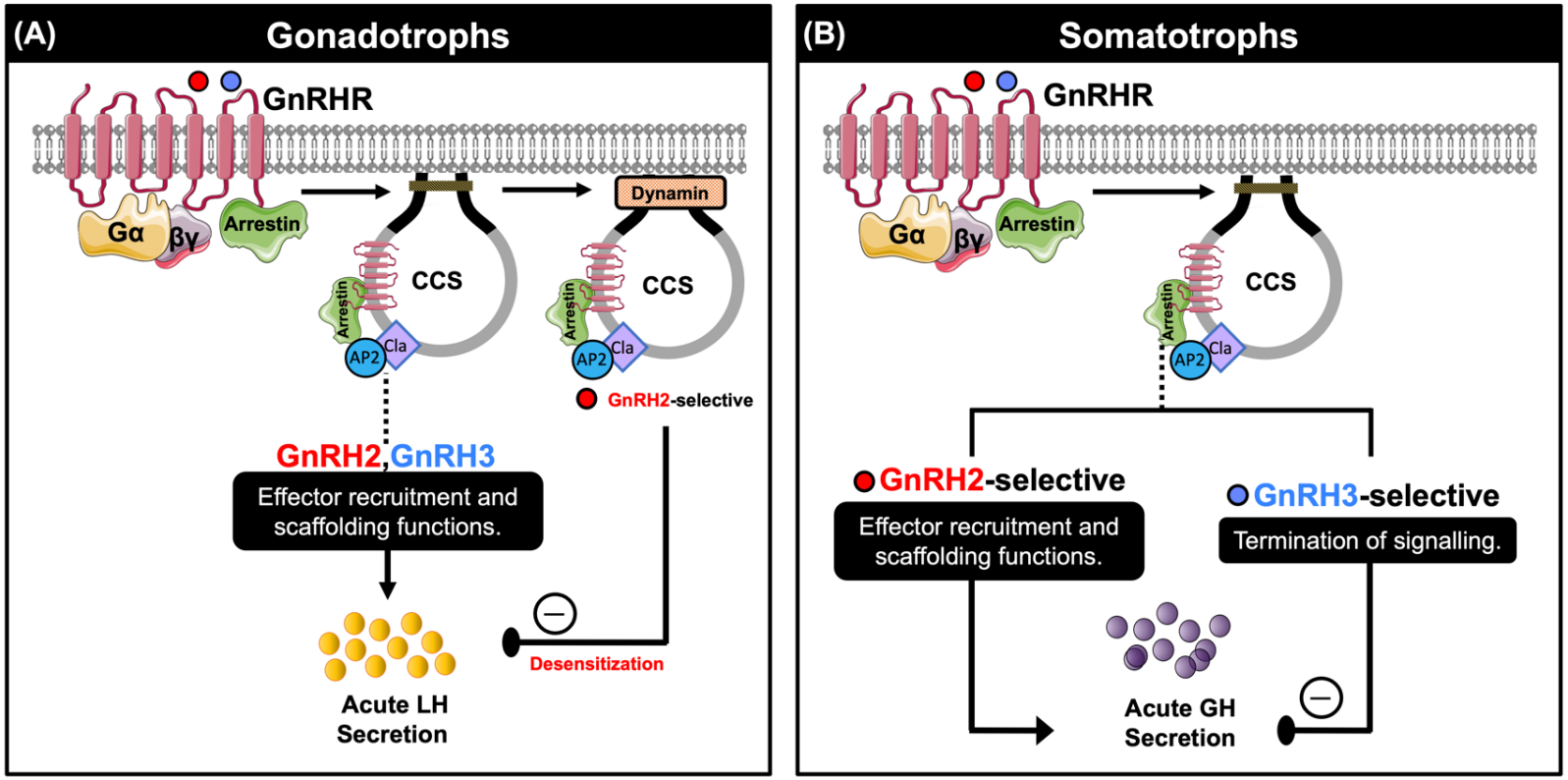

Basal LH
n.s.
(A)
Average %Pretreatment
160
140
120
100
80
60
40
20
0
Pretreatment
Dyngo4a
Basal GH
* (p<0.05)
(B)
Average %Pretreatment
160
140
120
100
80
60
40
20
0
Pretreatment
Dyngo4a

Figure 3.10. Proposed model for arrestin and dynamin actions during GnRH-stimulated hormone release from goldfish pituitary cells. Following receptor activation, GPCRs generally engage heterotrimeric G proteins composed of Gα and Gβγ subunits; G protein subunits mediate several aspects of signal transduction. In addition, for most GPCRs, arrestins are recruited following phosphorylation of intracellular loops and C-terminus tails of receptors (see Figure 1.5 for an overview of arrestin functions). In addition to receptor desensitization through steric hindrance of G proteins, arrestins also target GPCRs to clathrin-coated structures (CCS) through direct associations with the clathrin (Cla) adaptor AP2. The small molecule Barbadin selectively inhibits arrestin-AP2 interactions by occupying the arrestin binding site on AP2 (Figure 3.2), without interfering with arrestin recruitment/binding to receptors, allowing for the pharmacological dissection of arrestin function downstream of GnRHR activation. Based on this mechanism of action, results using Barbadin in column perifusion experiments suggest that arrestin-receptor complexes, localized to Cla-coated structures (CCS), recruit/engage downstream effectors which facilitate LH secretion from gonadotrophs (panel A). While GnRH2-dependent responses utilizing similar mechanisms in somatotrophs (left side, panel B), GnRH3-stabilized receptors in somatotrophs are directed to different fates, likely through receptor-degradation pathways (right side, panel B). In addition, results using Dyngo4a implicate dynamin GTPase in desensitization of the LH release response in gonadotrophs following stimulation with GnRH2, but not GnRH3, which suggests that GnRH2-stabilized GnRHRs in gonadotrophs are selectively internalized through dynamin-dependent scission of CCS (right side, panel A). Refer to section 1.5.2 for further information regarding both general and GnRHR-specific arrestin and dynamin functions. Figure adapted from: Nguyen et al., 2019; Wong et al., 2015; and Beautrait et al., 2017.


(A)
Gonadotrophs
GnRHR
Gα
βγ
Arrestin
CCS
Arrestin
AP2
Cla
Dynamin
CCS
Arrestin
AP2
Cla
GnRH2-selective
GnRH2, GnRH3
Effector recruitment and scaffolding functions.
Acute LH Secretion
Desensitization
(B)
Somatotrophs
GnRHR
Gα
βγ
Arrestin
CCS
Arrestin
AP2
Cla
GnRH2-selective
Effector recruitment and scaffolding functions.
GnRH3-selective
Termination of signalling.
Acute GH Secretion

Chapter Four

Role of G protein alpha subunits and GPCR kinases in GnRH-dependent hormone release

4.1 Introduction

Following GPCR activation, heterotrimeric G proteins are the initial mediators of GPCR signal transduction. Upon activation by the ligand-bound GPCR, and the exchange of a GDP molecule for GTP, Gα dissociates from the Gβγ complex of the heterotrimer, leading to selective modulation of different downstream signalling intermediates in a Gα-subtype specific fashion. Across vertebrate systems, including in the goldfish pituitary, GnRHRs are primarily considered to couple to the $G\alpha_{q/11}$ subtype (Grosse et al., 2000; White et al., 2008; Chang et al., 2012, 2009); however, $G\alpha_{q/11}$ subunit involvement has largely not been directly tested. Linkage to $G\alpha_{s}$ and $G\alpha_{i/o}$ proteins, which canonically modulate AC activity and subsequent cAMP production, is also described for several GnRHR model systems (Hawes, 1993; Liu et al., 2002b; Stanislaus et al., 1998). Although GnRH fails to elevate cAMP levels in goldfish pituitary cells in primary culture, suggesting the lack of a $G\alpha_{s}$ involvement, the potential coupling of goldfish GnRHRs to other non-$G\alpha_{q/11}$ proteins has not been conclusively ruled out (Chang et al., 1993). Additionally, as discussed in Chapter 1, other receptor-interacting effectors are involved in mediating GPCR function and are understudied in goldfish GnRH actions. Besides G protein α and βγ subunits, GRKs are recruited following receptor activation and initiate processes leading to receptor desensitization. Classically, desensitization is driven by released Gβγ subunits, which recruit cytosolic GRKs to the membrane localized GPCR. Here, GRKs can interact with both Gα and Gβγ subunits, and their kinase activity is directly stimulated by the GPCR, leading to subsequent GRK-dependent phosphorylation of residues on GPCR ICLs and C-terminus tails, the recruitment of β-arrestins, and ensuing desensitization (Gurevich and Gurevich, 2019a; Pitcher et al., 1998; Tesmer et al., 2005). In particular, patterns of phosphorylation imprinted by GRKs on GPCR residues determine downstream arrestin recruitment and subsequent actions (Bahouth and

Nooh, 2017). Given that goldfish GnRH actions involve β-arrestins (Chapter 3), determining the role of GRKs in the goldfish pituitary GnRHR system is prudent. In particular, GRK2 and GRK3 are well characterized for their ability to interact with both GPCRs and G protein subunits. Besides this canonical role, GRKs have also gained appreciation as a platform for signal transduction through multiple intracellular cascades, mediated by multiple protein- and lipid-interacting domains contained within these kinases (Penela et al., 2019). Due to the lack of C-terminus tail in prevalent mammalian models of GnRHR function, the study of GRKs, as with arrestins, has not been given much attention, although they are likely to be integral components of type II tailed-GnRHR actions which are known to internalize following receptor stimulation (Millar et al., 2004).

To investigate the role of receptor-interacting effectors, including the complement of Gα subunits and GPCR kinases, I utilized selective inhibitors of $G\alpha_{q/11,}$ $G\alpha_{i/o,}$ pan-Gα subunits, and a catalytic inhibitor of GRK2/3 activity in column perifusion experiments to examine the effects on acute GnRH-induced hormone release (Figure 4.1). Based on the prior observed involvement of phospholipase C, Ca^{2+}/CaM-dependent signalling and PKC in this system, which are all classical $G\alpha_{q/11}$ effectors, I hypothesize that $G\alpha_{q/11}$ subunits mediate GnRH2/3 actions in both gonadotrophs and somatotrophs. On the other hand, signalling to cascades such as the MAPKs and PI3Ks, which participate in goldfish GnRH actions, are not canonically linked to $G\alpha_{q/11}$ subunits, so I predict that additional non-$G\alpha_{q/11}$ Gα proteins, and/or GRKs, also participate in facilitating GnRH-induced responses. Similarly, interfering with GRK activity is likely to impair normal desensitization processes following GnRH stimulation, which may be reflected in signalling endpoints and/or hormone release responses.

4.2 Results

4.2.1 GnRH-induced hormone release involves $G\alpha_{q/11}$-dependent signal transduction

The involvement of $G\alpha_{q/11}$ subunits in acute GnRH-stimulated hormone secretion was investigated using the selective inhibitor YM-254890; this inhibitor had been shown to be highly selective for $G\alpha_{q/11}$ subunits in the micromolar range (Nishimura et al., 2010; Patt et al., 2021; Takasaki et al., 2004). Five min pulse-applications of maximally stimulatory concentrations of GnRH2 and GnRH3 (100 nM; Chang et al., 1990) reliably stimulated LH and GH release. YM-254890 (1 μM) application had no noticeable effects on basal secretion (Figures 4.2 and 4.3, grey traces) of either hormone; however, it significantly suppressed both LH and GH secretion responses to GnRH2 and GnRH3. Noticeably, this suppressive effect was more pronounced for GnRH3-stimulated LH and GH release and GnRH-2-induced LH secretion. In particular, GnRH2-stimulated GH release retained a YM-254890-insensitive component that was significantly different from both GnRH2 alone and YM-254890 alone treatments (Figure 4.3).

4.2.2 Broad Gα inhibition reveals disparate roles of these subunits in LH and GH release

To investigate the potential involvement of other Gα subunits in addition to $G\alpha_{q/11,}$ I also examined the effects of a pan-Gα inhibitor, BIM-46187, employing a 10 μM dose based on the reported IC_{50} of 1-3 μM for both inositol phosphate and cAMP accumulation across a panel of GPCRs (Ayoub et al., 2009). Basal levels of LH and GH release were elevated in the presence of this compound (Figures 4.4 and 4.5) and did not return to baseline following removal of the inhibitor, suggesting the involvement of some type(s) of Gα subunits in constitutive LH and GH secretion. BIM-46187 affected the LH and GH secretion response to GnRH differently. BIM-46187 abolished GnRH-induced LH release; however, in its presence, both GnRH isoforms were

still able to elicit GH secretion with net responses being comparable to those observed in GnRH treatments alone.

4.2.3 Pertussis-sensitive mechanisms are involved in GnRH3-dependent hormone release

Next, I examined whether $G\alpha_{i/o}$ subunits are part of the GnRH signalling components leading to LH and GH secretion using pertussis toxin (PTX). Due to the mechanisms of PTX uptake into cells and the slow onset of subsequent inhibition of $G\alpha_{i/o}$ subunits (Katada, 2012), studies using this toxin in examination of GPCR functions typically employ overnight incubations (Liu et al., 2002a; Robinson and Dickenson, 2001; Velarde et al., 1999). Accordingly, goldfish pituitary cells to be used in PTX alone and GnRH + PTX groups were cultured in the presence of 10 ng/mL PTX during the overnight cell culture period following pituitary cell dispersion, and PTX was additionally present during the 4-h acclimation period of the perifusion protocol as well as the testing period for these same groups (see also methods; Section 2.5). This treatment protocol prevented the evaluation of the effects of PTX treatment on basal hormone release within the same columns as in the above results sections in this chapter. However, in the presence of 10 ng/mL PTX, a dose sufficient to inhibit $G\alpha_{i/o}$ subunits in cell-based assays (Katada et al., 1982; Paramonov et al., 2020; Raze et al., 2006), both LH and GH hormone responses to GnRH3, but not those to GnRH2 were selectively suppressed (Figures 4.6 and 4.7). This suppressive effect of PTX was especially pronounced for GnRH3-evoked LH secretion.

4.2.4 GRK2/3 are differentially involved in GnRH-stimulated LH and GH release

The GRK2/3 dual inhibitor CMPD101 was employed at a dose of 3 μM, which is sufficient to inhibit endogenous GRK2-dependent actions in 3T3-L1 fibroblast cells (Cannavo et al., 2019), as well as in primary rat and mouse neurons (Lowe et al., 2015). CMPD101 application had negligible effects on basal LH and GH release (Figures 4.8 and 4.9, grey traces), but suppressed the acute LH responses to both GnRH isoforms by 50% or more (Figure 4.8). In somatotrophs, while the acute GH response to GnRH3 was not significantly affected by CMPD101, acute GnRH2-stimulated GH release was enhanced by another-fold in the presence of the GRK2/3 inhibitor (Figure 4.9). Additionally, the GnRH-stimulated GH secretion dynamics in the presence of CMPD101 were markedly prolonged and did not return to baseline following inhibitor washout, a finding not observed in gonadotrophs. Average GH values in both GnRH + CMPD101 groups were significantly higher than those in the CMPD101 alone group over the next 5 fractions following termination of the acute response quantification (70 to 90 min of the perifusion experiment; CMPD101: 109.24 ± 8.29 % pretreatment; GnRH2 + CMPD101: 151.04 ± 29.85 % pretreatment; GnRH3 + CMPD101: 167.71 ± 18.96 % pretreament; $P<0.05$ for both comparisons, Student's t-test).

4.2.5 Effects of $G\alpha_{q/11}$, pan-Gα, and GRK2/3 inhibitors on ERK phosphorylation

Consistent with previous findings (Klausen et al., 2008), stimulation of dispersed goldfish pituitary cells with 100 nM of either GnRH2 or GnRH3 for 5 min elevated phospho-ERK levels in protein extracts by 0.3 to 0.4 fold above unstimulated controls (Figure 4.10). YM-254890 treatment alone suppressed phospho-ERK levels, and GnRH stimulation in the presence of this compound did not restore phospho-ERK back to basal levels. In contrast, BIM-46187 (Figure 4.10 A) and CMPD101 (Figure 4.10 C) treatments elevated phospho-ERK above DMSO

controls, and GnRH stimulation similarly did not alter p-ERK levels beyond that of inhibitors alone.

4.3 Discussion

In this chapter, I examined whether receptor-interacting effectors (G-protein subunits and GRKs) participate in GnRH-stimulated LH and GH secretion from primary cultures of dispersed goldfish pituitary cells in a cell-type- and GnRH-isoform-selective fashion. Results reveal both ligand-selective and cell type-specific effects of this complement of proteins in mediating GnRHR actions. These novel findings indicate that G proteins and GRKs have substantial roles in propagating biased GnRHR transduction, and heavily implicate ligand-dependent coupling of goldfish GnRHRs to multiple Gα subunits in physiological contexts. Importantly, experiments utilizing $G\alpha_{q/11}$ selective inhibitors confirm the obligate requirement for these subunits in mediating goldfish acute GnRHR actions on pituitary hormone secretion.

4.3.1 $G\alpha_{q/11}$ subunits are obligate mediators of GnRH-induced hormone release

Despite clear demonstrations of the differential involvement of downstream second messengers in GnRH action on goldfish LH and GH secretion (Chang et al., 2000), information regarding the G-protein subunit repertoire involved in the control of hormone release is incomplete. This study is the first to employ direct inhibitors of Gα subunits to study their participation in GnRH-stimulated hormone secretion in a native and untransformed cell system. YM-254890 is a potent and selective $G\alpha_q$ inhibitor (Zhang et al., 2019). YM-254890 complexes with $G\alpha_q$ and stabilizes the GDP-bound state of Gα, preventing GDP exit from the nucleotide binding pocket which is the rate-limiting step in the GDP/GTP exchange process during the

initiation of G-protein signaling (Nishimura et al., 2010). Selectivity towards $G\alpha_q$ (and the related $G\alpha_{11}$ and $G\alpha_{14}$) has been well characterized in multiple cell lines; YM-254890 suppresses $G\alpha_q$-mediated second messenger production but does not interfere with signalling mediated by $G\alpha_s$, $G\alpha_{i/o}$ or $G\alpha_{15}$ when tested at concentrations up to 10 μM (Patt et al., 2021; Takasaki et al., 2004). The identified binding pocket and contact residues, which are conserved between $G\alpha_q$, $G\alpha_{11}$, and $G\alpha_{14}$ proteins, are 100% conserved among homologs of these G protein subunits in vertebrates (Figure 4.11). Results with YM-254890 indicate that $G\alpha_{q/11}$ subunits are obligate transducers of GnRHR signalling in the control of both LH and GH secretion from the goldfish pituitary (Figures 4.2 and 4.3). This provides the first direct evidence for these subunits being upstream of effectors classically linked to $G\alpha_{q/11}$ in this system, including PLC-β and PKC (Chang et al., 2000).

4.3.2 BIM-46187 reveals the role of non-$G\alpha_{q/11}$ Gα protein subunits in goldfish pituitary somatotrophs

Unlike YM-254890, BIM-46187's mechanism of action is less clear. Although BIM-46187 was initially reported to broadly silence all G-protein signalling (Ayoub et al., 2009), it was later shown to have varying selectivity towards $G\alpha_q$ *vs.* $G\alpha_s$/$G\alpha_{i/o}$ subunits depending on the cellular context; e.g., it was selective for $G\alpha_{q/11}$ in HEK and CHO cell lines but acted as a pan-Gα inhibitor in human skin cancer MZ7 cells (Schmitz et al., 2014; Zhang et al., 2019). However, in addition to receptor-G protein association studies in live cells, BIM-46187's specific interactions with Gα were also validated in a reconstituted cell-free system (Ayoub, 2018; Ayoub et al., 2009). As expected, the effects of BIM-461897 mirrored those of YM-254890 in gonadotrophs, with both being effective at attenuating GnRH-induced LH release.

Contrary to my hypothesis, however, BIM-46187 did not alter acute GnRH-stimulated GH secretion (Figure 4.5). Why BIM-46187 did not affect GnRH actions on GH release is unknown, but the presence of non-$G\alpha_{q/11}$ elements in regulating GnRH-induced GH release is a distinct possibility. A recent meta-analysis has revealed that GPCRs, including GnRH receptors, can activate more than a single subtype of G-proteins within a particular family (Hauser et al., 2022), which is likely to have functional consequences downstream (Masuho et al., 2015). In this regard, differential usage of PI3K p110β and p110γ subunits downstream of Gβγ subunit activation in the goldfish pituitary system has been shown (Pemberton et al., 2015; Pemberton and Chang, 2016). Not only do different Gα subunits preferentially associate with select Gβγ units to form distinct heterotrimers, but Gα subunits also play a role in dictating plasma membrane availability and distribution of the heterotrimer, as well as influencing association/dissociation kinetics of the complex (Mystek et al., 2019). Whether cell-specific differences in microdomain environment, such as lipid raft distribution, and in Gα subtype complement, as in the case of the dopamine D1 receptor (Mystek et al., 2016), also underlie the present observations between gonadotrophs and somatotrophs should be considered in the future.

The specific mechanisms of Gα inhibition by the two small molecules inhibitors are also different. While YM-254890 functions as a GDP dissociation inhibitor, stabilizing the inactive Gα-GDP state, BIM-46187 traps Gα in the "empty pocket configuration", following GDP exit but prior to GTP entry and binding (Ayoub, 2018; Schmitz et al., 2014). This distinction may have functional consequences, since agonist binding to the GPCR is likely a prerequisite for GDP release from Gα (Kumar et al., 2020). Thus, if the receptor-G-protein activation dynamics and/or the complement of Gα subtype differ between gonadotrophs and somatotrophs, the

mechanisms of inhibition by YM-254890 and BIM-46187 may underlie the observed differences in their abilities to modulate GnRH-induced LH and GH release.

Importantly, results from immunoblotting studies with YM-254890 mirrored the perifusion data for $G\alpha_q$ inhibition, with GnRH-driven signalling being suppressed in its presence (Figure 4.10 A; lanes 7-9). This is also consistent with findings using this inhibitor in conjunction with GnRH stimulation in GT1-7 cells (Higa-Nakamine et al., 2015), as well as with results from genome-deletion studies showing that G proteins are an obligate requirement to initiate ERK signalling downstream of GPCRs (Grundmann et al., 2018; O'Hayre et al., 2017). As with the hormone release experiments, the effects of BIM-46187 were different from YM-254890. First, BIM-46187 elevated phospho-ERK levels above that of the untreated control, and GnRH stimulation in the presence of the compound did not significantly raise phospho-ERK above BIM-46187 treatment alone (Figure 4.10 A; lanes 4-6). This is not surprising in light of BIM-46187's differential effects on GnRH-stimulated LH *vs.* GH release (Figures 4.4 and 4.5). Also, phospho-ERK levels were measured in protein extracts from mixed populations of dispersed pituitary cells; thus, specific phospho-ERK changes in gonadotrophs and somatotrophs may be masked when cell-specific effects are present.

4.3.3 PTX-sensitive inhibition of GnRH-induced hormone release

Results from PTX treatment in the present study revealed, for the first time in goldfish, a $G\alpha_{i/o}$-dependence in GnRH actions in gonadotrophs and somatotrophs; GnRH3-induced LH and GH release responses were selectively dampened in the presence of PTX. This toxin inactivates $G\alpha_{i/o}$ subunits by ADP-ribosylation, which stabilizes the GDP-bound form and selectively uncouples $G\alpha_{i/o}$ from GPCRs without altering rates of GDP/GTP exchange or interactions with

downstream $G\alpha_{i/o}$ effectors (Katada, 2012; Katada et al., 1986). PTX specifically ADP-ribosylates $G\alpha_{i/o}$ subunits at Cys^{351} residues near the C-terminus; importantly, all of $G\alpha_{i1,}$ $G\alpha_{i2,}$ $G\alpha_{i3,}$ and $G\alpha_{o}$ subunit isoforms and their homologs in fish possess this equivalent residue (Fig 4.12). The $G\alpha_{i3}$ isoform in fish has a substitution (Glu350 → Asp350) at the adjacent position; however, both are acidic amino acids with largely similar properties and changes in selectivity of PTX to this isoform may be minimal. Some other members of this family ($G\alpha_{t}$ and $G\alpha_{gust}$) also carry Cys^{351} but their expression is restricted to taste buds and the visual system, respectively (Hoon et al., 1995; Lerea et al., 1986), while the $G\alpha_{z}$ isoform has an neutral, nonpolar, uncharged Ile residue in the corresponding position and is PTX-insensitive (Fields and Casey, 1997). $G\alpha_{i1,}$ $G\alpha_{i2,}$ $G\alpha_{i3}$ are considered to be ubiquitously expressed, and $G\alpha_{o}$ has been found in neurons and neuroendocrine cell types; regardless, if present, these isoforms are likely to be PTX-sensitive. Additionally, non $G\alpha_{i/o}$ subunits, including $G\alpha_{q/11,}$ $G\alpha_{s,}$ $G\alpha_{12/13,}$ do not contain the equivalent cysteine residue targeted by PTX (Holbourn et al., 2006; Katada, 2012; Mangmool and Kurose, 2011; Milligan, 1988). Taken together, the above information strongly suggests a role for $G\alpha_{i1,}$ $G\alpha_{i2,}$ $G\alpha_{i3,}$ and/or $G\alpha_{o}$ subunit in mediating GnRH3, but not GnRH2, stimulation of goldfish LH and GH secretion.

PTX has previously been tested in goldfish pituitary cells, albeit in long term static incubation studies and in the absence of GnRH stimulation. PTX treatment elevated both basal LH and GH release from static cultures, and mirrored the effects of cholera toxin ($G\alpha_{s}$ activator), which leads to similar downstream increases in cAMP production (Chang et al., 1993). On the other hand, GnRH treatments do not elevate cAMP levels, and GnRH-induced LH and GH secretion responses are additive to responses to neuroendocrine regulators that stimulate hormone release through cAMP/PKA pathways or those of treatments elevating intracellular

cAMP levels (i.e., addition of cAMP analogs or a phosphodiesterase inhibitor; Chang et al., 2001, 1993; Wong et al., 1994, 1993). In addition, GnRH stimulation of hormone release is insensitive to inhibition of PKA (Jobin et al., 1996a). These observations suggest a lack of involvement of cAMP-dependent mechanisms in GnRH-dependent hormone release from goldfish gonadotrophs and somatotrophs. Thus, although $G\alpha_{i/o}$ inhibition elevates LH and GH release from static cultures, the present results with PTX indicate the participation of AC/cAMP-independent actions of $G\alpha_{i/o}$ in GnRH3-specific effects during acute stimulation. How this is mediated is presently unknown, but several possibilities exist.

In particular, for some GPCRs, PTX-sensitive mechanisms can increase cytosolic Ca^{2+} in a cAMP-independent fashion (Chavis et al., 1994; Koch et al., 1985). Additionally, the tyrosine kinase Src is also well characterized as a $G\alpha_{i/o}$ effector through direct binding interactions, which is presumably independent of cAMP formation (Luttrell and Luttrell, 2004; Ma et al., 2000). In the goldfish pituitary system, PLA_2-mediated activation of AA signalling is selectively involved in GnRH3-dependent LH release (Chang et al., 1991), and PLA_2 is a known effector of $G\alpha_{i/o}$-dependent signalling (Murray-Whelan et al., 1995); this reflects a possible mechanism for the PTX-sensitive inhibition of GnRH3-induced LH secretion in the present results. Furthermore, $G\beta\gamma$-dependent activation of PI3K p110β and p110γ subunits downstream of $G\alpha_{i/o}$-coupled GPCRs, such as those for complement proteins, formyl peptide, and leukotrienes is well established (Guillermet-Guibert et al., 2008; Rynkiewicz et al., 2020; Stephens et al., 1997; Suire et al., 2006; Vadas et al., 2013). Prior work from our laboratory has shown that $G\beta\gamma$ heterodimers are also essential for both GnRH2 and GnRH3 control of LH and GH release, and the involvement of these Class I PI3K subunits in GnRH stimulation of LH and GH release in goldfish has been demonstrated (Pemberton et al., 2015; Pemberton and Chang, 2016). In

addition, Gβγ complex liberated from $G\alpha_{i/o}$-coupled GPCRs can also activate PLC-β (Gurbel et al., 2015). Thus, it is conceivable that the present LH and GH results with GnRH3 and PTX can also be integrated with the previously identified involvement of Gβγ-dependent (p110β/p110γ and/or PLC-β) actions. However, the dissimilarities between GnRH2-induced responses during Gβγ *vs.* $G\alpha_{i/o}$ inhibition certainly warrant future investigations.

Interestingly, several GPCR-screening studies have revealed that a growing number of receptors have promiscuous couplings to multiple Gα types (Hauser et al., 2022; Masuho et al., 2015; Wan et al., 2018); in particular, $G\alpha_s$ and $G\alpha_{q/11}$-coupled receptors can often additionally engage $G\alpha_{i/o}$ subunits. Recently, a novel form of BRET (bioluminescent resonance energy transfer) methodology was used in HEK293 cells to show non-cognate binding to $G\alpha_{i/o}$ subunits by receptors that are $G\alpha_s$ (vasopressin V2 receptor, β2 adrenergic receptor, dopamine D1 receptor)- or $G\alpha_{q/11}$ (neurotensin receptor 1)-coupled. This coupling was PTX sensitive and a tripartite association was additionally shown between the GPCR, $G\alpha_{i/o}$, and β-arrestin, with this complex propagating signals to downstream effectors (Smith et al., 2021; Smith and Pack, 2021). In the same study, primary human smooth muscle cell responses to an AT1R β-arrestin-biased ligand (which does not promote canonical G protein signalling) were also inhibited by PTX, suggesting that this dependence on $G\alpha_{i/o}$ is also present in native cell contexts with physiological levels of receptor expression. It has also been proposed that such non-cognate interaction between $G\alpha_{i/o}$ and GPCRs is independent of nucleotide exchange (Smith and Pack, 2021) as has recently been shown for $G\alpha_{12/13}$ coupled-receptors (Okashah et al., 2020). This would greatly expand current models of G protein signal transduction, but further experimental characterization of non-cognate Gα functions is needed. Furthermore, emerging evidence suggests that the presence of $G\alpha_{q/11}$ subunits is required for $G\alpha_{i/o}$-linked GPCRs to transduce signals through Gβγ

subunits to PLC-β and Ca^{2+}-dependent mechanisms (Pfeil et al., 2020), indicative of a type of layered/cooperative mechanism between multiple classically "independent" pathways.

Regardless, the present results implying that goldfish GnRHRs utilize more than one Gα subtype are not at odds with the current understanding of GnRHR biology. As mentioned above (Section 4.1), GnRHR coupling to $G\alpha_s$ and $G\alpha_{i/o}$ have previously been described, and recent data from mammals has further elaborated on a physiological role for *in vivo* dual coupling. Mouse pituitary gonadotroph-specific deletions of $G\alpha_{q/11}$ or $G\alpha_s$ reveal that both subunits are required, albeit in different contexts, for mediating GnRH-induced responses of LH and FSH (Stamatiades et al., 2022). While deletions of $G\alpha_{q/11}$ resulted in infertility and a hypogonadotropic hypogonadism phenotype as previously shown in GnRH- or GnRHR-deficient mice (Cattanach et al., 1977; Pask et al., 2005), $G\alpha_s$-knockout mice showed impaired LH and FSH responses to gonadectomy, corresponding with decreases in pituitary gonadotrophin gene expression. Additionally, female mice of this genotype had reductions in LH surge amplitude, whereas males displayed blunted LH release responses to exogenous GnRH (Stamatiades et al., 2022). Also, in *in vitro* studies with LβT2 gonadotrophs, GnRH-induced increases in *Fshb* transcript levels were selectively reduced during low frequency GnRH stimulation in cells with $G\alpha_s$ knockdown, whereas $G\alpha_{q/11}$ knockdown resulted in similarly blunted *Fshb* transcript levels at low and high pulsatility (Stamatiades et al., 2022). These findings are also consistent with prior reports in LβT2 gonadotrophs that $G\alpha_s$-induced increases in cAMP were selectively brought about only during sustained GnRH stimulation (Larivière et al., 2007), and $G\alpha_s$-cAMP-PKA signalling is required for *Fshb* transcription in the same cells (Thompson et al., 2013). When all these results are viewed together, they strongly suggest that GnRHR interactions with more than one class of Gα is physiologically relevant and functionally selective. Future investigations in the goldfish

pituitary system are likely to reveal additional roles of non-cognate Gα types in GnRH control of pituitary cell functions over diverse timeframes and functional contexts.

4.3.4 GRK2/3 exert cell type-selective effects in GnRH-evoked LH and GH release

Besides recruiting cytosolic β-arrestins by phosphorylating activated GPCRs, leading to arrestin-mediated GPCR desensitization and arrestin-dependent signalling, GRKs also play a role in initiating a branch of "G-protein independent" signalling downstream of Class A GPCRs, including serving as a scaffolding for ERK activation (Gurevich et al., 2012; Gurevich and Gurevich, 2019a). Experiments in this thesis chapter sought to determine the roles of GRKs in goldfish pituitary cells using the GRK2/3 dual inhibitor CMPD101. GRKs are highly conserved between mammals and teleosts; in particular, CMPD101-interacting residues in the kinase domain are 100% conserved (Figure 4.13; Thal et al., 2011). Results in this chapter reveal the differential functions of GRK2/3 in GnRH-engaged receptors in gonadotrophs but not somatotrophs with CMPD101 treatment attenuating the LH, but not GH, secretion responses to both GnRHs (Figures 4.8 and 4.9). However, possibly due to the use of mixed populations of goldfish pituitary cells, immunoblotting indicated that CMPD101 did not alter overall GnRH2/3-induced ERK activity (Figure 4.10 C,D). This may also reflect divergence of receptor-propagated signals at the transducer level; while GRKs participate in driving signals leading to hormone release, these don't necessarily converge or interdict with MAPKs in the tested context.

Interestingly, the quantified acute GnRH2-induced GH release during GRK inhibition nearly doubled, while GnRH3-stimulated GH release was not impacted. The GH profiles in response to both GnRHs were also prolonged in the presence of CMPD101, i.e., the combination treatment took longer to return to baseline. These observations agree with the classical

desensitization function of GRKs (Gurevich and Gurevich, 2019); under catalytic inhibition, GRKs would be unable to dampen Gα-mediated signalling, leading to an extended response in the absence of this normal desensitization.

The blunting of LH responses to GnRH2/3 by CMPD101 suggests a GRK-dependent arm of positive signalling in LH secretion, for which there is support from various GPCR model systems (Penela et al., 2019). For example, GRK2 is known to complex with PI3Ks via their PIK/accessory domain, an interaction necessary for the local generation of PtdIns(3,4,5)P_3 phospholipids associated with GPCR endocytosis (Naga Prasad et al., 2002, 2001). In these studies, the conserved PIK domain isolated from p110γ could directly interact with GRK2 and wasn't dependent on Gβγ subunits, which are known to drive p110β and p110γ activation. In goldfish pituitary cells, Class I PI3K p110β and p110γ isoforms mediate GnRH2- and GnRH3-induced LH release, respectively. Thus, GnRH-engaged receptors in gonadotrophs may recruit Class I PI3K isoforms via GRK2-PH domains, either downstream or independent of Gβγ subunits. Taken together with data from prior arrestin studies (Chapter 3), while GRK2/3 likely act in concert with arrestins in somatotrophs and gonadotrophs, they can also be uncoupled from arrestin functions in some cases, at least in somatotrophs. While arrestin-AP2 inhibition attenuated the LH responses to both GnRHs (similar to GRK inhibition in this chapter), it blunted and potentiated the GnRH2- and GnRH3-induced GH secretion responses, respectively. Such uncoupling of functions within the typical "GRK-arrestin" paradigm has previously been observed for other members of the class A hormone-GPCR family. For example, dopamine D2 receptors and the delta opioid receptors are capable of undergoing arrestin-dependent desensitization without a requirement for GRK-dependent phosphorylation (Celver et al., 2013). Conversely, the $G\alpha_{q/11}$-coupled gastrin-releasing peptide receptor undergoes agonist-induced

desensitization that requires GRK phosphorylation, and can do so even in the absence of arrestins (Kroog et al., 1999). Similarly, as discussed in Chapter 3, dynamin-dependent and arrestin-independent receptor internalization can also be promoted by GRK activity (Von Moo et al., 2021). Independent of arrestin activity, GRKs are also a common target of several intracellular feedback loops, which fine-tune its spatiotemporal effects in the cell through post-translational modifications. Some examples include PKC, phosphorylation by which enhances GRK activity, whereas phosphorylation by ERK and Src kinases decrease GRK protein stability (Penela et al., 2019).

Interestingly, through protein-protein interactions mediated by their PH domains, GRKs are also known to sequester Gβγ subunits, and their downstream effects, following recruitment to the activated GPCR (Gurevich and Gurevich, 2019a). While these roles are consistent with the observed effects of Gβγ inhibition in goldfish pituitary cells during GnRH-stimulated LH release (Pemberton and Chang, 2016), the present results suggest diverging mechanisms occur in somatotrophs at the level of the GRK-Gβγ interactions. Indeed, GRKs can form complexes with Gα, Gβγ, and the GPCR, through simultaneous interactions with all three (Tesmer et al., 2005); thus, how interfering with GRK function specifically alters downstream responses of each of the other components likely involves complex effects and warrants future investigations. Overall, it is evident that the classical view of GRKs in receptor desensitization is insufficient, and heterogeneity of function is being continuously uncovered by emerging models of GRK actions (Chen and Tesmer, 2022), and this is further supported by results in this thesis chapter even downstream of a single population of GPCRs.

4.3.5 Effects of G protein inhibitors on basal hormone release

Exposure to BIM-46187, but not YM-254890, elevated basal LH and GH secretion over the duration of the perifusion. These results suggest that some unknown non-$G\alpha_{q/11}$ Gα subunits exert inhibitory influences over the control of basal release in both gonadotrophs and somatotrophs. The identity of these additional inhibitory components is unknown, but a potential role of $G\alpha_{i/o}$ subunits in this context may exist. In the goldfish, activation of dopamine D2 and somatostatin receptors are known to exert important inhibitory control on unstimulated LH and GH secretion, respectively; both receptor systems are coupled to $G\alpha_i$ (Schonbrunn, 2008; Yin et al., 2020), and as discussed above, inhibition of $G\alpha_{i/o}$ subunits by PTX in static pituitary cell cultures elevates both LH and GH release (Chang et al., 1993).

Effects of BIM-46187 on cytoskeletal dynamics were also recently described in $G\alpha_{q/11}$-deleted HEK293 cells (Küppers et al., 2020), which may also partially explain the elevated basal hormone release from both gonadotrophs and somatotrophs in our study. However, this observation is not at odds with the potential involvement of other Gα proteins in these functions. It is now known that besides activated GPCRs which are the canonical GEFs for Gα, other intracellular proteins can modulate activity of Gα subunits, even in the absence of agonist stimulation. One such group of proteins is the AGS (activator of G protein signalling) family, which can act as a GEF and stimulate GDP/GTP exchange on Gα, and other regulators with GEF and GAP activity also exist (Syrovatkina et al., 2016; Takesono et al., 1999). Thus, the pan-Gα inhibitor-sensitive effects on basal LH and GH secretion possibly reflect receptor-independent functions of these subunits in the control of basal hormone exocytosis. Additionally, the dissimilar effects of this inhibitor observed in LH *vs.* GH release when comparing actions of the same GnRH isoform likely rule out G-protein independent (i.e., off-target) effects as being major influences in the observed secretion patterns, at least on GnRH-stimulated hormone release.

4.4 Summary

Heterotrimeric G protein subunits are the primary transducers of GPCR signalling. Results from this thesis chapter identify for the first time the participation of multiple receptor-interacting effectors in the goldfish pituitary in a physiological model of hormone secretion (Figure 4.14). While second messenger readouts have implicated $G\alpha_{q/11}$-dependent mechanisms in goldfish GnRH actions, only recently have pharmacological tools become available in order to directly study these proteins, and the present results confirm their utilization downstream of both GnRH2 and GnRH3 actions in the control of LH and GH release. In addition, results utilizing pan-Gα- and $G\alpha_{i/o}$-selective inhibitors show that goldfish GnRHRs variably utilize non-canonical (i.e., non-$G\alpha_{q/11}$) mechanisms in the control of acute hormone secretion. How selectivity at the level of GnRHR-Gα interactions and the ensuing dynamics of Gα usage is manifested remains to be determined, but these findings are in agreement with coupling of GnRHRs to multiple G protein subtypes in mammalian systems, and with general Class A GPCR family-wide studies showing that increasing numbers of receptors display promiscuous couplings to more than one Gα subtype. In this vein, it is especially exciting that goldfish GnRHRs utilize both $G\alpha_{q/11}$ and $G\alpha_{i/o}$ (and possibly other) subunits in physiological contexts when responding to native ligand isoforms, but how variable engagement (and possible cooperation) of $G\alpha_{q/11}$ and $G\alpha_{i/o}$ might be achieved needs to be elucidated. Importantly, results from this chapter also highlight for the first time a role for GRKs in a native GnRHR system, both in facilitating GnRH-dependent hormone release responses and in mediating desensitization in both ligand- and cell-type specific fashions. Thus, in addition to $G\alpha_{q/11}$, Gβγ subunits (Pemberton and Chang, 2016) and β-arrestins, GRKs

and $G\alpha_{i/o}$ are also platforms for potential divergence of agonist-selective signalling in the goldfish GnRH system.

Figure 4.1. Pharmacological targeting of the receptor-interacting complement of G protein effectors and GPCR kinases examined in this chapter. Gα subunits, which are the primary transducers of GPCR actions, belong to four families ($G\alpha_s$, $G\alpha_{i/o}$, $G\alpha_{q/11}$, and $G\alpha_{12/13}$) and comprise 21 members in vertebrates. Classically, these are linked to discrete linear downstream signalling cascades (see also Figure 1.4), and individual receptors are typically considered to primarily utilize one Gα subtype to transduce agonist-specific signals, although considerable variation exists. In this chapter, a pan-selective Gα inhibitor (BIM-46187) was used alongside available subunit-specific inhibitors (YM-254890 and pertussis toxin) to dissect the involvement of Gα subtypes in GnRH2 and GnRH3 actions during stimulation of goldfish GnRHR(s). Additionally, GRKs are kinases that interact with G proteins and GPCRs, typically upstream of arrestin recruitment. The involvement of GRK2 and GRK3 isoforms, in particular, was examined utilizing a catalytic inhibitor of the kinase domain (CMPD101). See main text (Sections 4.2 and 4.3) for further details regarding the known mechanisms of action and/or selectivity for the employed pharmacological tools.

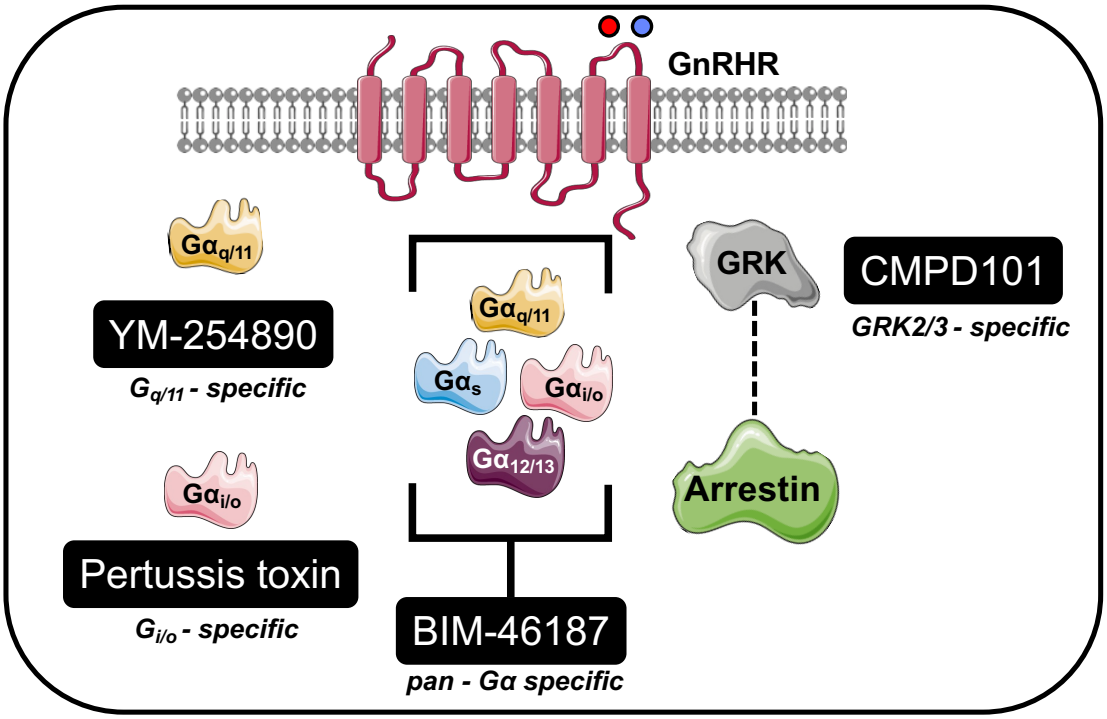
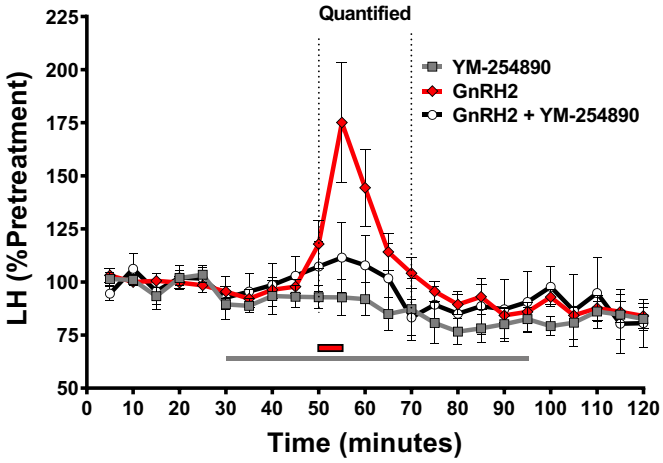
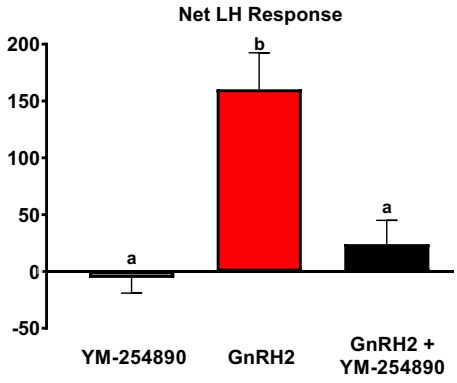
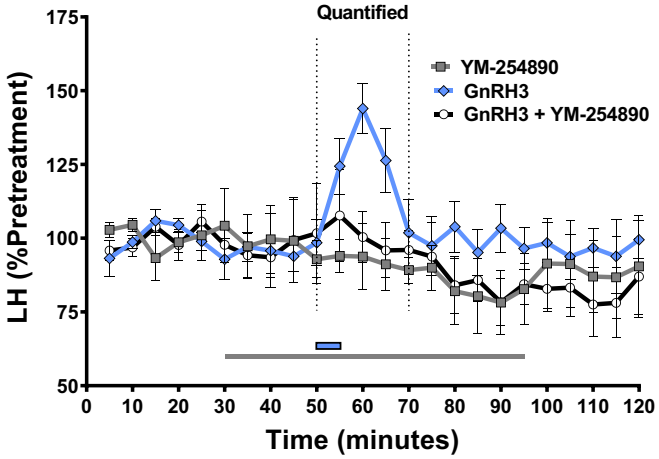

GnRHR
Gαq/11
YM-254890
Gq/11 - specific
Gαi/o
Pertussis toxin
Gi/o - specific
Gαq/11
Gαs
Gαi/o
Gα12/13
BIM-46187
pan - Gα specific
GRK
CMPD101
GRK2/3 - specific
Arrestin

Figure 4.2. Effects of the $G\alpha_{q/11}$-selective inhibitor YM-254890 (1 µM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are displayed on the left (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor) and the corresponding quantified net responses to agonist stimulation (fractions between vertical dotted lines) are presented on the right. The grey horizontal line underneath the hormone release profile indicates duration of inhibitor treatment, and the short coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 7.97 ± 0.48 ng/mL, n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from sexually matured (prespawning) goldfish and goldfish undergoing gonadal regression (May to August). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (Kruskal-Wallis followed by Dunn's multiple comparisons post-hoc test, panel A; one-way ANOVA followed by Fisher's LSD post-hoc test, panel B; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).


Quantified
YM-254890
GnRH2
GnRH2 + YM-254890
LH (%Pretreatment)
Time (minutes)


Net LH Response
a
b
a
YM-254890
GnRH2
GnRH2 + YM-254890


Quantified
YM-254890
GnRH3
GnRH3 + YM-254890
LH (%Pretreatment)
Time (minutes)

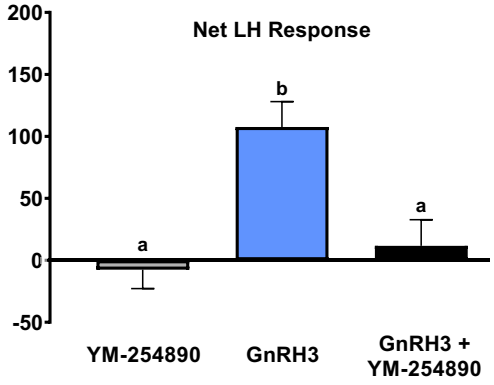

Net LH Response
a
b
a
YM-254890
GnRH3
GnRH3 + YM-254890

Figure 4.3. Effects of the $G\alpha_{q/11}$-selective inhibitor YM-254890 (1 µM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are displayed on the left (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor) and the corresponding quantified net responses to agonist stimulation (fractions between vertical dotted lines) are presented on the right. The grey horizontal line underneath the hormone release profile indicates duration of inhibitor treatment, and the short coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 11.67 ± 0.59 ng/mL, n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from sexually matured (prespawning) goldfish and goldfish undergoing gonadal regression (May to August). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

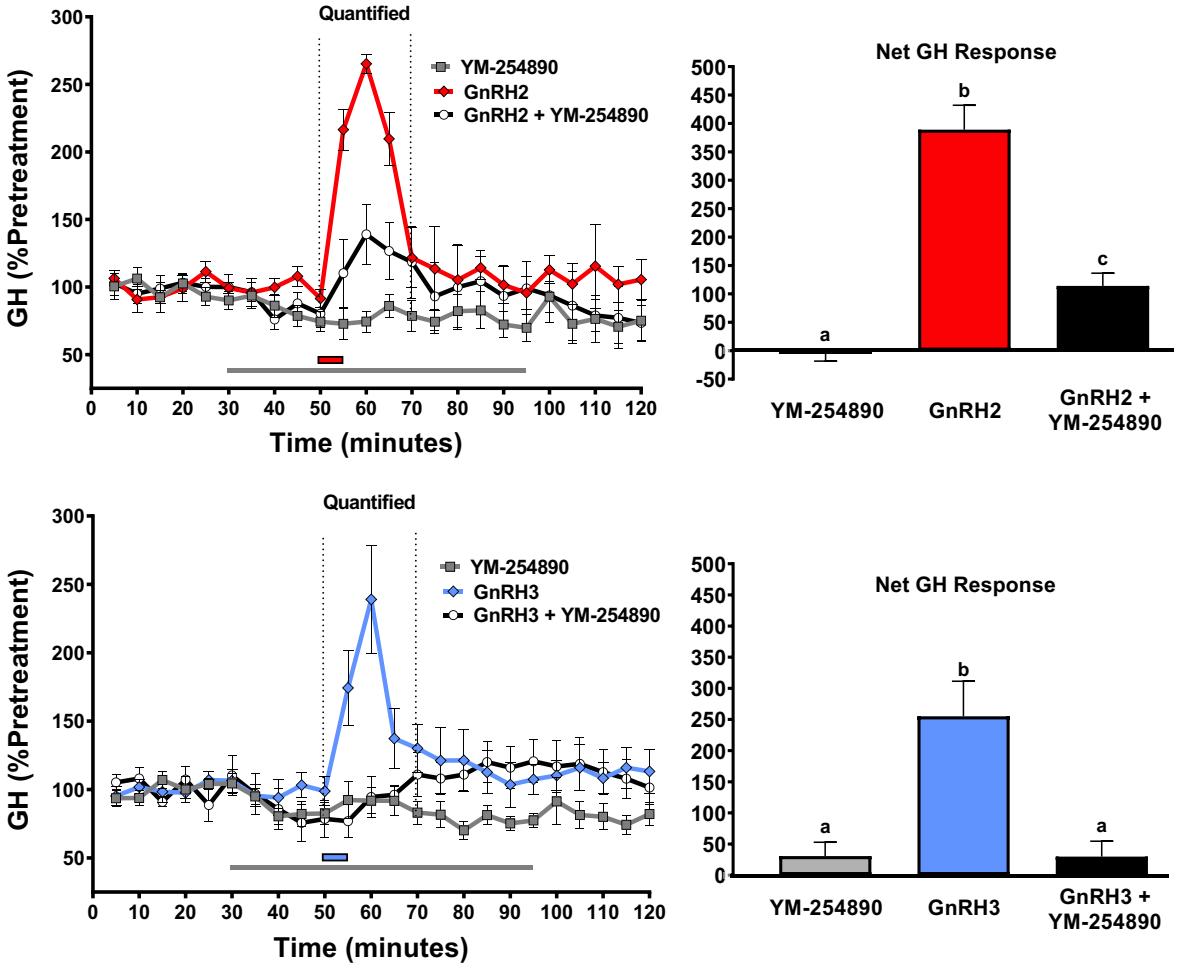

Quantified
YM-254890
GnRH2
GnRH2 + YM-254890
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
c
YM-254890
GnRH2
GnRH2 +
YM-254890
Quantified
YM-254890
GnRH3
GnRH3 + YM-254890
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
a
YM-254890
GnRH3
GnRH3 +
YM-254890

Figure 4.4. Effects of the Gα inhibitor BIM-46187 (10 μM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B; 100 nM). Hormone release kinetics are displayed on the left (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor) and the corresponding quantified net responses to agonist stimulation (fractions between vertical dotted lines) are presented on the right. The grey horizontal line underneath the hormone release profile indicates duration of inhibitor treatment, and the short coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 4.75 ± 0.07 ng/mL, n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from late recrudescent or sexually matured (prespawning) goldfish (April to early June). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

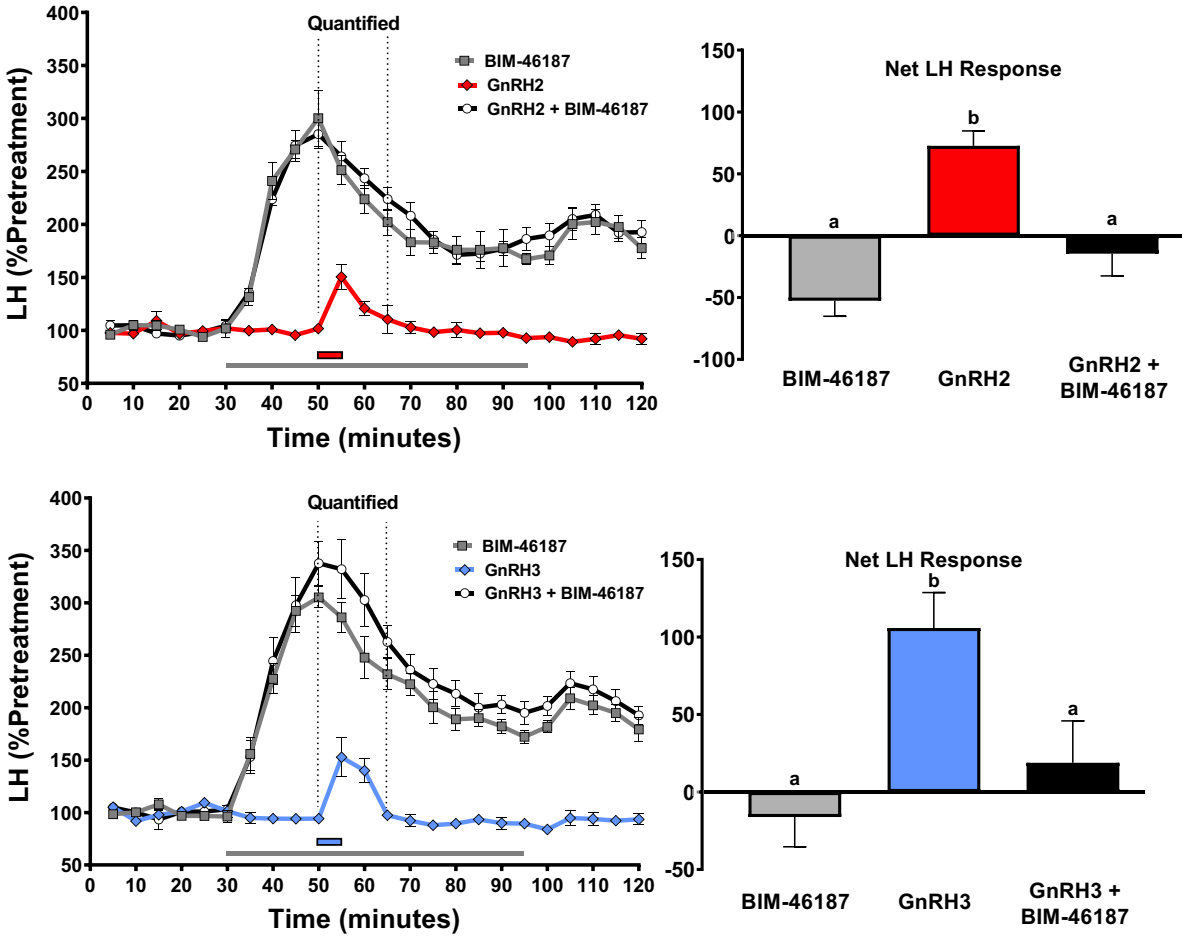

Quantified
BIM-46187
GnRH2
GnRH2 + BIM-46187
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
a
BIM-46187
GnRH2
GnRH2 +
BIM-46187
Quantified
BIM-46187
GnRH3
GnRH3 + BIM-46187
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
a
BIM-46187
GnRH3
GnRH3 +
BIM-46187

Figure 4.5. Effects of the Gα inhibitor BIM-46187 (10 µM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are displayed on the left (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor) and the corresponding quantified net responses to agonist stimulation (fractions between vertical dotted lines) are presented on the right. The grey horizontal line underneath the hormone release profile indicates duration of inhibitor treatment, and the short coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 10.31 ± 0.32 ng/mL, n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from late recrudescent or sexually matured (prespawning) goldfish (April to early June). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

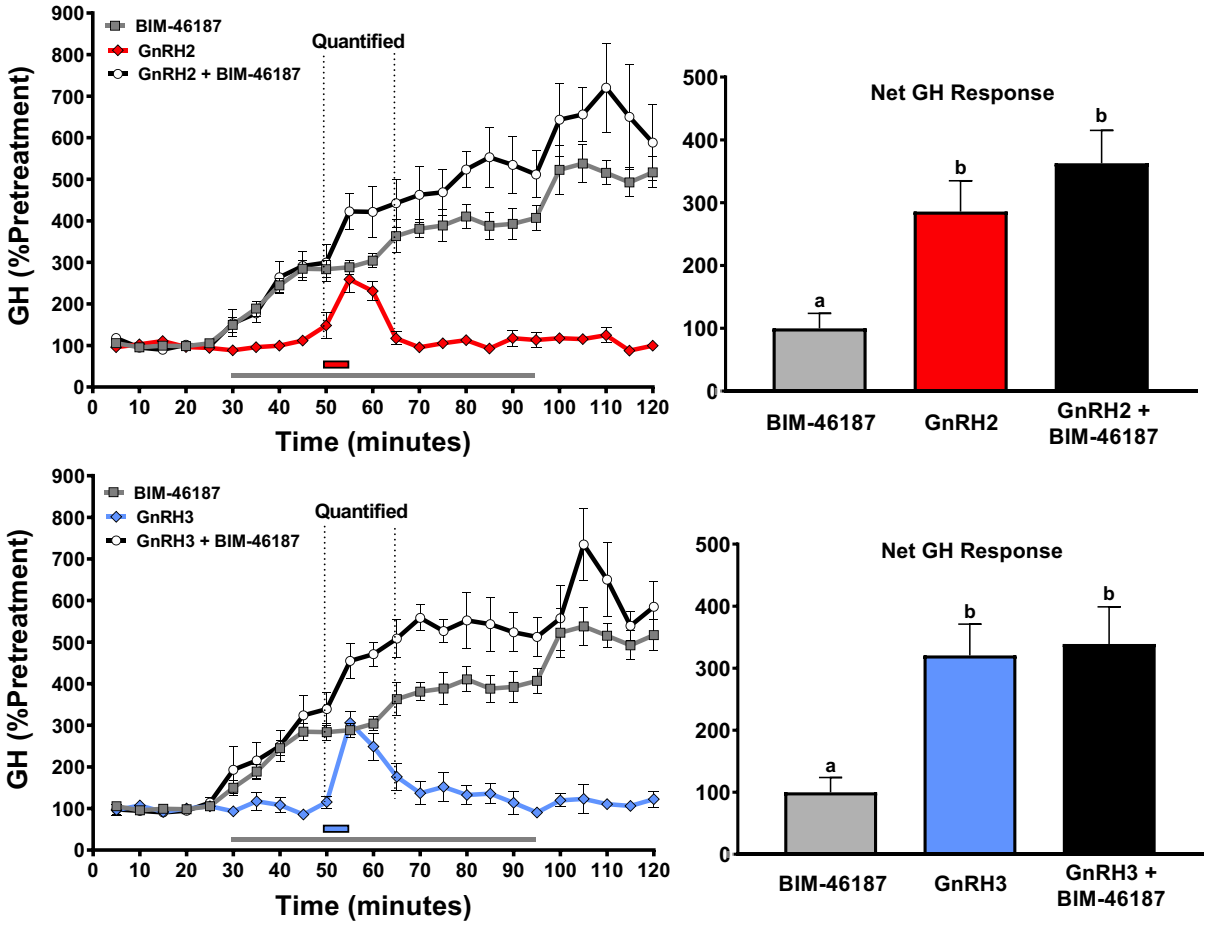

BIM-46187
GnRH2
GnRH2 + BIM-46187
Quantified
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
BIM-46187
GnRH2
GnRH2 +
BIM-46187
BIM-46187
GnRH3
GnRH3 + BIM-46187
Quantified
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
BIM-46187
GnRH3
GnRH3 +
BIM-46187

Figure 4.6. Effects of the $G\alpha_{i/o}$ inhibitor PTX (10 ng/mL) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are displayed on the left (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor) and the corresponding quantified net responses to agonist stimulation (fractions between vertical dotted lines) are presented on the right. For these experiments, cells in PTX alone and PTX + GnRH groups were exposed to the inhibitor for 16 h during overnight (O/N) culture and during the 4-h acclimation period (see Section 4.2.5). The grey horizontal line underneath the hormone release profile indicates duration of inhibitor treatment, and the short coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; GnRH alone groups: 2.03 ± 0.14 ng/mL, n = 16; PTX alone and PTX + GnRH groups: 1.80 ± 0.15 ng/mL, n = 32; from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish undergoing recrudescence (January to March). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

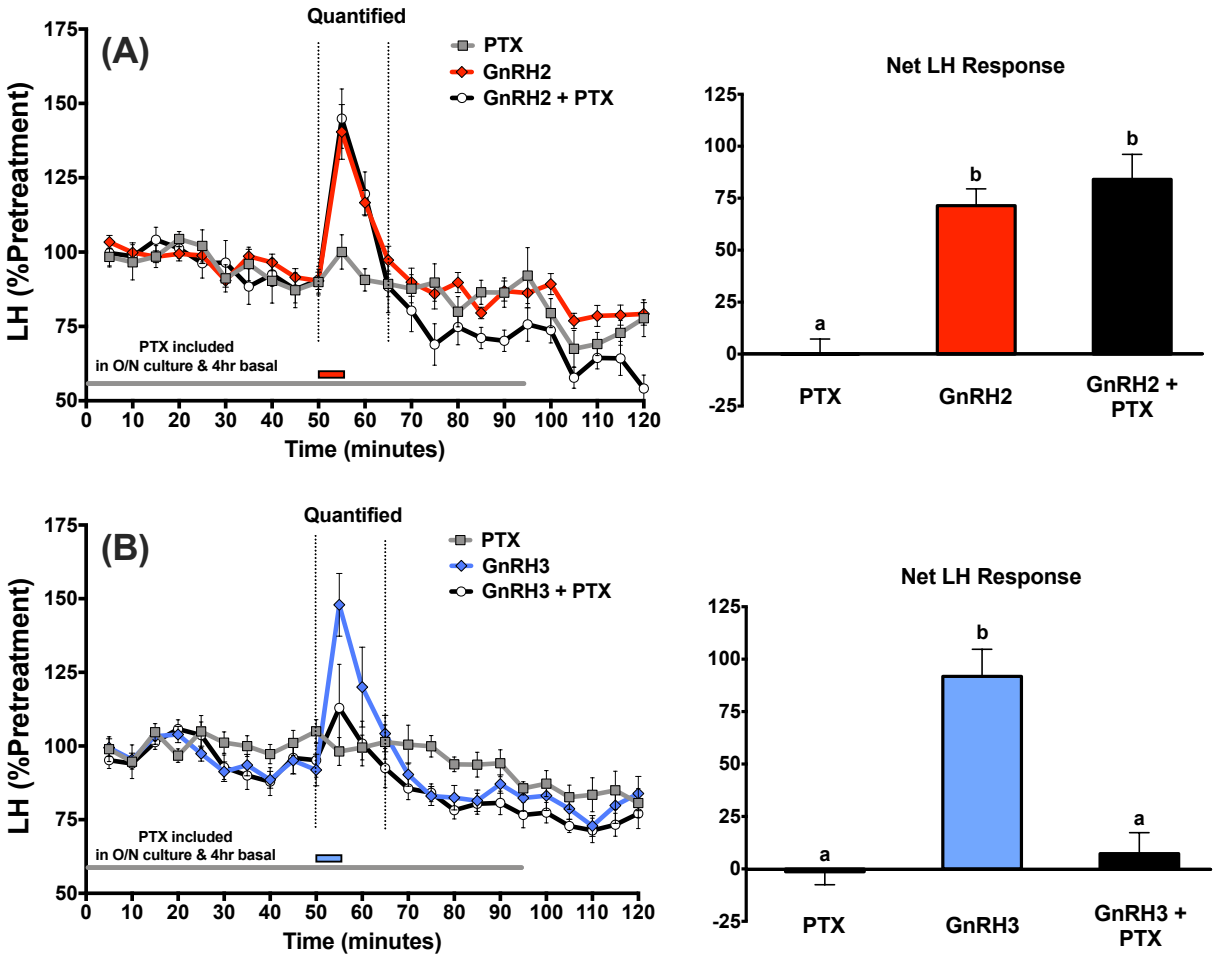

(A)
Quantified
PTX
GnRH2
GnRH2 + PTX
LH (%Pretreatment)
PTX included
in O/N culture & 4hr basal
Time (minutes)
Net LH Response
a
b
b
PTX
GnRH2
GnRH2 +
PTX
(B)
Quantified
PTX
GnRH3
GnRH3 + PTX
LH (%Pretreatment)
PTX included
in O/N culture & 4hr basal
Time (minutes)
Net LH Response
a
b
a
PTX
GnRH3
GnRH3 +
PTX

Figure 4.7. Effects of the $G\alpha_{i/o}$ inhibitor PTX (10 ng/mL) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are displayed on the left (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor) and the corresponding quantified net responses to agonist stimulation (fractions between vertical dotted lines) are presented on the right. For these experiments, cells in PTX alone and PTX + GnRH groups were exposed to the inhibitor for 16 h during overnight (O/N) culture and during the 4-h acclimation period (see Section 4.2.5). The grey horizontal line underneath the hormone release profile indicates duration of inhibitor treatment, and the short coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; GnRH alone groups: 7.56 ± 0.52 ng/mL, n = 16; PTX and PTX + GnRH groups: 8.25 ± 0.91 ng/mL, n = 32; from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish undergoing gonadal recrudescence (January to March). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

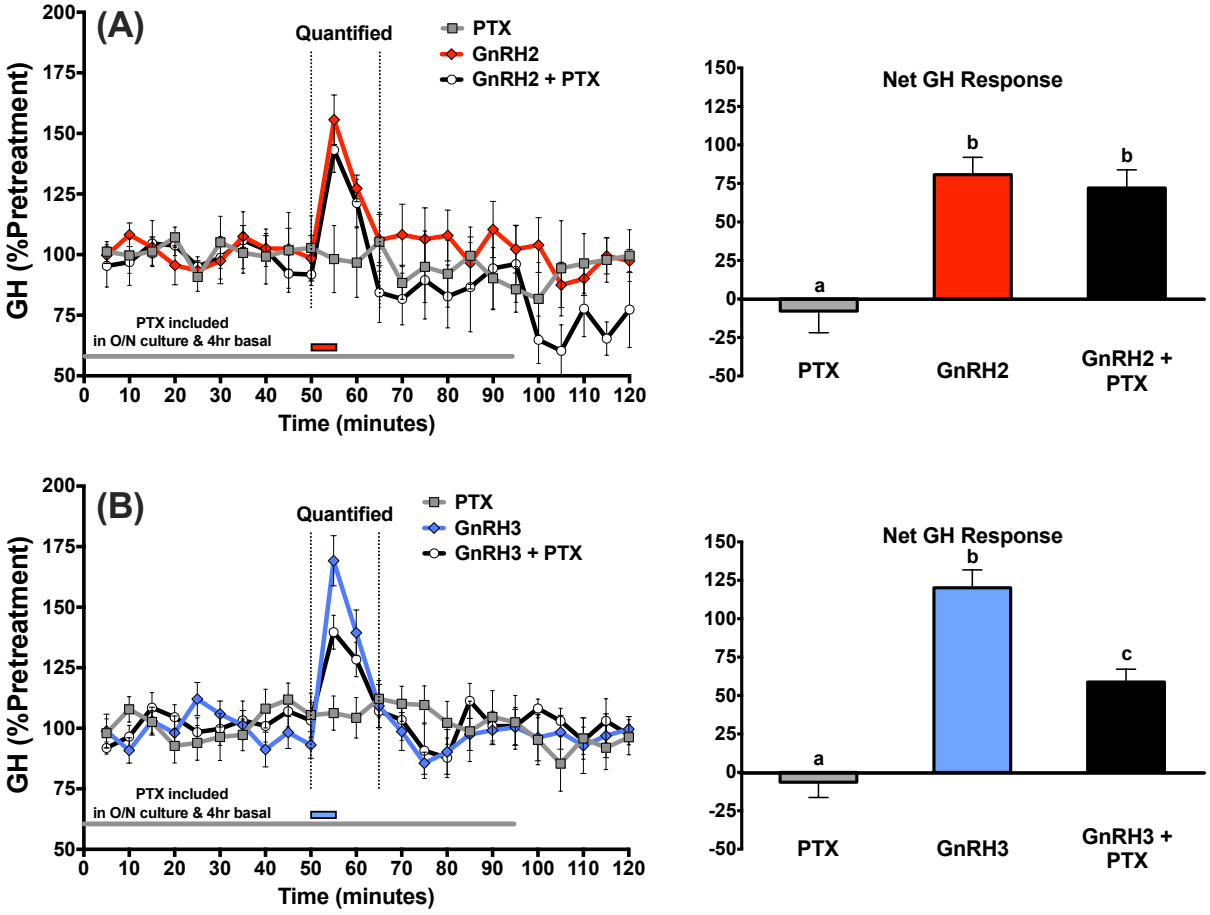

(A)
Quantified
PTX
GnRH2
GnRH2 + PTX
GH (%Pretreatment)
PTX included
in O/N culture & 4hr basal
Time (minutes)
Net GH Response
a
b
b
PTX
GnRH2
GnRH2 +
PTX
(B)
Quantified
PTX
GnRH3
GnRH3 + PTX
GH (%Pretreatment)
PTX included
in O/N culture & 4hr basal
Time (minutes)
Net GH Response
a
b
c
PTX
GnRH3
GnRH3 +
PTX

Figure 4.8. Effects of the GRK2/3 inhibitor CMPD101 (3 µM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are displayed on the left (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor) and the corresponding quantified net responses to agonist stimulation (fractions between vertical dotted lines) are presented on the right. The grey horizontal line underneath the hormone release profile indicates duration of inhibitor treatment, and the short coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 4.09 ± 0.13 ng/mL, n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish undergoing gonadal regression (June to July). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

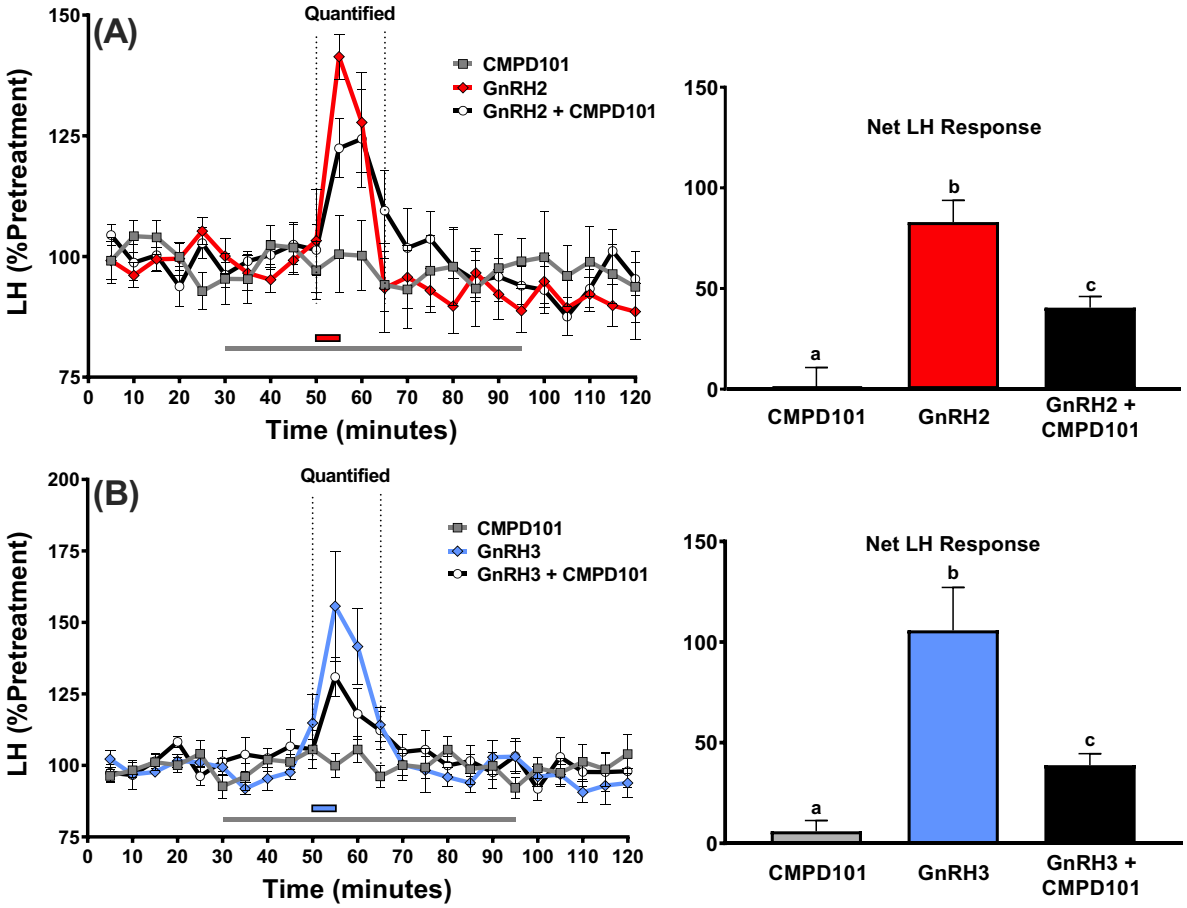

(A)
Quantified
CMPD101
GnRH2
GnRH2 + CMPD101
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
CMPD101
GnRH2
GnRH2 + CMPD101
(B)
Quantified
CMPD101
GnRH3
GnRH3 + CMPD101
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
CMPD101
GnRH3
GnRH3 + CMPD101

Figure 4.9. Effects of the GRK2/3 inhibitor CMPD101 (3 µM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are displayed on the left (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor) and the corresponding quantified net responses to agonist stimulation (fractions between vertical dotted lines) are presented on the right. The grey horizontal line underneath the hormone release profile indicates duration of inhibitor treatment, and the short coloured horizontal line represents a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results (mean ± SEM) are expressed as a percentage of pre-treatment values (average of the first five fractions; 10.24 ± 0.34 ng/mL, n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish undergoing gonadal regression (June to July). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

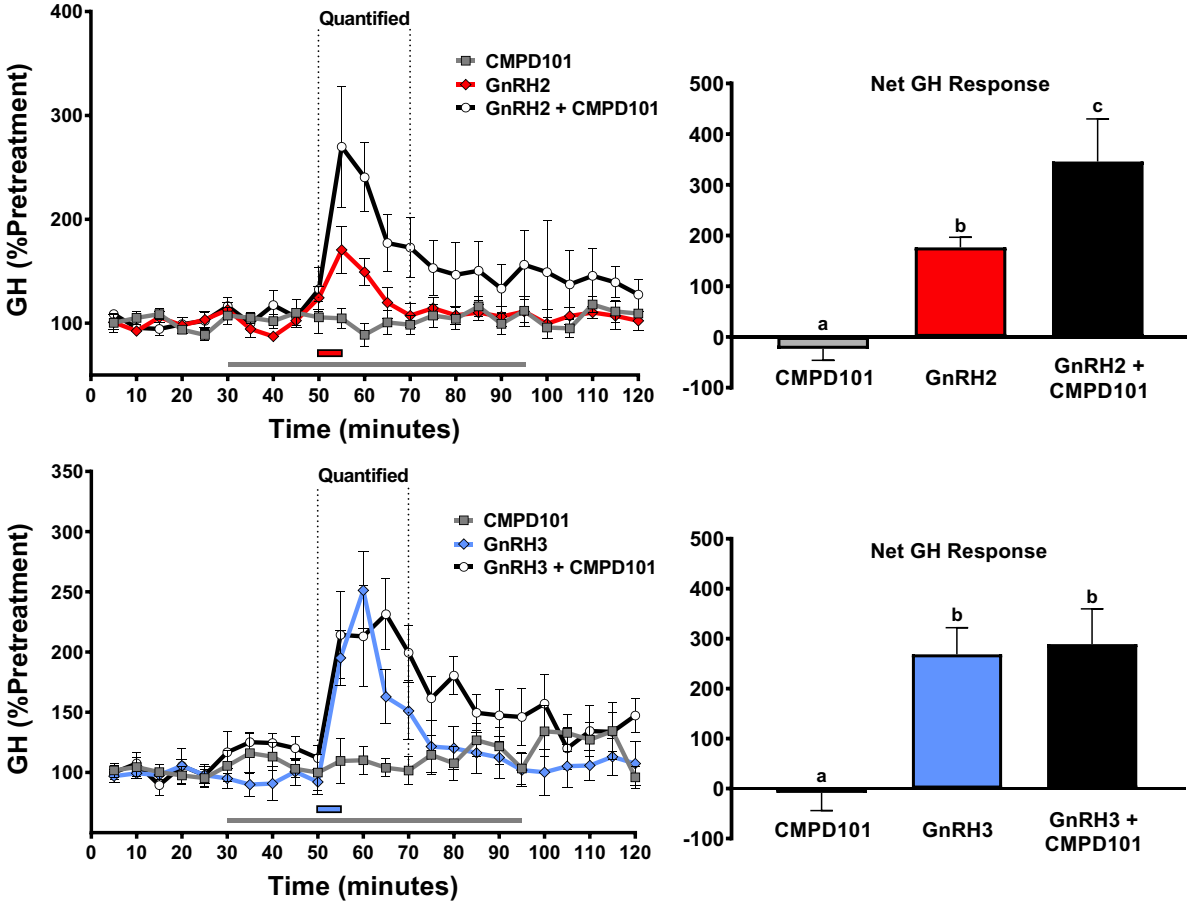

Quantified
CMPD101
GnRH2
GnRH2 + CMPD101
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
c
CMPD101
GnRH2
GnRH2 +
CMPD101
Quantified
CMPD101
GnRH3
GnRH3 + CMPD101
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
CMPD101
GnRH3
GnRH3 +
CMPD101

Figure 4.10. Effects of $G\alpha_{q/11}$, pan-Gα, and GRK2/3 inhibitors on ERK phosphorylation in dispersed cultures of goldfish pituitary cells. Following overnight culture, cells were pre-treated with select inhibitors (YM-254890, 1 μM; BIM-46187, 10 μM; CMPD101, 3 μM) or DMSO vehicle for 30 min, followed by addition of GnRH2 or GnRH3 (100 nM) for 5 min in the presence of DMSO or inhibitor. Cells were then harvested and lysed, and protein extracts probed for phospho-ERK (Thr202/Tyr204), total ERK, and β-actin. Representative blots are presented in panel A. Densitometry readings normalized to the unstimulated vehicle controls are presented in panel B (red: GnRH2; blue: GnRH3). Results (mean ± SEM) are pooled from 4 individual cell preparations each for CMPD101 (August-September; gonads at regressed states) and for YM-254890 and BIM-46187 experiments (Jan-March; during gonadal recrudescence). Total and p-ERK levels were normalized against actin levels to account for loading differences before the ratios of p-ERK to total ERK were calculated. Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$).

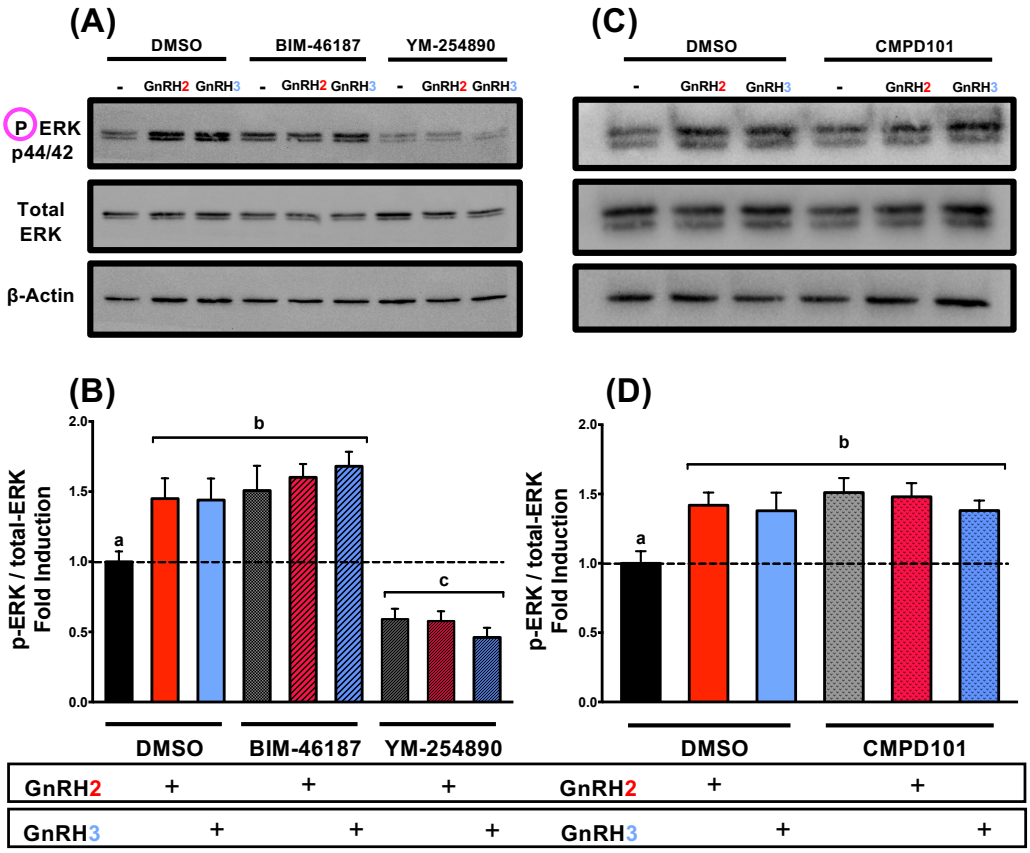

(A)
DMSO
BIM-46187
YM-254890
- GnRH2 GnRH3 - GnRH2 GnRH3 - GnRH2 GnRH3
P ERK p44/42
Total ERK
β-Actin
(C)
DMSO
CMPD101
- GnRH2 GnRH3 - GnRH2 GnRH3
(B)
p-ERK / total-ERK Fold Induction
2.0
1.5
1.0
0.5
0.0
a
b
c
DMSO
BIM-46187
YM-254890
GnRH2 + + +
GnRH3 + + +
(D)
p-ERK / total-ERK Fold Induction
a
b
DMSO
CMPD101
GnRH2 + +
GnRH3 + +

Figure 4.11. Primary amino acid sequence alignment and homology modelling of $G\alpha_q$ subunits. (A) Alignment corresponding to residues 1-240 of mouse $G\alpha_q$ demonstrates a high degree of conservation between mouse and teleosts based on confirmed sequences (zebrafish, *Danio Rerio*) and predicted sequences from genome assembly (goldfish, *Carassius auratus*). An asterisk (*) underneath the alignments represents a fully conserved residue, a colon (:) indicates conservation of residues with strongly similar properties (> 0.5 in the Gonnet PAM 250 matrix), and a period (.) indicates residues with weakly similar properties (between 0-0.5 in the Gonnet PAM 250 matrix). Importantly, regions involved in $G\alpha_q$ activation, such as Linker I and Switch I regions, as well as known YM-254890 contact sites (arrows), are 100% conserved between mouse and teleosts. (B) The structures of $G\alpha_q$ and the "YM-254890 binding pocket" are highly conserved between mammals and teleosts in predicted 3D homology models. *Left*, crystal structure of mouse $G\alpha_q$ in complex with GDP and YM-254890 (PDB 3AH8; Nishimura et al., 2010), with identified N and C termini and major protein domains. *Center*, goldfish $G\alpha_q$ (NCBI accession XP_026068289.1) homology-modelled onto mouse $G_{q/11}$ template (PDB 3AH8), and *right*, inhibitor binding pocket with known YM-254890-contact residues indicated in red (corresponding to arrows in panel A). Of note, the Switch 1 (pink) and Linker 1 (green) interdomain regions act as a hinge and play important roles in rearrangement of the helical domain with respect to the GTPase domain, leading to opening/closure of the guanine-nucleotide binding pocket. YM-254890 stabilizes the inactive $G\alpha_q$-GDP conformation through direct interaction with residues in these two regions, inhibiting GDP release by repressing the rearrangement of the GTPase and helical domains (Nishimura et al., 2010). See Section 2.8 for details regarding bioinformatics approaches.

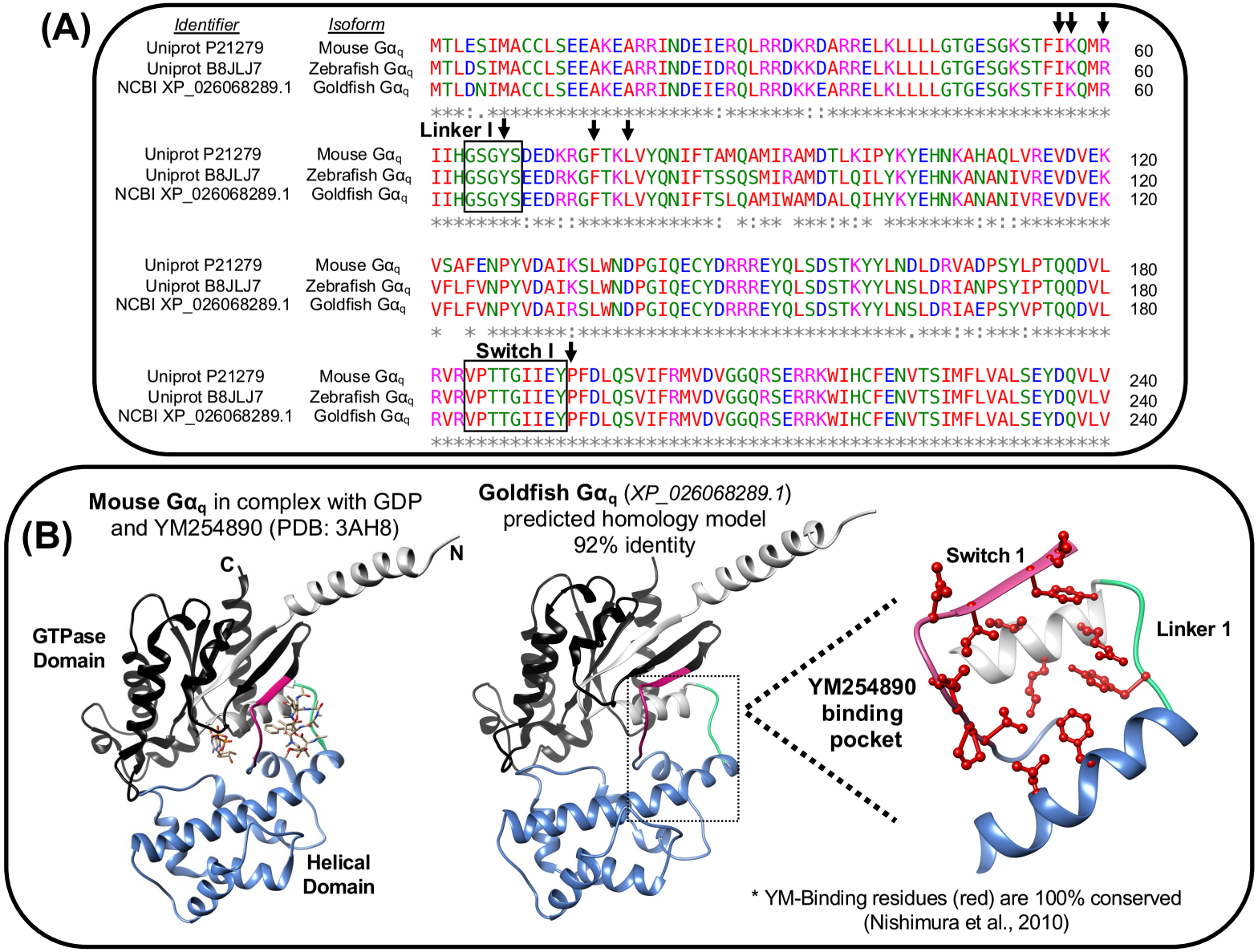

(A)
Identifier
Isoform
Uniprot P21279
Mouse Gαq
Uniprot B8JLJ7
Zebrafish Gαq
NCBI XP_026068289.1
Goldfish Gαq
MTLESIMACCLSEEAKEARRINDEIERQLRRDKRDARRELKLLLLGTGESGKSTFIKQMR 60
MTLDSIMACCLSEEAKEARRINDEIDRQLRRDKKDARRELKLLLLGTGESGKSTFIKQMR 60
MTLDNIMACCLSEEAKEARRINDEIERQLRRDKKEARRELKLLLLGTGESGKSTFIKQMR 60
Linker I
IIHGSGYSDEDKRGFTKLVYQNIFTAMQAMIRAMDTLKIPYKYEHNKAHAQLVREVDVEK 120
IIHGSGYSEEDRKGFTKLVYQNIFTSSQSMIRAMDTLQILYKYEHNKANANIVREVDVEK 120
IIHGSGYSEEDRRGFTKLVYQNIFTSLQAMIWAMDALQIHYKYEHNKANANIVREVDVEK 120
VSAFENPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLNDLDRVADPSYLPTQQDVL 180
VFLFVNPYVDAIKSLWNDPGIQECYDRRREYQLSDSTKYYLNSLDRIANPSYIPTQQDVL 180
VFLFVNPYVDAIRSLWNDPGIQECYDRRREYQLSDSTKYYLNSLDRIAEPSYVPTQQDVL 180
Switch I
RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 240
RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 240
RVRVPTTGIIEYPFDLQSVIFRMVDVGGQRSERRKWIHCFENVTSIMFLVALSEYDQVLV 240
(B)
Mouse Gαq in complex with GDP and YM254890 (PDB: 3AH8)
C
N
GTPase Domain
Helical Domain
Goldfish Gαq (XP_026068289.1) predicted homology model 92% identity
YM254890 binding pocket
Switch 1
Linker 1
* YM-Binding residues (red) are 100% conserved (Nishimura et al., 2010)

Figure 4.12. Alignment of carboxy-terminus residues of $G\alpha_{i/o}$ subunits between human and corresponding goldfish homologs. PTX specifically ADP-ribosylates the Cys residue located at the fourth position from the carboxy terminus in $G\alpha_{i1,}$ $G\alpha_{i2,}$ $G\alpha_{i3,}$ and $G\alpha_{o}$ subunits. Since the C-terminus domain of Gα proteins are involved in receptor interaction, PTX-mediated modification results in uncoupling of Gα from the receptor, hindering receptor signalling in a Gα-type specific fashion. Overall, $G\alpha_{i1,}$ $G\alpha_{i2,}$ $G\alpha_{i3,}$ and $G\alpha_{o}$ subunits display 91-97% conservation from humans to goldfish (full sequences not shown), and identifiers for the goldfish sequences with highest percent identity match are included for reference. Importantly, the equivalent residues in non $G\alpha_{i/o}$ proteins, such as $G\alpha_{q/11,}$ $G\alpha_{s,}$ and $G\alpha_{12/13}$ are not cysteines, the consequence of which is that these subunits are PTX-insensitive. A colon (:) underneath the alignments indicates conservation of residues with strongly similar properties (> 0.5 in the Gonnet PAM 250 matrix). See section 2.8 for details regarding bioinformatics approaches.

Identifier	***Isoform***	***Residues near C-terminus***
Uniprot P63096	**$G\alpha_{i1}$ Human**	**DVIIKNNLKDCGLF** [354]
NCBI XP_026065647.1	**$G\alpha_{i1}$ Goldfish**	**DVIIKNNLKDCGLF**
Uniprot P04899	**$G\alpha_{i2}$ Human**	**DVIIKNNLKDCGLF** [354]
NCBI XP_026121798.1	**$G\alpha_{i2}$ Goldfish**	**DVIIKNNLKDCGLF**
Uniprot P08754	**$G\alpha_{i3}$ Human**	**DVIIKNNLKECGLY** [354]
NCBI XP_026087140.1	**$G\alpha_{i3}$ Goldfish**	**DVIIKNNLKDCGLF**
Uniprot P09471	**$G\alpha_{o}$ Human**	**DIIIANNLRGCGLY** [354]
NCBI XP_026112991.1	**$G\alpha_{o}$ Goldfish**	**DIIIANNLRGCGLY**

Pertussis toxin site of ADP-ribosylation: Cys[351]

Figure 4.13. Primary amino acid sequence alignment and homology modelling of G-protein coupled receptor kinase 2 (GRK2). Alignment corresponding to residues 181-420 of bovine GRK2 kinase domain demonstrates a high degree of conservation between mammals and teleosts based on confirmed sequences (zebrafish, *Danio Rerio*) and predicted sequences from genome assembly (goldfish, *Carassius auratus*). An asterisk (*) underneath the alignments represents a fully conserved residue, a colon (:) indicates conservation of residues with strongly similar properties (> 0.5 in the Gonnet PAM 250 matrix), and a period (.) indicates residues with weakly similar properties (between 0-0.5 in the Gonnet PAM 250 matrix). Importantly, regions involved in GRK2 activation and opening/closure of the kinase domain, such as the phosphate-binding loop, activation loop, and hinge region, as well as identified CMPD101-binding residues (arrows), are 100% conserved between mammalian and teleost homologs of GRK2. (B) *Left,* known crystal structure of bovine (*Bos taurus*) GRK2 in complex with CMPD101 (PDB: 3PVU; Thal et al., 2011). The RGS-homology domain of GRK2 (purple) allows for interaction with $G\alpha_{q/11}$ subunits, while the PH domain (red) is implicated in membrane targeting, as well as binding to phospholipids, Gβγ subunits, and other targets such as PKC. The ATP-binding site is located at the interface between the small lobe (dark green) and large lobe (light green) of the bilobular "AGC kinase" domain. *Center*, goldfish GRK2 (NCBI accession XP_026076079.1) homology-modelled onto bovine GRK2 template (PDB: 1YM7) and *right*, CMPD101-binding pocket with residues known to interact with this inhibitor shown in red (corresponding to arrows in panel A). Notably, CMPD101 does not solely target the ATP-binding site, which is highly conserved between AGC kinases; rather, it stabilizes a unique inactive conformation of GRK2, lending this inhibitor its high selectivity towards GRK2 over that of the related PKA, PKG, and PKC, as well as over other GRK isoforms (Thal et al., 2011). See section 2.8 for details regarding bioinformatics approaches.

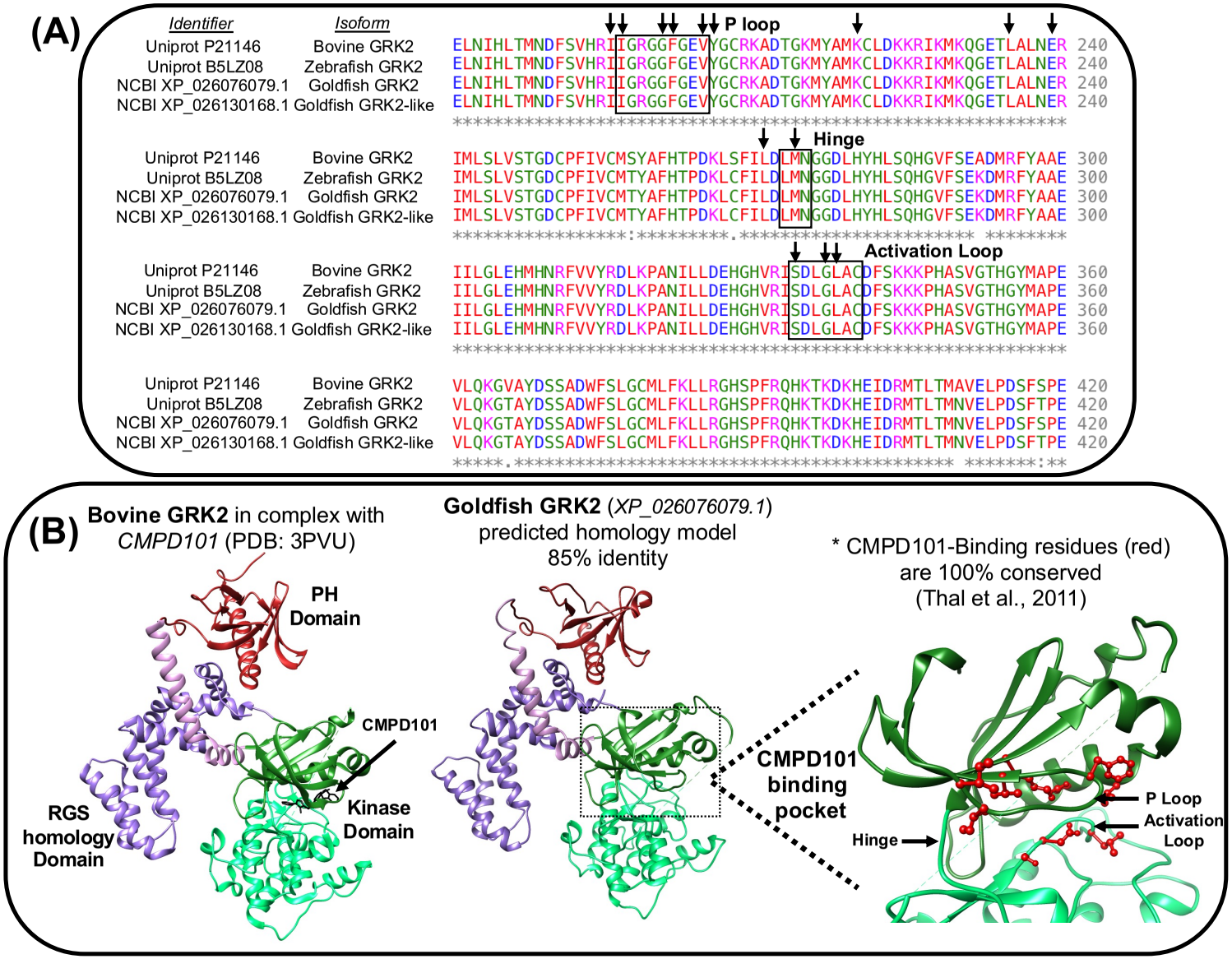
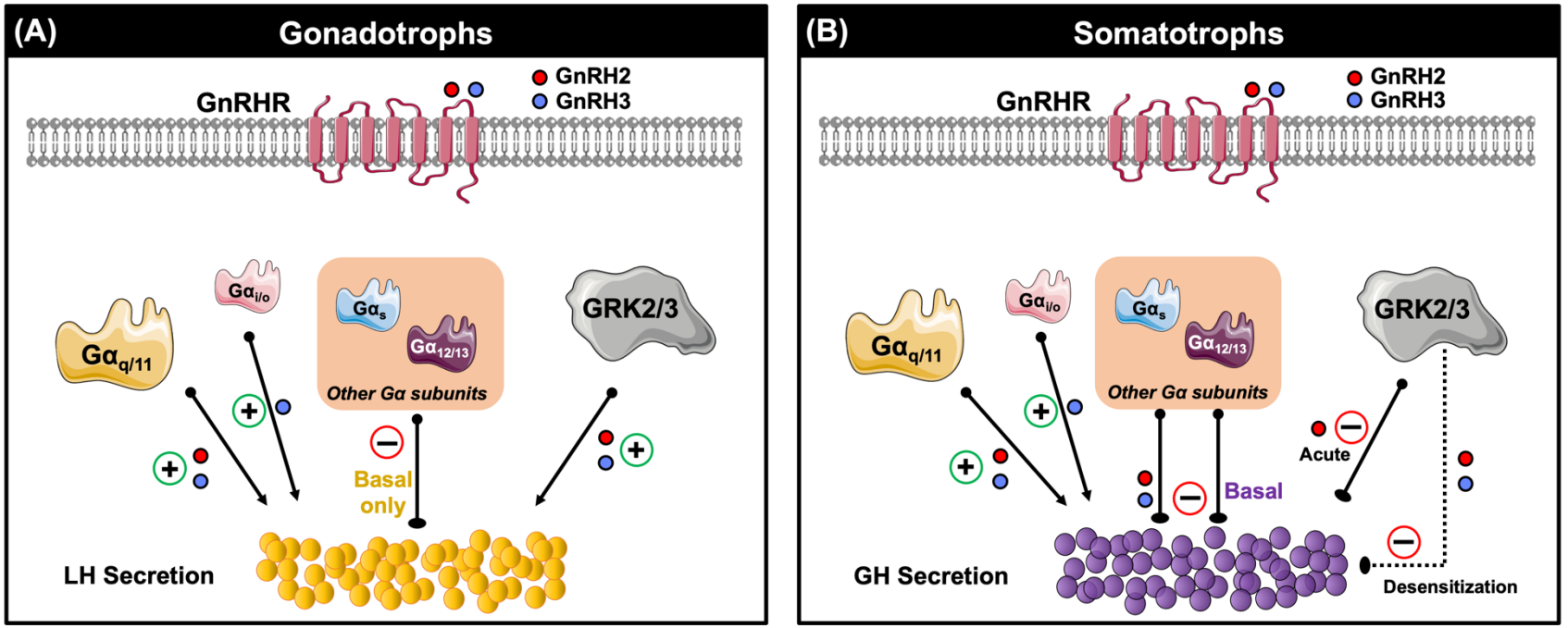

(A)
Identifier
Isoform
P loop
Hinge
Activation Loop
Uniprot P21146 Bovine GRK2
Uniprot B5LZ08 Zebrafish GRK2
NCBI XP_026076079.1 Goldfish GRK2
NCBI XP_026130168.1 Goldfish GRK2-like
ELNIHLTMNDFSVHRIIGRGGFGEVYGCRKADTGKMYAMKCLDKKRIKMKQGETLALNER 240
IMLSLVSTGDCPFIVCMSYAFHTPDKLSFILDLMNGGDLHYHLSQHGVFSEADMRFYAAE 300
IMLSLVSTGDCPFIVCMTYAFHTPDKLCFILDLMNGGDLHYHLSQHGVFSEKDMRFYAAE 300
IILGLEHMHNRFVVYRDLKPANILLDEHGHVRISDLGLACDFSKKKPHASVGTHGYMAPE 360
VLQKGVAYDSSADWFSLGCMLFKLLRGHSPFRQHKTKDKHEIDRMTLTMAVELPDSFSPE 420
VLQKGTAYDSSADWFSLGCMLFKLLRGHSPFRQHKTKDKHEIDRMTLTMNVELPDSFTPE 420
(B)
Bovine GRK2 in complex with CMPD101 (PDB: 3PVU)
PH Domain
CMPD101
RGS homology Domain
Kinase Domain
Goldfish GRK2 (XP_026076079.1) predicted homology model 85% identity
* CMPD101-Binding residues (red) are 100% conserved (Thal et al., 2011)
CMPD101 binding pocket
P Loop
Activation Loop
Hinge

Figure 4.14. Summary figure depicting the selective involvement of Gα subunits and GRKs in GnRH-evoked and basal LH and GH release. As indicated from prior inositol phosphate and Ca^{2+} second messenger data, as well as the involvement of PLC and PKC, $G\alpha_{q/11}$-dependent mechanisms are consistently involved in facilitating GnRH2/3-dependent stimulation of LH and GH release (A, B). $G\alpha_{i/o}$ subunits selectively mediate GnRH3-induced, but not GnRH2-elicited, LH and GH secretion. On the other hand, results with a pan-Gα inhibitor reveal additional negative influences of other Gα subunits (likely non-$G\alpha_{q/11}$/$G\alpha_{i/o}$) in the control of both basal LH and basal GH release (subunits superimposed on an orange square background). Finally, while GRK2/3 proteins facilitate GnRH2- and GnRH3-induced LH release responses (A), they selectively mediate desensitization-type effects in GnRH2 and GnRH3 control of GH release (dotted line, B), and additionally suppress the acute phase of GnRH2-dependent GH secretion (solid line, B).


(A)
Gonadotrophs
GnRHR
GnRH2
GnRH3
Gαi/o
Gαq/11
Gαs
Gα12/13
Other Gα subunits
GRK2/3
Basal only
LH Secretion
(B)
Somatotrophs
GnRHR
GnRH2
GnRH3
Gαi/o
Gαq/11
Gαs
Gα12/13
Other Gα subunits
GRK2/3
Acute
Basal
GH Secretion
Desensitization

Chapter Five

Small GTPase control of basal and GnRH-evoked hormone release

5.1 Introduction

In addition to the receptor-associating heterotrimeric G proteins examined in Chapter 4, another related group of G proteins involved in the regulation of a wide range of cellular functions is the monomeric small G proteins, also referred to as small GTPases. Similar to heterotrimeric G proteins, activity of small GTPases is controlled by alternating between active GTP-bound and inactive GDP-bound conformations, and type-specific molecules modulate these changes. The rate-limiting exchange of GDP for GTP (activation step) is facilitated by GEFs, whereas GAPs increase the intrinsic GTPase function leading to hydrolysis of GTP to GDP (deactivation). Specifically, some members of the Arf, Rho, and Ras families are linked to GPCR signalling cascades, and are also important players in the exocytotic pathway of secretory cell types, including many endocrine, neural, and immune cells (Collins, 2003; Gasman et al., 2003; Watson, 1999; also see Figure 1.7 schematic). However, knowledge on the role of these molecules in neuroendocrine systems and pituitary hormone secretion is limited, especially in studies utilizing basal vertebrates such as teleosts. In terms of GnRH-related functions, the Rho subfamily members RhoA and Rac1 are associated with GnRH-dependent associations to actin cytoskeletal dynamics in mammalian cell models (Aguilar-Rojas et al., 2012; Edwards et al., 2017; Navratil et al., 2007), and Arf GTPases are implicated in GnRH actions on LH and GH release in the goldfish pituitary as a PIP_3-dependent effector but whether this is the case has not been tested (Pemberton and Chang, 2016). Similarly, Ras is important in GnRH-induced activation of MAPK cascades in several mammalian study models (Harris et al., 2002; Naor et al., 2000; Okitsu-Sakurayama et al., 2021), and although a role for PKC inputs to MEK/ERK is known in the goldfish pituitary system (Klausen et al., 2008; Pemberton et al., 2013; also see Section 1.8.2), it remains to be seen whether and how the upstream elements which canonically

initiate this cascade play a role in goldfish GnRH actions. Just as importantly, while these GTPases are implicated in GnRH actions in capacities such as cell signalling and cell migration based on the literature, how these elements might impact the classical hypophysiotrophic functions of GnRH (i.e., hormone secretion) have not been directly examined in any model system thus far.

I hypothesized that these small GTPases are involved in the neuroendocrine control of hormone exocytosis from goldfish pituitary cells by GnRH and that that some of these elements are likely utilized in a cell-type- and GnRH-isoform-dependent fashion and might additionally be differentially involved in unstimulated and agonist-evoked release. To address these questions, the effects of specific inhibitors of Arf1/6, RhoA, Rac, and SOS-Ras interactions on basal and GnRH-stimulated LH and GH secretion from dispersed primary cultures of goldfish pituitary cells were examined in column perifusion experiments.

5.2 Results

5.2.1 Detection of small GTPase proteins in dispersed pituitary cell protein extracts

Since amino acid sequences of small GTPase proteins examined in this study are known to be highly conserved in vertebrates and presumably ubiquitously expressed (Uhlén et al., 2015) I first tested whether immunoreactive Arf1, Rac, RhoA and Ras are present in dispersed goldfish pituitary cell protein extracts using corresponding antibodies specific for their respective mammalian counterparts. Results showed the presence of Arf1, Rac, RhoA and Ras immunoactivity in protein extracts from goldfish pituitary cells lysates (Figure 5.1). Lysates prepared from a representative mammalian cell line, HEK-AD293, were used as positive controls for small GTPase protein expression (Abe et al., 2020; Müller et al., 2020; Rocca et al., 2013).

Immunoreactive bands for all four small GTPases were detected in dispersed goldfish pituitary cell extracts and generally corresponded to reference bands from HEK-AD293 lysates.

5.2.2 Arf1/6 inhibition selectively suppresses GnRH-dependent LH release

The involvement of Arf proteins was examined using a direct inhibitor of this GTPase, NAV-2729, at a concentration of 10 μM. This dose is reported to inhibit both spontaneous and GEF-dependent nucleotide exchange on Arf6, and is selective for Arf6 over other related small GTPases, including Rac1, RhoA, Ras, and Cdc42, at doses up to 50 μM, validated *in vitro* using recombinant proteins, and in HEK293T and Mel92.1/Mel202 cell lines (Yoo et al., 2016). Similar doses (5-10 μM) have been employed in mammalian primary cell and tissue culture studies (Huang et al., 2022; Wang et al., 2021). Although the initial characterization described selectivity of NAV-2729 for Arf6, a later study reported that it is a dual inhibitor of both Arf1 and Arf6 activity (Benabdi et al., 2017). In the present study, application of NAV-2729 led to an elevation in basal hormone secretion from both cell types (Figures 5.2 and 5.3, grey squares) prior to GnRH stimulation. GnRH application as a 5-min pulse of a maximally stimulatory 100 nM dose (Chang et al., 1990) increased LH and GH release. In the presence of NAV-2729, however, the net LH responses to the two GnRHs were not significantly different from that of inhibitor alone (Figure 5.2). On the other hand, in somatotrophs, GnRH application during NAV-2729 treatment still led to significant increases in the quantified GH response above values observed for inhibitor treatment alone (Figure 5.3).

5.2.3 Rac inhibition selectively enhances GnRH3-dependent GH secretion

The activity of Rac family GTPases was targeted using the inhibitor EHT 1864 at a concentration of 20 μM. Initial characterization of EHT 1864 in mammalian cell lines including NIH3T3 and 293T cells showed that it is specific for Rac GTPases, with the highest activity against Rac1 over the related Rac2 and Rac3 GTPases (Shutes et al., 2007). Importantly, this compound is specific for Rac over other Rho family GTPases, including RhoA and Cdc42, at doses up to 50 μM, and similar doses as in the present study have been utilized in both cell lines and primary tissue models (DiPaolo et al., 2013; Sakai et al., 2018; Sun et al., 2021). Similar to Arf1/6 inhibition, EHT 1864 treatment resulted in an elevation in basal LH and GH release within 5-10 min of application, an effect that was more pronounced in gonadotrophs (Figures 5.4 and 5.5, grey squares). Quantified net LH and GH responses to GnRH in the presence of EHT 1864 were significantly greater than the corresponding values in inhibitor alone treatment, and the net GnRH3-evoked GH secretion in presence of the inhibitor was also significantly greater than that of GnRH3 alone (Figure 5.5).

5.2.4 The Rho GTPase inhibitor Rhosin selectively enhances GnRH2-induced GH release

The involvement of Rho GTPases was tested using the inhibitor Rhosin, which targets the GEF-interacting site of these proteins, employing a 30 μM dose based on the original characterization in NIH-3T3 fibroblasts and PC12 neuroendocrine cells (Shang et al., 2012); similar concentrations have been employed in primary cell systems (Chiu et al., 2021; Sheshachalam et al., 2017). Rhosin is specific for RhoA (and the related RhoB and RhoC) binding surfaces but does not affect the related Rac1 and Cdc42 GTPases at concentrations up to 100 μM. Application of Rhosin in the present study also resulted in an elevation in basal release from both gonadotrophs and somatotrophs (grey squares, Figures 5.6 and 5.7). Like the effects of

Rac inhibition, the net GnRH-dependent LH and GH release responses were not attenuated in presence of Rhosin, whereas the net GnRH2-dependent GH release was significantly greater in the presence than in the absence of Rhosin (Figure 5.7).

5.2.5 Inhibiting SOS-Ras interactions leads to selective enhancement of GnRH-induced LH secretion but attenuates GnRH-elicited increases in ERK phosphorylation

The involvement of SOS-Ras interactions in pituitary hormone release was examined using the hotspot inhibitor BAY-293. BAY-293 selectively inhibits activation of KRas by preventing interactions with its upstream GEF, SOS, which generally control activation of the MEK/ERK cascade (Guo et al., 2020). A 5 μM dose of BAY-293 was chosen based on the initial characterization of this compound (Hillig et al., 2019); similar doses have been utilized in subsequently published independent studies (Plangger et al., 2021; Wang et al., 2022). In column perifusion experiments, treatment with BAY-293 selectively raised basal LH secretion, while basal GH release was unaffected (Figures 5.8 and 5.9, grey squares). In the case of agonist-stimulated effects, GnRH-evoked net release was generally comparable in GnRH alone *vs.* GnRH + inhibitor treatment groups, except for GnRH3-dependent LH release, which was greater in the presence than in the absence of BAY-293 (Figure 5.8).

To address the involvement of Ras in acute GnRH signalling to ERK, dispersed pituitary cells were treated with 5 μM BAY-293 (or DMSO vehicle control) for 30 min prior to 5-min stimulation with 100 nM GnRH2 or GnRH3 in either the presence or absence of the inhibitor. GnRH2 and GnRH3 treatments elevated phospho-ERK levels by 0.6 fold above vehicle control (Figure 5.10). BAY-293 alone treatment reduced basal phospho-ERK fold change values to 0.17

of DMSO control, and subsequent application of GnRH was unable to further elevate phospho-ERK levels above those of BAY-293 treatment alone ($p < 0.05$).

5.2.6 Effects of small GTPase inhibition on basal unstimulated hormone release

The effects of inhibitors on unstimulated LH and GH secretion were further compared using averaged hormone values before and during treatments with inhibitors of Arf1/6, Rac, RhoA, and SOS-RAS alone within perifusion columns (Figure 5.11; quantified net averages based on inhibitor alone perifusion columns, i.e., grey traces taken from Figures 5.2-5.9). The ranked order of averaged increases in basal LH secretion in the presence of GTPase inhibitors was Rac > Arf1/6 > SOS-Ras > RhoA inhibition (average responses of 198%, 169%, 129% and 114% pretreatment values, respectively) (Figure 5.11 A,B). In somatotrophs, SOS-RAS inhibition had no significant effect (Figure 5.11 C,D). The ranked order of effects of the other three GTPases on basal GH release was Arf1/6 > RhoA ≥ Rac (averaged responses of 147%, 129% and 125% of pretreatment, respectively; Figure 5.11 D), which was different from that for basal LH. Interestingly, hormone release from inhibitor alone treatment columns generally approached baseline levels of secretion following inhibitor washout, with the exception of Arf1/6 inhibition, which showed a "washout rebound" increase in hormone release. This effect was more pronounced for GH than in LH, and was observed in both inhibitor alone, as well as GnRH + inhibitor combination groups (Figures 5.2 and 5.3).

5.3 Discussion

In this thesis chapter, I examined the potential role of small GTPase proteins, in particular Arf1/6, RhoA, Rac, and Ras, in the regulation of hormone exocytosis from dispersed goldfish

pituitary cells during both unstimulated and agonist-dependent release. In general, there is limited knowledge about the role of these regulators in pituitary cell types, and information is particularly lacking from investigations in teleost model organisms. The small GTPase proteins investigated herein are highly conserved between humans and basal vertebrates, as observed from studies done in the closely related teleost species *Danio rerio* (Arf1: Petko et al., 2009; Ras: Liu et al., 2008; RhoA and Rac: Salas-Vidal et al., 2005). Similarly, primary amino acid sequences show between 95-100% similarity based on predicted goldfish sequences (Table 5.1). Accordingly, antibodies against mammalian Arf1/6, RhoA, Rac, and Ras reveal the presence of their immunoreactive counterparts of the corresponding molecular mass in goldfish pituitary cell lysates in the present study. Although the molecular mass of Arf1 is predicted to be ~21 kDa, other reports, including in HEK-293 cells, have shown this protein to resolve at 17-18 kDa (Beauchamp et al., 2020; Khater et al., 2021), which is consistent with the present results in both goldfish pituitary cell and HEK-AD293 lysates. The extra bands detected by anti-RhoA and anti-Ras antibodies may indicate the presence of additional RhoA- and Ras-like protein isoforms in goldfish pituitary, which is consistent with the known presence of duplicated small GTPases in 3R teleost genomes (Boureux et al., 2007; Salas-Vidal et al., 2005). Regardless, the general conservation of the small GTPases across eukaryote evolution also signifies the importance of this family of proteins in cellular homeostasis (Boureux et al., 2007). Since these proteins are known to modulate secretory vesicle trafficking and cytoskeletal dynamics, which represent important regulatory steps in hormone exocytosis, it is likely that they also play important roles in the neuroendocrine control of goldfish pituitary hormone exocytosis.

5.3.1 Small GTPase roles in unstimulated LH and GH secretion

In general, inhibition of the small GTPases resulted in elevated basal LH and GH release from pituitary gonadotrophs and somatotrophs, respectively, indicating that these monomeric G proteins exert a negative regulation over unstimulated hormone secretion, with the exception of Ras in somatotrophs. For Arf1/6 and Rac inhibition, these effects were more profound in altering basal LH release as compared to GH, from their respective cell types, while RhoA inhibition had stronger effects in elevating GH release. Together with differences in the rank order of effectiveness of the four GTPase inhibitors on basal LH *vs.* GH secretion, these observations indicate differential usage of small GTPases in gonadotrophs and somatotrophs during the control of unstimulated hormone exocytosis.

The observed increases in basal release in the presence of inhibitors of small GTPases are also consistent with prior observations in the goldfish pituitary cells study system utilizing PIT-1, an inhibitor of PtdIns(3,4,5)P_3-PH domain interactions (Pemberton and Chang, 2016), which is upstream of some activators (i.e., GEFs) of Arf1/6. Consistent with the present findings, expression of constitutively-active Arf6 mutants inhibits exocytosis in PC12 rodent neuroendocrine cells (Aikawa and Martin, 2003), and addition of non-hydrolysable GTP analogues also inhibit budding of secretory granules from the *trans*-Golgi network, where Arf1 is a well-characterized small G protein in this role (Takai et al., 2001; Tooze et al., 1990). Whether Arf1 likewise targeted events at the *trans*-Golgi network to inhibit basal hormone release from goldfish gonadotrophs and somatotrophs remains to be determined. Interestingly, pharmacological inhibition of RhoA or Rac1 in both mouse pancreatic islets and dispersed cells elevates basal glucagon and somatostatin secretion, whereas insulin secretion was only increased by RhoA inhibition (Ng et al., 2022), reflecting cell type-specific usage of these regulators within a mixed-endocrine tissue as in the present study. These roles in basal release, especially

for Rac and Rho GTPases, are also consistent with their known functions in promoting F-actin polymerization and remodelling (Schmidt and Hall, 1998; Spiering and Hodgson, 2011). Accordingly, evidence from research in islet cells suggests that dense cortical F-actin structures limit hormone-granule exocytosis, whereas agents that promote actin depolymerization restore and/or increase exocytosis (Hutchens and Piston, 2015; Jewell et al., 2008; Kolic et al., 2014; Pigeau et al., 2009). While the links between F-actin polymerization and exocytosis are not as well defined in the case of Arf1/6, these GTPases also promote cortical F-actin structures, and may work in concert with Rac to achieve this (Radhakrishna et al., 1999; Tanna et al., 2019). Thus, in the present study, inhibition of small GTPase activity might impede normal F-actin polymerization in goldfish pituitary cells, which likely explains, in part, the observed increases in basal hormone exocytosis.

Similar roles in inhibiting basal hormone exocytosis have also been described for the related Rab GTPases (Vázquez-Martínez and Malagón, 2011). Interestingly, the Rab18 isoform is proposed to be involved in facilitating two different secretory phenotypes in sub-populations of amphibian pituitary melanotrophs, which secrete melanotrophin. Hormone "storage" melanotrophs with low secretory profiles (both basal and stimulated) showed higher levels of Rab18 expression, whereas active melanotrophs with high basal secretory activity showed lower expression levels (Malagon et al., 2005; Peinado et al., 2002; Vazquez-Martinez et al., 2007). Known functions of Rab18 include vesicle formation, budding, transport, and fusion (Zerial and McBride, 2001), and many of these roles overlap with those of Arf and Rho family GTPases in particular. Rac1 can also be sequentially activated downstream of some Rab GTPases, and Rac1 can further recruit Arf6, as an example of coupling of multiple GTPases (Phuyal and Farhan, 2019). Whether similar intracellular mechanisms and interactions involving the small GTPases

examined in the present study also play a role in the control of seasonal changes in basal LH and GH secretion from goldfish pituitary cells (Chang et al., 2012; Johnson and Chang, 2002), are intriguing possibilities that warrant further investigation.

As discussed in Chapter 3, inhibition washout rebound such as the short-term hypersecretion of GH has been reported in the goldfish pituitary system following the removal of norepinephrine, somatostatin- and GnIH-mediated inhibition (Lee et al., 2001; Moussavi et al., 2014; Yu and Chang, 2010). Surprisingly, a further transient augmentation of the basal GH secretion, and to a smaller degree basal LH release, was observed following termination of the Arf1/6 inhibitor (Figure 5.2). The intracellular cellular mechanism(s) by which this effect of NAV-2729 removal is manifested is unknown but likely represents the result of complex changes in cellular homeostasis that need to be re-established. Nonetheless, this further augmentation of hormone release upon washout of GTPase inhibitor was only seen with the removal of NAV-2729 and not with the other GTPase inhibitors, indicating a degree of GTPase-specificity (Figure 5.11).

5.3.2 Small GTPase roles in GnRH-dependent LH and GH release

Despite generally exerting an inhibitory role on unstimulated basal LH and GH release, the manner in which these GTPases participate in mediating agonist (GnRH)-stimulated secretion shows GnRH isoform-, cell-type-, and GTPase-selectivity (Figure 5.12). Results indicate that while acute GnRH2- and GnRH3-induced LH release likely involve Arf1/6, GnRH3-elicited LH secretion is negatively modulated by a SOS-Ras component; however, SOS-Ras does not participate in GnRH2 action on LH release nor do Rac and RhoA in GnRH2 and GnRH3 stimulation of LH secretion. In contrast, neither Arf1/6 nor SOS-Ras are vital

components in mediating the GH responses to the two GnRHs while Rac and RhoA selectively exert negative modulatory influences on GnRH3 and GnRH2 stimulation of GH recreation, respectively. These observations, when taken together with the results of GTPase inhibitors on basal secretion, also add weight to previous findings that intracellular events controlling basal and ligand-stimulated goldfish pituitary hormone exocytosis can be uncoupled (Chang et al., 2012, 2009) and further reveal that small GTPases, in general, are part of the suite of such intracellular regulatory elements.

Whether the cellular mechanism(s) involved in Rac and Rho inhibitor treatments leading to the enhancement of basal GH release and their selective augmenting effects on GnRH3 and GnRH2 stimulation of GH secretion, respectively, are the same awaits further studies. Likewise, the intracellular component(s) targeted by Arf1/6 in mediating GnRH actions on hormone release from goldfish gonadotrophs is unknown. However, the divergence of Arf1/6's influence in the control of basal and GnRH-elicited LH and GH secretion is interesting given that PIT-1-sensitive PH domain interactions is upstream of Arf1/6. Although inhibition of PtdIns(3,4,5)P_3-PH domain interactions using PIT-1 similarly elevated basal hormone secretion from goldfish gonadotrophs and somatotrophs, it abolished GnRH-induced LH and GH responses (Pemberton and Chang, 2016) whereas inhibition of Arf1/6 does not attenuate the ability of the two GnRHs to induce GH secretion in the present study. Together, these findings suggest that a multitude of PH-domain-recruited effectors are involved in acute exocytosis in varying degrees and with varying effects in a cell-type- and context-dependent manner.

Results utilizing the Ras inhibitor BAY-293 also add to the body of work investigating the MAPK cascade in goldfish pituitary cells. Interestingly, although GnRH induction of ERK phosphorylation was inhibited by preventing SOS-dependent activation of Ras GTPase, GnRH-

induced hormone release was not impaired in the present study, whereas direct inhibition of the downstream MEK attenuated LH responses to both GnRH isoforms (Klausen et al., 2008), as well as the GH response to GnRH3, but not GnRH2 (Pemberton et al., 2013). Furthermore, PKC is known to activate ERK phosphorylation in dispersed pituitary cell cultures, possibly through direct phosphorylation of Raf kinase, which has been observed in NIH-3T3 and COS7 cells (Kolch et al., 1993; Marais et al., 1998). Thus, together with prior observations, the present findings suggest that while the Ras-Raf-MEK-ERK module is likely intact and functional in goldfish pituitary cells, the role of MEK/ERK in driving GnRH-stimulated acute hormone release is not downstream of SOS adaptors linking to Ras (Figure 5.12). This seems particularly so for GnRH stimulation of LH release, since the presence of BAY-293 enhanced the LH response to GnRH3. Differences in basal LH release profiles when comparing the present results of inhibiting SOS-Ras with prior experiments directly inhibiting MEK (Klausen et al., 2008), and the enhanced response in GnRH3-dependent LH release during upstream inhibition of Ras, likely also represents selective uncoupling of this axis in goldfish pituitary cells, similar to that observed for the PI3K-PDK1-Akt module (Pemberton et al., 2015, 2011).

Overall, the possible involvement of small GTPases linking to cytoskeletal actin machinery following GnRHR stimulation is consistent with known physiological roles of GnRH in pituitary cell types. Gonadotrophs in particular exhibit a high degree of plasticity and are known to undergo structural and positional changes throughout the reproductive cycle in mammalian models (Alim et al., 2012), and GnRH stimulates morphological rearrangements and development of cellular projections in these cells (Childs et al., 1983; Childs, 1985; Navratil et al., 2007). Interestingly, such cellular projections contain LH vesicles and extend towards blood vessels within the pituitary, providing a relevant physiological basis for GnRH-induced cell

remodelling (Alim et al., 2012; Childs, 2006). Furthermore, GnRH can engage the actin cytoskeleton rapidly (within 1 min of treatment), and treatment of primary mouse pituitary cells or αT3-1 gonadotrophs with agents that disrupt the actin cytoskeleton abolish GnRH-induced LH secretion (Navratil et al., 2014, 2007). These findings strongly indicate a role for actin in exocytosis of secretory vesicles in GnRH-stimulated gonadotrophs. Similarly, GnRH treatment elicits migration of gonadotrophs within *ex vivo* cultured mouse pituitary slices (Navratil et al., 2007). Altogether, these events involve dynamic remodelling of the actin cytoskeleton and are part of the coordinated cellular responses initiated by GnRH (Edwards et al., 2017). Whether such remodelling and/or cell migratory responses are elicited in goldfish pituitary cells is unknown, but the presence and conservation of small GTPase effectors, as well as the present results showing their involvement in GnRH-induced secretion indicate that this machinery is likely also conserved in basal vertebrates. Indeed, recent reports from teleost fish models support this hypothesis, showing that GnRH increases the formation of gonadotroph cell processes in an actin-dependent manner in primary culture of medaka pituitary cells, and imaging of whole medaka pituitaries further reveals that these processes may be utilized to facilitate cell-cell communication and/or to localize to the proximity of blood vessels (Fontaine et al., 2020; Grønlien et al., 2021). Thus, follow-up investigations can be carried out to address these potential roles of small GTPases in the totality of GnRH actions in the goldfish system.

It is also worth mentioning that with the exception of BAY-293, which targets a specific GEF interaction of Ras, all other inhibitors employed here directly target the small GTPase itself, without discriminating between inputs from several possible upstream GEFs. For example, at least 8 different GEFs for Arf6 have been identified in mammals, likely indicative of a high degree of spatiotemporal control in functions of this GTPase through distinct receptor-mediated

pathways (Casanova, 2007). Similarly, Ras activation may occur through alternate (non-SOS) GEFs such as Ras-GRP and p140Ras-GRF (Hennig et al., 2015) and represents areas for further investigation. Importantly, teleost pituitary cells are exposed to multiple hypothalamic neuromodulators which act through unique receptor systems. Gonadotrophs in particular may be regulated by over 20 distinct factors (Trudeau, 2018; Zohar et al., 2010), and at least 10 neuroendocrine regulators are known to affect goldfish somatotroph functions (Chang et al., 2012). Thus, whether the GTPases investigated in this chapter are selectively utilized downstream of other hormone-receptors within this multifactorial framework via the selective use of different upstream GEFs are questions that should be addressed in future investigations.

5.4 Summary

Results from this thesis chapter establish the participation of a highly conserved subset of small GTPase effectors in the control of pituitary hormone secretion, for the first time in any GnRH model system. Adding to work from other neuroendocrine and endocrine secretory cell types across taxa, the present findings reveal the constant involvement of small GTPases during hormone exocytosis from goldfish pituitary cells, and even transient pharmacological disruptions of their activity has substantial impacts on secretion profiles.

Furthermore, the data suggest differential involvement of GTPases in gonadotrophs and somatotrophs and variable engagement in a GnRH-isoform-dependent fashion, as well as segregation of functions during basal and agonist-stimulated cellular states (summary schematic in Figure 5.12). How such specificity is achieved is presently unknown, but likely involves the usage of unique GEFs and GAPs which coordinate spatiotemporal activities of small GTPases. Additionally, results with an inhibitor of SOS-Ras interactions in phosphorylation studies

highlights the importance of canonical players upstream of MEK-ERK during GnRHR signal transduction, and the usage of SOS additionally implicates recruitment of growth factor receptor cascades. Overall, the findings in this chapter enhance the understanding of biased GnRHR actions, as well as the control of basal LH and GH release, in the goldfish pituitary system by revealing the importance of select small GTPases, some of which potentially provide a link to exocytotic machinery through their known regulation of secretory pathways and cytoskeletal actin dynamics.

Table 5.1. Conservation of primary amino acid residues between human isoforms of small GTPases and predicted sequences of goldfish homologs. Compounds utilized in this study (Arf1/6: NAV-2729, Rac1: EHT 1864, RhoA: Rhosin, K-Ras: BAY-293) are designed against human isoforms of these GTPases. Human protein sequences for the GTPases were retrieved from Universal Protein Resource Knowledgebase (UniProtKB; https://www.uniprot.org), followed by organism-specific BLAST against the goldfish genome (*Carassius auratus*, NCBI Taxonomy ID 7956). Identifiers for the resulting goldfish sequences with highest percent identity match are presented.

Protein	% Identity	Human (UniProt ID)	Goldfish (NCBI accession)
Arf1	97.24%	P84077	XP_026057365.1
Arf6	98.29%	P62330	XP_026115546.1
Rac1	100%	P63000	XP_026064742.1
RhoA	95.85%	P61586	XP_026118769.1
K-Ras	96.99%	P01116	XP_026058800.1

Figure 5.1. Presence of immunoreactive small GTPases in dispersed goldfish pituitary cells. Representative immunoblots from one of three such experiments with lysates extracted from three independent dispersed goldfish pituitary cell cultures ("Pit"; cultures prepared from goldfish undergoing gonadal recrudescence, January-February) using antibodies against mammalian (A) Arf1, (B) Rac, (C) RhoA, and (D) K-Ras. Positive controls for GTPase expression were performed using lysates from the human epithelial-like cell line HEK-AD293 ("HEK"), which are known to express these four small GTPases.

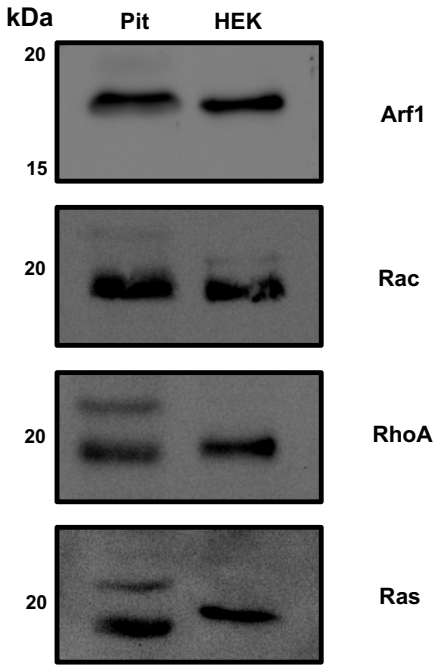
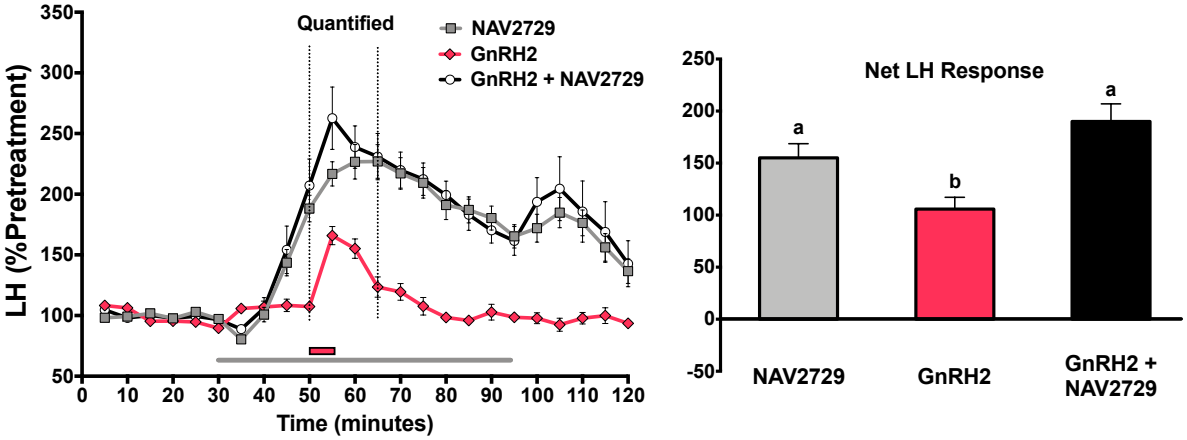

kDa
Pit
HEK
20
15
Arf1
20
Rac
20
RhoA
20
Ras

Figure 5.2. Effects of the Arf1/6 inhibitor NAV-2729 (10 µM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified net response to agonist stimulation (between indicated dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (3.23 ± 0.20 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from pre-spawning, sexually mature goldfish (March to April). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).


Quantified
NAV2729
GnRH2
GnRH2 + NAV2729
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
a
NAV2729
GnRH2
GnRH2 +
NAV2729

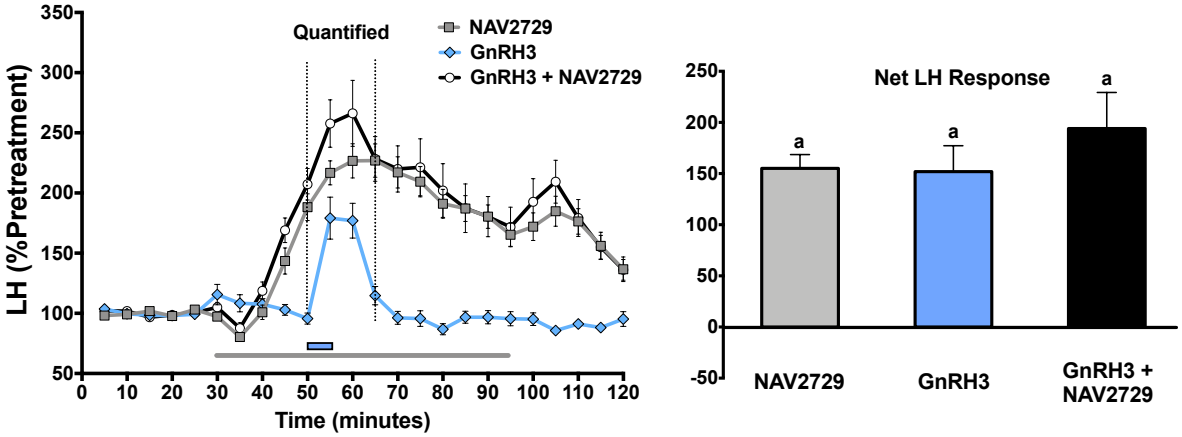

Quantified
NAV2729
GnRH3
GnRH3 + NAV2729
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
a
a
NAV2729
GnRH3
GnRH3 +
NAV2729

Figure 5.3. Effects of the Arf1/6 inhibitor NAV-2729 (10 µM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified net response to agonist stimulation (between indicated dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (9.36 ± 0.83 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from pre-spawning, sexually mature goldfish (March to April). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

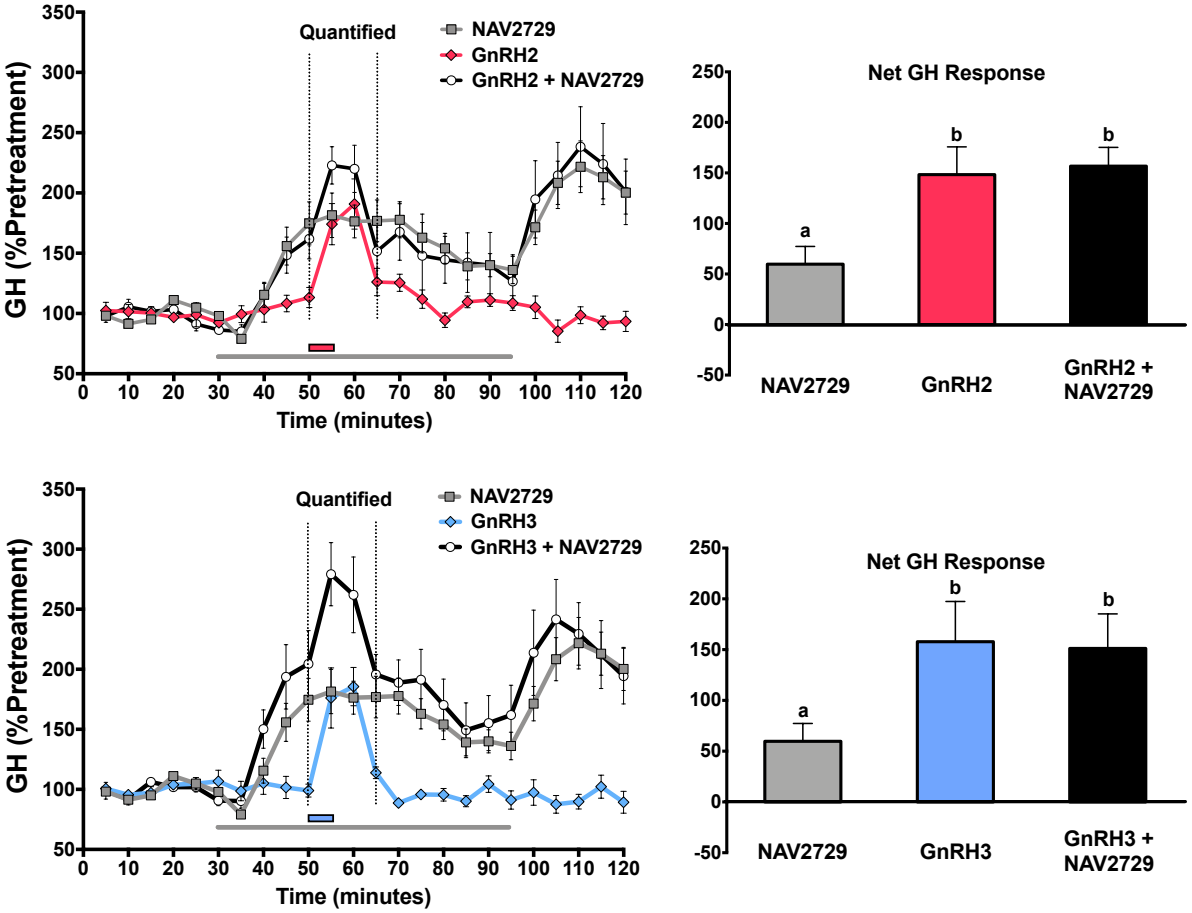
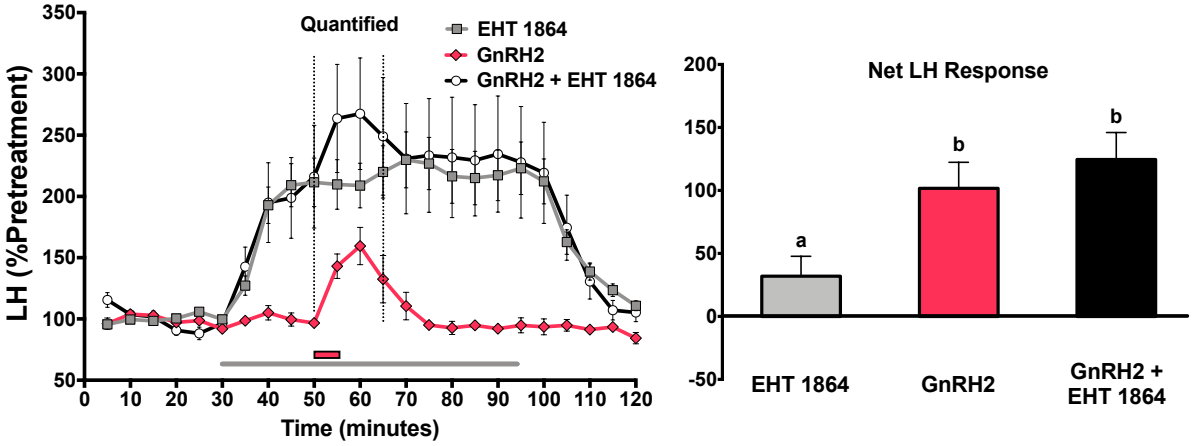

Quantified
NAV2729
GnRH2
GnRH2 + NAV2729
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
NAV2729
GnRH2
GnRH2 +
NAV2729
Quantified
NAV2729
GnRH3
GnRH3 + NAV2729
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
NAV2729
GnRH3
GnRH3 +
NAV2729

Figure 5.4. Effects of the Rac inhibitor EHT 1864 (20 µM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified net response to agonist stimulation (between indicated dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (1.65 ± 0.18 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish with regressed gonads (August to September). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).


Quantified
EHT 1864
GnRH2
GnRH2 + EHT 1864
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
EHT 1864
GnRH2
GnRH2 +
EHT 1864

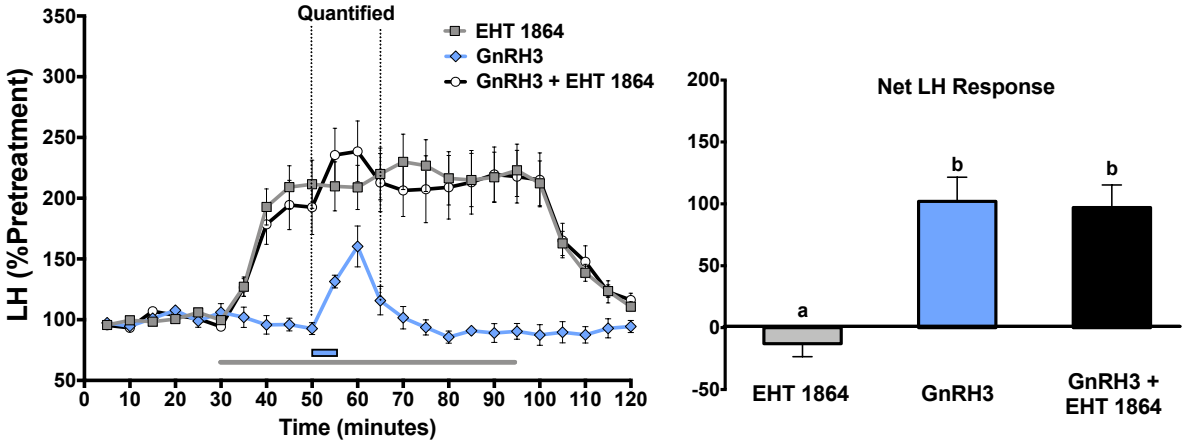

Quantified
EHT 1864
GnRH3
GnRH3 + EHT 1864
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
EHT 1864
GnRH3
GnRH3 +
EHT 1864

Figure 5.5. Effects of the Rac inhibitor EHT 1864 (20 μM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified net response to agonist stimulation (between indicated dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (16.44 ± 1.27 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish with regressed gonads (August to September). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

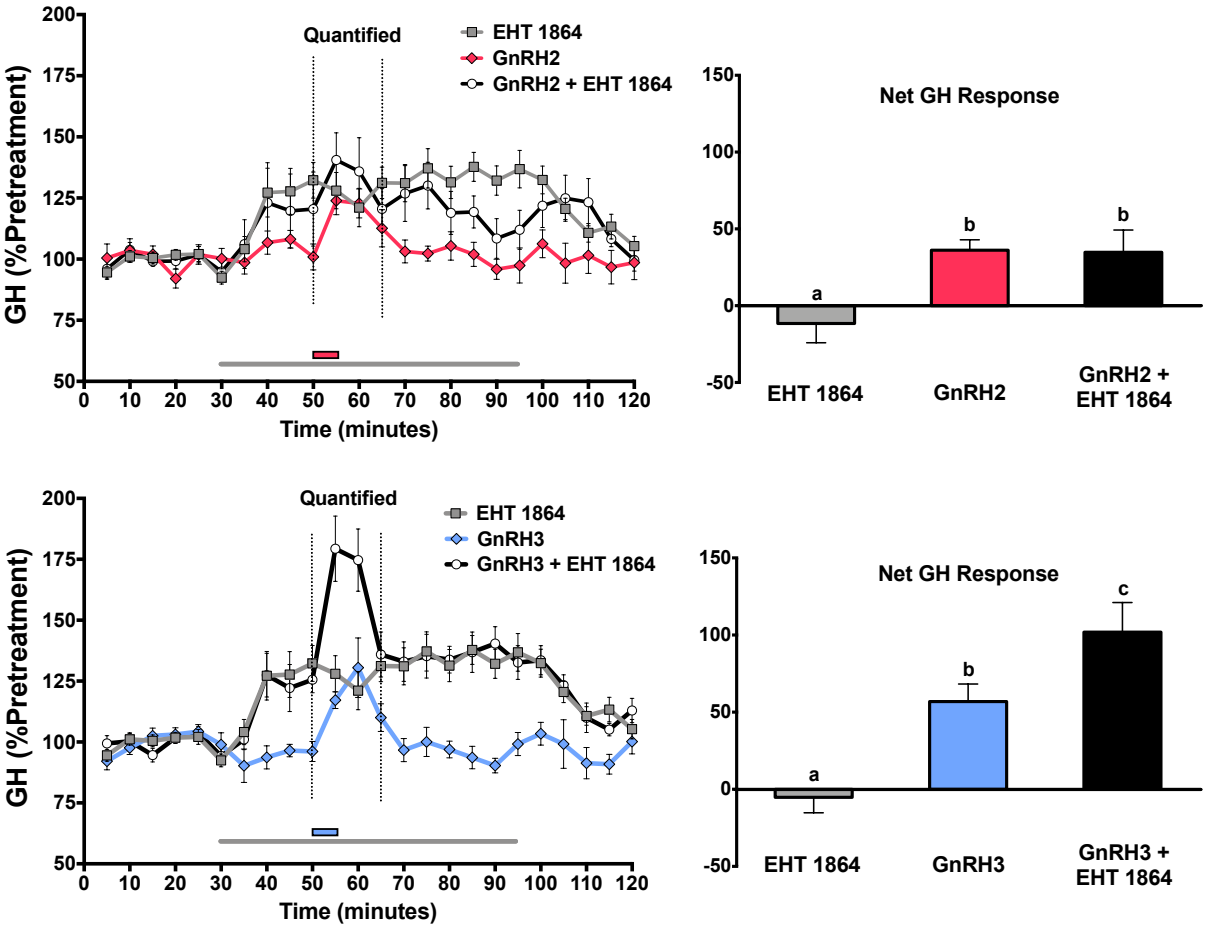

Quantified
EHT 1864
GnRH2
GnRH2 + EHT 1864
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
EHT 1864
GnRH2
GnRH2 +
EHT 1864
Quantified
EHT 1864
GnRH3
GnRH3 + EHT 1864
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
c
EHT 1864
GnRH3
GnRH3 +
EHT 1864

Figure 5.6. Effects of the RhoA inhibitor Rhosin (30 µM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified response to agonist stimulation (between indicated dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (2.57 ± 0.21 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish with regressed gonads or gonads at early recrudescence (September to October). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

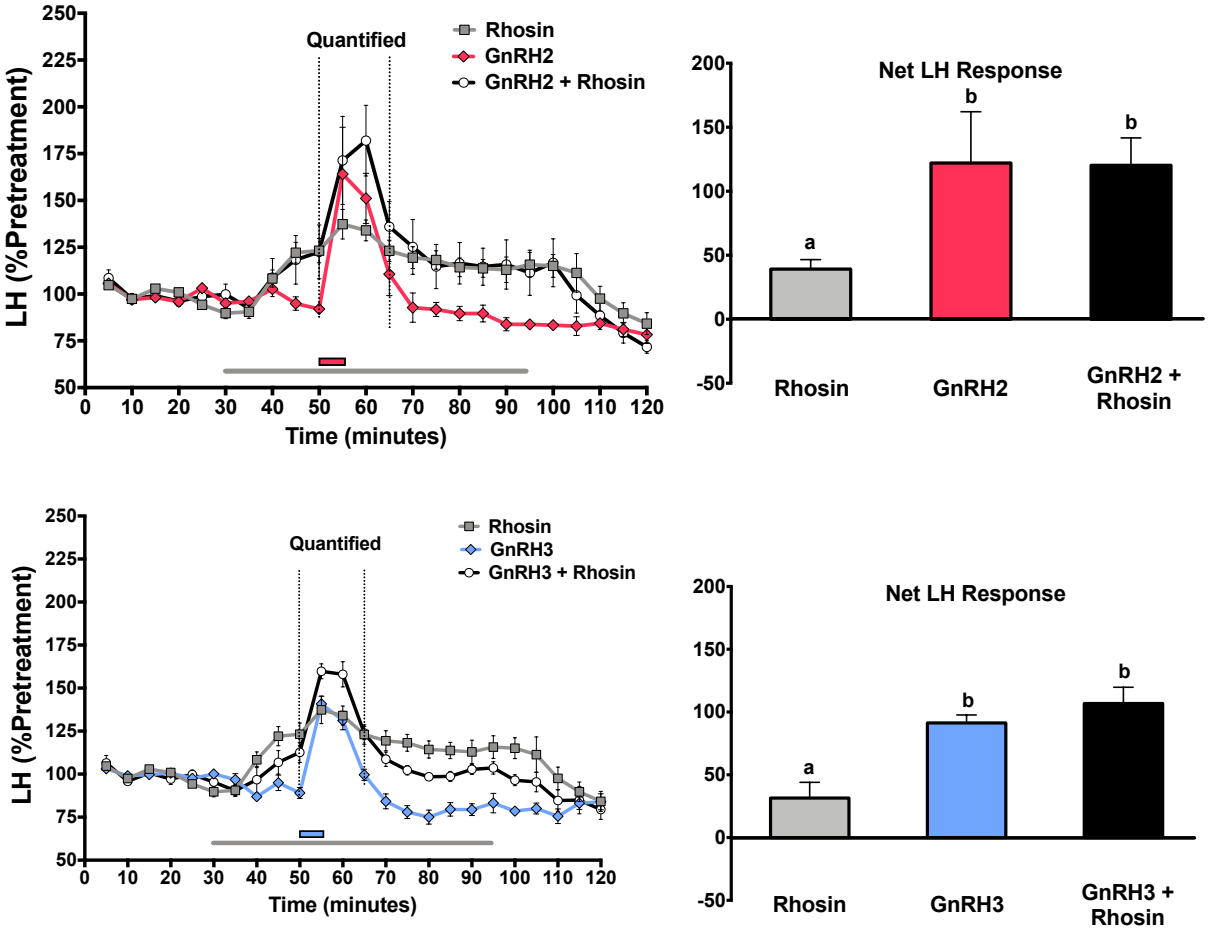

Quantified
Rhosin
GnRH2
GnRH2 + Rhosin
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
Rhosin
GnRH2
GnRH2 +
Rhosin
Quantified
Rhosin
GnRH3
GnRH3 + Rhosin
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
Rhosin
GnRH3
GnRH3 +
Rhosin

Figure 5.7. Effects of the RhoA inhibitor Rhosin (30 µM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified response to agonist stimulation (between indicated dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (21.43 ± 1.30 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish with regressed gonads or gonads at early recrudescence (September to October). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

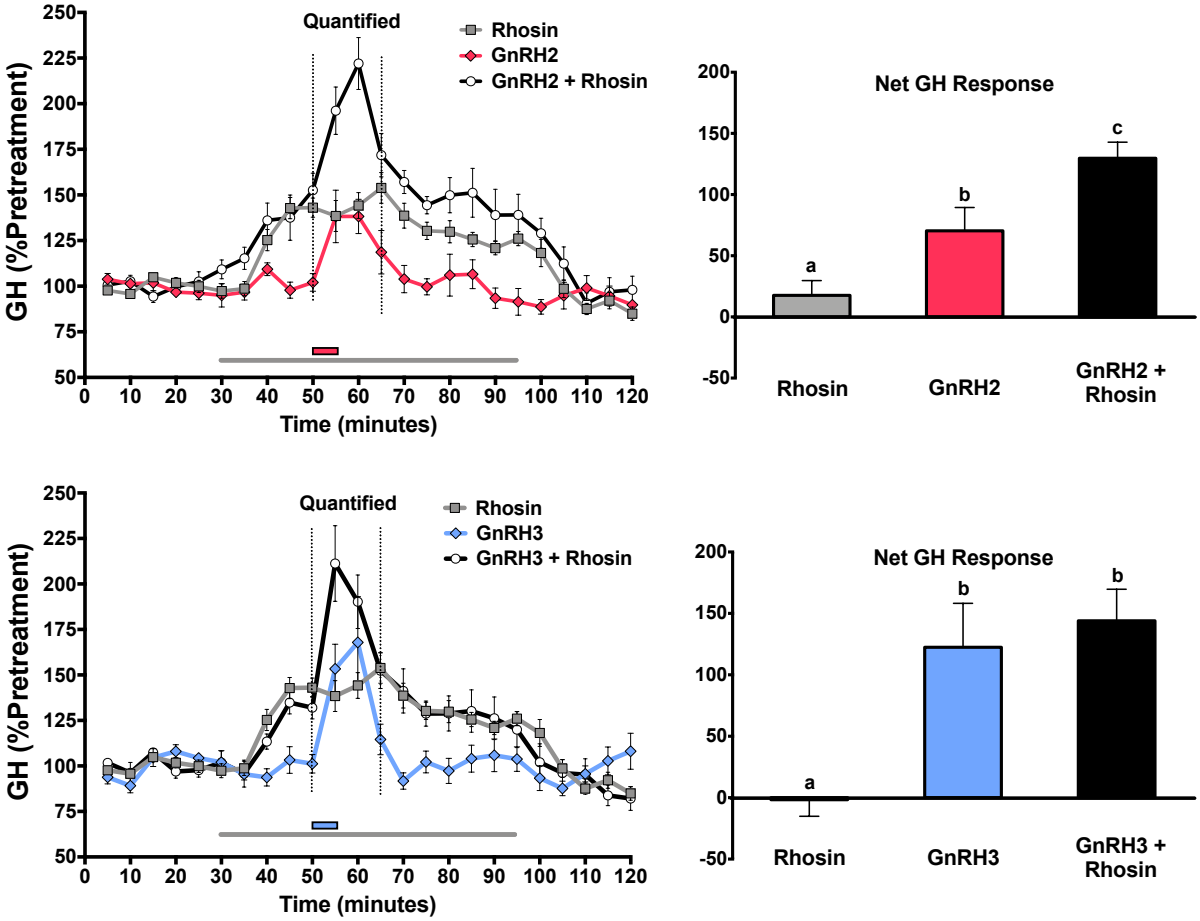
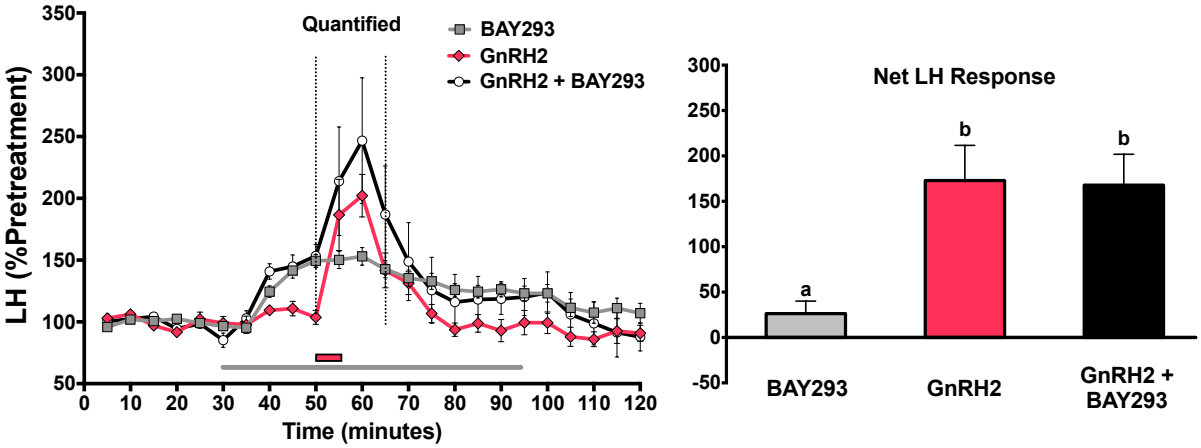

Quantified
Rhosin
GnRH2
GnRH2 + Rhosin
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
c
Rhosin
GnRH2
GnRH2 +
Rhosin
Quantified
Rhosin
GnRH3
GnRH3 + Rhosin
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
Rhosin
GnRH3
GnRH3 +
Rhosin

Figure 5.8. Effects of the SOS-Ras inhibitor BAY-293 (5 µM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified response to agonist stimulation (between indicated dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (1.18 ± 0.17 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish with regressed gonads (July to August). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).


Quantified
BAY293
GnRH2
GnRH2 + BAY293
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
BAY293
GnRH2
GnRH2 + BAY293

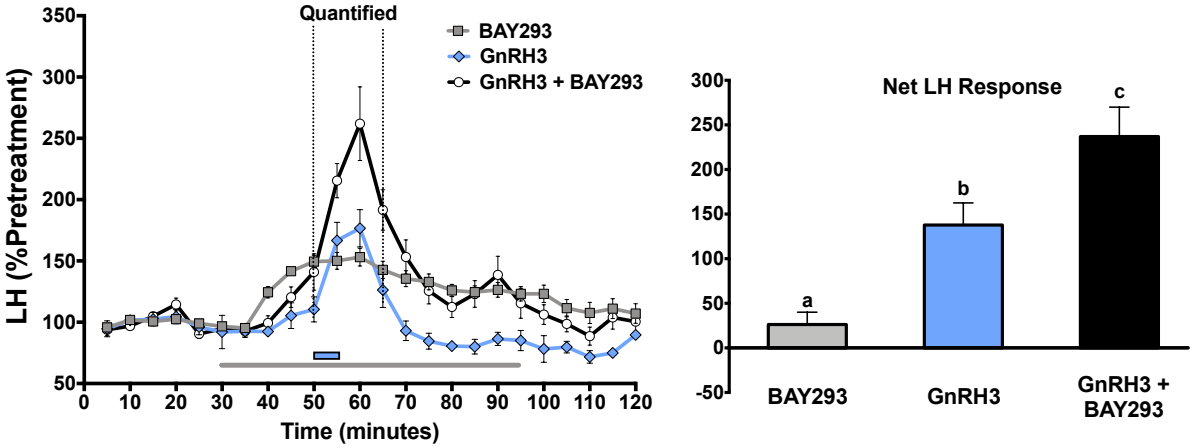

Quantified
BAY293
GnRH3
GnRH3 + BAY293
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
BAY293
GnRH3
GnRH3 + BAY293

Figure 5.9. Effects of the SOS-Ras inhibitor BAY-293 (5 µM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified response to agonist stimulation (between indicated dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (15.03 ± 1.84 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish with regressed gonads (July to August). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

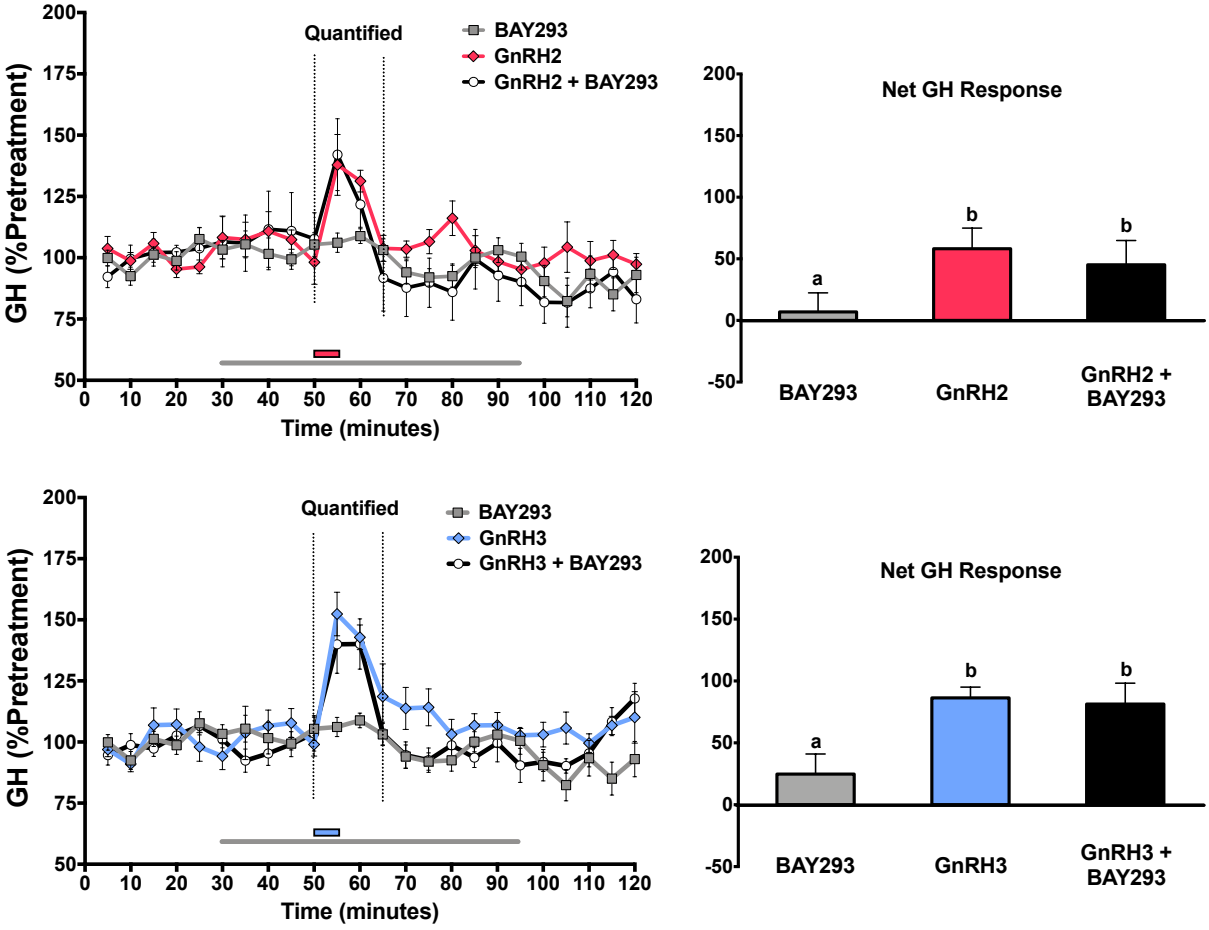

Quantified
BAY293
GnRH2
GnRH2 + BAY293
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
BAY293
GnRH2
GnRH2 +
BAY293
Quantified
BAY293
GnRH3
GnRH3 + BAY293
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
BAY293
GnRH3
GnRH3 +
BAY293

Figure 5.10. Effects of the SOS-Ras inhibitor BAY-293 on ERK phosphorylation in dispersed pituitary cells. Following overnight culture, dispersed pituitary cells were pre-treated with BAY-293 (5 μM) or DMSO vehicle for 30 min, followed by addition of GnRH2 or GnRH3 (100 nM) for 5 min in the presence of DMSO or Barbadin. Cells were then harvested and lysed, and protein extracts probed for phospho-ERK (Thr202/Tyr204), total ERK, and β-actin. Example blots are presented in panel A. Densitometry readings normalized to the unstimulated vehicle control are presented in panel B. Results (mean ± SEM) are pooled from n = 3 individual cell preparations from goldfish undergoing gonadal recrudescence (December-January). Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$).

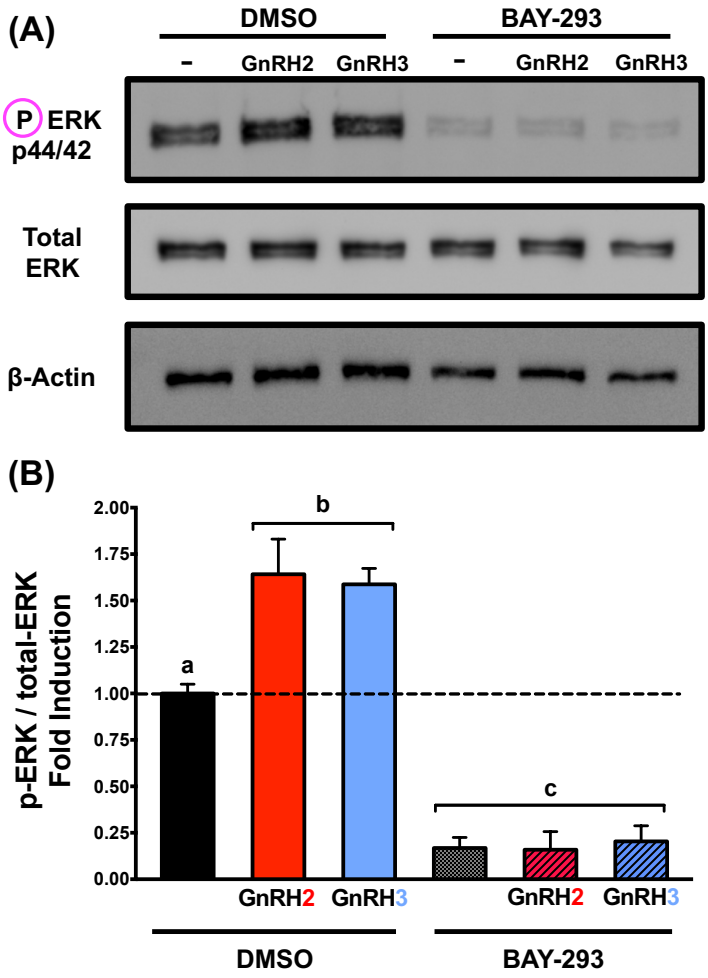

(A)
DMSO
BAY-293
-
GnRH2
GnRH3
-
GnRH2
GnRH3
P ERK p44/42
Total ERK
β-Actin
(B)
p-ERK / total-ERK Fold Induction
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
b
c
GnRH2
GnRH3
GnRH2
GnRH3
DMSO
BAY-293

Figure 5.11. Effects of small GTPase inhibitors of Arf1/6 (NAV-2729, 10 µM), Rac (EHT 1864, 20 µM), RhoA (Rhosin, 30 µM), SOS-Ras (BAY-293, 5 µM) on unstimulated LH (A, B) and GH (C, D) release. Inhibitor-alone hormone release profiles taken from perifusion experiments (from Figures 5.2 – 5.9) are shown on the left (A and C; green squares, Arf1/6 inhibitor, blue triangles, Rac inhibitor; red diamonds, RhoA inhibitor, orange circles, SOS-Ras inhibitor) and the corresponding quantified responses shown on the right (B and D). Basal hormone release prior to inhibitor treatment was quantified as the average of values from 0 to 20 min of the experiment (average % pretreatment; black horizontal line in A and C; corresponding quantified values in black vertical bar in B and D), during which cells are perifused with M199 media alone. The basal hormone release during inhibitor treatment was evaluated as the average of %pretreatment values over the duration of inhibitor treatment (30-95 min; grey horizontal line; corresponding quantified values in the other coloured vertical bars in B and D). An asterisk denotes the presence of responses during inhibitor treatment that were significantly different from basal release prior to inhibitor application (paired Student *t* test; $P < 0.05$; $n = 16$, from eight independent cell preparations per inhibitor).

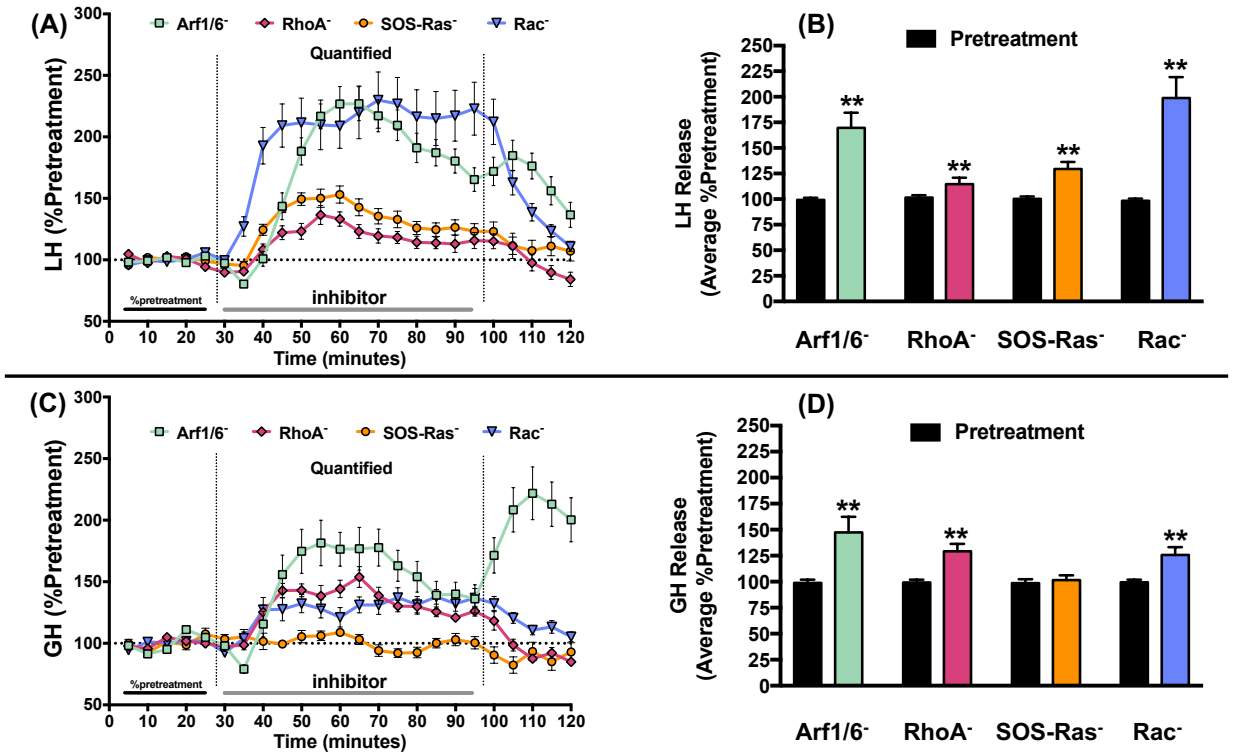
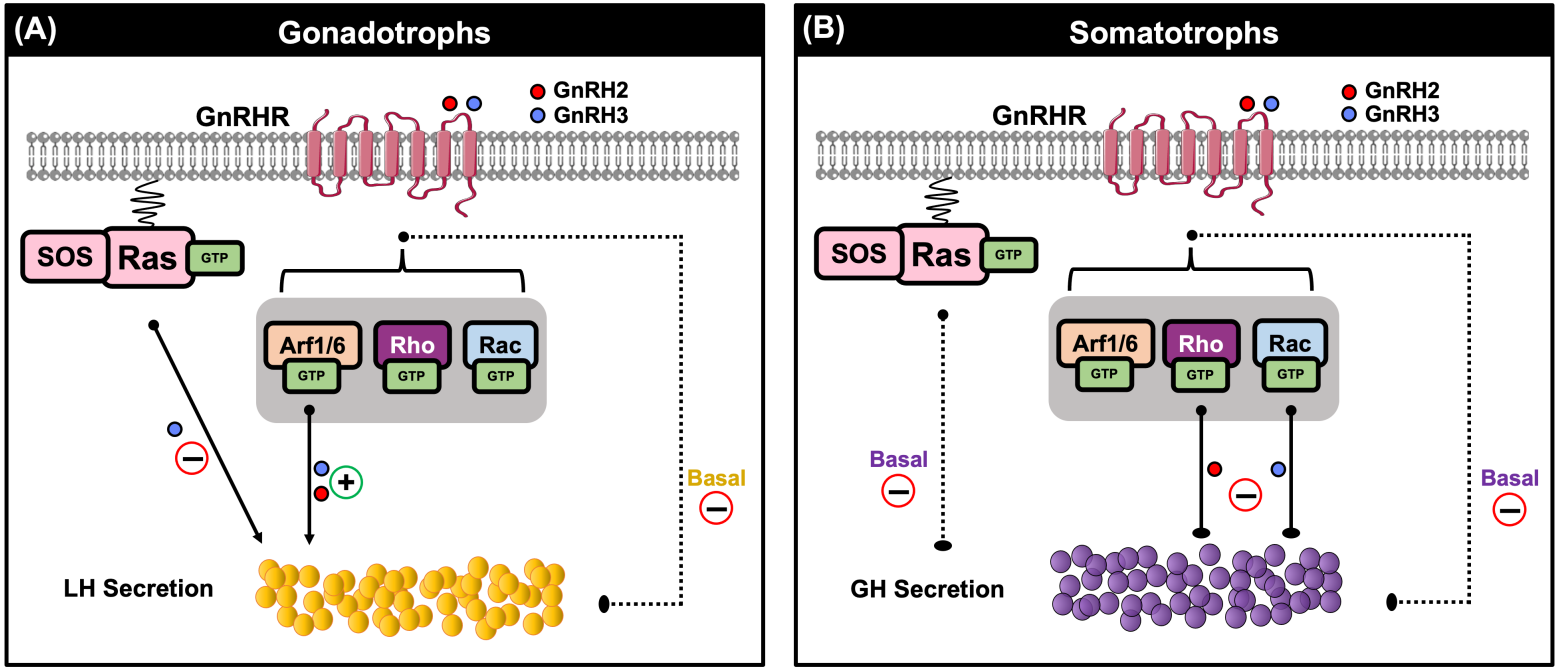

(A)
Arf1/6-
RhoA-
SOS-Ras-
Rac-
Quantified
LH (%Pretreatment)
%pretreatment
inhibitor
Time (minutes)
(B)
Pretreatment
LH Release
(Average %Pretreatment)
Arf1/6-
RhoA-
SOS-Ras-
Rac-
(C)
GH (%Pretreatment)
(D)
GH Release
(Average %Pretreatment)

Figure 5.12. Summary model of small GTPase actions in the control of basal and GnRH-evoked LH and GH release. Results from this chapter highlight the selective involvement of these proteins in basal *vs.* agonist-dependent functional contexts. In gonadotrophs (A), Arf1/6, Rho, and Rac GTPases inhibit basal LH release (dotted line), and Arf1/6 additionally mediates GnRH2/3-induced acute LH secretion. In somatotrophs (B), Arf1/6, Rho, Rac, and Ras all exert negative regulation over basal GH release (dotted lines), whereas Rho and Rac GTPases selectively inhibit GnRH2- and GnRH3-evoked acute GH secretion.


(A)
Gonadotrophs
GnRHR
GnRH2
GnRH3
SOS
Ras
GTP
Arf1/6
Rho
Rac
Basal
LH Secretion
(B)
Somatotrophs
GnRHR
GnRH2
GnRH3
SOS
Ras
GTP
Arf1/6
Rho
Rac
Basal
GH Secretion
Basal

Chapter Six

Involvement of matrix metalloproteinases and tyrosine kinase effectors in basal and GnRH-dependent control of hormone release

6.1 Introduction

Besides the activation of classical G protein-dependent pathways, many GPCRs can recruit protein tyrosine kinase signalling cascades, as well as utilize transactivation of growth factor receptors via MMP and/or other intracellular effectors, to achieve cellular outcomes (see Figure 1.8 for an overview). Involvement of these other signalling methods is often linked to the downstream activation of MEK/ERK by GPCRs (Wetzker and Böhmer, 2003).

In the case of GnRHRs, transactivation of the EGFR can be utilized to transmit part of, or the whole, GnRH-initiated signal leading to select intracellular responses (Roelle et al., 2003; Shah et al., 2003a). Importantly, such transactivation can occur rapidly within 2 min of GPCR stimulation (Koon et al., 2004; Santiskulvong and Rozengurt, 2003); thus, there is potential for these mechanisms to be involved in the acute GnRH control of hormone release in the timeframes examined throughout this thesis. Additionally, several intracellular protein tyrosine kinases have also been implicated in GnRH actions in mammalian model systems. Among these are members of the SFKs (e.g., Src tyrosine kinase; Shah et al., 2003a, 2003b), and other non-receptor tyrosine kinases such as the paralogs Pyk2 and FAK (Xie et al., 2008). Likewise, the previously described participation of Btk in goldfish GnRH actions implicates involvement of SFKs since they are largely required for complete activation of Btk (Pemberton and Chang, 2016; Takesono et al., 2002). Although how GnRHRs activate these tyrosine kinases are not well characterized, results from other GPCR systems indicate that recruitment and activation of SFKs can be achieved via direct interaction with some GPCRs (Fan et al., 2001), $G\alpha_{i/o}$-dependent mechanisms (Villaseca et al., 2022), or association with β-arrestins through SH3 domains (Yang et al., 2018). These mechanisms may also provide avenue(s) for the use of SFKs in the goldfish GnRH system especially given the involvement of $G\alpha_{i/o}$ and β-arrestin in some of the goldfish

GnRH-elicited hormone release responses (Chapters 3 and 4). Similarly, Pyk2 is a well-known effector of Ca^{2+}/CaM actions (following VGCC activation) and can also function downstream of PKC (Lev et al., 1995), and VGCCs, CaM and PKC are involved in GnRH2 and GnRH3 actions in stimulation of LH and GH release (Chang et al., 2000; also reviewed in Chapter 1). In mammalian models such as GT1-7 cells expressing endogenous GnRHRs, both CaMK and the SFK Fyn are needed for full activation of Pyk2; this provides an additional example of how GnRH-induced Ca^{2+} signalling, SFKs, and Pyk2 can interact and be linked to one another (Higa-Nakamine et al., 2020; Okitsu-Sakurayama et al., 2021).

While the aforementioned studies have explored GnRHR engagement of these mechanisms in signalling contexts, as well as the effects on gonadotrophin subunit gene expression in αT3-1 and LβT2 cells (Bonfil et al., 2004; Harris et al., 2003, 2002), no information is known to date about the relevance of these proteins in the control of hormone release in any study model. I hypothesized that, as in results from Roelle and colleagues (2003) and Shah and colleagues (2003), transactivation of EGFRs through MMP actions is part of the GnRH-elicited mechanisms in goldfish pituitary cells. Additionally, given the evidence for SFK and Pyk2/FAK involvement in mammalian GnRH systems and their links to MAPK/ERK and intracellular Ca^{2+} dynamics, I predicted that these intracellular effectors mediate GnRH-dependent hormone release, and may also be part of the intracellular link to transactivation machineries. To address these questions, inhibitors of EGFR, Src, Pyk2/FAK, and MMP activities were employed in studies with GnRH2 and GnRH3 in column perifusion hormone release and immunoblotting experiments (Figure 6.1).

6.2 Results

6.2.1 Effects of the MMP inhibitor GM6001 on hormone release

The broad-spectrum MMP inhibitor GM6001 was used to assess the potential contribution of MMP-dependent receptor transactivation to GnRH-dependent hormone release responses in column perifusion experiments. This compound has been widely utilized in studies exploring MMP-dependent receptor transactivation in cellular model systems across taxa (Cao et al., 2019; Peyton and Thomas, 2011; Santiskulvong and Rozengurt, 2003). GM6001 has broad selectivity for the family of MMPs, including MMP1 (collagenase), MMP2 (gelatinase A), MMP3 (stromelysin), MMP7 (matrilysin), MMP8 (neutrophil collagenase), MMP9 (gelatinase B), MMP12, MMP14, and MMP26, with K_is in the low or sub nanomolar range in biochemical *in vitro* assays (Galardy et al., 1994; Grobelny et al., 1992). In particular, MMP1, 2, 3, 7 and 14 are widely expressed, whereas MMP8, 9, 12 and 26 have restricted tissue expression. Importantly, goldfish homologs of these isoforms have conserved Zn^{2+} binding and cysteine switch motif regions, both of which interact with Zn^{2+} ions during the full activation of MMP enzymes (Vandenbroucke and Libert, 2014; Verma, 2012; Figure 6.2). GM6001 inhibition of MMPs involves chelation of Zn^{2+} (Hu et al., 2007; Wojtowicz-Praga et al., 1997), and cell-based assays probing GPCR-MMP functions have utilized this inhibitor in the 0.1 to 10 μM range (Chadzinska et al., 2008; Chen et al., 2010; Peyton and Thomas, 2011). Additionally, the presence of functional MMP isoforms has been shown in closely related cyprinids including common carp and zebrafish in contexts such as immune function, wound healing and regeneration (Wang et al., 2014; Xu et al., 2015; Zhang et al., 2003), and GM6001 has been leveraged as a tool to investigate MMP activity in many of these studies (Bai et al., 2005; Chadzinska et al., 2008; Xu et al., 2018).

Treatment of dispersed goldfish pituitary cells in column perifusion experiments with 0.2 μM GM6001 caused a transient spike in LH release, followed by a reduction in basal secretion levels over the duration of inhibitor treatment, and removal of inhibitor resulted in a gradual increase towards values observed prior to inhibitor application (Figure 6.3). In contrast, these prominent changes in basal secretion upon GM6001 application were not observed with GH release (Figure 6.4). On the other hand, the GnRH3-induced, but not GnRH2-evoked, LH and GH release responses were significantly attenuated by about half in the presence of GM6001, indicating a selective dependence of GnRH3 on MMP activities on hormone secretion from goldfish gonadotrophs and somatotrophs (Figures 6.3 and 6.4).

6.2.2 Effects of the EGFR inhibitor BIBW2992 on hormone release

BIBW2992 is a selective inhibitor of ErbB1 and ErbB2 receptors (commonly referred to as EGFR1 and EGFR2, respectively) over related tyrosine kinase receptors (vascular endothelial growth factor, VEGFR; and met/hepatocyte growth factor receptor) and intracellular protein tyrosine kinases (such as Src) (Li et al., 2008; Solca et al., 2012). BIBW2992 inhibits EGFRs via interactions with a methionine and a cysteine residue and these BIBW2992-targeted amino acids are conserved between human and goldfish ERBb1 molecules (Figure 6.5). Accordingly, BIBW2992 was employed in the present study at a dose of 1 μM based on the initial validations as well as subsequent studies demonstrating inhibitory effects on EGFR autophosphorylation and downstream cellular responses (Hoshi et al., 2017; Li et al., 2008; Suzawa et al., 2016). The acute LH release responses to the two GnRHs were not altered in presence of BIBW2992 (Figure 6.6). In contrast, while this inhibitor also did not affect GnRH2-induced GH release, its presence resulted in a significant increase (doubling) of the acute GnRH3-dependent GH secretion

response (Figure 6.7). On the other hand, in the presence of BIBW2992, a slight gradual decrease in basal secretion levels was observed for LH, but not GH, secretion.

6.2.3 Effects of the Src inhibitor DGY-06-116 on hormone release

The potential involvement of Src in GnRH actions was evaluated using the inhibitor DGY-06-116. This compound targets kinase activity of SFKs (including Src, Blk, Lck, Fyn, Fgr, and Yes) leading to inhibition of kinase activity in the low nanomolar range in biochemical studies (Du et al., 2020; Gurbani et al., 2020). Inhibition of individual SFKs is generally considered to be challenging due to the high degree of sequence homology within this kinase family; however, DGY-08-116 offers an added degree of selectivity towards Src and Yes over related SFKs by also forming covalent interactions with a non-conserved cysteine adjacent to the ATP-pocket, and specifically inhibits Src activity *in vitro* and *in vivo* in human cancerous cell lines and mouse models, respectively (Du et al., 2020; Gurbani et al., 2020; Heppner et al., 2018). Importantly, this cysteine is replaced in Fyn, and the residue is conserved in basal vertebrate homologs of Src and Yes, as are the drug-coordinating backbone residues as characterized for human c-Src specifically (Gurbani et al., 2020; Figure 6.8). Additionally, while the expression of Src, Fyn and Yes is widespread, expression of other SFKs (i.e., Blk, Fgr, Lyn, Lck) is typically constrained to cells of hematopoietic origin (Mukherjee et al., 2020), and both Src and Fyn are shown to be present in pituitary cell types (Levi et al., 1998; Okitsu-Sakurayama et al., 2019). Thus, this inhibitor can be employed to target Src with a degree of selectivity over related kinases, although potential interactions with the kinase Yes in this cellular background cannot be definitively ruled out.

In column perifusion experiments, treatments with 1 μM DGY-06-116 (a dose that inhibits Src signalling in cell-based assays; Du et al., 2020) led to a significant enhancement of LH release responses to both GnRH2 and GnRH3 (averaging 164% and 211% of GnRH alone values, respectively). In addition, the duration of the LH secretion responses to GnRH was prolonged, especially for GnRH2-dependent LH release (Figure 6.9). However for somatotrophs, inhibition of SFKs did not alter GnRH3-induced GH secretion, but significantly suppressed the GH release response to GnRH2 by about half (Figure 6.10). On the other hand, DGY-06-116 did not seem to have an appreciable effect on basal LH and GH release (Figures 6.9 and 6.10).

6.2.4 Effects of Pyk2/FAK inhibition on hormone release

PF-562271 is an ATP-competitive inhibitor of the related Pyk2 and FAK kinases with characterized biochemical IC_{50} of 1.5 and 14 nM, respectively (Guo et al., 2017; Mukherjee et al., 2020), and 1-10 μM doses of this small molecule inhibitor have been utilized in a range of cancerous and normal cellular models to suppress Pyk2/FAK functions (Chung et al., 2016; Hu et al., 2017; Wiemer et al., 2013). Human FAK and Pyk2 are 65% similar to each other, and goldfish homologs of FAK and Pyk2 have 80% and 60% sequence similarity to their human counterparts, respectively. Importantly, kinase domains of both are well conserved, especially the ATP-binding pocket which mediates PF-562271's inhibition of kinase activity; in addition, known interaction sites as determined for human FAK and Pyk2 are 100 and 90% conserved, respectively, in the predicted goldfish counterparts (Figure 6.11; Roberts et al., 2008). Treatment with PF-562271 (10 μM) resulted in significant reductions of nearly 50% in GnRH2- and GnRH3-dependent LH secretion, compared to treatments with GnRH alone (Figure 6.12). On the other hand, PF-562271 had insignificant effects on GnRH-induced GH release (Figure 6.13).

Despite the apparent lack of participation in GnRH-dependent secretion in somatotrophs, inhibition of Pyk2/FAK resulted in substantial and reversible elevations in both basal LH and GH release (Figures 6.12 and 6.13).

6.2.5 Comparison of effects of MMP, EGFR, Src and Pyk2/FAK inhibition on basal unstimulated hormone release

To further compare and evaluate the overall effects of inhibitors of MMP, EGFR, and tyrosine kinases on basal hormone release responses, the averaged hormone values before and during inhibitor application within each of the inhibitor alone treatment columns were quantified (Figure 6.14). Inhibition of MMPs, EGFR, and Src had significant suppressive effects on basal LH release (average responses during inhibitor application were 78%, 89%, and 91%, respectively, of those before inhibitor treatment), but these inhibitors did not affect basal GH secretion. On the other hand, Pyk2/FAK inhibition resulted in significant sustained increases in both basal LH and GH release (averaging 155% and 240%, respectively, of values seen prior to inhibitor application), before returning to levels observed prior to inhibitor treatment upon washout.

6.2.6 Effects of MMP, EGFR, Src and Pyk2/FAK inhibitors on ERK and/or Src phosphorylation

In parallel with hormone release studies, the effects of MMP, EGFR, Pyk2/FAK and Src inhibitors on engagement of ERK cascades were also assessed by monitoring the level of phospho-ERK. Interestingly, despite the reductions observed in basal LH and GnRH3-dependent LH and GH release upon MMP inhibition (Figures 6.3, 6.4 and 6.14), this treatment altered

neither basal nor GnRH-induced levels of ERK phosphorylation (Figure 6.15A). In contrast, in the presence of the EGFR inhibitor BIBW2992, basal phospho-ERK levels were slightly (though insignificantly) elevated and the phospho-ERK responses to the two GnRHs were not significantly different from values obtained with inhibitor alone treatment (Figure 6.15B). Treatment with the Pyk2/FAK inhibitor suppressed basal phosphor-ERK levels and inhibited the ability of GnRH to induce elevations in phospho-ERK (Figure 6.15C).

As to the effects of the Src inhibitor DGY-06-116, I first confirmed that this compound reduced phosphorylation levels of Tyr^{416}, a conserved site on Src which has been utilized as an index of enzyme activity (Okada, 2012; Figure 6.16). In comparison with the stimulation of ERK phosphorylation, GnRH treatment did not lead to an appreciable change in Src Tyr^{416} phosphorylation; however, basal phospho-ERK levels and GnRH-induced increases in ERK phosphorylation levels were significantly suppressed in the presence of DGY-06-116 (Figure 6.16).

6.3 Discussion

6.3.1 MMP and EGFR involvement in basal and GnRH-dependent hormone release

To investigate the involvement of MMP actions in goldfish GnRH actions, a broad inhibitor of the family of MMPs was utilized in column perifusion experiments. Interestingly, treatment with this inhibitor selectively attenuated GnRH3-dependent LH and GH release responses, suggesting the possibility of downstream EGFR transactivation as has been shown for mammalian GnRHRs (Roelle et al., 2003; Shah et al., 2003). However, my follow-up studies with a selective inhibitor of EGFR1 and EGFR2 not only did not recapitulate the effects of MMP inhibition (i.e., did not cause a reduction in GnRH3-stimulated LH and GH secretion), and

instead potentiated GnRH3-induced GH release, indicating the possible involvement of non-EGFR targets which may also exert differing modulatory effects on GnRH3 actions. Regardless, these observations indicate the involvement of MMP-mediated events are part of the suite of mechanisms engaged in biased signalling by these two GnRHs in goldfish pituitary cells.

In addition to EGF pro-ligands, a number of growth factors are sequestered in both the plasma membrane and the surrounding extracellular matrix (ECM), and these can be released by the remodelling activity of proteases such as MMPs and may serve as candidates for GnRH-induced transactivation. For example, GPCR-initiated cascades leading to MMP activation can cleave pro-ligands for TGF-β, which is secreted to the ECM as a latent molecule requiring proteolytic cleavage for activation; importantly, both TGF-β and its receptor are present in the human pituitary (Recouvreux et al., 2016). Likewise, VEGF is also bound to the ECM upon secretion and its subsequent release is dependent upon proteolytic activity, such as by MMP isoforms 3, 7, or 9 (Lee et al., 2005; Park et al., 1993), and this ligand-receptor pair is similarly present in the pituitary of sheep and rats (Alfer et al., 2015; Jabbour et al., 1997). Thus, receptors for VEGF and TGF-β represent potential areas of investigation to elucidate MMP actions downstream of GnRH. Although the expression of VEGF and TGF-β in the teleost pituitary has not been reported, members of TGF-β superfamily of hormones, such as Anti-Müllerian hormone and its receptor (Pfennig et al., 2015), as well as bone morphogenic protein BMP15 (Chen et al., 2012) are expressed in the pituitary of several teleost species. On the other hand, GM6001's lack of effects on GnRH-induced ERK activity (Figure 6.15) suggests that the target of MMP actions leading to alteration in GnRH3-induced hormone release is not a growth factor receptor but may instead involve ECM proteins and their interacting transmembrane protein partners.

Known ECM targets of MMP actions include components such as fibronectin, laminin, and collagen, all three of which are present during development of the mammalian pituitary gland and can modulate hormone secretion from both normal and cancerous pituitary cell models (Paez-Pereda et al., 2005). For example, addition of laminin to cultured GH3 cells modulates both basal and agonist-induced PRL secretion (Carvalho et al., 1989). Similarly, both laminin and type IV collagen have been shown to modulate basal PRL, LH, and FSH release from normal rat pituitaries. Besides these effects on basal release, laminin treatment also partially reduced the GnRH-induced FSH, but not LH, secretion response (Denduchis et al., 1994), and type IV collagen did the same for thyrotrophin-releasing hormone-evoked PRL secretion (Diaz et al., 2002), whereas laminin had the opposite effect on thyrotrophin-releasing hormone-stimulated PRL release (Carvalho et al., 1989). Since both GnRH and thyrotrophin-releasing hormone receptors are G-protein coupled, such effects of ECM components on agonist-evoked responses are suggestive of ECM proteins being integrated within GPCR signalling pathways in pituitary cell types (Denduchis et al., 1994; Diaz et al., 2002). Thus, the possibility exists that these ECM molecules may play a role in MMP-mediated GnRH actions, both in intact pituitaries where normal tissue architecture is maintained (such as cell-cell contacts and cell-ECM interactions), as well as in pituitary cell culture systems.

Integrin proteins are transmembrane receptors that are important components of focal adhesions, which serve as protein organizing scaffolds and mediate bidirectional communication between the intracellular cytoskeleton and ECM (Barczyk et al., 2010; Larsen et al., 2006). In addition to triggering the release of integrin receptor ligands in the form of cleaved ECM fragments (such as those of collagen, fibronectin, or laminin) leading to integrin activation, MMPs may act directly on integrins as in the case of MMP7 and MMP14 (Stamenkovic, 2003;

Niland and Eble, 2020), and MMP7-mediated cleavage of some integrin isoforms activates intracellular FAK signalling (Deryugina et al., 2002). Interestingly, GnRH stimulation of mammalian LβT2 gonadotrophs leads to the formation of integrin-containing focal adhesion complexes, which are used to spatially organize large multi-protein signalosomes containing Src Pyk2/FAK, PKC, ERK, and/or other effectors (Dobkin-Bekman et al., 2009). Also, GnRH stimulation of GnRHR-expressing HEK293 cells leads to increased cellular adherence to ECM within 1-2 min and remodelling of the actin cytoskeleton in an integrin-dependent manner (Davidson et al., 2004). Just as importantly, GnRH-induced focal adhesions in HEK293 cells are also associated with the recruitment of the signalling molecules Src, FAK, and ERK, and their formation was additionally dependent on the small GTPase Rac. Overall, it is thought that such rearrangement of the ECM and cytoskeleton, and integrin-dependent assembly of focal adhesion complexes, is part of the initiation mechanism of GnRH-dependent signalling, at least in some cellular contexts (Davidson et al., 2004); thus, the potential involvement of integrins and focal adhesion complexes are important areas for future investigations in understanding GnRH-induced MMP actions in hormone secretion.

Interestingly, the neural transmembrane cell adhesion molecule (NCAM) and N-cadherin, which both mediate cell-cell adhesion, have been implicated in active signalling in models of neurotransmission and their shedding from the membrane is MMP-sensitive (Conant et al., 2015, 2010). Both NCAM and N-cadherin are also present in mammalian pituitary cell types (Berardi et al., 1995) and have been shown to regulate GH secretion from human fetal and adult pituitaries (Rubinek et al., 2003). On the other hand, NCAM downregulates MMP expression in tumors (Edvardsen et al., 1993; Maidment et al., 1997), suggesting that some cell adhesion molecule systems and MMP functions can also be inversely regulated in certain contexts.

Nonetheless, given that the relevance of cell-cell contacts is well established in pituitary cell networks in both mammals and teleosts (Golan et al., 2016; Le Tissier et al., 2012), MMP-dependent actions on ECM-interacting systems may be important mechanisms in controlling pituitary hormone release.

It should be pointed out that goldfish pituitary cells utilized in the present experiments had undergone a trypsin enzymatic dispersion process prior to culture (see Methods Section 2.2), which raises the issue of whether such ECM components and intercellular contacts discussed above are preserved during experiments. Indeed, as shown using studies of porcine tissues, prolonged trypsin treatment can affect ECM components such as laminin and fibronectin, although collagen content is unaltered even after 24-h treatment with trypsin (Schenke-Layland et al., 2003). Similarly, rat pituitary cell cultures obtained using a dual trypsin/collagenase dispersion method show the presence of multiple types of collagen, in part due to *de novo* synthesis (Kaidzu et al., 2000). Thus, given the relatively short duration of trypsinization in our methodology, as well as the observations that dispersed goldfish pituitary cells form associations/clusters, both with Cytodex beads and between pituitary cells within 2 h of plating, it is evident that ECM components facilitating such contacts are intact. Regardless, this will need to be confirmed experimentally, and is an important consideration for any future evaluations of ECM components as putative MMP targets during goldfish GnRH actions.

Despite the apparent lack of involvement of EGFR downstream of MMP-mediated GnRH actions on hormone responses, the findings with EGFR inhibition (Figures 6.6 and 6.7) provide interesting insights into how growth factors may act as part of the neuroendocrine control of goldfish gonadotroph and somatotroph functions. There is a sizeable body of evidence from mammalian (human and rat) pituitary models for the involvement of both EGF and EGFRs

in multiple pituitary cell types and functions (Cooper et al., 2011). The reduction in basal goldfish LH secretion upon BIBW2992 treatment (Figure 6.14) is consistent with findings that EGF treatment stimulates LH secretion from dispersed rat pituitary cells in both static cultures and column superfusion experiments (Przylipiak et al., 1988). Interestingly, these reductions in unstimulated LH release following BIBW2992 application were similar to those observed during MMP inhibition (Figure 6.14), suggesting that basal MMP activities may regulate EGFR functions in goldfish pituitary cells. Furthermore, the ability of EGFR inhibition to enhance the GnRH3-induced GH responses (Figure 6.7), when taken together with the findings that EGF treatment inhibits GH synthesis in rodent GH3 cells (Johnson et al., 1980; Schonbrunn et al., 1980), indicates that the EGF-EGFR system provides a negative regulatory mechanisms to some pituitary cell types in both teleosts and mammals.

The inability of GM6001 to affect GnRH-induced increases in phospho-ERK level indicate that MMP is not part of the mechanisms for GnRH-GnRHR activation of ERK, whereas results with BIBW2992 blunting the ability of GnRH to significantly elevate phospho-ERK levels suggests that EGFRs may still have a role to play in GnRH downstream signalling (Figure 6.15). These observations may seem not to be entirely consistent with the roles of MMP and EGFRs in hormone release discussed above. However, besides playing a role in GnRH-induced hormone secretion, ERK activation also mediates the ability of GnRHs to affect gonadotrophin subunits and GH mRNA expression (Klausen et al., 2008, 2005) and these indices were not monitored in the present study. In addition, EGFR transactivation cannot be completely ruled out based on the present experiments inhibiting the intrinsic kinase activity of EGFRs. This has been shown for signalling downstream of the growth hormone receptor, where intracellular mechanisms can lead to tyrosine phosphorylation of EGFR C-tails even in the absence of a

functional EGFR kinase domain and the subsequent activation of MAPK (Yamauchi et al., 1997; Yamauchi et al., 1998). Regardless, how the EGF-EGFR system functions and how it integrates with GnRHR signalling in the control of goldfish pituitary hormone secretion would be interesting areas for further studies.

Overall, while MMPs and EGFRs have both been of considerable interest in the context of pituitary adenomas (Kawamoto et al., 1996; Onguru et al., 2004; Yang et al., 2018), the present results demonstrate functions for both proteins in normal pituitary cells in the control of hormone release.

6.3.2 Agonist and cell-type-dependent roles of Src in the control of GnRH-evoked hormone release and basal secretion.

As part of the aim to investigate tyrosine kinases in the regulation of goldfish pituitary LH and GH secretion responses, experiments employing the Src inhibitor DGY-06-116 interestingly revealed dual cell- and agonist-selective roles for Src in hormone release. While Src is not involved in GnRH3-dependent GH secretion, it plays a part in mediating GnRH2-induced GH release (Figure 6.10). In contrast, Src plays inhibitory roles for both GnRH isoforms in LH release from gonadotrophs (Figure 6.9). That Src can play both positive and negative roles in hormone release is supported by studies carried out in other mammalian endocrine cell types. For example, obestatin stimulation of GH secretion from rat tumor somatotrophs involves activation of Src (Pazos et al., 2009). On the other hand, results with three SFK inhibitors reveal that SFKs exert a tonic inhibitory effect on insulin secretion from isolated, primary rat pancreatic islets and from the insulin-secreting cell line INS-1 (Cheng et al., 2007). Similarly, results with the SFK inhibitor PP2 and overexpression of constitutively active Src in rodent neuroendocrine PC12

cells demonstrate that Src negatively regulates acute (over 2 to 20 min) Ca^{2+}-dependent release of both endogenous dopamine and transfected human growth hormone without affecting total transmitter/hormone levels (Ohnishi et al., 2001).

How Src exerts negative effects over GnRH-stimulated LH release from goldfish gonadotrophs is not known but actions on Ca^{2+} signalling and Ca^{2+}-dependent events appear likely. GnRH action in goldfish LH release is Ca^{2+}-sensitive and involves mobilization of Ca^{2+} from intracellular and extracellular sources (Chang et al., 2009). Results from studies on insulin secretion indicate that SFKs act distal to intracellular Ca^{2+} increases (Cheng et al., 2007). It has been proposed that at least part of these inhibitory influences is also through SFK-dependent regulation of the actin cytoskeleton in PC12 cells and remodelling of actin cytoskeleton is Ca^{2+}-sensitive (Ohnishi et al., 2001; Okamoto et al., 2007). In addition, information in the literature indicate that Src phosphorylation of plasma membrane Ca^{2+} channels can negatively regulate Ca^{2+} influx, and SFKs are also involved in promoting cytosolic Ca^{2+} clearance through SERCA or plasma membrane Ca^{2+} ATPases (Anguita and Villalobo, 2017; Ghosh et al., 2016; Vela et al., 2007). Whether one or more of these Ca^{2+}-related targets mediate Src's inhibitory influence on GnRH-stimulated goldfish gonadotrophs while exerting a positive control of basal secretion warrants further examination.

The LH release profile for the GnRH + DGY group (Figure 6.9) also indicates that Src may be part of the negative feedback loops and/or desensitization mechanisms in goldfish gonadotrophs following GnRHR activation. In support of this hypothesis, following activation of the $G\alpha_{q/11}$-coupled muscarinic M1 receptor, Src has been shown to directly phosphorylate tyrosine residues within the RH (regulator of G-protein signalling (RGS)-homology) domain of GRK2, which enhances GRK-$G\alpha_q$ associations, leading to inhibition of downstream Gα-PLC

signalling (Mariggiò et al., 2006; Ribas et al., 2007). Although direct inhibition of GRK catalytic activity in Chapter 4 resulted in reductions in GnRH-dependent LH release, the regulation of G protein subunits by GRKs is facilitated by several kinase-independent interactions mediated by both RH and PH domains, and it is not fully understood how tyrosine phosphorylation of RH domains may alter catalytic activity of GRK. Thus, GRKs may still be part of the events leading to the enhancement of LH release responses observed during Src inhibition.

In contrast to its effects on LH secretion, Src is part of the facilitative machinery leading to GH exocytosis, at least for GnRH2-stimulated cells. How this selective involvement of Src in GnRH2, but not GnRH3, actions in goldfish somatotrophs is achieved remains to be investigated but based on information in the literature some possible means of Src's recruitment and subsequent actions exist. While $G\alpha_{i/o}$ subunits are known regulators of SFKs (Ma et al., 2000), inhibition of $G\alpha_{i/o}$ subunits in Chapter 4 did not alter GnRH2-dependent GH secretion. On the other hand, β-arrestins may serve as the link to recruit Src during GnRH2 stimulation, as one of the earliest described "signalling" functions of β-arrestins is to link activated β2ARs to Src activation (Luttrell et al., 1999), and these interactions are mediated by binding of β-arrestin N-terminus PXXP motifs to Src's SH3 as well as SH2 catalytic domains (Peterson and Luttrell, 2017). Once recruited, facilitation of Ca^{2+} mobilization from intracellular stores may provide a basis for Src's involvement in mediating GnRH2-elicited GH release since activation of Src can lead to Ca^{2+} release from intracellular stores in Xenopus oocytes (Bates et al., 2014) and intracellular Ca^{2+} mobilization is part of GnRH2's mechanisms of action in goldfish somatotrophs (Chang et al., 2012). Furthermore, CaMK can function upstream of Src in the Ca^{2+} signalling mediated by several GPCRs (Della Rocca et al., 1997; Melien et al., 2002; Wang et al., 2003), likely through direct associations with Src (Stateva et al., 2015). Additionally,

potential interactions with PI3K subunits cannot be ruled out in this GnRH2-stimulated context. The PI3K isoforms p110β, p110δ, and p110γ are all involved in GnRH2-evoked GH release, and Src promotes PI3K-dependent lipid signalling through inhibition of the phosphatase PTEN, which antagonizes PI3K actions (Lu et al., 2003). Just as importantly, the agonist-selective effects of Src inhibition in somatotrophs in this chapter mirrored those of Btk inhibition observed previously (Pemberton and Chang, 2016) and Btk is a known effector of Src actions (Takesono et al., 2002), and Src and p110γ can coordinate to regulate the activity of Btk (Li et al., 1997). Regardless of how Src mediates GnRH2 action in goldfish somatotrophs, these kinases are likely not redundant in the control of GH secretion since Btk inhibition also altered basal GH secretion whereas inhibition of Src did not (Figure 6.14). Taken together, the above information suggest that Src is one of multiple inputs that converge at the level of Btk, and also that additional non-Btk effectors are involved downstream of Src in somatotrophs.

Despite the effects of the DGY-06-116 on hormone release responses (Figures 6.9 and 6.10), and the well-established ability of Src to link receptors to the MEK/ERK pathway (Della Rocca et al., 1997; Goldsmith and Dhanasekaran, 2007), GnRH treatments did not alter Y^{416} Src phosphorylation alongside the elevations in p-ERK, although inhibition of Src impaired the ability of GnRH to increase phospho-ERK levels (Figure 6.15). While this finding was unexpected, some possible explanations are worth considering. First, given the opposite effects of Src inhibition in LH and GH release, especially for GnRH2 (and the lack of effect for GnRH3-dependent GH release), it is conceivable that GnRH regulation of Src is different in the two cell types (i.e., positive in one cell-type and negative in another cell-type). If so, changes in Y^{416} phospho-index would likely be masked in the mixed cell lysates analyzed in immunoblotting studies. Second, it could also be that even cell-specific readouts may not reveal GnRH

stimulation of Y^{416} phosphorylation based on findings in mammalian GnRH models; furthermore, time and model system differences may be important. For example, while mammalian GnRH (LHRH) increased both phospho-ERK and Src Y^{416} levels in HEK293 cell line stably expressing type I GnRHR at 10 min (Davidson et al., 2004), experiments with GT1-7 neurons showed that while SFK activity (attributed to Src and Fyn) was required for GnRH activation of Pyk2, GnRH treatment did not alter levels of Src-Y^{416} during 5 or 10 min of stimulation, a time-frame in which ERK phosphorylation levels were reliably elevated just as in the present thesis study (Higa-Nakamine et al., 2015; Okitsu-Sakurayama et al., 2019). Likewise, another study using αT3-1 mouse gonadotrophs did not detect changes in Src-Y^{416} following treatment with the GnRH agonist buserelin, although downstream cellular responses were found to be sensitive to an inhibitor of SFKs (Navratil et al., 2014). On the other hand in LβT2 cells, buserelin stimulated Src Y^{416} phosphorylation at 5 min, a time when ERK phosphorylation was at its peak, but the peak of Src Y^{416} phosphorylation was delayed by another 25 min (Bonfil et al., 2004). Third, the detection endpoint for Src activation used may be inadequate or inappropriate. A study using LβT2 rat gonadotrophs showed that while GnRH induction of MAPK activity (10 min time-point) was robust and sensitive to SFK inhibition, only slight increases in Src phosphorylation were detected, although this measurement corresponded to overall phospho-Src status, which would include sites of phosphorylation utilized for negative regulation as well (Maudsley et al., 2007). In addition, GnRH-dependent enhancement of Src activity was reported in αT3-1 gonadotrophs by a different group; however, this was assayed using activity towards a downstream target of Src (enolase), and corresponding Src-Y^{416} levels were not measured (Levi et al., 1998). Furthermore, while Y^{416} phosphorylation has also been widely utilized as a marker for Src activity, some reports indicate that phosphorylation state of

this residue do not always correspond to activity of Src towards downstream targets, and this site may additionally be phosphorylated in inactive states of the kinase (Irtegun et al., 2013; Snyder et al., 1983). Thus, when all of the above possibilities are taken into consideration it is highly probable that despite the lack of detectable increases in Y^{416} phosphorylation, Src participates in goldfish pituitary GnRH actions as shown by both ERK phosphorylation studies and hormone-release responses.

While how GnRH-induced engagement of Src and subsequent signalling occurs independent of detectable changes in Y^{416} in goldfish pituitary cells remains unknown, other modes of regulation of Src and SFKs potentially exist. In particular, these events may be attributed to noncatalytic functions of Src facilitated by SH2 and SH3 domain-dependent interactions with effectors. In support of this idea, the tyrosine phosphatase Shp-2 can regulate Src activity independent of detectable changes in either Y^{416} or Y^{527} (site of negative regulation by CSK, C-terminal Src kinase), through non-enzymatic interactions with the SH3 domain (Walter et al., 1999). In addition, PRL receptors are able to link to Janus kinase (Jak) signalling through Src, and this signalling can proceed normally even with kinase-dead mutants of Src (García-Martínez et al., 2010). Another finding supporting the importance of such non-enzymatic interactions is that ATP-competitive catalytic inhibitors of SFKs can affect intermolecular interactions of SFKs with other binding partners/effectors through modulating accessibility of SH2 and SH3 domains, indicating that kinase-independent regulation is not a minor determinant of SFK activities (Leonard et al., 2014).

In examination of Src's participation during hormone release, consideration was also given to the potential for Src being part of the transactivation relay mechanism. While Src is considered one of the major "relay molecules" for GPCR-RTK transactivation in many GPCR

systems (see Figure 1.8), including for endogenous GnRHRs in GT1-7 cells (Shah et al., 2003a), a comparison of the effects between MMP and Src inhibition in GnRH3-dependent LH and GH release, as well in ERK phosphorylation, suggests that Src likely does not mediate such functions for goldfish GnRHRs.

6.3.3 Pyk2/FAK-dependent actions negatively regulate basal LH and GH secretion, but selectively mediate acute GnRH-dependent LH release

Although a role for Pyk2 as a target and sensor of GnRH-elicited intracellular Ca^{2+} signals has been described (Okitsu-Sakurayama et al., 2021, 2019; Xie et al., 2008), information on whether and how this impacts hormone secretion was lacking. The present results show a requirement for active Pyk2/FAK functions as part of the GnRH-elicited mechanisms in LH, but not GH, secretion (Figures 6.12 and 6.13). Consistent with prior observations in GT1-7, LβT2 and αT3-1 cells (Maudsley et al., 2007; Okitsu-Sakurayama et al., 2021; Xie et al., 2008), the present results also show a role for these kinases in activation of the MEK/ERK cascade in goldfish pituitary cells (Figure 6.14C). However, the individual contributions of Pyk2 and FAK cannot be isolated at this time as the compound utilized effectively inhibits both kinases at the concentration employed (Roberts et al., 2008). Although these two multidomain kinases are structurally related and share many common functions (McLean et al., 2005; Sasaki et al., 1995), recent work has uncovered non-overlapping roles of these proteins. In particular, while FAK is better associated with focal adhesion structures and RTK/integrin-dependent signalling scaffolds, Pyk2 is unique in its function as a direct Ca^{2+} sensor, which is mediated in part through associations with CaM (Momin et al., 2022). In its capacity as one of the predominant Ca^{2+}-binding proteins, CaM is a major regulator of spatiotemporal dynamics of Ca^{2+}-elicited signals

(Johnson and Chang, 2000a). Given the wealth of information regarding the complexities of Ca^{2+} stores and dynamics in the goldfish pituitary cell system (Chang et al., 2012, 2009), future investigations of how Pyk2 may integrate with these various channels and stores will no doubt reveal interesting information.

Despite playing a positive role in mediating GnRH stimulation of LH secretion, Pyk2/FAK exerts a negative influence on basal LH and GH secretion (Figure 6.14). Interestingly, Pyk2 is upstream of Akt in many systems (Guo et al., 2006; Tse et al., 2012; Xia et al., 2004) and the profound effects of Pyk2/FAK inhibition on basal LH and GH release are comparable to those of Akt inhibition observed previously (Pemberton and Chang, 2016) although the mechanisms linking PyK2 to Akt and how Pyk2/FAK and Akt inhibits basal hormone secretion has not been evaluated in this study. On the other hand, Pyk2/FAK kinases are known to exert direct control over cytoskeletal F-actin dynamics (Chapman and Houtman, 2014; Schaller, 2010), which is a key regulatory step in vesicle trafficking and fusion with the plasma membrane and is known to undergo active remodelling during hormone secretion as discussed in Chapter 5. Additionally, as discussed in Section 6.3.1, FAK-containing focal adhesions anchored to the cytoskeleton are known to scaffold effectors following GnRHR stimulation in LβT2 gonadotrophs and HEK293 cell types (Davidson et al., 2004; Dobkin-Bekman et al., 2009). These complexes may also be pre-formed in resting states, with rearrangement occurring following receptor activation. Interestingly, two *in vivo* studies on pancreatic islet cells showed that FAK may exert inhibitory effects on basal release while promoting the stimulated exocytotic process (Cai et al., 2012; Rondas et al., 2011), i.e., a dual role as in the present study. Whether these mechanisms of Pyk2 and/or FAK action occur in goldfish pituitary gonadotrophs and

somatotrophs should be of importance in future characterizations, as these are effective means to coordinate spatiotemporal responses of receptor signalling and basal hormone release.

6.4 Summary

Results presented in this chapter not only lends further support to the differential use of intracellular signalling mechanisms in GnRH isoform- and cell-type-specific hormone release as well as dissociation of the control of basal *vs.* agonist-elicited endocrine secretion, but also, importantly, reveal for the first time the participation of tyrosine kinase-dependent effectors in GnRH control of pituitary hormone secretion in a natural (untransformed) cell study system (Figure 6.17). In particular, experiments utilizing an inhibitor of MMPs opens up the possibility for the involvement of several downstream ECM targets and integrin molecules in GnRH actions, as have been reported in mammalian study systems. Similarly, the participation of EGFRs in basal and GnRH-dependent hormone release is identified, and how these are integrated with GnRH-dependent mechanisms will be an important area to address going forward. This thesis chapter also identifies Src and related SFKs as an important platform for mediating differential actions of GnRH, potentially linking to various Ca^{2+}-dependent mechanisms in goldfish pituitary cells, as well as in GnRH control of ERK cascades. Finally, the present results identify Pyk2/FAK, which are well-characterized links to focal adhesions and Ca^{2+}-CaM actions, as novel effectors of goldfish GnRHR actions in the control of LH release, and in the regulation of basal LH and GH release.

Figure 6.1. Schematic of pharmacological targeting of MMP, EGFR, Src and Pyk2/FAK proteins for experiments investigating their possible participation in GnRHR actions. MMP enzymes have a variety of membrane- and ECM-bound substrates, and one particular mechanism of interest in GPCR/GnRHR signalling is that of RTK transactivation through release of a pro-ligand. GM6001 targets several isoforms of MMPs, including MMP1, 2, 3, 7, 9 and 14. The possibility of EGFR transactivation in GnRH actions (GnRH2, red dot; GnRH3, blue dot) was investigated using BIBW2992, a selective catalytic inhibitor of ErbB1 and ErbB2 (EGFR and EGFR2) isoforms. Additionally, the involvement of the intracellular protein kinases Src and Pyk2/FAK in GnRH actions was explored using DGY-06-116, a selective inhibitor for Src over other SFK group proteins, and PF-562271, a dual inhibitor of Pyk2/FAK catalytic activity.

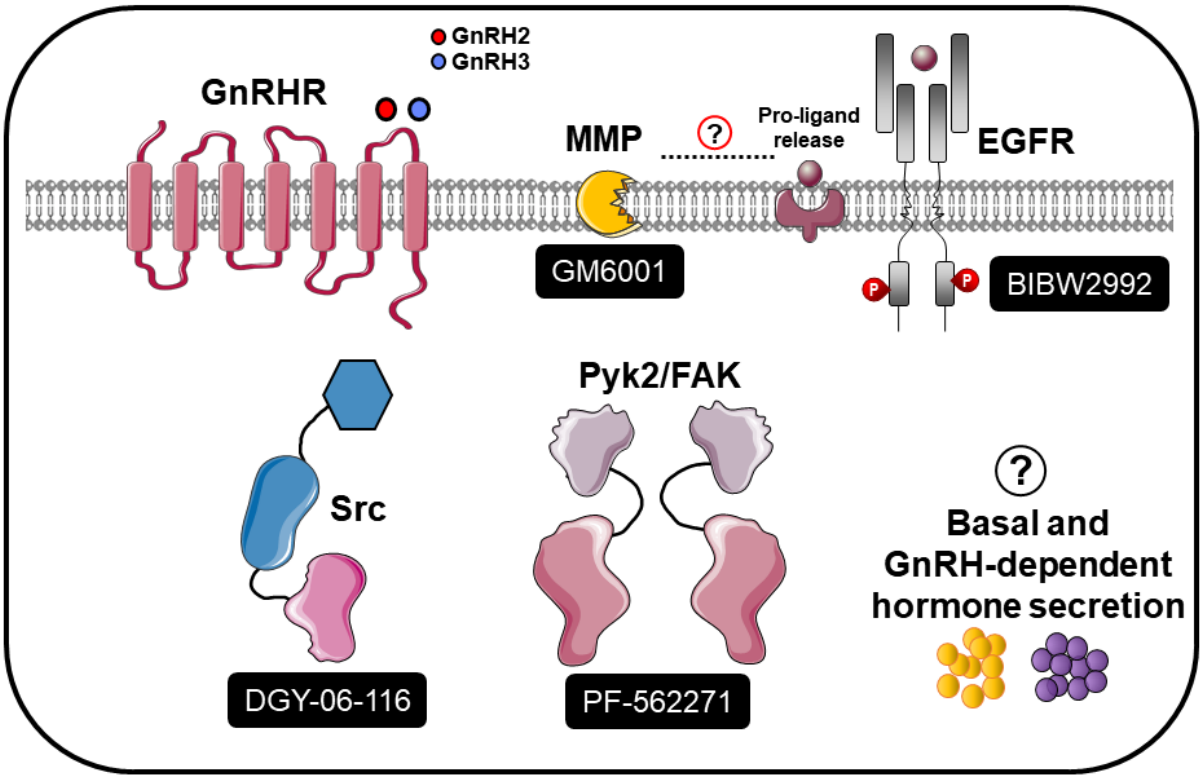

GnRH2
GnRH3
GnRHR
MMP
?
Pro-ligand release
EGFR
GM6001
BIBW2992
P
P
Pyk2/FAK
Src
?
Basal and GnRH-dependent hormone secretion
DGY-06-116
PF-562271

Figure 6.2. Comparison of catalytic and inhibitor interaction sites on predicted goldfish MMP isoforms of consideration relative to their mammalian counterparts. While other GM6001-interacting MMP isoforms are restricted to specific tissues, the ones listed in this figure generally have widespread expression, and MMP2 and MMP9 are of particular interest in pituitary cell types. Goldfish homologs corresponding to human MMP isoforms from the top half of the figure are shown below. Importantly, all isoforms contain the conserved cysteine switch and Zn^{2+} binding motifs, which act together to enable full activation of MMP functions. Broad spectrum hydroxamate-based inhibitors such as GM6001 target the catalytic Zn^{2+}-binding site formed by the three histidine residues and catalytic glutamate (position 4 in the same motif), a mechanism common to almost all MMP family members (Vandenbroucke et al., 2014; Verma, 2012). Human protein sequences for the MMPs were retrieved from Universal Protein Resource Knowledgebase (UniProtKB; https://www.uniprot.org), followed by organism-specific BLAST against the goldfish genome (*Carassius auratus*, NCBI Taxonomy ID 7956). Identifiers for the resulting goldfish sequences with highest percent identity match are presented.

Identifier	*Isoform*	*Cysteine switch*	*Zn2+ binding*
Uniprot P03956	**MMP1 (collagenase) Human**	**PRCGVPD**	**HELGHSLGLSH**
Uniprot P08253	**MMP2 (72 kDa type IV collagenase) Human**	**PRCGNPD**	**HEFGHAMGLEH**
Uniprot P08254	**MMP3 (stromelysin) human**	**PRCGVPD**	**HEIGHSLGLFH**
Uniprot P09237	**MMP7 (matrilysin) human**	**PRCGVPD**	**HELGHSLGMGH**
Uniprot P14780	**MMP9 (92 kDa type IV collagenase) human**	**PRCGVPD**	**HEFGHALGLDH**
Uniprot P50281	**MMP14 human**	**PRCGVPD**	**HELGHALGLEH**
NCBI XP_026074865.1	**MMP1/MMP3-like Goldfish**	**PRCGVPD**	**HEFGHSLGLSH**
NCBI XP_026071030.1	**72 kDa type IV collagenase-like Goldfish**	**PRCGVPD**	**HEFGHALGLEH**
NCBI XP_026060827.1	**MMP7-like goldfish**	**PRCGVSD**	**HEFGHALGLKH**
NCBI XP_026115270.1	**MMP9-like goldfish**	**PRCGVPD**	**HEFGHALGLDH**
NCBI XP_026060827.1	**MMP14-like goldfish**	**PRCGVPD**	**HELGHALGLEH**

Cysteine switch motif PRCGXPD
Zn^{2+} binding motif HEXGHXXGXXH

Figure 6.3. Effects of the MMP inhibitor GM6001 (0.2 μM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified net response to agonist stimulation (between indicated vertical dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (2.89 ± 0.16 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from pre-spawning, sexually mature goldfish (March). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

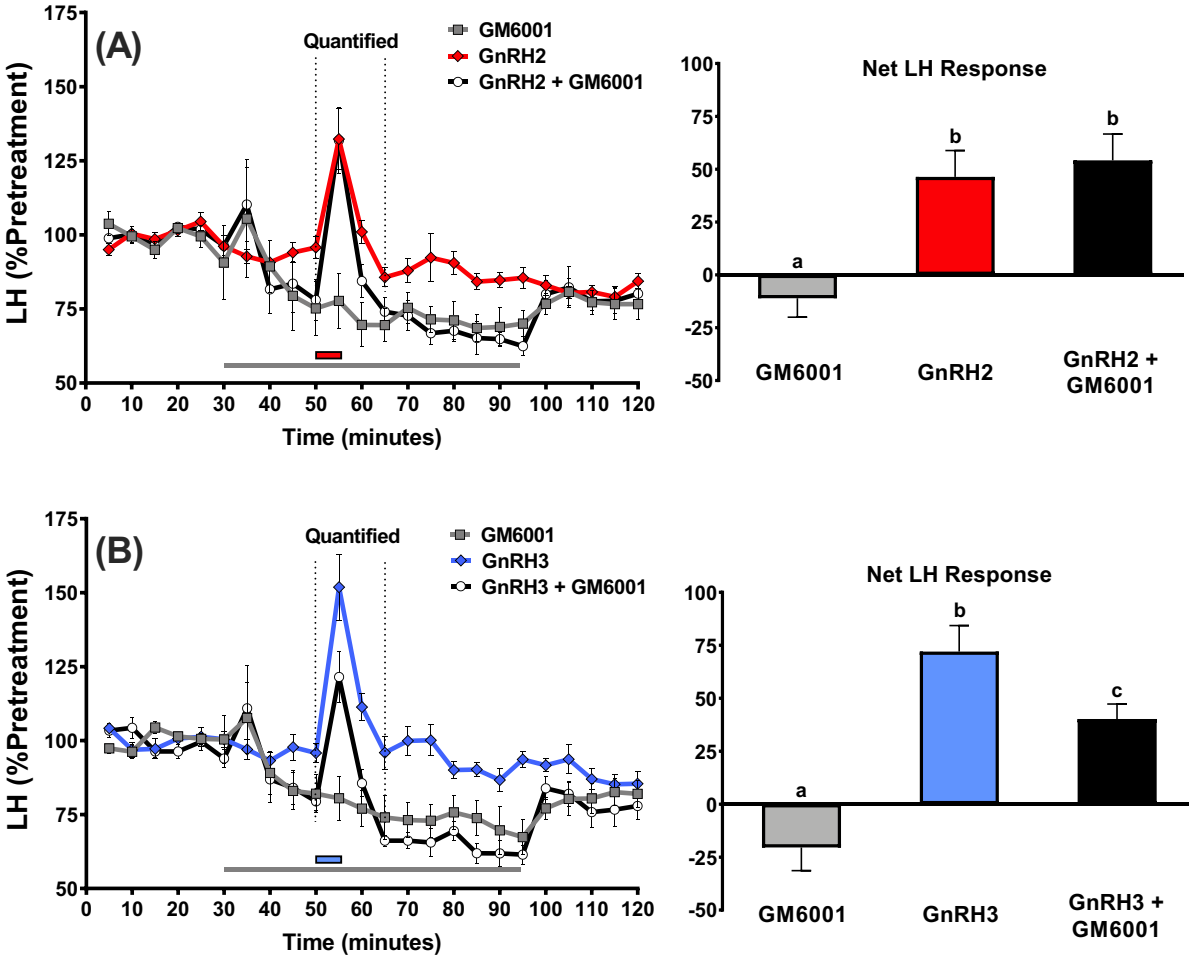

(A)
Quantified
GM6001
GnRH2
GnRH2 + GM6001
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
GM6001
GnRH2
GnRH2 +
GM6001
(B)
Quantified
GM6001
GnRH3
GnRH3 + GM6001
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
GM6001
GnRH3
GnRH3 +
GM6001

Figure 6.4. Effects of the MMP inhibitor GM6001 (0.2 μM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified net response to agonist stimulation (between indicated vertical dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (17.21 ± 1.08 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from pre-spawning, sexually mature goldfish (March). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

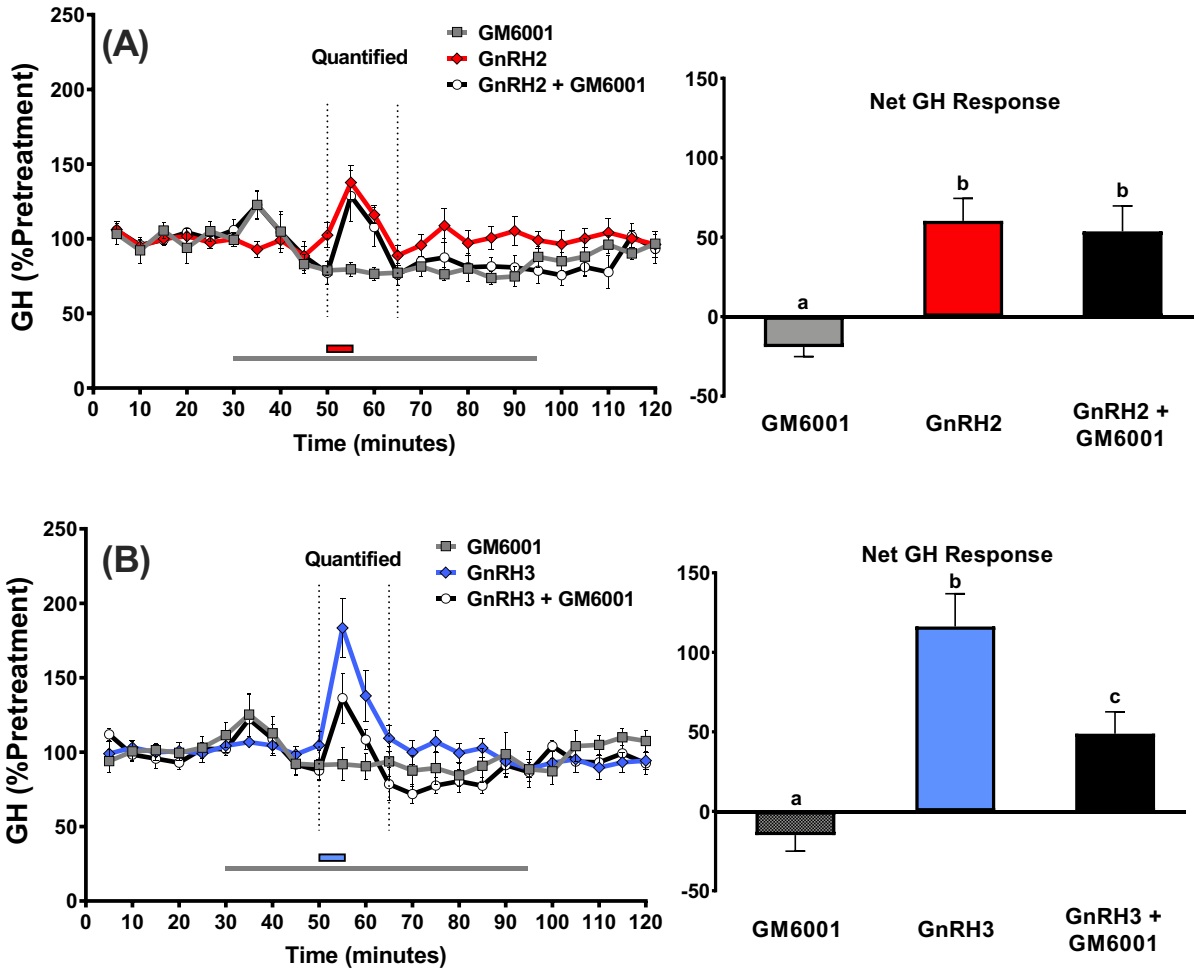
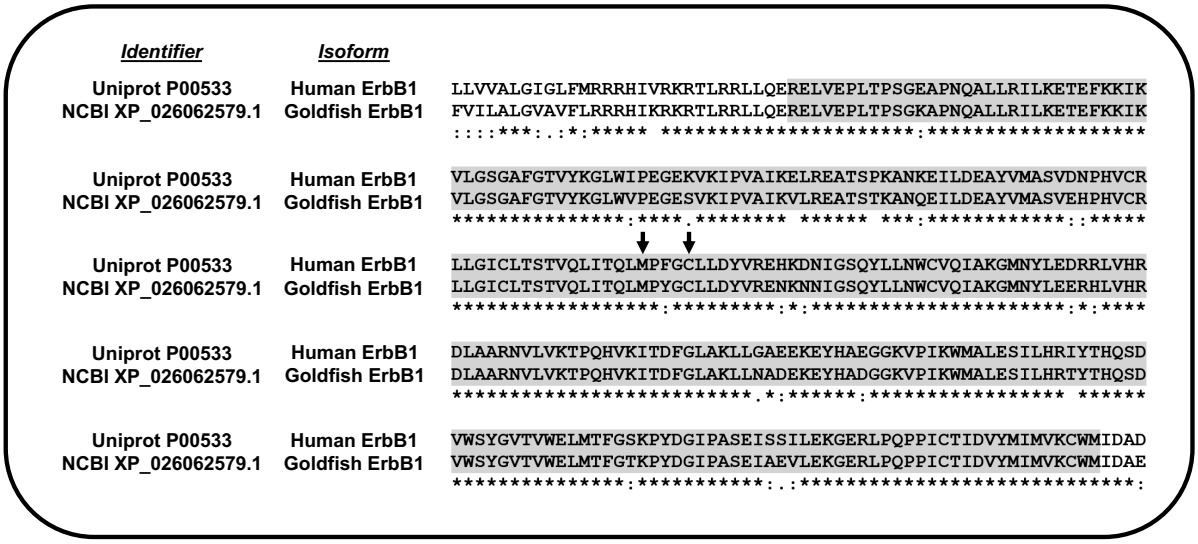

(A)
Quantified
GM6001
GnRH2
GnRH2 + GM6001
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
GM6001
GnRH2
GnRH2 + GM6001
(B)
Quantified
GM6001
GnRH3
GnRH3 + GM6001
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
c
GM6001
GnRH3
GnRH3 + GM6001

Figure 6.5. Primary amino acid sequence alignment of human and goldfish ErbB1 (EGFR). While teleost homologs of ErbB receptors vary considerably in their extracellular domains and regulatory C-terminus tails (not shown), the tyrosine kinase domains (highlighted grey) are well conserved. In particular, the small molecule inhibitor BIBW2992 binds to a cysteine residue (Cys797) and forms an additional hydrogen bond with Met793 in human ErbB1 (Li et al., 2008; Solca et al., 2012). Human protein sequences for the MMPs were retrieved from Universal Protein Resource Knowledgebase (UniProtKB; https://www.uniprot.org), followed by organism-specific BLAST against the goldfish genome (*Carassius auratus*, NCBI Taxonomy ID 7956). Identifiers for the resulting goldfish sequences with highest percent identity match are presented. Underneath the alignments, an asterisk (*) indicates positions which have a single, fully conserved residue, whereas a colon (:) indicates conservation of residues with *strongly* similar properties (> 0.5 in the Gonnet PAM 250 matrix). Lastly, a period (.) indicates conservation between groups with *weakly* similar properties (between 0 and 0.5 in the Gonnet PAM 250 matrix).


Identifier
Isoform
Uniprot P00533
Human ErbB1
LLVVALGIGLFMRRRHIVRKRTLRRLLQERELVEPLTPSGEAPNQALLRILKETEFKKIK
NCBI XP_026062579.1
Goldfish ErbB1
FVILALGVAVFLRRRHIKRKRTLRRLLQERELVEPLTPSGKAPNQALLRILKETEFKKIK
Uniprot P00533
Human ErbB1
VLGSGAFGTVYKGLWIPEGEKVKIPVAIKELREATSPKANKEILDEAYVMASVDNPHVCR
NCBI XP_026062579.1
Goldfish ErbB1
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Uniprot P00533
Human ErbB1
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NCBI XP_026062579.1
Goldfish ErbB1
LLGICLTSTVQLITQLMPYGCLLDYVRENKNNIGSQYLLNWCVQIAKGMNYLEERHLVHR
Uniprot P00533
Human ErbB1
DLAARNVLVKTPQHVKITDFGLAKLLGAEEKEYHAEGGKVPIKWMALESILHRIYTHQSD
NCBI XP_026062579.1
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Uniprot P00533
Human ErbB1
VWSYGVTVWELMTFGSKPYDGIPASEISSILEKGERLPQPPICTIDVYMIMVKCWMIDAD
NCBI XP_026062579.1
Goldfish ErbB1
VWSYGVTVWELMTFGTKPYDGIPASEIAEVLEKGERLPQPPICTIDVYMIMVKCWMIDAE
BIBW2992-interacting residues

Figure 6.6. Effects of the EGFR inhibitor BIBW2992 (1 μM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified net response to agonist stimulation (between indicated vertical dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (1.36 ± 0.08 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish with regressed gonads and those undergoing recrudescence (September to November). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

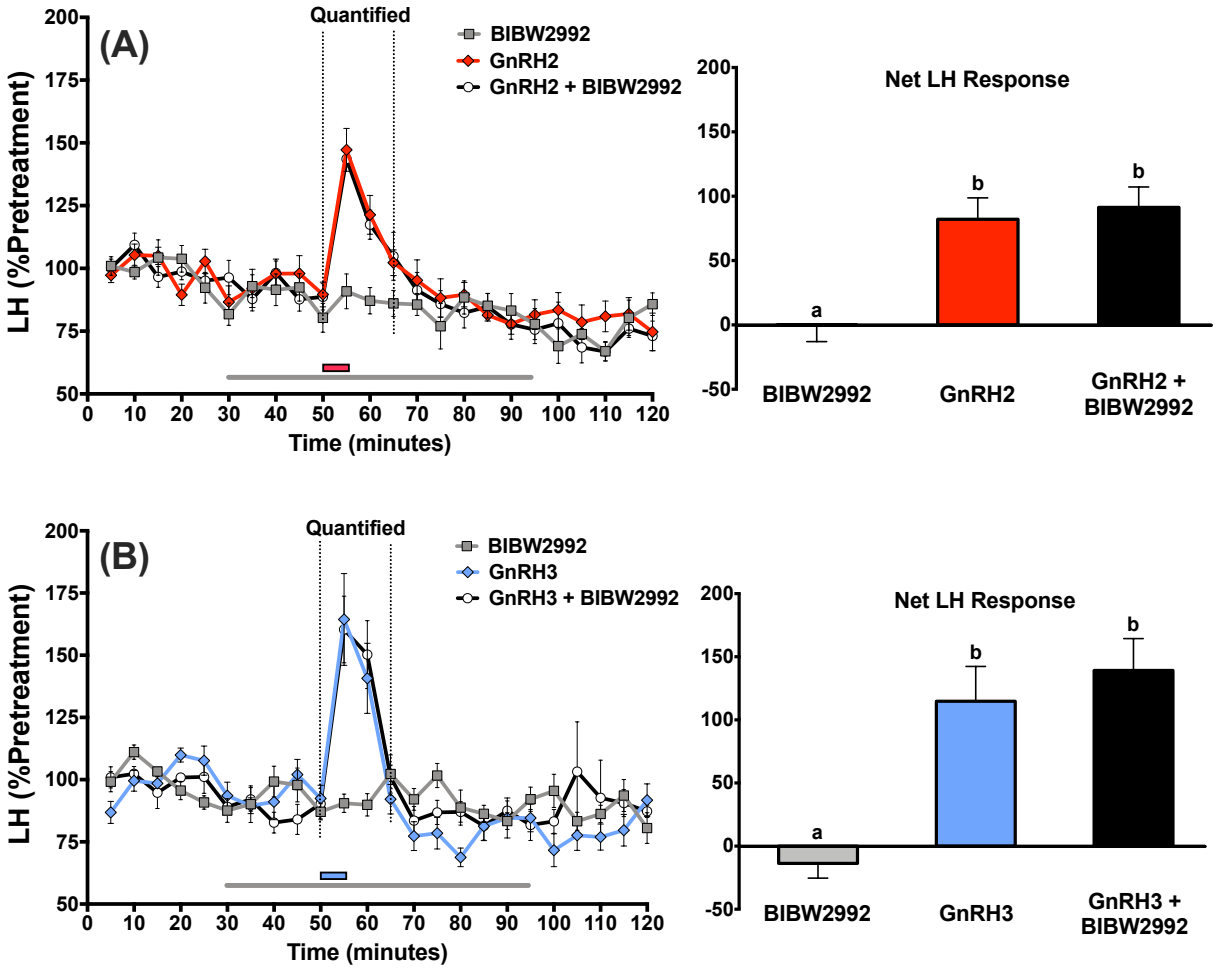

(A)
Quantified
BIBW2992
GnRH2
GnRH2 + BIBW2992
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
BIBW2992
GnRH2
GnRH2 + BIBW2992
(B)
Quantified
BIBW2992
GnRH3
GnRH3 + BIBW2992
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
b
BIBW2992
GnRH3
GnRH3 + BIBW2992

Figure 6.7. Effects of the EGFR inhibitor BIBW2992 (1 μM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified net response to agonist stimulation (between indicated vertical dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (6.13 ± 0.63 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish with regressed gonads and those undergoing recrudescence (September to November). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

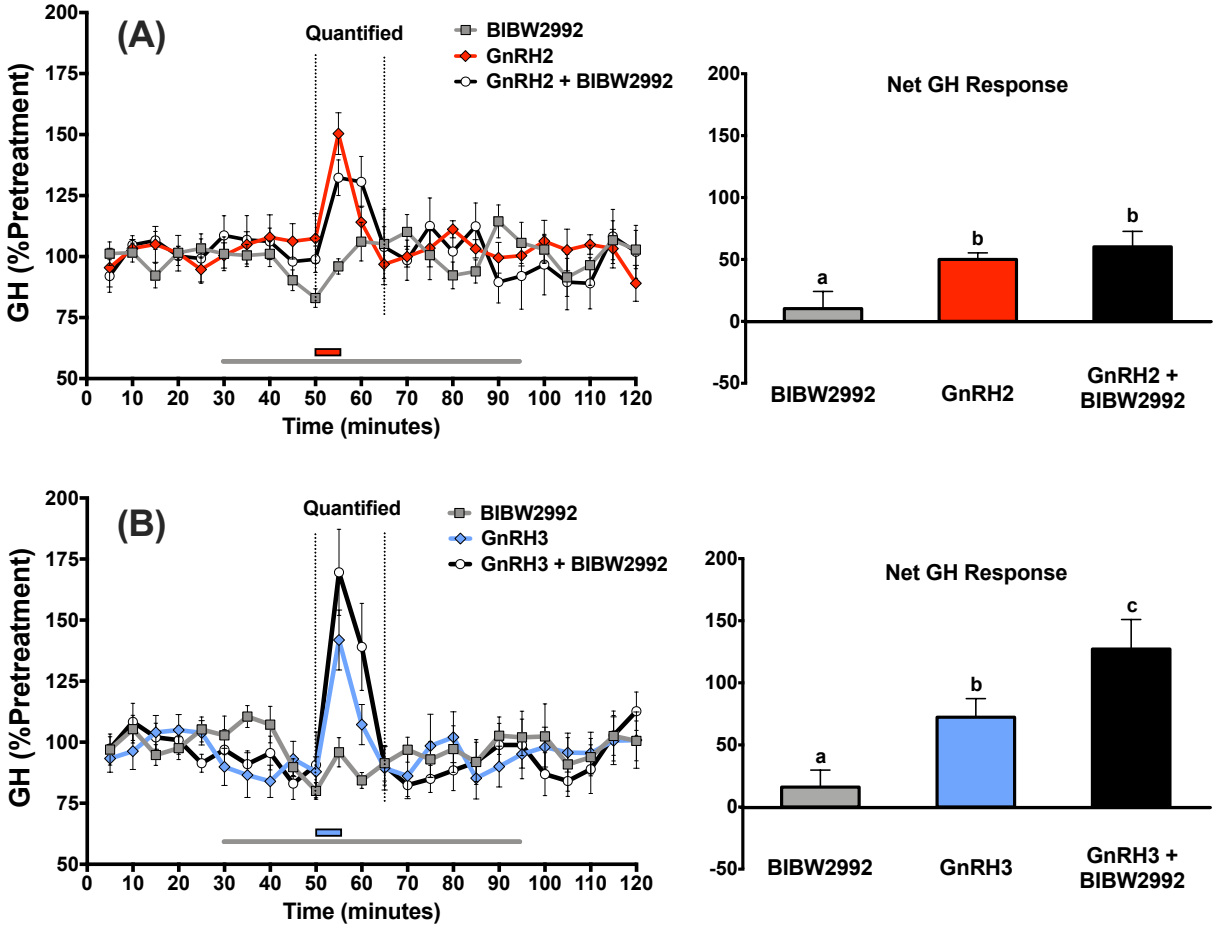
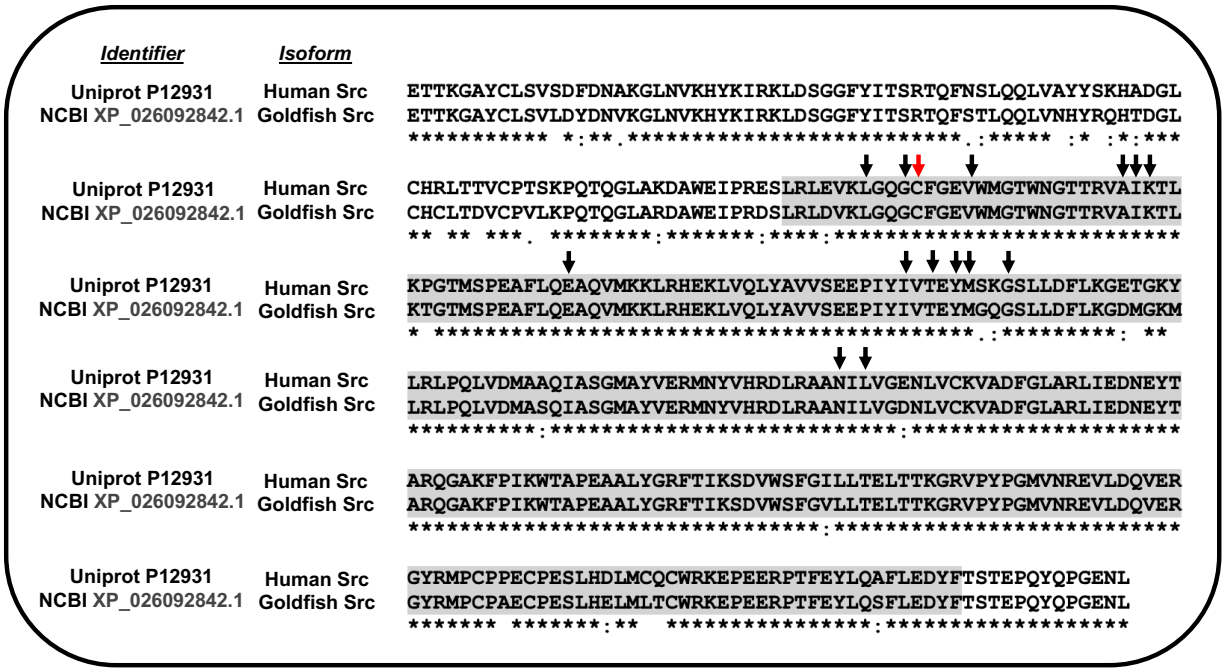

(A)
Quantified
BIBW2992
GnRH2
GnRH2 + BIBW2992
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
BIBW2992
GnRH2
GnRH2 +
BIBW2992
(B)
Quantified
BIBW2992
GnRH3
GnRH3 + BIBW2992
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
c
BIBW2992
GnRH3
GnRH3 +
BIBW2992

Figure 6.8. Conservation of the DGY-06-116-interacting amino acid residues between human Src and its corresponding goldfish homolog. Interaction sites within the kinase domain (grey) are indicated by arrows and the goldfish isoform with the highest percent identity match with the human sequence is presented. Importantly, the enhanced selectivity of DGY-06-116 for Src over other related SFKs is conferred in part by interactions with a cysteine residue (Cys 280, highlighted in red) in the glycine-rich P-loop region which is present in Src and Yes, but not in Fyn, of the three ubiquitously expressed SFKs (Gurbani et al., 2020). Human protein sequence for Src was retrieved from Universal Protein Resource Knowledgebase (UniProtKB; https://www.uniprot.org), followed by organism-specific BLAST against the goldfish genome (*Carassius auratus*, NCBI Taxonomy ID 7956). Underneath the alignments, an asterisk (*) indicates positions which have a single, fully conserved residue, whereas a colon (:) indicates conservation of residues with *strongly* similar properties (> 0.5 in the Gonnet PAM 250 matrix). Lastly, a period (.) indicates conservation between groups with *weakly* similar properties (between 0 and 0.5 in the Gonnet PAM 250 matrix).



DGY-06-116

Drug - Coordinating Residues **Cys^{280} Covalent Linker**

Figure 6.9. Effects of the Src inhibitor DGY-06-116 (1 µM; "DGY") on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified response to agonist stimulation (between indicated vertical dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (1.79 ± 0.13 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from pre-spawning, sexually mature goldfish (April to June). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

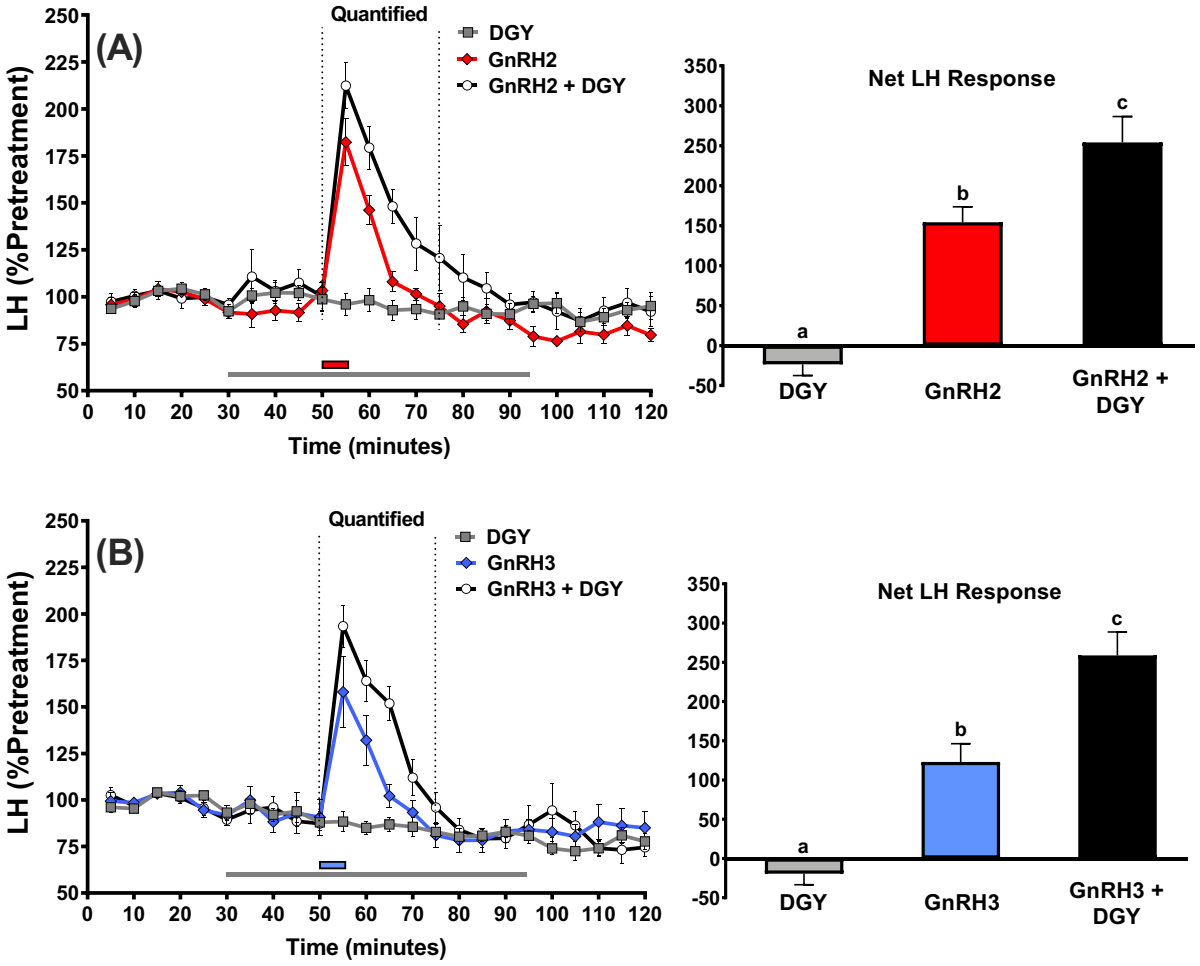

(A)
Quantified
DGY
GnRH2
GnRH2 + DGY
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
DGY
GnRH2
GnRH2 + DGY
(B)
Quantified
DGY
GnRH3
GnRH3 + DGY
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
DGY
GnRH3
GnRH3 + DGY

Figure 6.10. Effects of the Src inhibitor DGY-06-116 (1 µM; "DGY") on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified response to agonist stimulation (between indicated vertical dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to the 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (7.44 ± 0.68 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from pre-spawning, sexually mature goldfish (April to June). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

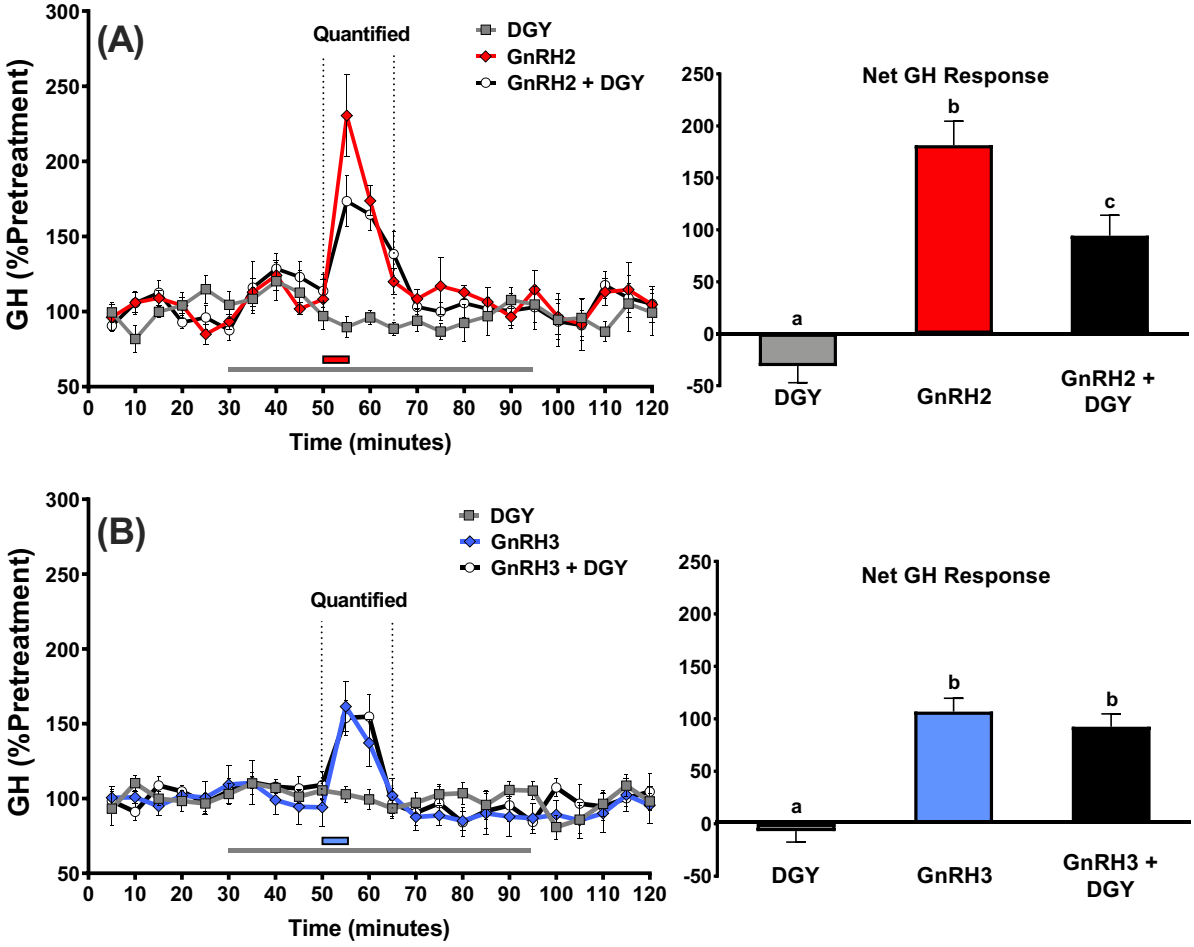

(A)
Quantified
DGY
GnRH2
GnRH2 + DGY
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
c
DGY
GnRH2
GnRH2 + DGY
(B)
Quantified
DGY
GnRH3
GnRH3 + DGY
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
DGY
GnRH3
GnRH3 + DGY

Figure 6.11. Primary amino acid sequence alignments of human and goldfish homologs of FAK (A) and Pyk2 (B) kinase domains (highlighted in grey). (A) Known PF-562271-interacting residues in the kinase domain and ATP-binding pocket (cyan) of FAK are indicated. Such interactions perturb conformation of the activation loop and disrupts ATP binding. (B) Of the 22 known PF-562271-interacting residues in human Pyk2, 2 are not conserved in goldfish homologs although these residues are not considered major determinants of high-affinity binding by the inhibitor, as indicated by comparatively very low buried surface area (BSA) values (Berger et al., 2021). The non-conserved P^{506} and H^{512} residues are indicated by red stars while all other conserved PF-562271-interacting residues are identified by black arrows, Human protein sequences for the MMPs were retrieved from Universal Protein Resource Knowledgebase (UniProtKB; https://www.uniprot.org), followed by organism-specific BLAST against the goldfish genome (*Carassius auratus*, NCBI Taxonomy ID 7956). Identifiers for the resulting goldfish sequences with highest percent identity match are presented. Underneath the alignments, an asterisk (*) indicates positions which have a single, fully conserved residue, whereas a colon (:) indicates conservation of residues with *strongly* similar properties (> 0.5 in the Gonnet PAM 250 matrix). Lastly, a period (.) indicates conservation between groups with *weakly* similar properties (between 0 and 0.5 in the Gonnet PAM 250 matrix). Structural information for PF-562271 interactions were obtained from two separate studies, Roberts et al., 2008 (original drug characterization for FAK binding) and Berger et al., 2021 (FAK and Pyk2).

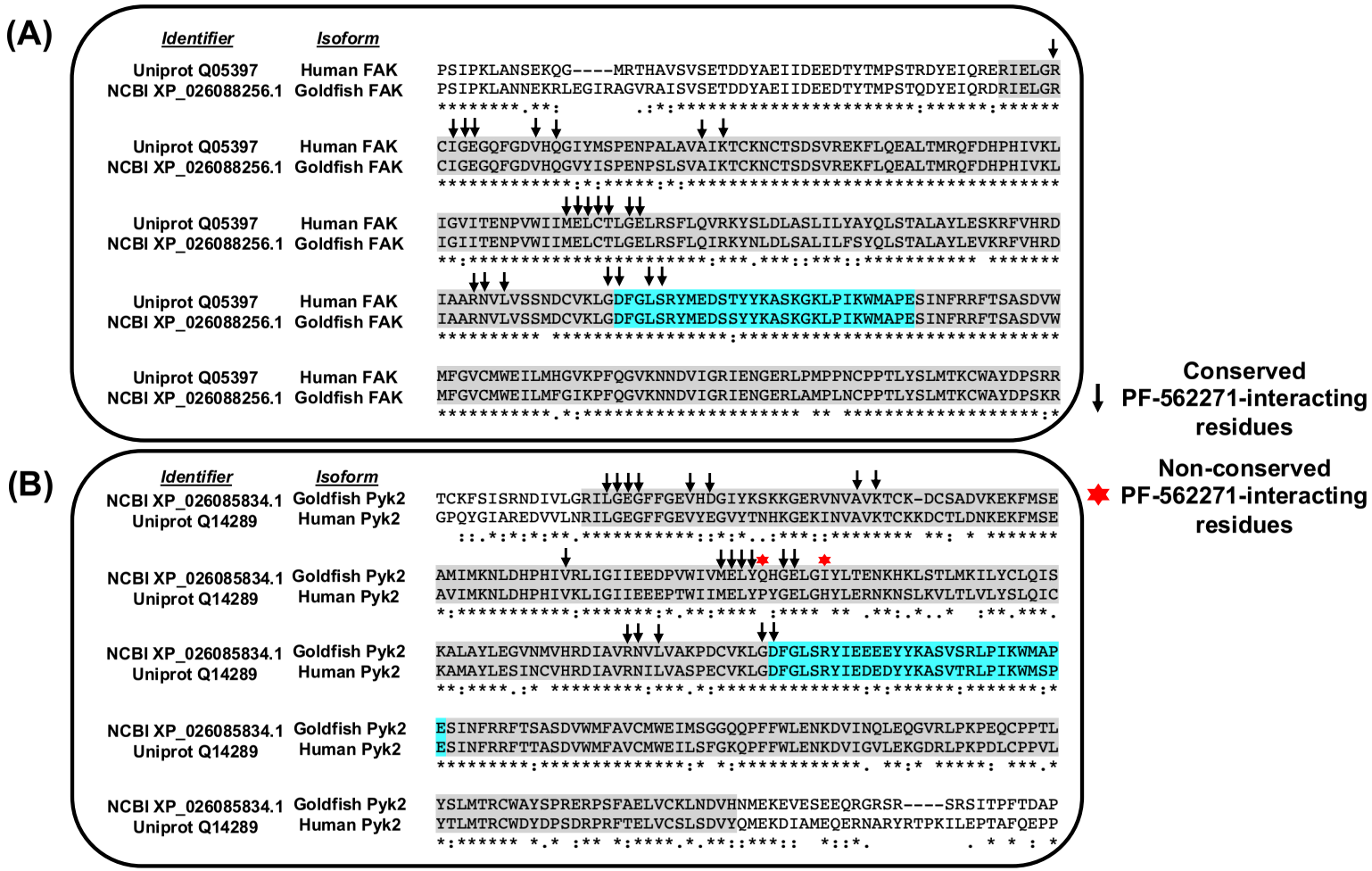
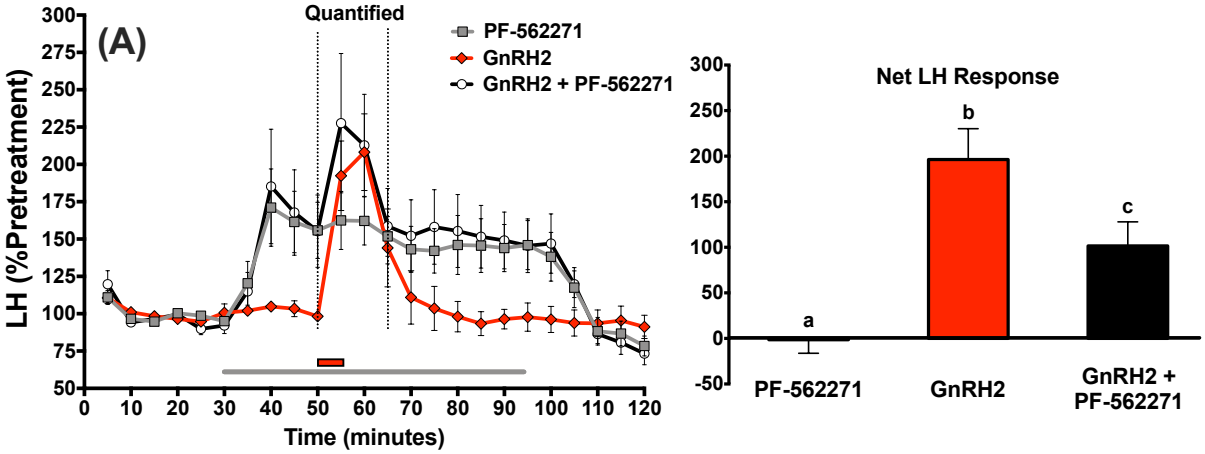

(A)
Identifier
Isoform
Uniprot Q05397 Human FAK PSIPKLANSEKQG----MRTHAVSVSETDDYAEIIDEEDTYTMPSTRDYEIQRERIELGR
NCBI XP_026088256.1 Goldfish FAK PSIPKLANNEKRLEGIRAGVRAISVSETDDYAEIIDEEDTYTMPSTQDYEIQRDRIELGR
Uniprot Q05397 Human FAK CIGEGQFGDVHQGIYMSPENPALAVAIKTCKNCTSDSVREKFLQEALTMRQFDHPHIVKL
NCBI XP_026088256.1 Goldfish FAK CIGEGQFGDVHQGVYISPENPSLSVAIKTCKNCTSDSVREKFLQEALTMRQFDHPHIVKL
Uniprot Q05397 Human FAK IGVITENPVWIIMELCTLGELRSFLQVRKYSLDLASLILYAYQLSTALAYLESKRFVHRD
NCBI XP_026088256.1 Goldfish FAK IGIITENPVWIIMELCTLGELRSFLQIRKYNLDLSALILFSYQLSTALAYLEVKRFVHRD
Uniprot Q05397 Human FAK IAARNVLVSSNDCVKLGDFGLSRYMEDSTYYKASKGKLPIKWMAPESINFRRFTSASDVW
NCBI XP_026088256.1 Goldfish FAK IAARNVLVSSMDCVKLGDFGLSRYMEDSSYYKASKGKLPIKWMAPESINFRRFTSASDVW
Uniprot Q05397 Human FAK MFGVCMWEILMHGVKPFQGVKNNDVIGRIENGERLPMPPNCPPTLYSLMTKCWAYDPSRR
NCBI XP_026088256.1 Goldfish FAK MFGVCMWEILMFGIKPFQGVKNNDVIGRIENGERLAMPLNCPPTLYSLMTKCWAYDPSKR
Conserved PF-562271-interacting residues
(B)
Identifier
Isoform
NCBI XP_026085834.1 Goldfish Pyk2 TCKFSISRNDIVLGRILGEGFFGEVHDGIYKSKKGERVNVAVKTCK-DCSADVKEKFMSE
Uniprot Q14289 Human Pyk2 GPQYGIAREDVVLNRILGEGFFGEVYEGVYTNHKGEKINVAVKTCKKDCTLDNKEKFMSE
NCBI XP_026085834.1 Goldfish Pyk2 AMIMKNLDHPHIVRLIGIIEEDPVWIVMELYQHGELGIYLTENKHKLSTLMKILYCLQIS
Uniprot Q14289 Human Pyk2 AVIMKNLDHPHIVKLIGIIEEEPTWIIMELYPYGELGHYLERNKNSLKVLTLVLYSLQIC
NCBI XP_026085834.1 Goldfish Pyk2 KALAYLEGVNMVHRDIAVRNVLVAKPDCVKLGDFGLSRYIEEEEYYKASVSRLPIKWMAP
Uniprot Q14289 Human Pyk2 KAMAYLESINCVHRDIAVRNILVASPECVKLGDFGLSRYIEDEDYYKASVTRLPIKWMSP
NCBI XP_026085834.1 Goldfish Pyk2 ESINFRRFTSASDVWMFAVCMWEIMSGGQQPFFWLENKDVINQLEQGVRLPKPEQCPPTL
Uniprot Q14289 Human Pyk2 ESINFRRFTTASDVWMFAVCMWEILSFGKQPFFWLENKDVIGVLEKGDRLPKPDLCPPVL
NCBI XP_026085834.1 Goldfish Pyk2 YSLMTRCWAYSPRERPSFAELVCKLNDVHNMEKEVESEEQRGRSR----SRSITPFTDAP
Uniprot Q14289 Human Pyk2 YTLMTRCWDYDPSDRPRFTELVCSLSDVYQMEKDIAMEQERNARYRTPKILEPTAFQEPP
Non-conserved PF-562271-interacting residues

Figure 6.12. Effects of the Pyk2/FAK inhibitor PF-562271 (10 µM) on the LH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified response to agonist stimulation (between indicated vertical dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (3.86 ± 0.23 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish undergoing gonadal recrudescence (October to November). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).


(A)
Quantified
PF-562271
GnRH2
GnRH2 + PF-562271
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
PF-562271
GnRH2
GnRH2 + PF-562271

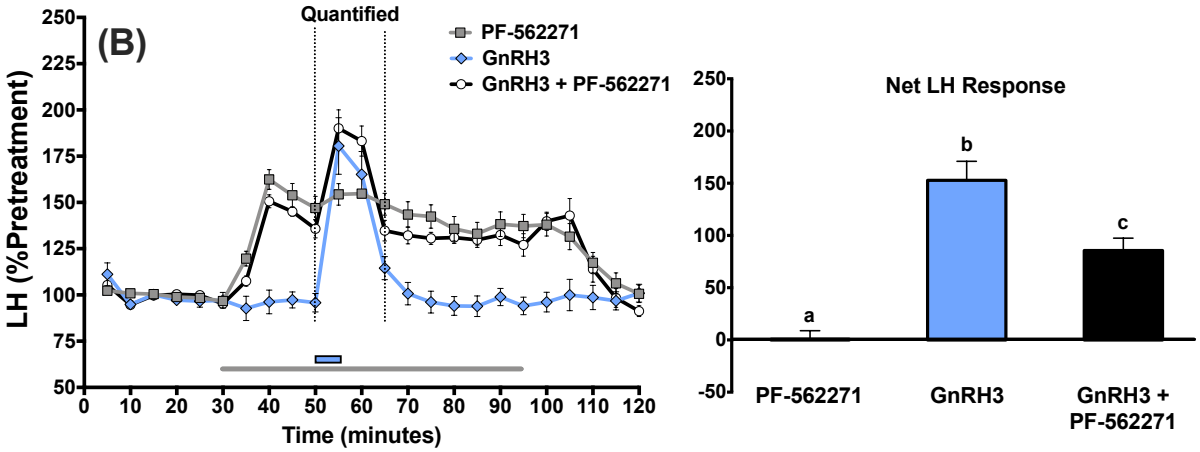

(B)
Quantified
PF-562271
GnRH3
GnRH3 + PF-562271
LH (%Pretreatment)
Time (minutes)
Net LH Response
a
b
c
PF-562271
GnRH3
GnRH3 + PF-562271

Figure 6.13. Effects of the Pyk2/FAK inhibitor PF-562271 (10 µM) on the GH secretion response to two endogenous GnRH isoforms (GnRH2, A; GnRH3, B). Hormone release kinetics are shown on the left for each panel (grey square, inhibitor alone; coloured diamond, GnRH alone; open white circle, GnRH + inhibitor), with the corresponding quantified response to agonist stimulation (between indicated vertical dotted lines) and the equivalents for the other treatment groups presented on the right. The grey horizontal bar indicates the duration of inhibitor exposure, and the red or blue bar denotes the exposure to a 5-min pulse of agonist (GnRH2, red; or GnRH3, blue; 100 nM) application. Results are expressed as a percentage of pretreatment values, which is an average of the first five fractions (19.82 ± 1.02 ng/mL; n = 48, from eight independent cell preparations). Experiments were carried out using dispersed cultures of goldfish pituitary cells prepared from goldfish undergoing gonadal recrudescence (October to November). Quantified net responses that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test; $p<0.05$; n = 8 for each treatment group obtained from four independent cell preparations).

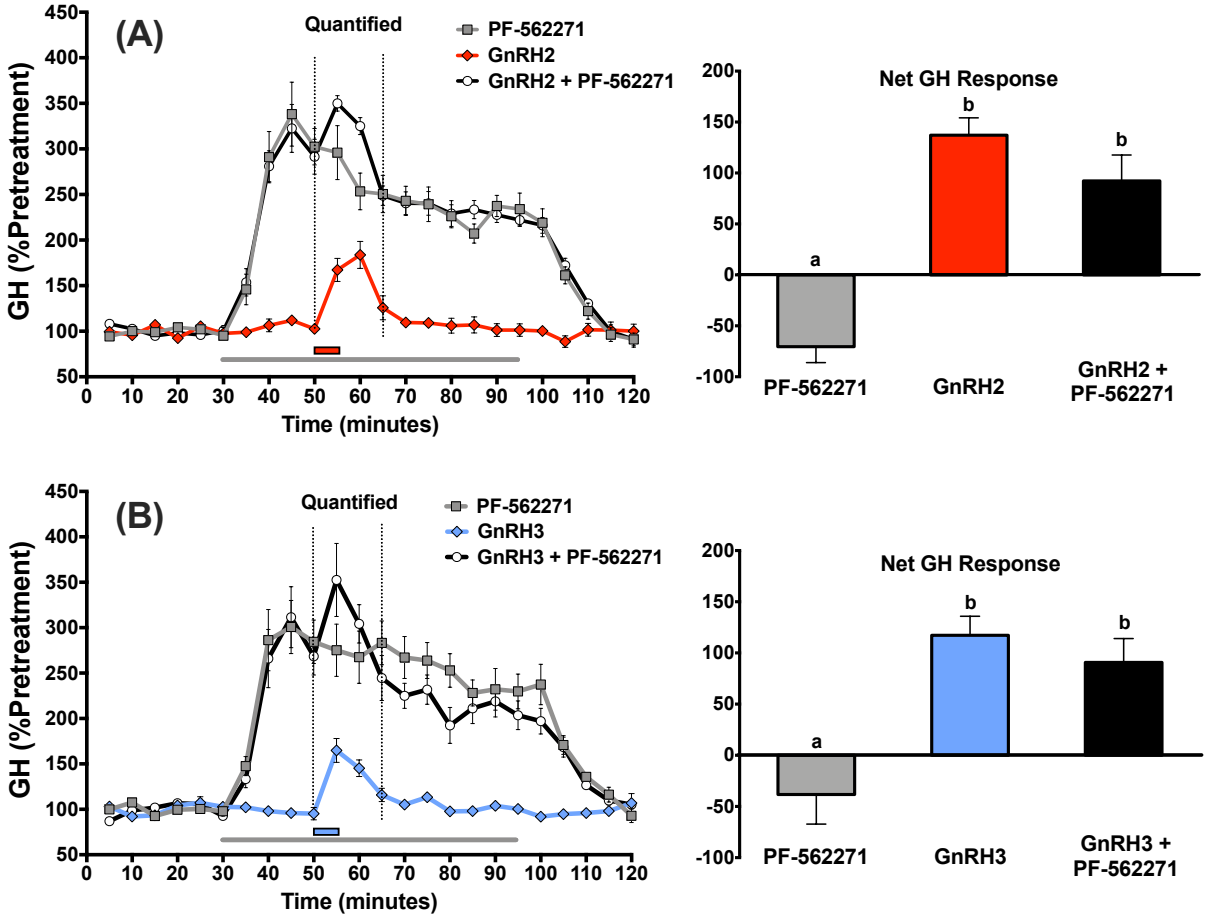

(A)
Quantified
PF-562271
GnRH2
GnRH2 + PF-562271
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
PF-562271
GnRH2
GnRH2 +
PF-562271
(B)
Quantified
PF-562271
GnRH3
GnRH3 + PF-562271
GH (%Pretreatment)
Time (minutes)
Net GH Response
a
b
b
PF-562271
GnRH3
GnRH3 +
PF-562271

Figure 6.14. Effects of small molecule inhibitors of MMPs (GM6001, 0.2 µM), EGFR (BIBW2992, 1 µM), Pyk2/FAK (PF-562271, 10 µM), Src (DGY-06-116, 1 µM) on unstimulated LH (A, B) and GH (C, D) release. Inhibitor-alone hormone release profiles taken from perifusion experiments are shown on the left (A and C; pink squares, MMP inhibitor; violet diamonds, EGFR inhibitor; blue circles, Src inhibitor; green triangles, Pyk2/FAK inhibitor) and the corresponding quantified responses shown on the right (B and D). Basal hormone release prior to inhibitor treatment was quantified as the average of values from 0 to 20 min of the experiment (average % pretreatment; black horizontal line in A and C; corresponding quantified values in black vertical bar in B and D), during which cells are perifused with M199 media alone. The basal hormone release during inhibitor treatment was evaluated as the average of %pretreatment values over the duration of inhibitor treatment (30-95 min; grey horizontal line; corresponding quantified values in the other coloured vertical bars in B and D). An asterisk denotes the presence of responses during inhibitor treatment that were significantly different from basal release prior to inhibitor application (paired Student *t* test; $P < 0.05$; $n = 16$, from eight independent cell preparations per inhibitor).

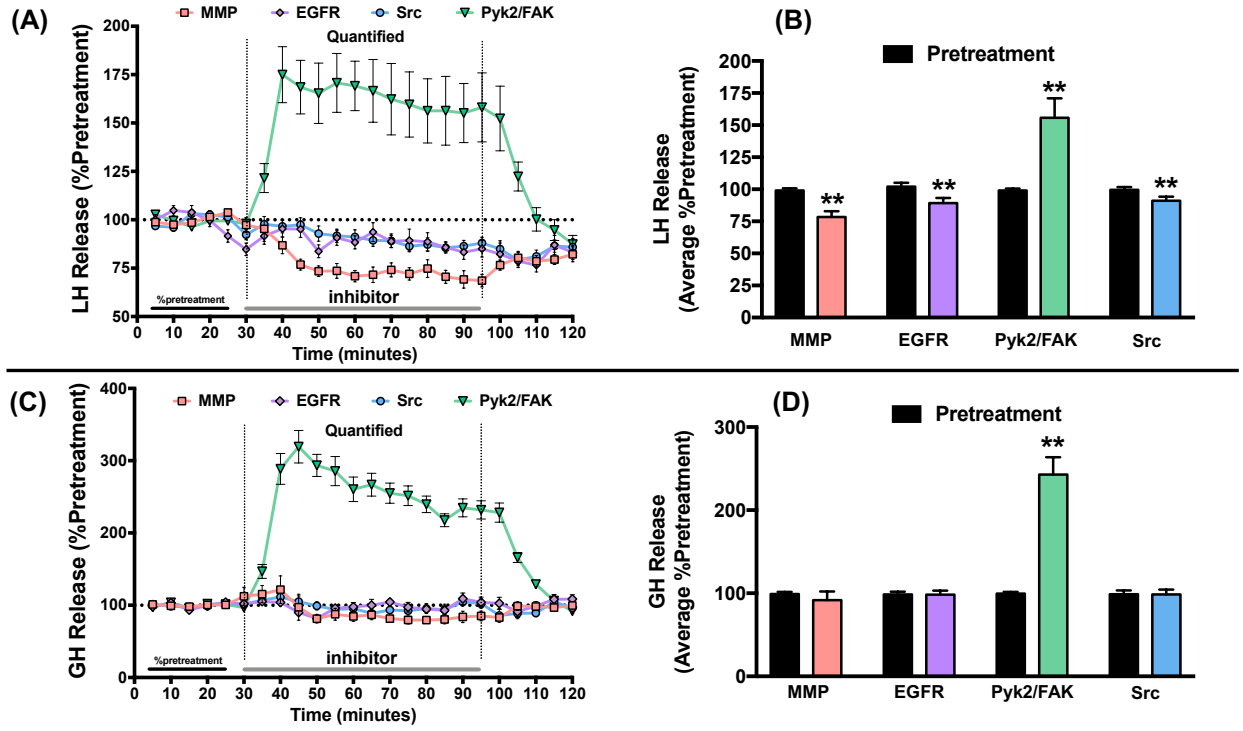

(A)
MMP
EGFR
Src
Pyk2/FAK
Quantified
LH Release (%Pretreatment)
%pretreatment
inhibitor
Time (minutes)
(B)
Pretreatment
LH Release (Average %Pretreatment)
MMP
EGFR
Pyk2/FAK
Src
(C)
GH Release (%Pretreatment)
(D)
GH Release (Average %Pretreatment)

Figure 6.15. Effects of MMP, EGFR, and Pyk2/FAK inhibitors on ERK phosphorylation in dispersed pituitary cells. Following overnight culture, dispersed pituitary cells were pre-treated with pharmacological inhibitors (A: GM6001, 0.2 μM; B: BIBW2992, 1 μM; C: PF-562271, 10 μM) or DMSO vehicle for 30 min, followed by addition of GnRH2 or GnRH3 (100 nM) for 5 min in the presence of DMSO or inhibitor. Cells were then harvested and lysed, and protein extracts probed for phospho-ERK (Thr^{202}/Tyr^{204}), total ERK, and β-actin. Example blots presented for each inhibitor are representative of 3 experiments from 3 independent cell preparations. Densitometry readings normalized to the unstimulated vehicle control are presented adjacent (C: Src and ERK) or below (A, B: ERK). Results (mean ± SEM) are pooled from n = 3 individual cell preparations from goldfish undergoing gonadal recrudescence (December-February; GM6001 and BIBW-2992) or from pre-spawning, sexually mature goldfish (March-April; PF-562271). Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$).

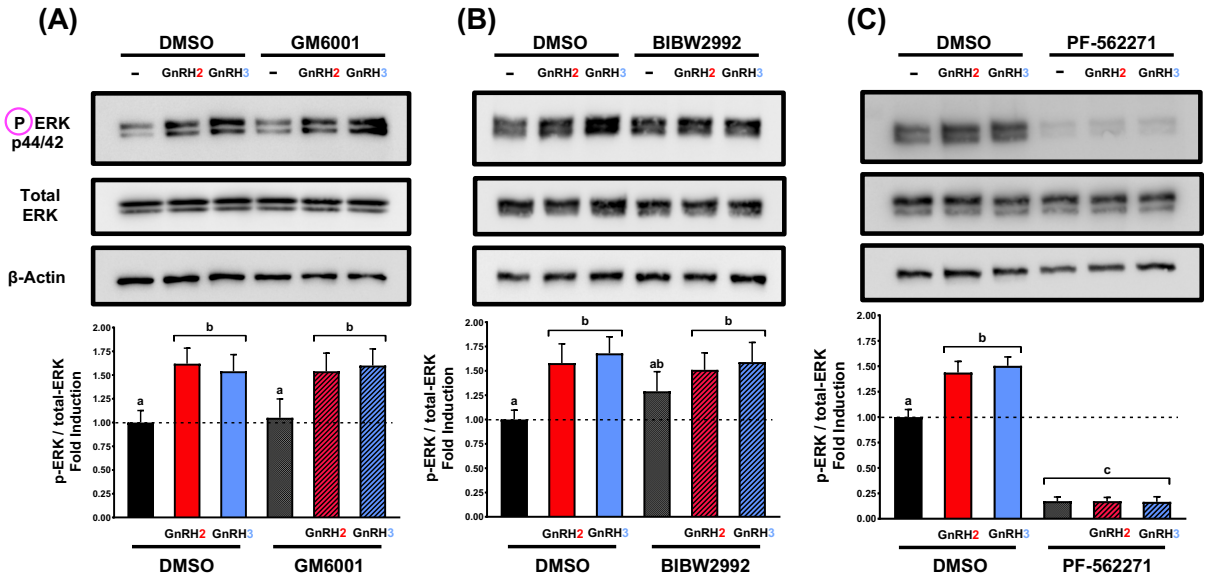

(A)
DMSO
GM6001
– GnRH2 GnRH3 – GnRH2 GnRH3
P ERK p44/42
Total ERK
β-Actin
p-ERK / total-ERK Fold Induction
a b a b
GnRH2 GnRH3 GnRH2 GnRH3
DMSO
GM6001
(B)
DMSO
BIBW2992
– GnRH2 GnRH3 – GnRH2 GnRH3
p-ERK / total-ERK Fold Induction
a b ab b
GnRH2 GnRH3 GnRH2 GnRH3
DMSO
BIBW2992
(C)
DMSO
PF-562271
– GnRH2 GnRH3 – GnRH2 GnRH3
p-ERK / total-ERK Fold Induction
a b c
GnRH2 GnRH3 GnRH2 GnRH3
DMSO
PF-562271

Figure 6.16. Effects of Src inhibitor DGY-06-116 on kinase phosphorylation in dispersed pituitary cells. Following overnight culture, dispersed pituitary cells were pre-treated with DGY-06-116 (5 μM) or DMSO vehicle for 30 min, followed by addition of GnRH2 or GnRH3 (100 nM) for 5 min in the presence of DMSO or inhibitor. Cells were then harvested and lysed, and protein extracts probed for phospho-ERK (Thr^{202}/Tyr^{204}), total ERK, phospho-Src (Tyr^{416}), total-Src, and β-actin. Example blots presented are representative of 3 experiments from 3 independent cell preparations. Densitometry readings for p-Src and p-ERK fold induction normalized to the unstimulated vehicle control are presented to the right. Results (mean ± SEM) are pooled from n = 3 individual cell preparations from goldfish undergoing gonadal recrudescence (December-February). Treatment groups that don't share a letter of the alphabet are significantly different from one another (one-way ANOVA followed by Fisher's LSD post-hoc test, $p<0.05$).

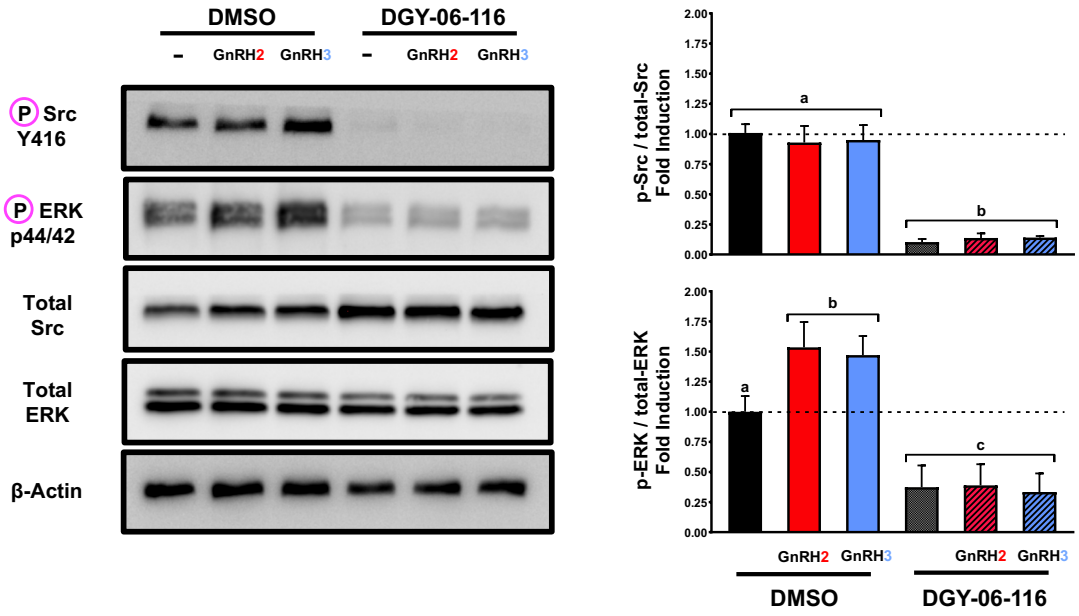
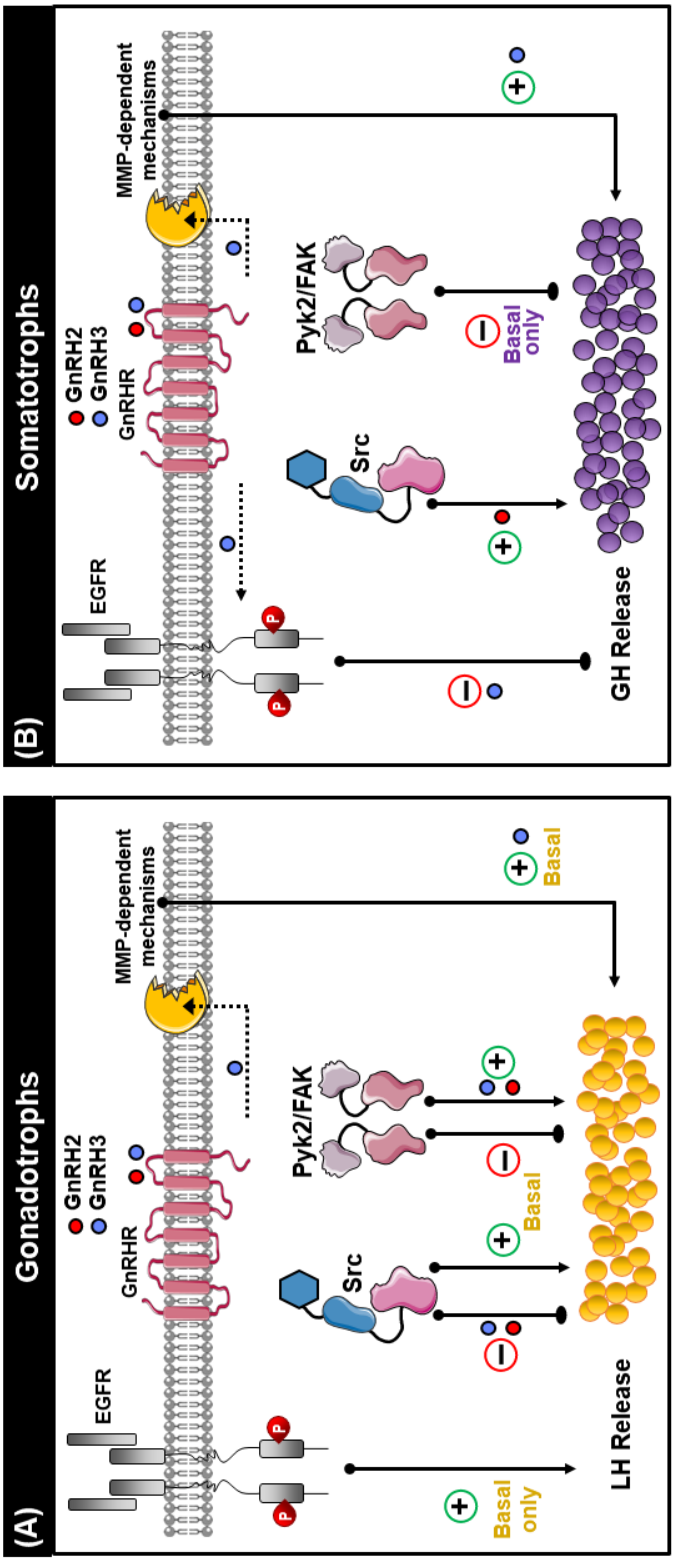

DMSO
DGY-06-116
–
GnRH2
GnRH3
–
GnRH2
GnRH3
P Src Y416
P ERK p44/42
Total Src
Total ERK
β-Actin
p-Src / total-Src Fold Induction
2.00
1.75
1.50
1.25
1.00
0.75
0.50
0.25
0.00
a
b
p-ERK / total-ERK Fold Induction
a
b
c
GnRH2
GnRH3
GnRH2
GnRH3
DMSO
DGY-06-116

Figure 6.17. Summary figure depicting the selective involvement of MMPs, EGFRs, and intracellular tyrosine kinases Src and Pyk2/FAK during basal and GnRH-evoked LH and GH release. Schematics for LH and GH are presented in panels A and B, respectively. MMP-dependent mechanisms are involved selectively in GnRH3 stimulation of acute LH and GH secretion, and exert a positive influence in the control of basal LH release. EGFR-sensitive mechanisms also promote basal LH release, but exert negative regulation over GnRH3-evoked acute GH secretion. The intracellular protein tyrosine kinase Src negatively influences GnRH2- and GnRH3-dependent LH release, but promotes basal LH secretion. On the other hand, Src kinase participates in mediating GnRH2-evoked GH release. Finally, while Pyk2/FAK proteins mediate GnRH2- and GnRH3-induced LH release responses, they are not involved in acute GnRH-stimulated GH secretion, but exert negative regulatory influences over both basal LH and GH release.


(A)
Gonadotrophs
GnRH2
GnRH3
GnRHR
EGFR
MMP-dependent mechanisms
Pyk2/FAK
Src
Basal only
Basal
LH Release
(B)
Somatotrophs
GnRH2
GnRH3
GnRHR
EGFR
MMP-dependent mechanisms
Pyk2/FAK
Src
Basal only
GH Release

Chapter Seven

General discussion and future perspectives.

7.1 Introduction

Overall, the findings from this thesis have identified the participation of a number of novel effectors in GnRH actions in the goldfish pituitary cell system. In particular, while a large body of work has previously characterized central effectors linking GnRHRs to the control of hormone secretion, the contribution of many upstream receptor-interacting and receptor-proximal mechanisms were understudied in comparison. Likewise, the potential for receptor transactivation and protein tyrosine kinase involvement in goldfish GnRH actions had not been adequately addressed. In addition, how small GTPases, which exert various effects on secretory vesicle trafficking and cytoskeletal dynamics, could be integrated into goldfish pituitary cell GnRH signalling cascades was not well understood. Results of my thesis research support the central hypothesis first laid out in Chapter 1 that several classical GPCR effectors mediate GnRH-induced responses while facilitating part of the functional selectivity observed in this system in response to the native isoforms GnRH2 and GnRH3 (Chapters 3 and 4), as well as the notion that these receptor-interacting effectors engage small GTPase effectors (Chapter 5) and protein tyrosine-kinase based signalling networks (Chapter 6) and to control GnRH-elicited hormone release responses in a GnRH isoform- and cell-type-specific manner. In the following sections of this general discussion, I will illustrate how the novel findings of the involvement of these receptor proximal components (G-protein subunits, β-arrestins, and GRKs) and their downstream signalling and cellular elements (e.g., dynamin, small G-proteins/GTPases, and tyrosine kinases), can be integrated into existing models of GnRH signalling mechanisms in the control of hormone release from the goldfish pituitary and highlight possible avenues for follow-up studies in this system.

7.2 Integrated model of GnRH2-dependent control of LH secretion

As discussed in Chapter 1, the functions of goldfish GnRHRs in pituitary hormone release have previously been largely attributed to PLC-β and PKC signalling, downstream of classical $G\alpha_{q/11}$ subunit activity, and novel contributions of Gβγ subunits were also described recently (Chang and Pemberton, 2018). Results from Chapters 3 and 4 identified additional receptor-interacting effectors, including β-arrestins and GRKs, as effectors utilized during acute GnRH2-evoked LH release. Importantly, the β-arrestin inhibitor Barbadin selectively perturbs β-arrestin-AP2 interactions, which classically link β-arrestin-bound receptors to CCS. Thus, based on the perturbed LH release responses during arrestin inhibition, β-arrestin-containing CCS likely scaffold effectors that organize signalling leading to GnRH2-dependent LH release (Figure 7.1). Possible effectors within this scaffolding complex include components of the Raf-MEK-ERK cascade, and the protein kinases PI3K and Src. While β-arrestin inhibition did not abrogate GnRH-induced ERK responses in mixed cell lysates (Chapter 3), MAPK cascade dynamics controlled by GPCRs are complex and β-arrestins may still represent a modality for controlling time- and context-dependent ERK responses (Gurevich and Gurevich, 2018; Gutkind and Kostenis, 2018). In addition, β-arrestins may also participate in the regulation of PI3K-dependent components of GnRH2-induced LH release. β-arrestins can associate with p85 regulatory subunits of class IA PI3Ks (p110α, p110β, p110δ) following GPCR stimulation (Cheung et al., 2009), of which p110β and p110δ play positive roles in GnRH2-dependent LH release (Pemberton et al., 2015). On the other hand, β-arrestins can also co-ordinate localized inhibition of PI3Ks through recruitment of PTEN lipid phosphatases (Wang and DeFea, 2006), and β-arrestin-dependent scaffolding is an emerging means of coordinating intracellular responses in both space and time to ensure specificity (Thomsen et al., 2018, 2016). Whether such a

mechanism is utilized to control PIP_3-dependent actions in GnRH control of hormone release certainly warrants further investigation.

Interestingly, in contrast to their classical desensitization functions (Krupnick and Benovic, 1998), GRKs played positive roles in mediating GnRH2-induced acute LH secretion, likely in concert with β-arrestin actions (Chapter 3). This positive role of GRKs is not entirely surprising given that positive regulation of effectors by GRKs has been shown for PI3K engagement following stimulation of β2ARs (Naga Prasad et al., 2002, 2001). In addition to β-arrestins and GRKs, results from Chapter 3 also revealed selective usage of dynamin-dependent mechanisms in GnRH2-evoked LH responses over a longer timeframe. The differing acute effects of Barbadin and the dynamin inhibitor suggests that dynamin-dependent internalization of GnRHRs may be involved in the normal termination of GnRH2-elicited responses. Interestingly, inhibition of the protein tyrosine kinase Src (Chapter 6) also resulted in enhancement of LH release responses which took longer to return to baseline, and the proline-rich regions in dynamin are known to associate with SH3-domain containing proteins including Src, an interaction which is thought to assist in localizing dynamin to CCP (Okamoto et al., 1997; Vallee and Okamoto, 1995). Whether such a mechanism establishes the link between GnRHR-activated Src- and dynamin-dependent receptor internalization during termination of GnRH2-dependent signalling would need to be confirmed in future studies. The Src/dynamin-mediated termination of GnRH2 stimulation of goldfish LH secretion may further involve negative regulation of intracellular Ca^{2+} signalling. Src kinases can oppose rises in cytosolic Ca^{2+} through direct inhibition of VGCCs, or promoting Ca^{2+} clearance through plasma membrane ATPases or SERCA pumps (Anguita and Villalobo, 2017; Ghosh et al., 2016; Vela et al., 2007). Alternatively, Src's negative influences on Ca^{2+}-dependent hormone release may be due to

actions distal to elevations in cytosolic Ca^{2+}, as observed for insulin secretion from primary rat pancreatic islets (Cheng et al., 2007); similar mechanisms for Src have been proposed during the secretion of glutamate from rat brain synaptosomes (Baldwin et al., 2006). Downstream of Ca^{2+} elevations, Src may modify actin cytoskeletal dynamics to negatively regulate exocytosis, as is the case for Ca^{2+}-dependent, but not basal, release in PC12 cells (Ohnishi et al., 2001). Since Rac and Rho GTPases did not participate in similar enhancements in GnRH2-dependent LH release, such a mechanism might be through direct actions of Src on actin-binding proteins such as cortactin, which modulates local levels of actin polymerized structures (Tehrani et al., 2007). While PI3K and ERK are also regulated by Src activity in several models of GPCR function, such an interaction is unlikely since these effectors generally facilitated LH release in prior studies (Klausen et al., 2008; Pemberton et al., 2015, 2013, 2011). Interestingly, β-arrestins are well-established conduits linking GPCRs to Src recruitment, and it would be expected that inhibiting either would result in similar outcomes, which was not the case in the present context. However, β-arrestins are also known to recruit several negative regulatory enzymes, bringing them into close proximity with their targets, such as phosphodiesterase (for cAMP), PTEN (for PI3K-PIP_3), protein phosphatase 2A (for Akt), and this is likewise the case for Src and other SFKs, through arrestin-dependent recruitment of the phosphatases SHP-1 and SHP-2 (Peterson and Luttrell, 2017). Thus, it might be that in GnRH2-stimulated gonadotrophs, β-arrestins negatively regulate Src through this clustering with phosphatases, resulting in a positive role of arrestin in mediating the LH secretion response; whereas similar recruitment in somatotrophs excludes incorporation of the negative regulators (see Section 7.4). Alternatively, apparent differences between β-arrestin actions may be due to selective actions of β-arrestin1 and β-

arrestin2 isoforms, which have been shown to have some non-redundant functions in GPCR signal transduction and trafficking (Srivastava et al., 2015).

The Arf1/6 small GTPases are identified as novel regulators facilitating acute LH secretion (Chapter 5), and likely represent downstream targets of PI3K and PIP_3-dependent actions, as predicted by prior work utilizing a PH-domain-selective inhibitor (Pemberton and Chang, 2016). A link to β-arrestins may also be proposed, as arrestin-containing scaffolds can recruit the Arf-GEF ARNO (Arf nucleotide-binding-site-opener) for localized activation of this GTPase (Peterson and Luttrell, 2017). Additionally, the Arf GTPases may form a link to MEK-ERK actions downstream of PIP_3, since this module is not downstream of PKC or SOS-activated Ras in GnRH-stimulated mechanisms in gonadotrophs (Chapter 5; Pemberton et al., 2013). On the other hand, other Ras GEFs such as Ras-GRP and p140 Ras-GRF may mediate canonical Ras-Raf-MEK-ERK activities due to Ca^{2+}/CaM-dependent activation, independent of PKC (Farnsworth et al., 1995; Fernández-Medarde and Santos, 2011), and the presence of Arf-GTP positively contributes to the lifetime of active Ras-ERK endosomal signalling platforms, whereas the conversion to Arf-GDP leads to a loss in signalling (Chen et al., 2022; Porat-Shliom et al., 2008).

Finally, Pyk2/FAK are identified as novel mediators of GnRH2-dependent LH release (Figure 7.1). In GT1-7 neurons, activation of these elements occurs downstream of GnRH-induced CaMK activation (Okitsu-Sakurayama et al., 2019), whereas only CaM, but not CaMK, is required in αT3-1 gonadotrophs; importantly, Pyk2 activity in αT3-1 gonadotrophs was nifedipine (i.e., L-type VGCC)-sensitive and a CaM-binding region was additionally mapped to the catalytic domain of Pyk2 (Xie et al., 2008). GnRH2 action on goldfish LH release involves increases in cytosolic Ca^{2+} through both VGCC entry and release from RyR-controlled stores,

and is also dependent on CaMK (Chang et al., 2009). Therefore, future studies may employ selective inhibitors of VGCCs and RyR channels, and of CaMK isoforms, in order to elucidate the specific links from GnRH-induced elevations in cytosolic Ca^{2+} to the activation of tyrosine kinase effectors (including Pyk2 and likely also Src) perhaps in phosphorylation assays.

7.3 Integrated model of GnRH3-dependent control of LH secretion

One of the major differences in GnRH3 actions in gonadotrophs, as compared to GnRH2, is the additional utilization of $G\alpha_{i/o}$ subunits in the control of acute LH release, as shown in Chapter 4 (also see summary Figure 7.2). While $G\alpha_{q/11}$, β-arrestins, GRKs, and βγ subunits (Pemberton and Chang, 2016) all evidently play similar roles in GnRH2- and GnRH3-elicited LH release, the selective involvement of $G\alpha_{i/o}$ indicates a novel mechanism for mediating agonist-dependent bias at the level of G protein subunit engagement. Interestingly, PLA_2 is one of the major targets of $G\alpha_{i/o}$ actions (Denson et al., 2005; Dickerson and Weiss, 1995), and arachidonic acid (AA) is selectively involved in GnRH3-dependent, but not GnRH2-dependent, LH secretion (Chang et al., 1991); together, this may indicate the selective usage of $G\alpha_{i/o}$ by GnRH3-stabilized receptors in the engagement of PLA_2-AA pathways. Interestingly, the involvement of $G\alpha_i$ may also implicate a role for non-selective cationic transient receptor potential canonical channels (TRPCs). $G\alpha_i$ can directly bind and activate TRPCs, especially TRPC4 and 5, following muscarinic receptor engagement without a requirement for Gβγ, and a binding-domain for $G\alpha_i$ has been mapped to the C-terminus region of TRPCs (Jeon et al., 2013, 2012). TRPCs mediate the GnRH-induced elevations in intracellular Ca^{2+} in mice gonadotrophs, in part through TRPC-induced-depolarization-dependent activation of L-type VGCCs (Beck et al., 2017; Götz et al., 2017). Since L-type VGCCs mediate hormone release responses to goldfish

GnRHs, the potential contributions of TRPCs during goldfish GnRH3 actions will need to be assessed in the future.

With regard to the selective usage of PI3K catalytic subunits in GnRH3 actions, the participation of PI3Kγ in mediating GnRH3-engaged receptors signalling may also involve $G\alpha_{i/o}$-coupling, since $G\alpha_{i/o}$-liberated βγ subunits are known to be very effective in the activation of PI3Kγ (Rynkiewicz et al., 2020; Suire et al., 2006; Vadas et al., 2013). This, in part, may explain the selective usage of PI3Kγ in GnRH3 stimulation of LH secretion, despite Gβγ subunits being utilized in both GnRH2- and GnRH3-elicited LH responses (Pemberton, 2015; Pemberton and Chang, 2016). Further investigations into the nature of $G\alpha_{q/11}$- and $G\alpha_{i/o}$-liberated βγ subunits following GnRHR activation, and the possible contributions of distinct βγ heterodimer assembly, will be necessary in order to shed further light on these agonist-specific mechanisms. In addition, MMP-dependent components were also identified in GnRH3 signal transduction (Chapter 6), and this mechanism may also explain in part the selective recruitment of PI3Kδ in gonadotrophs, possibly utilizing RTK and/or integrin receptor signalling following MMP activation, as discussed in Chapter 6 (Ferreira et al., 2006; Vanhaesebroeck et al., 2010).

In Chapter 5, inhibition of SOS-Ras interactions paradoxically enhanced GnRH3-stimulated LH release despite abolishing ERK phosphorylation, which is inconsistent with the ability of MEK-ERK inhibitors to suppress LH secretion (Klausen et al., 2008). How this is manifested is unknown at present, but it is clear that the roles of additional Ras-GEFs need to be clarified, as discussed above in Section 7.2. ERK also has a number of downstream targets that it exerts negative regulation over (Ullah et al., 2022), and it is possible that disrupting SOS-Ras activity upstream then releases ERK inhibition of its targets, in a manner different from direct perturbation of MEK. On the other hand, emerging evidence suggests that SOS isoforms have

different capacities for modulating the Ras-PI3K-Akt axis *vs.* the canonical Ras-Raf-MEK-ERK module (Baltanás et al., 2021), which may partly explain the apparent discrepancies in these findings. Disrupting SOS-Ras interactions did, however, abolish phospho-ERK activity in mixed pituitary cell lysates (Chapter 5), reinforcing the idea that while the canonical module is intact, context-specific inputs are possible either through alternate Ras-GEFs or direct modulation of Raf kinase. The possible existence of Src inputs to SOS-Ras is also indicated in Figure 7.2, since Src can stimulate the adaptor protein Shc, in turn leading to Ras activation via SOS (Goldsmith and Dhanasekaran, 2007), which would be consistent with Src's inhibitory effects on GnRH3-dependent LH release (Chapter 6). Interestingly, despite the consistent involvement of Src in both GnRH2 and GnRH3 actions in gonadotrophs, disruption of dynamin activity did not alter GnRH3-evoked LH release (Chapter 3). How Src's differential effects in GnRH2 and GnRH3 actions on gonadotrophs lead to distinct engagement of SOS and dynamin are interesting areas to address going forward. Just as importantly, since both GnRH2- and GnRH3-induced LH release responses are known to undergo desensitization (Habibi, 1991a, 1991b), the apparent lack of dynamin involvement for GnRH3-stabilized receptors might implicate alternate mechanisms of GnRHR internalization. These include receptor internalization through cholesterol-rich lipid rafts or caveolae, or novel clathrin-independent pathways such as FEME (fast endophilin-mediated endocytosis; Von Moo et al., 2021). The possible involvement of caveolae which contains caveolin rafts in the termination of GnRH3-GnRHR signalling is of particular interest since caveolin is known to negatively regulate NOS activity and NOS is a component of GnRH3 stimulation of LH release in this system (Meints et al., 2012).

Pyk2/FAK were similarly involved in both GnRH2 and GnRH3 control of acute LH secretion (Chapter 6). However, since FAK-dependent focal adhesions organize a number of

signalling effectors into localized complexes (Davidson et al., 2004; Dobkin-Bekman et al., 2009), differences may exist in the composition of these complexes/scaffolds downstream of GnRH2 *vs.* GnRH3 stabilized receptors. Future studies with more selective inhibitors of Pyk2/FAK may help disentangle these effects in addition to clarifying the specific effectors recruited to such localized complexes (see Section 7.7).

The involvement of Pyk2 and Arf1/6 in both GnRH2- and GnRH3-dependent LH release (Chapters 5 and 6) also implicate the participation of phospholipase D (PLD) in GnRH actions. In some systems, activation of Pyk2 is dependent on PLD (Banno et al., 2005). On the other hand, Arf activates PLD (Brown et al., 1993). PLD hydrolyzes phosphatidylcholine to generate the active signalling molecule phosphatidic acid, which is also interconvertible with diacylglycerol, the product of PLC actions (McDermott et al., 2004), and addition of PLD to static cultures of dispersed goldfish pituitary cells in prior studies elevated basal LH release (Chang et al., 1991). In particular, Arf-dependent PLD activation has been shown to regulate exocytosis in endocrine/neuroendocrine cell types through actions on secretory vesicle budding from the *trans*-Golgi (Chen et al., 1997; Chen and Shields, 1996; Jones et al., 1999), and Arf6-dependent PLD-induced production of phosphatidic acid is proposed to control aspects of fusion between secretory vesicles and the plasma membrane (Bader et al., 2004). Recent studies have also implicated Arf1 in post-Golgi trafficking to the plasma membrane (Adarska et al., 2021), although evidence from endocrine cell types is lacking. It is also likely that $G\alpha_{q/11}$-PKC signalling converges at the level of Arf/PLD to link extracellular signals to alterations in secretory vesicle trafficking (Frohman and Morris, 1996). This may provide a mechanism for GnRH2 and GnRH3 activation of Arf and then PLD (Figures 7.1 and 7.2). Interestingly, Ras can also activate PLD through Arf (del Peso et al., 1996; Luo et al., 1998); however, SOS/Ras exerts

a negative influence on GnRH3-induced LH release (Chapter 5). Thus how this potential SOS/Ras-Arf-PLD link may participate in GnRH3 actions on gonadotrophs is unknown (Figure 7.2).

7.4 Integrated model of GnRH2-dependent control of GH secretion

Results utilizing a pan-Gα inhibitor in Chapter 4 revealed that an inhibitory G-protein-dependent component is normally co-activated during GnRH2/3 stimulation of GH release. Consistent with this evidence, results from Chapter 5 showed that direct inhibition of Rho GTPases also enhanced acute GnRH2-dependent GH secretion. While Rho GTPases may also be activated through $G\alpha_{q/11}$-elicited mechanisms (Chikumi et al., 2002), the present results may be explained by GnRHR actions through utilization of $G\alpha_{12/13}$ subunits (Figure 7.3) since $G\alpha_{q/11}$ mediates, rather than suppresses, the hormone releasing activities of the two GnRHs (Chapter 4). Interestingly, the cholecystokinin receptor CCK1, which couples to several Gα subtypes in rat pancreatic acinar cells (Schnefel et al., 1990), utilizes $G\alpha_{q/11}$ and $G\alpha_{12/13}$ subunits to activate Rac and RhoA GTPases, respectively (Sabbatini et al., 2010). Future investigations into the specific Rho-GEFs involved in GnRH actions may help to clarify whether such differential G protein coupling underlies these observations in goldfish somatotrophs. Localized cellular concentrations of PIP_3 generated by PI3K isoforms are also capable of regulating small GTPase function; this is especially well characterized in the contexts of cell movements where phospholipid and GTPase dynamics modulate the distinct, yet coordinated, changes in leading and trailing edge cellular shapes and cytoskeletal structures (Dawes and Edelstein-Keshet, 2007). In particular, the RhoA effector ROCK (Rho-associated protein kinase) promotes actin polymerization, the prevention of which, as discussed in Chapter 5, causes the enhancement of hormone release as shown for many

secretory cell types (Bader et al., 2004; Streit et al., 2020). Importantly, given the apparent negative role of RhoA towards acute release, the participation of upstream Rho-GAPs, including p190RhoGAP and the family of ArhGAPs, as well as Rho-GDIs, warrant attention (Cherfils and Zeghouf, 2013; Mosaddeghzadeh and Ahmadian, 2021). However, many other downstream effectors of RhoA are also known, some independent of cytoskeletal functions (Bishop and Hall, 2000; Clayton and Ridley, 2020); these represent important targets for future investigations in GnRH action.

In contrast to their actions in gonadotrophs, disrupting GRK catalytic functions prolonged and enhanced the effects of GnRH2 on GH secretion, suggesting an overall negative role of GRKs in modulating GnRH2 actions in somatotrophs (Figure 7.3). In addition to the prolonged desensitization-type response which was present for both GnRH2 and GnRH3, the altered acute phase of GH secretion to GnRH2 stimulation suggests additional GRK-dependent signalling to effectors in the case of GnRH2 (Chapter 4). The GRK interactome is quite vast, including substrates modified in both positive and negative fashions, and GRKs are further regulated by multiple converging feedback loops from intracellular protein kinases, which can alter activity, stability, as well as localization of GRKs through post-translational modifications (Penela et al., 2019). Given the differences in GRK action between gonadotrophs and somatotrophs, it is likely that GRKs also differentially alter GnRHR phosphorylation barcodes which would lead to different modes of receptor/arrestin-induced responses as well as trafficking (Bahouth and Nooh, 2017; Nobles et al., 2011; Sente et al., 2018). To shed further light on this, reductionist *in vitro* biochemical approaches involving purified goldfish GnRHRs and GRK isoforms will be a good starting point for future investigations.

Additionally, in contrast to their functions in gonadotrophs, Src kinases played positive facilitative roles in the control of GnRH2-dependent GH release (Figure 7.3). In this context, Src may initially be recruited by arrestin-dependent scaffolds, possibly in concert with p110β/p110γ-generated PIP_3, with which Src co-ordinates to activate Btk (Afar et al., 1996; Pemberton and Chang, 2016). As discussed above in Section 7.2, Src kinases can also interact with Ca^{2+} handling mechanisms in multiple ways. In particular, the actions of Src in somatotrophs, especially for GnRH2-stimulated cells, may involve phosphorylation of known GnRH2 signal transduction elements such as plasma membrane-expressed L-type VGCCs and/or RyRs on intracellular stores (Chang et al., 2012), interactions which modulate channel opening and typically result in increases in cytosolic Ca^{2+} (Anguita and Villalobo, 2017). Interestingly, the involvement of both Src and Btk may indicate participation of PLC-γ in GnRH actions in somatotrophs (Figure 7.3), since PLC-γ is activated through phosphorylation by protein tyrosine kinases (Hajicek et al., 2019; Humphries et al., 2004; Law et al., 1996). Whether multiple PLC isoforms are integrated in GnRH2-elicited mechanisms in somatotrophs is unknown, but it is possible that tyrosine kinase-dependent actions underlie the prior observations showing PKC involvement. It should be noted that GnRH2 treatments do not elevate IP_3, but can increase other IP species in mixed population of dispersed goldfish pituitary cells, a finding not at variance with the possibility that different PLC isoform(s) may be utilized by GnRH2 in comparison with GnRH3 (Chang et al., 1995). Additionally, how the differential effects of Src are mediated between gonadotrophs and somatotrophs is unclear, but complex time-, cell context- and activity-dependent functions of SFKs on Ca^{2+} are well established (Anguita and Villalobo, 2017), and this may additionally be related to the differential integration of PI3K and Ca^{2+}-dependent signalling between the two cell types (Pemberton et al., 2011); thus, investigations of

distinct isoforms of plasma membrane and intracellular store Ca^{2+} channels, as well as those of CaMK, may be prudent.

7.5 Integrated model of GnRH3-dependent control of GH secretion

As with GnRH3-stimulated LH release, GnRH3 actions in somatotrophs also involved both $G\alpha_{q/11}$ and $G\alpha_{i/o}$ subunits (Chapter 4; Figure 7.4). Despite the sensitivity of GnRH3-dependent GH release to $G\alpha_{i/o}$ inhibition, unlike in gonadotrophs, the typical $G\alpha_{i/o}$ effectors such as PLA_2 and Src are not involved. Some evidence exists for $G\alpha_{i/o}$ regulation of PI3Ks and NOS isoforms (Liu et al., 2007; Wenzel et al., 2009; Wyckoff et al., 2001), which do participate in mediating GnRH3-elicited GH release, but further experimental confirmation will be needed. Similar to the situation in gonadotrophs, $G\alpha_{i/o}$ could also mediate activation of TRPCs (see Section 7.3), which contribute to intracellular Ca^{2+} responses following GHRH stimulation of mouse somatotrophs (Núñez et al., 2019). $G\alpha_{i/o}$ may also activate isoforms of PLC either shared or unique to those downstream of $G\alpha_{q/11}$. Alternatively, it is very possible that the targets of $G\alpha_{i/o}$ in mediating GnRH3 actions on GH release are as-yet uncharacterized.

A sensitivity of GnRH3-evoked GH release to MMP inhibition was also observed (Chapter 6), which may reflect a method of signal transduction leading to activation of PI3Kδ isoforms (Figure 7.4). On the other hand, β-arrestin-AP2 actions exerted strong negative influences over GH release (Chapter 3), indicating possible recruitment of negative regulators to β-arrestin-containing scaffolds, and/or trafficking to degradation pathways leading to termination of signalling (Figure 7.4). Given the specific negative influence of Akt to GnRH3-elicited GH secretion (Pemberton and Chang, 2016), and the known ability of β-arrestins to recruit and activate Akt through localized inhibition of its phosphatase PP2A (Kendall et al., 2011; Peterson

and Luttrell, 2017), such an interaction may underlie the effects on GnRH3-dependent GH secretion. Importantly, this proposed mechanism would also be consistent with the "uncoupled" nature of the PI3K-Akt axis in GnRH3-stimulated somatotrophs; i.e., PI3K or PIP_3-PH domain inhibition suppresses, whereas direct inhibition of Akt enhances, acute GH release (Pemberton et al., 2015; Pemberton and Chang, 2016). Alternatively, Akt recruitment may occur through EGFR-initiated mechanisms, which similarly suppress GH release from GnRH3-stimulated cells (Chapter 6), but how this might "bypass" the facilitative nature of PI3K actions is unknown at present. Similar to the differences in β-arrestin function between GnRH2- and GnRH3-stimulated somatotrophs cell states, GRKs did not have a selective acute component in GnRH3 actions but only mediated desensitization functions (Chapter 4; Figure 7.4). How this selectivity downstream of the presumably distinct stabilized GnRHR-states is achieved is unknown, but contributions of distinct GRK isoforms cannot be discounted in this regard. Since the dual inhibitor CMPD101 inhibits catalytic functions of both GRK2 and GRK3 (Thal et al., 2011), it is possible that these kinases mediate different functions downstream of activated GnRHRs. Regardless of which isoform(s) mediate which function(s), it is clear that the overall roles of GRK2/3 in GnRHR action vary considerably between goldfish gonadotrophs and somatotrophs.

In contrast to GnRH2-stimulated GH release, Rac, rather than RhoA, was the small GTPase which exerted negative regulation over acute hormone release (Chapter 5; Figure 7.4). Although the sub-family of Rho GTPases (which include Rho, Rac, and Cdc42) have many overlapping functions with regard to the control of actin and cytoskeletal dynamics, studies have indicated selective usage of these effectors to regulate distinct pools of intracellular actin and to control different types of remodelling (e.g., bundling of actin *vs.* polymerization), as well as effects on other structural elements including myosin (Burridge and Wennerberg, 2004;

Machesky and Hall, 1997). Regardless of how these effects are manifested, the involvement of Rac implicates downstream effectors which link this GTPase to modulators of the actin polymerization machinery, especially Wiskott-Aldrich syndrome protein family verprolin-homologous protein (commonly known as WAVE) and p21-activated kinase which link to the Arp2/3 (actin-related protein 2/3) complex; this in turn directly interacts with actin and regulates the processes of both polymerization and filament organization (Goley and Welch, 2006; Machesky and Gould, 1999). How Rac GTPases are recruited following GnRHR activation is unknown at present, but PI3K-initiated PIP_3 production and subsequent signalling represents a good candidate, given the coordinated actions of PI3K-dependent lipid remodelling and GTPase-dependent cytoskeletal actions in several cellular functions (Figure 7.4 and as discussed above in Section 7.4). Interestingly, PIP_3 can recruit both Rac-GEFs and Rac-GAPs via PIP_3-binding PH domains or polybasic regions (PBRs), leading to modulation of local activity of Rac GTPases in plasma membrane or endomembrane compartments (Campa et al., 2015). In the present context, given the prior evidence that PI3Ks mediate GnRH3-induced GH secretion (Pemberton et al., 2015, 2011), whereas Rac exerts negative regulation, PIP_3-dependent actions in GnRH3-stimulated somatotrophs may recruit Rac-GAPs, resulting in the overall promotion of actin depolymerization to facilitate hormone exocytosis. Thus, the potential involvement of Rac-GAPs such as the families of PBR-containing cdGAPs (Cdc42 GTPase-activating protein, which is active on both Cdc42 and Rac) and PH-domain-containing ArhGAPs (Rho GTPase-activating proteins) represent interesting areas for future investigation to definitively integrate PI3K and small GTPase functions in somatotrophs. Just as importantly, bi-directional interactions between Ca^{2+}-handling mechanisms and small GTPases are also known, and appear to vary in a cell-context dependent fashion (Aspenström, 2004; Figure 7.4). Since PI3K effects on GnRH3-

induced GH release is uniquely distal to rises in intracellular Ca^{2+} (Pemberton et al., 2011), it would be interesting to examine whether and how Ca^{2+} and PI3Ks might co-ordinate to regulate activity of Rac GTPase in this functional context.

7.6 Regulation of basal LH and GH exocytosis.

Throughout this thesis, column perifusion experiments with inhibitor-alone columns allowed for the assessment of effects on unstimulated release during pharmacological manipulation of various effectors in the absence of GnRH stimulation, and with results often indicating a different influence of these intracellular effector systems on induced *vs.* unstimulated release (as discussed in Chapters 3-6). Overall, most of the identified regulators exerted negative modulation of basal release, often with profound effects, whereas minor positive influences were noted for Src, MMP, and EGFRs, but only on LH secretion (Figure 7.5).

In general, unstimulated/basal release in secretory cell types (especially for hormones and neurohormones) is distinct from constitutive secretion ("bulk flow"), and is considered to arise from the same initial population of vesicles that make up the readily releasable pool, since levels of basal release can be further modulated by cell stimulation (Moore et al., 2002; Stojilkovic et al., 2005; Varro et al., 1996). This is corroborated in the goldfish pituitary model, at least for somatotrophs, by the finding that inhibition of protein synthesis does not modify basal release under 2 h (Johnson et al., 2002). However, the mechanisms controlling basal secretion have not received strong attention in this system. Given the number of effectors shown to modulate basal secretion just within the scope of this thesis, often with profound effects on release levels (especially for the complement of small GTPases and Pyk2/FAK in Chapters 5 and 6), it is clear

that these represent novel and important intracellular mechanisms that govern vesicle availability for exocytosis.

Results from Chapter 3 also revealed that β-arrestins and dynamin GTPase both suppress basal GH, but not LH, secretion. The agonist-independent functions of β-arrestins is consistent with their ability to regulate cytosolic ERK pools (Coffa et al., 2011; Hanson et al., 2007), and the ERK cascade modulates hormone release from somatotrophs (Pemberton et al., 2013). On the other hand, dynamin is known to regulate fusion pore dynamics during vesicle exocytosis in a range of secretory cell models (Holroyd et al., 2002; Trexler et al., 2016; Tsuboi et al., 2004). Understanding how these actions are selectively involved in somatotrophs, but not gonadotrophs, will require further study.

Results from this thesis also reveal that some non-$G\alpha_{11/q}$ subunits are important negative regulators of both basal LH and GH release (Chapter 4). This finding is consistent with the known actions of $G\alpha_{i/o}$ subunits (Chang et al., 1993) and when taken together suggest that $G\alpha_{i/o}$ is likely one of these basally active, negative regulatory subunits. In addition, isoforms of Arf, Rho, and Rac small GTPases regulate basal hormone release in both gonadotrophs and somatotrophs, whereas Ras actions are selective to gonadotrophs (Chapter 5). These small GTPases are known to influence cytoskeletal F-actin dynamics, which controls secretory vesicle access to the plasma membrane (Bader et al., 2004; Porat-Shliom et al., 2013; Streit et al., 2020). These cellular events are possible targets through which these small GTPases control agonist-independent basal LH and GH secretion. On the other hand, these small GTPases are likely also relevant to the control of potentially segregated hormonal vesicle pools, or distinct regulation of these same pools, thereby affecting hormone availability for release during the regulation by multiple known positive and negative neuroendocrine factors. These potential function(s) of small GTPases on

secretory vesicle availability in both basal and agonist-modulated control of secretion is a research question that is largely unexplored, especially when considering complex physiological interactions such as the differential, but concomitant, negative influences of somatostatin isoforms on both basal and agonist-stimulated GH secretion (Chang et al., 2012), as well as dopamine on basal and stimulated LH release (Chang et al., 1990). Interestingly, the magnitude of the attenuated basal LH secretion in the presence of inhibitors of Src, MMP, and EGFRs (Chapter 6) are similar to that observed with dopamine D2-mediated inhibition of basal LH release (Chang et al., 1990), suggesting that these signalling elements and their downstream targets are physiologically relevant, non-obligatory components of the basal exocytosis in gonadotrophs. Furthermore, the inhibitory actions of somatostatin on somatotrophs and dopamine on gonadotrophs can also be distal to GnRH-induced changes in second messengers including PKC, AA, Ca^{2+}, and cAMP (GH, Canosa et al., 2007; LH, Chang et al., 2009). Thus, small GTPases, tyrosine kinases, and other effectors modulating exocytotic events and associated cytoskeletal organizations represent attractive targets for investigation in these important functional contexts. Such selective control of releasable vesicle pools is likely even more important in mammalian gonadotrophs since these are typically multihormonal and secrete both LH and FSH, which are independently regulated in both basal and GnRH-stimulated states with varying episodic patterns (Constantin et al., 2022; McNeilly et al., 2003). Regardless, the mechanisms underlying small GTPase and tyrosine kinases in the control of such differential hormone release requires further investigation in pituitary cell types across taxa.

7.7 Other important future directions

From the above discussion Sections 7.2 to 7.6, it is clear that multiple layers of regulation and interactions exist for many of the identified players in GnRH control of acute LH and GH release from goldfish pituitary cells. Thus, while experiments from this thesis have shown the direct involvement of many effectors and some interesting areas for further investigations have been identified in order to consolidate/confirm some of the possible linkages between signalling cascades, several other related and impactful areas to consider going forward in the future can be identified.

One such important area of investigations includes the functional roles of the subcellular sequestration of effectors through the participation of multi-domain scaffolds, plasma membrane microdomains such as lipid rafts, as well as endomembrane compartments. As with the importance of intracellular receptor trafficking in determining the biological output of growth factor receptors (Tomas et al., 2014), recent evidence suggests that such spatial segregation is critical to both the efficiency and fidelity of GPCR signal transduction (Shen et al., 2018; Weinberg and Puthenveedu, 2019; West and Hanyaloglu, 2015), and a role for ligand-biased intracellular trafficking is also emerging (Girard et al., 2023; Molinari et al., 2010; Zheng et al., 2008). Accordingly, there is a clear need for higher resolution data in both space and time going forward to complement global cellular readouts, although monitoring concurrent physiological responses remains important. Prior single-cell analyses also indicate that GnRH-induced Ca^{2+} signals in goldfish pituitary cells are spatially specific, and have agonist-selective waveforms and kinetics (Johnson et al., 1999). To integrate these ideas with findings from this thesis, a focus on GRK/β-arrestin- and Src/FAK-dependent platforms may represent good starting points for future investigations, due to both their large multidomain structures with multiple protein- and lipid-interacting domains, as well as characterization in mammalian models of GnRH and other GPCR

functions (Alexander et al., 2020; Cance et al., 2013; Davidson et al., 2004; Dobkin-Bekman et al., 2009; Walkiewicz et al., 2015). β-arrestin-mediated GPCR trafficking to intracellular compartments is a novel and emerging paradigm for distinct phases of GPCR activity governing unique cellular functions (Thomsen et al., 2018), which also make β-arrestins an attractive target for mediating some of the discontinuous time-dependent effects of GnRH on hormone release and synthesis (Pemberton et al., 2014, 2013). Specifically, time-lapse imaging approaches can be leveraged to address how β-arrestin-GnRHR complexes are trafficked to intracellular compartments, to elucidate the identity of other effectors co-recruited to these compartments, as well as to evaluate the temporal dynamics and eventual fate of these complexes. Monitoring these readouts will shed further light on how intracellular signalling proceeds in specific intracellular compartments in co-ordination with mechanisms controlling desensitization and signal termination.

Furthermore, two GnRHR isoforms, GfA and GfB, are known to be expressed on goldfish pituitary gonadotrophs and somatotrophs (Illing et al., 1999). While it has been suggested that each isoform may individually mediate LH- and GH-releasing functions, the previous reported differences in GnRH2 and GnRH3 actions (reviewed in Chapter 1, Section 1.9), as well as results on GnRH-isoform and cell-type selectivity in the signalling leading to hormone release from my thesis research cannot be entirely accounted for by any potential differential expression of GfA and GfB on goldfish gonadotrophs and somatotrophs. Regardless, how and whether these receptor isoforms may variably interact with the complement of Gαβγ subunits, β-arrestins, and GRKs to mediate aspects of GnRH bias certainly needs to be addressed going forward in pituitary cell-specific contexts. While technically challenging and limited by the unavailability of commercial isoform-specific antibody reagents in the primary goldfish

pituitary system, heterologous expression systems can be used as complementary approaches to address the basic interactions between goldfish GnRHR isoforms and the complement of receptor-associating effectors. If undertaken, this avenue of research would help to clarify the fundamental molecular underpinnings of GnRH ligand bias through differential ligand- and time-dependent interactions with Gαβγ subunit isoforms and GRK/β-arrestins. Towards this general goal, the Chang lab has commissioned the generation of antibodies designed against unique extracellular domains of GfA and GfB in the hope that these will also act as receptor isoform-specific neutralizing antibodies to aid in functional and tracing studies.

The current thesis results on MMP actions also indicate the contribution of as yet unidentified cell-surface/ECM elements and receptor systems in mediating GnRH action on goldfish pituitary cells. While this highlights future avenues for investigation of these elements in regulating hormone secretion, these results also implicate the potential roles of other GnRH-dependent cellular functions such as cell remodelling and cell migration, especially when considered alongside the participation of small GTPases which link to F-actin dependent cytoskeletal reorganization. Studies in mammalian GnRH systems have described GnRH-dependent engagement of the actin cytoskeleton, which can be utilized to elicit changes in cell morphology, as well as cell movements (Alim et al., 2012; Edwards et al., 2017; Navratil et al., 2014). However, such mechanisms remain virtually unexplored in the goldfish pituitary neuroendocrine model. Whether goldfish GnRH2 and GnRH3 mediate aspects of these functions will be highly interesting to study, given that such cellular remodelling has implications for efficient pituitary hormone secretion *in vivo* (e.g., through dynamic repositioning of pituitary cells and processes in the proximity of vasculature) and represents part of the coordinated actions

of GnRH on pituitary cell function (Fontaine et al., 2020; Grønlien et al., 2021; Navratil et al., 2007).

Having now identified the involvement of classical GPCR effectors such as β-arrestins and GRKs in the goldfish pituitary system, these are also likely to be components mediating functions of other hypothalamic neuromodulators which control goldfish pituitary hormone release through GPCR-dependent pathways, such as dopamine, somatostatin, norepinephrine, PACAP, and ghrelin (Chang et al., 2012, 2009). Similarly, many of these neuroendocrine modulatory systems are likely to engage the conserved small GTPase machinery in the control of secretory vesicle trafficking and exocytosis, which can form the basis for multiple future studies. Such investigations will potentially improve our overall understanding of how multifactorial neuroendocrine regulation of secretion can be integrated and coordinated at the intracellular level.

7.8 Complexity of signal transduction during the control of hormone secretion

On the whole, the mechanisms underlying regulation of goldfish pituitary hormone secretion presented in the current thesis, as well as from prior findings (Chang and Pemberton, 2018), may appear to be unnecessarily complex. Furthermore, it may seem redundant to have two GnRH systems controlling LH and GH secretion through such intricate, and in many cases non-overlapping, signalling networks. However, it is important to recognize that the two GnRH neuronal populations originate, receive inputs from, as well as project to, distinct brain regions/centers in addition to the pituitary in teleost and other vertebrate species (Ogawa et al., 2021). In functional terms, while GnRH3 populations (or the equivalent GnRH1 system where GnRH1 is the primary hypophysiotropic regulator) can communicate with the olfactory and

visual sensory systems (Chi et al., 2017; Umatani and Oka, 2019), GnRH2 neurons have well-descibed links to feeding and energy centers in the brain (Kauffman and Rissman, 2004; Matsuda et al., 2008; Nishiguchi et al., 2012). Interestingly, some of this communication is bi-directional, such that GnRH neurons can also exert reciprocal control over the sensory systems, while also influencing other aspects of physiology such as sexual behaviour (Umatani and Oka, 2019). Such an organization allows for a co-ordinated physiological regulation of growth and reproduction in response to changes in environmental stimuli and nutritional status through the regulation of pituitary hormone secretion. In support of such a framework, recent functional evidence from studies in zebrafish indicates that during fasted conditions, while GnRH3 neurons undergo reductions in length and in pituitary innervation, GnRH2 neurons specifically increase in both length and abundance, and can functionally compensate for the depletions in GnRH3 (Marvel et al., 2021), providing a good justification for the apparent redundancy in having dual stimulatory inputs.

In light of these complex interactions at the level of the brain, with the two GnRHs linking to distinct physiological systems upstream, it is perhaps unsurprising that post-receptor transduction mechanisms for the two GnRHs can then vary significantly to regulate pituitary cell outcomes (although the contributions of GfA and GfB receptor isoforms cannot be entirely discounted, as discussed in Section 7.7). The complexity (and multiplicity) of the GnRH post-receptor transduction system also allows for modulation of the hormone secretion response by other neuroendocrine factors important for regulating gonadotroph and somatotroph functions given the multifactorial nature of their neuroendocrine control (see Section 7.7). Just as importantly, the control of basal/spontaneous LH and GH exocytosis has also been shown to be different from agonist-evoked release in both results from this thesis as well as prior studies in

the goldfish system (Chang and Pemberton, 2018). This is also unsurprising when considering the specific physiological needs of organisms as maintained by basal levels of pituitary hormones, as opposed to the specific, timed increases required to facilitate pronounced changes in physiology in response to upstream brain inputs.

Also relevant to this discussion is the information that GnRH-GnRHR is an evolutionarily ancient system, dating back at least to the origin of vertebrates, and possibly further back to the common ancestor of protostomes and deuterostomes, given the recently described relationships to the invertebrate adipokinetic hormone and corazonin neuropeptides and their receptor systems (Dufour et al., 2020; Zandawala et al., 2018). Thus, GnRH, as well as several other neuropeptide-GPCR systems have had considerable time to evolve alongside subsets of eukaryotic cellular machineries (Elphick et al., 2018), especially for the more "evolutionarily advanced" lineages of teleost species such as the goldfish (~11 million years ago; Chen et al., 2019; Xu et al., 2019). However, detailed studies of natural GnRH signal transduction mechanisms in more primitive species such as basal chordates and invertebrates are sparse/lacking, and future investigations addressing such questions would be needed in order to shed light on the evolutionary developments in GnRH's engagement of diverse subsets of intracellular machinery in regulating cellular functions.

7.9 Summary

The findings from this thesis have identified the role of several novel effectors in GnRH control of pituitary hormone secretion in a basal vertebrate model. Through the use of primary untransformed cells and naturally occurring ligands, in conjunction with physiological cellular outputs, the importance of these proteins to normal neuroendocrine control of goldfish pituitary

cell functions has been clearly demonstrated. Furthermore, the findings add to our understanding of coordinated regulation of the reproductive and growth endocrine axes through shared upstream hypothalamic regulators. In particular, studies of receptor-interacting effectors in Chapters 3 and 4 have made headway in identifying the underpinnings of natural ligand bias occurring at the initial steps of intracellular transducer engagement, and demonstrated how multiple proximal elements can variably affect GnRH-dependent responses. From Chapter 5, proteins from the small GTPase superfamily are revealed to be important regulators of basal pituitary hormone release, while being selectively recruited in GnRH-elicited pathways. Lastly, effectors controlling both basal and GnRH-dependent hormone release are identified in Chapter 6, including both intracellular protein tyrosine kinases and other cell-surface elements recruited through transactivation. While some of these effectors have been implicated in mammalian GnRH signalling networks, physiological readouts in native cellular contexts were largely lacking. Thus, crucially, the studies in this thesis have established the roles of these effectors in the predominant hypophysiotrophic function controlled by GnRH (i.e., pituitary hormone secretion). Overall, this thesis has contributed novel information from a lower vertebrate model, providing comparative insights into the evolution of neuropeptide control of pituitary hormone release, while also adding knowledge to the field of biased GPCR signalling mechanisms at large.

Figure 7.1. Integrated summary schematic of GnRH2-dependent mechanisms in the control of acute LH release. GnRH2 control of pituitary LH secretion in goldfish utilizes the canonical $G\alpha_{q/11}$ effector PLC-β, with subsequent liberation of DAG leading to activation of PKC isoforms. Although the GnRH2-induced rises in cytosolic calcium are not sensitive to inhibition of IP_3 receptors (not shown), they are dependent on actions of ryanodine receptors (RyR) and extracellular Ca^{2+} influx through VGCCs. In addition, recent work has highlighted the selective use of PI3Kδ and PI3Kβ isoforms, the latter of which is likely activated by G protein βγ subunits. Results from this thesis directly identify $G\alpha_{q/11}$ subunit participation, as well as that of the receptor-interacting protein β-arrestins, which are classically recruited through receptor phosphorylation by GRKs. Together with $G\alpha_{q/11}$, GRK2/3 isoforms and β-arrestins play facilitative roles in GnRH2-induced LH release, likely through scaffolding of protein kinase effectors in clathrin-coated structures (CCS). Arrestin, through its interaction with the adaptor protein AP2 is known to be important for the incorporation of receptors into CCS. On the other hand, dynamin participates in desensitization of GnRH2-evoked LH release responses; this may be downstream of Src kinases, which also exert negative influences on LH release. Arf1/6 small GTPases are also identified as novel effectors in facilitating GnRH2-dependent LH release, and their activation is likely mediated by PI3K-dependent synthesis of the rare phospholipid $PtdIns(3,4,5)P_3$ (PIP_3) which modulates the activities of specific Arf-GEFs and Arf-GAPs; in turn, Arf1/6 GTPase involvement implicates the participation of phospholipase D (PLD). Additionally, Pyk2/FAK also facilitate acute LH secretion, which is expected to be downstream of rises in intracellular Ca^{2+} and calmodulin (CaM) activity, but proximal to Raf-MEK-ERK activation. Perturbation of SOS-Ras interactions did not alter LH release, and given that PKC is not upstream of MEK-ERK in GnRH-dependent LH release based on previous work, alternate Ras-GEFs such as Ras-GRP and p140 Ras-GRF are implicated in initiating this cascade, whereas Arf1/6 GTPases likely also coordinate its activity indirectly through their actions on phospholipid synthesis. See text for full details.

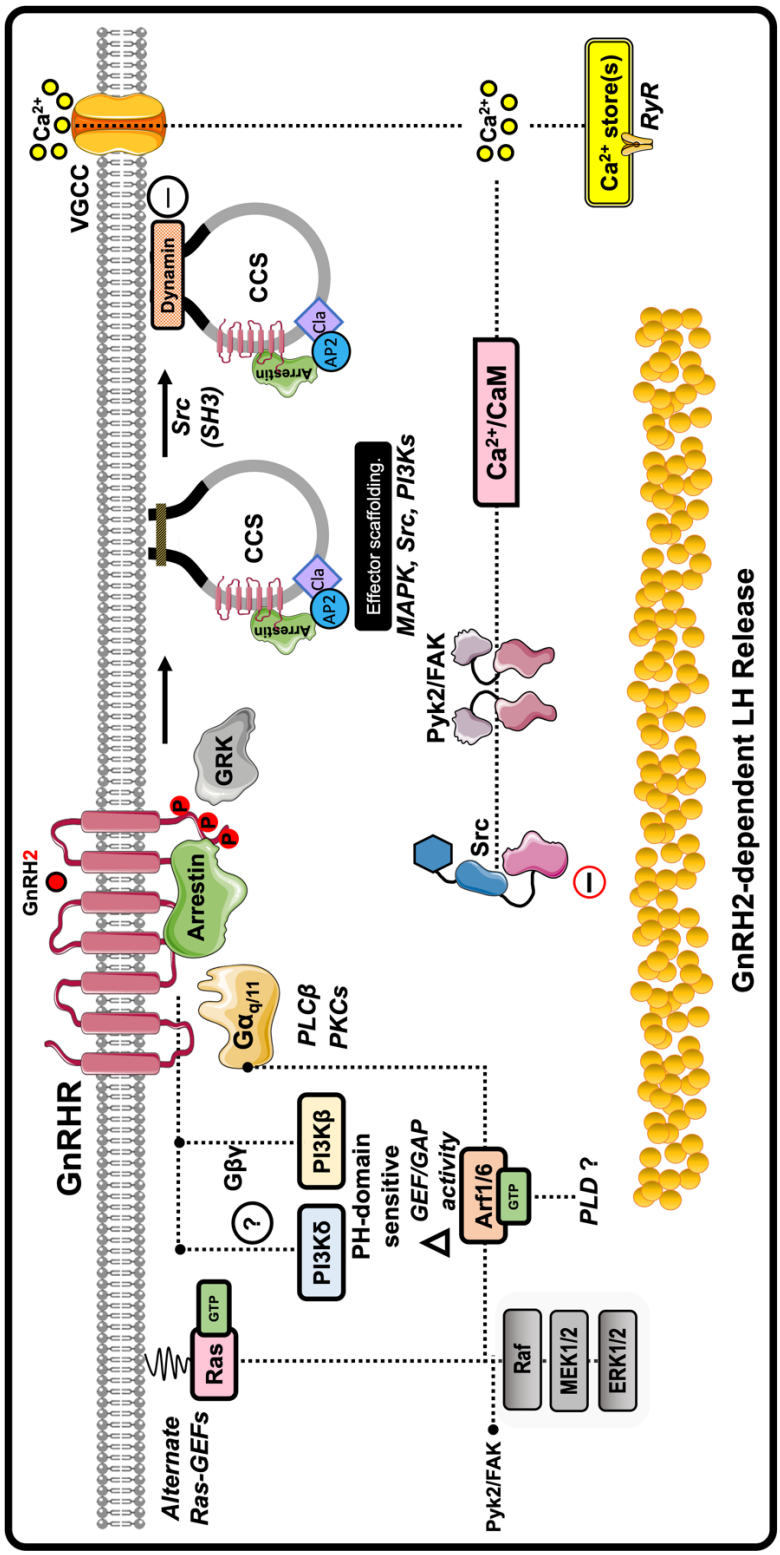

GnRHR
GnRH2
Arrestin
GRK
Gαq/11
PLCβ
PKCs
Gβγ
PI3Kβ
PI3Kδ
PH-domain
sensitive
GEF/GAP
activity
Arf1/6
GTP
PLD ?
Alternate
Ras-GEFs
Ras
GTP
Raf
MEK1/2
ERK1/2
Pyk2/FAK
Src
Pyk2/FAK
Ca2+/CaM
Ca2+
Ca2+ store(s)
RyR
VGCC
Ca2+
Dynamin
CCS
Arrestin
AP2
Cla
Src
(SH3)
Effector scaffolding.
MAPK, Src, PI3Ks
GnRH2-dependent LH Release

Figure 7.2. Integrated summary schematic of GnRH3-dependent mechanisms in the control of acute LH release. GnRH3 control of pituitary LH secretion in goldfish utilizes the canonical $G\alpha_{q/11}$ effector PLC-β, with subsequent liberation of DAG leading to activation of PKC isoforms and IP_3 production. Activation of IP_3 receptor (IP_3R) Ca^{2+} channels together with ryanodine receptor (RyR)-mediated intracellular Ca^{2+} release channels allows for mobilization of Ca^{2+} from intracellular stores which in addition to extracellular Ca^{2+} entry through VGCCs, lead to increases in cytosolic Ca^{2+}. In addition, recent work has indicated the selective use of PI3Kδ and PI3Kγ isoforms. While PI3Kγ is likely downstream of G protein βγ subunits, PI3Kδ may be activated by the MMP-dependent actions identified in this thesis. MMP actions may also be mediated by as yet untested effects on MMP substrates such as extracellular matrix (ECM) and integrin, and membrane-tethered proligands of receptor tyrosine kinase (RTKs). My thesis results also directly identify $G\alpha_{q/11}$ subunit participation, as well as that of the receptor-interacting protein β-arrestins, which are classically recruited through receptor phosphorylation by GRKs. Together with $G\alpha_{q/11}$, GRK2/3 isoforms and β-arrestins play facilitative roles in GnRH3-induced LH release, likely through scaffolding of protein kinase effectors in clathrin-coated structures (CCS). Arrestin, through its interaction with the adaptor protein AP2 is known to be important for the incorporation of receptors into CCS. In addition, GnRH3 control of LH release uniquely involves AA, and the selective usage of $G\alpha_{i/o}$ subunits may stimulate activity of PLA_2 enzymes to mobilize AA. Additionally, $G\alpha_{i/o}$ can directly bind and activate isoforms of non-selective cationic TRPCs, which may directly contribute to cytosolic Ca^{2+} increases, or activate VGCCs through local depolarization following cation influx. Arf1/6 small GTPases are also identified as novel effectors in facilitating GnRH-dependent LH release, and their activation is mediated by PI3K-dependent synthesis of the rare phospholipid PtdIns(3,4,5)P_3 (PIP_3) leading to changes in activity of specific Arf-GEFs and Arf-GAPs. In turn, Arf1/6 GTPase involvement implicates the participation of phospholipase D (PLD). Additionally, Pyk2/FAK also facilitate acute LH secretion, which is expected to be downstream of rises in intracellular Ca^{2+} and calmodulin (CaM) activity, but proximal to Raf-MEK-ERK activation. Ca^{2+}/CaM-dependent mechanisms, through activation of nitric oxide synthase (NOS) lead to the recruitment of nitric oxide-dependent mechanisms in GnRH3-stimulated gonadotrophs. SOS-Ras interactions exert negative influences over acute LH release, which may be driven by Src activation of SOS through Shc adaptors (not shown). In addition, GnRH3-induced LH release is known to undergo desensitization, but results from this thesis demonstrated that it is not dynamin-dependent (not shown).

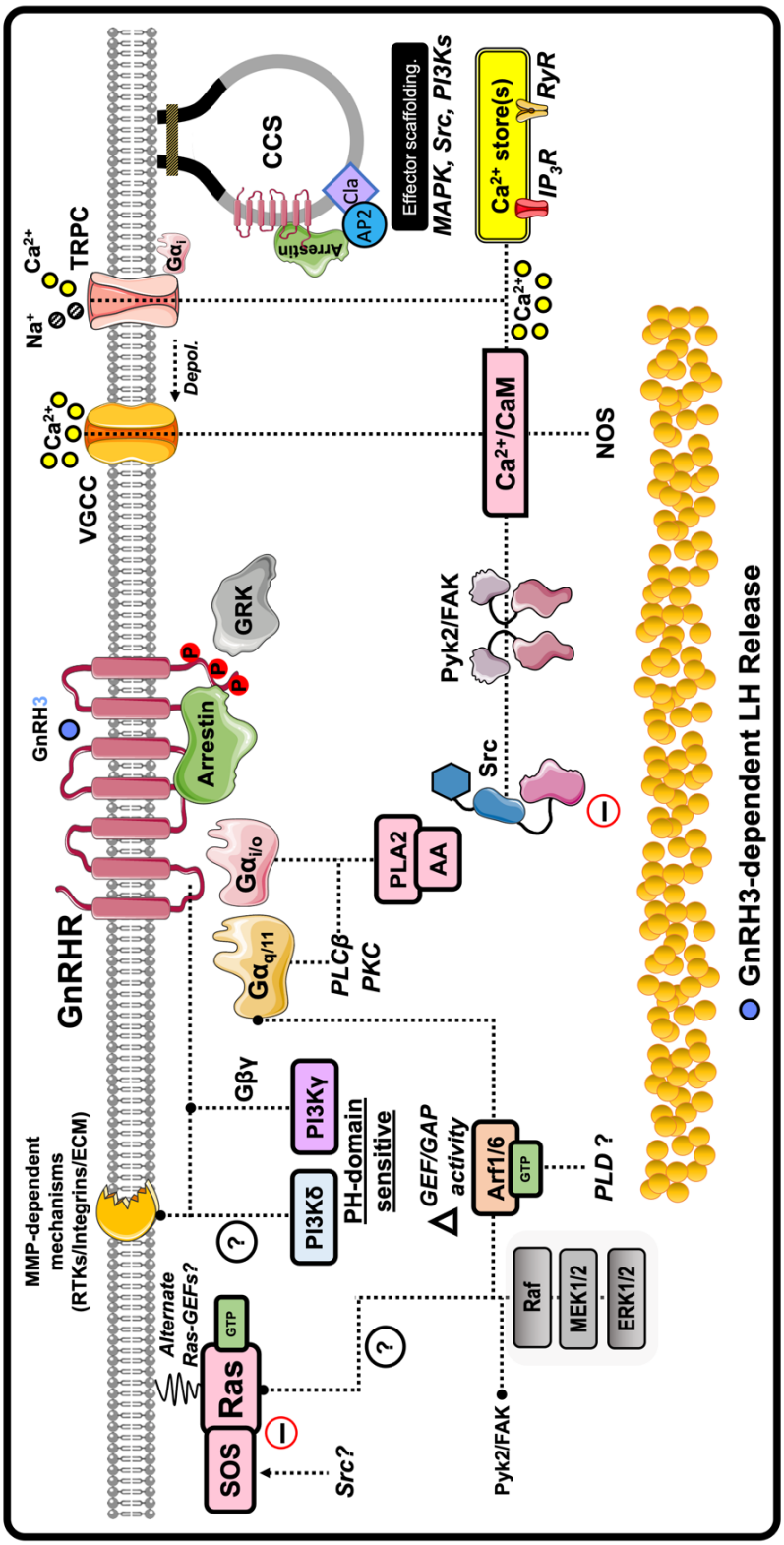

MMP-dependent mechanisms (RTKs/Integrins/ECM)
GnRHR
GnRH3
Alternate Ras-GEFs?
SOS
Ras
GTP
Src?
Gβγ
PI3Kγ
PI3Kδ
PH-domain sensitive
GEF/GAP activity
Arf1/6
GTP
PLD ?
Pyk2/FAK
Raf
MEK1/2
ERK1/2
Gαi/o
Gαq/11
PLCβ
PKC
PLA2
AA
Src
Pyk2/FAK
Ca2+/CaM
NOS
Ca2+
Ca2+ store(s)
IP3R
RyR
Effector scaffolding.
MAPK, Src, PI3Ks
CCS
Cla
AP2
Arrestin
GRK
Gαi
Depol.
Na+
Ca2+
TRPC
VGCC
Ca2+
GnRH3-dependent LH Release

Figure 7.3. Integrated summary schematic of GnRH2-dependent mechanisms in the control of acute GH release. GnRH2 control of pituitary GH secretion in goldfish utilizes the canonical $G\alpha_{q/11}$ effector PLC-β, with subsequent liberation of DAG leading to activation of PKC isoforms, although the rises in cytosolic calcium are not sensitive to inhibition of IP_3 receptors (not shown), but instead are dependent on actions of ryanodine receptors (RyR) and extracellular Ca^{2+} influx through VGCCs. MEK-ERK activities (not shown) uniquely do not contribute to GnRH2-dependent GH secretion. In addition, recent work has highlighted the selective involvement of PI3Kβ and PI3Kγ isoforms, which can both be activated by G protein βγ subunits. Results from this thesis directly identify $G\alpha_{q/11}$ subunit participation, as well as that of the receptor-interacting protein β-arrestins, which are classically recruited through receptor phosphorylation by GRKs. In contrast to actions in gonadotrophs, GRK2/3 isoforms exert negative influences over the acute phase of GnRH-dependent release and mediate desensitization functions. Also, results with a pan-Gα inhibitor suggest the co-participation of other Gα subtypes exerting negative regulation over acute GH release. Rho small GTPases are also identified as novel negative regulators of GnRH-dependent GH release, and their activation is likely affected by PI3K-dependent synthesis of the rare phospholipid PtdIns(3,4,5)P_3 (PIP_3), and/or through $G\alpha_{12/13}$-dependent mechanisms, leading to changes in the activities of specific Rho-GEFs and Rho-GAPs. Lastly, the protein tyrosine kinase Src facilitates GnRH2-dependent GH release, which could be downstream of β-arrestin-AP2-dependent events (which lead to the incorporation of receptors into clathrin-coated structures, CCS) and/or Ca^{2+}/CaM activities; the latter is also upstream of nitric oxide synthase (NOS), a known component of GnRH2-induced GH secretion. Src is known to co-ordinate with PI3Ks for the activation of the previously identified effector Btk; together, the involvement of Src and Btk in facilitating GH release strongly support the involvement of PLC-γ isoforms in GnRH2 actions in somatotrophs.

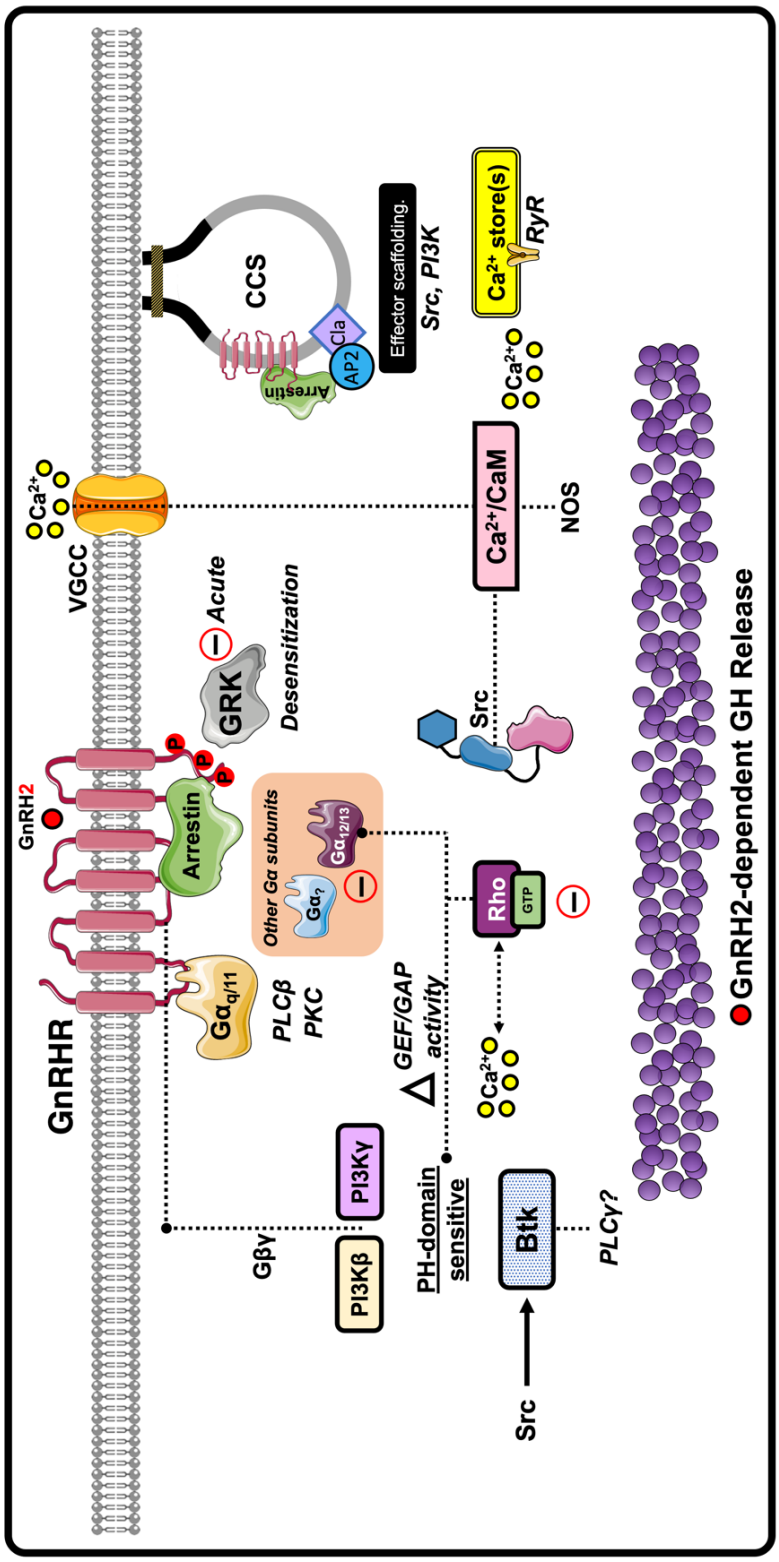

GnRHR
GnRH2
Gαq/11
PLCβ
PKC
Arrestin
P
P
P
GRK
Acute
Desensitization
Other Gα subunits
Gαi
Gα12/13
GEF/GAP
activity
Rho
GTP
Gβγ
PI3Kγ
PI3Kβ
PH-domain
sensitive
Src
Btk
PLCγ?
Ca2+
VGCC
Ca2+/CaM
NOS
Src
Ca2+
Ca2+ store(s)
RyR
CCS
Arrestin
AP2
Cla
Effector scaffolding.
Src, PI3K
GnRH2-dependent GH Release

Figure 7.4. Integrated summary schematic of GnRH3-dependent mechanisms in the control of acute GH release. GnRH3 control of pituitary GH secretion in goldfish utilizes the canonical $G\alpha_{q/11}$ effector PLC-β, with subsequent liberation of DAG leading to activation of PKC isoforms and IP_3 production. Activation of IP_3 receptor (IP_3R) Ca^{2+} channels and VGCCs lead to increases in cytosolic Ca^{2+}. Among its many effects, Ca^{2+}/CaM action on nitric oxide synthase (NOS) can lead to nitric oxide-dependent signalling. In addition, recent work has highlighted the participation of PI3Kδ, PI3Kβ, and PI3Kγ isoforms in GnRH3-stimulated GH secretion. While the latter two can be engaged by G protein βγ subunits, PI3Kδ is likely downstream of MMP-dependent mechanisms in GnRH3-stimulated cells revealed in the present study. MMP actions may also be mediated by as yet untested effects on MMP substrates such as extracellular matrix (ECM) and integrin, and membrane-tethered proligands of receptor tyrosine kinase (RTKs). Results from this thesis further directly identify $G\alpha_{q/11}$ subunit participation, as well as that of the receptor-interacting protein β-arrestins, which are classically recruited through receptor phosphorylation by GRKs. In addition, $G\alpha_{i/o}$ subunits may co-ordinate with $G\alpha_{q/11}$ to regulate phospholipase C activities, as well as interact with other downstream effectors. $G\alpha_{i/o}$ can also directly bind and activate isoforms of non-selective cationic TRPCs, which may directly contribute to cytosolic Ca^{2+} increases, or activate VGCCs through local depolarization following cation influx. β-arrestins exert negative regulation over acute GnRH-dependent secretion. β-arrestins through interactions with the adaptor protein AP2 and subsequent incorporation of receptors into clathrin-coated structures (CCS) may participate in receptor downregulation and/or provide a scaffolding for the recruitment of Akt which is known to inhibit GH secretion. Akt inhibition of GH release is also consistent with the known ability of Akt to inhibit Raf kinase, whereas PKC inputs to Raf mediate its known stimulatory effects on GH release through MEK-ERK. While GRK2/3 isoforms mediate a prolonged desensitization type effect for GnRH3-dependent GH release (possibly via recruitment of arrestin-sensitive downstream components), they do not participate in the acute phase of stimulation. Rac small GTPases are also identified as novel negative regulators of GnRH-dependent GH release, and their activation is likely affected by PI3K-dependent synthesis of the rare phospholipid PtdIns(3,4,5)P_3 (PIP_3), leading to changes in the activities of specific Rho-GEFs and Rho-GAPs. Finally, EGFRs inhibited acute GnRH3-induced GH release, although activation of these RTKs does not involve a MMP component.

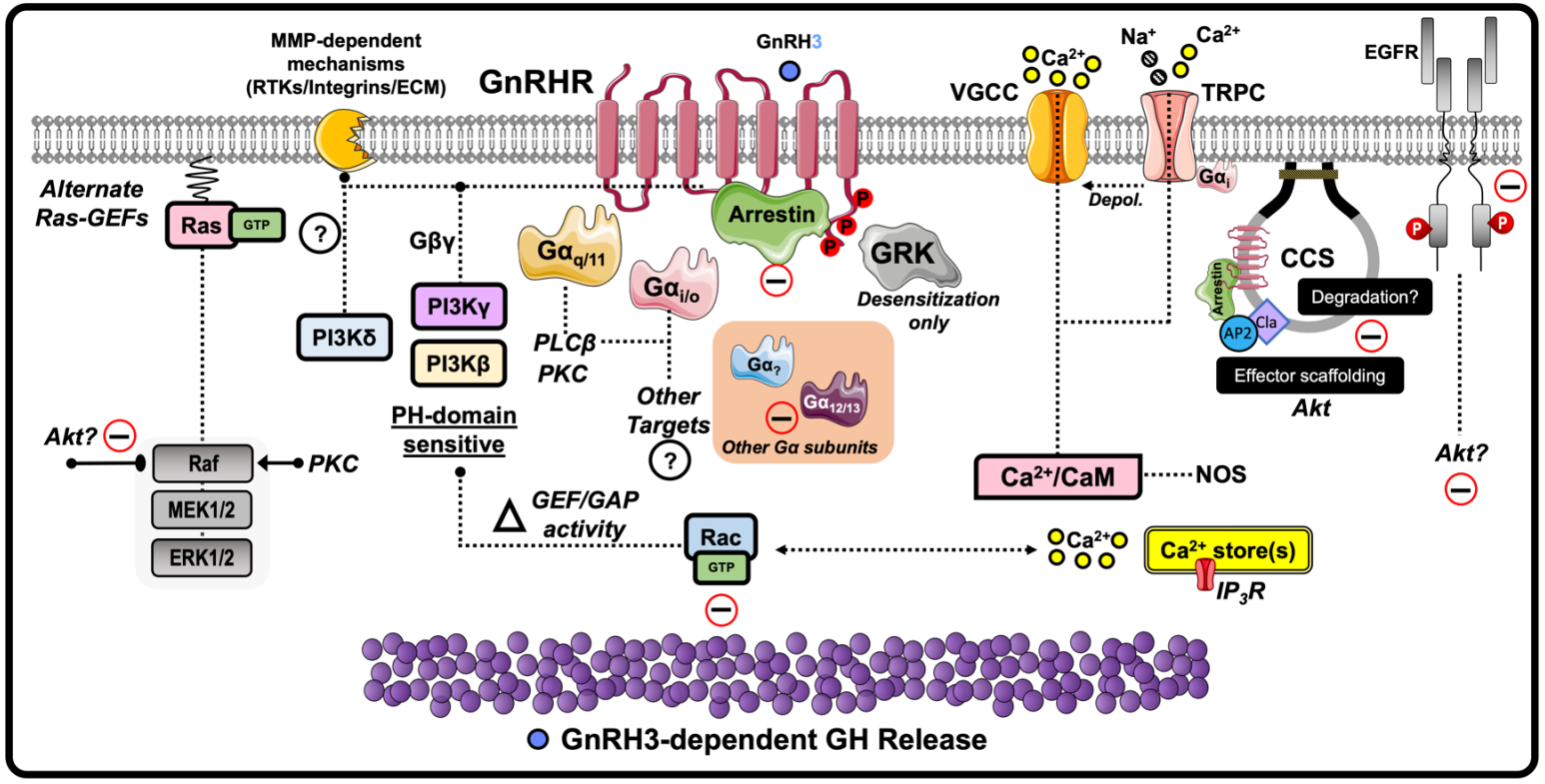
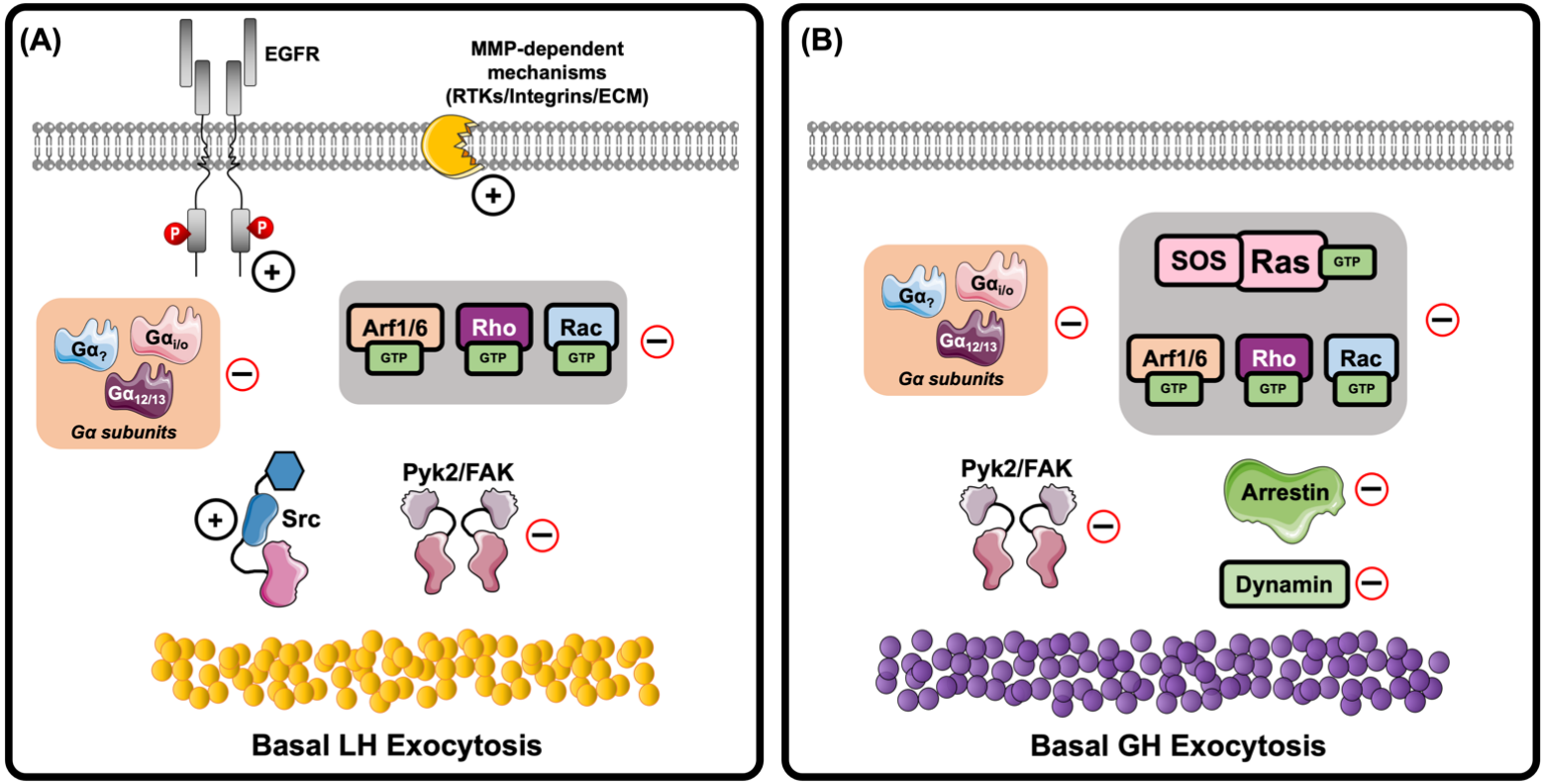

MMP-dependent mechanisms (RTKs/Integrins/ECM)
GnRHR
GnRH3
VGCC
Ca2+
Na+
Ca2+
TRPC
EGFR
Alternate Ras-GEFs
Ras
GTP
Gβγ
Gαq/11
Gαi/o
Arrestin
GRK
Desensitization only
Depol.
Gαi
CCS
Degradation?
Arrestin
AP2
Cla
Effector scaffolding
Akt
PI3Kδ
PI3Kγ
PI3Kβ
PH-domain sensitive
PLCβ
PKC
Other Targets
Gα?
Gα12/13
Other Gα subunits
Akt?
Raf
PKC
MEK1/2
ERK1/2
GEF/GAP activity
Rac
GTP
Ca2+/CaM
NOS
Ca2+
Ca2+ store(s)
IP3R
Akt?
GnRH3-dependent GH Release

Figure 7.5. Summary of effector involvement in the control of basal (unstimulated) LH and GH release. Results from this thesis have identified the involvement of several proteins that regulate basal hormone release from gonadotrophs (A) and somatotrophs (B). Often, these roles in basal release are uncoupled from actions during GnRH-stimulated cell states. The small GTPases Arf1/6, Rho, and Rac were consistently involved in the negative control of basal release, while SOS-Ras activity had selective functions only in basal GH secretion. Similarly, non-$G\alpha_{q/11}$ Gα subunits (definitive identity currently unknown), and the tyrosine kinases Pyk2/FAK, exerted strong negative regulation over both LH and GH release. Src, EGFR, and MMP activities positively modulate basal LH release, whereas β-arrestins and dynamin exert inhibitory influence on basal GH secretion.


(A)
EGFR
MMP-dependent
mechanisms
(RTKs/Integrins/ECM)
P
P
+
+
Gα?
Gαi/o
Gα12/13
Gα subunits
−
Arf1/6
GTP
Rho
GTP
Rac
GTP
−
+
Src
Pyk2/FAK
−
Basal LH Exocytosis
(B)
Gα?
Gαi/o
Gα12/13
Gα subunits
−
SOS
Ras
GTP
Arf1/6
GTP
Rho
GTP
Rac
GTP
−
Pyk2/FAK
−
Arrestin
−
Dynamin
−
Basal GH Exocytosis

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